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# β-Carotene inhibits neuroblastoma tumorigenesis by regulating cell differentiation and cancer cell stemness

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ABSTRACT

Neuroblastoma (NB) is the most common extracranial solid cancer in young children and malignant NB cells have been shown to possess cancer stem cell (CSC) characteristics. Thus, the successful elimination of CSCs represents a strategy for developing an effective preventive and chemotherapeutic agent. CSCs are characterized by differentiation and tumorigenicity. β-Carotene (BC) has been associated with many anticancer mechanisms, although the efficacy of BC on CSCs remains unclear. In the present study, the effects of BC on tumor cell differentiation and tumorigenicity was investigated using a xenograft model. Mice were pretreated with BC for 21 days, then received a subcutaneous injection of SK-N-BE(2)C cells. Both tumor incidence and tumor growth were significantly inhibited for mice that received BC supplementation compared to the control group. Treatment with BC has also been shown to induce tumor cell differentiation by up-regulating differentiation markers, such as vimentin, peripherin, and neurofilament. Conversely, BC treatment has been shown to significantly suppress tumor stemness by down-regulating CSC markers such as Oct 3/4 and DLK1. BC treatment also significantly down-regulated HIF1-α expression and its downstream target, vascular endothelial growth factor (VEGF). Taken together, these results suggest that BC is a potential chemotherapeutic reagent for the treatment of NB, and mediates this effect by regulating the differentiation and stemness of CSCs, respectively.



## 1. Introduction

Neuroblastoma (NB) is the most common extracranial solid cancer diagnosed in young children, and derives from multipotent crest cells of the sympathetic nervous system (SNS) that are localized to the adrenal glands and in paraspinal locations from the neck to the pelvis [1]. It has been reported that NB cells have the capacity to differentiate when properly stimulated, and higher tumor cell differentiation stages are related to a positive clinical outcome. Based on significant similarities that exist between the gene expression profiles of normal neuroblasts and malignant NB, these cells have been used for study of cancer stem cells (CSCs) characteristics [2]. However, the treatment of high-risk NB remains a challenge. For example, patients older than one year of age with stage 4 NB have a poor prognosis and a survival rate of 30–40% [3]. Furthermore, more than half of children with high-risk NB experience relapse due to CSCs.

A small cell population of cells termed, CSCs, or cancer-initiation cells, are able to undergo differentiation, self-renewal, and tumorigenicity. Moreover, CSCs have the potential to enhance tumor growth and induce metastasis, thereby leading to the relapse of cancers following treatment [4]. Therefore, targeting of CSCs may represent a successful therapeutic strategy for the treatment of cancer and the prevention of tumor relapse. Tumorigenic cells can be distinguished from nontumorigenic cells based on various stem cell markers. Among these, drosophila delta-like 1 homologue (DLK1) is a member of the epidermal growth factor-like homeotic protein family and is known to modulate differentiation signaling in adipocytes and several types of stem cells. Consequently, it is recognized as a stem cell gene in neuronal tumors [5].

Decreased oxygenation, or hypoxia, is a well-known characteristic of solid tumors and is a key prognostic factor for tumor progression and poor clinical outcome. A number of studies have reported that hypoxia has the potential to regulate tumor cell differentiation thereby facilitating the maintenance of CSC characteristics that can lead to malignancy [6]. Hypoxia-inducible factor-1 (HIF-1) is a heterodimeric transcription factor that plays a key role in the response of tumor cells to hypoxia, and consists of an α- and β-subunit [7]. Upon stabilization of the α-subunit, HIF-1α

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translocates to the nucleus to form an HIF-1α/β heterodimer and transactivate target gene expression. One of these targets includes vascular endothelial growth factor (VEGF) [8]. Thus, regulation of hypoxia may represent an important therapeutic strategy for targeting malignant cancers.

β-Carotene (BC) is a well-known vitamin A precursor that is found in many fruits and vegetables that are dark green or orange in color. Numerous observational studies have found that a high dietary intake of fruits and vegetables rich in BC is associated with a reduced cancer risk for several types of cancers. Accumulating evidence also suggests that BC induces the differentiation of cancer cells, including melanoma and leukemia cancer cells [9,10]. In our recent study, BC was found to induce the differentiation, and suppress the cancer stemness, of malignant SK-N-BE(2)C [BE(2)C] NB cells *in vitro* [2]. The anti-metastatic effect of BC was also demonstrated both *in vitro* and *in vivo* for NBs [11]. However, little is known about the role of BC in the tumorigenesis of CSCs. Therefore, in the present study, the effect of BC on tumor cell differentiation and tumorigenicity was investigated using a xenograft model.

## 2. Materials and methods

### 2.1. Cell culture

The human NB cell line, BE(2)C, was purchased from American Type Culture Collection (ATCC, Rockville, MD) and cultured in a 1:1 mixture of Minimum Essential Medium (MEM, Welgene, Daegu, Korea) and Ham's F-12 (Welgene, Daegu, Korea) medium containing 10% fetal bovine serum (FBS, Hyclone, Logan, UT) and 1% penicillin–streptomycin (100 U/ml and 100 μg/ml, respectively; Invitrogen, Carlsbad, CA). The cells were maintained in humidified air at 37 °C with 5% $CO_2$. β-Carotene was purchased from Sigma Chemical Co. (St. Louis, MO).

### 2.2. In vivo tumor xenograft model

Male five-week-old BALB/c nude mice were purchased from Central Lab, Animal Inc. (Seoul, Korea) and were maintained under specific pathogen-free conditions (College of Pharmacy, Ewha Womans University, Seoul, Korea). Weight and food intake were monitored twice a week. After a one-week acclimation period, mice were randomly assigned to three groups (10 animals per group) as follows; (a) tumor control group (Ctrl): oral supplementation with corn oil as a vehicle; (b) BC 2 group (BC 2): oral supplementation with 2 mg/kg b.w. of BC; (c) BC 6 group (BC 6): oral supplementation with 6 mg/kg b.w of BC, respectively. The mice received either corn oil, or each dose of BC in corn oil, orally twice a week for seven weeks. On day 21 of the BC treatment regimen, mice were subcutaneously injected with $4.0 \times 10^5$ BE(2)C cells suspended in growth factor-reduced matrigel (BD Bioscience Laboratory, Bedford, MA) into the right flank region. After the tumors were established, tumor volume [volume = length (mm) × width$^2$ (mm$^2$) × 0.5] was measured twice a week using digital calipers. Mice were sacrificed four weeks later, with pimonidazole (60 mg/kg) administered 1 h prior to sacrifice. Tumors were counted and excised. After measuring the size and weight of each tumor, they were immediately frozen or immersed in 4% neutral buffered formaldehyde. Animal care and experimental protocols for this study were approved by the Animal Care and Use Committee of Ewha Womans University (IACUC approval No: 2013-01-028).

### 2.3. Reverse transcription-PCR and quantitative PCR analysis

PCR amplification of cDNA was performed using Taq polymerase (TAKARA, Tokyo, Japan). The resulting PCR products were separated in a 2% agarose gel containing ethidium bromide. For each sample, quantitative PCR analysis detected β-actin as an internal control. The primers used included: 5′-AGCA CCC ATG GCA GAA GG-3′ (forward) and 5′-CTC GAT TGG ATG GCA GTA CT-3′ (reverse) for human VEGF; and 5′-ATT GGC AAT GAG CGG TTC-3′ (forward) and 5′-GGA TGC CAC AGG ACT CCA T-3′ (reverse) for β-actin.

### 2.4. Western blot analysis

Western blot assays were performed as described previously [12]. Primary antibodies raised against peripherin (Millipore, Billerica, MA), vimentin (Cell Signaling Technology, Boston, MA), Oct 3/4 (Santa Cruz Biotechnology, Santa Cruz, CA), HIF-1α (Novus, Littleton, CO), and α-tubulin (Sigma Aldrich, St. Louis, MO) were used for the experiment.

### 2.5. Immunohistochemistry

Immunohistochemistry staining for neurofilament (DAKO, Ely, UK), DLK1 (Millipore, Billerica, MA), and HIF-1α antibody (Novus, Littleton, CO) was performed as described previously [11].

### 2.6. Statistical analysis

All data are presented as the mean ± standard error of the mean (SEM) and were analyzed using GraphPad PRISM software (GraphPad Software, San Diego, CA). Pearson's Chi-square or one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test was performed. A *P*-value less than 0.05 was considered statistically significant.

## 3. Results

### 3.1. BC supplementation reduces tumor volume and tumor incidence

At the time of sacrifice, 10 out of 10 Ctrl mice developed tumors, while only 4 out of 10 mice in BC 6 groups developed tumors (Table 1). Thus, BC supplementation inhibited tumorigenesis by 60%. Furthermore, the tumors formed in the BC 6 group were smaller than those formed in Ctrl and BC 2 mouse groups. For example, the mean tumor volume for the Ctrl group was 997.6 ± 703.0 mm$^3$ compared with 226.2 ± 335.2 mm$^3$ for the BC 6 group. This represented a 77% decrease in tumor volume for the BC 6 group. The BC 2 group also exhibited a smaller tumor volume compared with the Ctrl group, however, the difference was not statistically significant. These data suggest that BC supplementation may inhibit tumor growth *in vivo*.

### 3.2. BC supplementation induces cell differentiation in vivo

It was previously demonstrated that BC inhibited cancer cell stemness by inducing the differentiation of NB cells *in vitro* [2]. To investigate the anti-tumorigenic effect of BC supplementation *in vivo*, the cell differentiation markers, peripherin and vimentin were assayed. In the BC 6 tumor tissues, expression of the neuronal differentiation marker, peripherin was significantly up-regulated (Fig. 1A). Vimentin, a marker of cytodifferentiation, was also up-regulated in the BC 6 tumor tissues compared to the Ctrl tissues (Fig. 1B). Similarly, expression of peripherin and vimentin were higher in BC 2 tissues compared to the Ctrl tissues, although the differences were not statistically significant.

To confirm these results, expression of neurofilament was analyzed by immunohistochemistry. Neurofilament is a protein which exhibits limited expression only in mature neurons. In Ctrl tumor tissues, levels of neurofilament were very low (Fig. 1C). In contrast, BC 2 and BC 6 tumor tissues expressed higher levels of

**Table 1**
BC suppresses tumor incidence and final tumor volume[1].

| Treatment group | Total number of mice | Tumor incidence | Final tumor volume ($mm^3$) |
|---|---|---|---|
| TC | 10 | 10/10 | 997.60 ± 703.02[a] |
| BC 2 | 8 | 8/8 | 563.90 ± 504.71[a] |
| BC 6 | 10 | 4/10 | 226.22 ± 335.22[b] |

The different groups with different alphabets are significantly different ($P < 0.05$).
[1] Number of mice with tumor incidence and final tumor volume of mice injected with BE(2)C cells. Comparisons among groups were conducted by chi-square ($P < 0.05$).

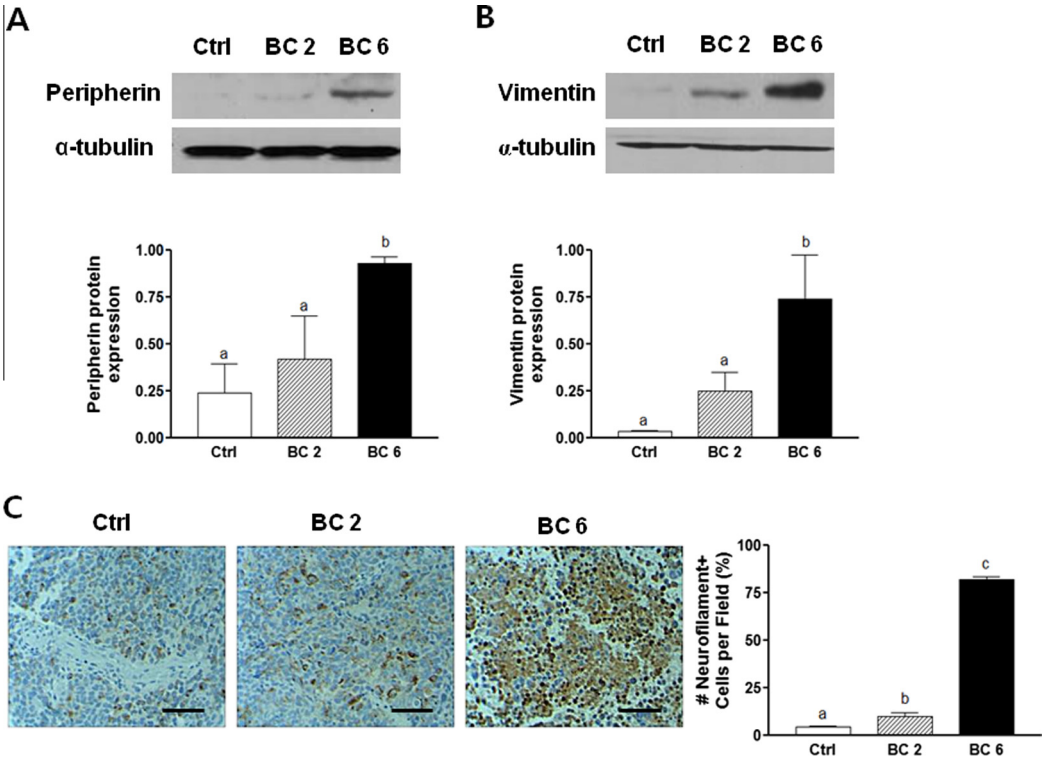


**Fig. 1.** BC supplementation induces cell differentiation *in vivo*. (A and B) Expression levels of peripherin (A) and vimentin (B) were detected in tumor tissue extracts by Western blot. Detection of α-tubulin was used as a loading control. Representative figures are shown (upper panel). Quantification of peripherin levels is shown (lower panel). (C) Immunohistochemical staining of neurofilament was performed for the tumor sections indicated. Stained slides were imaged at 400× magnification. The scale bar represents 4 μm. Neurofilament positive cells were counted and quantified. Bars represent the mean ± SEM. Bars labeled with different letters differ significantly ($P < 0.05$). Ctrl, tumor control; BC 2, tumor injection + BC 2 mg/kg b.w; BC 6, tumor injection + BC 6 mg/kg b.w.

neurofilament. Taken together, these results suggest that BC suppresses tumor formation by NB cells by inducing cell differentiation via regulation of the differentiation markers, peripherin, vimentin, and neurofilament.

### 3.3. BC suppresses CSC marker expression by NB cells in vivo

CSCs have the potential to induce tumor growth and progression [4]. Correspondingly, it has been reported that BC inhibits CSC characteristics, such as self-renewal, in NB cells *in vitro* [2]. To understand the effect of BC on cancer cell stemness in NB cells *in vivo*, the expression of several CSC markers were analyzed using Western blot and immunohistochemistry assays (Fig. 2). Expression levels of Oct 3/4, a hallmark for CSC-like cells, were significantly lower in BC 6 group compared to Ctrl group (Fig. 2A). In contrast, BC 2 group exhibited lower levels of Oct 3/4 expression compared to the Ctrl group, although the difference was not statistically significant. DLK1 is a neuronal stem cell marker which is important for the maintenance of stem cells [13]. Immunohistochemical detection of DLK1 showed that the number of DLK1 positive-stained cells were significantly lower in both BC 2 and BC 6 group compared with those in Ctrl group (Fig. 2B). These results suggest that BC supplementation suppresses cancer cell stemness by down-regulating several CSC markers.

### 3.4. BC supplementation suppresses the expression of HIF-1α and its downstream target VEGF in vivo

NB typically grow under hypoxic conditions and hypoxic tumors exhibit more malignant characteristics [14]. Correspondingly, hypoxia has been shown to promote tumor growth and to inhibit differentiation [14]. To investigate whether BC supplementation suppresses tumor growth by regulating levels of HIF-1α, immunohistochemistry and Western blot assays were performed. In tumors, HIF-1α positive cells were significantly higher in Ctrl group compared with BC 6 group (Fig. 3A). Quantification of HIF-1α positive cells represented that BC 6 supplementation suppressed HIF-1α expression. Consistent with the immunohistochemistry results, higher levels of HIF-1α expression were also detected in Ctrl tumor tissue extracts compared to BC 6 tissue extracts that were analyzed by Western blot (Fig. 3B). While BC 2 group also exhibited lower levels of HIF-1α, the difference from Ctrl group was not statistically significant. *VEGF* is a hypoxia-inducible gene [13]. mRNA levels of *VEGF* were analyzed using

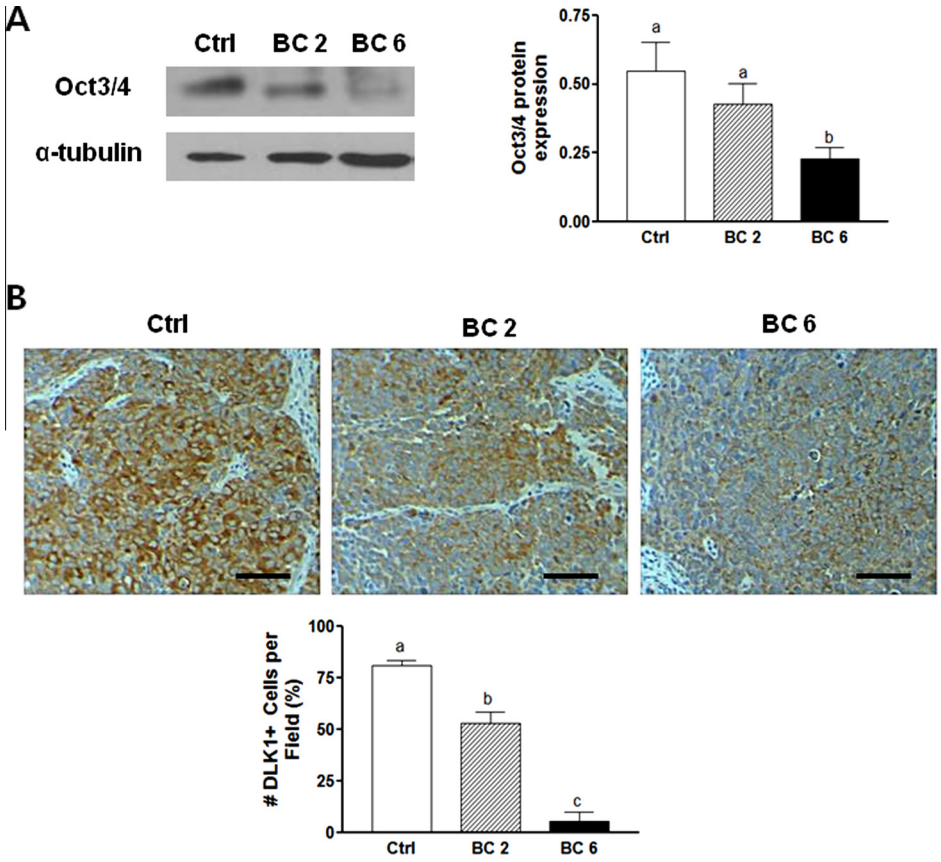


**Fig. 2.** BC suppresses expression of CSC markers by NB *in vivo*. (A) Expression levels of Oct3/4 were detected in tumor extracts by Western blot. Detection of α-tubulin was used as a loading control (left panel). Quantification of Oct3/4 levels is shown (right panel). (B) Expression of DLK1 was detected immunohistochemically in tumor tissue sections at 400× magnification. The number of DLK1 positive cells present was quantified. The scale bar represents 4 μm. The bars represent show the mean ± SEM. Bars labeled with different letters differ significantly ($P < 0.05$). Ctrl, tumor control; BC 2, tumor injection + BC 2 mg/kg b.w; BC 6, tumor injection + BC 6 mg/kg b.w.

quantitative PCR. Significantly lower levels of VEGF mRNA were detected in the BC 6 group compared to the Ctrl group. Taken together, these results suggest that BC suppresses NB tumor growth by regulating levels of HIF-1α and its downstream target gene, *VEGF*.

## 4. Discussion

BC is a well-known antioxidant and precursor of retinoic acid (RA), and both induce differentiation [15,16]. Similarly, Gross et al. [15] reported that both BC and lutein induced HL-60 cells to undergo differentiation, while Noguchi et al. [16] successfully induced the differentiation of Neuro2a cells by treating them with β-cryptoxanthin. More recently, BC was used to induce the differentiation of BE(2)C cells, thereby resulting in the identification of anti-CSC characteristics for this cell line, which include suppression of self-renewal capacity, such as clonogenicity and sphere formation. BC treatment has also been found to resensitize NB cells to cisplatin-mediated cytotoxicity by directly targeting CSCs [2]. However, despite the high antioxidants potential of BC, the anti-carcinogenic effect of BC on various cancers has been inconsistent.

In the present study, BC supplementation (6 mg/kg b.w.) inhibited NB tumorigenesis and affected markers of cell differentiation and cancer cell stemness. Regarding the administration of BC at 6 mg/kg b.w. (1.7 mg/kg b.w./day) twice a week, which corresponds to about 6–7 mg/day for a 70-kg person [17]. High (2.4 mg/kg b.w./day) and low (0.43 mg/kg b.w./day) doses of BC that were administered to a ferret model resulted in differing effects on lung tumorigenicity [18]. For example, the low dose (0.43 mg/kg b.w./day) suppressed lung carcinogenesis induced by exposure to smoke, while a high dose of BC (2.4 mg/kg b.w./day) did not. This difference may be related to the instability of BC in the free radical-rich environment of smoke-exposed lungs. It was also previously demonstrated that administration of 16 mg/kg b.w. BC twice a week suppressed the growth of prostate tumor in nude mice [17], and 20 mg/kg b.w. BC inhibited lung metastasis events for SK-Hep 1 human hepatoma cells [19]. For mice supplemented with either 2 mg/kg BC or 22 mg/kg BC for 36 weeks, a significant reduction in the number of colon tumor induced by 1,2-dimethylhydrazine (DMH), and the time to appearance, were observed at both doses [20]. Moreover, Kim et al. reported that 2 mg/kg b.w. BC administered twice a week inhibited NB cell invasion and metastasis, and also suppressed liver metastasis *in vivo*, by suppressing MMPs and regulating HIF-1α [11]. Although the duration of BC supplementation was shorter and the dose was smaller than that for other studies, the effect of BC on NB tumor incidence and final tumor volume was greater. These results indicate that BC may exert a greater anti-carcinogenesis effect on NB CSCs than other cancers. Taken together, these results indicate that BC supplementation administered prior to tumor growth is highly effective in inhibiting NB tumor growth which possesses CSC characteristics.

It is a possible that the mechanism underlying the antitumorigenic activities of BC involves the neuronal differentiation of BE(2)C cells *in vivo*. The cytoskeletons undergoes changes during the NB differentiation process [21], and several proteins are involved. For example, peripherin is associated with the peripheral nervous system and has been found to be up-regulated in a NB cell line in the presence of a differentiation inducing agent [20]. When

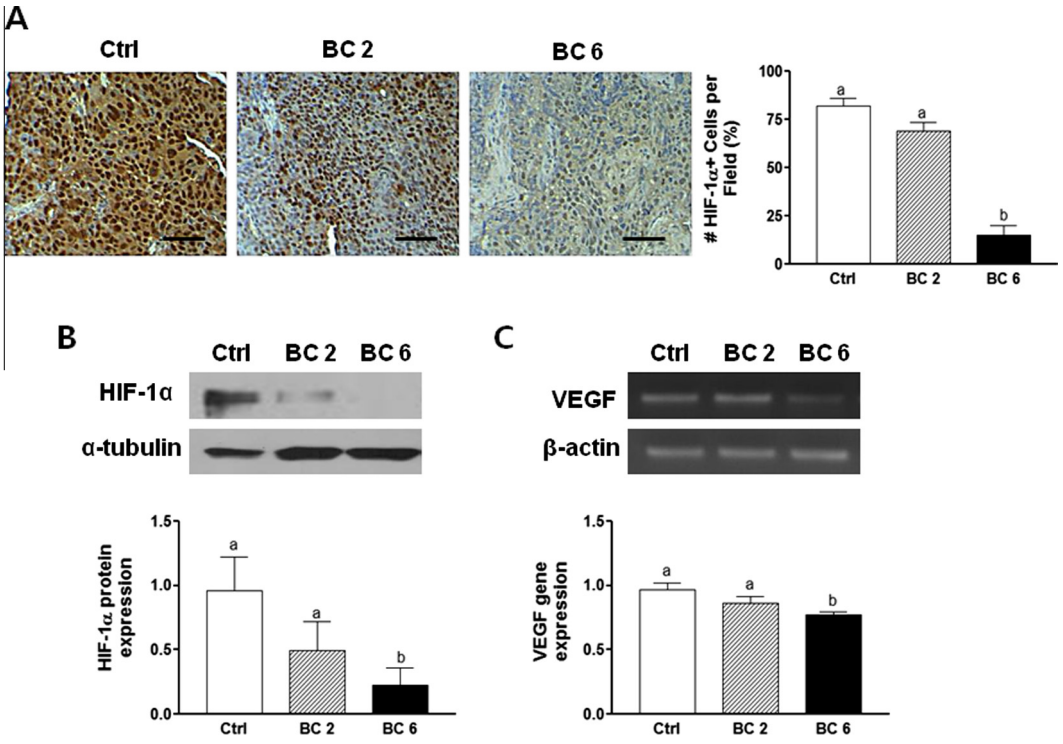


**Fig. 3.** BC supplementation suppresses expression of HIF-1α and its downstream target, VEGF, *in vivo*. (A) Immunohistochemical detection of HIF-1α was performed for the tumor sections indicated. Stained slides were imaged at 400× magnification. The scale bar represents 4 μm. HIF-1α-positive cells were quantified. Bars represent the mean ± SEM. Bars labeled with different letters differ significantly ($P < 0.05$). (B) Expression levels of HIF-1α were detected in tumor tissue extracts by Western blot. Detection of α-tubulin was used as a loading control (upper panel). Quantification of HIF-1α levels is shown (lower panel). (C) Using RT-PCR, mRNA levels of *VEGF* were analyzed. Detection of β-actin mRNA was used as a loading control (upper panel). Quantification of *VEGF* levels is shown (lower panel). The bars represent the mean ± SEM. The bars with labeled with different letters differ significantly ($P < 0.05$). Ctrl, tumor control; BC 2, tumor injection + BC 2 mg/kg b.w; BC 6, tumor injection + BC 6 mg/kg b.w.

a NB cell line was treated with a new agent for induction of NB differentiation, synthesis of vimentin, a marker of cells undergoing cytodifferentiation to a Schwann phenotype in NB, was observed [22]. Lastly, neurofilament is an intermediate filament protein that is characterized by limited expression only in mature neurons [23]. In the present study, expression levels of peripherin, vimentin, and neurofilament were found to be significantly up-regulated during BC supplementation. Previously, it was demonstrated that BC supplementation induced NB cell differentiation concomitant with up-regulation of β-tubulin III and increased phosphorylation of ERK1/2 (p42/p44) [2]. Taken together, these results confirm the anti-tumorigenesis properties of BC that mediate an induction of neuronal differentiation.

Another possible mechanism underlying the anti-tumorigenic activities of BC involves the inhibition of CSCs, which can affect tumor growth and therapy resistance [4]. Oct 4 is considered a biomarker for CSC-like cells and its expression has been found to be related to the dedifferentiation state of cancer cells [24]. In the present study, expression of Oct 3/4 was found to be significantly lower in the BC 6 group compared to the Ctrl group. This result is consistent with the observation that inoculated breast cancer cells that expressed higher levels of Oct 4 were associated with a higher incidence of tumors (83.3%) compared with inoculated tumor cells that expressed lower levels of Oct 4 (16.6%) [25]. DLK1 is a member of the notch/delta/serrate family and is strongly expressed in immature embryonic cells [5]. It is hypothesized that DLK1 enhances cancer cell stemness and suppresses tumor differentiation [13], while DLK1 has been shown to be required for glioblastoma growth *in vitro* [26]. Furthermore, Begum et al., have reported that xenograft tumors derived from NB cell over-expressing DLK1 exhibited significantly lower levels of neuronal differentiation marker expression [27]. In the present study, the number of DLK1-positive cells was significantly reduced in the BC 6 group, which also expressed higher levels of neuronal differentiation markers.

Hypoxia is an important environmental factor that regulates the cell differentiation of many stem and progenitor cells. It also plays an important role in many aspects of tumor development and growth [12]. It has been reported that NB cells that are exposed to hypoxia acquire an undifferentiated neural crest-like phenotype, and that pre-treated NB cells exhibit a slight increase in growth rate in a xenograft model [28]. Since HIF-1α is stabilized when oxygen levels are low, tumor cells induce expression of HIF-1α when they are exposed to hypoxic conditions [29]. Previously, treatment with 10 μM BC was found to dramatically inhibit HIF transcriptional activity under hypoxic conditions *in vitro* [11], and *in vivo*, HIF-1α protein expression was down-regulated in the liver tissue of a metastatic NB model [11]. Correspondingly, 6 mg/kg b.w BC supplementation in the present study was associated with a significant down-regulation of HIF-1α expression and its downstream gene, *VEGF*, in tumor tissues. VEGF is a potent growth factor for blood vessel endothelial cells and facilitates both cell migration and proliferation [30]. It has been reported that BC inhibits tumor-specific angiogenesis by regulating VEGF stimulated by TNF-α, IL-1β, and IL-6 in B16F-10 melanoma cells [30]. Induced activation of chemotaxis by human endothelial progenitor cells has also been observed for BC treatments [31].

In summary, the results of the present study demonstrated that BC supplementation inhibits tumorigenesis in NB *in vivo* by inducing neuronal differentiation, inhibiting cancer stemness, and regulating hypoxia-related HIF-1α and its down-stream target, *VEGF*. Additional studies are needed to elucidate the mechanism of action of BC in other tumor microenvironments. However, for advanced

NB, it appears that BC has the potential to mediate chemotherapeutic effects.

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