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Original Article

BCAT1 knockdown-mediated suppression of melanoma cell proliferation and migration is associated with reduced oxidative phosphorylation

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Abstract: Malignant melanoma has a high mutational rate. As a result, resistance to current therapies is common. Consequently, there is an unmet medical need to develop novel therapies. Recent data suggest that branched-chain amino acid transaminase 1 (BCAT1) is overexpressed in multiple cancers, and such overexpressed BCAT1 is necessary for individual cancer progression. Therefore, BCAT1 appears to be a good target in cancer treatment. Additionally, because its expression in healthy tissues is highly restricted in adults and is limited to the brain, ovary, and placenta, BCAT1 is especially an ideal target in cancer therapies. Currently, the function of BCAT1 in malignant melanoma has not been demonstrated. Therefore, we investigated the role of BCAT1 in the proliferation and migration of malignant melanomas using human samples and mouse malignant B16 melanoma cell line. Our data showed that BCAT1 was overexpressed in malignant melanoma tissues both in humans and mice. Besides, BCAT1 knockdown suppressed melanoma cell proliferation and migration, which was associated with reduced oxidative phosphorylation. Collectively, our data indicate that BCAT1 is a promising therapeutic target for the treatment of malignant melanomas.

Keywords: Malignant melanoma, branched-chain amino acid transaminase 1, branched-chain amino acids, oxidative phosphorylation, glycolysis, proliferation, migration

Introduction

Malignant melanoma is predominantly a cutaneous disease. In the United States, its incidence continues to increase [1]. With the advent of recently developed novel therapies, stage IV metastatic melanoma patients' survival rate increased significantly [2]. However, because of a high mutational rate [2], resistance to current therapies is common [3]. For this reason, there is an unmet medical need to develop novel therapies.

To develop effective therapies, we reason that it is necessary to understand better the mechanisms underlying malignant melanoma progression. In this regard, recent data suggest that branched-chain amino acid transaminase 1 (BCAT1) is overexpressed in multiple cancers, and such overexpressed BCAT1 is necessary for individual cancer progression [4-6]. Therefore, BCAT1 appears to be a good target in cancer treatment. Additionally, because its expression in healthy tissues is highly restricted in adults and is limited to the brain, ovary, and placenta [7], BCAT1 is especially an ideal target in cancer therapies. Currently, the function of BCAT1 in malignant melanoma has not been demonstrated. Based on the previous reports, we hypothesized that understanding

BCAT1's roles in metastatic malignant melanoma would reveal potential novel therapeutic targets.

BCAT has two isoforms, i.e., BCAT1 and BCAT2. BCAT1 is present in cytosol, whereas BCAT2 is present in mitochondria [8]. Concerning its biological functions, BCAT1 is the enzyme that catalyzes the first step of branched-chain amino acid (BCAA) metabolism (BCAAs include leucine, isoleucine, and valine) [9-11]. It has been demonstrated that BCAT1 can convert either the BCAAs to branched-chain keto acids (BCKAs) or the BCKAs back to BCAAs.

Interestingly, previous findings showed that overexpressed BCAT1 catabolized BCAAs in some cancers [4] but synthesized BCAAs in others [6]. Additionally, BCAT1 was essential for non-small cell lung carcinoma's disease progression but not pancreatic ductal adenocarcinoma [12]. The above data indicate that BCAT1's function is tissue-specific. Hence, we must precisely understand the BCAT1's role in metastatic malignant melanoma to design specific targeted therapies.

This article will present the data to support that BCAT1 is critical for the progression of metastatic malignant melanomas by enhancing oxidative phosphorylation.

Materials and methods

Human subjects

The human tissue microarray (ME2082c) was purchased from Xi'an Alena Biotechnology Ltd., Co. (Xi'an, Shaanxi Province, China).

Animals

C57BL/6 mice were purchased from the Animal Center of Zhengzhou University. Mice of 6 to 8 weeks age were used for all the experiments. This study was approved by the Animal Care and Use Committee at Zhengzhou University.

Cell lines

The mouse melanoma cell line B16 was donated by Dr. Zhenyu Ji (Henan Medical Science Research Institute). Other melanoma cell lines, including B16-F10, Melan-α, A375, A2058, and M14, were purchased from Wuhan Biofavor Biotechnology Service Co., Ltd. The above melanoma cell lines were cultured in RPMI-1640 cell culture medium supplemented with 10% fetal bovine serum (FBS) at 37°C and 5% CO_2.

Analysis of BCAT1 expression in B16 melanoma

In vivo melanoma was induced by subcutaneous injection of 1×10^6 B16 cells. After two weeks, melanoma and normal tissues were collected, fixed with 4% paraformaldehyde, and analyzed for BCAT1 expression using immunohistochemistry.

Generation of stable BCAT1 knockdown B16 cell lines

BCAT1 was knocked down using small hairpin RNA (shRNA). Briefly, shRNA targeting BCAT1 and a negative control shRNA were purchased from Hanbio Biotechnology Co., Ltd. (Shanghai, China). The shRNA sequences used in this study are shown below: control-shRNA: 5'-TTCTCCGAACGTGTCACGTAA-3'; sh1-*BCAT1*: 5'-GGATCAAGAATGGGTCCCATATTCA-3'; sh2-*BCAT1:* 5'-GTCCCAAGTATGTAAGAGCCTGGAAA-3'.

The generation of lentiviruses and virus transduction have been described previously [13]. Briefly, the lentiviruses were generated by transfecting HEK293T cells with the pHB-U6-MCS-CMV-ZsGreen-PGK-PURO plasmid, together with the pSPAX2, the pMD2G and the Lipofiter™ Transfection reagent. The lentiviruses were subsequently used for transducing the B16 cells.

Before virus transduction, the B16 cells were added into a 6-well plate at a density of 2×10^5 cells/well and incubated at 37°C and 5% CO_2. After reaching 70% confluence, the cells were transduced with the control-shRNA (NC), the sh1-BCAT1 (sh1), or the sh2-BCAT1 (sh2). Puromycin was added at 2 mg/ml to select positively transduced cells. The positively transduced cells were monitored by GFP expression under an inverted fluorescence microscope. Stably-transduced cells were confirmed by western blotting.

Western blot analysis

Cells were lysed using radioimmunoprecipitation assay (RIPA) buffer (Solarbio, Beijing, China) containing phenylmethylsulfonyl fluo-

ride (PMSF) (a serine protease inhibitor, Solarbio, Beijing, China). Proteins were separated on a 10% SDS-PAGE gel and transferred to a PVDF membrane. The membrane was blocked for 1.5 hours in non-fat powdered milk (BBI Life Sciences, Shanghai, China) and then incubated with primary antibodies at 4°C overnight. Primary antibodies used included anti-BCAT1 antibody (BBI Life Sciences, Shanghai, China), anti-GAPDH antibody (BBI Life Sciences, Shanghai, China), and anti-citrate Synthase rabbit polyclonal antibody (Proteintech, Wuhan, Hubei, China), anti-E-cadherin (E-ca) (20874-1-AP, Proteintech Group Inc., Wuhan, Hubei, China), and anti-vimentin (Vim) (ab8978, abcam, Cambridge, CB2 OAX, UK). The membrane was washed three times with Tris-buffered saline with Tween-20 (TBST) and incubated with TBST diluted secondary antibody (HRP-conjugated Goat Anti-Rabbit IgG) for 2 hours. The membrane was washed three times with TBST. Protein bands were detected using the ECL chemiluminescence system. In addition, mitochondrial functional markers, i.e., cytochrome c oxidase IV (COX IV), voltage-dependent anion channel (VDAC), pyruvate dehydrogenase (PDH), citrate synthase (CS), and succinate dehydrogenase complex subunit A (SDHA) were analyzed by western blot using a mitochondrial marker antibody sampler kit (Cat#: 8674T, Cell Signaling Technology, Danvers, MA, USA).

Cell proliferation assay

The proliferation ability of B16 cells was examined using the xCELLigence Real-Time Cell Analyzer (RTCA)-MP system (Acea Biosciences/Roche Applied Science). This platform can measure cellular growth status in real-time. Briefly, 50 uL of culture medium was added in each well of E-Plate 16 (Roche Applied Science) to obtain equilibrium. The transduced cells were incubated in 25 cm^2 culture plates for 24 h and seeded into the E-Plate 16 as 5×10^3 cells/well. The E-Plate 16 was locked in RTCA-MP device and cultured at 37°C, 5% CO_2. Changes in electrical impedance were presented as a cell index that directly reflected the cellular proliferation on biocompatible microelectrode coated surfaces. Cell index was read automatically every 5 minutes, and the corded curve was shown as cell index ± SEM.

Colony formation assay

B16 cells were plated into a 6-well plate at a density of 2000 cells per well. The cells were fixed seven days later with 4% paraformaldehyde and stained with 0.5% crystal violet. Finally, colony numbers were manually counted.

Wound healing assay

Parallel lines were made at the bottom of a 6-well plate. Then, the transduced B16 cells (2×10^5) in serum-free medium were seeded in each well of the 6-well plate. After the cells were grown to 80% confluence, the medium in the wells was discarded, and wounds were drawn using a 200 uL pipette. Briefly, three parallel lines were scratched in the middle of each well, and the scratched space was cleaned with PBS so that no residual cells were present. The plate was cultured at 37°C and 5% CO_2. After 24 and 48 hours, the cells were photographed using a CKX53 microscope (Olympus, Tokyo, Japan), and migrated cells were counted.

Immunohistochemistry (IHC)

The IHC was performed as described previously [9]. Briefly, paraffin-embedded tissue slides (4 μm) were prepared, and deparaffinized. The tissue slides were then baked in a microwave oven at 60°C for 30 minutes, and antigens in the tissues were retrieved. Subsequently, the endogenous peroxidase was blocked using 3% H_2O_2-methanol, and the tissue slides were incubated with the primary anti-BCAT1 antibody at 4°C overnight.

The tissue slides were washed with 0.1% Tween-20 PBS and incubated with the secondary antibody at room temperature for 30 minutes on the second day. After being rewashed with the 0.1% Tween-20 PBS, the tissue slides were incubated with 3,3-diaminobenzidine (DAB) for 5 minutes. Finally, the tissue slides were washed thoroughly with H_2O and counterstained with hematoxylin.

The staining intensity was determined according to the color intensity of the cells: "-" means no coloration, "+" means light brown, "++" means brown, and "+++" means sepia. Besides, the number of positive cells was recorded as follows: "-" referred to positive cells less

than 10%, "+" referred to positive cells between 10% to 25%, "++" referred to positive cells between 26% to 49%, and "+++" referred to positive cells above 50%. At least 5~10 high-power-fields were randomly observed, and their mean values were taken. "1", "2", "3", and "4" corresponded to "-", "+", "++", "+++".

Cell cycle assay

Cell cycle was analyzed using a cell cycle detection kit (KeyGEN BioTECH Co., Ltd.) following the provided instruction. Briefly, cells were collected and pelleted by centrifugation (2000 rpm for 5 min). The cells were adjusted to 1 × 10^6 cells/mL, pelleted, and supernatants discarded. The cells were reconstituted in 500 μL of 70% Fix in cold ethanol and stored at 4°C overnight. The cells were added with 1 mL of PBS and pelleted by centrifugation (1000 rpm for 3 min). The cells were resuspended with 500 μL RNaseA/PI (1:9) staining working solution and incubated at room temperature for 30~60 minutes while avoiding the light. The cells were then analyzed by fluorescence-activated cell sorting (FACS).

Glycolysis stress test

Extracellular acidification rate (ECAR) was analyzed using the Seahorse XF Glycolysis Stress Test Kit on the Seahorse XF^e 96 Extracellular Flux Analyzer (Seahorse Bioscience). Experiments were performed according to the manufacturer's protocols. Briefly, to determine the optimal cell concentration in the glycolysis stress test, the cells were seeded into a Seahorse XF 96 cell culture microplate as 0.5X, 1X, 2X, 4X (X=10,000) cells per well. After baseline measurements, glucose, the oligomycin (oxidative phosphorylation inhibitor), and 2-DG (glycolytic inhibitor) were sequentially injected into each well at indicated time points. Data were assessed by Seahorse XF-96 Wave software. ECAR is shown in mpH/minute.

Mitochondrial stress test

Oxygen consumption rate (OCR) was examined using the Seahorse XF Cell Mito Stress Test Kit. Firstly, FCCP (reversible inhibitor of oxidative phosphorylation) was divided into 0 μM, 0.125 μM, 0.25 μM, 0.5 μM, 1.0 μM, 2.0 μM to determine the optimum dosing concentration. After baseline measurements, oligomycin, carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazone (FCCP), antimycin A (mitochondrial complex I inhibitor), and rotenone (mitochondrial complex III inhibitor) were sequentially injected into each well at indicated time points. Data were assessed by Seahorse XF-96 Wave software. OCR is shown in pmols/minute.

Targeted metabolomics-energy metabolite analysis

1 × 10^7 cells of NC and sh2 cell samples were collected and pelleted by centrifugation. The cell pellet was added with 200 μL pre-cooled water. After homogenization, the cells were added with 800 μL methanol/acetonitrile (2:2, v/v), mixed by vortex, and soaked in the ice bath for 60 min in ice. The precipitated protein was incubated at 20°C for 1 hour and centrifuged at 14,000 g for 10 min at 4°C. The supernatant was taken and lyophilized. For mass spectrometry, The protein was reconstituted in 100 uL of aceonitrile-water solution (1:1, v/v), then centrifuged at 14,000 g for 15 min at 4°C, and the injection was analyzed. The samples were separated using a Vanquish Ultra High-Pressure Liquid Chromatograph (UHPLC, Thermo Fisher Scientific). Mass spectrometry was performed in positive/negative ion mode using a TSQVantageTM mass spectrometer (Thermo Fisher Scientific).

Statistical analysis

Student's t-test and ANOVA were performed using GraphPad Prism 6.0. The difference was considered significant at $P<0.05$.

Results

Compared to normal healthy skin tissues, malignant melanoma tissues expressed significantly higher BCAT1 levels in humans and mice

Firstly, we asked whether BCAT1 was overexpressed in human malignant melanoma tissues. To answer this question, we analyzed BCAT1 expression in malignant melanoma tissues from 183 melanoma patients and 16 matched control skin tissues using a tissue array kit as described in Materials and Methods. Our data showed that BCAT1 expression was significantly higher in malignant melanoma tissues than the control skin tissues (**Figure 1A** and **1B**).

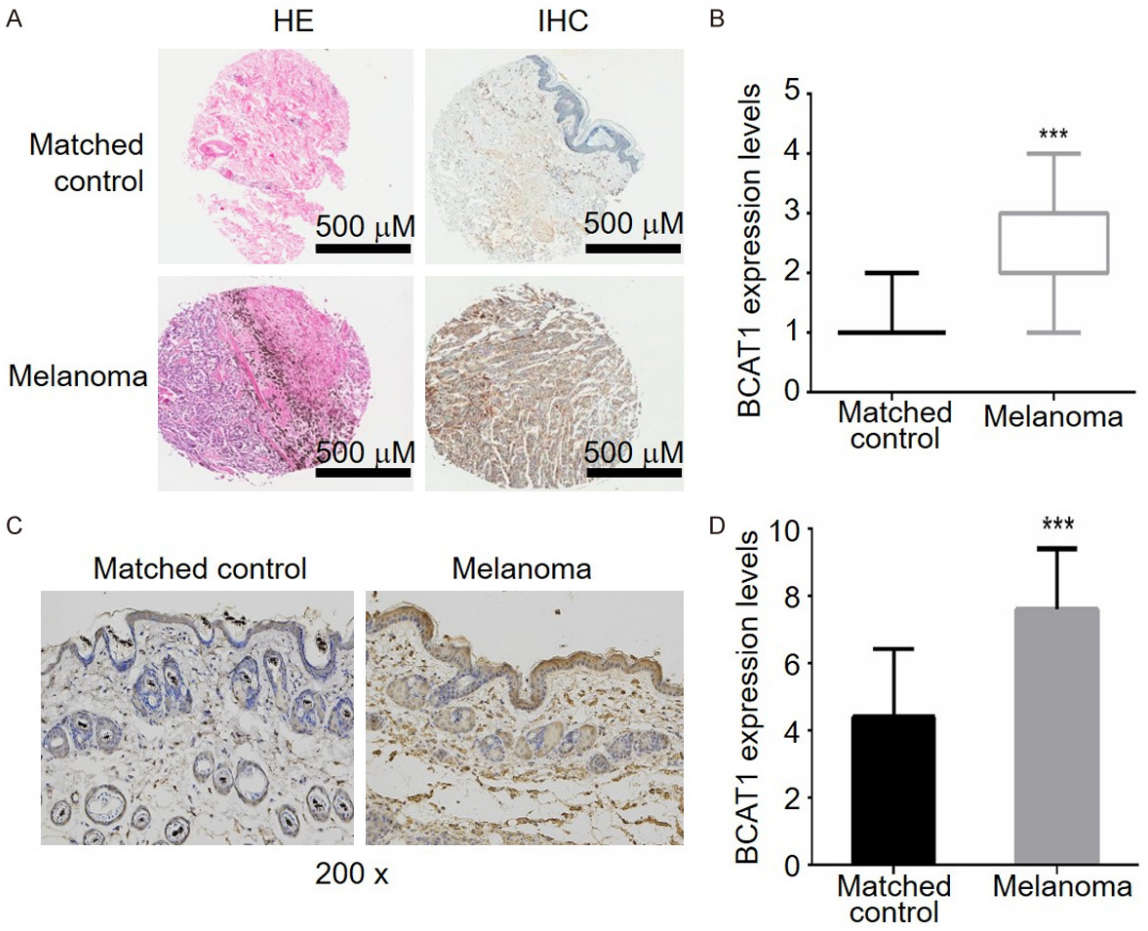

Figure 1. Compared to normal healthy skin tissues, malignant melanoma tissues expressed significantly higher BCAT1 levels in both humans and mice. (A) Representative images of HE (left panel) and BCAT1 immunohistochemistry (right panel) staining of human melanoma and matched control tissues. (B) One hundred eighty-three human melanoma tissues and 16 matched control tissues were analyzed for BCAT1 expression by immunohistochemistry. Data were means ± SEM. ***P<0.001. t-test. (C) BCAT1 expression in mouse B16 melanoma and matched control tissues were analyzed by immunohistochemistry. Representative images were shown. (D) Cumulative data from (C). Data were means ± SEM. ***P<0.001. t-test. N=5.

Additionally, we analyzed BCAT1 expression in malignant B16 melanoma tissues because B16 is a highly aggressive metastatic malignant melanoma in mice [14, 15]. Consistent with the findings in human malignant melanoma patients, mouse malignant B16 melanoma tissues, compared to the control skin tissues, expressed significantly higher levels of BCAT1 (**Figure 1C** and **1D**).

BCAT1 knockdown inhibited the proliferation of malignant B16 melanoma cells

To investigate the role of BCAT1 in malignant melanoma, we determined to select a suitable melanoma cell line for further analysis. To do this, we screened BCAT1 protein expression in mouse (B16, B16-F10, and Melan-α) and human (A375, A2508, and M14) malignant melanoma cell lines. Our data showed that most of these malignant melanoma cell lines displayed overexpression of BCAT1 protein (**Figure 2A** and **2B**). B16 and B16-F10 cells expressed much higher BCAT1 levels among the cell lines tested than other cell lines. We hence decided to study the B16 cell line further.

To determine BCAT1 function in B16 cells, we decided to knockdown BCAT1 using small hairpin RNA (shRNA) as described in Materials and Methods. Puromycin was used to select positively transduced cells. Additionally, because the shRNAs also expressed the green fluorescence protein (GFP), positively transduced cells can be visualized under a fluorescence microscope. Our data showed that the three

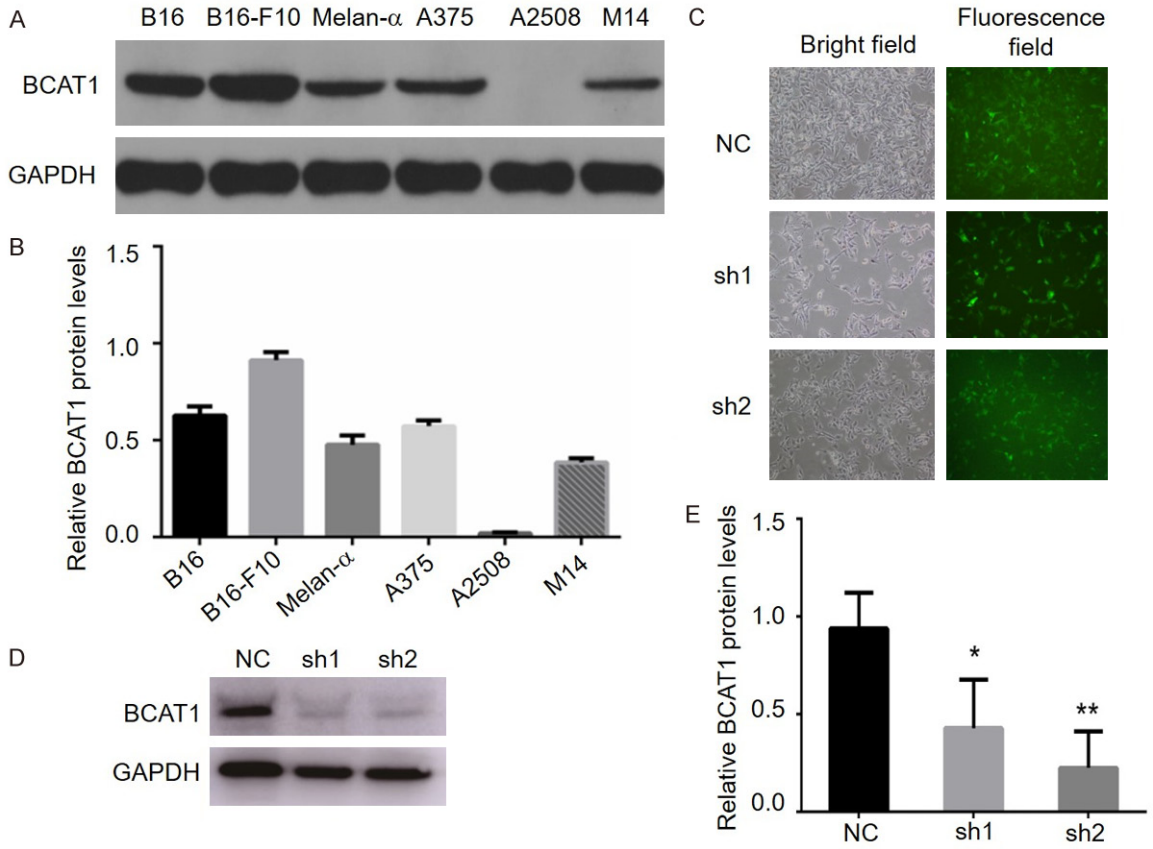

Figure 2. B16 melanoma cells expressed a high level of BCAT1 that could be knocked down using shRNA. (A) The expression of BCAT1 in six melanoma cell lines was measured by Western Blot. Representative images were shown. GAPDH (Glyceraldehyde 3-phosphate dehydrogenase): a housekeeping gene. (B) Quantitative data of (A). (C) B16 cells were transduced with a control shRNA (NC) or a BCAT1 shRNA (sh1 or sh2). Transduction efficiency was examined under a fluorescence microscope. (D) The BCAT1 protein expression in the transduced B16 cells in (C) was analyzed by Western Blot. (E) Quantitative data from (D). Data were means ± SEM. *P<0.05; **P<0.01. t-test.

B16 cell lines' transduction rates were close to 100% (GFP^+) (**Figure 2C**). Furthermore, the two shRNA (sh1 and sh2), when compared to the control shRNA (NC), significantly reduced BCAT1 expression in B16 cells (**Figure 2D** and **2E**).

We then investigated the effects of BCAT1 knockdown on the proliferation of B16 cells. Our data showed that BCAT1 knockdown significantly suppressed the proliferation of B16 cells (**Figure 3A**). Consistent with the decreased proliferation, colony formation was also reduced considerably in BCAT1-knockdown B16 cells (**Figure 3B** and **3C**).

BCAT1 knockdown led to G2/M cell cycle arrest in malignant B16 melanoma cells

Previous data suggest that BCAT1 regulates the cell cycle. It was shown that BCAT1 knockdown led to cell cycle arrest at the G1 phase in glioblastoma [4] and the S phase in ovarian cancer [5]. We, therefore, studied cell cycle distribution in BCAT1-knockdown B16 cells. Our data showed that BCAT1 knockdown significantly increased cell numbers at the G2/M phase (G2/M arrest) (**Figure 4**). Hence, our data suggest that BCAT1 is also essential for cell cycle progression in malignant B16 melanoma cells. Combined with our findings in the proliferation experiment (**Figure 3**), our data demonstrate that BCAT1 is crucial for malignant melanoma cells' proliferation.

BCAT1 knockdown decreased the migration ability of malignant B16 melanoma cells

We next asked whether BCAT1 played a role in the migration of B16 cells because migration is essential for melanoma metastasis. We used a

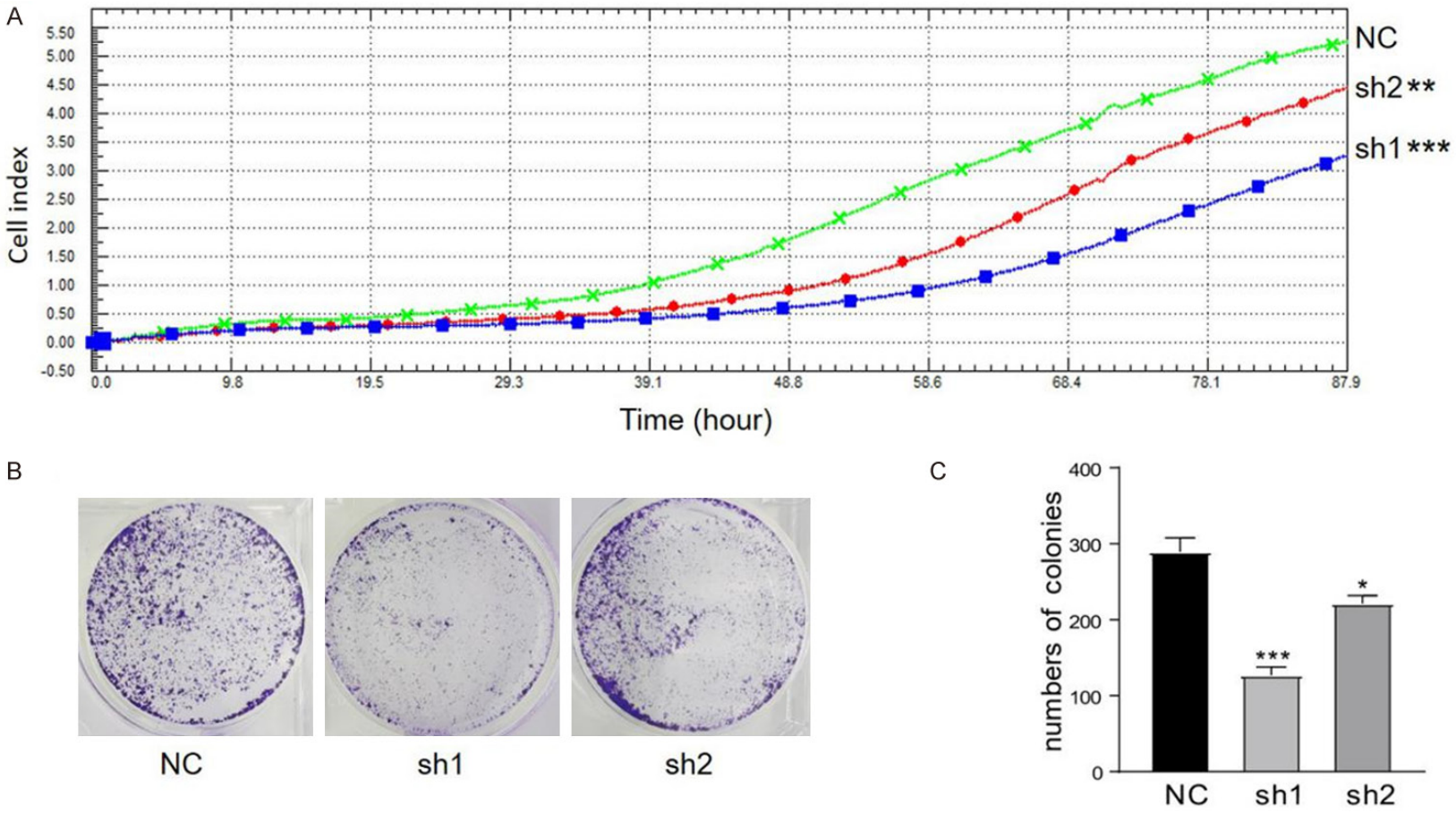

Figure 3. BCAT1 knockdown inhibited the proliferation of malignant B16 melanoma cells. (A) BCAT1 in B16 melanoma cells was knocked down, as shown in **Figure 2**. The proliferation (cell index) of the BCAT1-knockdown (sh1 and sh2) and control (NC) B16 cells was examined using the xCELLigence Real-Time Cell Analyzer as described in Materials and Methods. Data showed cell index over time in cultures. **P<0.01. ***P<0.001. One-way ANOVA test. (B) Colony formation of the BCAT1-knockdown (sh1 and sh2) and control (NC) B16 cells was determined. Representative images were shown. (C) Cumulative data from (B). *P<0.05. ***P<0.001. One-way ANOVA test. Where applicable, data were presented as means ± SEM.

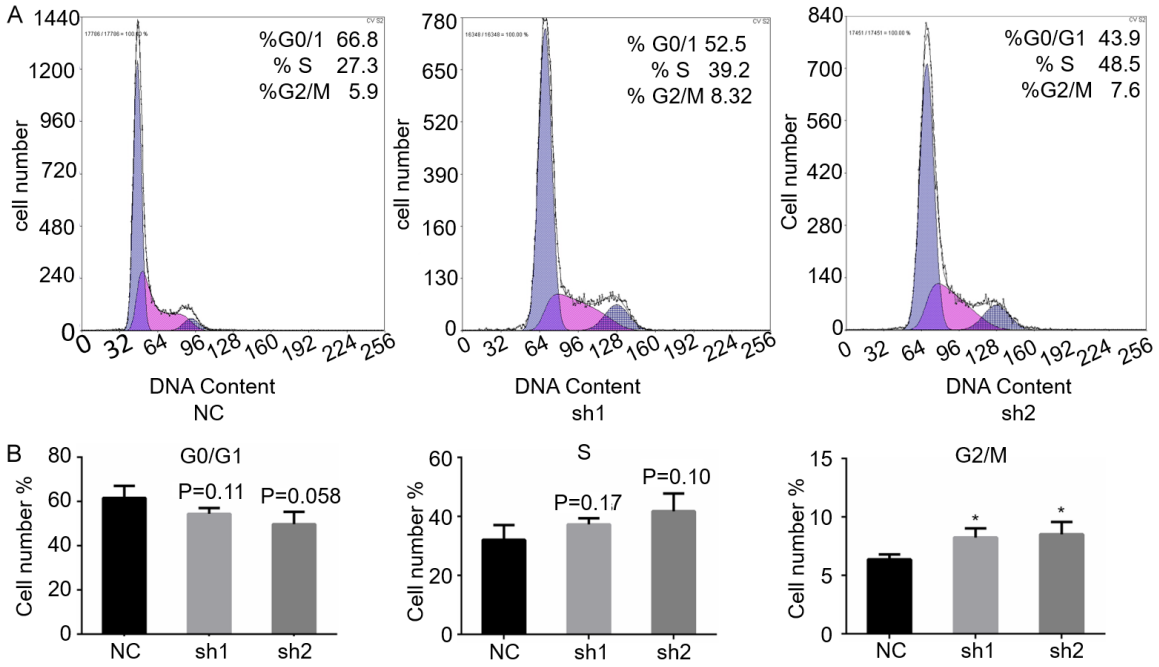

Figure 4. BCAT1 knockdown led to G2/M arrest in malignant B16 melanoma cells. (A) BCAT1 in B16 melanoma cells was knocked down as shown in **Figure 2**. Cell cycle profiles in the BCAT1-knockdown (sh1 and sh2) and control (NC) B16 cells were analyzed by FACS. Data showed representative FACS plots. (B) Cumulative data from (A) showed percentages of cells in G0/G1, S, and G2/M stages. *P<0.05. ANOVA test.

wound-healing assay for evaluating B16 cell migration. Our data showed that BCAT1-knockdown B16 cells displayed significantly reduced migration ability than the control cells (**Figure 5A** and **5B**).

Previous reports have demonstrated that the loss of E-cadherin [16] and the overexpression of vimentin [17] are associated with malignant melanoma metastasis. We, therefore, investigated the effects of BCAT1 knockdown on the expression of E-cadherin and vimentin. Our data showed that BCAT1 knockdown significantly increased the E-cadherin expression while decreased vimentin expression (**Figure 5C** and **5D**). Hence, our data suggest that BCAT1 promotes malignant melanoma metastasis by downregulating the E-cadherin expression and upregulating the vimentin expression.

BCAT1 knockdown suppressed oxidative phosphorylation but enhanced glycolysis in malignant B16 melanoma cells

Cancer growth requires energy. Energy can be produced by either oxidative phosphorylation or glycolysis. To understand the energy sources that BCAT1 used to promote the proliferation and migration of malignant B16 melanoma cells, we investigated the effects of BCAT1 knockdown on oxidative phosphorylation and glycolysis. Our data showed that BCAT1 knockdown significantly suppressed oxidative phosphorylation (**Figure 6**). Additionally, BCAT1 knockdown significantly enhanced glycolysis (**Figure 7**). Therefore, our data suggest that BCAT1 promotes the proliferation and migration of malignant B16 melanoma cells by augmenting oxidative phosphorylation.

We further investigated the molecules involved in cell energy metabolism. Our data showed that BCAT1 knockdown significantly reduced the amount of the molecules involved in oxidative phosphorylation. The downregulated molecules included acetyl coenzyme A, adenosine, flavin mononucleotide (FMN), nicotinamide adenine dinucleotide (NAD), cytochrome c oxidase IV (COX IV), voltage-dependent anion channel (VDAC), pyruvate dehydrogenase (PDH), citrate synthase (CS), and succinate dehydrogenase complex subunit A (SDHA) (**Figure 8**). Therefore, the molecular analysis of cell energy metabolism supports that BCAT1 promotes oxidative phosphorylation in malignant B16 melanoma.

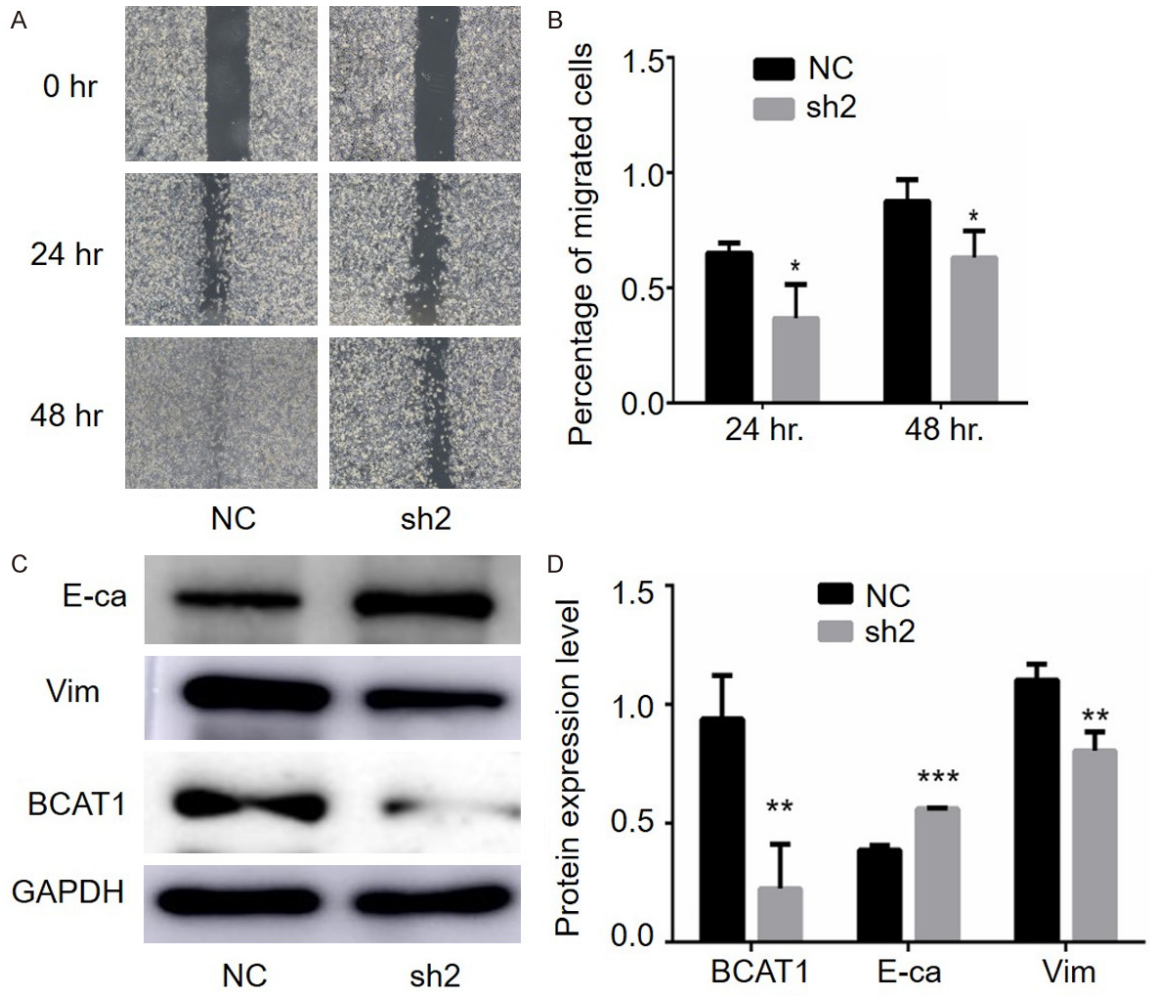

Figure 5. BCAT1 knockdown decreased the migration ability of malignant B16 melanoma cells. (A) BCAT1 in B16 melanoma cells was knocked down as shown in **Figure 2**. The migration ability of the BCAT1-knockdown (sh2) and control (NC) B16 cells was analyzed in vitro using the wound healing assay described in Materials and Methods. Representative images at 0, 24, and 48 hours were shown. (B) Cumulative data of the percentages of migrated cells in (A) were shown. *P<0.05. t-test. Data were means ± SEM. (C) The expressions of E-cadherin (E-ca), vimentin (Vim), and BCAT1 were determined using Western Blot. Representative images were shown. (D) Cumulative data of protein expression levels in (C) were shown. **P<0.01, ***P<0.001. t-test. Data were means ± SEM.

Discussion

Recent data have demonstrated that BCAT1 is overexpressed in a broad range of malignancies. The malignancies that carry overexpressed BCAT1 include glioblastoma carrying wild-type isocitrate dehydrogenase 1 [4], non-small cell lung carcinoma [12], epithelial ovarian cancer [5], myeloid leukemia [6], urothelial carcinomas [18], hepatocellular carcinoma [19], and gastric cancer [9] among others. A caveat is that not all cancers carry overexpressed BCAT1. For example, although the glioblastomas carrying wild-type isocitrate dehydrogenase 1 had overexpressed BCAT1, those with mutant isocitrate dehydrogenase 1 showed significantly decreased BCAT1 expression [4]. Another example was that, although both non-small cell lung carcinoma and pancreatic ductal adenocarcinoma were driven by Kras activation, BCAT1 expression was increased in non-small cell lung carcinoma but decreased in pancreatic ductal adenocarcinoma [12]. For the first time, our data showed that BCAT1 was overexpressed in malignant melanoma in both humans and mice (**Figure 1**), suggesting a potential role of BCAT1 in the progression of malignant melanoma.

A general finding from previous studies was that the overexpressed BCAT1 enhanced tumor growth and invasiveness [4-6, 9, 18, 19].

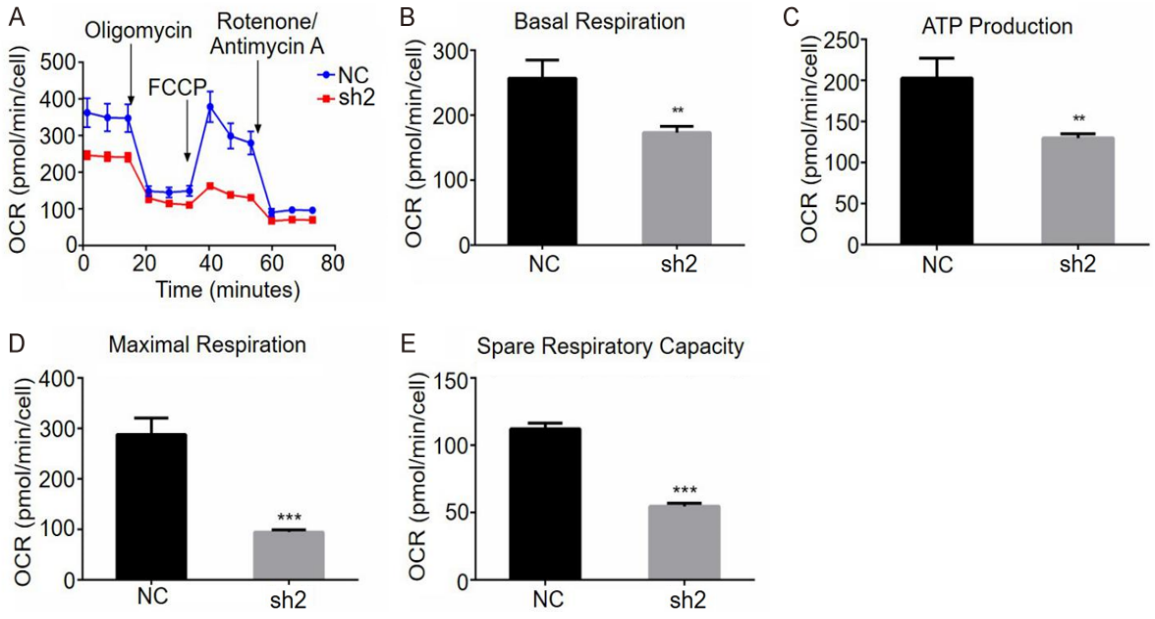

Figure 6. BCAT1 knockdown suppressed oxidative phosphorylation in malignant B16 melanoma cells. A. BCAT1 in B16 melanoma cells was knocked down as shown in **Figure 2**. The oxidative phosphorylation, measured as oxygen consumption rate (OCR), in BCAT1-knockdown (sh2) and control (NC) B16 cells were examined using the Agilent Seahorse XF Cell Mito Stress Test as described in Materials and Methods. Data showed the OCR profiles after the addition of modulators, i.e., oligomycin, carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazone (FCCP), and rotenone/antimycin A. B. Quantitative data of basal respiration. C. Quantitative data of ATP production. D. Quantitative data of maximal respiration. E. Quantitative data of spare respiratory capacity. Data were presented as means ± SEM. **P<0.01, ***P<0.001. Student's t test.

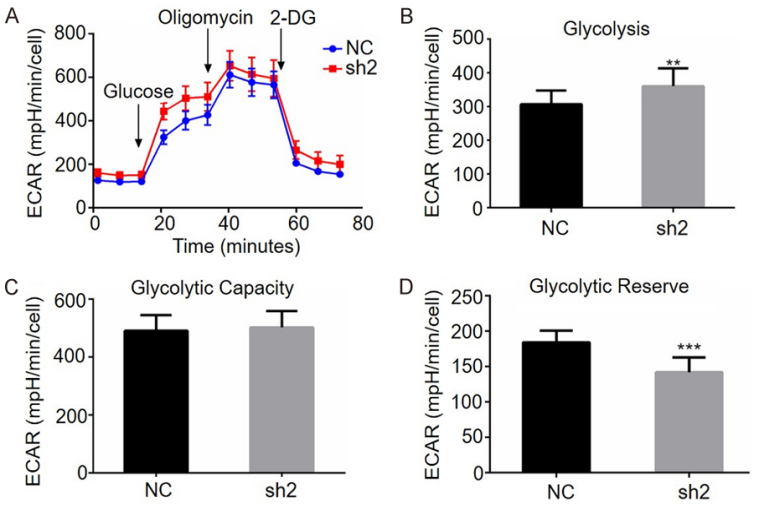

Figure 7. BCAT1 knockdown enhanced glycolysis in malignant B16 melanoma cells. A. BCAT1 in B16 melanoma cells was knocked down, as shown in **Figure 2**. Glycolysis, measured as extracellular acidification rate (ECAR), in BCAT1-knockdown (sh2) and control (NC) B16 cells were examined using the Agilent Seahorse XF Glycolysis Test as described in Materials and Methods. Data showed the ECAR profiles after the addition of modulators, i.e., glucose, oligomycin, and 2-deoxy-glucose (2DG). B. Quantitative data of glycolysis. C. Quantitative data of glycolytic capacity. D. Quantitative data of glycolytic reserve. Data were presented as means ± SEM. **P<0.01; ***P<0.001. Student's t test.

Consistent with these previous reports, our BCAT1 knockdown data suggest that BCAT1 promotes malignant melanoma cells' proliferation and migration (**Figures 3**-**5**). Therefore, our data indicate that BACT1 is a potential therapeutic target for malignant melanoma. However, one should be cautious if BCAT1 inhibitors are used to treat malignant melanoma because activated immune cells also use similar metabolic pathways to generate energy [20]. Because BCAT1 expression in normal healthy tissues is restricted to the brain, ovary, and placenta [7], the overexpressed BCAT1 in malignant melanoma cells can be potentially targeted through immunotherapies.

The importance of BCAT1 for malignant melanoma progres-

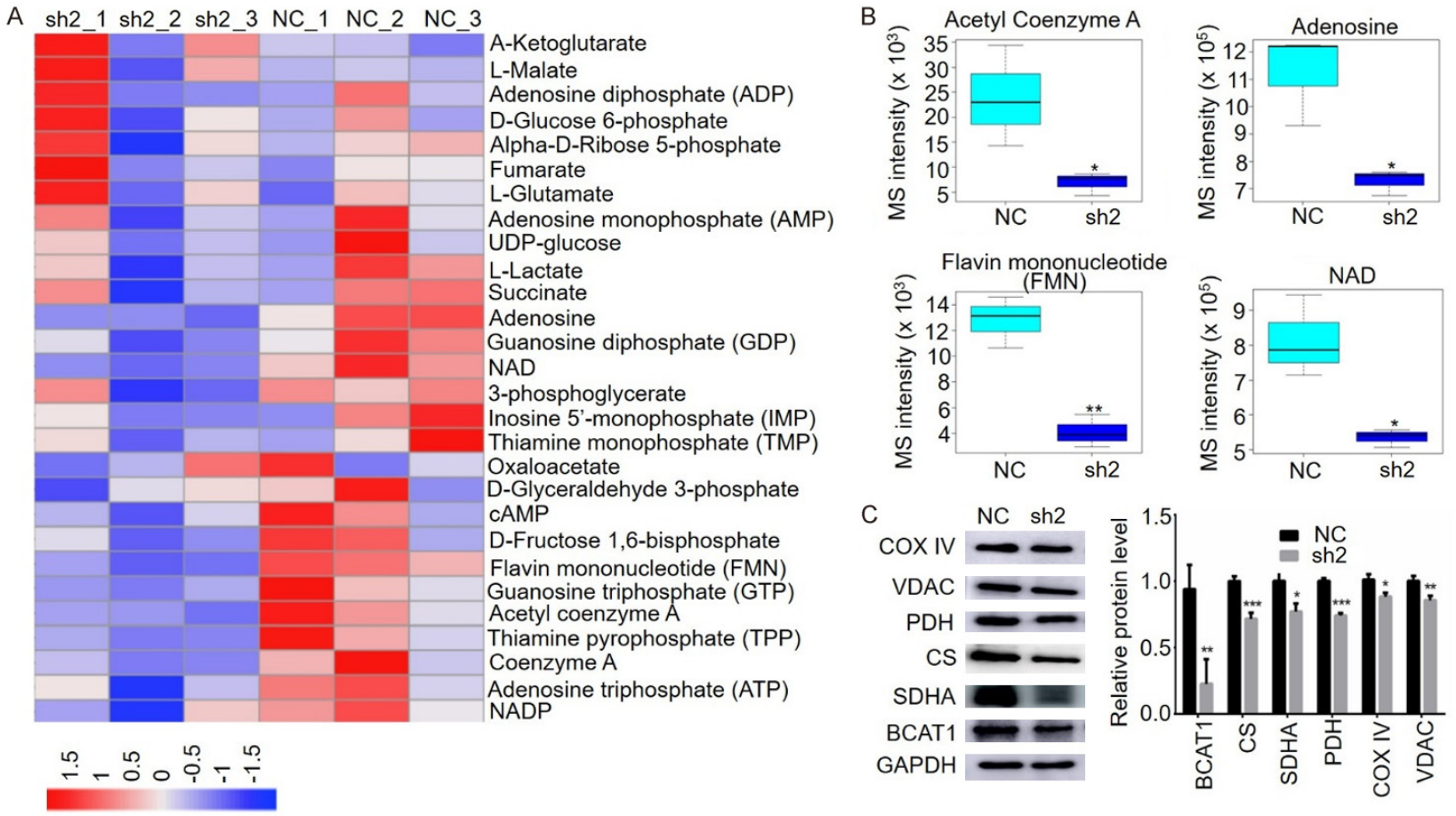

Figure 8. BCAT1 knockdown significantly reduced the amount of molecules associated with oxidative phosphorylation. (A) Common metabolites associated with cell energy metabolism were analyzed as described in Materials and Methods. Heat map showed quantities of the metabolites from three BCAT1-knockdown (sh2_1, sh2_2, and sh2_3) and three control samples (NC_1, NC_2, and NC_3). (B) Significantly altered metabolites in (A) included acetyl coenzyme A, adenosine, flavin mononucleotide (FMN), and nicotinamide adenine dinucleotide (NAD) were displayed using box-plot. (C) Western blot analysis of the molecules associated with oxidative phosphorylation, i.e., cytochrome c oxidase IV (COX IV), voltage-dependent anion channel (VDAC), pyruvate dehydrogenase (PDH), citrate synthase (CS), and succinate dehydrogenase complex subunit A (SDHA) were analyzed in BCAT1-knockdown (sh2) and control (NC) B16 melanomas. Left panel: representative western blot data were shown. Right panel: cumulative data. Where applicable, data were means ± SEM. *P<0.05, **P<0.01, ***P<0.001. Student's t test.

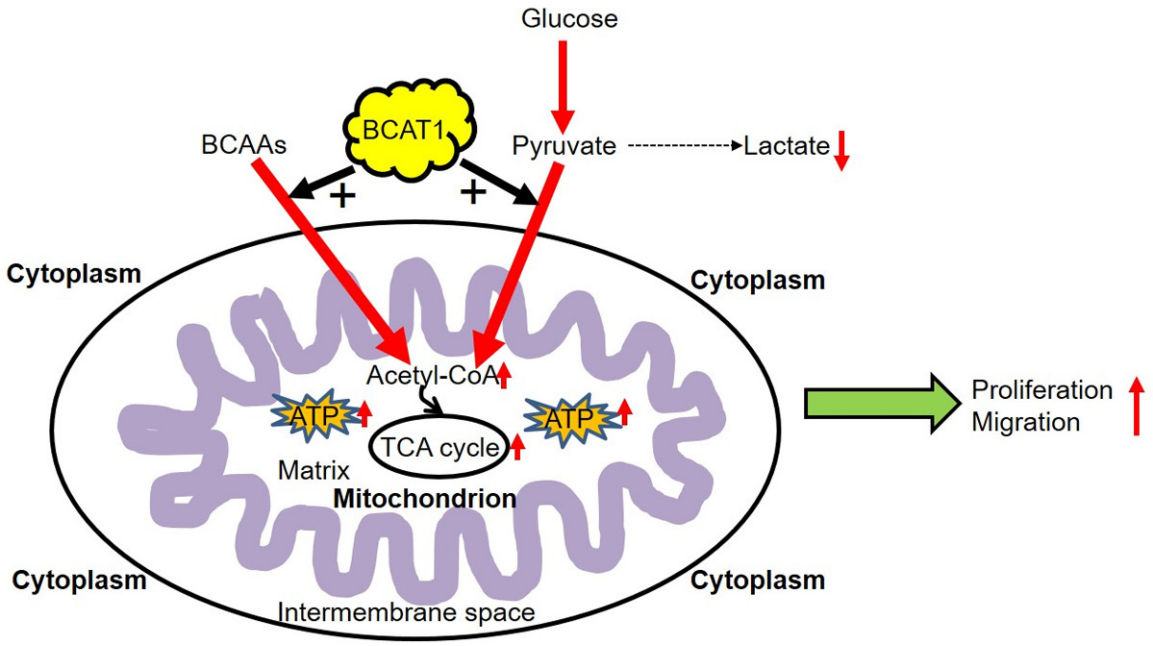

Figure 9. A model for the potential functions of the overexpressed BCAT1 in malignant melanomas. BCAT1 promotes oxidative phosphorylation by increasing acetyl-CoA production. The acetyl CoA production is enhanced mainly through two mechanisms. One mechanism is that BCAT1 catabolizes BCAAs to generate acetyl CoA. The other is that BCAT1 reduces the cellular amount of BCAAs and releases the BCAA-mediated inhibition of pyruvate oxidation, which increases the conversion of pyruvate into acetyl CoA. The acetyl CoA then enters into the tricarboxylic acid (TCA) cycle to increase ATP production, thereby augmenting oxidative phosphorylation. Consequently, the proliferation and migration of malignant melanoma cells are enhanced.

sion is not surprising because BCAT1 is a direct target of c-Myc that is a proto-oncogene [21]. In this regard, c-Myc is also overexpressed in malignant melanoma cells and essential for metastasis [22, 23]. Therefore, one can extrapolate that BCAT1 is one of the downstream mechanisms by which c-Myc enhances melanoma progression.

Interestingly, our data showed that BCAT1 knockdown decreased oxidative phosphorylation (**Figures 6** and **8**) but increased glycolysis (**Figure 7**). The data suggest that BCAT1 promotes melanoma cell proliferation and migration by enhancing oxidative phosphorylation. Our data support the current view that mitochondria are essential for cancer initiation, progression, and response to other treatments [24, 25].

Based on our findings, we now propose a model for the potential functions of the overexpressed BCAT1 in malignant melanomas (**Figure 9**). BCAT1 promotes oxidative phosphorylation by increasing acetyl-CoA production. The acetyl CoA production is enhanced mainly through two mechanisms. One mechanism is that BCAT1 catabolizes BCAAs to generate acetyl CoA [26]. The other is that BCAT1 reduces the cellular amount of BCAAs and releases the BCAA-mediated inhibition of pyruvate oxidation [27], which increases the conversion of pyruvate into acetyl CoA. The acetyl CoA then enters into the tricarboxylic acid (TCA) cycle to increase ATP production, thereby augmenting oxidative phosphorylation. Consequently, the proliferation and migration of malignant melanoma cells are enhanced.

A limitation of this study was that all experiments were performed in vitro. The results presented in this article need to be verified in vivo using appropriate animal models. However, our in vitro data laid a solid foundation for future studies on the role of BCAT1 in the progression and treatment of malignant melanoma.

In conclusion, in this study, we, for the first time, reported the BCAT1 overexpression in both human and mouse melanomas. Additionally, we presented evidence that BCAT1 enhanced the proliferation and migration of

malignant melanoma cells. Finally, we showed that BCAT1 enhanced oxidative phosphorylation in malignant melanoma cells. Our findings laid a foundation for the future development of novel BCAT1-based therapies for malignant melanomas.

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Disclosure of conflict of interest

None.

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