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Berberine-induced AMPK activation inhibits the metastatic potential of melanoma cells via reduction of ERK activity and COX-2 protein expression

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ABSTRACT

Berberine is clinically important natural isoquinoline alkaloid that affects various biological functions, such as cell proliferation, migration and survival. The activation of AMP-activated protein kinase (AMPK) regulates tumor cell migration. However, the specific role of AMPK on the metastatic potential of cancer cells remains largely unknown. The present study investigated whether berberine induces AMPK activation and whether this induction directly affects mouse melanoma cell migration, adhesion and invasion. Berberine strongly increased AMPK phosphorylation via reactive oxygen species (ROS) production. 5-Aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR), a well-known AMPK activator, also inhibited tumor cell adhesion and invasion and reduced the expression of epithelial to mesenchymal transition (EMT)-related genes. Knockdown of AMPKα subunits using siRNAs significantly abated the berberine-induced inhibition of tumor cell invasion. Furthermore, berberine inhibited the metastatic potential of melanoma cells through a decrease in ERK activity and protein levels of cyclooxygenase-2 (COX-2) by a berberine-induced AMPK activation. These data were confirmed using specific MEK inhibitor, PD98059, and a COX-2 inhibitor, celecoxib. Berberine- and AICAR-treated groups demonstrated significantly decreased lung metastases in the pulmonary metastasis model *in vivo*. Treatment with berberine also decreased the metastatic potential of A375 human melanoma cells. Collectively, our results suggest that berberine-induced AMPK activation inhibits the metastatic potential of tumor cells through a reduction in the activity of the ERK signaling pathway and COX-2 protein levels.

1. Introduction

Skin cancer is a common form of cancer worldwide; it is especially prevalent among Caucasians and other light-skinned races [1]. Melanoma is the leading cause of death from skin disease because of its propensity to metastasize [2]. Ultraviolet (UV) radiation is a risk factor for the development of skin cancer, including melanoma. Exposure to UV irradiation up-regulates cyclooxygenase-2 (COX-2) expression and increases the production of its primary product, prostaglandin E2 (PGE_2), in the skin [3]. The two major isoforms of COX, COX-1 and COX-2, have distinct physiological functions. COX-1 is constitutively expressed in most tissues, and it is involved in normal physiological functions. COX-2 is an inducible enzyme that is activated by extracellular stimuli, such as UV radiation, hormones, cytokines and tumor promoters [3–5]. COX-2 and its products, prostaglandins (PGE_s), play a central role in multiple events of cancer invasion, metastasis and tumor development [2,6,7].

Many plant-derived agents are potential alternative therapies for invasive cancers [8,9]. Among these agents, berberine, an isoquinoline alkaloid, is found in the rhizome, roots and stem bark of a number of important medicinal plants, the *Berberis* species. Berberine exhibits multiple pharmacological activities, including anti-cancer and anti-diabetes mellitus effects [10–12]. A recent report indicated that berberine inhibits melanoma cell migration, which is an essential step in tumor invasion, by reducing the expression of COX-2, PGE_2 and PGE_2 receptors [2]. Although evidence of the beneficial effects of berberine is expanding, uncertainty of the association between the metastatic potential and the metabolic field remains.

AMP-activated protein kinase (AMPK) is a heterotrimer with a catalytic subunit (α) and two regulatory subunits (β and γ) that primarily functions as an energy sensor [13]. The activation of AMPK inhibits ATP-consuming metabolic pathways but facilitates the activation of energy-producing pathways for cellular protection. AMPK is phosphorylated by upstream kinases, LKB1 and

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calmodulin-dependent protein kinase kinase β (CaMKKβ). Also, several tumor suppressor proteins such as LKB1, tuberous sclerosis complex 2 (TSC2) and p53 involved in the AMPK signal network contribute to the regulation of cell growth and death [14]. Recent studies indicated that the increasing phosphorylation of cytoplasmic linker protein-170 by AMPK regulates cell migration through dynamic microtubule assembly [15]. Moreover, the knockdown of E-cadherin using RNAi led to loss of LKB1 localized to adherens junctions, and AMPK activation in MDCK cells and LKB1–AMPK–S6K axis inhibits the cell migration and invasion in breast cancer cells [16,17]. Berberine strongly induces AMPK activity via reactive oxygen species (ROS) production and LKB1 activation [18], and AMPK is known to play a role as a regulatory factor in cell migration [12,17,19–21]. However, the relationship between AMPK activation and metastatic potential remains controversial.

We investigated the role of AMPK in the berberine-induced inhibitory effects on the metastatic potential of melanoma cells. Berberine increased AMPK activity via ROS production, and its activation performed a critical function in the inhibition of metastatic potential through a reduction in the activity of the ERK signaling pathway and COX-2 protein levels.

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and 2′,7′-dichlorofluorescein diacetate (DCF-DA) were obtained from Gibco/Invitrogen (Carlsbad, CA). Berberine, PD98059, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), *N*-acetylcysteine (NAC), 3-[4,5-dimethylthiazol-2-thiazolyl]-2,5-diphenyltetrazolium bromide (MTT) and fibronectin were obtained from Sigma–Aldrich (St. Louis, MO). 5-Aminoimidazole-4-carboxaminde-1-β-D-ribofuranoside (AICAR), antibodies recognizing the phosphospecific forms of AMPKα-Thr^{172} and ERK (Thr^{202}/Tyr^{204}) and an antibody against ERK were purchased from Cell Signaling Technology (Boston, MA). Antibodies against α-actinin and AMPKα were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). An antibody against COX-2 was purchased from Cayman Chemical (Ann Arbor, MI).

2.2. Mice and cell cultures

B16F10 murine melanoma cells and A375 human melanoma cells (ATCC, Manassas, VA) were maintained in DMEM supplemented with 10% heat-inactivated FBS and 100 U/ml penicillin/100 μg/ml streptomycin (Gibco/Invitrogen) at 37 °C in a 5% CO_2 humidified incubator. For animal experiments, specific pathogen-free female C57BL/6J mice were purchased from the Central Laboratory Animal Inc. (Seoul, Republic of Korea) and maintained in our animal facility for at least 2 weeks prior to use. The mice were housed under specific pathogen-free conditions in a barrier facility with 12 h light–dark cycles. Experimental procedures were performed at 6–8 weeks of age.

2.3. In vitro cell growth and soft agar colony formation assays

For the *in vitro* cell growth analysis, cells were seeded in a six-well plate and treated with 0–40 μM of berberine for 24–72 h. The cells were harvested and counted using the trypan blue exclusion test. For a determination of anchorage-independent cell growth, 1×10^3 melanoma cells were suspended in 1.5 ml of the medium containing 0.3% agar, 10% FBS and 0–40 μM berberine and applied onto pre-solidified 0.5% agar (1.5 ml) in six-well plates. After a 2-week incubation, colonies on soft agar were observed under a phase-contrast microscope and photographed. Colony numbers were assessed visually. The colonies that contained >50 cells and appeared normal were counted.

2.4. Determination of in vitro tumor cell migration and invasion

The *in vitro* migration and invasion assays were performed using a 24-well transwell unit with polycarbonate filters that have a diameter of 6.5 mm and a pore size of 8.0 μm (Corning Costar, Cambridge, MA). For the invasion assay, the lower part of the transwell was filled with DMEM plus 10% FBS as a chemoattractant, and the transwell was coated with 20 μl of a 1:2 mixture of Matrigel:DMEM (Matrigel; BD Biosciences, Bedford, MA), which was assembled as the intervening invasive barrier in a 24-well plate. The cells (5×10^4) were suspended in serum-free DMEM with 20 μM berberine or 1 mM AICAR, added to the upper part of the transwell and incubated for 48 h at 37 °C. The cells that attached to the upper surface of the filter were completely removed by wiping with a cotton swab, and the filters were stained with a 0.2% crystal violet/20% methanol (w/v) solution. The stain was eluted, and absorbance was measured at 540 nm. The migration assay was performed using a transwell culture system without Matrigel coating.

2.5. Wound healing assay

The cells were seeded in six-well plates and incubated for 12 h in starvation medium. The cellular monolayer was wounded with a sterile 200 μl-pipette tip and washed with starvation medium to remove detached cells from the plates. The cells were pretreated with 0–40 μM berberine for 30 min, and then incubated in the presence or absence of 40 ng/ml TPA for 24 or 36 h. The medium was replaced with phosphate-buffered saline, and the cells were photographed using a phase-contrast microscope.

2.6. Adhesion assay

The cells were pretreated with 20 μM berberine or 1 mM AICAR for 24 h and plated (5×10^4 cells/well) in 10 mg/cm^2 fibronectin-coated wells in a 96-well plate followed by one hour's incubation at 37 °C (5% CO_2). Adherent cells were fixed with 3.7% paraformaldehyde for 10 min and stained with a 0.2% crystal violet/20% methanol (w/v) solution. The stain was eluted, and absorbance was measured at 540 nm.

2.7. Measurement of reactive oxygen species (ROS) generation

The cells were treated with 20–40 μM berberine or 5 mM NAC for 1 h and incubated with 10 μM DCF-DA for 30 min. The adherent cells were trypsinized and collected in a 15 ml tube. After washing twice with PBS, the intensity of DCF-DA fluorescence was determined using a FACSCanto^{TM}II flow cytometer (BD Bioscience, San Jose, CA) with an excitation wavelength of 480 nm and an emission wavelength of 530 nm.

2.8. RNA extraction and reverse transcription-PCR (RT-PCR)

Total RNA was extracted from the cells using TRIzol reagent (Invitrogen, Carlsbad, CA) following the manufacturer's instructions and reverse transcribed to complementary DNA using M-MLV reverse transcriptase (Promega, Madison, WI) and oligo(dT) primers. Complementary DNA aliquots of 5 μg RNA were analyzed using semi-quantitative PCR. PCR products were electrophoresed on 1% agarose gels containing ethidium bromide.

2.9. Protein preparation

For the preparation of whole-cell lysates, the cells were lysed on ice in the PRO-PREP™ Protein Extraction Solution (iNtRON Biotechnology, Seoul, Republic of Korea) for 30 min. Supernatant fractions were recovered by centrifugation (14,000 × *g* × 20 min, 4 °C), and the protein concentration was determined using the Bradford protein assay. Proteins on the immunoblots were visualized using an enhanced chemiluminescence system (Amersham Pharmacia Biotech, Piscataway, NJ) and a LAS-3000 or LAS-4000 imaging system (FUJIFILM Corporation, Tokyo, Japan). The band intensity of Western blot data was analyzed by Quantity One software (Bio-Rad Laboratories, Hercules, CA).

2.10. Knockdown of AMPKα1 and 2 subunits using AMPK-siRNA transfection

AMPKα1/2 siRNA (m) (sc-45313) was purchased from Santa Cruz Biotechnology as described previously [22]. Fluorescein-conjugated control siRNA (sc-36869) was used as a negative control and an indicator of transfection efficiency. Transfection of siRNAs was performed according to the manufacturer's instruction.

2.11. Cell apoptosis and viability

Cell apoptosis was assessed using a fluorescence-activated cell sorter (FACS). In brief, cells were pretreated with berberine or AICAR for 24 h and suspended in PBS for 1 h at room temperature. Suspended cells were reseeded in complete medium in a culture plate and incubated for 24 h. Total cells were harvested by trypsinization and washed with PBS. After fixation in 70% ethanol, the cells were resuspended in PBS containing 10 μg/ml propidium iodide. The intensity of fluorescence was determined using a FACSCanto™II flow cytometer. Cell viability was assessed using the MTT assay. The cells were treated with a 5 μg/ml MTT solution and incubated for 3 h. The cells were dissolved in DMSO, and the absorbance was measured at 570 nm.

2.12. In vivo metastasis assay

The experimental metastatic potential of B16F10 cells was measured using the lung colonization assay. In brief, B16F10 cells (3×10^5 cells/0.2 ml PBS) were pretreated with 20 μM berberine or 1 mM AICAR for 24 h and injected into the tail vein of C57BL/6J mice. The mice were euthanized 26 days later and metastatic colonies on the lung surface were quantified macroscopically. For H&E staining, lung tissues from mice were embedded in a frozen section compound (Leica Microsystems, Bannockburn, IL), frozen rapidly in liquid nitrogen. The cryosections of frozen tissues to a thickness of 16 μm were prepared at −20 °C and allowed to dry at 37 °C for 10 min. The slides were fixed in acetone for 10 min, air-dried for 30 min and then stained for 5 min in hematoxylin (Sigma). After washing with distilled water, slides were submerged in methanol with 1% HCl and dipped in water with 0.1% NH_4OH. The tissues were stained in alcoholic eosin (Sigma) for 2 min,

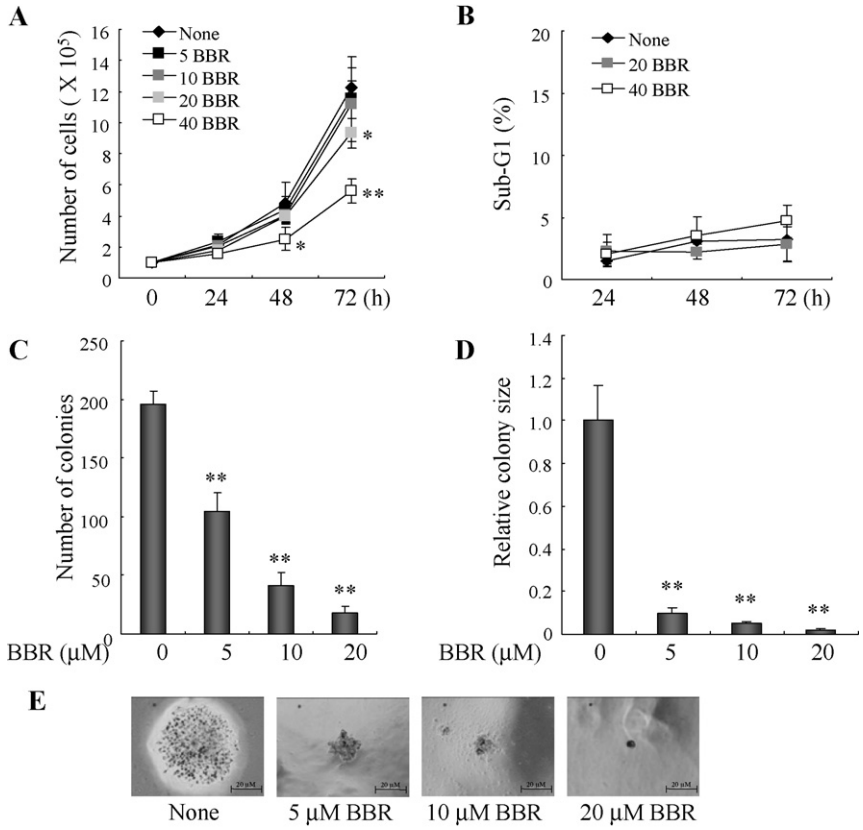

Fig. 1. Berberine inhibits the anchorage-independent growth of B16F10 cells. (A) B16F10 cells were treated with berberine (BBR) for 24–72 h at concentrations from 5 to 40 μM, and the cells were quantified. (B) The cells were treated with the indicated concentrations of berberine for 24–48 h and analyzed for apoptosis using FACS analysis, and the percentages of sub-G1 cells are shown. (C) B16F10 cells (1×10^3) were suspended in 1.5 ml of medium containing 0.3% agar, 10% FBS and the indicated concentrations of berberine and applied onto pre-solidified 0.5% agar (1.5 ml) with the indicated concentrations of berberine in a six-well culture plate. After incubation for 2 weeks, the colony numbers on soft agar were assessed visually. (D) The diameter of 15–20 representative colonies was measured. (E) The colonies were quantified visually under a phase-contrast microscope. $^*P < 0.05$ and $^{**}P < 0.01$, compared with untreated cells (1-way ANOVA test with Newman–Keuls multiple comparison test).

followed by dehydration with 95 and 100% ethanol for 2 min and then mounted. The tissues were photographed using a phase-contrast microscope.

2.13. Statistical analysis

The results are presented as the means ± SD. All experiments were repeated at least three times, and the data were analyzed for statistical significance using GraphPad Prism 5 software (GraphPad Software, La Jolla, CA). Significant differences were analyzed using 1-way ANOVA test followed by Newman–Keuls multiple comparison test for over 3 groups or unpaired *t*-test for 2 groups. *P* values of <0.05 were considered significant.

3. Results

3.1. Berberine treatment decreases the anchorage-independent growth of B16F10 cells

The colony formation assay examined the effect of berberine on the phenotypic changes of B16F10 melanoma cells. The ability of cells to form colonies in a semisolid medium is a marker of anchorage independence, which is positively correlated with metastatic potential [23]. As shown in Fig. 1A, berberine did not significantly affect the growth of B16F10 melanoma cells for 48 h at concentrations from 5 to 20 μM, but reduced their growth after 72 h at concentrations from 20 to 40 μM. However, it did not significantly affect cell apoptosis for 24–72 h at concentrations from 20 to 40 μM (Fig. 1B). Untreated B16F10 cells formed sizable colonies and exhibited rapid proliferation in soft agar. However, berberine-treated B16F10 cells showed a significant dose-dependent decrease in the ability to form soft agar colonies, including fewer sizable colonies and a reduction in colony size (Fig. 1C–E). Moreover, cells that were treated with 40 μM of berberine rarely formed soft agar colonies (data not shown).

3.2. Berberine inhibits the migration, adhesion and invasion of B16F10 melanoma cells

In vitro migration, wound healing, adhesion and invasion assays were performed to further evaluate the effect of berberine on metastatic activity. Berberine treatment of B16F10 cells for 48 h resulted in a significant reduction in migration (Fig. 2A). Similar results were noted in the wound healing assay (Fig. 2B). Cell adhesion assays were performed using fibronectin as an adhesion substrate. Berberine-treated B16F10 cells showed a significant reduction in adhesion to fibronectin compared with untreated controls (Fig. 2C). Next, B16F10 cells were treated with and without berberine for 48 h to determine whether berberine also inhibits melanoma cell invasion. The treatment of B16F10 cells with 20 μM of berberine decreased invasive activity (Fig. 2D). Collectively, these results showed that berberine inhibited the migration, adhesion and invasion of B16F10 melanoma cells.

3.3. AMPK activation is critical for the berberine-induced inhibition of adhesion and invasion of B16F10 cells

The activation of AMPK by adiponectin inhibits cell adhesion, migration and invasion via the modulation of the LKB1–AMPK–S6K axis [17]. We investigated the association of the metastatic

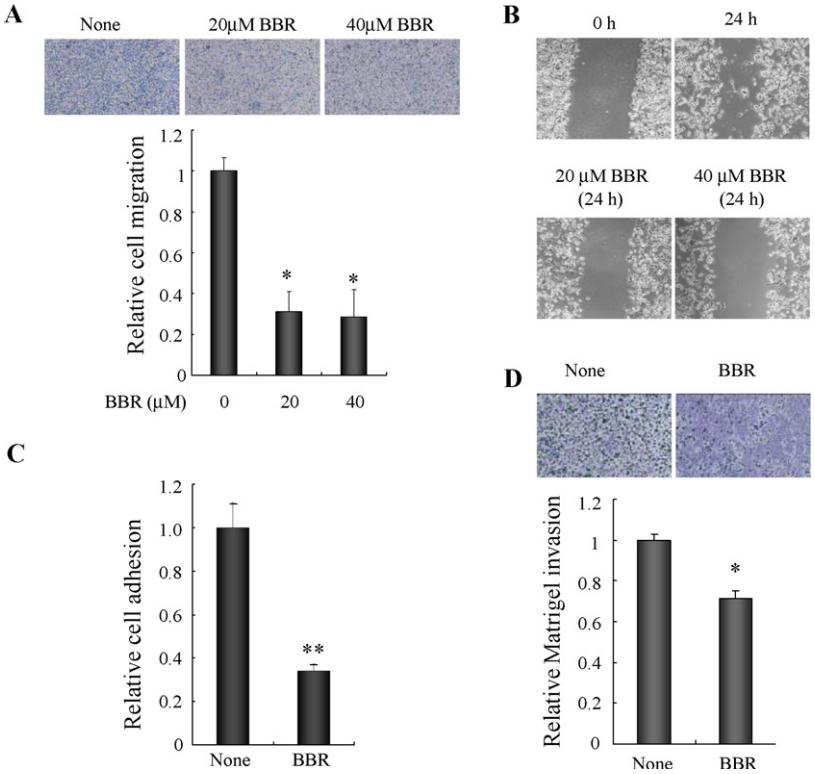

Fig. 2. Effects of berberine on the migration, adhesion and invasion of B16F10 cells. (A) A fixed number of cells were suspended in serum-free medium with berberine and added to the upper part of the transwell; the media compartment of the lower chamber contained 10% FBS. After incubation for 48 h, cells that migrated to the lower surface of the membrane were stained with 0.2% crystal violet and observed using phase-contrast microscope. The stain was eluted, and absorbance was measured at 540 nm. (B) B16F10 cells were grown to confluence, scratched with a pipette tip and photographed immediately after scratching (0 h). After incubation for 24 h, the plates were observed with a phase-contrast microscope. (C) B16F10 cells were pretreated with 20 μM berberine for 24 h and plated in 10 $\mu g/cm^2$ fibronectin-coated wells in a 24-well plate for 1 h. The adherent cells were measured using crystal violet staining. (D) A fixed number of cells were plated with berberine (20 μM) onto the upper part of the Matrigel-coated transwell chamber. After incubation for 48 h, invasive cells were characterized as described in A. $^{*}P < 0.05$ and $^{**}P < 0.01$, compared with untreated cells (unpaired *t*-test).

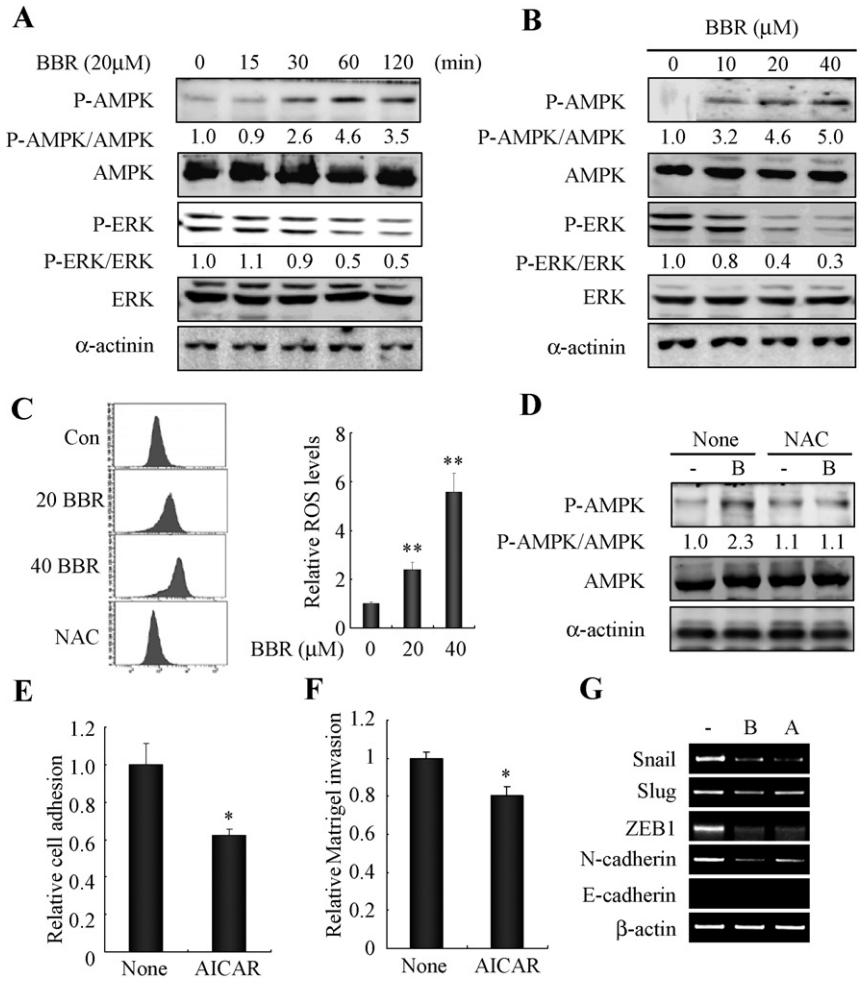

Fig. 3. AMPK activation by berberine affects the adhesion and invasion of B16F10 cells. (A, B) B16F10 cells were exposed to berberine (20 μM) for the indicated time or exposed to the indicated concentrations of berberine for 30 min. Under these conditions, the total cell extracts were subjected to Western blot assays using anti-phosphospecific AMPKα (P-AMPK), anti-AMPKα (AMPK), anti-phosphospecific ERK (P-ERK), anti-ERK (ERK) and anti-α-actinin antibodies. (C) After incubation with berberine for 1 h, ROS levels in B16F10 cells were determined using a DCF-DA dye. The data are expressed as the means ± S.E. for 3 determinations in triplicate. (D) After exposure of B16F10 cells to berberine (20 μM, 1 h) in the presence of 5 mM *N*-acetylcysteine (NAC), the phosphorylation level of AMPK was measured. (E) B16F10 cells were pretreated with 1 mM AICAR for 24 h and plated in 10 μg/cm^2 fibronectin-coated wells in a 24-well plate for 1 h. The adherent cells were measured using crystal violet staining. (F) A fixed number of cells were plated with berberine (20 μM) onto the upper part of the Matrigel-coated transwell chamber. After incubation for 48 h, invasive cells were characterized as described in Fig. 2A. (G) B16F10 cells were exposed to 20 μM berberine or 1 mM AICAR for 18 h. The mRNA levels of Snail, Slug, Twist, ZEB1, SIP1 and β-actin were determined via RT-PCR. $^*P < 0.05$ and $^{**}P < 0.01$, compared with untreated cells (unpaired *t*-test).

potential of melanoma cells and AMPK activation on the inhibition of metastatic potential by berberine treatment. Berberine rapidly activated AMPK in B16F10 cells in a time- and dose-dependent manner (Fig. 3A and B), which was evidenced by the enhanced phosphorylation levels at Thr^{172} in the active site of AMPKα [24]. In contrast, berberine decreased the phosphorylation level of ERK in B16F10 cells. The levels of ROS were measured to elucidate the mechanisms of berberine-induced AMPK activation because AMPK is sensitively activated by ROS [24,25]. Berberine increased ROS generation in B16F10 cells in a dose-dependent manner (Fig. 3C), and NAC, an anti-oxidant, blocked berberine-induced AMPK activation (Fig. 3D). These results indicated that ROS is a mediator of AMPK activation by berberine. The effect of AICAR, an AMPK activator, on B16F10 cell adhesion and invasion was examined to determine whether AMPK activation is necessary for the inhibition of metastatic potential. Treatment with AICAR inhibited adhesion and invasion of B16F10 cells (Fig. 3E and F). In addition, we investigated whether the phenotypic changes are associated with mRNA expression level of epithelial to mesenchymal transition (EMT) markers because increased motility and invasiveness of cancer cells are often associated with EMT [26]. In fact, AMPK activators markedly induced the reduction in the mRNA level of some EMT-related genes (e.g., Snail, Slug, ZEB1 and N-cadherin) (Fig. 3G).

3.4. AMPK-knockdown attenuates the berberine-induced inhibitory effects on ERK activation and invasion of B16F10 cells

The effects of AMPKα1 and 2 subunit knockdown using specific siRNAs on B16F10 cell invasion were investigated to confirm the function of AMPK activity in berberine-induced inhibition of tumor cell invasion. B16F10 cells were transfected with control siRNA or AMPKα-specific siRNA for 48 h and treated with berberine. Western blot analysis revealed that the knockdown of AMPKα subunits slightly increased p-ERK levels and rescued the reduction of p-ERK levels by berberine (Fig. 4A). Matrigel invasion assays also demonstrated that the knockdown of AMPKα subunits significantly abated the berberine-induced inhibition of tumor cell invasion, but AMPKα knockdown did not influence basal invasion potential (Fig. 4B). These results suggested that berberine-induced

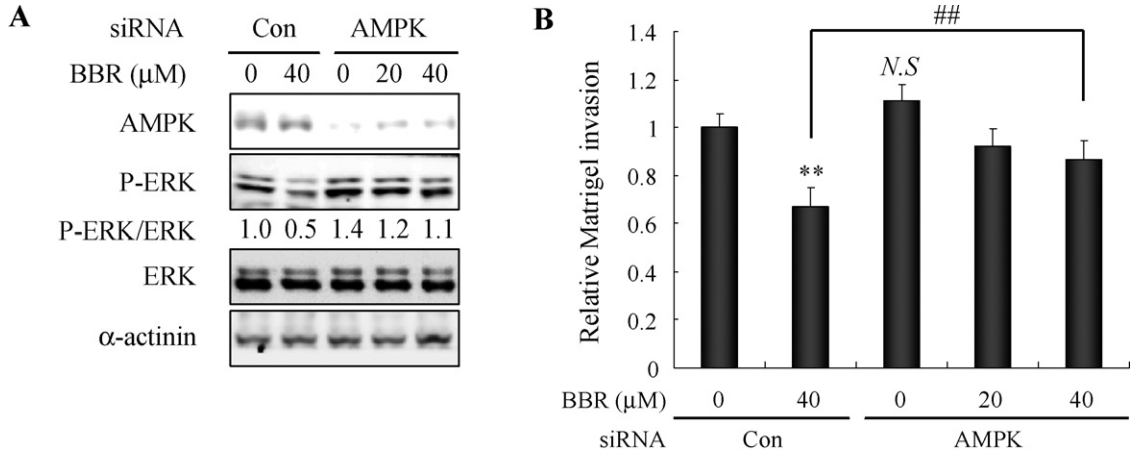

Fig. 4. Effect of AMPKα knockdown by siRNA on the Matrigel invasion of B16F10 cells. (A) B16F10 cells were transfected with control or AMPKα1 and 2 subunit siRNAs for 48 h. The cell lysates were prepared after berberine treatment for 1 h and subjected to Western blot assays using anti-AMPKα, anti-phosphospecific ERK, anti-ERK and anti-α-actinin antibodies. (B) A fixed number of cells were plated with berberine onto the upper part of the Matrigel-coated transwell chamber. After incubation for 48 h, invasive cells were characterized as described in Fig. 2A. The data are expressed as the means ± S.E. for 3 determinations in triplicate. *N.S*, not significant and $^{**}P < 0.01$, compared with control siRNA alone, $^{\#\#}P < 0.01$, compared with control siRNA plus berberine (1-way ANOVA test with Newman–Keuls multiple comparison test).

AMPK activity regulates the activity of ERK and inhibits the invasion of B16F10 cells.

3.5. AMPK activation by berberine regulates cyclooxygenase-2 expression

The inhibition of COX-2 expression by berberine inhibits cell migration [2], and the activation of AMPK by selenium decreases COX-2 expression and ERK activity [27]. Because treatment of skin with TPA stimulates the levels of COX-2 expression [28,29], we determined the effects of TPA on the berberine-induced inhibition of B16F10 cell invasion. Interestingly, B16F10 cells treated with TPA exhibited an increase in COX-2 protein expression and ERK phosphorylation, and the stimulation of AMPK by berberine or AICAR abrogated TPA-induced ERK phosphorylation and COX-2 protein expression (Fig. 5A). The activation of AMPK by berberine

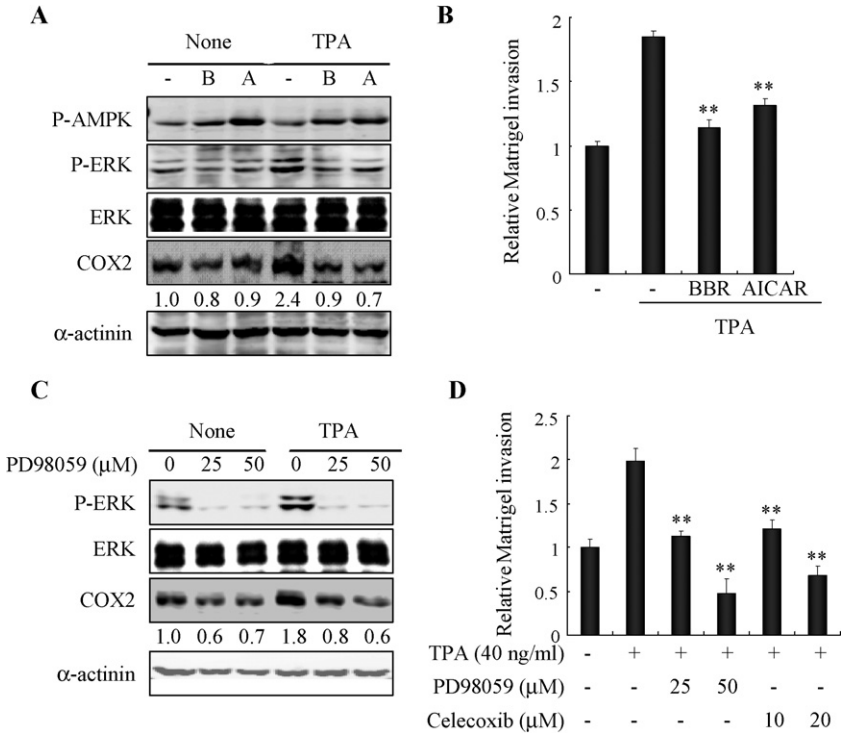

Fig. 5. AMPK activation by berberine and AICAR dramatically abrogates the elevated protein levels of COX-2 in TPA-treated B16F10 cells. (A) B16F10 cells were pretreated with 20 μM berberine and 1 mM AICAR for 30 min and exposed to 40 ng/ml TPA for 6 h. The total cellular extracts were subjected to Western blot assays using anti-phosphospecific AMPKα, anti-phosphospecific ERK, anti-ERK, anti-COX-2 and anti-α-actinin antibodies. (B) A fixed number of cells were plated with berberine and AICAR onto the upper part of the Matrigel-coated transwell chamber for 30 min and exposed to 40 ng/ml TPA. After incubation for 48 h, invasive cells were characterized. (C) The cells were pretreated with PD98059 and exposed to 40 ng/ml TPA for 6 h. The total cellular extracts were subjected to Western blot assays using anti-phosphospecific ERK, anti-ERK, anti-COX-2 and anti-α-actinin antibodies. (D) A fixed number of cells were plated with PD98059 and celecoxib onto the upper part of the Matrigel-coated transwell chamber for 30 min and exposed to 40 ng/ml TPA. After incubation for 48 h, invasive cells were characterized as described in Fig. 2A. The data are expressed as the means ± S.E. for 3 determinations in triplicate. $^{**}P < 0.01$, compared with TPA alone (1-way ANOVA test with Newman–Keuls multiple comparison test).

or AICAR also decreased the TPA-induced invasion of B16F10 cells (Fig. 5B). Furthermore, the treatment of B16F10 cells with PD98059, an MEK inhibitor, reduced basal and TPA-induced COX-2 protein levels and ERK activation, which verified the association of ERK activity and COX-2 expression (Fig. 5C). These results indicated that ERK signaling is necessary for the TPA-induced expression of COX-2 protein. The inhibition of ERK activity by PD98059 also abrogated the TPA-induced invasion of B16F10 cells (Fig. 5D). Similar to the inhibition of ERK, addition of celecoxib, a COX-2 inhibitor, also inhibited the TPA-induced invasion of B16F10 cells.

3.6. Berberine-induced AMPK activation inhibits pulmonary metastasis in vivo

The ability of B16F10 cells to colonize the lung after intravenous injection was examined to clarify the role of berberine-induced AMPK activation in tumor metastasis *in vivo*. B16F10 cells were pretreated with berberine or AICAR for 24 h and suspended in PBS for 1 h at room temperature to ensure that the cells received the same treatment conditions for the measurement of *in vivo* metastasis. The cells in suspension were reseeded in complete medium in a culture plate and incubated for 24 h. Cell viability assays demonstrated that the treatment of B16F10 cells with berberine or AICAR did not significantly affect cell viability for 24 h (Fig. 6A and B). In parallel, when B16F10 cells were pretreated with berberine or AICAR for 24 h and injected into the tail vein of C57BL/6J mice, the berberine- and AICAR-treated cells produced significantly fewer colonies in the lung compared to untreated control cells (Fig. 6C and D). Similarly, microscopic evaluation of the histology sections showed that the lung from mice injected with berberine- or AICAR-treated cells was occupied by fewer melanoma colonies compared to the lung from mice injected with control cells (Fig. 6E).

3.7. AMPK activation by berberine reduces the metastatic potential of A375 human melanoma cells

The inhibitory effect of berberine on the metastatic potential of A375 human melanoma cells was determined. As shown in Fig. 7A and B, berberine significantly reduced the colony number and size of A375 cells in a semisolid medium. Berberine or AICAR treatment of A375 cells for 48 h inhibited both basal levels of migration and TPA-induced migration of human melanoma cells (Fig. 7C). Similar results were noted in the wound healing assay (Fig. 7D). Berberine or AICAR treatment also reduced melanoma cell invasion (Fig. 7E).

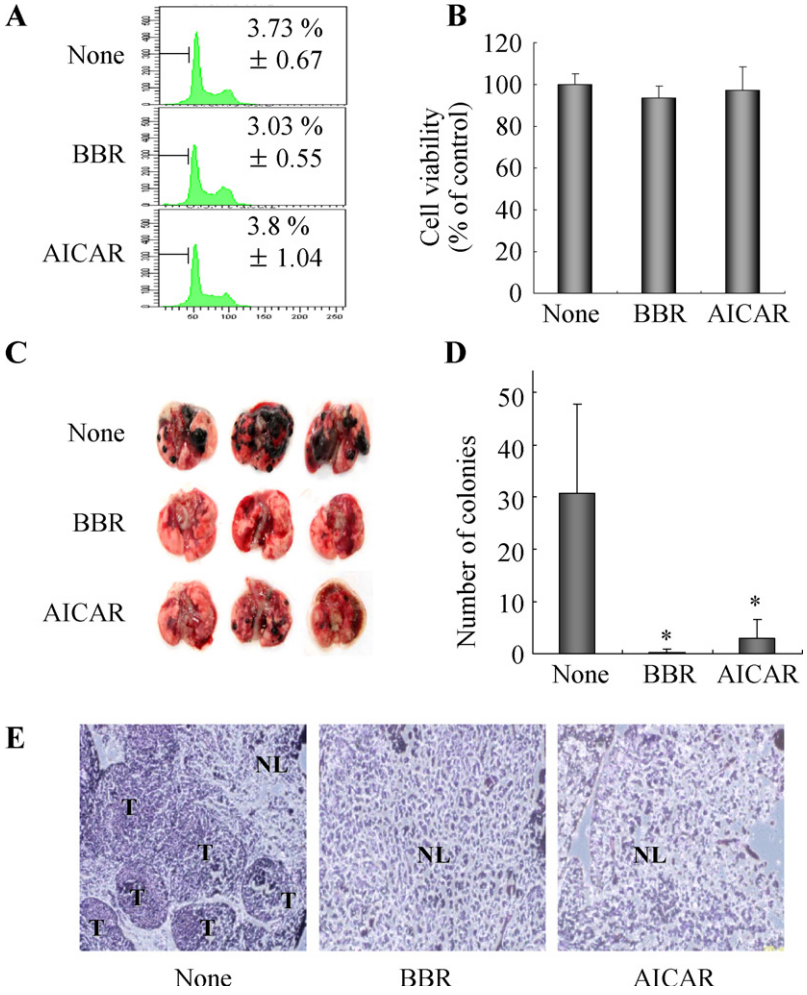

Fig. 6. *In vivo* anti-metastatic effects of berberine-induced AMPK activation. (A) B16F10 cells were pretreated with berberine or AICAR for 24 h and suspended in PBS for 1 h at room temperature. Suspended cells were reseeded with complete medium in a culture plate and incubated for 24 h. The cells were analyzed for apoptosis using FACS analysis, and the percentages of sub-G1 cells are shown. (B) Under the same conditions as in A, the cells were analyzed for their viability using MTT assay. The data are expressed as the means ± S.E. for 3 determinations in triplicate. (C, D) B16F10 cells (3×10^5 cells/0.2 ml PBS) were pretreated with 20 μM berberine or 1 mM AICAR for 24 h and injected into the tail vein of C57BL/6J mice. Three mice were assigned to each group. The metastatic colonies (black dots) on the lung surface were quantified macroscopically 26 days later. $^*P < 0.05$, compared with control (1-way ANOVA test with Newman–Keuls multiple comparison test). (E) Histological photographs of lung tissue sections stained with hematoxylin and eosin. NL, normal lung tissue; and T, metastatic tumor lesion.

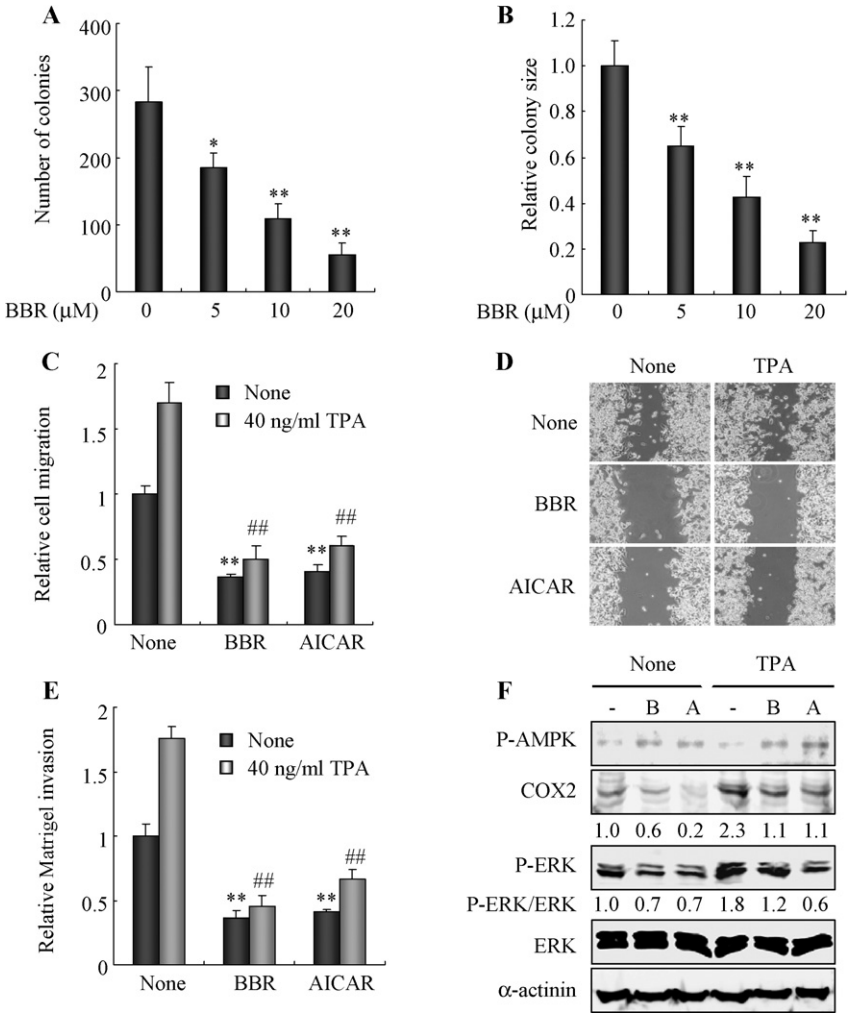

Fig. 7. Effect of berberine-induced AMPK activation on metastatic potential of A375 human melanoma cells. (A) A375 cells (1×10^3) were suspended in 1.5 ml of medium containing 0.3% agar, 10% FBS and the indicated concentrations of berberine and applied onto pre-solidified 0.5% agar (1.5 ml) with the indicated concentrations of berberine. After incubation for 2 weeks, the colonies were counted. (B) The diameter of 10–15 representative colonies was measured. (C) A fixed number of A375 cells were pretreated with 20 μM berberine or 1 mM AICAR for 30 min in the upper part of the transwell chamber. These cells were treated with or without TPA. After incubation for 48 h, migration of tumor cells was analyzed as described in Fig. 2A. The data represent the means ± S.E. in triplicate. (D) A375 cells were grown to confluence, scratched with a pipette tip and photographed immediately after scratching. After incubation for 30 h, the plates were observed under a phase-contrast microscope. (E) A fixed number of cells were pretreated with 20 μM berberine or 1 mM AICAR for 30 min in the upper part of the Matrigel-coated transwell chamber. The cells were treated with or without TPA. After incubation for 48 h, invasion was analyzed. The data are expressed as the means ± S.E. for 3 determinations in triplicate. (F) A375 cells were pretreated with 20 μM berberine and 1 mM AICAR for 30 min and exposed to 40 ng/ml TPA for 6 h. The total cellular extracts were subjected to Western blot assays using anti-phosphospecific AMPKα, anti-phosphospecific ERK, anti-ERK, anti-COX-2 and anti-α-actinin antibodies. $^{*}P < 0.05$ and $^{**}P < 0.01$, compared with control and $^{\#\#}P < 0.01$, compared with TPA alone (1-way ANOVA test with Newman–Keuls multiple comparison test).

Alteration in ERK/COX-2 expression by AMPK regulation was investigated to confirm the effect of AMPK activation on COX-2 in A375 human melanoma cells. The activation of AMPK by berberine or AICAR abrogated TPA-induced ERK phosphorylation and COX-2 protein expression (Fig. 7F), which suggested that berberine exerted a similar activity on ERK activation and COX-2 expression in mouse and human melanoma cells.

4. Discussion

This study investigated the effect of berberine on the metastatic potential of B16F10 cells using an intracellular energy balancing system, AMPK. Our data clearly demonstrated that berberine was an effective inhibitor of the metastatic potential of cancer cells. In addition, the activity of the ERK/COX-2 pathway, which was reduced by AMPK activation, was important for the suppressive effect of berberine on the migration, adhesion and invasion of B16F10 cells.

The suppressive effect of berberine on the invasion of multiple tumor cells is associated with many important signaling pathways. IKK- and NF-κB-mediated pathways and ERK and p38 MAPK signaling mediate the anti-invasive effect of berberine in SCC-4 tongue squamous cancer cells and MDA-MB-231 human breast cancer cells [30,31]. Moreover, berberine exerts an inhibitory effect on tumor cell migration and invasion through a reduction in COX-2 levels, PGE_2 and PGE_2 receptors or MMP-1, -2 and -9 expression [2,32]. In the present study, the involvement of a previously unknown signaling pathway, AMPK signaling, in the berberine-induced suppressive effect on B16F10 cell invasion was demonstrated. Furthermore, our results demonstrated that berberine inhibited metastatic potential through an activation of AMPK and the subsequent reduction of ERK activity and protein levels of COX-2.

A number of studies have revealed a relationship between AMPK and ERK signal pathway [33–35]. Glucose deprivation-induced AMPK activation suppresses ERK signal pathway via

inducing activity of dual-specificity protein phosphatases in HCT116 carcinoma cells [33]. AMPK activation by AICAR reduced IGF-1-induced ERK activity [34]. Moreover, AMPK activation by AICAR or selenium down-regulates the ERK activity, COX-2 and production of prostaglandin E2 [27]. In our study, ERK signaling was necessary for the TPA-induced expression of COX-2 protein. The inhibition of ERK activity by berberine-induced AMPK activation or PD98059 abrogated the TPA-induced COX-2 expression. Thus, we hypothesized that AMPK reduces ERK activity via dual-specificity protein phosphatases and COX-2 expression is reduced via ERK activity decreased by AMPK activation.

AMPK is a pivot point between cell survival and apoptosis, and it is a novel therapeutic target for cancer and metabolic diseases [36–38]. AMPK also acts as a regulator of cell motility. For example, the activation of CaMKKβ–AMPK by lysophosphatidic acid increases the migration of ovarian cancer cells through dynamic cytoskeletal rearrangements [20]. AMPK activation also increases human umbilical vein endothelial cell migration and contributes to transendothelial lymphocyte migration [39,40]. However, AMPK activation by adiponectin inhibits the migration of breast cancer cells and adventitial fibroblast cells, and the deletion of AMPKα1 and 2 subunits in MEF cells leads to the expression of MMP-9 and the augmentation of TNF-α effects on MMP-9 expression [17,19,41]. Compound C, an AMPK inhibitor, inhibits vascular smooth muscle cell (SMC) migration in an AMPK-independent fashion, but AICAR, an AMPK activator, also inhibits the migration of SMCs [21]. Accordingly, the role of AMPK in cell migration may be dependent on the type of stimulus or cell that is used in the assay. Because the role of AMPK in migration of tumor cells is unclear, it is crucially important to understand the identity of the factors that trigger AMPK activation and subsequent inhibition of tumor cell migration. Interestingly, berberine-induced reduction of COX-2 expression inhibits the migration of melanoma cells [2]. In addition, AMPK activation down-regulates COX-2 expression [27,42]. In our study, berberine rapidly increased AMPK phosphorylation by the production of ROS, and this activation down-regulated COX-2 expression, which decreased the migration of B16F10 melanoma cells. Therefore, we hypothesized that AMPK activation by berberine was a negative regulator of B16F10 cell migration.

Tumor metastasis is a multi-step process that includes migration, invasion, adhesion, proliferation and angiogenesis. During cancer progression, some tumor cells acquire new characters represented by an expression of mesenchymal markers and loss of epithelial markers. EMT is characterized by the loss of cell–cell adhesion and increase in cell motility, and it is key step in the progression of tumor metastasis and invasion. Thus, the suppression of EMT through regulation of EMT-related genes such as Slug, Snail, ZEB1, N- or E-cadherin is essential for the prevention of tumor metastasis [26]. AMPK activity is involved in the cell–cell interaction by E-cadherin-dependent adhesion [16]. In our study, the activation of AMPK by berberine or AICAR markedly decreased the mRNA levels of several EMT-related genes (e.g., Snail, Slug, ZEB1 and N-cadherin). Increased oxidative stress and ROS production are associated with many human metastatic tumors [43,44]. Although several studies have investigated the effects of ROS on cell migration and invasion, variable results have been reported depending on the dose, type of ROS (superoxide, hydrogen peroxide or hydroxyl radical) and tissue type of cells [45–48]. ROS differentially regulates cancer cell migration and invasion; the hydroxyl radical plays a promoting role, and superoxide and hydrogen peroxide have an inhibitory role in lung carcinoma cells [49]. In the present study, berberine-induced ROS generation stimulated AMPK and inhibited the metastatic potential of melanoma cells. Because AMPK is sensitively activated by exogenous hydrogen peroxide [25], we proposed that AMPK activation by berberine-induced ROS may have contributed to the inhibitory effect on the metastatic potential of cancer cells.

In conclusion, the results from this study identified a major role of AMPK activation by berberine in the attenuation of the metastatic potential of melanoma cells through inhibitory effects on ERK/COX-2 signaling. Furthermore, our study suggests that berberine should be developed as a pharmacological agent for use in combination with other anti-cancer or anti-metastatic drugs in the treatment of metastatic melanoma.

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