

# Roles of p38 $\alpha$ and p38 $\beta$ mitogen-activated protein kinase isoforms in human malignant melanoma A375 cells

SU-YING WEN<sup>1,2</sup>, SHI-YANN CHENG<sup>3,4</sup>, SHANG-CHUAN NG<sup>5</sup>, RITU ANEJA<sup>6</sup>,  
CHIH-JUNG CHEN<sup>7</sup>, CHIH-YANG HUANG<sup>8-12\*</sup> and WEI-WEN KUO<sup>5\*</sup>

<sup>1</sup>Department of Dermatology, Taipei City Hospital, Renai Branch, Taipei 106; <sup>2</sup>Department of Health Care Management, National Taipei University of Nursing and Health Sciences, Taipei 112; <sup>3</sup>Department of Medical Education and Research and Department of Obstetrics and Gynecology, China Medical University Beigang Hospital, Yunlin 65152;

<sup>4</sup>Obstetrics and Gynecology, School of Medicine, China Medical University; <sup>5</sup>Department of Biological Science and Technology, College of Biopharmaceutical and Food Sciences, China Medical University, Taichung 404, Taiwan, R.O.C.;

<sup>6</sup>Department of Biology, Georgia State University, Atlanta, GA 30303, USA; <sup>7</sup>Division of Breast Surgery, Department of Surgery, China Medical University Hospital; <sup>8</sup>Graduate Institute of Biomedical Sciences, China Medical University,

Taichung 404; <sup>9</sup>Cardiovascular and Mitochondrial Related Disease Research Center, Hualien Tzu Chi Hospital, Buddhist Tzu Chi Medical Foundation; <sup>10</sup>Center of General Education, Buddhist Tzu Chi Medical Foundation,

Tzu Chi University of Science and Technology, Hualien 970; <sup>11</sup>Department of Medical Research, China Medical University Hospital, China Medical University, Taichung 404; <sup>12</sup>Department of

Biotechnology, Asia University, Taichung 413, Taiwan, R.O.C.

Received March 29, 2019; Accepted September 12, 2019

DOI: 10.3892/ijmm.2019.4383

**Abstract.** Skin cancer is one of the most common cancers worldwide. Melanoma accounts for ~5% of skin cancers but causes the large majority of skin cancer-related deaths. Recent discoveries have shown that the mitogen-activated protein kinase (MAPK) signaling pathway is critical for melanoma development and progression. Many oncogenic pathways that cause melanoma tumorigenesis have been identified, most of which are due to RAF/MEK/ERK (MAPK) pathway activation. However, the precise role of p38 remains unclear. Using specific short hairpin (sh) RNA to silence p38 $\alpha$  and p38 $\beta$ , the present findings demonstrated that p38 $\alpha$  was a crucial factor in regulating cell migration in the A375 melanoma cell line. Silencing p38 $\alpha$  downregulated the expression of epithelial-mesenchymal transition markers, such as matrix metalloproteinase (MMP) 2, MMP9, twist family bHLH transcription factor 1, snail family transcriptional repressor 1 and

vimentin, while mesenchymal-epithelial transition markers, such as E-cadherin, were upregulated. Of note, the results also demonstrated that p38 $\alpha$  silencing impaired vascular endothelial growth factor expression, which regulates tumor angiogenesis. Furthermore, p38 $\alpha$  knockdown inhibited cell proliferation in melanoma cells. In addition, silencing p38 $\alpha$  induced senescence-like features, but not cell cycle arrest. Expression of the senescence markers p16, p21, p53 and  $\beta$ -galactosidase was upregulated, and an increase in the number of senescence-associated  $\beta$ -galactosidase-positive cells was observed in a p38 $\alpha$  knockdown stable clone. However, no significant difference was found between control and p38 $\beta$  stable knockdown cells. Taken together, the present results suggested that p38 $\alpha$  knockdown impaired migration and proliferation, and increased senescence, in A375 melanoma cells. However, p38 $\beta$  may not be involved in melanoma tumorigenesis. Therefore, targeting p38 $\alpha$  may be a valuable approach towards inhibiting tumor growth and metastasis in patients with melanoma.

*Correspondence to:* Professor Wei-Wen Kuo, Department of Biological Science and Technology, College of Biopharmaceutical and Food Sciences, China Medical University, 91 Hsueh-Shih Road, Taichung 404, Taiwan, R.O.C.  
E-mail: wwkuo@mail.cmu.edu.tw

\*Contributed equally

**Key words:** p38 $\alpha$ , epithelial-mesenchymal transition, senescence, melanoma

## Introduction

Skin cancer is the most common cancer in western countries, and it was one of the ten leading cancer types when estimating new cases and deaths in the United States in 2017 (1). The main cause of all types of skin cancer is exposure to ultraviolet radiation from the sun and other sources (2). Skin cancer is divided into two categories: Melanoma and non-melanoma. Non-melanoma skin cancer includes basal cell carcinoma and squamous cell carcinoma. Basal cell carcinoma (BCC) accounts for ~75% of skin cancer cases, squamous cell

carcinoma (SCC) accounts for ~20% of cases, and melanoma accounts for only ~5% of cases (3). In comparison with other skin cancers, the incidence rate of melanoma is the lowest, but it is the deadliest.

Mitogen-activated protein kinase (MAPK) signal transduction pathways are highly conserved among eukaryotes during evolution and can regulate different cellular functions, such as proliferation, differentiation, apoptosis, cell-cell adhesion, inflammation, migration and invasion, in response to various environmental signals (4). There are four types of P38 MAPK isoforms in mammalian cells: p38 $\alpha$  (also known as MAPK14), p38 $\beta$  (also known as MAPK 11), p38 $\gamma$  (also known as MAPK 12), and p38 $\delta$  (also known as MAPK13) (5). Regarding structural similarity, p38 $\alpha$  and p38 $\beta$  are 75% similar, while p38 $\gamma$  and p38 $\delta$  are 70% similar (5). P38 $\alpha$  and p38 $\beta$  are universally expressed, while p38 $\gamma$  and p38 $\delta$  are more tissue-specific (6). Regarding kinase inhibition, p38 $\alpha$  and p38 $\beta$  can be targeted by a class of pyridinyl imidazole drugs, but these drugs cannot inhibit p38 $\gamma$  and p38 $\delta$  (7). However, this type of chemical inhibition does not allow us to distinguish whether functions are mediated by p38 $\alpha$  or p38 $\beta$ . In the present study, specific short hairpin (sh)RNA or small interfering (si)RNA were used in order to knockdown p38 $\alpha$  or p38 $\beta$  and to clearly differentiate their biological functions.

P38 $\alpha$  is an essential protein during embryonic development, and it can regulate various cellular functions. Notably, multiple proteins can be directly phosphorylated by p38 $\alpha$ . Additionally, the p38 $\alpha$  pathway can control the production of different extracellular signaling molecules, such as growth factors, cytokines and chemokines (8). The p38 $\alpha$  protein can regulate different cellular functions during tumor formation at different stages of development and for different types of cancers. P38 $\alpha$  is reported to act as a tumor suppressor in the initial stages (9) but promotes tumor activity in the later stages of tumorigenesis (10,11). In the initial stages, p38 $\alpha$  can regulate cell homeostasis by balancing cell apoptosis, proliferation and differentiation (12). In the later stages, it can facilitate tumor cell survival and dissemination (13). Therefore, p38 $\alpha$  can have different roles in different types of cancers.

Epithelial-mesenchymal transition (EMT) is a biological process that allows a polarized epithelial cell to undergo certain biological changes, such as polarity stimulation or loss, thus shifting the cell to a more aggressive phenotype; these altered cells resemble mesenchymal cells (14). Mesenchymal cells gain migratory and invasion abilities, develop apoptosis resistance and have increased extracellular matrix (ECM) component production (15,16). Once mesenchymal cell formation occurs, the cells can migrate away from their origin. There are several biomarkers for evaluating EMT, such as twist family bHLH transcription factor 1 (Twist), snail family transcriptional repressor 1 (Snail, also known as Snail) and vimentin. By contrast, E-cadherin, zonula occludens-1 (ZO-1) and cytokeratin serve as mesenchymal-epithelial transition (MET) markers (17). Snail and Twist can transcriptionally activate the downstream targets of various signaling pathways, thus regulating EMT (18). A previous study has shown that certain transcription factors, such as Snail, Slug, Twist, zinc finger E-box binding homeobox (ZEB) 1, ZEB2 and transcription factor 3 (TCF3), can regulate EMT by transcriptionally inhibiting E-cadherin expression via suppressing its

promoter activity (19). In addition, vimentin, which serves as a cytoskeleton marker of EMT, is extremely abundant in various cancer cells, and its expression is highly correlated with cancer invasion and poor prognosis (20). Furthermore, matrix metalloproteinases (MMPs) are involved in the development and progression of different cancers. MMPs, such as the gelatinases MMP2 and MMP9, can degrade type IV collagen, a major component of the ECM, to regulate cancer cell metastasis (21). Whether p38 $\alpha$  or p38 $\beta$  could be involved in EMT or MET in A375 melanoma cells remains unknown.

A previous study indicated that p38 $\alpha$  may act as a tumor promoter and enhance melanoma cell metastasis under cytokine stimulation (22,23). Therefore, p38 $\alpha$  can regulate the migration abilities of tumor cells, as well as EMT processes (24). In other types of cancer, p38 $\alpha$  has been reported to regulate cell proliferation, differentiation, apoptosis and numerous cellular processes, but in melanoma, its precise role remains unknown. In addition, p38 $\beta$  has been demonstrated to regulate cell apoptosis, differentiation and metabolism (25-27). However, the functions of p38 $\beta$ , which shares 75% structural similarity with p38 $\alpha$ , have not been investigated in melanoma. Therefore, the goal of the present study was to identify the biological functions of p38 $\alpha$  and p38 $\beta$ .

The present study investigated the roles of p38 $\alpha$  and p38 $\beta$  by ablating their gene functions using specific shRNA in A375 melanoma cells. Based on proliferation, apoptosis, migration and aging assay analyses, the data identified that p38 $\alpha$ , but not p38 $\beta$ , may regulate melanoma cell proliferation and migration. Thus, p38 $\alpha$  might be a valuable therapeutic target in patients with melanoma.

## Materials and methods

**Cell culture and treatment.** A375 human melanoma cells and B16F0 mouse melanoma cells were obtained from the Bioresource Collection & Research Center (Hsinchu, Taiwan) and 293T cells were purchased from the American Type Culture Collection; cat. no. CRL-3216). Both cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (FBS; HyClone; GE Healthcare Life Sciences), 2 mM glutamine, 2 mM sodium pyruvate, 100  $\mu$ g/ml penicillin, and 100  $\mu$ g/ml streptomycin in humidified air (5% CO<sub>2</sub>) at 37°C.

**Gene silencing using shRNA.** shRNA-encoding plasmids were purchased from the National RNAi Core Facility (Academia Sinica, Taipei, Taiwan). 293T cells were co-transfected with three plasmids: pMD. G (0.5  $\mu$ g), pCMV $\Delta$ r89.1 (4.5  $\mu$ g) and p38 $\alpha$  (5  $\mu$ g) or p38 $\beta$  (5  $\mu$ g) shRNA plasmid (cloned in the pLKO.1 vector). The plasmids were transfected using the PureFection transfection reagent (cat. no. LV750A-1), according to the manufacturer's instructions (System Biosciences LLC). After transfection for 24 h, the medium was replaced with fresh medium. The lentiviral particle-containing medium was then harvested after 24 and 48 h, and used to transduce the A375 cells. After 48 h of infection, 2  $\mu$ g/ml puromycin was added to the media for 48 h to remove the non-transfected cells. The stable cells were then routinely cultured in the presence of 1  $\mu$ g/ml puromycin.

The shRNA oligoribonucleotide sequences were as follows: p38 $\alpha$  #1, GTTCAGTTCCTTATCTACCAA; p38 $\alpha$  #2, CCATGAGGCAAGAACTATAT; p38 $\alpha$  #3, GCCGTATAG GATGTCAGACAA; p38 $\beta$  #1, GCCACGTCCATCGAGGAC TTC; p38 $\beta$  #2, CCTGTCTCTTCTGGCTACTG; and p38 $\beta$  #3, CGGCTCCGTCTGTTCCGGCCTA.

**siRNA silencing.** B16F0 cells were cultured to reach 80% confluence on the day of transfection. siRNAs (10 nM) were transfected using the PureFection transfection reagent, according to the manufacturer's instructions (System Biosciences). p38 $\alpha$  siRNA (cat. no. MAPK14\_Hs01\_00018467), p38 $\beta$  siRNA (cat. no. MAPK11\_Hs01\_00071113) and siRNA universal negative control #1 (cat. no. SIC001) were purchased from Sigma-Aldrich (Merck KGaA). At 48 h post-transfection, subsequent experiments were performed.

**Western blot analysis.** Cultured A375 human melanoma cells were scraped, washed twice with PBS and centrifuged at 23,000  $\times$  g (28). The cell pellets were lysed in buffer containing 50 mM Tris (pH 7.5), 0.5 M NaCl, 1.0 mM EDTA (pH 7.5), 10% glycerol, 1 mM BME, 1% IGEPAL-630 and protease inhibitor cocktail (Roche Molecular Diagnostics). After incubation for 30 min on ice, the supernatants were collected by centrifugation at 23,000  $\times$  g for 10 min at 4°C. The Bradford method was performed to determine the protein concentrations. Samples containing the same amounts of protein (10  $\mu$ g) were analyzed by western blotting. Proteins were separated on 10% or 12% SDS-PAGE gels (depending on the molecular weight) and transferred onto PVDF membranes (EMD Millipore). Non-specific protein binding was prevented by incubating the membranes with blocking buffer (5% non-fat dry milk, 20 mM Tris-HCl pH 7.6, 150 mM NaCl and 0.1% Tween-20) for at least 1 h at room temperature. Then, the membranes were incubated with the following specific primary antibodies: Anti-p38 $\alpha$  (clone A1F7; cat. no. sc-33688; Santa Cruz Biotechnology, Inc.), anti-p38 $\beta$  (clone N-14; cat. no. sc-15918; Santa Cruz Biotechnology, Inc.), anti-E-cadherin (cat. no. sc-8426; Santa Cruz Biotechnology, Inc.), anti-Twist (cat. no. sc-81417; Santa Cruz Biotechnology, Inc.), anti-vimentin (clone RV202; cat. no. sc-32322; Santa Cruz Biotechnology, Inc.), anti-Snail (clone T-18; cat. no. sc-10433; Santa Cruz Biotechnology, Inc.), anti-MMP2 (clone 8B4; cat. no. sc-13595; Santa Cruz Biotechnology, Inc.), anti-MMP9 (clone M-17; cat. no. sc-6841; Santa Cruz Biotechnology, Inc.), anti-vascular endothelial growth factor (VEGF; clone 147; cat. no. sc-507; Santa Cruz Biotechnology, Inc.), anti-p53 (cat. no. sc-1311; Santa Cruz Biotechnology, Inc.), anti-p21 (cat. no. sc-6246; Santa Cruz Biotechnology, Inc.), anti-pl6 (cat. no. 10883-1-AP; ProteinTech Group, Inc.) and anti- $\beta$ -actin (clone C4; cat. no. sc-47778; Santa Cruz Biotechnology, Inc.). The dilution of primary antibodies was 1:1,000. All membranes with primary antibodies were placed on an orbital shaker at 4°C overnight. The membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies (1:3,000; all from Santa Cruz Biotechnology, Inc.) mouse anti-rabbit immunoglobulin (Ig) G (cat. no. sc-2357), mouse-IgGk light chain binding protein (cat. no. sc-516102) and mouse anti-goat IgG (cat. no. sc-2354) for 2 h at room temperature. Densitometric analysis of the immunoblots was performed using a Fuji LAS 3000 imaging system and the Image Lab Software (version 5.2.1, Bio-Rad Laboratories, Inc.).

**Cell viability assay.** A375 human melanoma cells (shp38 $\alpha$  #1 and shp38 $\beta$  #2 stable clones;  $2 \times 10^4$  cells/ml) were inoculated in 96-well plates in 200  $\mu$ l of medium containing 10% FBS. After 12, 24, 36, 48 and 72 h, 20  $\mu$ l of MTT solution (5 mg/ml) was added to each well for 3 h. The MTT solution was removed, and dimethyl sulfoxide (DMSO) was added. Cell viability was measured at 570 nm using a spectrophotometer.

**Senescence-associated  $\beta$ -galactosidase (SA- $\beta$ -gal) staining.** The assay was performed using the Senescence  $\beta$ -Galactosidase Staining kit (cat. no. 9680; Cell Signaling Technology, Inc.), according to the manufacturer's instructions (29). SA- $\beta$ -gal staining was performed to determine the percentage of SA-Gal-positive cells. A375 human melanoma cells (shp38 $\alpha$  #1 and shp38 $\beta$  #2 stable clones;  $1 \times 10^5$  cells/ml) were seeded on 6-well plates for 24 h. The cells were then fixed with 4% paraformaldehyde at room temperature for 30 min. After fixation, the cells were washed with PBS three times and incubated with X-Gal at pH 6.0 overnight at 37°C. After washing, the cells were counted using bright-field light microscopy (DP73; Olympus Corporation) at  $\times 100$  magnification.

**RNA extraction and reverse transcription-quantitative PCR (RT-qPCR).** A375 non-infected control, shp38 $\alpha$  and shp38 $\beta$  cell lines were collected by trypsinization. RNA was then extracted using the Direct-zol<sup>TM</sup> RNA MiniPrep kit (Zymo Research Corporation). RNA samples (1  $\mu$ g) were reverse transcribed into cDNA. An aliquot of RNA was incubated with 0.5  $\mu$ g of oligo dT (MD Bio.). Following incubation at 70°C for 15 min, 0.25 mM dNTPs (MD Bio.), 20 U of RNasin I Plus RNase Inhibitor (Promega Corporation) and 20 U of M-MLV Reverse Transcriptase (Promega Corporation) were added and incubated at 42°C for 90 min for cDNA synthesis. Then, a GeneAmp PCR system 2400 (PerkinElmer, Inc.) was used to amplify the cDNA. qPCR analysis was performed using SYBR-Green I Master Mix (Bio-Rad Laboratories, Inc.). The total reaction volume used for PCR was 10  $\mu$ l, and included 2  $\mu$ l of cDNA, 5  $\mu$ l of SYBR-Green, 0.5  $\mu$ l of 10  $\mu$ M forward primer and reverse primer, and 2  $\mu$ l of ddH<sub>2</sub>O. The reactions were incubated in Applied Biosystems MicroAmp Optical 8-Tube Strips with 8-Cap Strips (0.1 ml) at 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec, 55°C for 15 sec and 72°C for 30 sec, and all data were collected in triplicate. An Applied Biosystems QuantStudio<sup>TM</sup> 3 Real-Time PCR System was used to detect mRNA expression. All reactions were run in triplicate. The cycle number at which the reaction crossed the threshold cycle (Cq) was determined for each gene and the relative amount of each gene to GAPDH was described using the equation  $2^{\Delta Cq}$  where  $\Delta Cq = Cq_{\text{interest gene}} - Cq_{\text{GAPDH}}$  (30). The primers were as follows: p38 $\alpha$ , forward ACCTGTCTCCAG TGGGCTCT and reverse CACGTAACCCCGTTTTTGTG; p38 $\beta$ , forward CATCTTCCGTGGAGCCAAC and reverse CACTGTCCAGCACCAGCAT; and GAPDH, forward CCA GCCGAGCCACATCGCTC and reverse ATGAGCCCCAGC CTTCTCCAT.

**Migration assay.** To determine the cell migration ability, A375 human melanoma cells (shp38 $\alpha$  #1 and shp38 $\beta$  #2 stable clones) were seeded at a density of  $3 \times 10^4$  cells/well in a 48-well Boyden chamber (Neuro Probe Inc.) plate; the polycarbonate

membrane filters had 8  $\mu$ m pores. The lower compartment was supplemented with DMEM and 10% FBS. Cells were seeded in the upper part of the Boyden chamber in serum-free medium and incubated for 24 h. Cotton swabs were used to remove the cells on the upper surface (non-migrated cells). Next, the cells on the lower surface of the membrane filters were fixed with methanol and stained with a 0.05% Giemsa solution for 1 h. The filters were then rinsed twice with distilled water until no additional stain remained, and air-dried for 20 min. The cells that migrated to the lower side of the filter were observed under a light microscope at x100 magnification. Four fields were selected randomly and counted for each filter. Three independent experiments were performed.

**Cell cycle analysis.** For flow cytometry analysis of cell cycle phase distributions, cells were seeded at 80% confluency in 10 cm dishes, and after 24 h, the cells were trypsinized and washed twice with PBS. Next, 70% ethanol was used to fix the cells at 4°C for 1 h. After that, the cells were stained with a propidium iodide (Sigma-Aldrich; Merck KGaA) solution (20  $\mu$ g/ml PI, 0.1% Triton-X, 0.2 mg/ml RNase in ice-cold PBS) and incubated at room temperature for 15 min. Data analysis was conducted using flow cytometry (BD Biosciences). MODFIT™ LT 3.0 software (Verity Software House, Inc.) was used to analyze the cell cycle contribution. Three independent experiments were performed.

**Wound healing assay.** To examine whether p38 $\alpha$  or p38 $\beta$  knockdown can reduce the migration ability of A375 melanoma cells, A375 cells were plated in a six-well plate and grown for 24 h. Cells were scratched with a pipette tip and washed with PBS to remove the non-adhered floating cells. Mitomycin C (5  $\mu$ g/ml) was added to inhibit cell proliferation. Cells were maintained in 10% FBS medium. The scratches were photographed at 0 and 24 h, and the relative migration was analyzed by ImageJ software with 64-bit Java 1.8.0\_112 (National Institutes of Health).

**Statistical analysis.** Each experiment was repeated at least three times. Statistical analyses were performed using GraphPad Prism version 5 (GraphPad Software, Inc.). The data were analyzed by one-way ANOVA. Significance between the individual means was determined by Tukey's test. Imaging results were quantified by ImageJ and processed with Adobe Photoshop (Adobe Systems, Inc).  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**MAPK p38 $\alpha$  and p38 $\beta$  silencing efficiency in the A375 melanoma cell line.** To determine the silencing efficiency, the mRNA and protein expression levels of p38 $\alpha$  and p38 $\beta$  were measured following knockdown using shRNA (Fig. 1). The silencing specificity of the shRNA against p38 $\alpha$  and p38 $\beta$  was evaluated by RT-qPCR to determine mRNA levels and by western blotting to determine protein levels. As shown in Fig. 1A and C, p38 $\alpha$  and p38 $\beta$  protein levels were significantly decreased in p38 $\alpha$  and p38 $\beta$  shRNA stable clone cells. Similar results were obtained for the mRNA expression levels (Fig. 1B and D). The clonal lines shp38 $\alpha$  #1 and shp38 $\beta$  #2 had

the highest knockdown efficiencies, and therefore these were selected for subsequent experiments. A shRNA scrambled control was also used in A375 melanoma cells to confirm the transduction and knockdown efficiency (Fig. S1). Notably, the results demonstrated that the shRNA sequences were specific, with the p38 $\alpha$  shRNA not affecting the p38 $\beta$  mRNA and protein levels, and the p38 $\beta$  shRNA not affecting the p38 $\alpha$  levels (Fig. 1).

**Knockdown of p38 $\alpha$ , but not p38 $\beta$ , reverses EMT and impairs VEGF expression in A375 melanoma cells.** It has been reported that p38 $\alpha$  regulates the production of different extracellular signaling molecules, such as growth factors, cytokines and chemokines. These signaling molecules promote EMT and increase cell migration and invasion abilities (17). To examine EMT and MET markers, western blot analysis was performed. The results demonstrated that the protein expression levels of the MET marker E-cadherin were significantly increased after p38 $\alpha$  silencing. Furthermore, the protein levels of EMT markers, including Twist, Snail, vimentin, MMP2 and MMP9, were decreased after p38 $\alpha$  silencing. Of note, VEGF, which acts as an angiogenesis and metastasis mediator, was downregulated after p38 $\alpha$  shRNA transduction (Fig. 2A). By contrast, there were no obvious changes in MET, EMT or angiogenesis markers in cells transduced with p38 $\beta$  shRNA compared with those in control cells (Fig. 2B). Furthermore, the EMT markers MMP2 and MMP9 were downregulated in p38 $\alpha$ , but not p38 $\beta$ , shRNA stable clone cells when compared with the scrambled control in A375 melanoma cells (Fig. S1). Taken together, these results demonstrated that the knockdown of p38 $\alpha$  triggered a molecular switch from a mesenchymal phenotype to an epithelial-like phenotype in A375 melanoma cells. However, p38 $\beta$  was not involved in the regulation of EMT and angiogenesis-related gene expression.

**Effects of silencing p38 $\alpha$  or p38 $\beta$  on A375 cell migration.** p38 $\alpha$  silencing has been shown to inhibit melanoma cell motility under IL-6 stimulation (22,31); however, no study has investigated the migration ability after p38 $\alpha$  silencing and without cytokine stimulation. The present study examined the migration ability of p38 $\alpha$  shRNA and p38 $\beta$  shRNA A375 stable clonal cell lines by wound healing and Transwell assays. As presented in Fig. 3A and B, silencing p38 $\alpha$  via shRNA significantly reduced the migration ability of A375 cells. By contrast, in the p38 $\beta$  shRNA stable clone, the migratory ability did not change significantly compared with the control cells (Fig. 3A and B). To further confirm these results, transient transfection was performed with p38 $\alpha$  and p38 $\beta$ -specific siRNAs into B16F0 melanoma cells, and the knockdown efficiencies were analyzed by western blotting (Fig. S2A). Knockdown of p38 $\alpha$ , but not p38 $\beta$ , inhibited B16F0 melanoma cell migration (Fig. S2B). These findings indicated that p38 $\alpha$ , but not p38 $\beta$ , was crucial for the migration ability of A375 human melanoma cells.

**Knockdown of p38 $\alpha$  inhibits cell proliferation but not cell cycle progression.** To analyze cell proliferation, an MTT assay was performed. The results demonstrated that the p38 $\alpha$  shRNA stable clone had a lower proliferation rate compared with control cells (Fig. 4A). By contrast, no significant changes

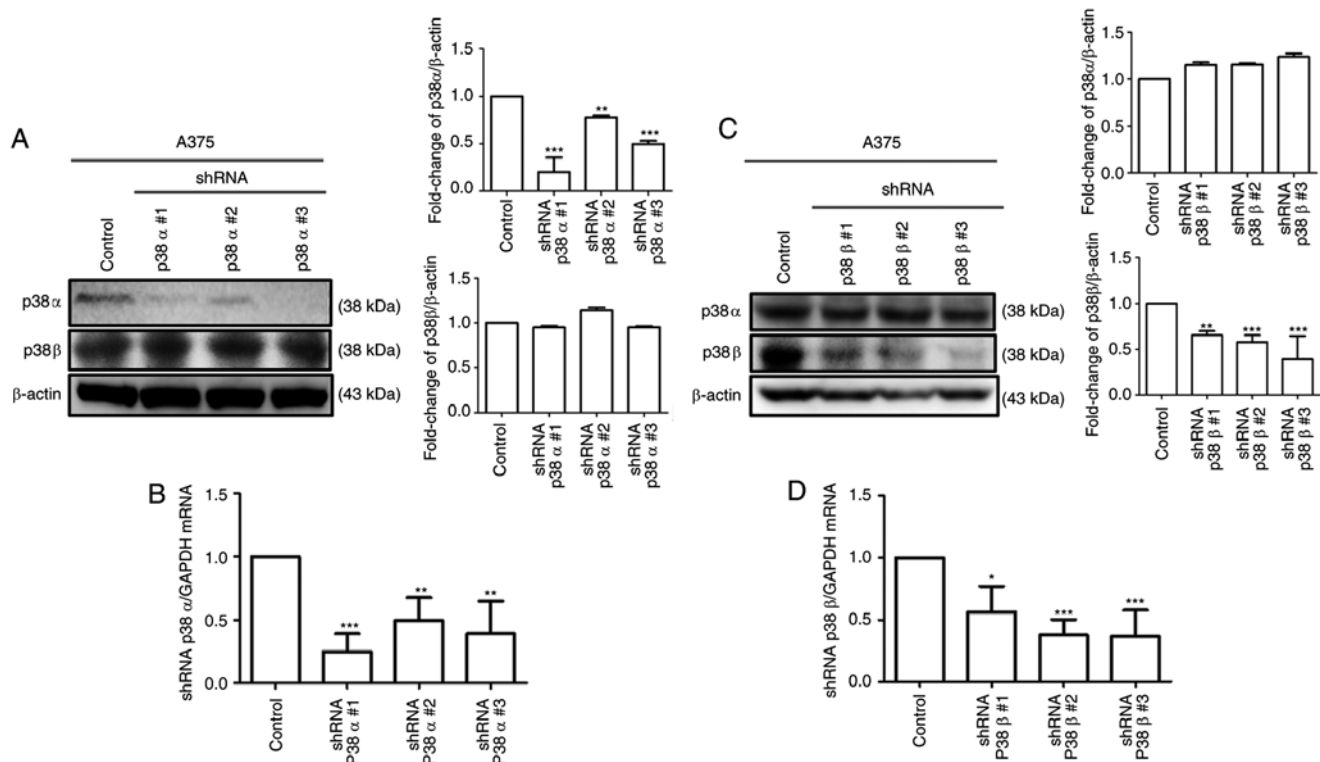


Figure 1. p38α and p38β silencing efficiency in the A375 melanoma cell line. A375 cells were infected with lentiviruses containing p38α-specific shRNAs #1, #2 and #3 or p38β-specific shRNAs #1, #2, and #3. (A) Protein expression levels of p38α. (B) mRNA expression levels of p38α. (C) Protein expression levels of p38β. (D) mRNA expression levels of p38β. Data are presented as means ± SD (n=3). \*P<0.05, \*\*P<0.01 and \*\*\*P<0.001 vs. control cells. sh, short hairpin.

in the p38β shRNA stable clone proliferation were observed (Fig. 4A). To understand how p38α regulates cell growth, the cell cycle phase distribution was analyzed by flow cytometry. There were no significant changes in the cell cycle phase distribution between control, p38α shRNA and p38β shRNA stable clones (Fig. 4B). Taken together, these data suggested that knockdown of p38α partially reduced the proliferation of A375 melanoma cells, while p38β was not involved in regulating cell proliferation.

*Knockdown of p38α induces senescence-like features but not cell cycle arrest to reduce growth in A375 cells.* To understand whether the knockdown of p38α or p38β could induce cell senescence, a SA-β-gal assay was performed and the protein expression of senescence-related markers p16, p21, p53 and β-gal was detected by western blotting. In the p38α shRNA stable clones, a significant increase was observed in the numbers of SA-β-gal-positive cells (Fig. 5B) and in the expression levels of p16, p21, p53 and β-gal (Fig. 5A). By contrast, no positive staining for SA-β-gal was observed in the p38β shRNA stable clone (Fig. 5B). These data suggested that p38α knockdown induced cellular senescence, while p38β was not involved in regulating cell senescence.

## Discussion

The current study revealed that, between p38α and p38β, only p38α was significantly associated with EMT marker and VEGF expression, cellular migration and proliferation in A375 melanoma cells. This finding is important because high p38α expression is associated with cancer development in

melanoma (32). Therefore, targeting p38α may be a potential treatment approach for patients with melanoma.

While there have been reports about the contradictory roles of p38 MAPK in cancers, the significance of the p38 MAPK signaling pathway in regulating various cancers is widely recognized (5,33-35). In cancer cells, the activation of p38 MAPK leads to growth inhibition, but it stimulates cell growth after activation in some other types of cancer (36,37). Furthermore, p38 MAPK activation has also been shown to be associated with metastasis and tumorigenesis. Conversely, several studies have reported that p38 MAPK may be a negative regulator of metastasis and tumorigenesis (38-40). In addition, p38 MAPK has opposite roles in regulating cell death, and it can mediate either cell survival or cell death depending on, not only the type of stimulus, but also the type of cell. The important question that remains to be answered is, therefore, whether the p38 MAPK isoforms have a pro-oncogenic or tumor-suppressive role in different cancer cells. To this end, the present study aimed to identify the specific roles of p38α and p38β in melanoma.

A crucial step in melanoma progression is EMT, a process that regulates melanoma migration, invasion and metastasis (41,42). The present study demonstrated that p38α regulated melanoma EMT. Previous studies have reported that high expression of p38α in melanoma enhances metastasis (22,23); the current findings, therefore, together with previous literature, support a tumor-promoting role of p38α in melanoma. However, contradictory results have been reported in other types of cancer, such as breast cancer (9,43,44), lung cancer (11,45) and liver cancer (46,47), where p38α has been found to act as a tumor suppressor in the initial step of tumor formation and to act as a



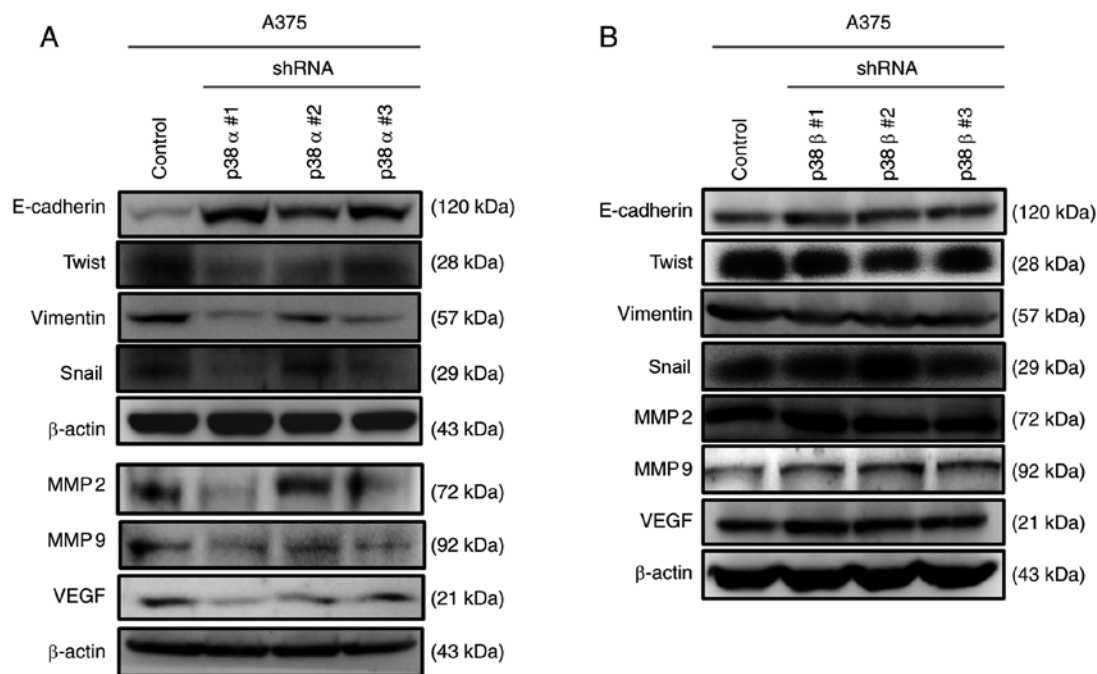


Figure 2. Effects of p38 $\alpha$  and p38 $\beta$  knockdown on EMT marker and VEGF expression in A375 melanoma cells. (A) The protein expression levels of EMT and angiogenesis markers were determined in p38 $\alpha$ -silenced clonal cells and in (B) p38 $\beta$ -silenced clonal cells by western blotting.  $\beta$ -actin served as a loading control. Twist, twist family bHLH transcription factor 1; Snail, snail family transcriptional repressor 1; MMP, matrix metalloproteinase; VEGF, vascular endothelial growth factor; sh, short hairpin.

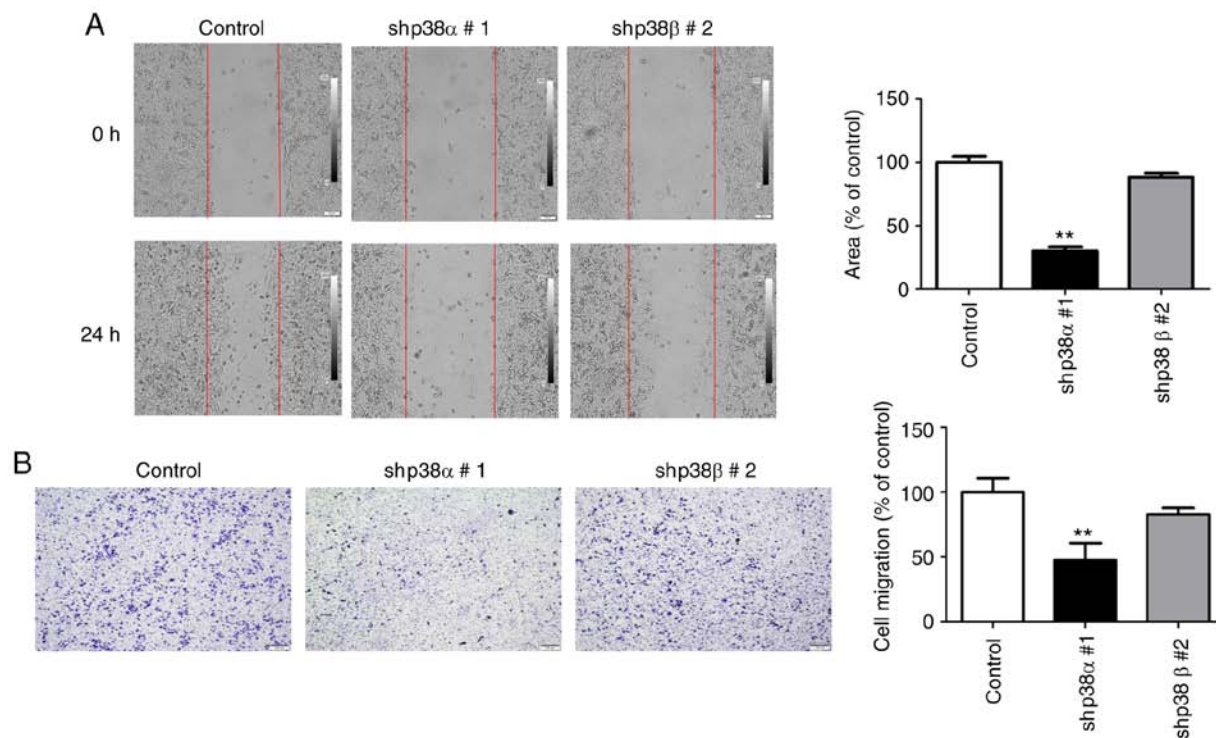


Figure 3. Effects of p38 $\alpha$  or p38 $\beta$  knockdown on cell migration abilities in A375 melanoma cells. (A) A single scratch was created in confluent monolayers of shp38 $\alpha$  #1 or shp38 $\beta$  #2 stable clone A375 cells. Mitomycin C (5  $\mu$ g/ml) was added to inhibit cell proliferation. The scratches were photographed at 0 and 24 h. The relative migration was analyzed by ImageJ software. (B) Migration capacity was determined by Transwell assay in shp38 $\alpha$  #1 and shp38 $\beta$  #2 stable clone A375 cells. Representative photographs and quantification of the migrated cells are shown. Data are presented as means  $\pm$  SD (n=3). \*\*P<0.01 vs. control cells. sh, short hairpin.

tumor promoter in the later stages of cancer to enhance metastasis. The present study demonstrated that p38 $\alpha$ , but not p38 $\beta$ , regulated melanoma migration by upregulating the protein

expression of EMT markers, including Twist, Snail, vimentin, MMP2 and MMP9, and by downregulating the protein expression of the MET marker E-cadherin. Furthermore, the results of

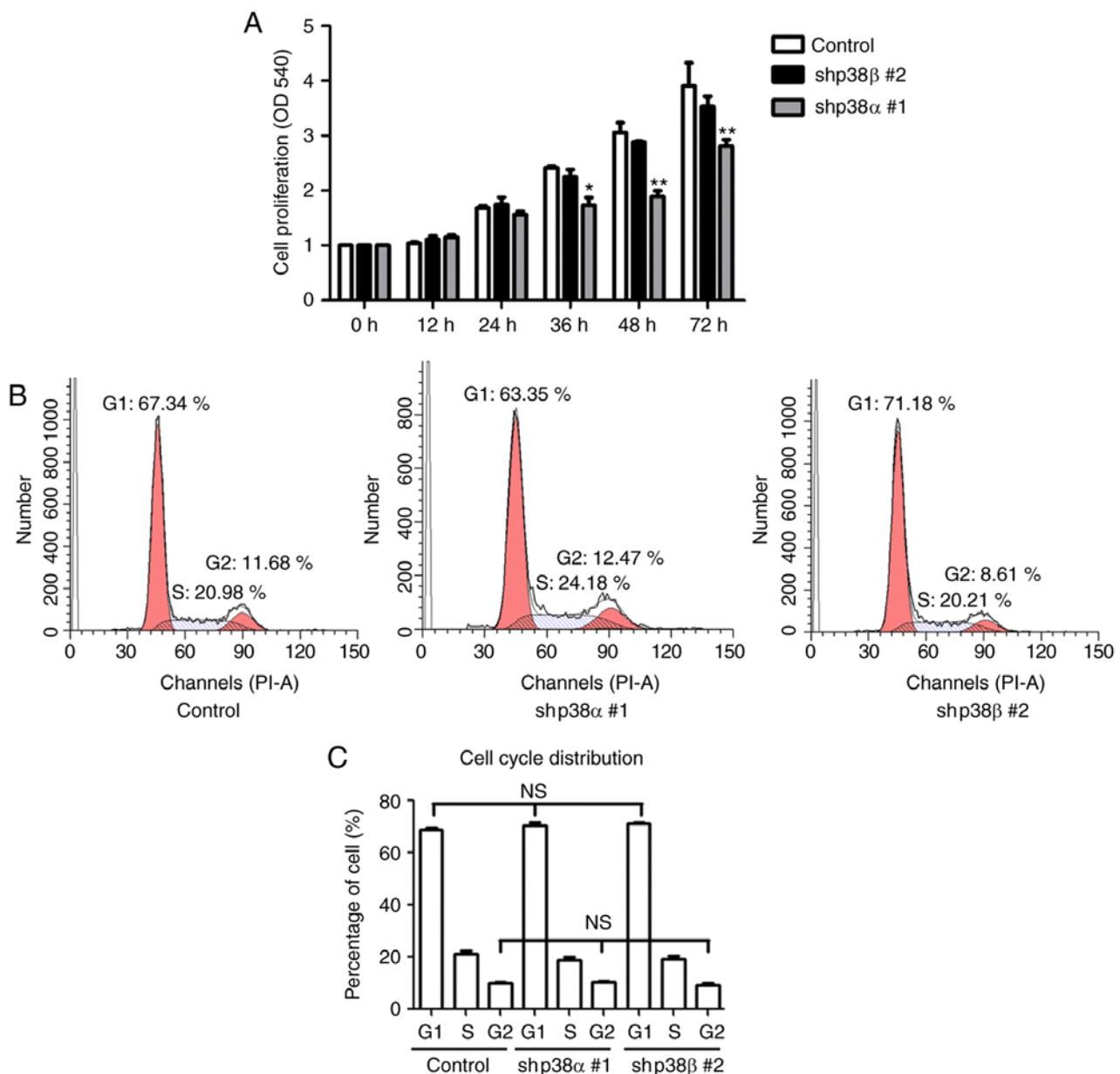


Figure 4. Knockdown of p38 $\alpha$  reduces cell proliferation but does not affect cell cycle arrest. (A) Cell proliferation was measured by MTT assay at different times as indicated in shp38 $\alpha$  #1 and shp38 $\beta$  #2 stable clone A375 cells. (B) Representative plots and (C) quantification of cell cycle phase distribution analysis in shp38 $\alpha$  #1 and shp38 $\beta$  #2 stable A375 cells. Data are presented as means  $\pm$  SD (n=3). \*P<0.05 and \*\*P<0.01 vs. control cells. sh, short hairpin; OD, optical density; PI, propidium iodide; ns, not significant.

wound healing and Transwell assays showed that the migration ability was impaired in shp38 $\alpha$ , but not in shp38 $\beta$ , stable clones. Taken together, these results indicated that p38 $\alpha$  enhanced melanoma EMT and migration ability.

Angiogenesis contributes to providing adequate blood supply to the tumor and subsequently enhances tumor growth and progression (48). VEGF is a pro-angiogenic stimulator that can bind specifically to different receptor tyrosine kinases, such as VEGFR1/2/3, and send angiogenic signals (49). A previous study has shown that p38 activation can enhance endothelial cell migration and tumor formation (50). However, the precise effect of different p38 isoforms on VEGF expression in melanoma remained unknown to date. The present study demonstrated that the knockdown of p38 $\alpha$ , but not p38 $\beta$ , decreased the expression levels of VEGF. In addition to promoting angiogenesis, p38 $\alpha$

has been shown to participate in regulating pro-inflammatory cytokines and chemokines (22,31). Whether p38 $\alpha$  enhances angiogenesis by upregulating pro-inflammatory cytokines or chemokines requires further study.

Uncontrolled proliferation is a characteristic of cancer (51). Therefore, cell cycle arrest or cellular senescence, which are considered a barrier to tumorigenesis, may inhibit proliferation (52,53). Having discovered that silencing p38 $\alpha$ , but not p38 $\beta$ , reduced cell proliferation, it was hypothesized that the inhibition of p38 $\alpha$  or p38 $\beta$  could affect the cell cycle. To examine this hypothesis, flow cytometry was used for cell cycle phase distribution analysis. However, the results demonstrated that neither the shp38 $\alpha$  nor the shp38 $\beta$  stable clonal cells had any changes in their percentages of cells in the G1/S and G2/M phases, indicating that cell cycle arrest was not the primary

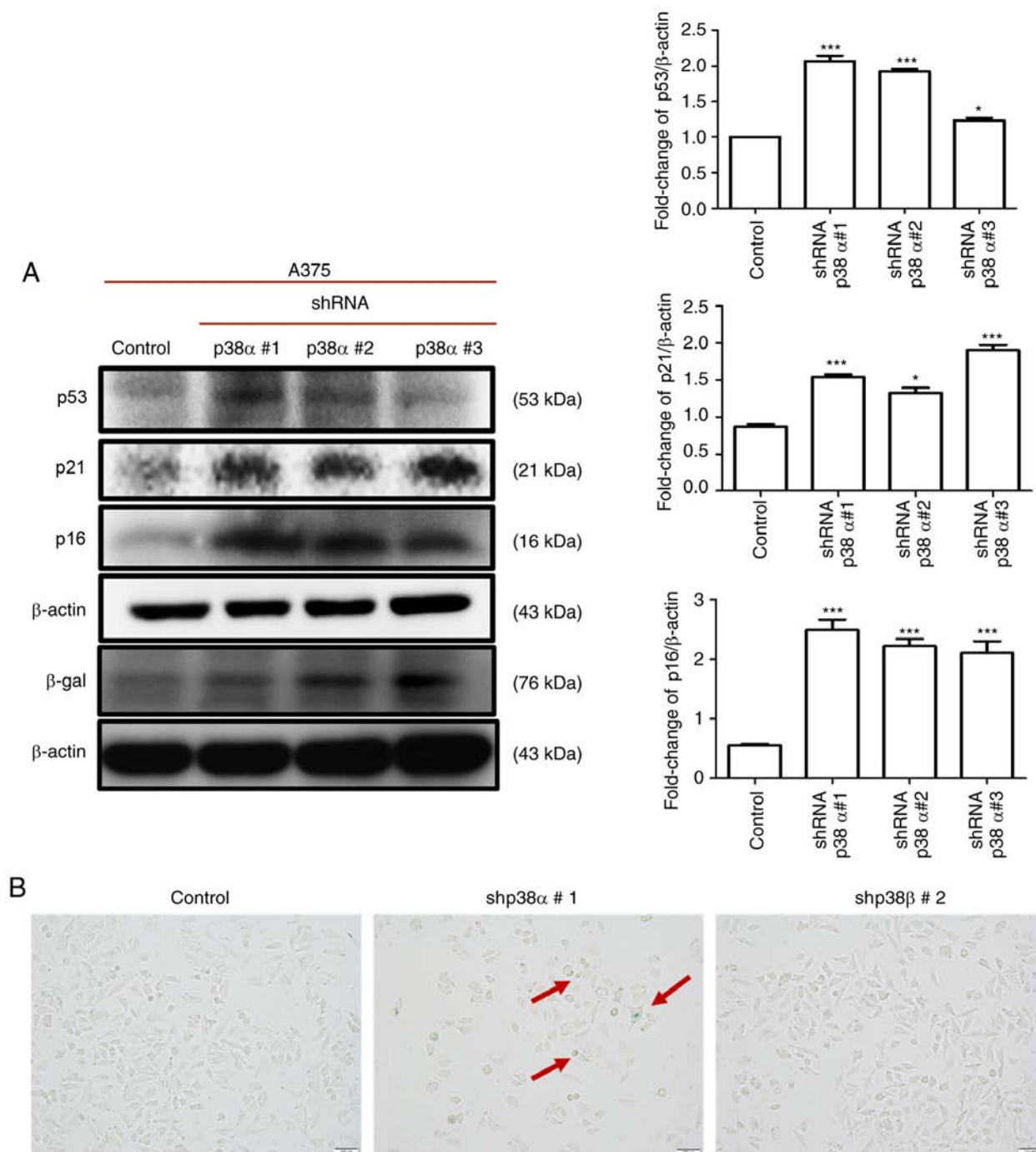


Figure 5. Knockdown of p38 $\alpha$  induces senescence-like features but not cell cycle arrest in A375 cells. (A) Western blot analysis of senescence markers in shp38 $\alpha$  stable clone cells.  $\beta$ -actin served as a loading control. (B) Cells (5,000 per well) were plated in triplicate and stained to detect senescence-associated  $\beta$ -gal activity. Positively stained cells (blue) are indicated by the red arrows. Data are presented as means  $\pm$  SD (n=3). \*P<0.05 and \*\*\*P<0.001 vs. control cells. sh, short hairpin;  $\beta$ -gal,  $\beta$ -galactosidase.

mechanism of growth reduction caused by p38 $\alpha$  knockdown. Thus, other mechanisms, that may be involved in proliferation reduction, need to be examined. In a previous study, overexpression of p16 and p14, which are markers of senescence, inhibited melanoma A375 cell proliferation, migration and invasion, and promoted apoptosis (54). Therefore, it was hypothesized that senescence activation in shp38 $\alpha$  stable clonal cells may be another mechanism that caused proliferation and EMT inhibition. The present results demonstrated that p38 $\alpha$  knockdown, but not p38 $\beta$ , induced cellular senescence. Together, these

findings suggested that p38 $\alpha$  served a crucial role in regulating cell proliferation and cellular senescence.

In conclusion, when comparing the roles of p38 $\alpha$  and p38 $\beta$ , only p38 $\alpha$  was identified to be significantly associated with regulating EMT and senescence in melanoma cells. In the present *in vitro* study, shRNA was used to specifically knockdown p38 $\alpha$  or p38 $\beta$  in the A375 melanoma cell line and the results revealed that only p38 $\alpha$  was a crucial factor in regulating cell proliferation and migration, suggesting that p38 $\alpha$  may have an oncogenic-maintaining role. The present



study highlighted the distinct and often opposing functions of the individual p38 MAPK isoforms in melanoma. These novel findings indicated that targeting p38 $\alpha$  may provide a potential strategy in treating melanoma.

## Acknowledgements

Not applicable.

## Funding

This study was supported by China Medical University Hospital (grant no. DMR-108-137).

## Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

## Authors' contributions

SYW and SCN conceived and designed the study. CJC, CYH and WWK performed the experiments. SCN wrote the manuscript. All authors have read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy and integrity of any part of the work are appropriately investigated and resolved.

## Ethics approval and consent to participate

Not applicable.

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare they have no competing interests.

## References

1. Siegel RL, Miller KD and Jemal A: Cancer statistics, 2017. *CA Cancer J Clin* 67: 7-30, 2017.
2. Fogel AL, Jaju PD, Li S, Halpern-Felsher B, Tang JY and Sarin KY: Factors influencing and modifying the decision to pursue genetic testing for skin cancer risk. *J Am Acad Dermatol* 76: 829-835, 2017.
3. Bradford PT: Skin cancer in skin of color. *Dermatol Nurs* 21: 170-177, 2009.
4. Koul HK, Pal M and Koul S: Role of p38 MAP kinase signal transduction in solid tumors. *Genes Cancer* 4: 342-359, 2013.
5. Cuenda A and Rousseau S: P38 MAP-kinases pathway regulation, function and role in human diseases. *Biochim Biophys Acta* 1773: 1358-1375, 2007.
6. Cuadrado A and Nebreda AR: Mechanisms and functions of p38 MAPK signalling. *Biochem J* 429: 403-417, 2010.
7. Patel SB, Cameron PM, O'Keefe SJ, Frantz-Wattley B, Thompson J, O'Neill EA, Tennis T, Liu L, Becker JW and Scapin G: The three-dimensional structure of MAP kinase p38beta: Different features of the ATP-binding site in p38beta compared with p38alpha. *Acta Crystallogr D Biol Crystallogr* 65: 777-785, 2009.
8. Katz M, Amit I and Yarden Y: Regulation of MAPKs by growth factors and receptor tyrosine kinases. *Biochim Biophys Acta* 1773: 1161-1176, 2007.
9. Bulavin DV and Fornace AJ Jr: P38 MAP kinase's emerging role as a tumor suppressor. *Adv Cancer Res* 92: 95-118, 2004.
10. Igea A and Nebreda AR: The stress kinase p38 $\alpha$  as a target for cancer therapy. *Cancer Res* 75: 3997-4002, 2015.
11. Wagner EF and Nebreda AR: Signal integration by JNK and p38 MAPK pathways in cancer development. *Nat Rev Cancer* 9: 537-549, 2009.
12. Gupta J, del Barco Barrantes I, Igea A, Sakellariou S, Pateras IS, Gorgoulis VG and Nebreda AR: Dual function of p38 $\alpha$  MAPK in colon cancer: Suppression of colitis-associated tumor initiation but requirement for cancer cell survival. *Cancer Cell* 25: 484-500, 2014.
13. Chiacchiera F, Matrone A, Ferrari E, Ingravalle G, Lo Sasso G, Murzilli S, Petruzzelli M, Salvatore L, Moschetta A and Simone C: P38alpha blockade inhibits colorectal cancer growth in vivo by inducing a switch from HIF1alpha- to FoxO-dependent transcription. *Cell Death Differ* 16: 1203-1214, 2009.
14. Kalluri R and Weinberg RA: The basics of epithelial-mesenchymal transition. *J Clin Invest* 119: 1420-1428, 2009.
15. Kalluri R and Neilson EG: Epithelial-mesenchymal transition and its implications for fibrosis. *J Clin Invest* 112: 1776-1784, 2003.
16. Hay ED: An overview of epithelio-mesenchymal transformation. *Acta Anat (Basel)* 154: 8-20, 1995.
17. Lee JM, Dedhar S, Kalluri R and Thompson EW: The epithelial-mesenchymal transition: New insights in signaling, development, and disease. *J Cell Biol* 172: 973-981, 2006.
18. Zeisberg M and Neilson EG: Biomarkers for epithelial-mesenchymal transitions. *J Clin Invest* 119: 1429-1437, 2009.
19. Voutsadakis IA: The network of pluripotency, epithelial-mesenchymal transition, and prognosis of breast cancer. *Breast Cancer (Dove Med Press)* 7: 303-319, 2015.
20. Liu CY, Lin HH, Tang MJ and Wang YK: Vimentin contributes to epithelial-mesenchymal transition cancer cell mechanics by mediating cytoskeletal organization and focal adhesion maturation. *Oncotarget* 6: 15966-15983, 2015.
21. Bauvois B: New facets of matrix metalloproteinases MMP-2 and MMP-9 as cell surface transducers: Outside-in signaling and relationship to tumor progression. *Biochim Biophys Acta* 1825: 29-36, 2012.
22. Linnskog R, Jonsson G, Axelsson L, Prasad CP and Andersson T: Interleukin-6 drives melanoma cell motility through p38 $\alpha$ -MAPK-dependent up-regulation of WNT5A expression. *Mol Oncol* 8: 1365-1378, 2014.
23. Yan Q, Bach DQ, Gatla N, Sun P, Liu JW, Lu JY, Paller AS and Wang XQ: Deacetylated GM3 promotes uPAR-associated membrane molecular complex to activate p38 MAPK in metastatic melanoma. *Mol Cancer Res* 11: 665-675, 2013.
24. del Barco Barrantes I and Nebreda AR: Roles of p38 MAPKs in invasion and metastasis. *Biochem Soc Trans* 40: 79-84, 2012.
25. Lee WH, Liu FH, Lee YL and Huang HM: Interferon-alpha induces the growth inhibition of human T-cell leukaemia line jurkat through p38alpha and p38beta. *J Biochem* 147: 645-650, 2010.
26. Hale KK, Trollinger D, Rihaneck M and Manthey CL: Differential expression and activation of p38 mitogen-activated protein kinase alpha, beta, gamma, and delta in inflammatory cell lineages. *J Immunol* 162: 4246-4252, 1999.
27. Kuma Y, Campbell DG and Cuenda A: Identification of glycogen synthase as a new substrate for stress-activated protein kinase 2b/p38beta. *Biochem J* 379: 133-139, 2004.
28. Lee MR, Lin C, Lu CC, Kuo SC, Tsao JW, Juan YN, Chiu HY, Lee FY, Yang JS and Tsai FJ: YC-1 induces G0/G1 phase arrest and mitochondria-dependent apoptosis in cisplatin-resistant human oral cancer CAR cells. *Biomedicine (Taipei)* 7: 12, 2017.
29. Su D, Zhu S, Han X, Feng Y, Huang H, Ren G, Pan L, Zhang Y, Lu J and Huang B: BMP4-Smad signaling pathway mediates adriamycin-induced premature senescence in lung cancer cells. *J Biol Chem* 284: 12153-12164, 2009.
30. Piazza VG, Bartke A, Miquet JG and Sotelo AI: Analysis of different approaches for the selection of reference genes in RT-qPCR experiments: A case study in skeletal muscle of growing mice. *Int J Mol Sci* 18: E1060, 2017.
31. Linnskog R, Mohapatra P, Moradi F, Prasad CP and Andersson T: Demonstration of a WNT5A-IL-6 positive feedback loop in melanoma cells: Dual interference of this loop more effectively impairs melanoma cell invasion. *Oncotarget* 7: 37790-37802, 2016.
32. Liu K, Yu D, Cho YY, Bode AM, Ma W, Yao K, Li S, Li J, Bowden GT, Dong Z and Dong Z: Sunlight UV-induced skin cancer relies upon activation of the p38 $\alpha$  signaling pathway. *Cancer Res* 73: 2181-2188, 2013.

33. Liu H, He J and Yang J: Tumor cell p38 MAPK: A trigger of cancer bone osteolysis. *Cancer Cell Microenviron* 2: e464, 2015.
34. Gonzalez-Villasana V, Fuentes-Mattei E, Ivan C, Dalton HJ, Rodriguez-Aguayo C, Fernandez-de Thomas RJ, Aslan B, Del C Monroig P, Velazquez-Torres G, Previs RA, *et al*: Rac1/Pak1/p38/MMP-2 axis regulates angiogenesis in ovarian cancer. *Clin Cancer Res* 21: 2127-2137, 2015.
35. Loesch M and Chen G: The p38 MAPK stress pathway as a tumor suppressor or more? *Front Biosci* 13: 3581-3593, 2008.
36. Ding XZ and Adrian TE: MEK/ERK-mediated proliferation is negatively regulated by P38 map kinase in the human pancreatic cancer cell line, PANC-1. *Biochem Biophys Res Commun* 282: 447-453, 2001.
37. Cocolakis E, Lemay S, Ali S and Lebrun JJ: The p38 MAPK pathway is required for cell growth inhibition of human breast cancer cells in response to activin. *J Biol Chem* 276: 18430-18436, 2001.
38. Grossi V, Peserico A, Tezil T and Simone C: p38 $\alpha$  MAPK pathway: A key factor in colorectal cancer therapy and chemoresistance. *World J Gastroenterol* 20: 9744-9758, 2014.
39. Tai TW, Su FC, Chen CY, Jou IM and Lin CF: Activation of p38 MAPK-regulated Bcl-xL signaling increases survival against zoledronic acid-induced apoptosis in osteoclast precursors. *Bone* 67: 166-174, 2014.
40. Dreissigacker U, Mueller MS, Unger M, Siegert P, Genze F, Gierschik P and Giehl K: Oncogenic K-Ras down-regulates Rac1 and RhoA activity and enhances migration and invasion of pancreatic carcinoma cells through activation of p38. *Cell Signal* 18: 1156-1168, 2006.
41. Thiery JP and Sleeman JP: Complex networks orchestrate epithelial-mesenchymal transitions. *Nat Rev Mol Cell Biol* 7: 131-142, 2006.
42. Nakamura M and Tokura Y: Epithelial-mesenchymal transition in the skin. *J Dermatol Sci* 61: 7-13, 2011.
43. Campbell RM, Anderson BD, Brooks NA, Brooks HB, Chan EM, De Dios A, Gilmour R, Graff JR, Jambrina E, Mader M, *et al*: Characterization of LY2228820 dimesylate, a potent and selective inhibitor of p38 MAPK with antitumor activity. *Mol Cancer Ther* 13: 364-374, 2014.
44. Pereira L, Igea A, Canovas B, Dolado I and Nebreda AR: Inhibition of p38 MAPK sensitizes tumour cells to cisplatin-induced apoptosis mediated by reactive oxygen species and JNK. *EMBO Mol Med* 5: 1759-1774, 2013.
45. Ventura JJ, Tenbaum S, Perdiguero E, Huth M, Guerra C, Barbacid M, Pasparakis M and Nebreda AR: p38alpha MAP kinase is essential in lung stem and progenitor cell proliferation and differentiation. *Nat Genet* 39: 750-758, 2007.
46. Hui L, Bakiri L, Mairhorfer A, Schweifer N, Haslinger C, Kenner L, Komnenovic V, Scheuch H, Beug H and Wagner EF: p38alpha suppresses normal and cancer cell proliferation by antagonizing the JNK-c-Jun pathway. *Nat Genet* 39: 741-749, 2007.
47. Sakurai T, He G, Matsuzawa A, Yu GY, Maeda S, Hardiman G and Karin M: Hepatocyte necrosis induced by oxidative stress and IL-1 alpha release mediate carcinogen-induced compensatory proliferation and liver tumorigenesis. *Cancer Cell* 14: 156-165, 2008.
48. Rajabi M and Mousa SA: The role of angiogenesis in cancer treatment. *Biomedicines* 5: E34, 2017.
49. Mehrad B, Keane MP and Strieter RM: Chemokines as mediators of angiogenesis. *Thromb Haemost* 97: 755-762, 2007.
50. Yoshizuka N, Chen RM, Xu Z, Liao R, Hong L, Hu WY, Yu G, Han J, Chen L and Sun P: A novel function of p38-regulated/activated kinase in endothelial cell migration and tumor angiogenesis. *Mol Cell Biol* 32: 606-618, 2012.
51. Fouad YA and Aanei C: Revisiting the hallmarks of cancer. *Am J Cancer Res* 7: 1016-1036, 2017.
52. Cairney CJ, Bilsland AE, Evans TR, Roffey J, Bennett DC, Narita M, Torrance CJ and Keith WN: Cancer cell senescence: A new frontier in drug development. *Drug Discov Today* 17: 269-276, 2012.
53. Prieur A and Peeper DS: Cellular senescence in vivo: A barrier to tumorigenesis. *Curr Opin Cell Biol* 20: 150-155, 2008.
54. Bai M, Yu NZ, Long F, Feng C and Wang XJ: Effects of CDKN2A (p16INK4A/p14ARF) over-expression on proliferation and migration of human melanoma A375 Cells. *Cell Physiol Biochem* 40: 1367-1376, 2016.



This work is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International (CC BY-NC-ND 4.0) License.