Roles of p38α and p38β mitogen-activated protein kinase isoforms in human malignant melanoma A375 cells

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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 p38α and p38β, the present findings demonstrated that p38α was a crucial factor in regulating cell migration in the A375 melanoma cell line. Silencing p38α downregulated the expression of epithelial-mesenchymal transition markers, such as matrix metallopeptidase (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 p38α silencing impaired vascular endothelial growth factor expression, which regulates tumor angiogenesis. Furthermore, p38α knockdown inhibited cell proliferation in melanoma cells. In addition, silencing p38α induced senescence-like features, but not cell cycle arrest. Expression of the senescence markers p16, p21, p53 and β-galactosidase was upregulated, and an increase in the number of senescence-associated β-galactosidase-positive cells was observed in a p38α knockdown stable clone. However, no significant difference was found between control and p38β stable knockdown cells. Taken together, the present results suggested that p38α knockdown impaired migration and proliferation, and increased senescence, in A375 melanoma cells. However, p38β may not be involved in melanoma tumorigenesis. Therefore, targeting p38α may be a valuable approach towards inhibiting tumor growth and metastasis in patients with melanoma.

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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: p38α (also known as MAPK14), p38β (also known as MAPK 11), p38γ (also known as MAPK 12), and p38δ (also known as MAPK13) (5). Regarding structural similarity, p38α and p38β are 75% similar, while p38γ and p38δ are 70% similar (5). P38α and p38β are universally expressed, while p38γ and p38δ are more tissue-specific (6). Regarding kinase inhibition, p38α and p38β can be targeted by a class of pyridinyl imidazole drugs, but these drugs cannot inhibit p38γ and p38δ (7). However, this type of chemical inhibition does not allow us to distinguish whether functions are mediated by p38α or p38β. In the present study, specific short hairpin (sh)RNA or small interfering (si)RNA were used in order to knockdown p38α or p38β and to clearly differentiate their biological functions.

P38α is an essential protein during embryonic development, and it can regulate various cellular functions. Notably, multiple proteins can be directly phosphorylated by p38α. Additionally, the p38α pathway can control the production of different extracellular signaling molecules, such as growth factors, cytokines and chemokines (8). The p38α protein can regulate different cellular functions during tumor formation at different stages of development and for different types of cancers. P38α 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, p38α 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, p38α 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 (Snai1, 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 metallopeptidases (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 p38α or p38β could be involved in EMT or MET in A375 melanoma cells remains unknown.

A previous study indicated that p38α may act as a tumor promoter and enhance melanoma cell metastasis under cytokine stimulation (22,23). Therefore, p38α can regulate the migration abilities of tumor cells, as well as EMT processes (24). In other types of cancer, p38α has been reported to regulate cell proliferation, differentiation, apoptosis and numerous cellular processes, but in melanoma, its precise role remains unknown. In addition, p38β has been demonstrated to regulate cell apoptosis, differentiation and metabolism (25-27). However, the functions of p38β, which shares 75% structural similarity with p38α, have not been investigated in melanoma. Therefore, the goal of the present study was to identify the biological functions of p38α and p38β.

The present study investigated the roles of p38α and p38β 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 p38α, but not p38β, may regulate melanoma cell proliferation and migration. Thus, p38α 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 μg/ml penicillin, and 100 μg/ml streptomycin in humidified air (5% CO_2) 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 μg), pCMVΔr89.1 (4.5 μg) and p38α (5 μg) or p38β (5 μg) 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 μg/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 μg/ml puromycin.

The shRNA oligoribonucleotide sequences were as follows: p38α #1, GTTCAGTTCCTTATCTACCAA; p38α #2, CCATGAGGCAAGAAACTATAT; p38α, #3 GCCGTATAG GATGTCAGACAA; p38β #1, GCCACGTCCATCGAGGAC TTC; p38β #2 CCTGTCCTCTTCTGGCTACTG; and p38β, #3, CGGCTCCGTCTGTTCGGCCTA.

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). p38α siRNA (cat. no. MAPK14_Hs01_00018467), p38β 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 x 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 x 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 μg) 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-p38α (clone A1F7; cat. no. sc-33688; Santa Cruz Biotechnology, Inc.), anti-p38β (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-p16 (cat. no. 10883-1-AP; ProteinTech Group, Inc.) and anti-β-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-IgGκ 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 (shp38α #1 and shp38β #2 stable clones; $2x10^4$ cells/ml) were inoculated in 96-well plates in 200 μl of medium containing 10% FBS. After 12, 24, 36, 48 and 72 h, 20 μl 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 β-galactosidase (SA-β-gal) staining. The assay was performed using the Senescence β-Galactosidase Staining kit (cat. no. 9680; Cell Signaling Technology, Inc.), according to the manufacturer's instructions (29). SA-β-gal staining was performed to determine the percentage of SA-Gal-positive cells. A375 human melanoma cells (shp38α #1 and shp38β#2 stable clones; $1x10^5$ cells/ml) were seeded on 6-well plates for 24 h. The cells were then fixed with 4% para-formaldehyde 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 x100 magnification.

RNA extraction and reverse transcription-quantitative PCR (RT-qPCR). A375 non-infected control, shp38α and shp38β cell lines were collected by trypsinization. RNA was then extracted using the Direct-zol™ RNA MiniPrep kit (Zymo Research Corporation). RNA samples (1 μg) were reverse transcribed into cDNA. An aliquot of RNA was incubated with 0.5 μg 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 μl, and included 2 μl of cDNA, 5 μl of SYBR-Green, 0.5 μl of 10 μM forward primer and reverse primer, and 2 μl of ddH$_2$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™ 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 $\Delta Cq=Cq_{interest\ gene}-Cq_{GAPDH}$) (30). The primers were as follows: p38α, forward ACCTGTCTCCAG TGGGCTCT and reverse CACGTAACCCCGTTTTTGTG; p38β, 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 (shp38α #1 and shp38β #2 stable clones) were seeded at a density of $3x10^4$ cells/well in a 48-well Boyden chamber (Neuro Probe Inc.) plate; the polycarbonate

membrane filters had 8 μm 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 μg/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 p38α or p38β 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 μg/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 p38α and p38β silencing efficiency in the A375 melanoma cell line. To determine the silencing efficiency, the mRNA and protein expression levels of p38α and p38β were measured following knockdown using shRNA (Fig. 1). The silencing specificity of the shRNA against p38α and p38β was evaluated by RT-qPCR to determine mRNA levels and by western blotting to determine protein levels. As shown in Fig. 1A and C, p38α and p38β protein levels were significantly decreased in p38α and p38β shRNA stable clone cells. Similar results were obtained for the mRNA expression levels (Fig. 1B and D). The clonal lines shp38α #1 and shp38β#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 p38α shRNA not affecting the p38β mRNA and protein levels, and the p38β shRNA not affecting the p38α levels Fig. 1).

Knockdown of p38α, but not p38β, reverses EMT and impairs VEGF expression in A375 melanoma cells. It has been reported that p38α 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 p38α silencing. Furthermore, the protein levels of EMT markers, including Twist, Snai1, vimentin, MMP2 and MMP9, were decreased after p38α silencing. Of note, VEGF, which acts as an angiogenesis and metastasis mediator, was downregulated after p38α shRNA transduction (Fig. 2A). By contrast, there were no obvious changes in MET, EMT or angiogenesis markers in cells transduced with p38β shRNA compared with those in control cells (Fig. 2B). Furthermore, the EMT markers MMP2 and MMP9 were downregulated in p38α, but not p38β, 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 p38α triggered a molecular switch from a mesenchymal phenotype to an epithelial-like phenotype in A375 melanoma cells. However, p38β was not involved in the regulation of EMT and angiogenesis-related gene expression.

Effects of silencing p38α or p38β on A375 cell migration. p38α silencing has been shown to inhibit melanoma cell motility under IL-6 stimulation (22,31); however, no study has investigated the migration ability after p38α silencing and without cytokine stimulation. The present study examined the migration ability of p38α shRNA and p38β shRNA A375 stable clonal cell lines by wound healing and Transwell assays. As presented in Fig. 3A and B, silencing p38α via shRNA significantly reduced the migration ability of A375 cells. By contrast, in the p38β 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 p38α and p38β-specific siRNAs into B16F0 melanoma cells, and the knockdown efficiencies were analyzed by western blotting (Fig. S2A). Knockdown of p38α, but not p38β, inhibited B16F0 melanoma cell migration (Fig. S2B). These findings indicated that p38α, but not p38β, was crucial for the migration ability of A375 human melanoma cells.

Knockdown of p38α inhibits cell proliferation but not cell cycle progression. To analyze cell proliferation, an MTT assay was performed. The results demonstrated that the p38α shRNA stable clone had a lower proliferation rate compared with control cells (Fig. 4A). By contrast, no significant changes
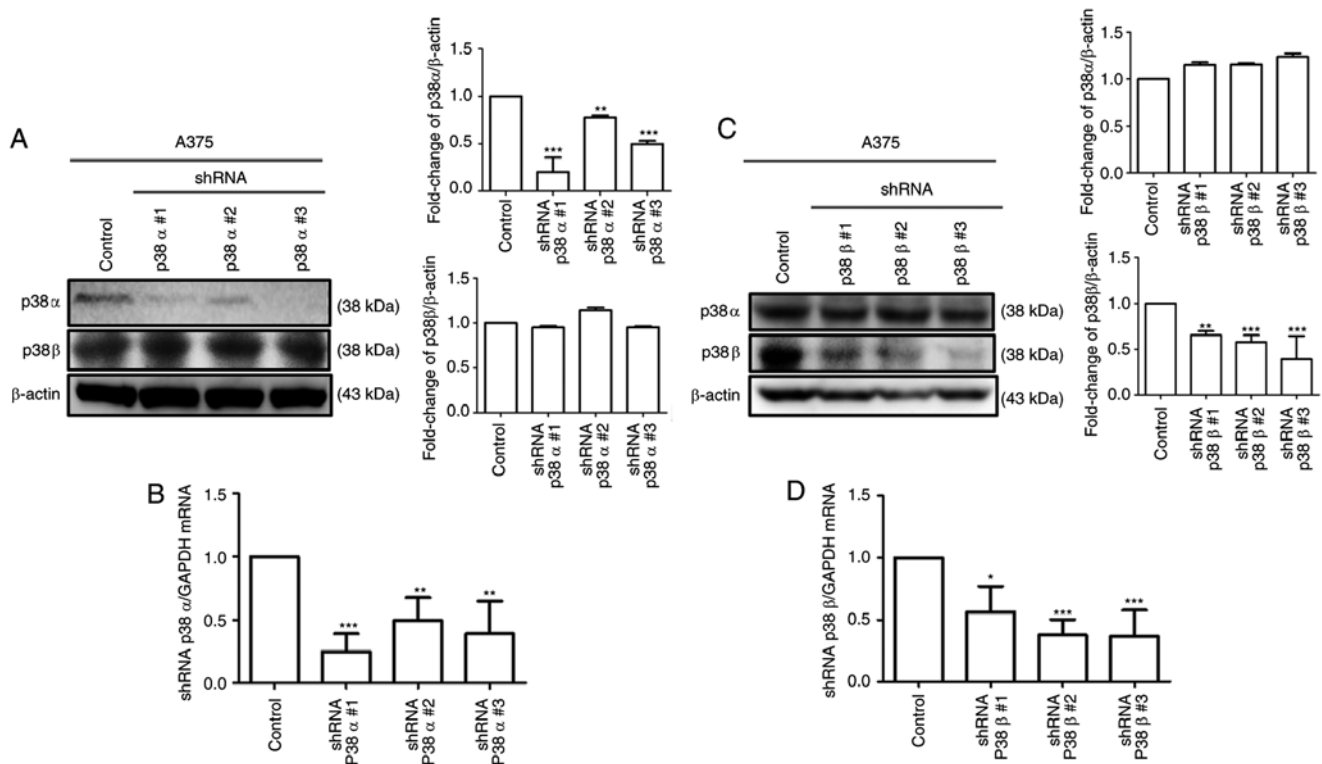
Figure 1. p38α and p38β silencing efficiency in the A375 melanoma cell line. A375 cells were infected with lentiviruses containing p38α-specific shRNAs #1, #2 and #3 or p38β-specific shRNAs #1, #2, and #3. (A) Protein expression levels of p38α. (B) mRNA expression levels of p38α. (C) Protein expression 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

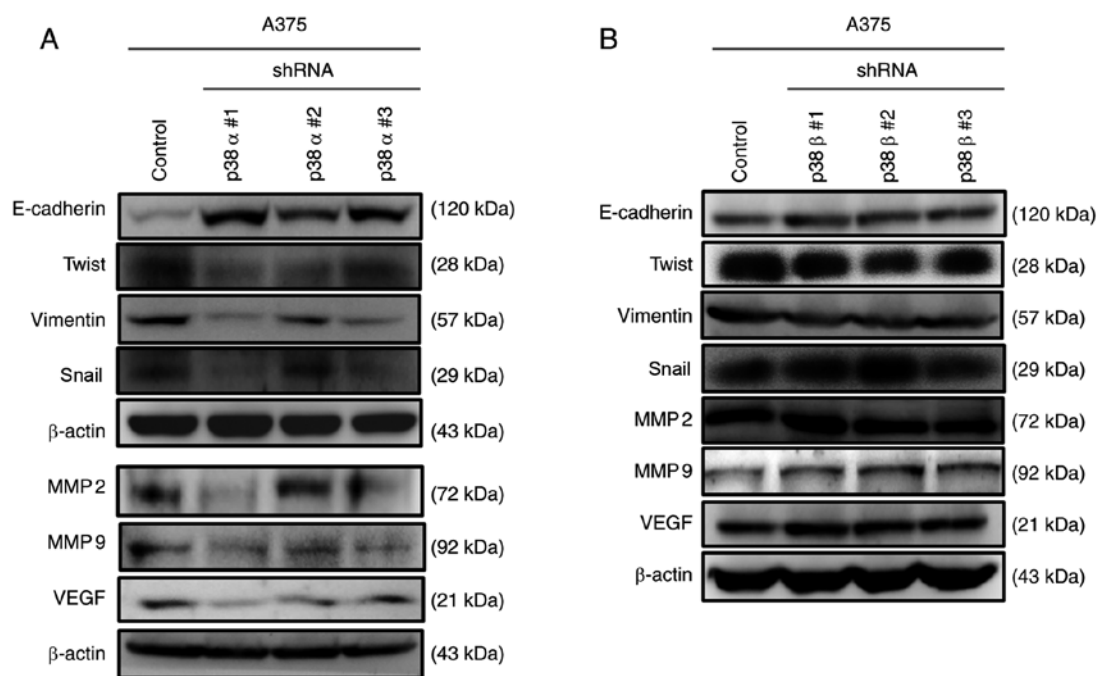Figure 2. Effects of p38α and p38β knockdown on EMT marker and VEGF expression in A375 melanoma cells. (A) The protein expression levels of EMT and angiogenesis markers were determined in p38α-silenced clonal cells and in (B) p38β-silenced clonal cells by western blotting. β-actin served as a loading control. Twist, twist family bHLH transcription factor 1; Snai1, snail family transcriptional repressor 1; MMP, matrix metallopeptidase; VEGF, vascular endothelial growth factor; sh, short hairpin.
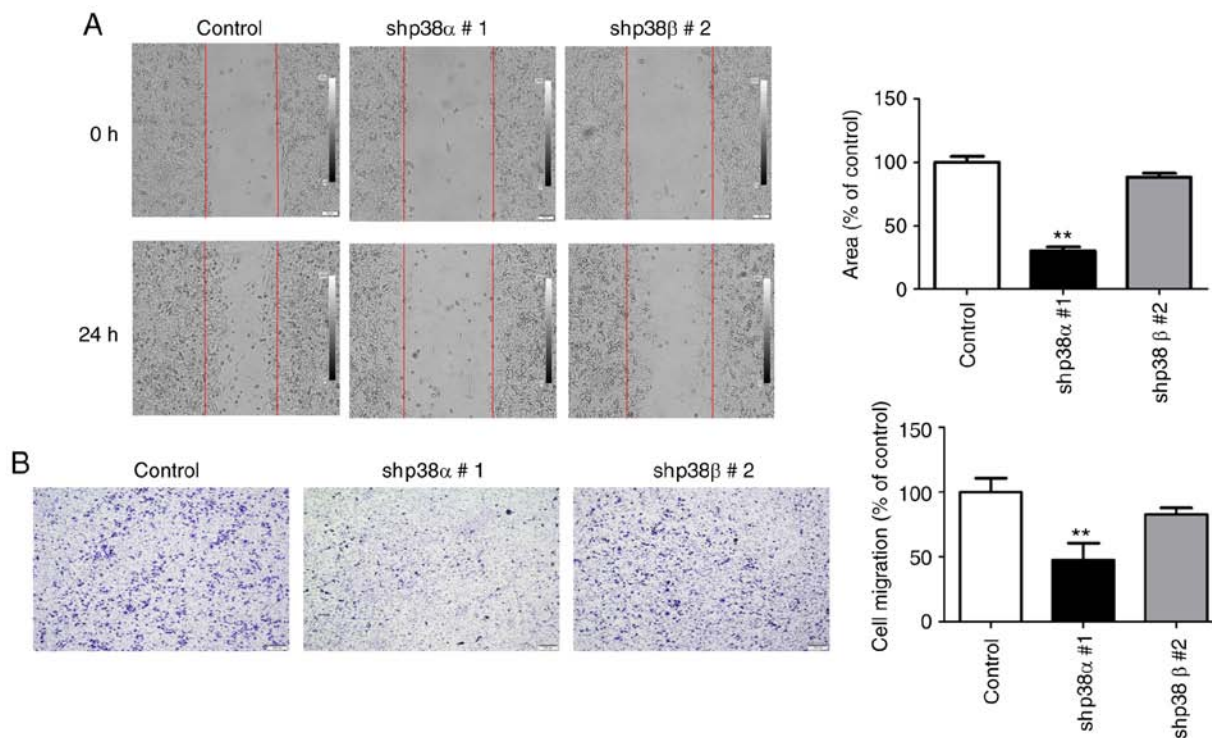

Figure 3. Effects of p38α or p38β knockdown on cell migration abilities in A375 melanoma cells. (A) A single scratch was created in confluent monolayers of shp38α #1 or shp38β #2 stable clone A375 cells. Mitomycin C (5 µg/ml) was added to inhibit cell proliferation. The scratches were photographed at 0 and 24 h. The relative migration was analyzed by ImageJ software. (B) Migration capacity was determined by Transwell assay in shp38α #1 and shp38β #2 stable clone A375 cells. Representative photographs and quantification of the migrated cells are shown. Data are presented as means ± 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 p38α, but not p38β, 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
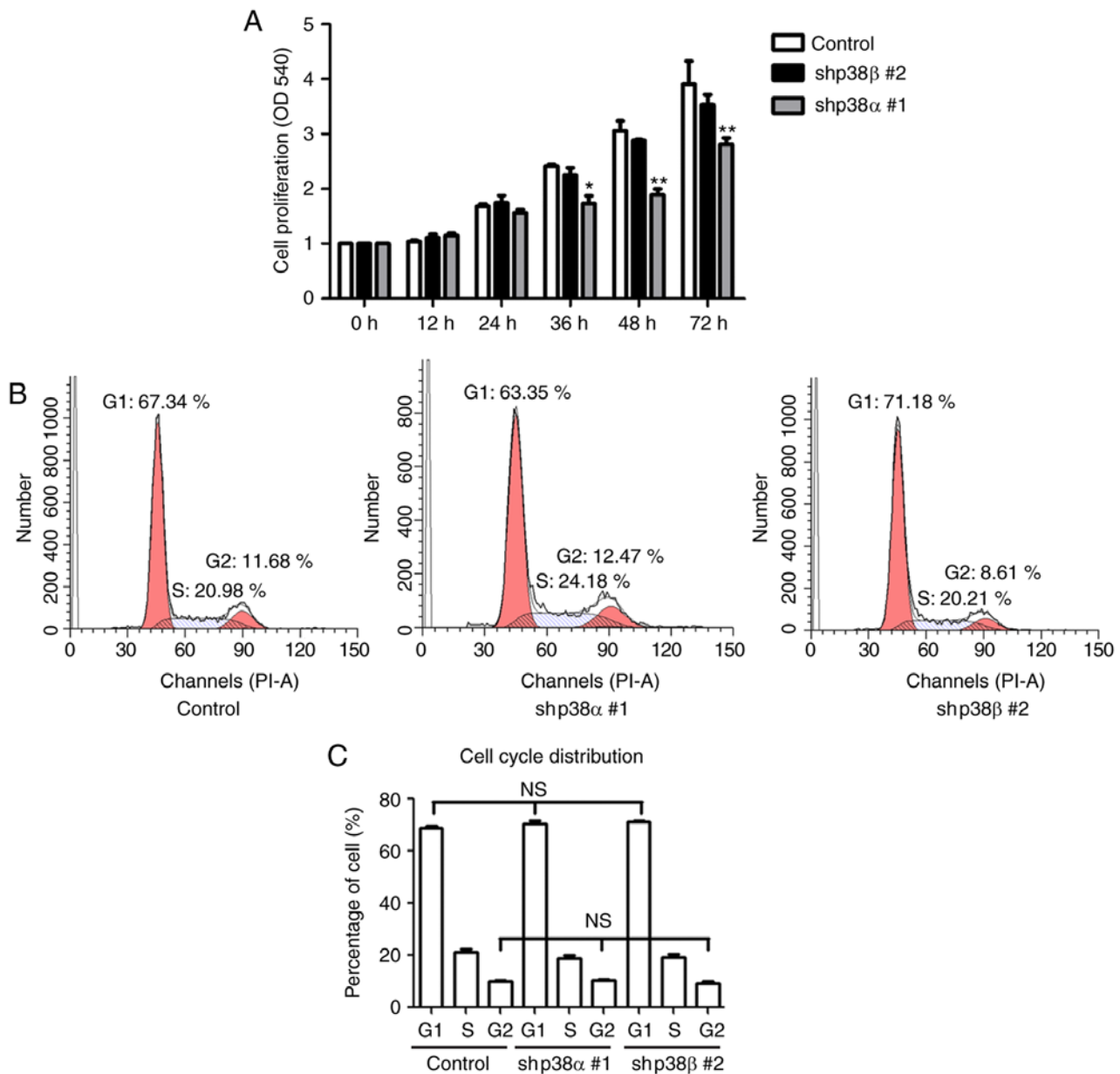
Figure 4. Knockdown of p38α reduces cell proliferation but does not affect cell cycle arrest. (A) Cell proliferation was measured by MTT assay at different times as indicated in shp38α #1 and shp38β #2 stable clone A375 cells. (B) Representative plots and (C) quantification of cell cycle phase distribution analysis in shp38α #1 and shp38β #2 stable A375 cells. Data are presented as means ± 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 shp38α, but not in shp38β, stable clones. Taken together, these results indicated that p38α 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 p38α, but not p38β, decreased the expression levels of VEGF. In addition to promoting angiogenesis, p38α

has been shown to participate in regulating pro-inflammatory cytokines and chemokines (22,31). Whether p38α 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 p38α, but not p38β, reduced cell proliferation, it was hypothesized that the inhibition of p38α or p38β 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 shp38α nor the shp38β 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
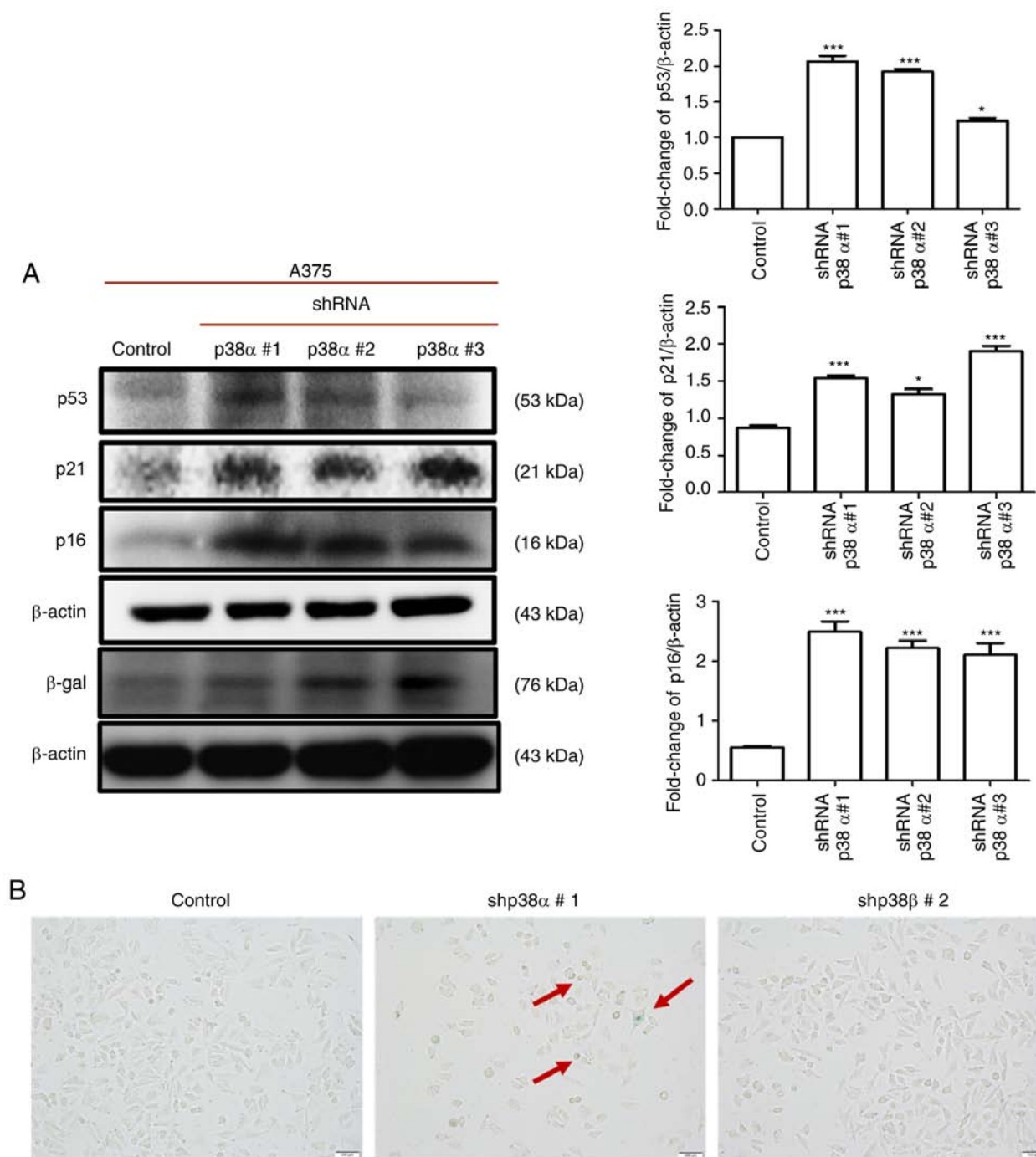
Figure 5. Knockdown of p38α induces senescence-like features but not cell cycle arrest in A375 cells. (A) Western blot analysis of senescence markers in shp38α stable clone cells. β-actin served as a loading control. (B) Cells (5,000 per well) were plated in triplicate and stained to detect senescence-associated β-gal activity. Positively stained cells (blue) are indicated by the red arrows. Data are presented as means ± SD (n=3). *P<0.05 and ***P<0.001 vs. control cells. sh, short hairpin; β-gal, β-galactosidase.

mechanism of growth reduction caused by p38α 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 shp38α stable clonal cells may be another mechanism that caused proliferation and EMT inhibition. The present results demonstrated that p38α knockdown, but not p38β, induced cellular senescence. Together, these

findings suggested that p38α served a crucial role in regulating cell proliferation and cellular senescence.

In conclusion, when comparing the roles of p38α and p38β, only p38α 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 p38α or p38β in the A375 melanoma cell line and the results revealed that only p38α was a crucial factor in regulating cell proliferation and migration, suggesting that p38α 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 p38α may provide a potential strategy in treating melanoma.

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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.

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