Overexpression of Dock180 and Elmo1 in melanoma is associated with cell survival and migration

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Abstract

Melanoma is one of the most aggressive and metastatic skin cancers, of these metastatic melanoma is a lethal disease with invasive behavior. Although overexpression of Dock180 and Elmo1 has been identified in various cancers, including glioma, ovarian cancer, hepatocellular carcinoma, and breast cancer, the expression and functions of them in melanoma remain unknown. Therefore, the purpose of this study is to confirm the expression of Dock180 and Elmo1, their underlying mechanisms, and roles in melanoma. Both immunohistochemical staining and Western blotting were used to confirm expression of Dock180 and Elmo1 in human melanoma and normal skin. To identify roles of Dock180 and Elmo1 in cell survival, apoptosis and migration, downregulation of Dock180 or Elmo1 in melanoma cells treated with siRNA was performed in Cell viability assay, Phase contrast images, DAPI staining, Cell cycle analysis, Apoptosis assay, Wound healing assay, Colony formation assay, and Western blotting. We identified overexpression of Dock180 and Elmo1 in human melanoma compared to normal skin *ex vivo*. Inhibition of Dock180 or Elmo1 following siRNA treatment in melanoma cells reduced cell viability and increased apoptosis as supported by increased proportion of cells with Annexin V-PE(+) staining and sub-G_0/G_1 peak in cell cycle analysis. Moreover, inhibition of Dock180 or Elmo1 regulated apoptosis-related proteins, showing downregulaton of Bcl-2, caspase-3, and PARP and upregulation of Bax, PUMA, cleaved caspase3, and cleaved PARP. Furthermore, knockdown of Dock180 and Elmo1 in melanoma cells reduced cell migration and changed cellular signaling pathways including ERK and AKT. Vemurafenib treatment decreased cell viability in a concentration-dependent manner, while transfection with Dock180- or Elmo1-specific siRNA in melanoma cells significantly reduced cell viability compared to non-transfected cells. Collectively, these findings suggest that both Dock180 and Elmo1 may be associated with cancer progression such as cell survival and migration in melanoma, and can be potential targets for treatment of melanoma.

Introduction

Melanoma is one of the most aggressive and metastatic skin cancers. Melanoma can undergo rapid systemic dissemination, resulting in metastatic melanoma that is usually lethal. Although the average five-year survival rate for all stages of melanoma was 92 percent in United States, it was reduced to 64 and 23 percent when melanoma migrated to the lymph nodes and distant organs, respectively [1]. The five-year survival rate of patients with metastatic melanoma is less than 15 percent [2]. Melanoma possesses migration and invasion mechanisms to avoid detection by the body's surveillance system [3], thus facilitating further metastatic spread. In addition, there have been reports suggesting that metastasis of melanoma is involved in genetic mutations and microenvironment of cancer cells, induced by upregulation of proteins related to cancer invasion and infiltration [4–9].

The Rho family proteins, including Rac1, are small guanosine triphosphatase (GTPases). As key regulators of actin cytoskeletal dynamics, they play important roles in transducing various signals from many stimuli to downstream factors which control cell migration and invasion [10]. The dedicator of the cytokinesis I (Dock180) superfamily of proteins has been known as a novel guanine nucleotide exchange

factor (GEF) for Rho GTPases [11]. Nucleotide exchange of Rac1 is provoked by Dock180 via its unconventional Docker GEF domain [12–14]. However, it is necessary for binding to the engulfment and cell motility protein 1 (Elmo1) to accomplish the exchange of guanosine diphosphate/guanosine triphosphate (GDP/GTP) on Rac1[12]. A previous study reported that Elmo1 inhibited the ubiquitination of Dock180 [15]. Moreover, the complex of Dock180 and Elmo1 acts upstream of Rac1 to facilitate cell migration [16]. Also, it has been suggested that Dock180 and Elmo1 can be associated with cell survival [17]. Recently, some studies reported that Dock180 and Elmo1 were implicated in cancers, demonstrating that Dock180 and Elmo1 promoted human glioma cell invasion, and overexpression of Dock180 was related to aggressive phenotype of ovarian cancer and poor survival [18–20]. In addition, it was shown that inhibition of Dock180 suppressed cell invasion in melanoma cells [21]. However, there have been no reports covering all of roles involving the relationship between Dock180 and Elmo1 in cell proliferation, apoptosis and migration, in melanoma.

Therefore, we hypothesized that Dock180 and Elmo1 would be associated with cancer progression by altering cell survival and facilitating the migration of melanoma, allowing it to metastasize. The purpose of this study was to assess the expression levels of Dock180 and Elmo1 in melanoma tissues and to investigate the roles of Dock180 and Elmo1 in cancer progression using the human melanoma cells.

Materials And Methods

Tissue sample collection and preparation

A total of six normal skin tissues and six melanoma tissues were obtained from patients who underwent surgery between December 2015 and November 2018 in the Department of Plastic and Reconstructive Surgery of Soonchunhyang University Hospital, Korea. The Institutional Review Board of Seoul and Bucheon Soonchunhyang University Hospital approved the research protocol regarding the use of the tissue samples. All of the melanoma tissues were examined both by conventional pathological evaluation and immunohistochemical (IHC) staining to confirm the diagnosis. The others of remainder of the specimens were frozen in liquid nitrogen immediately after resection and stored at –70°C for Western blot analysis.

Cell culture

The human melanoma cell lines G361 and SK-MEL-2 were purchased from the American Type Culture Collection (CRL-1424 and HTB-68TM; ATCC, Rockville, MD, USA). G361 cells were incubated in Dulbecco’s Modified Eagle’s Medium (DMEM) containing 5% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C in a 5% CO2 incubator. SK-MEL-2 cells were incubated in complete Eagle's Minimum Essential Medium (EMEM) containing 10% FBS.

Small interfering RNA (siRNA) transfection

RNA interference of Dock180 and Elmo1 was performed using a Dock180- and Elmo1-specific small interfering RNA (siRNA) duplex obtained from Invitrogen (HSS102871 and HSS145324, respectively; Invitrogen, Carlsbad, CA, USA). and we followed the methods of Lee et al. [22].

Western blot analysis

The tissue samples and total cell lysates were extracted with a 1X RIPA buffer and western blot analysis was performed according to the methods of Lee et al. [23]. Antibodies to phospho-ERK1/2 (p-ERK) (Cat. no. 9101), ERK (Cat. no. 9102), phospho-AKT (p-AKT) (Cat. no. 9271), AKT (Cat. no. 9272), caspase-3 (Cat. no. 14220), cleaved caspase-3 (Cat. no. 9664), PARP (Cat. no. 9542), cleaved PARP (Cat. no. 9541), Mcl-1 (Cat. no. 5453), Bcl-2 (Cat. no. 2870), Bax (Cat. no. 5023) and PUMA (Cat. no. 4976) were purchsed form Cell Signaling Technology, Inc.(Danvers, MA, USA). Antibodies to Dock180 (SC-13163) and Rac1 (SC-217) were purchsed from Santa Cruz Biotechnology, Inc. and antibodied to Elmo1 (ab2239) was purchased from Abcam.(Cambridge, UK.). The secondary antibodies used were HRP conjugated anti-rabbit IgG (sc-2004), anti-goat IgG (sc-2020), and anti-mouse IgG (sc-2005) were purchsed from Santa Cruz Biotechnology, Inc. The human malignant melanoma cells G361 were used as a positive control for antioxidant expression.

IHC analysis

IHC analysis was performed according to the previously described method [22]. Assessment of IHC was evaluated by the percentage of stained cells (<25%; 25-75%, and >75%) and scored as 0, 1+ (mild), 2+ (moderate), and 3+ (strong). The final assessment was made by several independent investigators.

Cell viability assay

We followed the methods of Lee et al. [23]. Briefly, cells were seeded into 96-well microtiter plates, followed by transfection with 20 nM siRNA targeting Dock180 and Elmo1 (si-Dock180 and si-Elmo1) or StealthTM siRNA control (si-Ctrl) plus vemurafenib (A10739; Adooq Bioscience, Irvine, CA, USA) as for 24 h, 48 h, or 72 h.

DAPI staining

Nuclear condensation and fragmentation were observed by nucleic acid staining with DAPI. Cells were treated with si-Dock180 and si-Elmo1 or si-Ctrl, harvested by trypsinization, and fixed in 100% methanol at room temperature for 20 min. The cells were spread on slides, stained with DAPI solution (2 μg/mL), and analyzed under a FluoView confocal fluorescent microscope (FluoviewFV10i; Olympus Corporation, Tokyo, Japan).

Cell cycle analysis

The percentages of cells in G1, S, and G2/M phases were measured by quantifying the DNA content in PI-stained cells according to the method of Lee et al. [23].

Apoptosis assay

The apoptotic cell distribution was determined using the MuseTM Annexin V and Dead Cell kit (Catalog No. MCH100105, Merck Millipore, Billerica, MA, USA) according to the method of Lee et al. [23].

Wound healing assay

Cells were seeded into 6-well cell culture plates, cultured overnight, and transfected with si-Dock180 and si-Elmo1. At 24 hours post-transfection, the cells were grown to near confluence and then wounded by dragging a 10 μl pipette tip through the monolayer. The cells were then washed with pre-warmed 1 × PBS to remove cellular debris. The cells were then left to migrate for an additional 48 hours. Cell migration images were captured soon after the wound was introduced (0 h) and at a designated time (48 h after wounding) under a light microscope.

Colony formation assay

The G361 and SK-MEL-2 cells were pre-treated with si-Dock180 and si-Elmo1. One thousand cells were seeded into 6-well plates in 2 mL culture medium containing 10% FBS. After 14 days of incubation in DMEM containing 10% FBS at 37^{O}C in a humidified incubator with 5% CO2 atmosphere to promote colony formation, the colonies were counted. The cells were washed twice with PBS, stained with Giemsa, and the colonies containing > 50 cells were counted. The colony formation efficiency (%) was defined as: (the number of clones / the number of seed cells) × 100%.

Statistical analysis

undefinedThe data are presented as means ± standard deviations (SD). The Mann–Whitney U test was used to compare non-normally distributed variables. Data from the Raytest TINA software to quantify Western blot were analyzed with SPSS 17.0 (IBM Corp., Armonk, NY, USA). Statistical significance was set at $p < 0.05$.

Results

Expression of Dock180 and Elmo1 in melanoma tissues and normal skin tissues

Both IHC staining and Western blot analysis demonstrated that expressions of both Dock180 and Elmo1 were significantly higher in melanoma tissues than in normal skin tissues (Fig. 1). The IHC staining for Dock180 and Elmo1 was performed in the paraffin sections of human pathologic tissue specimens. As shown in Fig. 1A, the IHC results showed 2+ (moderate) positive staining of Dock180 and Elmo1 in four melanoma tissues and 1+ (mild) positive staining of them in two melanoma tissues. 0 case had fewer than 25% of stained cells, two cases had 25% to 75% of stained cells, and four cases had more than 75% of stained cells in both Dock180 and Elmo1 positive cases of melanoma. However, four and two of normal skin tissues showed 0 and 1+ (mild) positive staining of Dock180, respectively, while five of normal skin tissues showed negative staining with 1+ (mild) positive staining of Elmo1 in one normal

skin tissues. Two of Dock180 positive cases in normal skin had fewer than 25% of stained cells, while one of Elmo1 positive case in normal skin showed fewer than 25% of stained cells. Consistent with IHC staining, the Western blot analysis demonstrated overexpression of both Dock180 and Elmo1 proteins in melanoma tissues compared to in normal skin tissues (Fig. 1B). The results of the Mann-Whitney U test demonstrate a median (interquartile range) of 0.9182 (0.7940~0.9506) and 1.1964 (1.1236~1.2630) for Dock180 in normal skin tissues and melanoma tissues, respectively. Also, a median (interquartile range) for Elmo1 is 0.4002 (0.2808~0.7721) in normal skin tissues with 0.9539 (0.8762~1.0324) in melanoma tissues.

Knockdown of Dock180 and Elmo1 by siRNA reduces cell proliferation and increases cell death of G361 and SK-MEL-2 cells by regulating apoptosis-related proteins, Rac1, and signaling pathways including ERK and AKT

First, results of the MTT assay showed that the proliferation of G361 and SK-MEL-2 cells was decreased by the knockdown of Dock180 and Elmo1 in a time-dependent manner (Fig. 2A). At 48 h after siRNA transfection, phase contrast images of cells showed that the cell shapes dramatically changed and some cells were detached (Fig. 2B). As shown in Fig. 2C, the analysis of nuclei using DAPI staining showed increased proportion of adherent cells with chromatin condensation and nuclear fragmentation in G361 and SK-MEL-2 cells following treatment of si-Dock180 or si-Elmo1. As shown in Fig. 3A, the Western blotting demonstrated that the G361 and SK-MEL-2 cells treated with Dock180-specific siRNA downregulated caspase-3, PARP, and Bcl2, while they upregulated cleaved caspase-3, cleaved PARP, and pro-apoptotic proteins, including Bax and PUMA. Treatment with the Elmo1 siRNA decreased expression of caspase-3, PARP, and Bcl2, while it increased the expression of cleaved caspase-3, cleaved PARP, Bax, and PUMA. Pro-apoptotic and anti-apoptotic proteins were significantly changed in the G361 and SK-MEL-2 cells following simultaneous treatment with both Dock180- and Elmo1-siRNAs. As shown in Fig. 3B, it was demonstrated that treatment of G361 and SK-MEL-2 cells with si-Dock180 significantly reduced the expression of Dock180 and Elmo1 compared to Rac1. In addition, si-Elmo1 treatment markedly suppressed the expression of both Elmo1 and Rac1 compared to the Dock180 protein levels. When the cells were treated with si-Dock180 and si-Elmo1 simultaneously, the expressions of Dock180, Elmo1, and Rac1 decreased. The levels of p-ERK, ERK, p-AKT, and AKT were measured by Western blotting following transfection with si-Dock180 and si-Elmo1. The treatment of G361 and SK-MEL-2 cells with the siRNAs decreased the phosphorylation of ERK1/2 and AKT, both of which were augmented by treatment with the combination of si-Dock180 and si-Elmo1.

Knockdown of Dock180 and Elmo1 affects cell cycles and increases apoptosis of melanoma cells

In the flow cytometric analysis, it showed the presence of a sub-G_0, implying apoptosis, was increased after transfection with either specific siRNA (Fig. 4A). After si-Dock180 and si-Elmo1 transfection, the percentage of cells in the G_1 and S phases tended to decrease compared to that in the control cells. However, the percentage of cells in the G_2/M phase was increased after siRNA transfection. These results indicated that inhibition of Dock180 and Elmo1 might promote cell apoptosis in melanoma cells. Three

different populations of cells were detected in the apoptosis assay after Annexin V-PE staining of G361 and SK-MEL-2 cells, a live cell group (lower left panel), an early apoptotic cell group (lower right panel), and a late apoptotic cell group (top right panel). The apoptotic cell population was increased after siRNA treatment (Fig. 4B). Interestingly, when Dock180 and Elmo1 were simultaneously knocked down by both siRNAs in G361 and SK-MEL-2 cells, the degree of apoptosis markedly increased, while cell proliferation was inhibited compared to those in the Dock180 or Elmo1 siRNA-transfected cells.

Knockdown of Dock180 and Elmo1 reduces cell migration and colony formation

Cell migration and invasion are characteristics of metastasis. Therefore, we examined the effect of Dock180 and Elmo1 knockdown on cell migration using a scratch wound healing assay. The silencing of both Dock180 and Elmo1 highly decreased the wound filling ability of G361 and SK-MEL-2 cells (Fig. 5A). Cells treated with the si-Dock180 or si-Elmo1 showed much lower wound filling ability than the cells treated with si-Ctrl, indicating that both Dock180 and Elmo1 can promote cancer cell migration. Furthermore, both melanoma cells treated with both Dock180- and Elmo1-siRNA showed significantly impeded cell migration compared to cells treated with si-Dock180 or si-Elmo1 alone. To confirm that Dock180 and Elmo1 protein contributed to cancer progression in melanoma, a colony formation assay was performed. Colony formation was decreased in G361 and SK-MEL-2 cells after transfection with si-Dock180 or si-Elmo1 (Fig. 5B).

BRAF inhibitor decreased significantly melanoma cell survival, following knockdown of Dock180 and Elmo1 in G361 and SK-MEL-2 cells

Results of the MTT assay demonstrated that cell survival in melanoma cells following treatment of vemurafenib, which were known as BRAF inhibitor, was significantly decreased by the knockdown of Dock180 and Elmo1 (Fig. 6). Vemurafenib treatment reduced cell viability of G361 and SK-MEL-2 cells in a concentration-dependent manner, and transfection with Dock180- and Elmo1-specific siRNA, alone or in combination, decreased more significantly cell viability compared to si-Ctrl. In SK-MEL-2, reduction of cell viability was more evident compared to G361 cells.

Discussion

Dock180 protein is one of the dedicator of the cytokinesis superfamily which plays a role as a GEF for Rho GTPases [11]. Moreover, Rac1 is a member of the Rho GTPases which control signal transduction pathways in eukaryotic cells and contributes to cell migration and invasion resulting from a variety of stimuli [10, 24]. Eleven members of the Dock family have been identified, including Dock-A (Dock180, Dock2, Dock5), Dock-B (Dock3, Dock4), Dock-C (Dock6-8), and Dock-D (Dock9-11) [25]. While most other Dock members use the conserved Docker domain, Dock180 uses the characteristic Dbl homology domain to regulate the GDP/GTP exchange on Rho GTPases [11]. Furthermore, Elmo1 is necessary for the nucleotide exchange in Rac1 as well as Dock180 [12].

Elmo1, initially known as a mammalian homolog of *C. elegans* Ced-12, is essential for the regulation of cell migration and the engulfment of dying cells [26]. Also, Elmo1 interacts with Crk and Dock180 functionally to promote phagocytosis and morphological changes, including filopodia formation associated with cell motility [16, 26, 27]. Moreover, Elmo1, which inhibits the ubiquitination of Dock180 [15], attaches to Dock180 and functions as an unconventional bipartite GEF for Rac1 [12]. Previous studies reported that the small GTPase RhoG interacted with Elmo1 directly. Rac1 can be activated by a complex including Dock180 and Elmo1, resulting in cell migration, phagocytosis, and neurite outgrowth [28, 29]. Also, Dock180 and Elmo1 are known to be conserved proteins contributing to multiple biological processes associated with cell migration [26, 30, 31].

The upregulation of Dock180 protein has been reported to be involved in the invasion of some cancers, including human glioma and ovarian cancer [19, 32, 33]. Moreover, it has been suggested that overexpression of Elmo1 also may induce cell migration and invasion in human cancers, such as hepatocellular carcinoma and breast cancer [17, 34]. However, the effects of Dock1 and Elmo1 and the relationship between them have not been studied well in melanoma. In the present study, we extended these observations to both *ex vivo* and *in vitro* human melanoma models and found that Dock1 and Elmo1 were important for promoting cancer cell proliferation and migration. Our results showed that Dock180 and Elmo1 were overexpressed in melanoma tissues compared to normal skin tissues using Western blotting and IHC staining. We also found that the inhibition of Dock180 and Elmo1 suppressed proliferation and increased apoptosis in melanoma cells. These findings suggest that both Dock180 and Elmo1 may have a role in the carcinogenesis of melanoma.

To clarify the relationship between apoptosis and the silencing of Dock180 and Elmo1 in melanoma cells, we investigated changes in apoptosis-related proteins using Western blotting. Our data showed that silencing of Dock180 and Elmo1 increased pro-apoptotic proteins, including Bax, PUMA, and cleavage of caspase-3 and PARP, while it decreased anti-apoptotic proteins, including Bcl-2. Consistent with a previous report that Dock180 and Elmo1 inhibited apoptosis in endothelial cells [35], these findings support that overexpression of Dock180 and Elmo1 may protect melanoma cells from apoptosis via regulating pro-apoptotic and anti-apoptotic proteins.

In addition, we demonstrated that inhibition of Dock180 and Elmo1 attenuated the invasive behavior of melanoma cell lines. Some studies have reported that both Dock180 and Elmo1 were associated with cell motility in cancers. It has been suggested that changes in gene and protein expression of Dock1, Elmo1, and Rac1 may be responsible for the migratory and invasive behavior of glioma cells [36–39]. Wang et al. [40] reported that Dock180 and Elmo1 synergistically activated Rac1 and promoted cell motility in ovarian carcinoma. Elmo1 expression was associated with lymph node and distant metastasis, whereas knockdown of Elmo1 impaired metastasis to the lung in breast cancer [41]. Consistent with the findings of these previous studies, our data suggest that Dock180 and Elmo1 may promote cancer progression by enhancing cell migration and invasion in melanoma.

Furthermore, we investigated the interaction between Dock180 and Elmo1 in G361 cells. In our studies, Dock180 influenced the expression of Elmo1 in melanoma cells while Elmo1 didn't affect the expression of Dock180. Moreover, some studies have reported that Dock180 and Elmo1 were associated with Rac1. In breast cancer cells transfected with si-Dock180 and si-Elmo1 respectively, inhibition of Dock180 suppressed expression of Elmo1 and GTP-bound Rac1 (Rac1-GTP) while inhibition of Elmo1 reduced expression of Dock180 and Rac1-GTP [17]. However, in glioma cell transfected with si-Dock180 and si-Elmo1 respectively, although downregulation of Elmo1 affected expression of Dock180 and Rac1-GTP, Dock180 inhibition reduced expression of Rac1-GTP except Elmo1 protein [18]. Moreover, downregulation of Elmo1 decreased Rac1 expression in hepatocellular carcinoma cells treated with si-Elmo1 [34]. Our data demonstrated that the expression of Rac1 was significantly changed by silencing Elmo1 rather than silencing Dock180 in G361 cells. Collectively, these results indicate that the interaction between Dock180, Elmo1, and Rac1 may vary depending on the cancer type.

In addition, some studies have reported that Dock180 and Elmo1 may be involved in activation of the MEK/ERK1/2 and/or PI3K/AKT pathways in glioma cells, endothelial cells, and macrophages [18, 35, 42]. Consistent with previous reports, silencing of Dock180 and Elmo1 inhibited phosphorylation of ERK and AKT in melanoma cells. These results demonstrate that both Dock180 and Elmo1 can cross-communicate via the MEK/ERK1/2 and/or PI3K/AKT pathways, which are important for cancer progression, including cell survival, proliferation, and migration in melanoma cells.

Drugs such as BRAF inhibitors have been known to extend overall survival of patients with melanoma having a BRAF mutation [43–46]. According a previous report, BRAF mutant melanoma cells such as G361 are intrinsically resistant to BRAF inhibitors [47]. In addition, SK-MEL-2, one of human melanoma cell lines expressing wild-type BRAF, is also resistant to BRAF inhibitors [48]. In our experiments, we found that vemurafenib treatment reduced more significantly cell viability of melanoma cells transfected with Dock180- and Elmo1-specific siRNA alone or in combination compared to si-Ctrl. These findings suggest that Dock180 and Elmo1 may be associated with drug resistance in melanoma.

Collectively, the results of the present investigation, together with the findings in previous studies, suggest that cell proliferation and migration are regulated by Dock180 and Elmo1. Furthermore, our study showed that both Dock180 and Elmo1 were overexpressed in melanoma tissues and might be associated with cell proliferation and migration via the downregulation of apoptosis-related proteins and the activation of MEK/ERK1/2 and/or PI3K/AKT pathways in human melanoma cells. Moreover, our results demonstrated that both Dock180 and Elmo1 might be related to drug resistance in melanoma. Therefore, Dock180 and Elmo1 might be potential targets for the development of therapeutics to treat and/or prevent melanoma.

Abbreviations

GTPases: Guanosine triphosphatase; Dock180: Dedicator of the cytokinesis I; GEF: Guanine nucleotide exchange factor; Elmo1: Engulfment and cell motility protein 1; GDP: Guanosine diphosphate: GTP: Guanosine triphosphate; IHC: Immunohistochemical; ATCC: American Type Culture Collection; DMEM:

Dulbecco's Modified Eagle's Medium; FBS: fetal bovine serum; siRNA: Small interfering RNA; p-ERK: phospho-ERK1/2; p-AKT: phospho-AKT; ABC: Avidin-biotin complex; PBS: Phosphate buffered saline; si-Dock180: siRNA targeting Dock180; si-Elmo1: siRNA targeting Elmo1; si-Ctrl: StealthTM siRNA control; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; DMSO: Dimethyl sulfoxide; SD: Standard deviations; Rac1-GTP: GTP-bound Rac1

Declarations

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Authors' contributions

Study conception and design : MKC

Acquisition of data : YJL, DCC, SHL

Analysis and interpretation of data : YJL, HSN, JYH, DSK

Drafting of manuscript : YJL, MKC

Critical revision : MKC, SHL, YSC, SYK, SMN

All authors discussed the results, commented on the manuscript, and approved the final manuscript.

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Availability of data and materials

Data generated or analysed during this study are included in this article. Raw data used and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

The research protocol of this study regarding the use of the tissue samples was approved by the Institutional Review Board of Seoul and Bucheon Soonchunhyang University Hospital. Signed, written informed consents were obtained.

Consent for publication

Not applicable

Competing interests

The authors declare that there are no competing interests in this manuscript.

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Figures

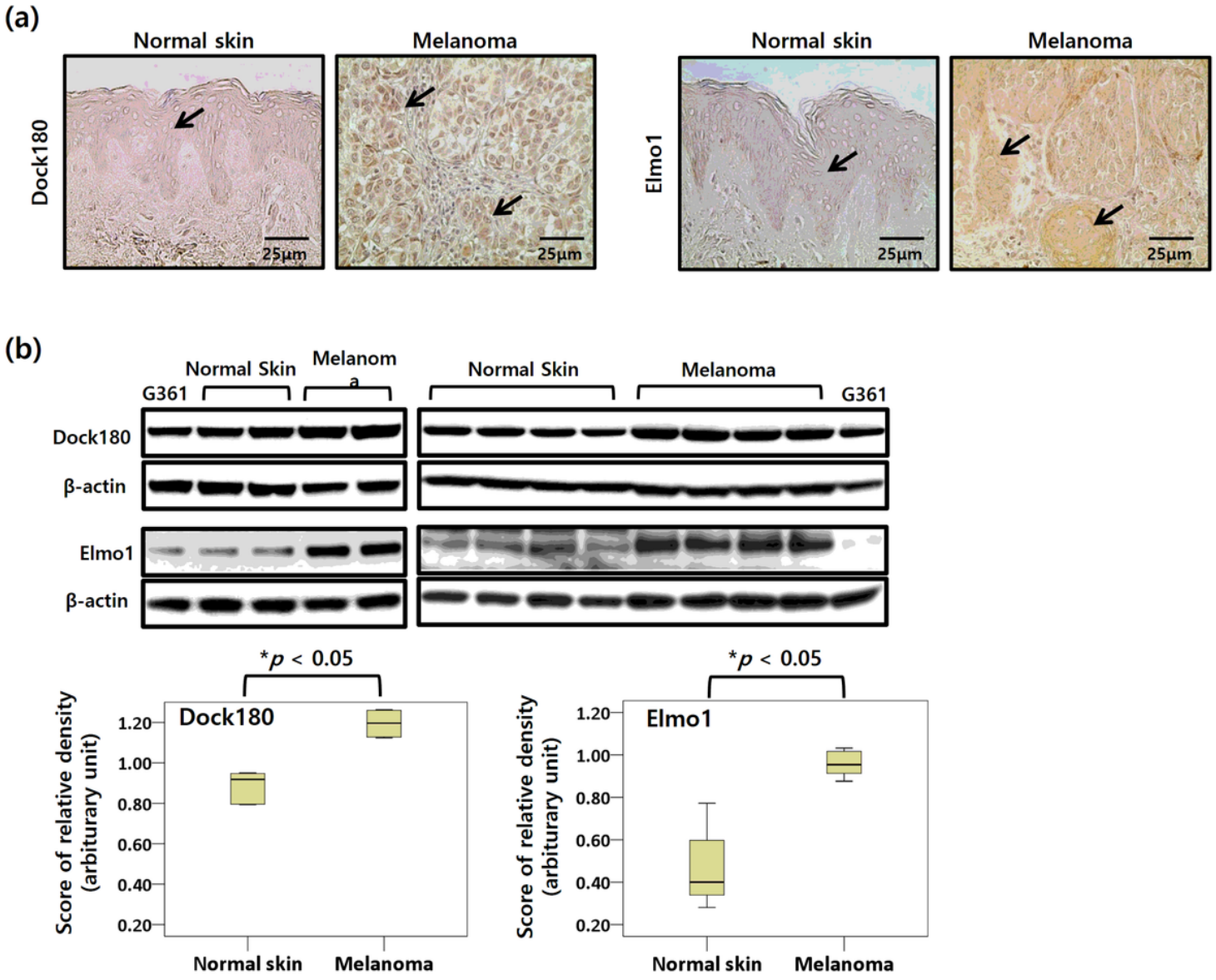

Figure 1

Dock180 and Elmo1 protein expression in paraffin-embedded normal skin and melanoma tissues. (**a**) Representative immunohistochemical staining. Weakly positive staining of Dock180 and Elmo1 in normal skin tissues (×400). Moderately positive staining of Dock180 and Elmo1 in melanoma tissues (×400). (**b**) Western blot analysis. Expression of Dock180 and Elmo1 was high in melanoma tissues. β-actin used as a loading control. The human melanoma G361 cells served as a positive control for Dock180 and Elmo1 expression. The median values of normal skin and melanoma tissues were measured by the Mann-Whitney test (n=12, *p□0.05).

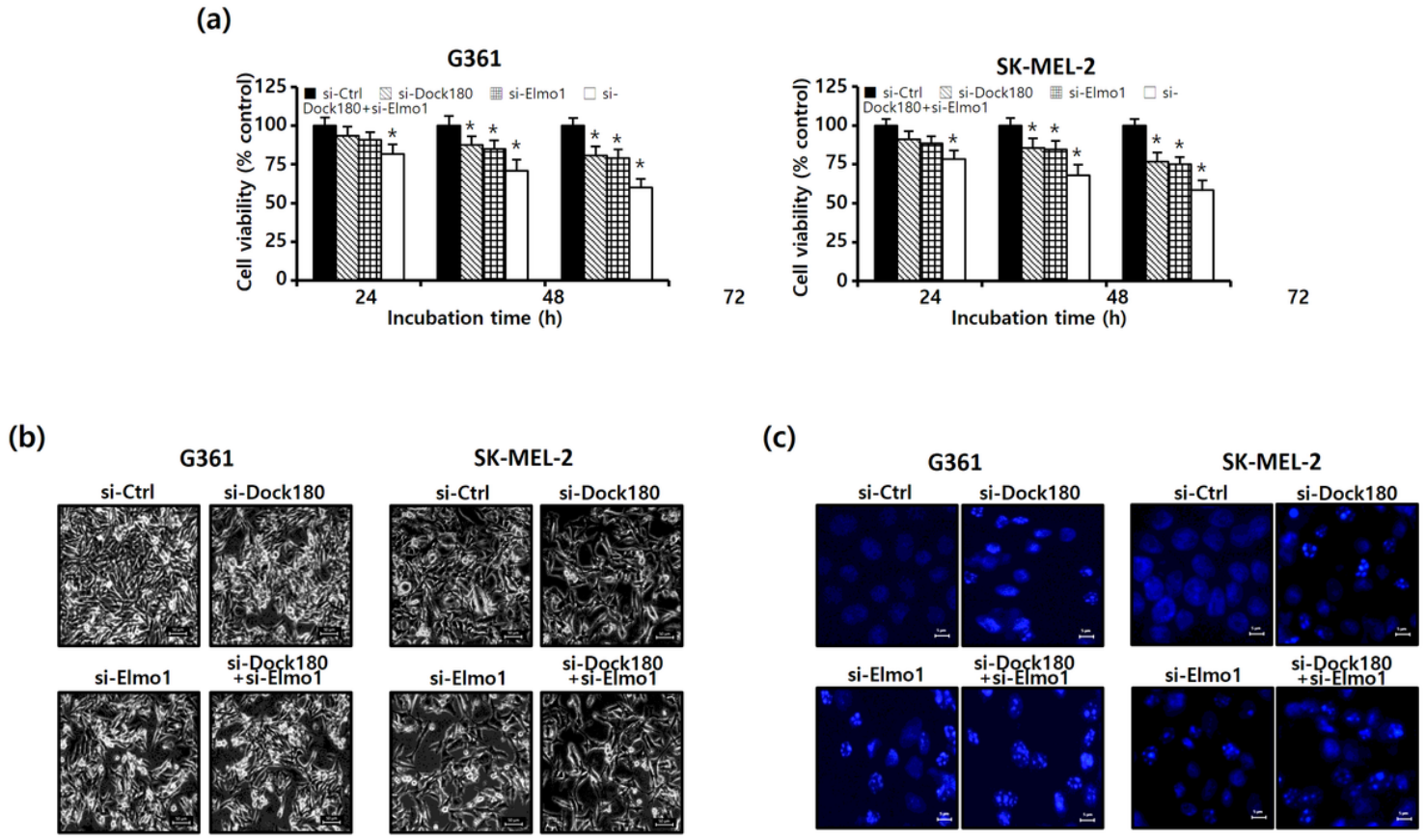

Figure 2

Apoptotic effects following knockdown of Dock180 and Elmo1 in G361 and SK-MEL-2 cells. Cells were treated with Dock180- or Elmo1-specific siRNA. (**a**) The percentage of cell viability was measured by MTT assay as mean ± SD for three independent experiments. *p<0.05 compared to untreated controls. (**b**) Phase contrast images of G361 and SK-MEL-2 cells. (**c**) In DAPI staining, both chromatin condensation and nuclear fragmentation were increased in G361 and SK-MEL-2 cells following treatment of si-Dock180 or si-Elmo1.

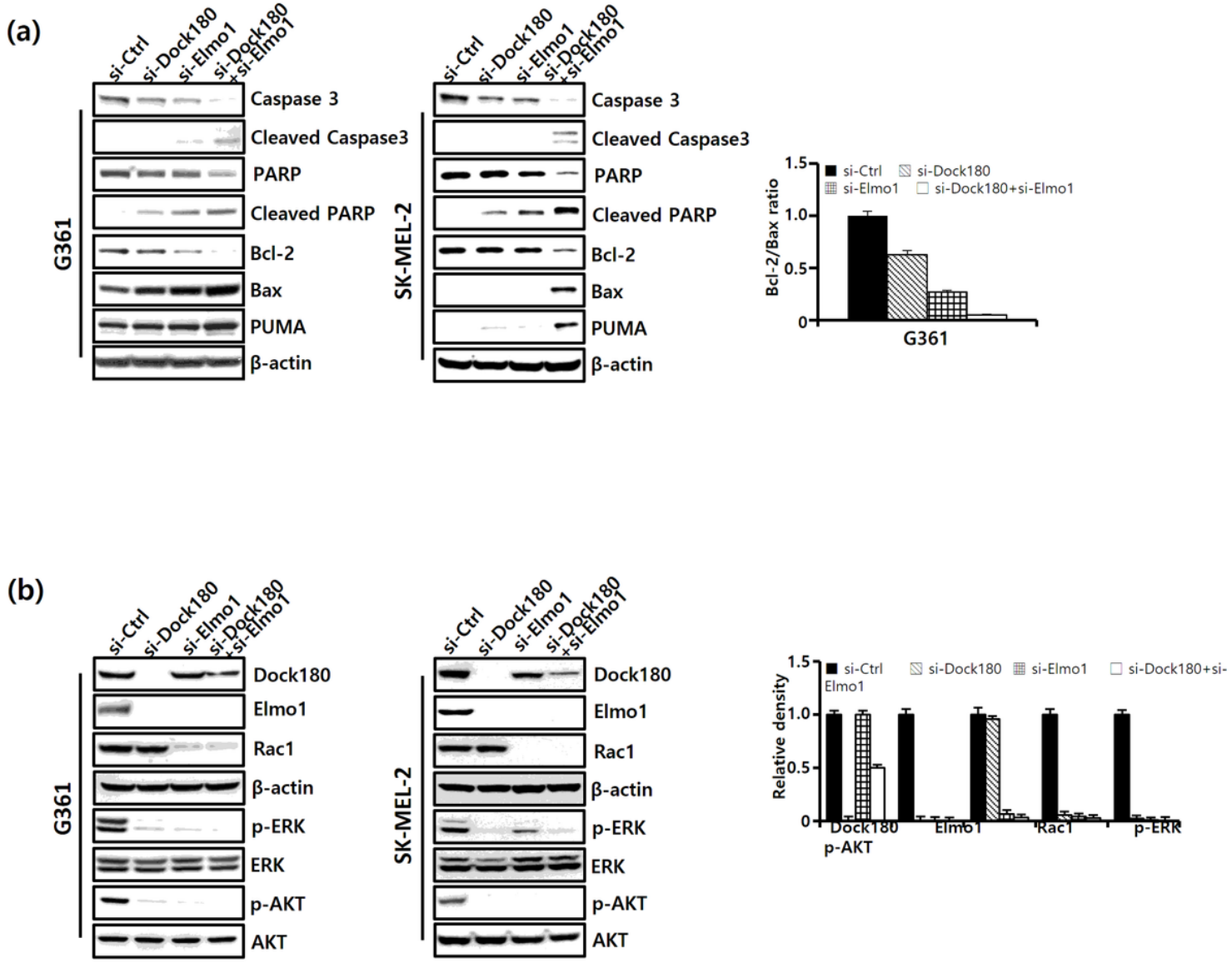

Figure 3

(a) The expression of pro- and anti-apoptotic proteins were measured by Western blotting. β-actin was used as a loading control. The Bcl-2/Bax ratio was shown at 48 hours of incubation time. **(b)** The expression of Dock180, Elmo1, and Rac1 as well as the levels on phosphorylation of ERK and AKT were measured by Western blotting after Dock180- or Elmo1-specific siRNA treatment. β-actin was used as a loading control. Data are mean ± SD of three independent experiments in triplicates.

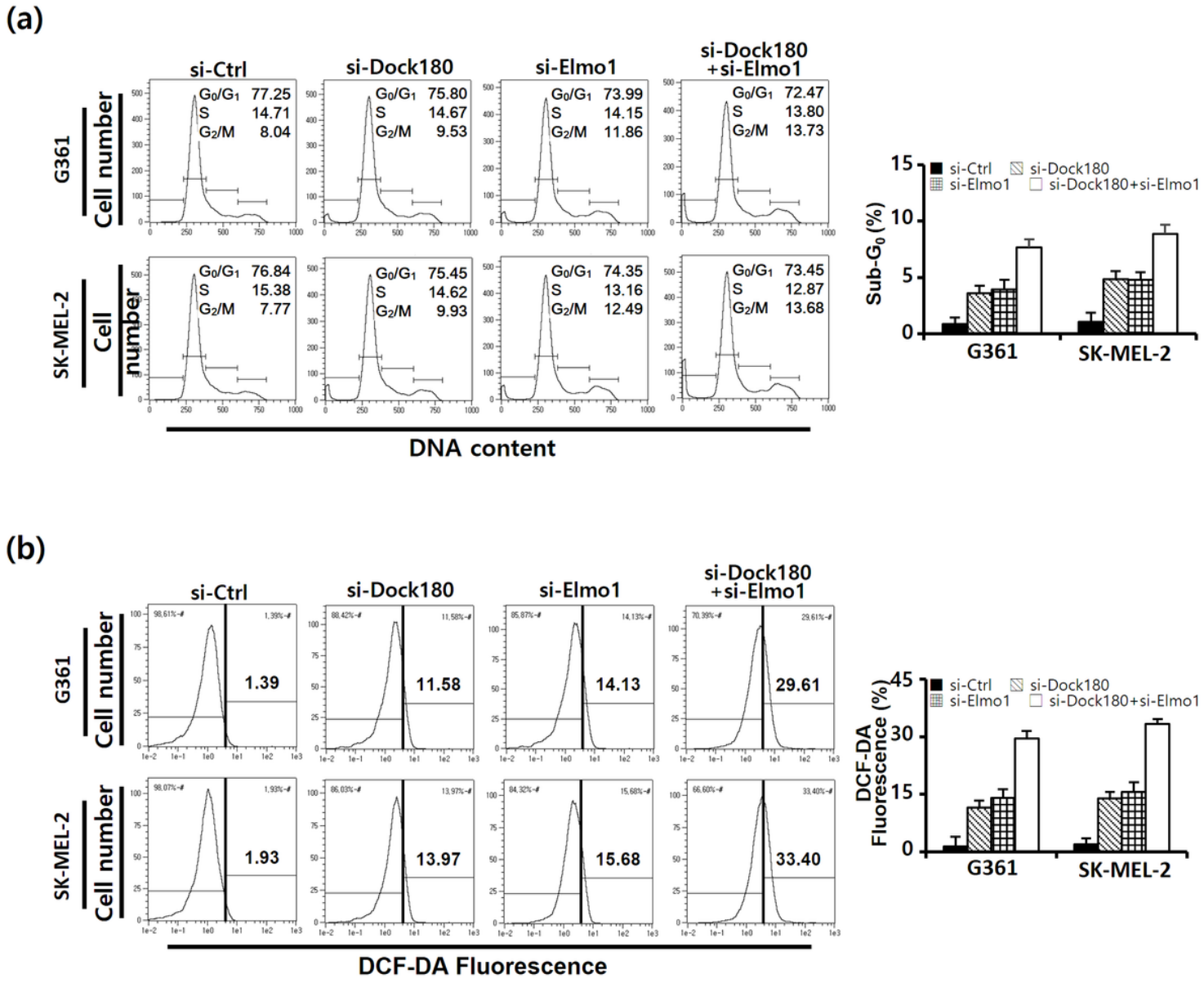

Figure 4

Inhibition of Dock180 and Elmo1 changes cell cycles and apoptosis of G361 and SK-MEL-2 cells. (**a**) Cell distribution at G_0/G_1, S, and G_2/M phases was analyzed at 48 hours of incubation time using flow cytometry after staining with propidium iodide (20 μg/mL). (**b**) The percentage of apoptotic cells after Annexin V-PE binding was analyzed at 48 hours of incubation time using a Muse cell analyzer. The quantitative data were shown as mean ± SD for three independent experiments. *p<0.05 compared to untreated controls.

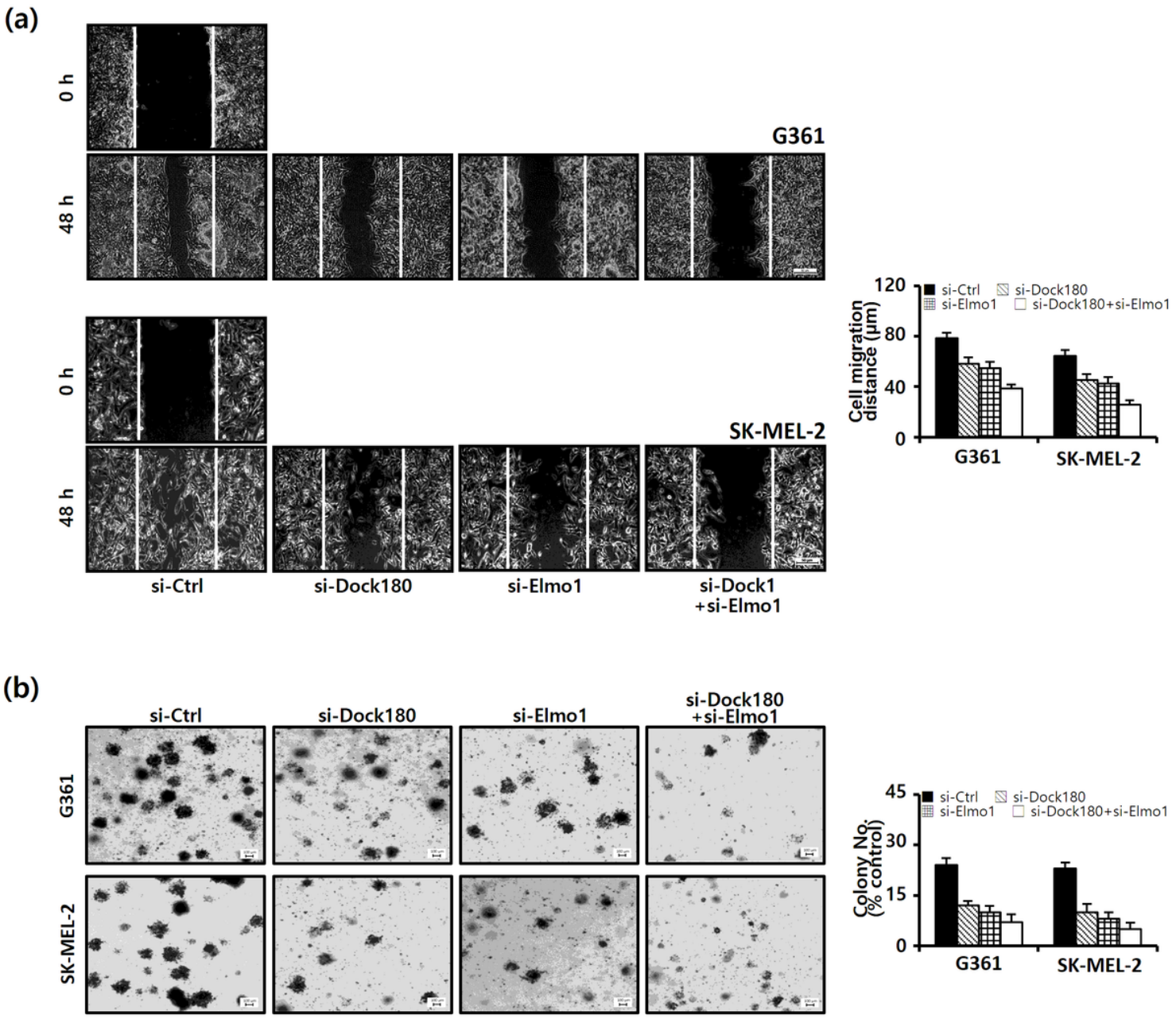

Figure 5

Inhibition of migration and colony formation in G361 and SK-MEL-2 cells by knockdown of Dock180 and Elmo1. (**a**) Transfection with Dock180- or Elmo1-specific siRNA induced the inhibition of melanoma cell migration. At 24 hours post transfection, the cells were grown to near confluence, and then wounded by dragging a 20 μl pipette tip through the monolayer. Cell migration images were captured soon after the wound was introduced (0 h) and at a designated time (48 h after wounding) under a microscope. (**b**) In 2 weeks after transfection with Dock180- or Elmo1-specific siRNA, colony formation of the G361 cells was suppressed. Immunoblot analysis of Dock180, Elmo1, Rac1 and phosphorylation of ERK and AKT in G361 and SK-MEL-2 cells treated with Dock180- or Elmo1-specific siRNA. *p<0.05 compared to untreated controls.

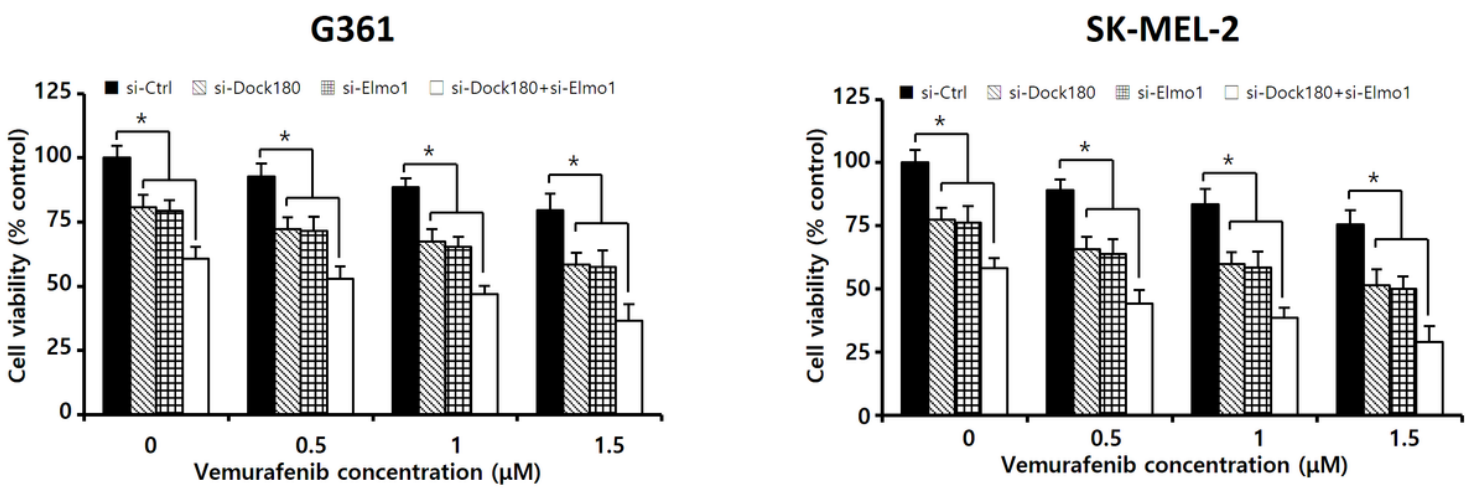

Figure 6

Treatment of vemurafenib in melanoma cells treated with either Dock180- or Elmo1-specific siRNA. Vemurafenib decreased cell viability of melanoma cells in a concentration-dependent manner. Knockdown of Dock180 or Elmo1 decreased markedly cell viability in melanoma cells following treatment of vemurafenib. quantitative data were shown as mean ± SD for three independent experiments. *p<0.05 compared to untreated controls.