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Original Article

Muc1 promotes migration and lung metastasis of melanoma cells

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Abstract: Early stages of melanoma can be successfully treated by surgical resection of the tumor, but there is still no effective treatment once it is progressed to metastatic phases. Although growing family of both melanoma metastasis promoting and metastasis suppressor genes have been reported be related to metastasis, the molecular mechanisms governing melanoma metastatic cascade are still not completely understood. Therefore, defining the molecules that govern melanoma metastasis may aid the development of more effective therapeutic strategies for combating melanoma. In the present study, we found that muc1 is involved in the metastasis of melanoma cells and demonstrated that muc1 disruption impairs melanoma cells migration and metastasis. The requirement of muc1 in the migration of melanoma cells was further confirmed by gene silencing in vitro. In corresponding to this result, over-expression of muc1 significantly promoted the migratory of melanoma cells. Moreover, down-regulation of muc1 expression strikingly inhibits melanoma cellular metastasis in vivo. Finally, we found that muc1 promotes melanoma migration through the protein kinase B (Akt) signaling pathway. To conclude, our findings suggest a novel mechanism underlying the metastasis of melanoma cells which might serve as a new intervention target for the treatment of melanoma.

Keywords: Melanoma, metastasis, muc1, Akt

Introduction

Melanoma is a highly aggressive and the main cause of death in patients with skin cancer caused by malignant transformation of melanocytes. At the early-stage malignant melanoma, most melanoma patients can be treated successfully by surgical removal of the tumor. However, currently, there is still no curative treatment for melanoma once the disease has progressed to metastatic phases [1]. Cancer metastasis is the spread of malignant tumor cells from a primary site to distant tissue and formation of clinically significant secondary tumors [2]. Metastasis is a series of biologic events that can be envisioned a succession of cell biological changes, including cancer cells separating from original tumor, local invasion through surrounding tissues, intravasation into and transferring through the blood stream, arresting in the parenchyma of distant tissues, formation of small nodules (micro-metastasis), and finally, growth of micro-metastatic lesions into macroscopic tumors [3]. Although a variety of metastasis-promoting genes have been recently identified to be related to the metastasis of melanoma, the molecular mechanisms governing this metastasis process are still not completely understood and the treatment efficiency of metastatic melanoma has not been significantly improved [4, 5]. Hence, further study to uncover related underlying mechanism is urgently required to find new potential target for the treatment of melanoma.

Muc1 is a member of the mucin family and encodes a membrane bound, glycosylated phosphoprotein [6]. Recently, the highly conserved cytoplasmic domain of muc1 has gained interest, as a number of proto-oncogenes were

identified to interact with muc1, such as epidermal growth factor receptor (EGFR), c-src and beta-catenin [7]. These proteins are associated with a mesenchymal phenotype, characterized by increased motility and invasiveness of tumor cells. In addition, muc-1 has been determined to be one of the most frequently expressed surface markers in metastatic breast cancers. Muc-1 cooperates with receptor tyrosine kinases and promotes an invasive phenotype in breast tumorigenesis [8]. Furthermore, muc-1 confers an invasive phenotype and drives progression of primary lung cancer and enhances their metastatic capability via activation of the phosphatidylinositol 3 kinase (PI3K)-Akt pathway [9]. Activation of Akt has been linked to mitogenesis, differentiation, survival, migration, invasion, and actin cytoskeletal reorganization. The Akt pathway is a major regulator of signal transducer and activator of transcription 3 (STAT3), which can promote oncogenesis by being constitutively active through various pathways, and matrix metallopeptidase-2/9 (MMP-2/9) activity. Collectively, these results point to a role for muc1 in controlling cell mobility and, alternatively, cell metastasis into the tumor microenvironment [10].

In the present work, we reported the identification of a novel role of muc1 whose disruption impaired the metastasis of melanoma B16 and B16BL6 cells. By silencing and over-expression of muc1, we further confirmed the role of muc1 in the migration of melanoma cells in vitro. Moreover, reduction and increasing of muc1 expression in melanoma B16 and B16BL6 cells significantly impaired and promoted their metastasis in vivo. Further investigation demonstrated that Akt signaling pathway is involved in muc1-promoted melanoma migration and metastasis.

Materials and methods

Cell culture and reagents

Melanoma A375, B16, CHL-1, SK-MEL-2, SK-MEL-5, B16BL6 cells, and adult human epidermal melanocytes NHEM-a cells were purchased from Cell Resource Center, Shanghai Institutes for Biological Sciences. Cells were cultured in DMEM or 1640 with 10% fetal calf serum (FCS) (Gibco, Invitrogen, USA), and 1% Penicilin/Streptomycin mix (Gibco, Invitrogen, USA) and maintained at 37°C in a humidified atmosphere containing 5% CO_2. Specific inhibitor for Akt (GSK690693) was obtained from Selleck (USA). Lipofectamine 2000 was purchased from Invitrogen (USA).

Plasmids and DNA constructs

The pDisrup retroviral vector was constructed based upon MMLV retroviral vector pLNCX as backbone. The splicing donor and acceptor were designed according to human adenovirus type 2 major late mRNA intron sequence [11]. The short small interfering RNA (siRNA) was constructed with sequence specifically targeted to mouse muc1 extracellular domain gene: (#1: 5'-CAAGGAGTTAGTTAAATGC-3') or (#2: 5'-AAGACTGATGCCAGTAGCACT-3') [12]. Target and scrambled control oligonucleotides duplexes were cloned into pSilencer4.1-CMV vector (Ambion, USA) according to the manufacturer's instructions. The muc1 colony was cloned into the sites of EcoRI and XhoI of pIRES2-EGFP vector (Clontech, USA) with gene specific primers. The primer used was as follows: muc1 (5'-GGCCACTGATTGTGCCTTAT-3' and 5'-TGCAACCTTGAAGTGGTCAG-3'). A constitutively active mutant D2Akt (T308D/S473D) plasmid was a gift from Peter Vogt (Addgene plasmid # 49192) [13]. Transient transfection was performed using the Lipofectamine RNAi MAX reagent (Invitrogen) and following the manufacturer's instructions. For pDisrup clone selections, cells were selected with Blasticidin S.HCl at 25 μg/ml (Invitrogen, USA).

One solution cell proliferation assay

The cell viability was determined by CellTiter 96® Aqueous One Solution cell proliferation assay (Promega, Madison, WI, USA). Briefly, cells were seeded in 96-well cell culture plates and treated with indicated agents. After incubation for indicated time period, 20 μL of One Solution reagent were added to each well and incubation was continued for additional 4 h. The absorbance was measured at 490 nm using Synergy™ HT Multi-Mode Microplate Reader (Bio-Tek, Winooski, VT, USA). The effect of siRNA on cell viability was assessed as the percent of cell viability compared with vehicle-treated control cells, which were arbitrarily assigned 100% viability. The data were presented as mean ± SE. Differences in the results of different groups were evaluated using either two-tailed Student's t test or one-way ANOVA followed by post hoc Dunnett's test.

Wound healing assay

Briefly, B16 and B16BL6 melanoma cells were seeded in 60 mm dishes and cultured at 37°C overnight to produce a confluent monolayer. After starvation in serum-free medium for 24 hours, a wound was created by scratching the monolayer with a 200 μl sterile pipette tip. The wounded monolayer was then washed three to remove cell debris and incubated with fresh medium. The area of cell-free scratch was photographed at 0 h and 48 h after scratching respectively. The wound healing effect was determined by measuring the percentage of the remaining cell-free area compared with the area of the initial wound [14]. The data were presented as mean ± SE. Differences in the results of different groups were evaluated using either two-tailed Student's t test or one-way ANOVA followed by post hoc Dunnett's test.

Migration assay

Migration of cells was determined by BD Transwell Migration Chamber (BD Biosciences, USA) assay in vitro according to the manufacturer's instructions. In brief, 1×10^5 cells with 500 μl in serum-free medium were added into the upper chamber and 750 μl of cells conditioned medium was added into the lower chamber. After incubation in humidified tissue culture incubator, 37°C, 5% CO_2 atmosphere for 24 h, the non-migration cells in the upper surface of the membrane were removed by "scrubbing" with cotton tipped swab and the cells migrating to the lower surface of the membrane were fixed and stained with 0.5% crystal violet for 30 minutes. Cell counting was then carried out by photographing the membrane through the microscope. Five random fields under microscope were taken and migration cells were quantified [15]. The data were presented as mean ± SE. Differences in the results of different groups were evaluated using either two-tailed Student's t test or one-way ANOVA followed by post hoc Dunnett's test.

Western blot

After washing with PBS (3.2 mM Na2HPO4, 0.5 mM KH2PO4, 1.3 mM KCl and 140 mM NaCl, pH 7.4) twice, cells were extracted with cold lysis buffer (20 mM Tris, 100 mM NaCl, 5 mM EDTA, 1 mM EGTA, 5 mM MgCl2, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM b-glycerolphosphate, 1 mM Na3VO4, 1 mM PMSF, and Roche complete protease inhibitors) and centrifuged at 15,000 g for 15 min at 4°C. Protein concentration of the supernatants was determined with Bradford assay (Biorad, USA). 10-40 μg of samples was separated by electrophoresis on 8-16% SDS-PAGE and transferred to Polyvinylidene fluoride membrane (Millipore, USA). After blocking with 5% skimmed milk for 1 h, membranes were incubated with different specific primary antibodies in either 5% skimmed milk or 5% bovine serum albumin (BSA) (anti-muc1, FAK, phospho-FAK^{Tyr397}, ERK1/2, phospho-ERK1/2^{Thr202}, Akt, phospho-Akt^{Ser473}, Src, and phospho-Src^{Tyr416} from Cell Signaling Technology). After washing with TBST for 30 min, the membranes were further incubated with corresponding HRP-conjugated secondary antibodies and developed with Pierce's West Pico chemiluminescence substrate (Millipore, USA). All results were obtained from 3 independent experiments [16].

Real-time PCR

Total RNA was isolated using TRIzol according to the manufacturer's instructions (Invitrogen, USA) and the concentration of total RNA was detected by spectrophotometry at OD260. Reverse transcription (RT) was carried out using superscript III reverse transcriptase (Invitrogen, USA) as described in the manufacturer's manual. The real-time PCR was performed on ABI Prism 7500 Sequence detection system (Applied Biosystems, CA) with the KAPA SYBR® qPCR Kit (KAPA Biosystems, USA) according to the manufacturer's instructions. The primers used was as follow: muc1 (Forward: 5'-GGCCACTGATTGTGCCTTAT-3', Reverse: 5'-TGCAACCTTGAAGTGGTCAG-3'), β-actin (Forward: 5'-GCT CTT TTC CAG CCT TCCTT-3', Reverse: 5'-TGA TCC ACA TCT GCT GGAAG-3'). The target mRNA level of control cells normalized to the level of β-actin mRNA, was defined as 1. Results were obtained from three independent experiments. The data were presented as mean ± SE. Differences in the results of different groups were evaluated using either two-tailed Student's t test or one-way ANOVA followed by post hoc Dunnett's test.

Immunofluorescence analysis

The effects of muc1 silencing and over-expression on the expression of Akt in cells were

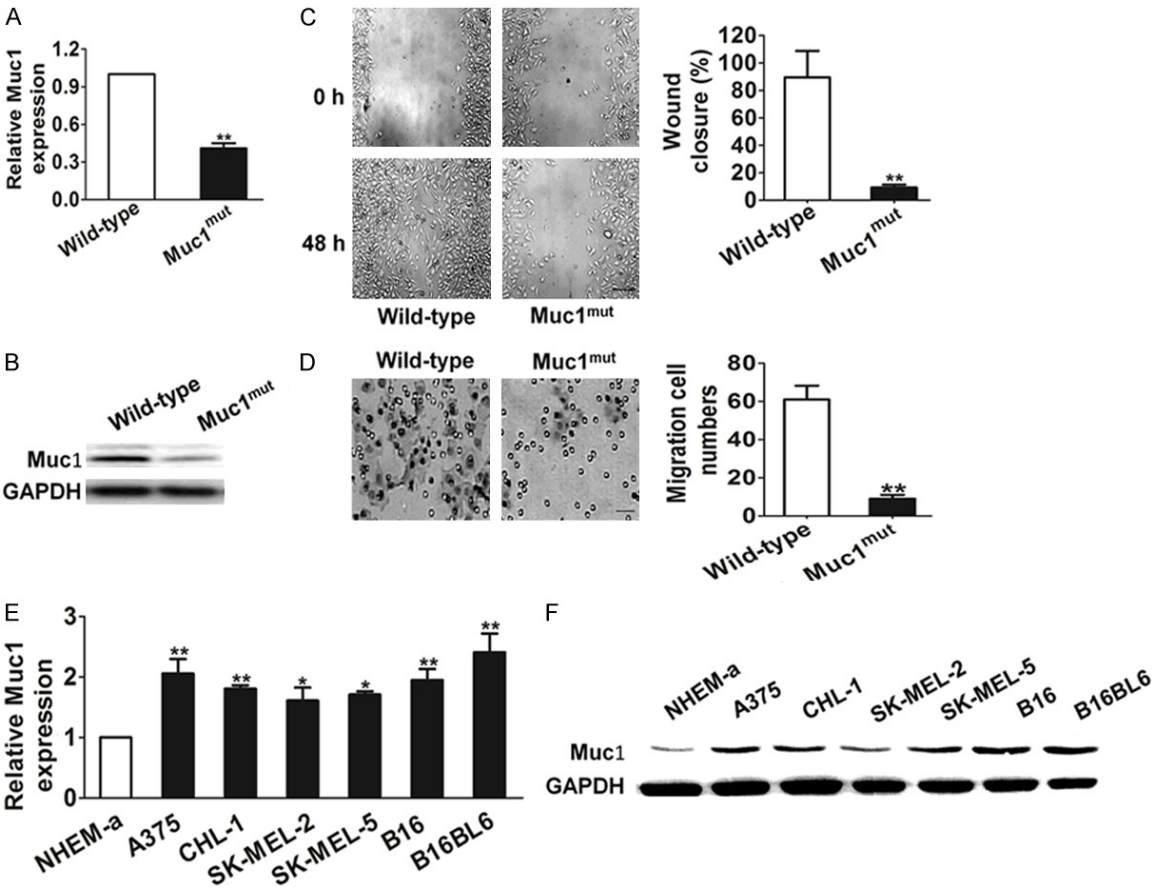

Figure 1. Identification of a novel role of muc1 in the metastasis of melanoma B16 cells. A and B. Muc1 expression in muc1^{mut} cells was analyzed by real-time PCR and Western blotting. C. Wound healing of control and muc1^{mut} cells was performed and representative pictures of the wound distance were taken at each time point as indicated. Scale bars: 100 μm. D. The cell motility was evaluated by transwell assay. Representative pictures were taken after staining with crystal violet. Scale bars: 50 μm. Data were collected from three independent experiments and were average ± S.E. values. $^{**}P < 0.01$, compared to wild type cells. E. Quantification of muc-1 mRNA levels in melanoma-derived cell lines by qRT-PCR analysis. All melanoma cells had significant up-regulation of muc-1 mRNA compared with that in the NHEM-a (Bars were represented as the mean ± S.E, n = 3, *P < 0.05 and **P < 0.01 versus NHEM-a). F. Immunoblotting analysis of muc-1 protein in the melanoma cell lines and melanocyte cell. Muc-1 protein expressions were up-regulated in all metastatic melanoma cell lines examined compared with that in the melanocyte cell.

examined using an immunocytochemical method. For immunofluorescent labeling, anti-Akt antibody was used as primary antibody and goat anti-rabbit IgG-FITC was used as a secondary antibody. Fluorescence cells were observed and photographed under a laser scanning confocal microscope (LEICA TCS SP5, Mannheim, Germany).

Animals and experimental metastasis assay

Female C57BL/6 mice at 6-8 weeks old (15-20 g) were purchased from Shanghai Slack laboratory animal co., LTD. Mice were maintained at dark/light cycles of 12 h duration with food and water available. 12 animals were randomly divided into two experiment groups. For experimental metastasis analysis, the mice were injected at the lateral tail vein with (5×10^5) cells carrying control and muc1 siRNA plasmids or vector and muc1. Mice were sacrificed 2 weeks after inoculation and all organs were examined for the presence of macroscopic metastases. Lung and liver metastatic nodules were determined under a dissecting microscope [17]. Animal handling and experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Huazhong University of Science and Technology. The data were presented as mean ± SE. Differences in the results of different treatment groups were evaluated using either two-tailed

Student's t test or one-way ANOVA followed by post hoc Dunnett's test.

Xenograft model

After muc1 knockdown, 3×10^6 B16 cells or B16BL6 cells were subcutaneously implanted into female, C57BL/6 mice to build melanoma xenograft [18]. Tumor volume and mice body weight were measured every 3 days. Tumor volume was calculated as $mm^3 = 0.5 \times \text{length } (mm)^3 \text{ width } (mm)^2$. Animal handling and experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Huazhong University of Science and Technology. The data were presented as mean ± SE. Differences in the results of two groups were evaluated using either two-tailed Student's t test or one-way ANOVA followed by post hoc Dunnett's test.

Statistical analysis

The data were presented as mean ± SE. Differences in the results of two groups were evaluated using either two-tailed Student's t test or one-way ANOVA followed by post hoc Dunnett's test. The differences with $P < 0.05$ were considered statistically significant.

Result

Identification of muc1 as a novel melanoma metastasis regulator

The melanoma cell line B16 is a widely used model to study the metastasis of melanoma for its high metastatic potential [19]. To identify the key genes involved in melanoma cell metastasis, we transfected B16 cells with pDisrup vector to randomly produce insertions into the genomic DNA, followed by selection with blasticidin to obtain mutated B16 cell clones. Cellular motility and migration are key hallmarks that distinguish benign from malignant tumors, enabling cells to cross tissue boundaries, disseminate in blood and lymph and establish metastases at distant sites. The mobility and migration ability of the selected mutant cell clones was then determined by wound healing and Transwell migration assay. Finally, cell clones with increased or decreased migration potential were further analyzed by the RT-PCR and 3' RACE to identify the genes disrupted by pDisrup vector. With this strategy, several candidate genes were identified, including a gene named muc1 and this candidate was designated as $muc1^{mut}$ which exhibited decreased metastasis potential. To verify whether the gene identified by this method was indeed disrupted in melanoma B16 cells, real-time PCR was carried out to determine muc1 gene expression. As shown in **Figure 1A** and **1B**, the expression of muc1 was greatly reduced in this cell clone compared to control cells. To determine if loss function of muc1 affects B16 migration, we performed wound healing and Transwell assay to evaluate the cell motility. As shown in **Figure 1C**, 48 h after scratching, the area of wound recovered by the migration of $muc1^{mut}$ cells was not significant and only less than half of that for control ones. 48 h later, wild type cells had almost closed up the wound, but not $muc1^{mut}$ cells. Consistently, there were less $muc1^{mut}$ cells that migrated across the membrane of the Transwell chamber compared to the wide type cells (**Figure 1D**). In summary, disruption of muc1 led to reduced B16 cells mobility and significantly impaired the migration of melanoma cell B16.

To investigate the mRNA expression of muc1, we performed quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) analysis in normal human melanocyte cell line NHEM-a and a panel of melanoma cell lines. Muc1 mRNA was up-regulated in all metastatic cell lines compared with that in the melanocyte cell line NHEM-a (**Figure 1E**). We also performed western blot analysis to investigate the muc1 protein expression status in the melanoma cells and melanocyte cell. A significant increase in muc1 protein expression was seen in metastatic cell lines compared with NHEM-a (**Figure 1F**). These analyses indicated that both transcription and translational products of muc1 were highly expressed in metastatic melanoma cell lines. The expression level of muc1 genes in melanoma was also analyzed using Oncomine (https://www.oncomine.org/). For this, we compared clinical specimens of cancer vs. normal patient from the Haqq Melanoma database [20]. We analyzed the results for their *p*-values and fold change. Oncomine analysis of neoplastic vs. normal tissue showed that muc1 was significantly over-expressed in melanoma (Figure S1).

To ascertain muc1 was indeed responsible for reduced migration in melanoma cells, we investigated whether reduced melanoma B16 and B16BL6 cells migration could be reproduced by

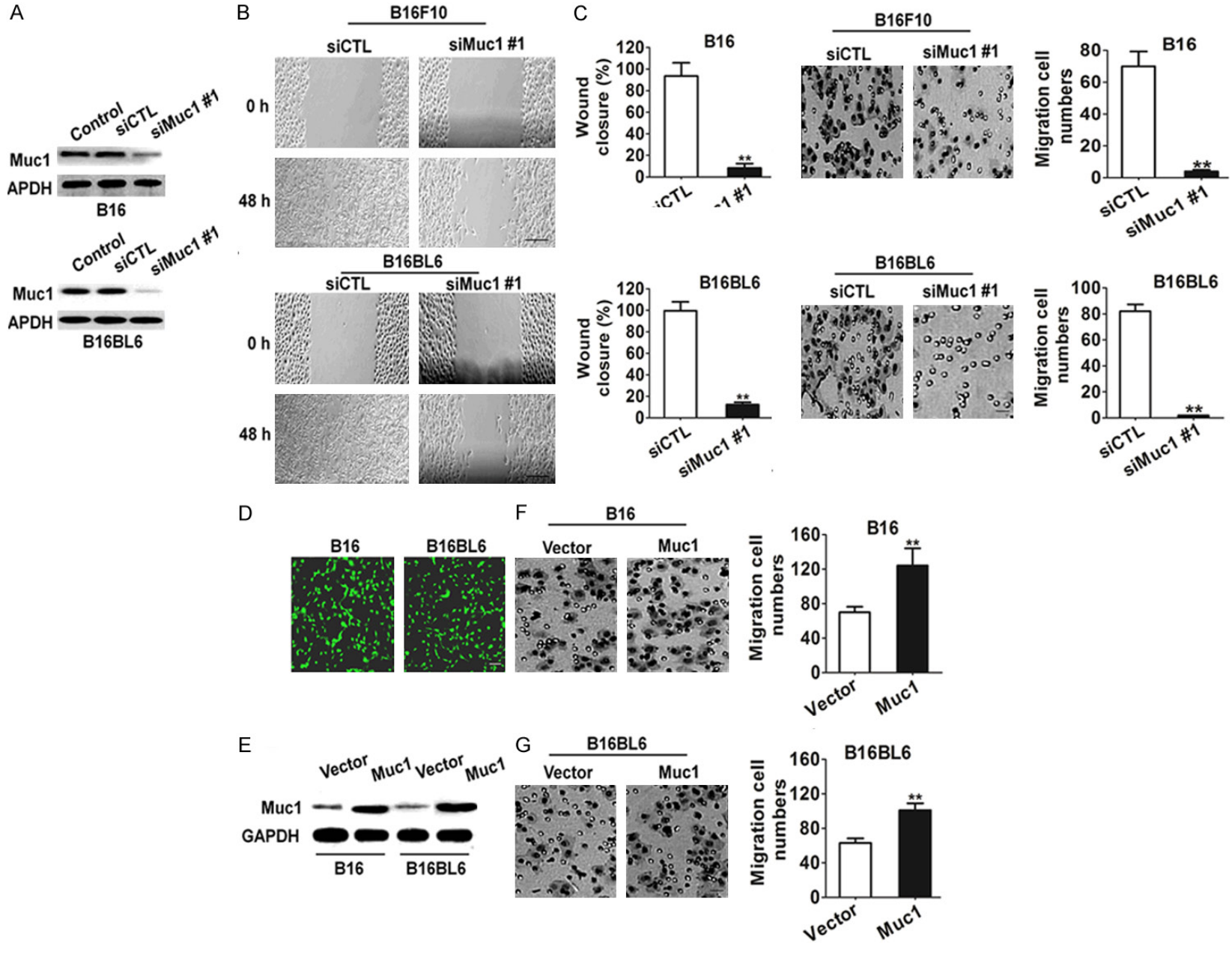
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Figure 2. Muc1 silencing decreases the migration of melanoma B16 and B16BL6 cells. A. Western blot shows that the muc1 was elevated in control cells, siMuc1 #1 and siCTL transfected cells. GAPDH was used as a loading control. B. Both siMuc1 and siCTL #1 transfected cells were plated in 6 cm dishes. Wound healing assay was performed to determine the metastatic potential of cells and representative pictures of the wound distance were taken at 0 and 48 h post scratching as indicated. The percentage of wound closure was quantified. C. The cell motility was evaluated by transwell assays. Data were collected from three independent experiments and were average ± S.E. values. **P < 0.01, compared to control cells. D. Muc1 was cloned into pIRES2-EGFP vector and transfected into melanoma B16 and B16BL6 cells. The cells transfected with an empty vector were used as control. E. The transfection efficiency was evaluated by the expression of green fluorescence protein (GFP) and the expression of muc1 was determined western blot with muc1 antibody. Scale bars: 100 μm. GAPDH was used as a loading control. F and G. The cell motility was evaluated by Transwell assay and the number of migration cells was quantified. Scale bars: 50 μm. Data were from three independent experiments and are average ± S.E. values. *P < 0.01, compared to control cells.

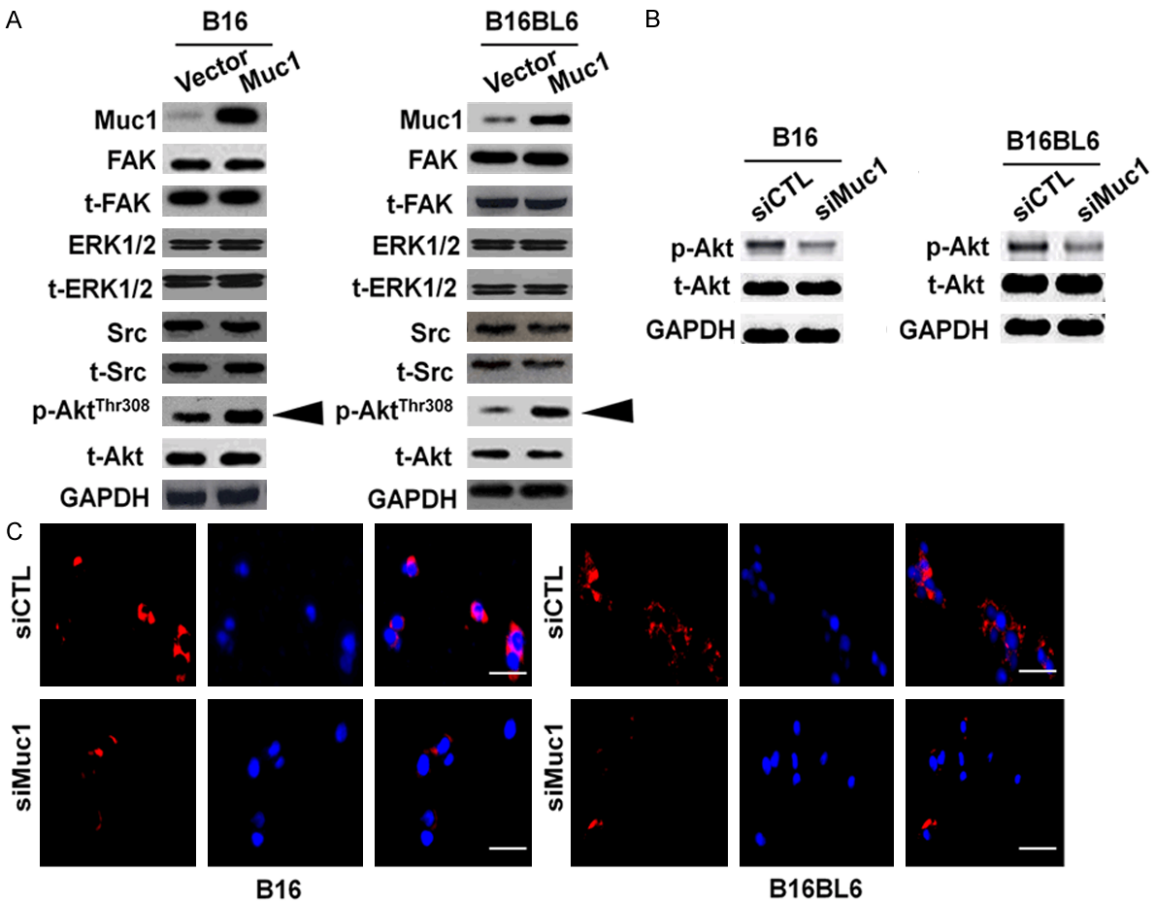

Figure 3. Muc1 regulates Akt pathway in melanoma cells. A. Western blot shows that the phosphorylation of Akt was elevated in cells transfected with pIRES-EGFP-muc1. B. The phosphorylation of Akt was perturbed in cells transfected with muc1 siRNA plasmid. C. Immunofluorescent staining analysis of the effect of muc1 gene silencing and over-expression on intracellular Akt expression in B16 and B16BL6 cells. Red color was detected for Akt, while nuclei were counterstained with blue color using DAPI (scale bar represents 50 μm).

gene silencing with two independent siRNAs specific for murine muc1 (the sequences of siRNAs are listed in Materials and Methods: Plasmids and DNA Constructs). To perform this experiment, we silenced the expression of muc1 with two siRNA-incorporated plasmids. As shown in **Figure 2A**, the expression of muc1 in B16 and B16BL6 transfected with muc1-specific siRNAs (siMuc1 #1) was significantly decreased compared with the cells transfected with scrambled siRNA (siCTL). Then, the transfected cells were subjected to wound healing and Transwell assay to evaluate their migratory potential. Both B16 and B16BL6 cells transfected with siCTL were able to close a wound by 48 h. However, the wound inflicted on cells

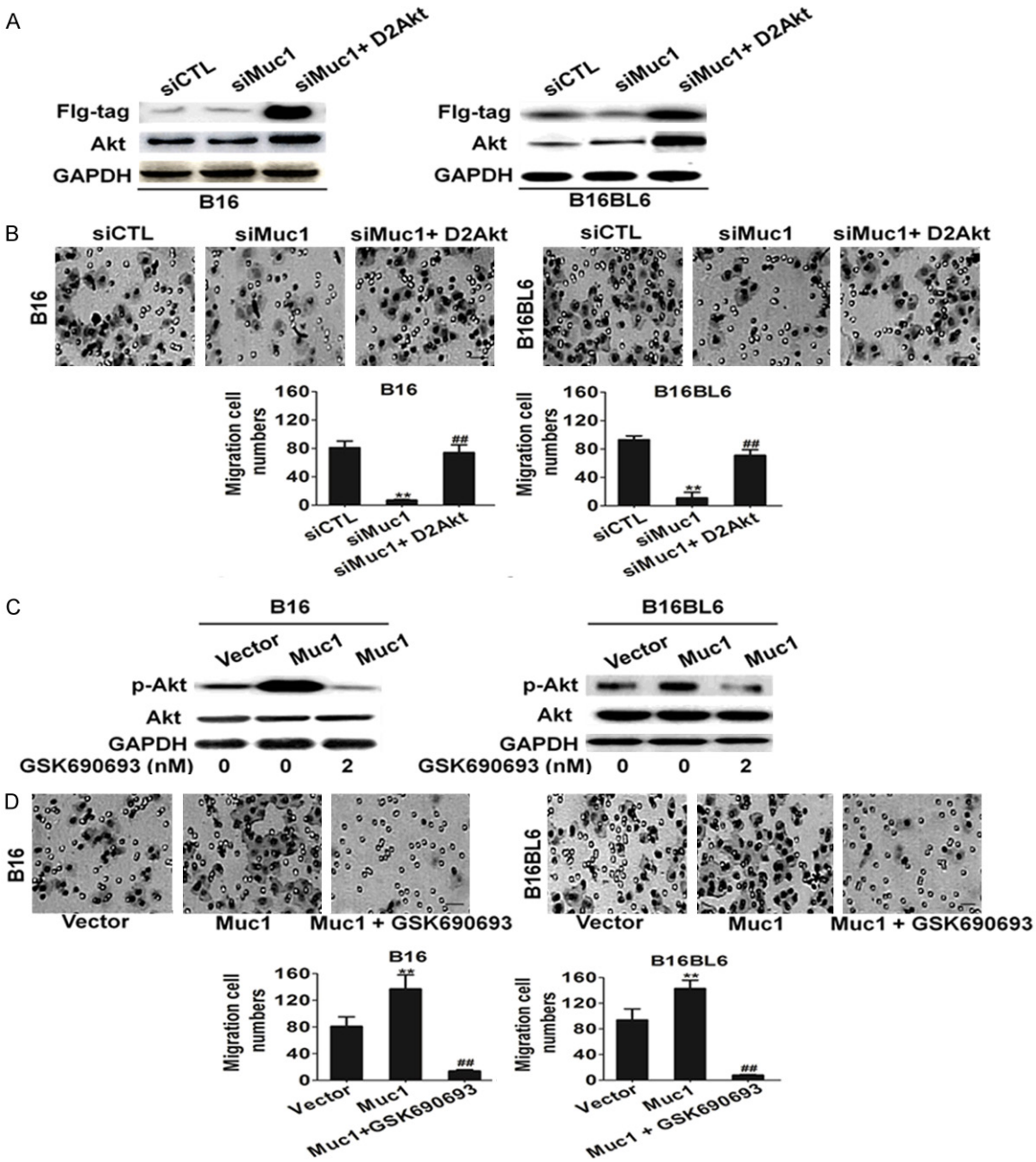

Figure 4. Muc1 facilities the migration of melanoma cells via Akt pathway. A. Expression of D2Akt was confirmed by western blot with antibody against Flag-tag and Akt, and GAPDH was used as loading control. B. Transwell assay was performed to determine the motility of cells co-transfected with muc1 silencing plasmid and D2Akt plasmid. Columns were data collected from three independent experiments. Representative pictures were taken after staining with crystal violet. Scale bar represents 50 μm. Data were from three independent experiments and are average ± S.E. values. $^{**}P < 0.01$, compared to siCTL cells; $^{\#\#}P < 0.01$, compared to siMuc1 cells. C. In the presence of GSK690693 (2 nM), B16 and B16BL6 cells were incubated for 1 h, protein extracts were analyzed by western blot with antibodies against phosphorylated Akt (S473) or Akt. D. In the presence of GSK690693 (2 nM), transwell migration assay was conducted to evaluate the cell motility after transfection. Scale bars: 50 μm. Data were from three independent experiments and were average ± S.E. values. $^{**}P < 0.01$, compared to transfected vector cells; $^{\#\#}P < 0.01$, compared to transfected muc1 cells.

transfected with muc1 siRNA #1 had not yet closed up at 48 h (**Figure 2B**). Consistently, transwell assay results also showed that the number of siMuc1 cells moved across the

membrane was fewer than the siCTL cells (**Figure 2C**). We tested another specific siRNA for muc1 (muc1 siRNA #2) to rule out the off-target effects. The muc1 siRNA #2 showed essentially the same effects on muc1 expression and on cell migration (Figure S2). Because muc1 down-regulation was stronger in the muc1 siRNA #1 cells, we employed B16-siMuc1 #1 and B16BL6-siMuc1 #1 cells to perform the following studies. These cells are labeled as "siMuc1" cells in the figures. Taken together, these results indicated that silencing of muc1 could reproduce the effect of muc1 disruption by pDisrup 8 plasmid and drastically reduced melanoma cells motility.

To further confirm the role of muc1 in melanoma cells motility, we cloned muc1 into pIRES-EGFP vector and transfected it into melanoma B16 and B16BL6 cells. The transfection efficiency was confirmed by the expression of green fluorescence protein (GFP) (**Figure 2D**). We also confirmed the over-expression of muc1 by western blot with muc1 antibody (**Figure 2E**). The migration of muc1 over-expressed B16 and B16BL6 cells was then examined by migration assay. As shown in **Figure 2F** and **2G**, there were more muc1 over-expressed cells migrated across the membrane compared with cells transfected vector. Invasion is the hallmark of tumor metastasis. Using invasion analysis, we investigated the effects of muc1 siRNA on invasion in tumor cells. In accordance with migration result, we found that silencing of muc1 in B16 or B16BL6 resulted in significantly suppressed invasion (Figure S3).

Akt mediates the role of muc1 in melanoma cell migration

To determine the signaling pathways which are involved in muc1-mediated melanoma cell migration, multiple potential signaling pathways related to migration and invasion of cancer cells were screened. As shown in **Figure 3A**, only the basal level of Akt activation was found to be significantly up-regulated in cells over-expressing muc1. In contrast, no obvious difference could be observed for many other signaling pathways, such as Focal Adhesion Kinase (FAK), extracellular regulated protein kinases (ERK) and Src. Consistently, when muc1 was silenced by siRNA in melanoma cells, Akt activation was also down regulated in B16 and B16BL6 cells (**Figure 3B**). Additionally, the activity of Akt was explored by immunofluorescence staining and confocal microscopy. As illustrated in **Figure 3C**, muc1 gene silencing hardly decreased Akt activity. Consistently, muc1 over-expression promoted Akt activity. In combination, these results strongly suggest that muc1 facilitates the activation of Akt signaling pathway in melanoma cells.

To confirm the role of Akt in muc1-mediated cell migration, constitutively active form of Akt (D2Akt) was introduced into muc1-silenced cells. The expression of D2Akt was confirmed by western blot with anti-Flag and anti-Akt antibody (**Figure 4A**). The migration of cells was then examined by migration assay. As expected, active Akt largely restored the impaired migration in muc1-silenced B16 and B16BL6 cells (**Figure 4B**). Furthermore, GSK690693, an Akt specific inhibitor was also employed to dissect the role of Akt signaling in cell migration [21]. As shown in **Figure 4C**, muc1-mediated phosphorylation of Akt was completely blocked by GSK690693. As a result, muc1-promoted migration in B16 and B16BL6 cells were also abolished by GSK690693, as shown by the transwell migration assay (**Figure 4D**). To conclude, these data indicate that Akt signaling is involved in muc1 promoted metastasis of melanoma cells.

Muc1 promotes lung metastasis of melanoma in vivo

To further investigate the role of muc1 in the metastasis of melanoma cells in vivo, an experimental metastasis assay was performed. Control and muc1 knock down cells were injected into the lateral tail vein of C57BL/6J mice. 2 weeks post inoculation, animals were sacrificed and all the major organs were checked for the generation of tumor metastasis. The tumor metastasis was mainly observed in the lungs as previously reported. We found that injection of B16 and B16BL6 control cells resulted in the formation of numerous lung colonies whereas silencing of muc1 significantly suppressed pulmonary metastasis (**Figure 5A**) and only generated one third of lung colonies (**Figure 5B**). To further confirm the role of muc1 in the metastasis of melanoma cells in vivo, experimental metastasis assay was performed. Control and muc1 over-expression cells were injected into

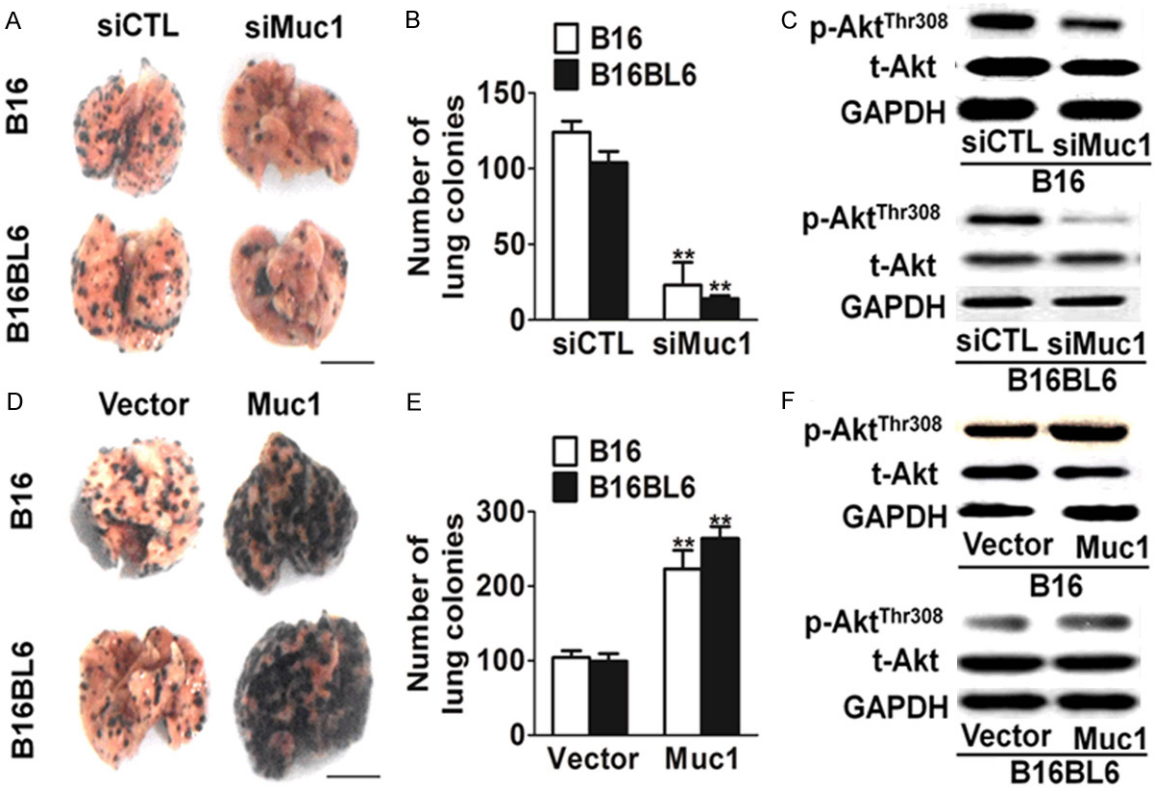

Figure 5. The metastasis of melanoma B16 and B16BL6 is impaired by muc1 silencing in vivo. A. siCTL and siMuc1 cells were injected into the lateral vein of C57BL/6 mice respectively. Representative pictures of lungs from mice were taken after 2 weeks of injection with control cells or with muc1 knock-down cells. B. Numbers of lung metastasis were quantified and showed by each data point. Data were from three independent experiments and were average ± S.E. values. n = 6, $**P < 0.01$, compared to mice injected with siTCL cells. C. Western blot showed that the phosphorylation of Akt was inhibited in lung tissue from mice injected with muc1 siRNA cells. D. Control vector transfected and muc1 over-expression cells were injected into the lateral vein of C57BL/6 mice respectively. Representative pictures of lungs from mice were taken after 2 weeks of injection with control cells or with muc1 over-expression cells. E. Numbers of lung metastasis were quantified and showed by each data point. Data were from three independent experiments and were average ± S.E. values. $**P < 0.01$, compared to mice injected with transfected vector cells. Scale bars: 1 cm. F. Western blot shows that the phosphorylation of Akt was increased in lung tissue from mice injected with muc1 over-expression melanoma cells.

the lateral tail vein of C57BL/6J mice. 2 weeks post inoculation, animals were sacrificed and we found that injection of B16 and B16BL6 over-expression cells resulted in the formation of more lung colonies than control cells (**Figure 5D** and **5E**). In addition, muc1 ove-rexpression cells produced nodules that occupied a higher percentage of the total lung area, while metastatic nodules of control cells generated discrete black foci (**Figure 5D**). These results implied that muc1 indeed perturbed the metastasis of melanoma cells not only in vitro but also in vivo. Muc1 has been identified as a key regulator in the Akt and influences cell migration and invasion in vitro. To investigate muc1-mediated metastasis in vivo was through regulation of Akt, Akt activity in lung tissues was assayed by western blot. Mice injected with muc1 knockdown cells showed a significant reduction of phospho-Akt expression in lung (**Figure 5C**). We also assessed the phospho-Akt levels in lung from mice injected with muc1 over-expression cells. Consistently, up-regulation of Akt activity was detected (**Figure 5F**).

Muc1 knockdown inhibits growth of melanoma cells

As Akt activity regulates many processes considered to be hallmarks of cancer, the Akt pathway has become an important new therapeutic target. Akt activation promotes resistance to standard chemotherapy and radiation therapy, and inhibition of Akt signaling induces apoptosis and decreased growth of tumor cells dependent on elevated Akt signaling for survival and

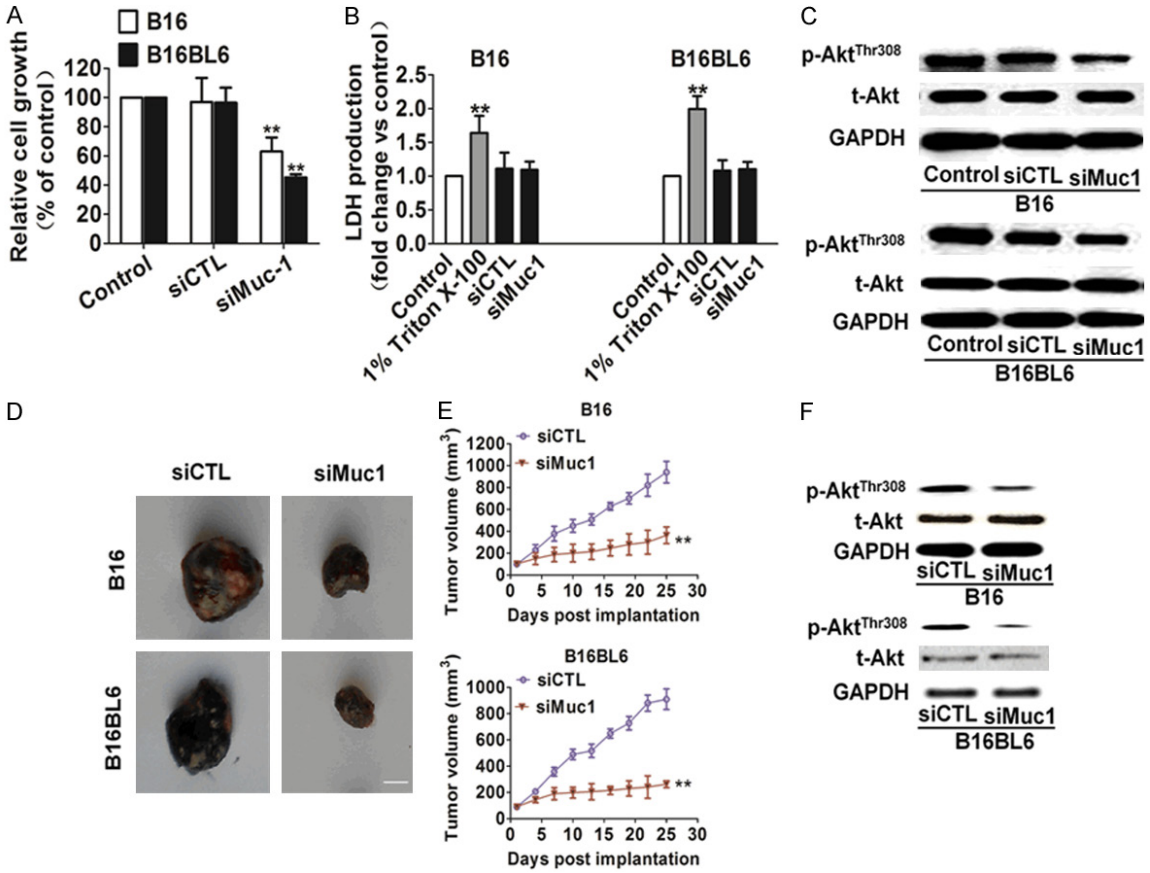

Figure 6. Muc1 siRNA inhibits tumor growth in vitro and in vivo. A. One solution cell proliferation assay analyzed cell proliferation in B16-siMuc1, B16BL6-siMuc1 and control cells. Data were from three independent experiments and were average ± S.E. values. $^{**}P < 0.01$, compared to control cells. B. Muc1 siRNA did not result in LDH release, indicating muc1 siRNA brought little toxic effects on melanoma cells. Data were from three independent experiments and were average ± S.E. values. $^{**}P < 0.01$, compared to control cells. C. Western blot showed that the phosphorylation of Akt was inhibited in cells transfected with muc1 siRNA. D. B16-siMuc1 and B16BL6-siMuc1 cells (3×10^6) were subcutaneously implanted into female, C57BL/6 mice to build melanoma xenograft. On day 25 post injection, tumors were removed and photographed. Representative pictures of tumor sizes in the B16 and B16BL6 groups. Scale bars: 0.5 cm. E. Muc1 siRNA resulted in significantly tumor growth inhibition versus control mice. Data were from three independent experiments and were average ± S.E. values. n = 6, $^{**}P < 0.01$, compared to mice injected with siTCL cells. F. Akt expression in tumor sections from different group was detected by western blot with the anti-Akt antibody and anti-phospho-Akt^{Ser473} antibody.

growth. We observed cell proliferation of the cell lines after transfected. In two cell lines tested, muc1 knockdown lead to a statistically significant decrease in growth compared to siCTL treated cells (**Figure 6A**). To validate whether the growth inhibition of muc1 siRNA was due to its toxicity effects on melanoma cells, LDH cytotoxicity assay was carried out. As shown in **Figure 6B**, Triton X-100 significantly increased LDH release, and muc1 knockdown as well as siCTL treated brought little toxic effects on two melanoma cells when compared to vehicle control. To elucidate its mechanism of action, we assessed the effect of muc1 knockdown on Akt. B16 and B16BL6 cells were transfected with siCTL or muc1 siRNA and western blot analysis was performed. As shown in **Figure 6C**, muc1 knockdown was associated with a decline in Akt activity.

To evaluate the effects of muc1 knockdown on melanoma growth in vivo, we further constructed an experiment using melanoma cells xenograft mouse model. Representative mice with B16 or B16BL6 xenografts and tumor masses were shown in **Figure 6D**. It was found that muc1 knockdown dramatically suppressed tumor volumes compared with the siCTL con-

trol group (**Figure 6E**). Furthermore, muc1 knockdown was well tolerated, and there was no significant difference in mice implantation with muc1 knockdown cells between control group (Figure S4). To further examine whether muc1 knockdown suppress melanoma cells growth via Akt, Akt activity in tumor tissues were assayed by western blot analysis with specific antibody against p-Akt^{Thr308}. Mice implantation with muc1 knockdown cells showed a significant reduction of p-Akt^{Thr308} in tumors (**Figure 6F**). All the results demonstrated that muc1 knockdown inhibited growth of melanoma cells via suppressed Akt signaling pathway.

Discussion

Malignant melanoma is the skin cancer with the highest risk of death for its highly metastatic potential. However, there is currently no effective treatment for metastatic melanoma partly due to the complicated mechanism underlying its metastasis. In the present study, we identified a novel role for muc1 in the metastasis of murine melanoma cells. We found that muc1 promotes the metastasis of melanoma cells in vitro and in vivo through the Akt signaling pathway. Our results may provide a new target for intervention in the melanoma treatment and may improve the future treatment of melanoma.

Muc1 is a membrane-bound glycoprotein consisting of a large extracellular subunit of a 20 amino acid tandem repeat domain, a small extracellular domain subunit, a transmembrane domain and a cytoplasm tail [22]. Previous studies showed that muc1 is frequently over-expressed in various cancers including breast, ovarian, lung, and colon cancer. It is also considered as a potential diagnostic, prognostic, and therapeutic biomarker of pancreatic cancer [23]. Over-expression of muc1 has been observed in many types of adenocarcinoma and correlated with lymph node metastasis and poor prognosis in patients. Increased levels and altered glycosylation of muc1 facilitate invasive growth and metastasis of tumor cells [24]. Muc1 cytoplasmic tail domain is involved in a wide range of intracellular signaling by association with numerous kinases, cell adhesion molecules, transcription factors and chaperones that are implicated in malignant transformation [25]. Recently studies shown muc1 contributes to the invasive and metastatic properties of adenocarcinomas by mediating the epithelial to mesenchymal transition (EMT), the mechanism by which polarized epithelial cells acquire mesenchymal cell properties with an enhanced potential for migration, and modulating both adhesive and anti-adhesive properties of tumor cells. In addition, muc1 in association with other molecules such as β-catenin, NF-kB p65 or EGFR, regulates transcription of several genes responsible for progression and invasiveness of cancer [26]. So far, no investigation has been carried out on the involvement of muc1 in melanoma metastasis. In this work, we firstly reported a novel role for muc1 in the metastasis of melanoma cells. We found that the metastasis of melanoma cells was significantly inhibited, when the muc1 gene was disrupted by insertional mutagenesis. Further investigation with gene silencing of muc1 showed that the metastasis of melanoma cells was significantly decreased as revealed by the migration assay and this effect is specific to skin cancer cells. In contrast, the over-expression of muc1 in murine B16 and B16BL6 melanoma cells greatly enhanced the migration of melanoma cells. More convincingly, muc1 silencing markedly impaired the lung metastasis of murine melanoma cells in vivo. All these data presented that muc1 is involved in the metastasis of melanoma cells. In the past study from Fengli Wang, et al [27], authors investigated the effects of muc1 on cancer-related characteristics of B16 cells by stable expression of the human full-length muc1 in cells and found up-expression of human full-length muc1 inhibit migration of mouse melanoma cell line. However, they transfected full-length human muc1 into murine derived melanoma cells B16, it may merely be equivalent to transfection of the mouse muc1 cytoplasmic tail [27], Structurally, muc1 contains two domains believed to be of functional significance, the large extracellular domain of muc1 and muc1 cytoplasmic tail. The muc1 extracellular domain engages in signal transduction through several residues that can be phosphorylated by receptor tyrosine kinases (and other kinases) [12, 27]. In our current manuscript, we investigated the effects and related mechanisms of muc1 on cancer cells by specific siRNA muc1 extracellular domain and found down-expression of muc1 inhibit migration of mouse

melanoma cells. Although the results are inconsistent, we suspect the function of muc1 extracellular domain and muc1 cytoplasmic tail is opposite in melanoma. Whatever, contradiction in this term throws out light into the double-edged sword in cancer. In drug development, blocking antibody should specific for muc1 extracellular domain to inhibit melanoma cells metastasis. In addition, we proofed muc1 is up-expression in cancer using cell lines and cancer public database (Oncomine), which is consistent with previous results [28, 29]. Based on these findings, we propose that muc1 may play an important role in regulating melanoma cells migration.

The underlying molecular mechanism for muc1-regulated melanoma metastasis is identified to be related to Akt signaling pathway. Our results showed that over-expression of muc1 in B16 and B16BL6 murine melanoma cells induces up-regulation of Akt phosphorylation and muc1 silencing leads to reduced Akt phosphorylation. Furthermore, restored Akt activity by an active form Akt plasmid could rescue the impaired migration of melanoma cells induced by muc1 silencing. In the presence of GSK690693, a specific inhibitor of Akt signaling pathway, muc1 over-expression promoted migration were significantly inhibited as revealed by the Transwell migration assay. The pro-metastatic potential of Akt pathway can be supported by the involvement of up-regulation of phosphorylated Akt in severely dysplastic nevi and metastatic melanomas compared with normal or mildly dysplastic nevi. Moreover, activation of Akt pathway is also required in human breast cancer cells and ovarian cancer cells to promote cellular invasion and metastasis. However, the detail mechanisms underlying muc1 regulating the metastasis of melanoma cells is still not clear. We speculated that the mechanism leading to this specificity involves the ability of muc1 to associate with Akt activity.

Due to the important roles of muc1 in the metastasis of melanoma cells, it may serve as an attractive target for molecular targeting cancer therapy. In the further work, it is worthwhile to elucidate the precise roles of muc1 in regulating the Akt signaling thus mediating the metastasis in melanoma cells.

Oncomine analysis

The expression level of muc1 genes in the melanoma was analyzed using Oncomine [1]. For this, we compared clinical specimens of cancer vs. normal patient from the Haqq Melanoma database [2]. In order to reduce our false discovery rate, we selected $P < 0.01$ as a threshold. We analyzed the results for their p-values, fold change, and cancer subtype.

Lactate dehydrogenase (LDH) toxicity assay

The LDH released into cell cultures is an index of cytotoxicity and evaluation of the permeability of cell membrane. HUVECs were seeded in 96-well plate at a density of 3×10^3 cells per well. After incubation with vehicle (0.1% DMSO), 1% Triton X-100 or various concentrations of rhamnazin for 24 h, cell supernatants were collected and analyzed for LDH activity using LDH cyto-toxicity assay kit from Keygen biotech [3]. The absorbance of formed formazan was read at 490 nm on a microplate reader. Differences in the results of two groups were evaluated using either two-tailed Student's t test or one-way ANOVA followed by post hoc Dunnett's test.

Invasion assay

Assay was performed with Matrigel-coated chambers from a BioCoat Matrigel Invasion Chamber Kit (BD Biosciences). Cells with 500 μl in serum-free medium were added into the upper chamber and complete medium was added into the lower chamber. After incubation for 24 h, non-invasive cells in the upper surface of the membrane were removed and the cells invasion to the lower surface of the membrane was fixed. Invasion cells was stained with cell membrane green fluorescent probe (3,3'-dioctadecyloxacarbocyanine perchlorate, DiO, Beyotime Biotechnology). Cell counting was then carried out by photographing the membrane through the microscope [4] and five random fields were taken. The data were presented as mean ± SD. Differences in the results of two groups were evaluated using either two-tailed Student's t test or one-way ANOVA followed by post hoc Dunnett's test.

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Disclosure of conflict of interest

None.

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References

[1] Mort RL, Jackson IJ, Patton EE. The melanocyte lineage in development and disease. Development 2015; 142: 620-632.

[2] Gonsalves CF, Eschelman DJ, Thornburg B, Frangos A, Sato T. Uveal Melanoma Metastatic to the Liver: Chemoembolization With 1,3-Bis-(2-Chloroethyl)-1-Nitrosourea. AJR Am J Roentgenol 2015; 23: 1-5.

[3] Aris M, Barrio MM. Combining immunotherapy with oncogene-targeted therapy: a new road for melanoma treatment. Front Immunol 2015; 6: 46.

[4] Olszanski AJ. Current and future roles of targeted therapy and immunotherapy in advanced melanoma. J Manag Care Spec Pharm 2014; 20: 346-356.

[5] Azijli K, Stelloo E, Peters GJ, VAN DEN Eertwegh AJ. New developments in the treatment of metastatic melanoma: immune checkpoint inhibitors and targeted therapies. Anticancer Res 2014; 34: 1493-1505.

[6] Kufe DW. MUC1-C oncoprotein as a target in breast cancer: activation of signaling pathways and therapeutic approaches. Oncogene 2013; 32: 1073-1081.

[7] Kesari MV, Gaopande VL, Joshi AR, Babanagare SV, Gogate BP, Khadilkar AV. Immunohistochemical study of MUC1, MUC2 and MUC5AC in colorectal carcinoma and review of literature. Indian J Gastroenterol 2015; 34: 63-67.

[8] Park JY, Hiroshima Y, Lee JY, Maawy AA, Hoffman RM, Bouvet M. MUC1 Selectively Targets Human Pancreatic Cancer in Orthotopic Nude Mouse Models. PLoS One 2015; 10: e0122100.

[9] Tréhoux S, Duchêne B, Jonckheere N, Van Seuningen I. The MUC1 oncomucin regulates pancreatic cancer cell biological properties and chemoresistance. Implication of p42-44 MAPK, Akt, Bcl-2 and MMP13 pathways. Biochem Biophys Res Commun 2015; 456: 757-762.

[10] Banerjee D, Fernandez HR, Patil PB, Premaratne P, Quiding Järbrink M, Lindén SK. Epithelial MUC1 promotes cell migration, reduces apoptosis and affects levels of mucosal modulators during acetylsalicylic acid (aspirin)-induced gastropathy. Biochem J 2015; 465: 423-431.

[11] Miller AD, Buttimore C. Redesign of retrovirus packaging cell lines to avoid recombination leading to helper virus production. Mol Cell Biol 1986; 6: 2895-2902.

[12] Pochampalli MR, el Bejjani RM, Schroeder JA. MUC1 is a novel regulator of ErbB1 receptor trafficking. Oncogene 2007; 26: 1693-701.

[13] Aoki M, Batista O, Bellacosa A, Tsichlis P, Vogt PK. The akt kinase: molecular determinants of oncogenicity. Proc Natl Acad Sci U S A 1998; 95: 14950-14955.

[14] Seong I, Min HJ, Lee JH, Yeo CY, Kang DM, Oh ES, Hwang ES, Kim J. Sox10 controls migration of B16F10 melanoma cells through multiple regulatory target genes. PLoS One 2012; 7: e31477.

[15] Hu S, Cao B, Zhang M, Linghu E, Zhan Q, Brock MV, Herman JG, Mao G, Guo M. Epigenetic silencing BCL6B induced colorectal cancer proliferation and metastasis by inhibiting P53 signaling. Am J Cancer Res 2015; 5: 651-662.

[16] Hambright HG, Meng P, Kumar AP, Ghosh R. Inhibition of PI3K/AKT/mTOR axis disrupts oxidative stress-mediated survival of melanoma cells. Oncotarget 2015; 6: 7195-7208.

[17] Song Z, Lin J, Sun Z, Ni J, Sha Y. RNAi-mediated downregulation of CDKL1 inhibits growth and colony-formation ability, promotes apoptosis of human melanoma cells. J Dermatol Sci 2015; 79: 57-63.

[18] Mittal A, Tabasum S, Singh RP. Berberine in combination with doxorubicin suppresses growth of murine melanoma B16F10 cells in culture and xenograft. Phytomedicine 2014; 21: 340-347.

[19] Netland PA, Zetter BR. Metastatic Potential of B16 Melanoma Cells after In Vitro Selection for Organ-specific Adherence. J Cell Biol 1985; 101: 720-724.

[20] Haqq C, Nosrati M, Sudilovsky D, Crothers J, Khodabakhsh D, Pulliam BL, Federman S, Miller JR 3rd, Allen RE, Singer MI, Leong SP, Ljung BM, Sagebiel RW, Kashani-Sabet M. The gene expression signatures of melanoma progression. Proc Natl Acad Sci U S A 2005; 102: 6092-6097.

[21] Salt MB, Bandyopadhyay S, McCormick F. Epithelial-to-mesenchymal transition rewires the molecular path to PI3K-dependent proliferation. Cancer Discov 2014; 4: 186-199.

[22] Tholey RM, Lal S, Jimbo M, Burkhart RA, Blanco FF, Cozzitorto JA, Eisenberg JD, Jiang W, Iacobuzio Donahue CA, Witkiewicz AK, Glbert M, Yeo CJ, Brody JR, Sawicki JA, Winter JM. MUC1 Promoter-Driven DTA as a Targeted Therapeutic Strategy against Pancreatic Cancer. Mol Cancer Res 2015; 13: 439-448.

[23] Mori Y, Akita K, Tanida S, Ishida A, Toda M, Inoue M, Yashiro M, Sawada T, Hirakawa K, Nakada H. MUC1 protein induces urokinase-type plasminogen activator (uPA) by forming a complex with NF-κB p65 transcription factor and binding to the uPA promoter, leading to enhanced invasiveness of cancer cells. J Biol Chem 2014; 289: 35193-35204.

[24] Kharbanda A, Rajabi H, Jin C, Tchaicha J, Kikuchi E, Wong KK, Kufe D. Targeting the oncogenic MUC1-C protein inhibits mutant EGFR-mediated signaling and survival in non-small cell lung cancer cells. Clin Cancer Res 2014; 20: 5423-5434.

[25] Gronnier C, Bruyère E, Lahdaoui F, Jonckheere N, Perrais M, Leteurtre E, Piessen G, Mariette C, Seuningen I. The MUC1 mucin regulates the tumorigenic properties of human esophageal adenocarcinomatous cells. Biochim Biophys Acta 2014; 1843: 2432-2437.

[26] Pinzaglia M, Montaldo C, Polinari D, Simone M, La Teana A, Tripodi M, Mancone C, Londei P, Benelli D. EIF6 over-expression increases the motility and invasiveness of cancer cells by modulating the expression of a critical subset of membrane-bound proteins. BMC Cancer 2015; 15: 131.

[27] Wang F, Li Q, Ni W, Fang F, Sun X, Xie F, Wang J, Wang F, Gao S, Tai G. Expression of human full-length MUC1 inhibits the proliferation and migration of a B16 mouse melanoma cell line. Oncol Rep 2013; 30: 260-268.

[28] Lan MS, Batra SK, Qi WN, Metzgar RS, Hollingsworth MA. Cloning and sequencing of a human pancreatic tumor mucin cDNA. J Biol Chem 1990; 265: 15294-15299.

[29] Bafna S, Kaur S, Batra SK. Membrane-bound mucins: the mechanistic basis for alterations in the growth and survival of cancer cells. Oncogene 2010; 29: 2893-904.

[30] Gasparini P, Fassan M, Cascione L, Guler G, Balci S, Irkkan C, Paisie C, Lovat F, Morrison C, Zhang J, Scarpa A, Croce CM, Shapiro CL, Huebner K. Androgen receptor status is a prognostic marker in non-basal triple negative breast cancers and determines novel therapeutic options. PLoS One 2014; 9: e88525.

[31] Haqq C, Nosrati M, Sudilovsky D, Crothers J, Khodabakhsh D, Pulliam BL, Federman S, Miller JR 3rd, Allen RE, Singer MI, Leong SP, Ljung BM, Sagebiel RW, Kashani-Sabet M. The gene expression signatures of melanoma progression. Proc Natl Acad Sci U S A 2005; 102: 6092-6097.

[32] Yu Y, Cai W, Pei CG, Shao Y. Rhamnazin, a novel inhibitor of VEGFR2 signaling with potent antiangiogenic activity and antitumor efficacy. Biochem Biophys Res Commun 2015; 458: 913-919.

[33] Liang J, Li X, Li Y, Wei J, Daniels G, Zhong X, Wang J, Sfanos K, Melamed J, Zhao J, Lee P. LEF1 targeting EMT in prostate cancer invasion is mediated by miR-181a. Am J Cancer Res 2015; 5: 1124-1132.

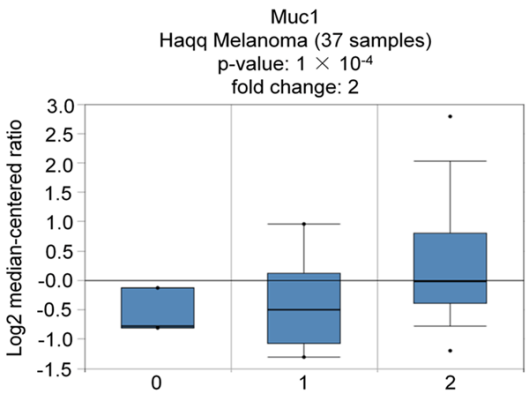

Figure S1. Analysis of muc1 expression in normal skin versus melanoma from the Haqq Melanoma database. Left plot (normal, n = 3); Middle plot (non-neoplastic nevus, n = 9) and right plot (melanoma, n = 25).

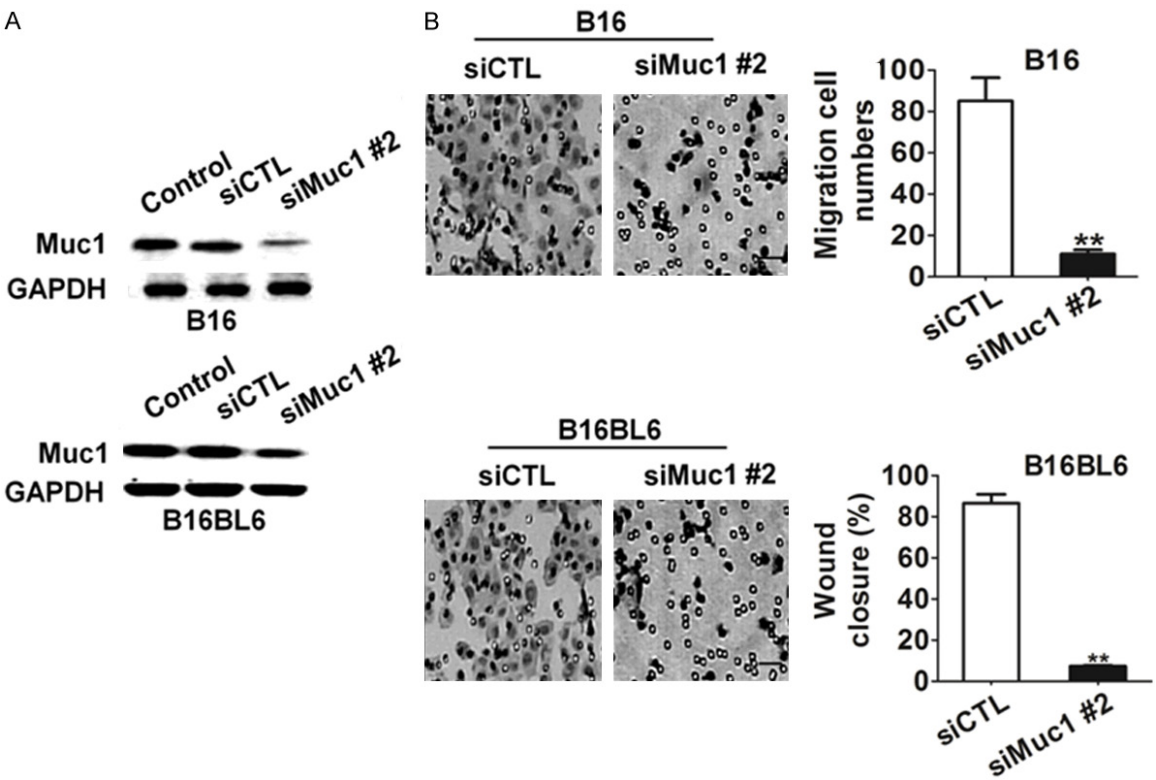

Figure S2. Muc1 siRNA #2 decreases the migration of melanoma B16 and B16BL6 cells. A. Western blot shows that the muc1 was elevated in control cells, siMuc1 #2 and siCTL transfected cells. GAPDH was used as a loading control. B. Both siMuc1 #2 and siCTL transfected cells were performed to migration assays to evaluated migration ability. Data were collected from three independent experiments and were average ± S.E. values. **P < 0.01, compared to control cells. Scale bars: 50 μm.

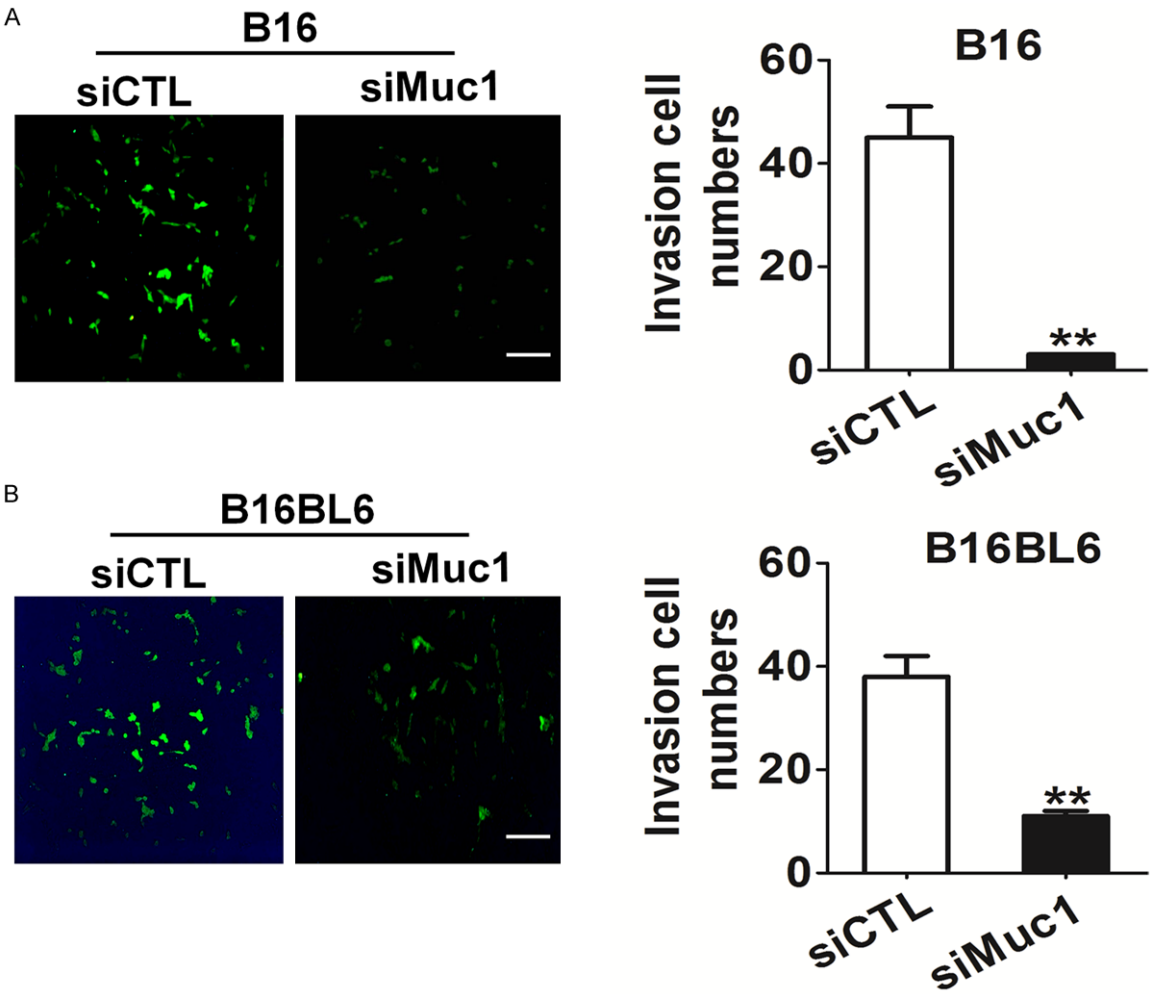

Figure S3. The cell invasion was evaluated by Transwell invasion assays. Data are collected from three independent experiments and are average ± S.E. values. $^{**}P < 0.01$, compared to control cells. Muc1 siRNA inhibited the invasion of melanoma B16 (A) and B16BL6 cells (B). Scale bars: 50 μm.

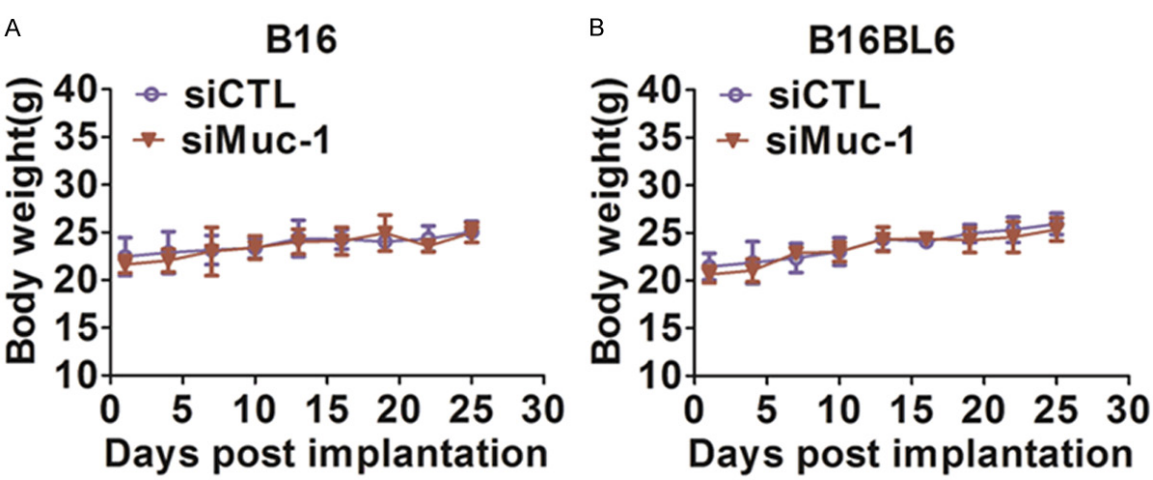

Figure S4. Body weight changes in C57BL/6 mice mice injected with siCTL and siMuc1 B16 cells (A) or siCTL and siMuc1 B16BL6 cells (B). There was no significant difference in body weight between siCTL and siMuc1 group. Bars are represented as the mean ± S.E, n = 6.