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Original Paper

MicroRNA-29a Inhibits Growth, Migration and Invasion of Melanoma A375 Cells in Vitro by Directly Targeting BMI1

Ying Xiong[a] Liqian Liu[a] Ying Qiu[b] Lili Liu[b]

[a]Department of Dermatology, Linyi People's Hospital, Linyi, [b]Department of Pathology, Linyi People's Hospital, Linyi, China

Key Words
Melanoma • miR-29a • BMI1 • Tumor suppressor • Wnt/β-catenin pathway • NF-κB pathway

Abstract
Background/Aims: Melanoma is one of the most aggressive malignant tumors, with increasing incidence, poor prognosis, and lack of any effective targeted therapies. Abnormal expression of miR-29a has been found in several types of cancers, including melanoma. In this study, experiments were performed to investigate the role of miR-29a in melanoma, and the molecular mechanism by which miR-29a represses melanoma. ***Methods:*** miR-29 and Bmi1 expression was examined by quantitative real-time polymerase chain reaction (qRT-PCR). The cell viability, apoptosis, migration and invasion were respectively determined by Cell Counting Kit-8 assay, Propidium iodide (PI) fluorescein isothiocynate (FITC)-Annexin V staining assay, wound healing assay and transwell assay. Luciferase reporter assay was performed to determine a target gene of miR-29a. Western blot was used to analyze protein expression of apoptosis-related proteins, Bmi1, Wnt/β-catenin and Nuclear factor-κB (NF-κB) pathway target genes. ***Results:*** miR-29a was down-regulated in all tested melanoma cell lines. Up-regulation of miR-29a effectively inhibited cell viability, migration, and invasion, but promoted apoptosis in A375 cells. Bmi1 was a direct target gene of miR-29a. Transfection with miR-29a mimic decreased cell migration and invasion and Bmi1 expression in Malme-3M cells, SK-MEL-2, SK-MEL-5, and M14 cell lines. Moreover, miR-29a might suppress growth, migration and invasion of A375 cells by negatively regulating Bmi1. In addition, our results demonstrated that transfection with miR-29a mimic effectively blocked Wnt/β-catenin and NF-κB pathways via down-regulating Bmi1. ***Conclusion:*** miR-29a could be functioned as a potential tumor suppressor through direct regulation of Bmi1 in melanoma cells.

Introduction

Malignant melanoma, an aggressive and deadly skin cancer, accounted for a majority of skin cancer-related deaths and keeps a stable mortality rate [1]. Despite the progresses in

Lili Liu
Department of Pathology, Linyi People's Hospital
No. 27, Jiefang East Road, Linyi, Shandong 276003 (China)
E-Mail liulili823@126.com

chemotherapy, surgery, and radiotherapy to limit the mortality it causes, the situation is still not optimistic because melanoma incidence has doubled since 1950s in Europe, Singapore, Canada, and United State [2-4]. Consequently, the further research of melanoma, such as the mechanisms underlying its pathogenesis, is important to identify its occurrence and metastasis early and provide new potential targets for melanoma treatment.

microRNAs (miRNAs), a class of short (19-24 nucleotides in length) endogenous non-protein-coding single-stranded RNAs, can regulate ~1/3 of mRNAs in the human genome by binding the 3' untranslated regions (3'UTRs) of target mRNAs and then inducing either translational repression or transcript degradation [5]. Recently, abundant studies have already reported the importance of miRNAs in an increasing number of cancers [5, 6]. In melanoma, some miRNAs have been found abnormally expressed, such as miR-153-3p [7], miR-211 [8], miR-196a [9], miR-30b, and miR-30d [10]. They have been revealed as oncogenes or tumor suppressor genes in melanoma. For instance, ectopic expression of miR-211 in melanoma cell lines resulted in significant inhibition of growth and invasion compared to parental cells [8]. Contrary to miR-211, miR-30b and miR-30d were reported to have functions as potential tumor promoters, as evidenced by simultaneously promoting cellular invasion and immunosuppression in melanoma cells [10].

Aberrant expression of miR-29a has been found in several types of cancers, indicating that miR-29a might play a critical role in occurrence and development of tumors [11, 12]. Interestingly, previous studies revealed that miR-29a demonstrated anti-tumor functions as a tumor suppressor in hepatocellular carcinoma [13], pancreatic cancer cells [14], pediatric acute myeloid leukemia [15], prostate cancer [16], papillary thyroid carcinoma [11] and myeloma [17]; while miR-29a surprisingly demonstrated tumor-promoting functions in breast cancer [18], nasopharyngeal carcinoma [12] and in oral squamous cell carcinoma [19]. Thus, results showed that miR-29a might have different functions in different cancer cells. Based on that, experiments were performed to investigate the effects of miR-29a in melanoma cells.

Schmitt *et al.* reported that melanoma cell lines with lower miR-29a showed an increased proliferation rate compared to lines with higher basal miR-29a levels [20]. However, the effects of miR-29a on melanoma cell viability, apoptosis, migration, and invasion was unknown. Besides, the underlying mechanisms by which miR-29a affects melanoma are still unclear. Thus, this study focused on the role of miR-29a on cell growth, migration, and invasion of melanoma in different melanoma cell lines, as well as functions of miR-29a in A375 cells. The regulation of miR-29a and Bmi1 were emphatically detected to reveal whether Bmi1 was involved in the anti-melanoma role of miR-29a in A375 cells.

Materials and Methods

Cell line and cell culture

Normal human melanocyte HACAT and foreskin fibroblast HFF, as well as human melanoma cell lines A375, Malme-3M, SK-MEL-2, SK-MEL-5, and M14 were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were maintained in Dulbecco's modified Eagle medium (DMEM; Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS; Gibco, Carlsbad, CA, USA), 100 U/mL penicillin, and 100 mg/mL streptomycin (Gibco). Cells were maintained in a 5% CO_2 humidified incubator at 37°C.

Transfection assay

The Bmi1 overexpression was conducted by constructing the full-length Bmi1 sequences into cells via pEX-2 plasmids (GenePharma, Shanghai, China). pEX-2 plasmids contains coding zones but no 3'UTR. miR-29a mimic, miR-29a inhibitor, and their negative controls (NC) were synthesized by GenePharma Co. (Shanghai, China). Lipofectamine 3000 reagent (Invitrogen) was used for all transfections following the manufacturer's protocol. After 48 h of transfection, cells were collected for subsequent tests.

Cell Counting Kit-8 (CCK-8) assay

In vitro cell viability was monitored using a CCK-8 assay (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) according to the manufacturer's instructions. Transfected cells were collected and seeded into 96-well plates at a density of 3, 000 cells/well. Quantification of cell viability was performed after 2 days. A 20 μL volume of CCK-8 assay solution was added to wells prior to incubation at 37°C for 2 h in a cell incubator. The absorbance of each well was determined at 450 nm using a spectrophotometer (ColorFlex EZ, HunterLab, Virginia, USA). Each sample was determined in triplicate.

Apoptosis assay

A375 cells which were transfection groups and control groups were seeded in 6-well culture plate. After 48 h culture, cells were collected, washed with phosphate-buffered saline (PBS), and resuspended in 1 × binding buffer with a concentration of 1×10^6 cells/mL. Then, 5 μL Annexin V- fluorescein isothiocynate (FITC)and 5 μL propidium iodide (PI)were added to the cell suspension and incubated for 15 min in the dark at room temperature. Cell apoptosis was assessed by flow cytometry (Becton Dickinson, San Jose, CA, USA).

Wound healing assay

The cell migratory capacity was assessed by wound healing assay. Equal numbers of cells were cultured in 6-well plates until reaching 95% confluence. Wound gaps were created by scratching cell sheets with a sterile 200 μL-pipette tip. The floating cells were removed by washing wells with PBS. Changes of the scratched areas were observed daily for 4 days by an inverted microscope (Leica, Germany). The wound widths were measured for calculating cell migratory capacity.

Transwell invasion assay

Cell invasion was assessed using the 24-well Millicell Hanging Cell Culture inserts with 8 μm poly (ethylene terephthalate) (PET) membranes (Millipore, Bedford, Massachusetts, USA) according to the manufacturer's instructions. After cell transfection, 5×10^4 cells from each group were suspended in serum-free medium and seeded into the upper chamber, while complete medium containing 10% FBS was added to the lower chamber. After incubation at 37°C for 48 h, non-invading cells were removed from the upper surface of the filter carefully. The invaded cells in the lower chamber (below the filter surface) were fixed in 100% methanol, stained with 0.1 mg/mL crystal violet solution (Beyotime Biotechnology, Shanghai, China), and counted under a microscope. Five random visual fields were counted for each well, and the average value was determined.

Dual-luciferase activity assay

The pmirGLO luciferase reporter plasmids Bmi1-3'UTR wild type (wt) and Bmi1-3'UTR mutant (mt) were purchased from Shanghai GenePharma Co., Ltd. Position 660-666 of Bmi1 wt 3' UTR: 5'... AUUCGCUUUUGUAAGAAUCAGAU... and also Bmi1mt: 5' ...AUUCGCUUUUGUAAGGUAGGAAU.... A375 cells were seeded into 12-well plates and cultured until the cell density reached 90%. Cells were co-transfected with miR-29a mimic (or NC) and constructed vectors using Lipofectamine 2000 (Invitrogen). After 48 h of transfection, firefly luciferase activity and Renilla luciferase activity were detected by Dual-Luciferase Reporter Assay system (Promega Corporation, Madison, WI, USA) following to the manufacturer's information. The firefly luciferase activity was normalized to the Renilla luciferase activity.

Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted by TRIzol reagent (Invitrogen). To detect miR-29a expression, total RNA was treated with the First-Strand cDNA Synthesis kit (TaKaRa Dalian, China) following the manufacturer's protocol. To detect Bmi1 expression, total RNA was converted into cDNA by reverse transcription kit (Promega, Madison, USA). RT-PCR was performed on a 7500 real-time-PCR System (Applied Biosystems, Carlsbad, CA, USA) using Taqman probes for miR-29a (Applied Biosystems, 4427975) and primers for Bmi1. The sequence of the primers: Primer sets (5'-3') were as follows: Bmi1: CACCAGAGAGATGGACTGACAA

and AGGAAACTGTGGATGAGGAGAC [21]. The $2^{-\Delta\Delta Ct}$ method was used to calculate expression levels. U6 small nuclear RNA and GAPDH were respectively used as endogenous controls for miR-29a and Bmi1.

Western blot

Total protein was extracted from cells with lysis buffer (Beyotime Biotechnology, Shanghai, China). Proteins (40 μg) were subjected to Sodium dodecyl sulfate-polyacrykamide gel electrophoresis (SDS-PAGE) with 10% polyacrylamide gels and transferred to Polyvinylidene Difluoride (PVDF) membranes. These were blocked with 5% non-fat milk for 2 h at room temperature, prior to incubation with primary antibodies to Bmi1 (ab38295), Bcl-2 (ab32124), Bax (ab32503), pro caspase-3 (ab4051), cleaved caspase-3 (ab32042), pro caspase-9 (ab32539), cleaved caspase-9 (ab32539), Wnt3a (ab28472), Wnt5a (ab72583), β-catenin (ab6302), p-p65 (ab86299), p65 (ab16502), inhibitor of NF-κB (IκBα) (ab32518), p-IκBα (ab32518), and GAPDH (ab9485, Abcam, Cambridge, MA) at 4°C overnight. The membranes were washed thrice and incubated with secondary antibody conjugated with horseradish peroxidase for 2 h. Protein bands were detected by enhanced chemiluminescence (ECL) method and visualized using VersaDoc-MP Imaging Systems (Bio-Rad, CA, USA). Protein levels were normalized to GAPDH.

Statistical analysis

Data were expressed as mean ± standard deviation (SD) from at least three separate experiments. Statistical analysis was using SPSS version 18.0 (SPSS Inc., Chicago, IL, USA). Experimental results were assessed using t-test or ANOVA. *P* values < 0.05 were considered statistically significant.

Results

miR-29a inhibited viability, migration and invasion, and promoted apoptosis of melanoma A375 cells

First, we detected miR-29a expression in normal cell lines and various melanoma cell lines. Results exhibited that miR-29a was significantly down-regulated in all tested melanoma cell lines A375, Malme-3M, SK-MEL-2, SK-MEL-5, M14 ($P < 0.05$ or $P < 0.01$). The lowest expression of miR-29a was in A375, Malme-3M, and M14 cell lines (Fig. 1A). To examine whether miR-29a regulates the growth of A375 cells, A375 cells were transfected with miR-29a mimic or miR-29a inhibitor. As shown in Fig. 1B, the overexpression of miR-29a by transfection with miR-29 mimic and the downexpression of miR-29 by transfection with miR-29a inhibitor were observed based on qRT-PCR analysis. CCK8 analysis was conducted to evaluate the effect of miR-29a on cell viability, which displayed that viability of A375 cells was significantly inhibited with transfection with miR-29a mimic ($P < 0.05$), but enhanced with transfection with miR-29a inhibitor ($P < 0.05$, Fig. 1C). Flow cytometry detection results given in Fig. 1D showed that, apoptotic cell rate was increased by transfection with miR-29a mimic ($P < 0.01$), while no significant effect was observed after transfection with miR-29a inhibitor. The results of Western blot showed a notable decrease in the Bcl-2 expression level, increase in the Bax level, as well as activation of caspase-3 and capsase-9 after transfection with miR-29a mimic. However, no obvious changes were showed after transfection with miR-29a inhibitor (Fig. 1E). All data suggested that miR-29a effectively inhibited viability but promoted apoptosis of melanoma A375 cells, which indicated that miR-29a suppressed cell growth of A375.

Wound healing assay and transwell assay were respectively conducted to verify the effects of miR-29a on migration and invasion of A375 cells (Fig. 1F and 1G). A375 cells transfacted with miR-29a mimic or inhibitor were cultured for 4 days and the migration ability was daily detected. As shown in Fig. 1F, cell migration were persistently suppressed by transfection with miR-29a mimic as comparison to NC group. Especially on 4th day, the migration of A375 cells were largely inhibited. However, transfection withmiR-29a inhibitor had no significant impact on cell migration throughout the testing period. Invasion ability of A375 cells was detected after transfaction with miR-29a mimic or inhibitor. As shown in Fig. 1G, transfection with miR-29a mimic significantly inhibited cell invasion ($P < 0.05$), whereas transfection withmiR-29a inhibitor significantly promoted cell invasion ($P < 0.05$). Above data indicated miR-29a inhibited cell migration and cell invasion.

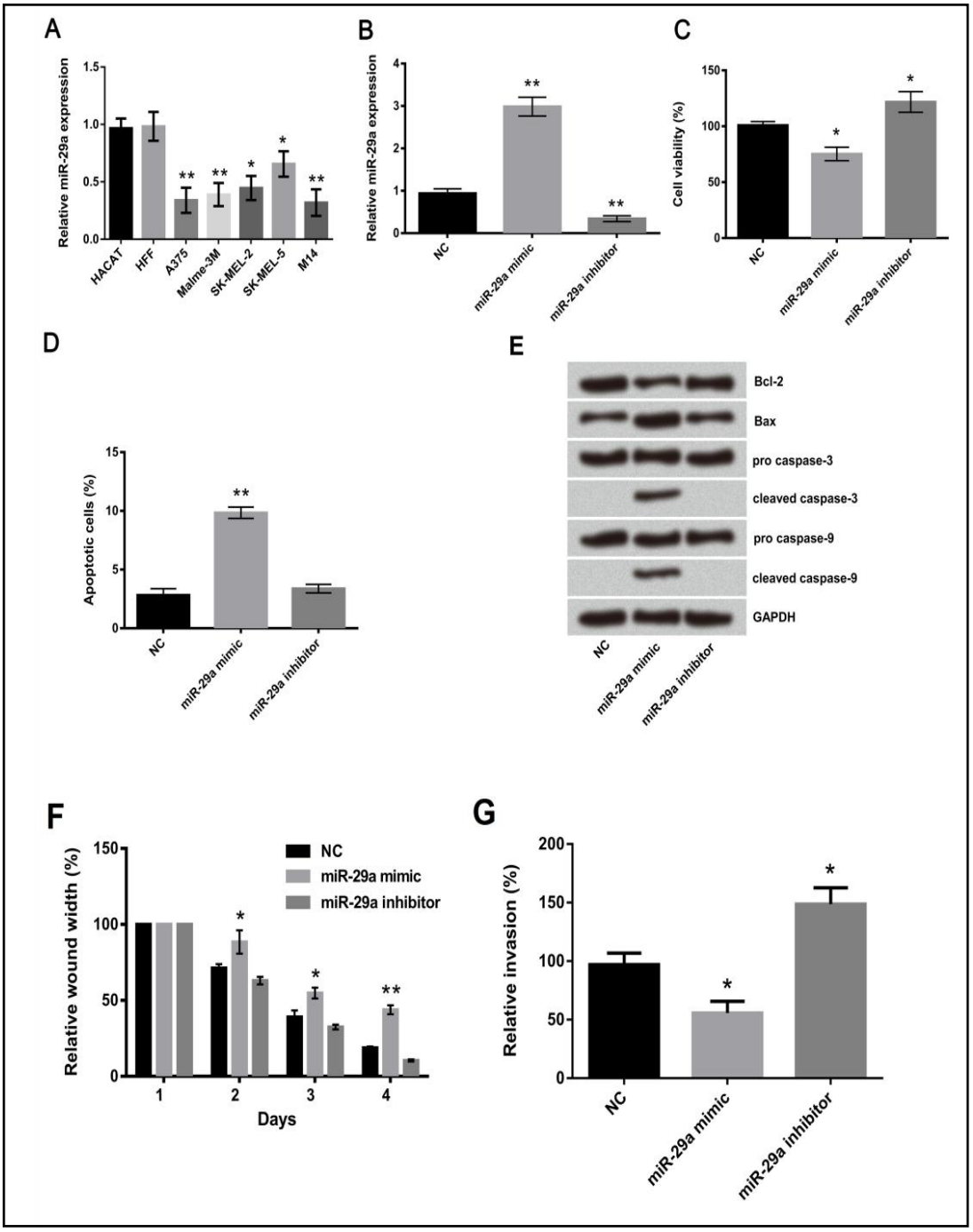

Fig. 1. Up-regulating microRNA-29a (miR-29a) inhibited cell viability, migration and invasion, and promoted apoptosis in A375 cells, whereas down-regulating miR-29a exhibited the contrary effect. (A) The relative expression of miR-29a was analyzed by quantitative real time polymerase chain reaction (qRT-PCR) in various melanoma cell lines. After transfection with miR-29a mimic or inhibitor, (B) the mRNA expression level of miR-29a was analyzed by qRT-PCR, (C) the cell viability was analyzed by Cell Counting Kit (CCK-8) method, (D) cell apoptosis was evaluated by Annexin V- fluorescein isothiocynate (FITC)/propidium iodide (PI) double staining method, (E) cell apoptosis-related proteins were detected by Western blot, (F-G) Cell migration and invasion were respectively analyzed by wound healing assay and transwell assay. Data were expressed as mean ± standard deviation (SD). * P<0.05, ** P<0.01.

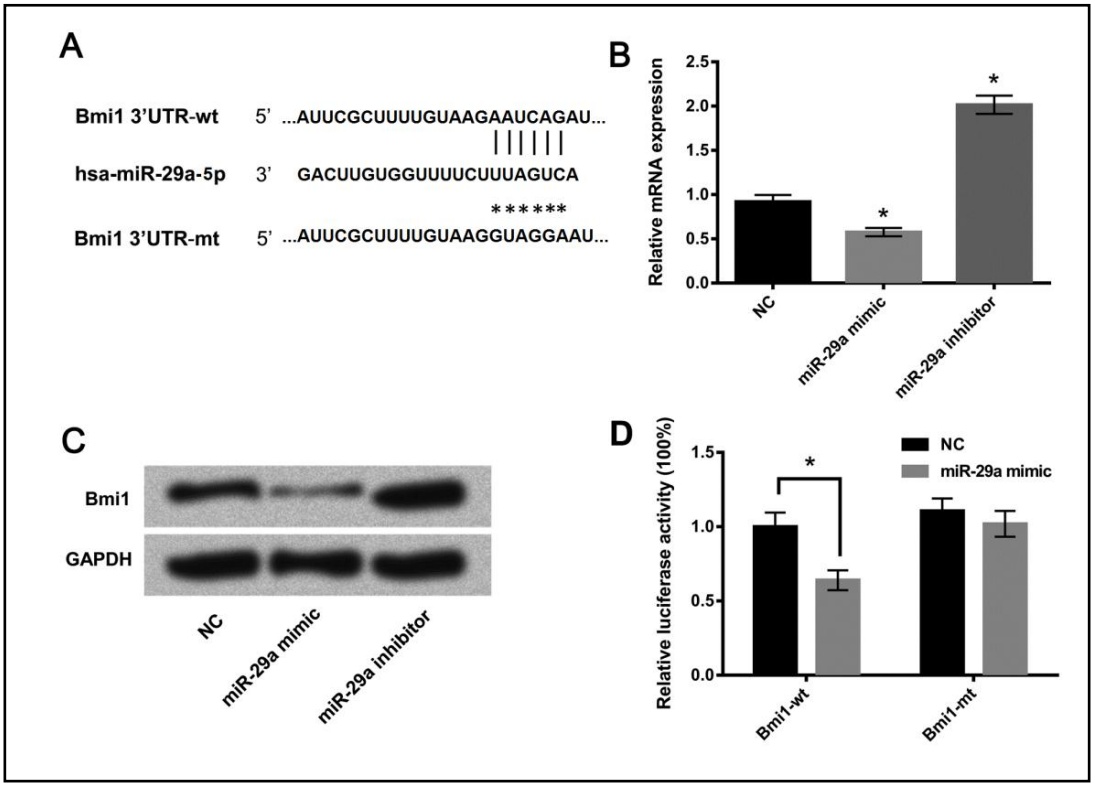

Fig. 2. Bmi1 was a direct target gene of microRNA-29a (miR-29a) in A375 cells. (A) Prediction of Bmi1 3'UTR contained a target site for miR-29a. (B) The mRNA and (C) protein level expressions of Bmi1 after transfected miR-29a mimic and miR-29a inhibitor were evaluated by quantitative real time polymerase chain reaction (qRT-PCR) and Western blot. (D) Luciferase reporter assay was conducted after cells were transfected with Bmi1-wt (or Bmi1-mt) and miR-29a mimic. Data were expressed as mean ± standard deviation (SD). * P< 0.05.

Bmi1 is a target of miR-29a in melanoma A375 cells

Considering that Bmi1 is highly expressed in melanoma cell lines and down-regulation of Bmi1 has been reported to significantly inhibit the aggressive behavior of melanoma [22], we intended to explore whether miR-29a could affect the expression of Bmi1 in this study. As results shown in Fig. 2A, Bmi1 has a relevant binding site for miR-29a. Fig. 2B and 2C showed that Bmi1 was significantly down-regulated both on mRNA level and protein level after miR-29a mimic transfected ($P < 0.05$), while was up-regulated after miR-29a inhibitor transfected ($P < 0.05$). We further speculated that Bmi1 might be a direct target of miR-29a. To further validate whether miR-29a directly interacted with the Bmi1 mRNA 3′UTR and subsequently interfered with the translation process, the dual-luciferase reporter assay was conducted. NC or miR-29a mimic was individually co-transfected with Bmi1-wt or Bmi1-mt. Results displayed that the co-transfection of miR-29a and Bmi1-wt obviously decreased the luciferase activity compared with NC groups (Fig. 2D), indicating that Bmi1 is a direct target of miR-29a. Collectively, these data indicated that miR-29a directly bound to Bmi1 and regulated Bmi1 expression.

Transfection with miR-29a mimic decreased cell migration and invasion and Bmi1 expression in Malme-3M cells, SK-MEL-2, SK-MEL-5 and M14 cell lines

To explore whether the same or similar results were also observed in other cell lines, same assays were performed with additional melanoma cell lines, in Malme-3M cells, SK-MEL-2, SK-MEL-5 and M14. Cell migration and invasion were respectively analyzed by wound healing assay and transwell assay. Results showed that cell migration (Malme-3M cells, SK-

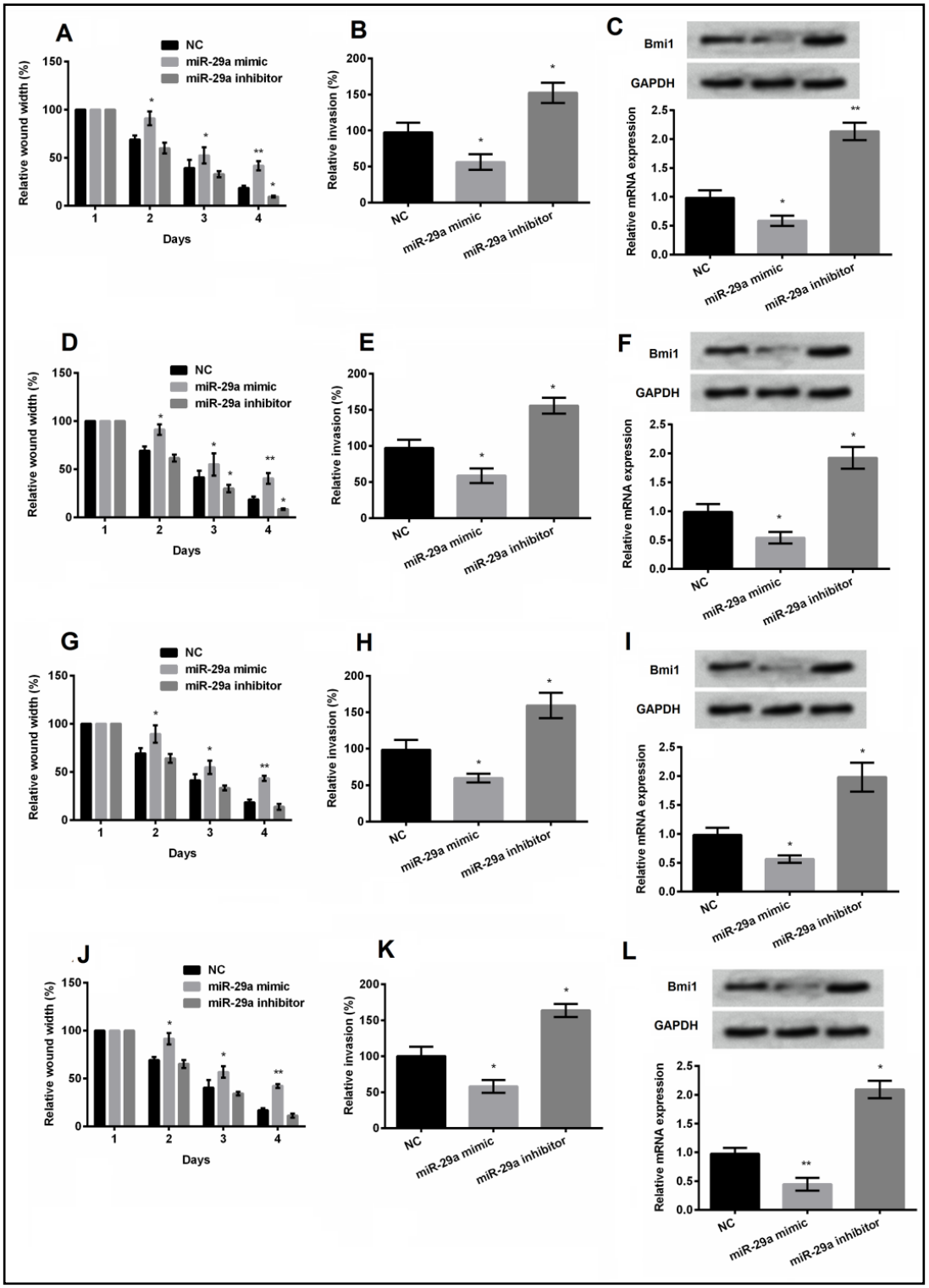

Fig. 3. Transfection with microRNA-29a (miR-29a) mimic decreased cell migration and invasion and Bmi1 expression in Malme-3M cells, SK-MEL-2, SK-MEL-5, and M14, respectively. (A, D, G, J) Cell migration and (B, E, H, K) invasion were respectively analyzed by wound healing assay and transwell assay, respectively. (C, F, I, L)The mRNA and protein level expressions of Bmi1 after transfected miR-29a mimic and miR-29a inhibitor were evaluated by quantitative real time polymerase chain reaction (qRT-PCR) and western blot, respectively. Data were expressed as mean ± standard deviation (SD). * P<0.05, ** P<0.01.

MEL-2, SK-MEL-5 and M14, respectively) was persistently inhibited by transfection with miR-29a mimic as comparison to NC group (Fig. 3A, 3D, 3G and 3J). However, no significant impact on cell migration was found by transfection withmiR-29a inhibitor throughout the testing period (Fig. 3A, 3D, 3G and 3J). In addition, cell invasion (Malme-3M cells, SK-MEL-2, SK-MEL-5 and M14, respectively) was decreased by transfection with miR-29a mimic while increased by transfection with miR-29a inhibitor (Fig. 3B, 3E, 3H and 3K). The mRNA and protein level expressions of Bmi1 after transfected miR-29a mimic and miR-29a inhibitor

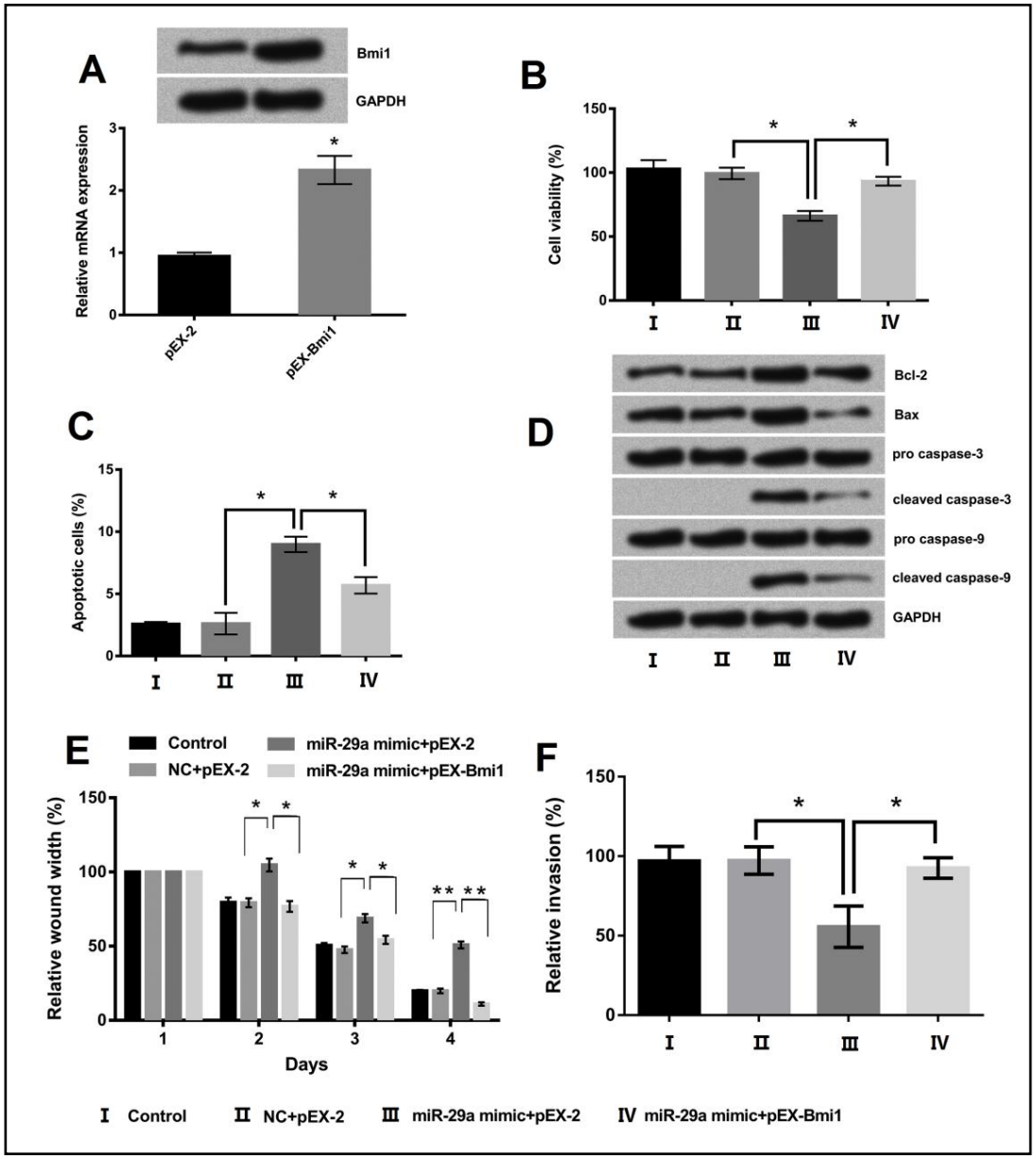

Fig. 4. microRNA-29a (miR-29a) suppressed growth of A375 cells via regulation of Bmi1. After A375 cells were transfected with pEX-Bmi1, (A) mRNA expression level and (B) protein level of Bmi1 were elevated by quantitative real time polymerase chain reaction (qRT-PCR) and Western blot. After A375 cells were co-transfected with pEX-Bmi1 and miR-29a mimic, (C) cell viability, (D) apoptotic cell rate, and (E) the expressions of apoptosis-associated proteins were respectively assessed by Cell Counting Kit (CCK-8), flow cytometry, and western blot. (E-F) Cell migration and invasion were respectively obtained by wound healing assay and transwell assay. Data were expressed as means ± standard deviation (SD). * P<0.05 ** P<0.01.

were evaluated by qRT-PCR and western blot, respectively. Results demonstrated that Bmi1 was significantly downregulated both in mRNA level and protein level by transfection with miR-29a mimic ($P < 0.05$ or $P < 0.01$) while upregulated by transfection with miR-29a inhibitor (all $P < 0.05$, Fig. 3C, 3F, 3I and 3L). Taken together, transfection with miR-29a mimic decreased cell migration and invasion and Bmi1 expression in Malme-3M cells, SK-MEL-2, SK-MEL-5 and M14, which revealed the similar trend with cell line A375.

miR-29a inhibited viability, migration and invasion, and promoted apoptosis of A375 cells by negatively regulating Bmi1

To further explore whether the functional effects of miR-29a on A375 cells were exerted via Bmi1, functional assays were performed following transfection with pEX-Bmi1 or its control pEX-2. The transfection efficiency was measured using qRT-PCR and western blot analysis. The expression level of Bmi1 was markedly enhanced in cells transfected with pEX-Bmi1 compared with the pEX-2 group (Fig. 4A). Subsequent assays demonstrated that co-transfection with miR-29a mimic and pEX-Bmi1 impaired cell viability-inhibiting effect and cell apoptosis-promoting effect of miR-29a overexpression (all $P < 0.05$, Fig. 4B and 4C). Furthermore, compared with co-transfection with miR-29a mimic and pEX-2 group, the pro-apoptosis proteins, Bax, cleaved caspase-3, and cleaved caspase-9 were down-regulated after co-transfection with miR-29a mimic and pEX-Bmi1 group (Fig. 4D). Results suggested that miR-29a inhibited growth of A375 cells by down-regulation of Bmi1.

The role of Bmi1 involved in migration and invasion of miR-29a was explored. Cell migration ability was detected and results were displayed in Fig. 4E. Co-transfection with miR-29a and pEX-Bmi1 abolished the migration-suppressing effect of transfection with miR-29a mimic ($P < 0.05$ or $P < 0.01$). Finally, cell invasion was analyzed and results showed that cell invasion was inhibited by miR-29a; however, was then recovered after transfection with pEX-Bmi1 (both $P < 0.05$, Fig. 4F). Thus co-transfection with miR-29a and

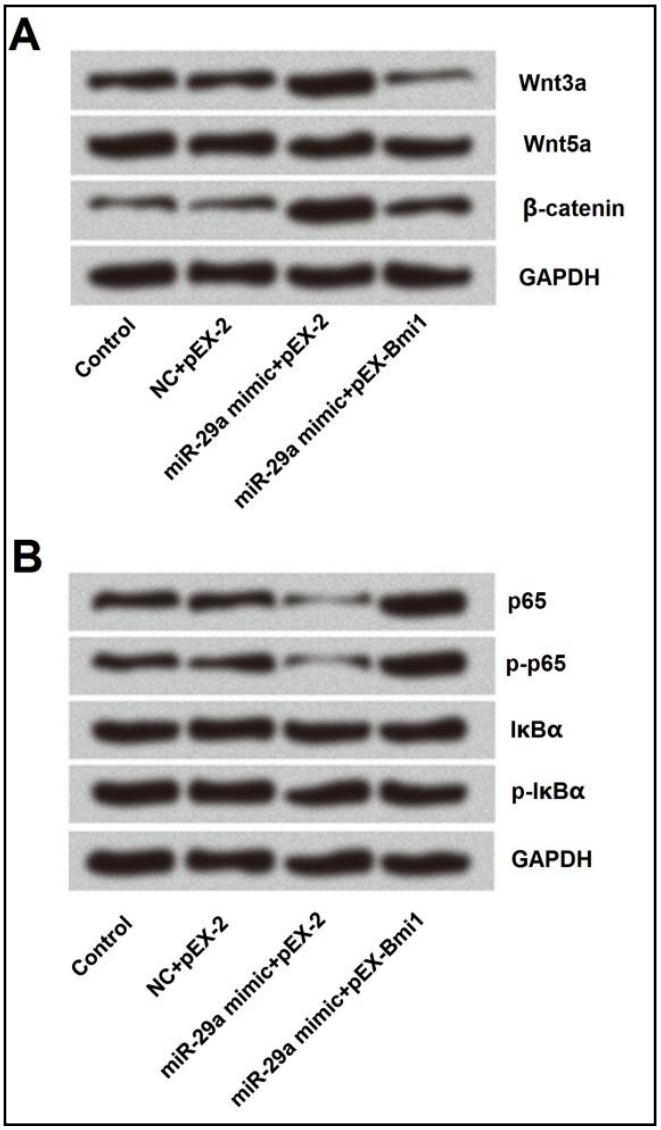

Fig. 5. microRNA-29a (miR-29a) inactivates Wnt/β-catenin and nuclear factor κB (NF-κB) pathways by down-regulating Bmi1. The protein expression of key components of (A) Wnt/β-catenin and (B) NF-κB signaling pathways were determined by Western blot after cells were co-transfected with miR-29a mimic and pEX-Bmi1.

pEX-Bmi1 impaired the cell invasion-inhibiting effect of transfection with miR-29a mimic. These data further indicated that Bmi1 might be a functional target of miR-29a in A375 cells and involved in miR-29a inhibition effects on A375 cells.

miR-29a blocked Wnt/β-catenin and NF-κB signal pathways by negative regulating Bmi1 in melanoma A375 cells

In the following experiment, we explored the function of miR-29a and Bmi1 in Wnt/β-catenin and NF-κB signal pathways. It was reported that activations of Wnt/β-catenin and NF-κB pathways were closely related to melanoma development [23, 24], thereby we examined whether miR-29a and Bmi1 regulated their activities. Western blot was used to analyze the protein expression of key components of Wnt/β-catenin and NF-κB signaling pathways. Co-transfection with miR-29a mimic and pEX-2 activated Wnt/β-catenin via up-regulation of Wnt3a and Wnt5a expressions compared with NC (Fig. 5A), and inactivated NF-κB pathways by down-regulation of p-p65 and IκBα (Fig. 5B). However, overexpression of miR-29a and Bmi1 by co-transfection with miR-29a mimic and pEX-Bmi1 significantly reversed these effects. Therefore, our data indicated that miR-29a might activate Wnt/β-catenin and inactive NF-κB signal pathways by targeting Bmi1 in melanoma A375 cells.

Discussion

Melanoma is one of the most dangerous form of skin cancers, being largely resistant to conventional therapies especially at advanced stages [25]. Understanding the mechanisms behind melanoma might be the key for the development of novel therapeutic strategies. miRNAs post-transcriptionally control gene expression, thereby regulating a variety of cellular signaling pathways were involved in the initiation and progression of different cancers, including melanoma [26]. Based on that, the function of miR-29a on melanoma A357 cells was investigated in this study.

miR-29 family, including miR-29a, miR-29b-1, miR-29b-2, and miR-29c, have been proved to be function as regulators of specific gene expression and associated with tumorigenesis and cancer progression [27]. A small number of previous studies suggested the tumor-promoting effect of miR-29a in breast cancer [18, 28]. Adversely, quite a number of researches indicated its tumor-suppressing effect against various cancer types [19, 29, 30]. For instance, Pei *et al.* reported that miR-29a overexpression promoted breast cancer proliferation and metastasis, as well as accelerated cell cycle progression [18]. Contrary to its effect on breast cancer, overexpression of miR-29a suppressed the growth and migration of hepatocellular carcinoma [29]. Consistent with most studies, the anti-tumor role of miR-29a was confirmed in our study. Up-regulation of miR-29a inhibited cell viability, migration, invasion and increased cell apoptosis.

Bmi1, which is the first member of the polycomb group gene family identified in mammals, played an essential role in the self-renewal of stem cells. Previous studies revealed that various miRNAs affected tumor growth through targeting or regulation of Bmi1. For example, miR-200c inhibits melanoma progression through down-regulation of Bmi1 [31], and miR-203 inhibits the proliferation of esophageal cancer stem-like cells by suppressing Bmi1 [32]. In addition, miR-29a exerted activities via regulation of its target genes like claudin-1(CLDN1) [29], phosphatase and tensin homolog (PTEN) [33], matrix metalloprotein (MMP)-2 [19], and etc. To investigate by which miR-29a repressed melanoma A357 cells, we hypothesized that effects of miR-29 were via regulation of Bmi1. Surprisingly, we found Bmi1 was a direct target gene of miR-29a. miR-29a negatively regulated the expression of Bmi1. Elevated expression of miR-29a in A357 cells decreased Bmi1 expression level. Furthermore, we validated that miR-29a repressed melanoma in A375 cells by down-regulating Bmi1.

Many studies reported that Bmi1 functioned as an oncogene in human malignancies [34]. Bmi1 overexpression was found to promote the metastasis of some cancers, such as nasopharyngeal carcinoma [35], endometrial cancer [36], and breast cancer [37]. Bmi1 was

expressed higher in cell lines from metastatic melanoma in comparison with cell lines from primary melanoma and it might be involved in the metastatic behavior of melanoma cells [38]. It was reported that Bmi1 could affected epithelial-mesenchymal transition (EMT) in melanoma [39]. Liu *et al.* found that knockdown of Bmi1 largely inhibited the aggressive behavior of melanoma by reversing EMT-like changes [22], which was consistent with our result that Bmi1 acted as an oncogene.

In cancer, Wnt/β-catenin signaling pathway is ubiquitously referred to as an "oncogenic" pathway and promotes tumor progression [23]. In melanoma, Wnt/β-catenin signaling directly regulates the expression of microphthalmia transcription factor (MITF), which is a major determinant of both melanocyte development and melanoma development [40-42]. Our data displayed that Wnt/β-catenin pathway was inhibited through miR-29a overexpression, which was consistent with the previous study that inhibition of human melanoma cell growth was through disruption of Wnt/β-catenin signaling [43]. We speculated that up-regulating miR-29a possibly repressed melanoma via indirect regulation of MITF and thereby further study about this should be conducted for penetrating investigating the anti-melanoma mechanism of miR-29a. Also, NF-κB pathway has been implicated in the pathogenesis of malignant melanoma. It was reported some medicines realized the melanoma-inhibiting effect through inactivating NF-κB pathway [24, 44]. Previous studies have shown that activation of NF-κB can inhibit apoptosis induced by a number of stimuli [45]. Similarly, miR-29a overexpression suppressed melanoma growth by inducing cell apoptosis via inactivating NF-κB pathway.

Conclusion

In conclusion, our study proposed that effects of miR-29a in melanoma A357 cells through regulation of Bmi1. We found that miR-29a suppressed cell growth, migration and invasion partly by reducing Bmi1expression though binding to its 3'UTR. Thus, we highlighted the interaction between miR-29a and Bmi1 in melanoma development, which provided novel target for exploring new therapeutic strategies.

Disclosure Statement

The authors declare no potential conflicts of interest.

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