European Review for Medical and Pharmacological Sciences 2019; 23: 965-971

Suppression of connexin 43 expression by miR-106a promotes melanoma cell proliferation

J.-L. WANG, H. LI, J.-B. ZHANG, C.-H. ZHANG, X.-Q. HOU

Department of Burn and Plastic Surgery, Cangzhou Central Hospital, Cangzhou City, Hebei Province, China

Abstract. – OBJECTIVE: Connexin 43 (Cx43), a vital gap junction protein is reported to be involved in melanoma progression. The aim of the study is to investigate the regulatory role of Cx43 in melanoma.

MATERIALS AND METHODS: Western blot assay was used to detect the protein expression of Cx43 in melanoma cells and the human epidermal melanocytes (HEMn). MTT cell proliferation and cell colony formation assays were used to assess cell proliferation. Bioinformatics prediction, luciferase reporter assay, Western blot and qRT-PCR assays were applied to demonstrate that Cx43 was a direct target of miR-106a in melanoma cells.

RESULTS: Connexin 43 (Cx43) was lower expressed in melanoma cells compared with human epidermal melanocytes (HEMn). Cx43 overexpression significantly inhibited melanoma cell proliferation and colony formation ability *in vitro*. However, knockdown of Cx43 had opposite effects on cell proliferation and colony formation. Bioinformatics prediction and luciferase reporter assays demonstrated that miR-106a targeted the 3' untranslated region (3'UTR) of Cx43 and regulated its mRNA and protein expression levels in melanoma cells. MiR-106a was upregulated in melanoma cells, and its overexpression attenuated the effects caused by upregulating Cx43 expression.

CONCLUSIONS: Thus, our results indicated that Cx43 was downregulated in melanoma cells and may be a potential target of melanoma treatment.

Key Words:
Melanoma, Connexin 43, miR-106a, Cell proliferation.

Introduction

Melanoma is the most dangerous form of skin cancer and accounts for more than 70% of skin tumor-related deaths[1]. In 2016, approximately 76380 new melanoma cases are diagnosed[2]. Due to tumor recurrence and metastasis, the overall survival rate of melanoma patients is still poor. Approximately 10,130 cases die from metastatic melanoma every year[3,4]. Thus, the molecular mechanisms underlying melanoma process need to be investigated.

Connexin 43 (Cx43) a vital gap junction protein in the tumor microenvironment (TME) and its lower expression significantly associates with increased malignancy in multiple cancers[5]. For instance, miR-125b promotes glioma cell line growth and clone formation by inhibiting connexin43 expression[6]. MiR-20a inhibitor suppresses the proliferation of human prostate cancer by suppressing the Cx43 expression[7]. In melanoma, Connexin 43 upregulation by dioscin inhibits melanoma progression via suppressing malignancy and inducing M1 polarization[8]. However, the regulator role of Cx43 in melanoma is still not well known.

In this study, we found that connexin 43 (Cx43) was poor expressed in melanoma cells. Gain function assays demonstrated that Cx43 overexpression significantly suppressed cell proliferation of melanoma. Moreover, we demonstrated that miR-106a targeted the 3' untranslated region (3'UTR) of Cx43 and regulated its mRNA and protein expression levels in melanoma cells. Thus, our results indicated that Cx43 may be a potential target of melanoma treatment.

Materials and Methods

Cell Line Culture

Human malignant melanoma cell lines A375 and A2058 and the human epidermal melanocytes (HEMn) were purchased from American Type Culture Collection (Manassas, VA, USA). Cells were cultured in Dulbecco's modified Eagle's (DMEM) medium (Invitrogen, Carlsbad,

Corresponding Author: Junling Wang, MD; email: wangjunling1994@yeah.net

CA, USA) with 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA, USA) and maintained at 37°C with 5% CO_2 in a humidified incubator.

Cell Transfection

The siRNA targeting Cx43 and pcDNA3.1-Cx43 plasmid were constructed and purchased from Ribobio (Guangzhou, China). MiR-106a mimic or miR-negative control was constructed and purchased from Ribobio (Guangzhou, China). The cells transfection was performed using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Cells were harvested at 48 hours after transfection. A plasmid designed to express full-length Cx43 mRNA sequence was subcloned from cDNA into the Xba I and Kpn I sites within pcDNA3.1 (Ribobio, Guangzhou, China).

Cell Proliferation Assay

A 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay was performed to detect the cell viability. The transfected A375 or A2058 cells (3000 cells/well) were plated into 96-well plates. After cell transfection at 0, 24 h, 48 h, and 72 h, the MTT solution (5 mg/ml MTT in PBS; Sangon Biotech Co., Ltd., Shanghai, China) was added to each well and cultured for 4 hours. Cells were detected using a microplate reader (Bio Tek Instruments, Inc., Winooski, VT, USA) and the absorbance was at 490 nm.

Cell Colony Formation Assay

For cell colony formation assay, the transfected A375 or A2058 cells (300 cells/well) were seeded into a 3 cm culture dish (BD Biosciences, Franklin Lakes, NJ, USA). Cells were cultured for 7 days at 37°C with 5% CO_2 in a humidified incubator. Next, cells were fixed with 100% methanol, stained with 1% crystal violet and were counted.

Quantitative Real-Time PCR (QRT-PCR)

Total RNA was extracted by using TRIzol Reagent (TaKaRa, Dalian, China) according to the manufacturer's instructions. A 500 ng RNA was reverse to cDNA using PrimeScript RT reagent Kit (TaKaRa, Dalian, China) according to the manufacturer's instruction. For mRNA analysis, SYBR Premix ExTaq kit (TaKaRa, Dalian, China) was used to detect the miR-106a and Cx43 expression on ABI 7500 Thermocycler (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA). GAPDH or U6 gene was used as internal reference. The relative expression was analyzed by the $2^{-\Delta\Delta Ct}$ method. The primer sequences were as follow: Cx43-forward: 5'-GGCGTGACTTCACTACTTTTAAGCA-3', and Cx43-reverse: 5'-CAGTTGAGTAGGCTTGAACCTTGTC-3'. GAPDH-forward, 5'-AGCCACATCGCTCAGACAC-3', and GAPDH-reverse, 5'-GCCCAATACGACCAAATCC-3'.

Western Blot Analysis

Cells were lysed using Radio Immuno Precipitation Assay Buffer (Cell Signaling Technology, Inc., Danvers, MA, USA). The concentration of protein was detected by using a Bicinchoninic Protein Assay kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Equal proteins were separated in 10-12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels and then transferred to polyvinylidene membranes. The membranes were blocked with 5% skim milk for 1 h and were incubated with primary antibodies against Cx43 (1:1000, Cell Signaling Technology, Danvers, MA, USA) or GAPDH (1:1000, Cell Signaling Technology, Danvers, MA, USA) at 4°C overnight. The membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (1:1000, Cell Signaling Technology, Danvers, MA, USA) at room temperature for 1 h. The blots were detected using enhanced chemiluminescence (ECL) reagents (Pierce, Rockford, IL, USA). GAPDH was used as an internal control.

Luciferase Reporter Assays

The wild and mutant sequence of the 3'-UTR of Cx43 was cloned downstream of the firefly luciferase gene in the pmirGLO Vector (Promega, Madison, WI, USA). HEK293 T cells were co-transfected with 15 pmol of the miR-106a mimic, miR-186 control or wild and mutant sequence of the 3'-UTR of Cx43 and 5 ng pRL-TK TKRenilla plasmid (Promega, Madison, WI, USA) using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA). The luciferase activity was assessed at 48 h following transfection by using the dual luciferase assay kit (Beyotime Biotechnology Institute of Biotechnology, Inc.) according to the manufacturer's instructions.

Statistical Analysis

All statistical analyses were analyzed using SPSS 19.0 (IBM, Armonk, NY, USA). The Student's *t*-test was applied to assess the significance of the differences between groups. A $p<0.05$ was considered as statistically significant.

Results

Cx43 Overexpression Inhibits Cell Proliferation and Cell Colony Formation of Melanoma

We detected the expression of Cx43 in human malignant melanoma cell lines A375 or A2058 and the human epidermal melanocytes (HEMn). The Western blot analysis results demonstrated that Cx43 expression in melanoma cell was significantly downregulated compared to HEMn cells (Figure 1A). To investigate the role of Cx43 expression in melanoma development, we performed the gain-and-loss function assays in A375 and A2058 cells using pcDNA3.1-Cx43 or siRNA-Cx43 (Figure 1B). The MTT assays results demonstrated that increased Cx43 expression in A375 cells significantly suppressed cell growth, but Cx43 knockdown dramatically promoted cell growth in A2058 cells (Figure 1C-1D). Meanwhile, cell colony formation assays results revealed that cell colony formed number was reduced after Cx43 was overexpressed in A375 cells. However, cell colony formed number was increased after Cx43 was knocked down in A2058 cells (Figure 1E-1F). Thus, these results indicated that Cx43 overexpression inhibited cell proliferation and cell colony formation of melanoma.

Cx43 is a Target of miR-106a in Melanoma Cells

To explore the underlying mechanism that affected cell proliferation induced by Cx43 in melanoma. By searching online predicting software miRanda and Targetscan, we found that Cx43 was a putative target of miR-106a (Figure 2A). We then constructed the wild and mutant type sequence of the 3'-UTR of Cx43 and cloned downstream of the firefly luciferase gene in the pmirGLO vectors. HEK 293T cells were cotransfected with miR-106a mimic, miR-106a control and wild-type (WT) and mutant type (MUT) sequences of the 3'-UTR of Cx43 and 5 ng pRL-TK TKRenilla plasmids. The results showed that miR-106a mimic significantly reduced the luciferase activity of wild-type (WT) of 3'-UTR of Cx43, but did not change the mutant type (MUT) of 3'-UTR of Cx43 luciferase activity (Figure 2B). Thus, the results indicated that Cx43 was a target of miR-106a. Moreover, we demonstrated that overexpressed miR-106a mimic reduced the mRNA and protein expression of Cx43 in A375 cells, but the mRNA and protein expression of Cx43 were enhanced when miR-106a was downregulated in A2058 cells (Figure 2C-2F). Therefore, these results indicated that Cx43 was a target of miR-106a and was regulated by miR-106a in melanoma cells.

Cx43 Overexpression Reverses the Effects of miR-106a on Cell Proliferation of Melanoma

We also detected the expression of miR-106a in melanoma and HEMn cells. Results showed that miR-106a was higher expressed in two melanoma cells compared to HEMn cell (Figure 3A). We investigated whether Cx43 mediated the effects of miR-106a on cell proliferation of melanoma. The miR-106a was overexpressed by transfected with miR-106a mimic in A375 cells (Figure 3B). It was demonstrated that Cx43 overexpression inhibited cell proliferation rate of melanoma using MTT assay, but was reversed by cotransfection of miR-106a mimic and pcDNA3.1-Cx43 plasmid in A375 cells (Figure 3C). Cell colony formation assay showed that cell colony formation number was decreased when A375 cells were treated with pcDNA3.1-Cx43 plasmid, but was reversed by cotransfection of miR-106a mimic and pcDNA3.1-Cx43 plasmid in A375 cells (Figure 3D). Thus, these results indicated that Cx43 overexpression reversed the effects of miR-106a on cell proliferation.

Discussion

In the previous studies, Cx43 was found to function as a tumor suppressor. Such as, reduced Connexin 43 expression is associated with malignant tumor behaviors and biochemical recurrence-free survival of prostate cancer[9]. Connexin 43 suppresses tumor angiogenesis by down-regulation of vascular endothelial growth factor via hypoxic-induced factor-1α[10]. Connexin-43 can delay early recurrence and metastasis in patients with hepatitis B-related hepatocellular carcinoma and low serum alpha-fetoprotein after radical hepatectomy[11]. Overexpression of connexin 43 reduces A549 lung adenocarcinoma cells proliferation and metastatic capacity[12]. In melanoma, specific inhibition of Cx43 channel activity accelerated melanoma cell proliferation, whereas overexpression of Cx43 increased GJ coupling and reduced cell growth[13]. In our study, we demonstrated that Cx43 was lower expressed in melanoma cells compared to the human epidermal melanocytes (HEMn). Overexpression of Cx43 significantly

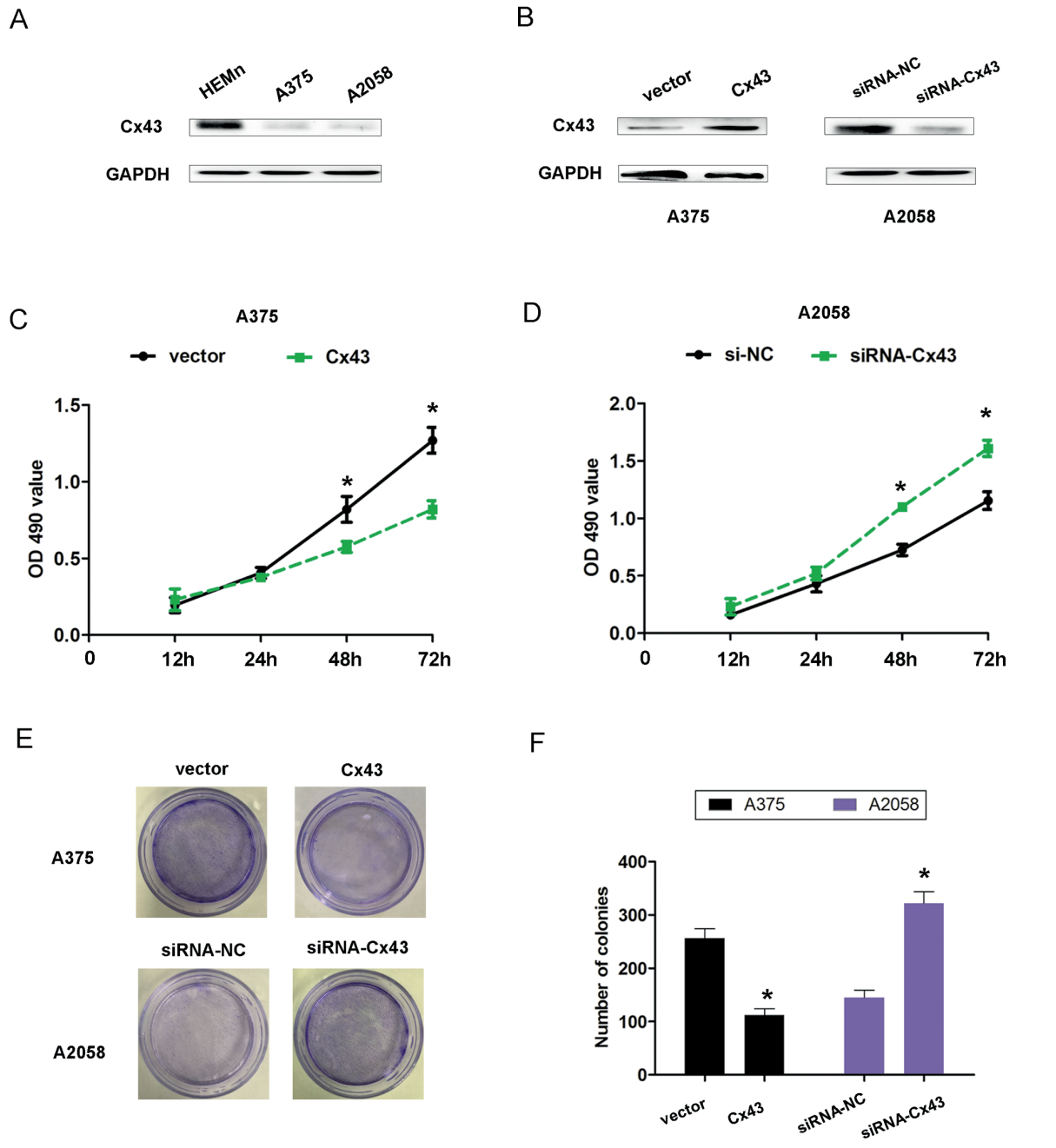

Figure 1. Cx43 overexpression inhibits cell proliferation and colony formation ability of melanoma. ***(A)*** Cx43 expression was detected by using western blot analysis in A375 or A2058 cells and HEMn cells. GAPDH was used as the internal control. ***(B)*** Cx43 expression was detected by using western blot analysis after A375 cells transfected with pcDNA3.1-vector or pcDNA3.1-Cx43 or in A2058 cells transfected with siRNA-NC or siRNA-Cx43. GAPDH was used as the internal control. ***(C-D)*** MTT assay were used to assess cell proliferation after A375 cells transfected with pcDNA3.1-vector or pcDNA3.1-Cx43 plasmid or in A2058 cells transfected with siRNA-NC or siRNA-Cx43. ***(E-F)*** Cell colony formation assay were used to assess cell proliferation after A375 cells transfected with pcDNA3.1-vector or pcDNA3.1-Cx43 or A2058 cells transfected with siRNA-NC or siRNA-Cx43. All of experiments were repeated at least three times, *$p<0.05$.

inhibited cell proliferation and colony formation ability. However, the reduced Cx43 had a promoting role for melanoma cell proliferation. Thus, these results indicated that Cx43 could inhibit cell proliferation of melanoma. Furthermore, we demonstrated that miR-106a was higher expressed in melanoma cells compared to the human epidermal melanocytes (HEMn). The luciferase activity

results showed that miR-106a mimic significantly reduced the luciferase activity of wild-type (WT) of 3'-UTR of Cx43, but did not change the mutant type (MUT) of 3'-UTR of Cx43, which suggested that miR-106a targeted the 3'UTR region of Cx43. MiR-106a was identified as an oncogene in some

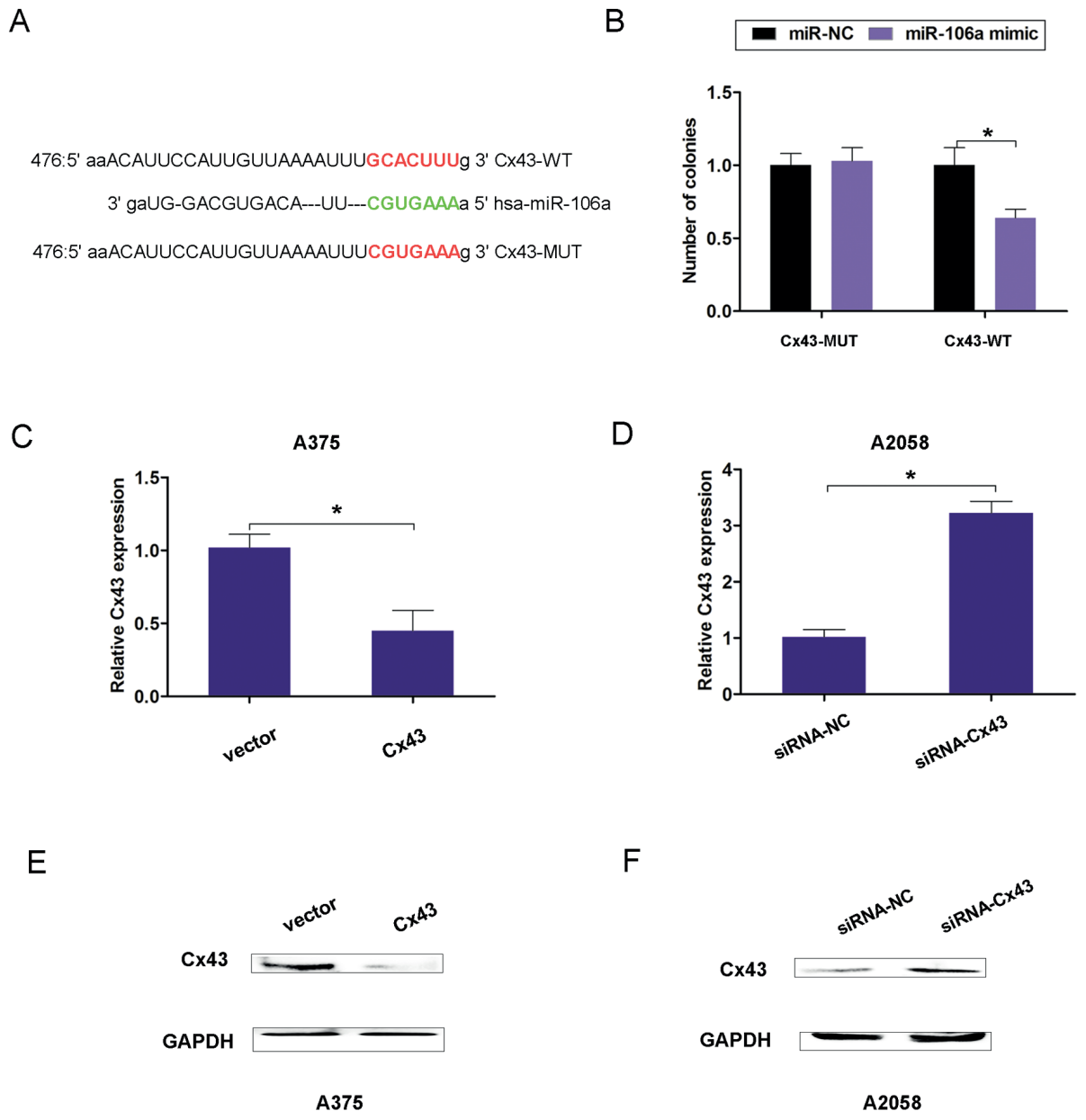

Figure 2. Cx43 is a direct target of miR-106a. ***(A)*** By searching online predicting software miRanda and Targetscan, the results showed that Cx43 was a putative target of miR-106a. The wild and mutant type sequence of the 3'-UTR of Cx43 and cloned downstream of the firefly luciferase gene in the pmirGLO vectors. ***(B)*** MiR-106a mimic significantly reduced the luciferase activity of wild type (WT) of 3'-UTR of Cx43, but did not change the mutant type (MUT) of 3'-UTR of Cx43 luciferase activity. ***(C-D)*** The mRNA expression of Cx43 was detected using qRT-PCR analysis after A375 cells transfected with pcDNA3.1-vector or pcDNA3.1-Cx43 or in A2058 cells transfected with siRNA-NC or siRNA-Cx43.was detected using qRT-PCR analysis after A375 cells transfected with pcDNA3.1-vector or pcDNA3.1-Cx43 or in A2058 cells transfected with siRNA-NC or siRNA-Cx43. ***(E-F)*** The protein expression of Cx43 was detected using qRT-PCR analysis after A375 cells transfected with pcDNA3.1-vector or pcDNA3.1-Cx43 or in A2058 cells transfected with siRNA-NC or siRNA-Cx43. All of experiments were repeated at least three times, *$p<0.05$.

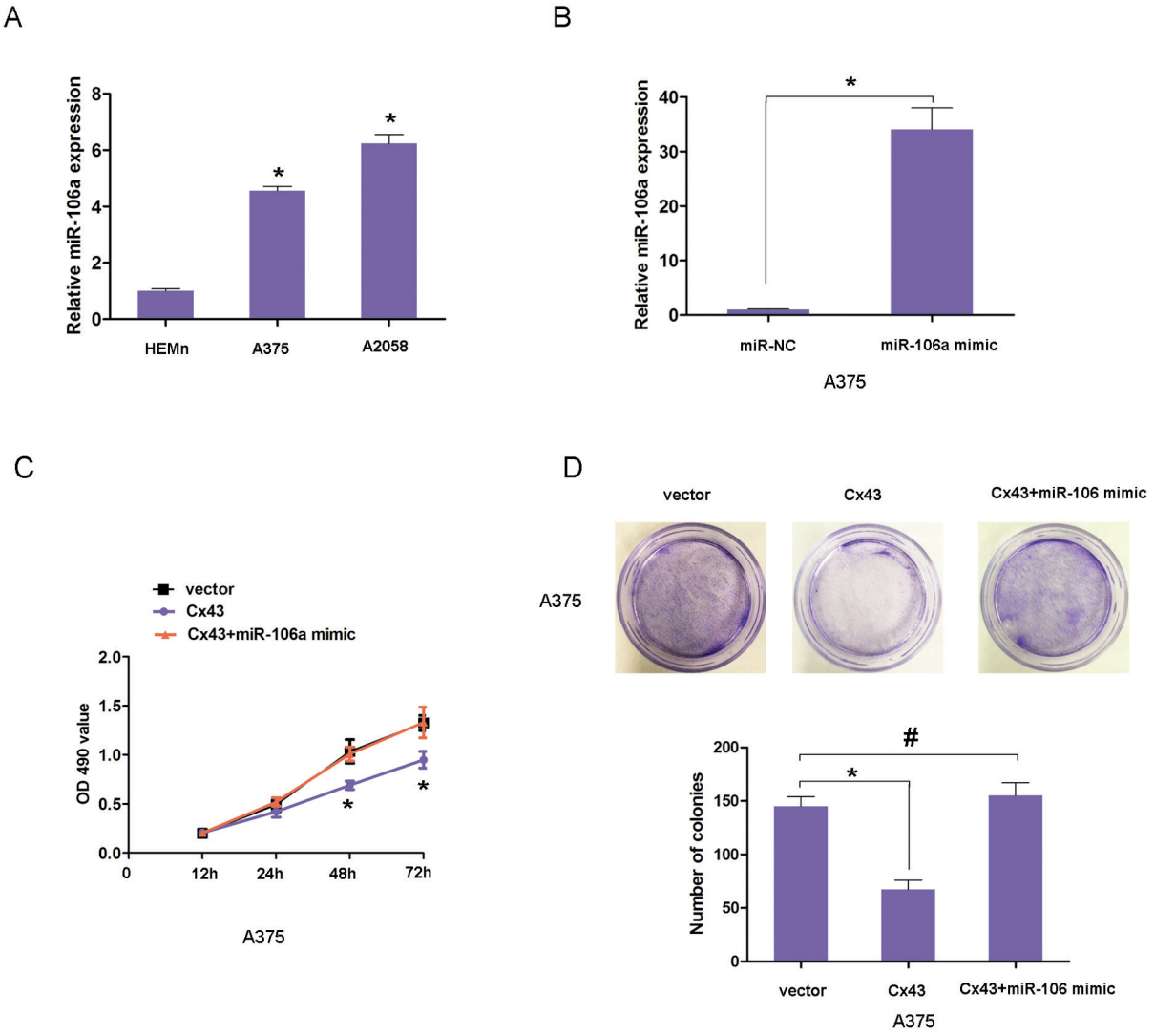

Figure 3. Cx43 overexpression reverses the effects of miR-106a on cell proliferation of melanoma. ***(A)*** The relative miR-106a expression was detected using qRT-PCR in A375 or A2058 cells and HEMn cells. ***(B)*** The relative miR-106a expression was detected using qRT-PCR after A375 cells were transfected with miR-106a mimic or miR-negative control (NC). ***(C)*** MTT assay were used to assess cell proliferation after A375 cells transfected with pcDNA3.1-vector, pcDNA3.1-Cx43 or pcDNA3.1-Cx43+miR-106a mimic. ***(D)*** Cell colony formation assay were used to assess cell proliferation after A375 cells transfected with pcDNA3.1-vector, pcDNA3.1-Cx43 or pcDNA3.1-Cx43+miR-106a mimic. All of experiments were repeated at least three times, *$p<0.05$, #, not significance.

tumors. For instance, microRNA-106a functions as an oncogene in human gastric cancer and contributes to proliferation and metastasis *in vitro* and *in vivo*[14]. MiR-106a promotes growth and metastasis of non-small cell lung cancer by targeting PTEN[15]. Upregulated expression of miR-106a by DNA hypomethylation plays an oncogenic role in hepatocellular carcinoma[16]. Oncogenic miR-106a enhances the invasiveness of human glioma stem cells by directly targeting TIMP-2[17]. Here, we demonstrated that miR-106a regulated the expression of Cx43. Cx43 overexpression could reverse the effects of miR-106a on cell proliferation by performing MTT cell proliferation and cell colony formation.

Conclusions

We demonstrated that Cx43 suppressed cell proliferation of melanoma. Cx43 was a direct target of miR-106a and mediated the effects indu-

ced by miR-106a on cell proliferation. Thus, Cx43 may serve as a target of melanoma treatment.

Conflict of Interest

The Authors declare that they have no conflict of interest.

References

1) Olszanski AJ. Current and future roles of targeted therapy and immunotherapy in advanced melanoma. J Manag Care Spec Pharm 2014; 20: 346-356.
2) Achkar T, Tarhini AA. The use of immunotherapy in the treatment of melanoma. J Hematol Oncol 2017; 10: 88.
3) Siegel RL, Miller KD, Jemal A. Cancer statistics, 2016. CA Cancer J Clin 2016; 66: 7-30.
4) Fattore L, Costantini S, Malpicci D, Ruggiero CF, Ascierto PA, Croce CM, Mancini R, Ciliberto G. MicroRNAs in melanoma development and resistance to target therapy. Oncotarget 2017; 8: 22262-22278.
5) Ming J, Zhou Y, Du J, Fan S, Pan B, Wang Y, Fan L, Jiang J. miR-381 suppresses C/EBPalpha-dependent Cx43 expression in breast cancer cells. Biosci Rep 2015; 35:
6) Jin Z, Xu S, Yu H, Yang B, Zhao H, Zhao G. miR-125b inhibits Connexin43 and promotes glioma growth. Cell Mol Neurobiol 2013; 33: 1143-1148.
7) Li X, Pan JH, Song B, Xiong EQ, Chen ZW, Zhou ZS, Su YP. Suppression of CX43 expression by miR-20a in the progression of human prostate cancer. Cancer Biol Ther 2012; 13: 890-898.
8) Kou Y, Ji L, Wang H, Wang W, Zheng H, Zou J, Liu L, Qi X, Liu Z, Du B and Lu L. Connexin 43 upregulation by dioscin inhibits melanoma progression via suppressing malignancy and inducing M1 polarization. Int J Cancer 2017; 141: 1690-1703.
9) Xu N, Chen HJ, Chen SH, Xue XY, Chen H, Zheng QS, Wei Y, Li XD, Huang JB, Cai H, Sun XL. Reduced Connexin 43 expression is associated with tumor malignant behaviors and biochemical recurrence-free survival of prostate cancer. Oncotarget 2016; 7: 67476-67484.
10) Wang WK, Chen MC, Leong HF, Kuo YL, Kuo CY, Lee CH. Connexin 43 suppresses tumor angiogenesis by down-regulation of vascular endothelial growth factor via hypoxic-induced factor-1alpha. Int J Mol Sci 2014; 16: 439-451.
11) Wang ZS, Wu LQ, Yi X, Geng C, Li YJ, Yao RY. Connexin-43 can delay early recurrence and metastasis in patients with hepatitis B-related hepatocellular carcinoma and low serum alpha-fetoprotein after radical hepatectomy. BMC Cancer 2013; 13: 306.
12) Yu M, Zhang C, Li L, Dong S, Zhang N, Tong X. Cx43 reverses the resistance of A549 lung adenocarcinoma cells to cisplatin by inhibiting EMT. Oncol Rep 2014; 31: 2751-2758.
13) Tittarelli A, Guerrero I, Tempio F, Gleisner MA, Avalos I, Sabanegh S, Ortiz C, Michea L, Lopez MN, Mendoza-Naranjo A, Salazar-Onfray F. Overexpression of connexin 43 reduces melanoma proliferative and metastatic capacity. Br J Cancer 2015; 113: 259-267.
14) Zhu M, Zhang N, He S, Yan R, Zhang J. MicroRNA-106a functions as an oncogene in human gastric cancer and contributes to proliferation and metastasis in vitro and in vivo. Clin Exp Metastasis 2016; 33: 509-519.
15) Xie X, Liu HT, Mei J, Ding FB, Xiao HB, Hu FQ, Hu R, Wang MS. miR-106a promotes growth and metastasis of non-small cell lung cancer by targeting PTEN. Int J Clin Exp Pathol 2015; 8: 3827-3834.
16) Yuan R, Zhi Q, Zhao H, Han Y, Gao L, Wang B, Kou Z, Guo Z, He S, Xue X, Hu H. Upregulated expression of miR-106a by DNA hypomethylation plays an oncogenic role in hepatocellular carcinoma. Tumour Biol 2015; 36: 3093-3100.
17) Wang Z, Wang B, Shi Y, Xu C, Xiao HL, Ma LN, Xu SL, Yang L, Wang QL, Dang WQ, Cui W, Yu SC, Ping YF, Cui YH, Kung HF, Qian C, Zhang X, Bian XW. Oncogenic miR-20a and miR-106a enhance the invasiveness of human glioma stem cells by directly targeting TIMP-2. Oncogene 2015; 34: 1407-1419.