

Targeted silencing of the ADP-ribosyltransferase 3 gene inhibits the migration ability of melanoma cells

JIE HE^{1,2*}, YONGYUN LI^{1,2*}, YING WANG^{1,2*}, HE ZHANG^{1,2}, SHENGFANG GE^{1,2} and XIANQUN FAN^{1,2}

¹Department of Ophthalmology; ²Shanghai Key Laboratory of Orbital Diseases and Ocular Oncology, Shanghai Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai 200011, P.R. China

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Abstract. Melanoma is the most common primary intraocular malignancy and metastasis of melanoma to other organs often results in a poor prognosis. ADP-ribosyltransferase 3 (ART3) is involved in cell division and DNA repair. However, its biological function in melanoma remains unclear. In the present study, it was identified that ART3 is highly expressed in melanoma cells and melanoma tissues compared with the normal RPE cell line, and adjacent normal tissue, respectively. Small interfering RNA and short hairpin RNA were used to silence ART3 gene expression, and the results revealed that the silencing of ART3 inhibits the migratory ability of melanoma cells. The present study indicates that ART3 serves a notable role in the metastasis of melanoma and provides a potential therapeutic target for this disease.

Introduction

Melanoma is the most common malignancy of the eye, with an incidence of 5-7 cases per million according to global statistics in 1988 (1). Melanoma has a high rate of metastasis, which primarily spreads to the liver through the blood stream (2,3). Despite advances in the technology used to treat melanoma, the mortality rate of metastatic disease remains unchanged, and ~50% of all patients with melanoma eventually succumb to mortality due to metastatic disease (4). As the molecular mechanisms of melanoma aggressiveness are yet to be clearly understood, metastatic melanoma cannot be effectively treated (5,6). Understanding the crucial signals that contribute

to the invasive and metastatic potential of melanoma is necessary in order to identify novel therapeutic targets.

Mono-ADP-ribosylation is a post-translational protein modification involved in the transfer of the ADP-ribose moiety from NAD⁺ to a specific amino acid in a target protein, resulting in the alteration of the functional properties of the target protein (7). This enzymatic reaction was originally identified as the pathogenic mechanism of bacterial toxins, including cholera, pertussis and diphtheria toxins (8). ADP-ribosyltransferases (ARTs) regulate endogenous protein functions, including DNA repair, cell differentiation and cell cycle progression by attaching ADP-ribose to specific amino acid residues in membrane proteins (9). ART3 is a member of the ART family, and is involved in cell division and the regulation of inflammatory responses (10). Shi *et al* (11) revealed that ART3 may contribute to the pathophysiological and biochemical progression of a neural lesion. In one previous study, it was identified that genetic variation of ART3 may result in a functional defect in the process of spermatogenesis (12).

ARTs have been reported to be involved in tumorigenesis. Xiao *et al* (13) confirmed that the knockdown of ART1 increased the apoptosis of CT26 cells in transplanted tumor types. However, the biological function of ART3 in melanoma progression has not previously been studied, to the best of our knowledge. In the present study, it was revealed that ART3 was abnormally expressed in melanoma tissues and melanoma cells. Then, following the silencing of ART3 by small interfering RNA (siRNA) and short hairpin RNA (shRNA) in order to study its function in melanoma, the results revealed that ART3 knockdown may inhibit the migration ability of melanoma cells.

Correspondence to: Professor Shengfang Ge or Professor Xianqun Fan, Department of Ophthalmology, Shanghai Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine, 833 ZhiZaoJu Road, Huangpu, Shanghai 200011, P.R. China
E-mail: geshengfang@sjtu.edu.cn
E-mail: fanxq@sh163.net

*Contributed equally

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Materials and methods

Cell lines and cell culture. Human melanoma cell lines OCM1, OM431 and OCM1A were provided by Professor John F. Marshall (Tumor Biology Laboratory, Cancer Research UK Clinical Center, John Vane Science Centre, London, UK). A human retinal pigment epithelium (RPE-19) cell line was provided by the Department of Ophthalmology, Ruijin Hospital, Shanghai Jiao Tong University School of Medicine (Shanghai, China). The melanoma and RPE cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA)

supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.; cat. no. 10099141), 100 U penicillin and 100 mg/ml streptomycin (Gibco; Thermo Fisher Scientific, Inc.; cat. no. 10378016). The cultures were maintained in a humidified incubator at 37°C with 5% CO₂.

Patients. A set of melanoma tissues (n=18) paired with adjacent normal tissue (n=18) were obtained from patients diagnosed with melanoma from Shanghai Ninth People's Hospital, Shanghai Jiao Tong University, School of Medicine (Shanghai, China) from 2010 to 2015. There were 10 males and 8 females, their age ranged from 23-63, with a mean age of 41±8.23, after surgical resection of the tumor, the tissues were put into the cryopreservation tube and immediately preserved in the liquid nitrogen. Ethical approval obtained from the Independent Ethics Committee of Shanghai Ninth People's Hospital, Shanghai Jiao Tong University, School of Medicine and written informed consent was obtained from all patients involved.

ART3 siRNA oligonucleotides. ART3 siRNA oligonucleotides were as follows: Si-ART3-1, GACAUGGCAGAU AUGCAU dTdT, si-ART3-2, CACAGUUUGGGAUGGUCAUdTdT and si-ART3-3, CUGUAUUGAGAACCUAGAA dTdT. A random homologous sequence of all human genes was set as si-NC: UUCUCCGAACGUGUCACGUTTdTdT.

siRNA transfection. OCM1 and OM431 cells were seeded in 6-well plates at a density of 2x10⁵ cells/well. When the cells reached 70% confluency, cells were transfected with 50 nM of each siRNA using Lipofectamine[®] 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. After 6-8 h, the transfection medium was replaced with DMEM with 10% FBS. After 48 h of transfection, cells were collected for RNA extraction and protein extraction.

shRNA-expressing plasmid construction. The two shRNA sequences (shART3-1: 5'-CACCGACATGGCAGATATGCA TCGAAATGCATATCTGCCATGTC-3'; shART3-3: 5'-CAC CGCTGTATTGAGAACCTAGAACGAATTCTAGGTTCT CAATACAG-3') that target sh-ART3 were cloned into pGIPZ lentivirus vector (System Biosciences, Palo Alto, CA, USA).

Lentivirus package. The 293 T cells were cultured in Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% (vol/vol) fetal bovine serum and maintained at 37°C at a concentration of 6,000,000 cells and transfected using Lipofectamine 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) with 3 µg GIPZ-shART3, 3 µg pMD2.D, and 6.0 µg PsPax. After incubation overnight with 293 T cells, the media was replaced with 5 ml fresh medium. The virus-containing supernatants were collected at 48 and 72 h after transfection and then mixed and filtered through a 0.45 µm cellulose acetate filter (Sartorius AG, Göttingen, Germany). The viral supernatants were concentrated with Amicon Ultra-15 Centrifugal Filter Units (EMD Millipore, Schwalbach, Germany) at 4°C and spun at 3,913 x g for 30 min. viral supernatants were added into OCM1 and OM431 cells for 48 h and then replaced by fresh cell-culture medium. Then the colonies were selected

for subsequent culture after incubation with 4 g/ml puromycin for 2 weeks.

Western blotting. Western blotting was performed as previously described (14). Western blotting results were repeated three times and quantitative analysis of western blotting results were performed with Image J1.47 (National Institutes of Health, Bethesda, MD, USA). Protein was extracted from cells by RIPA Lysis and Extraction Buffer (Thermo Fisher Scientific, Inc.; cat. no. 89900). Antibodies used were as follows: Anti-ART3 polyclonal antibody (Wuhan Sanying Biotechnology, Wuhan, China; cat. no. 15930-1-AP; dilution, 1:1,000), GAPDH antibody (Wuhan Sanying Biotechnology; cat. no. 10494-1-AP; dilution, 1:5,000) and horseradish peroxidase-conjugated goat anti-rabbit secondary antibodies was used for western blot (Rabbit IgG, Abcam, Cambridge, UK; cat. no. ab6721; dilution, 1:5,000).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted using Trizol[®] (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol, and cDNA was synthesized using the PrimeScript RT reagent kit (Takara Bio, Inc., Otsu, Japan). qPCR analysis was performed using the RT qPCR Power SYBR[™] Green Master mix (Applied Biosystems; Thermo Fisher Scientific, Inc.; cat. no. 4367659) using Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.), and amplified qPCR products were quantified and normalized using GAPDH as a control (15). GAPDH and ART3 primers were as follows: GAPDH forward, 5'-ACTTCAACAGCGACA CCCACTC-3' and reverse, 5'-GTCCACCACCCTGTTGCT GTAG-3'; ART3 forward, 5'GCAACCATGATTCTAGTG GAC-3' and reverse, 5'-CTTTAGCAGTTGGGGAACG-3'. Thermocycling conditions for ART3 expression were as follows: 45 cycles of denaturation at 95°C for 30 sec, 60°C for 30 sec, extension at 72°C for 30 sec and a final extension at 72°C for 5 min.

Migration assay. The migration assay was based on the migration of melanoma cells seeded in an upper chamber of transwell insert through a membrane with an 8 µm pore size (EMD Millipore). Cells were seeded into 6-well plates at a density of 2x10⁵ cells/well, cultured for 18-24 h and then transfected with 50 nM of each siRNA Lipofectamine 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.). After 6-8 h, the medium was replaced with fresh medium.

A total of 24 h after transfection, cells were detached and collected. The upper chamber was seeded with 1x10⁵ cells of OCM1 and OM431 treated with siART3-1, siART3-3, the cells stably expressing shART3-1 and shART3-3 were contained in DMEM with 1% FBS (200 µl). The lower chamber contained 800 µl DMEM and 20% FBS was added to each well of the 24-well plate. Following incubation at 37°C for 48 h, the upper chamber was fixed with 100% methanol and stained with 0.1% crystal violet for 1 h as described previously (14). Then, the stained chambers were observed and photographing in microscope using a CCD camera (Visitron Systems GmbH, Puchheim, Germany) on a Zeiss Axioplan 2 imaging microscope (Zeiss AG, Oberkochen, Germany). Magnification: x4.

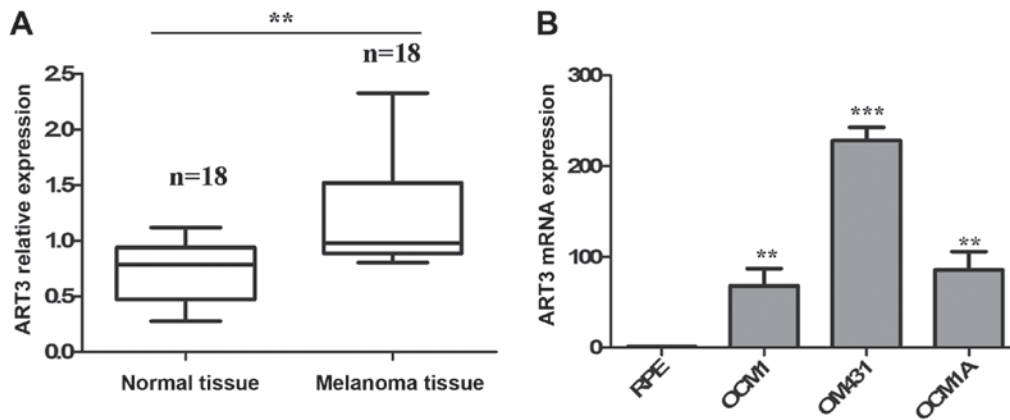


Figure 1. ART3 was overexpressed in melanoma tissues and melanoma cells. (A) ART3 expression levels in normal tissues (n=18) and melanoma tissues (n=18) were analyzed using Kolmogorov-Smirnov tests in order to assess normality, and were followed by a paired Student's t-test to assess differences. ART3 was significantly more highly expressed in melanoma tissues compared with normal tissues (P=0.0018). **P<0.01 vs. normal tissue. (B) Results of reverse transcription-polymerase chain reaction detection of ART3 expression levels in melanoma cells (OCM1, OM431 and OCM1A) and RPE cells. Expression of ART3 in different cell lines were evaluated using a one-way analysis of variance test with Tukey's honest significant difference post-hoc test. **P<0.01 and ***P<0.001 vs. RPE cells. ART3, ADP-ribosyltransferase 3; RPE, human retinal pigment epithelium cells.

Immunofluorescence staining assays. Cells were grown on glass coverslips for 24 h, then fixed with 4% paraformaldehyde in room temperature for 30 min and permeabilized with 0.5% Triton X-100 for 5 min. Following blocking with 10% FBS in DMEM in room temperature for 30 min, cells were incubated with the following antibodies in 4°C overnight according to the manufacturer's protocol: Anti-human ART3 (Wuhan Sanying Biotechnology; cat. no. 15930-1-AP; dilution, 1:1,000), GAPDH Rabbit mAb (Cell Signaling Technology; cat. no. 5174; dilution, 1:1,000) and Cy2-labeled goat anti-rabbit secondary antibodies were used to visualize primary antibodies (Jackson Immuno Research Laboratories, Inc., West Grove, PA, USA; cat. no. 111-225-006 dilution, 1:5,000). Cell micrographs were obtained using a CCD camera (Visitron Systems GmbH) on a Zeiss Axioplan 2 imaging microscope (Zeiss AG, Oberkochen, Germany). Magnification: x20.

Statistical analysis. All statistical analyses were performed using GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, CA, USA). Results were expressed as the mean \pm standard deviation. Parametric and non-parametric Kolmogorov-Smirnov tests were applied to assess normality, and the expression of ART3 in melanoma tissues was analyzed using a paired Student's t-test. Data were evaluated using a one-way analysis of variance with a Tukey's honest significant difference post-hoc test for comparisons between groups. P<0.05, P<0.01 and P<0.001 were considered to indicate a statistically significant, markedly significant and very significant difference, respectively. Western blotting results were analyzed using gray scale with Image J1.47 (National Institutes of Health, Bethesda, MD, USA).

Results

ART3 is overexpressed in melanoma tissues and melanoma cells. To investigate the association between the abnormal expression of ART3 and the occurrence of melanoma, a set of melanoma tissues (n=18) paired with adjacent normal tissue (n=18) were obtained from diagnosed patients and used

to examine the expression of ART3. It was revealed that the expression levels of ART3 were significantly increased in melanoma tissues compared with adjacent normal tissues (P<0.01; Fig. 1A). To further verify the clinical significance of ART3, ART3 expression in three melanoma cell lines (OCM1, OM431 and OCM1A) and one normal cell line (RPE) was examined using RT-qPCR. Consistent with the results in melanoma tissues, the melanoma cell lines expressed significantly higher levels of ART3 compared with the RPE cells (P<0.01; Fig. 1B). These data indicate the clinical importance of ART3 in melanoma.

Targeted gene interference of ART3 with siRNA and shRNA. As ART3 is highly expressed in melanoma cells and melanoma tissues, it was speculated that ART3 may serve a function in the occurrence of melanoma. siRNA-mediated interference of ART3 expression was performed in OCM1 and OM431 cells, in order to exclude off-target effects. OCM1 and OM431 cells were transfected with three si-ART3 and si-negative control (NC). The RNA interference efficiency was tested using RT-qPCR, among which the interference efficiency of si-ART3-1 and si-ART3-3 reached >50%, revealing a significant difference compared with the NC (P<0.05; Fig. 2A and B). Next, one siRNA (si-ART3-1) was selected for the construction of pGIPZ ART3-shRNA plasmids. The pGIPZ ART3-shRNA vectors with an empty vector (mock) were then packaged into lentiviruses, and transduced into OCM1 and OM431 cells. Following transduction, the cells were screened using puromycin for the stable expression of sh-ART3. The expression levels of ART3 in stable cell clones were then detected using RT-qPCR (Fig. 2C and D). It was revealed that ART3 expression levels were significantly knocked down in the two shART3-expressing cell lines, OCM1 (P<0.05; Fig. 2C) and OM431 (P<0.05; Fig. 2D).

Detecting the knockdown of ART3 in melanoma cells by western blotting and immunofluorescence staining. To further verify the knockdown of ART3 in melanoma cells, western blotting (Fig. 3A and B) and immunofluorescence staining

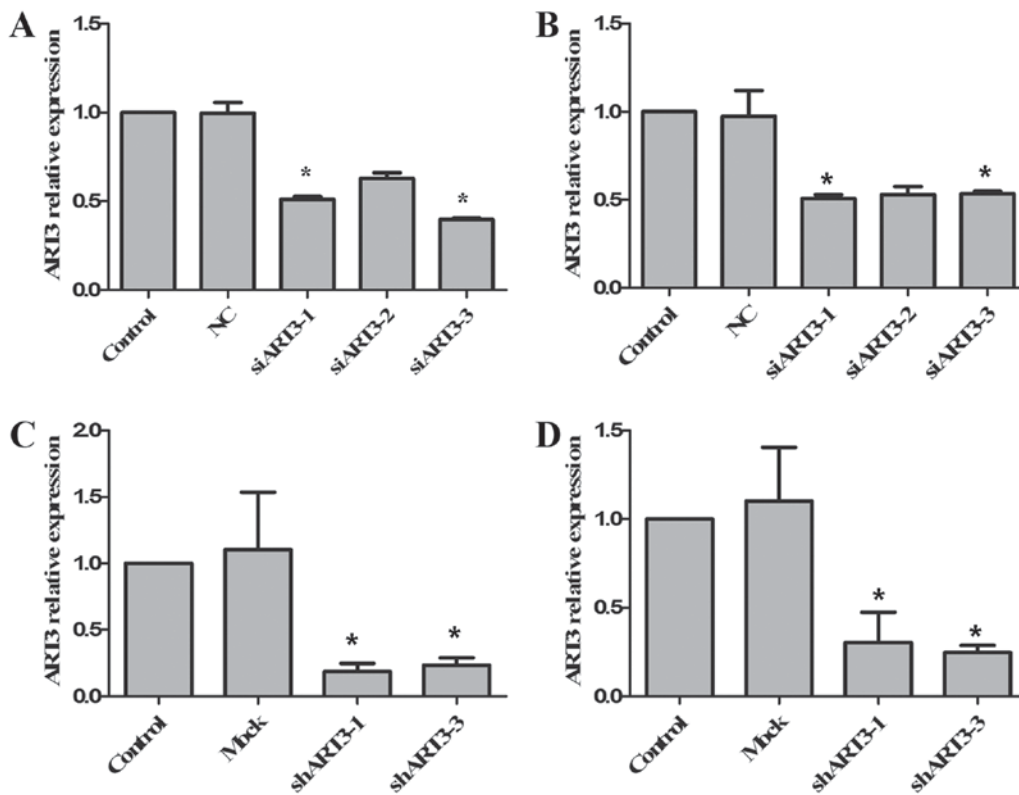


Figure 2. Targeted gene interference of ART3 with siRNA and shRNA. The expression of ART3 were analyzed using a one-way analysis of variance followed by the Tukey's honest significant difference post-hoc test. ART3 gene expression levels were detected using RT-qPCR in (A) OCM1 cells and (B) OM431 cells, 48 h after the transfection of three si-ART3, si-NC and control. * $P < 0.05$ vs. NC. RT-qPCR analysis of ART3 gene expression levels in (C) OCM1 cells and (D) OM431 cells that stably expressed sh-ART3 and an empty vector (mock). * $P < 0.05$ vs. mock. ART3, ADP-ribosyltransferase 3; siRNA, small interfering RNA; shRNA, short hairpin RNA; RT-qPCR, reverse transcription-polymerase chain reaction; NC, transfected with siNC; Control, untransfected.

(Fig. 3C) was used to examine the ART3 expression in shART3 expressing cells. As expected, the quantified results of the western blotting and immunofluorescence revealed that the expression levels of ART3 in stable cell clones was significantly knocked down compared with the empty vector transfected cells (mock; Fig. 3D and E).

Knockdown of ART3 inhibits the migration of melanoma cells. To study the effect of ART3 on migration, si-ART3-1 and si-ART3-3 transfected OCM1 and OM431 cells were harvested subsequent to being cultured for 48 h, stained with 0.1% crystal violet and imaged. The result of the Transwell chamber assay indicated that the number of si-ART3-1 and si-ART3-3 cells migrating through the filtration membrane was markedly lower compared with that of the controls (Fig. 4A). This indicated that the interference of ART3 may inhibit the migratory ability of melanoma cells. In addition, a markedly decreased migratory rate in shART3-expressing OCM1 and OM431 cells compared with that of the controls after 48 h was observed (Fig. 4B). These data indicate that ART3 serves a regulatory role in melanoma cell migration and may serve as a novel therapeutic target.

Discussion

ADP ribosylation is a notable posttranslational protein modification, regulating a host of critical cellular processes, including tumorigenesis and cell apoptosis (16). Momii and Koide (17)

suggested that mouse testicular cells possess an ART that catalyzes the transfer of the ADP-ribose moiety from NAD⁺ to an unknown acceptor protein. Genetic variation of ART3 may result in a functional defect in the process of spermatogenesis. Okada *et al* (18) revealed that ART3 was identified as a susceptible gene underlying non-obstructive azoospermia. The biological function of ART3 varies in different tissues and organs (19); however, the effect of ART3 function on melanoma tumorigenesis has yet to be reported.

In the present study, it was revealed that there was an abnormal expression of ART3 in melanoma cells and melanoma tissues, which indicated the potential function of ART3 in melanoma tumorigenesis. To gain a better understanding of the biological function of ART3, siRNA and shRNA were used to silence the ART3 gene in melanoma cell lines, the results revealed the efficient inhibition of ART3. The effects of ART3 silencing on cell migration were further examined. The results revealed that ART3 knockdown notably inhibited melanoma migration.

Considering that melanoma is a highly metastatic disease and ~1/2 of all patients with melanoma succumb to mortality from metastases (20,21), the treatment of melanoma faces numerous challenges. Single-agent therapies (mono-therapies) have not yet demonstrated a clear clinical benefit (22). Selumetinib provides a significant advantage in increased progression-free survival rates, but with an insignificant impact on improved overall survival (23). The use of histone deacetylase inhibitors to induce the differentiation of

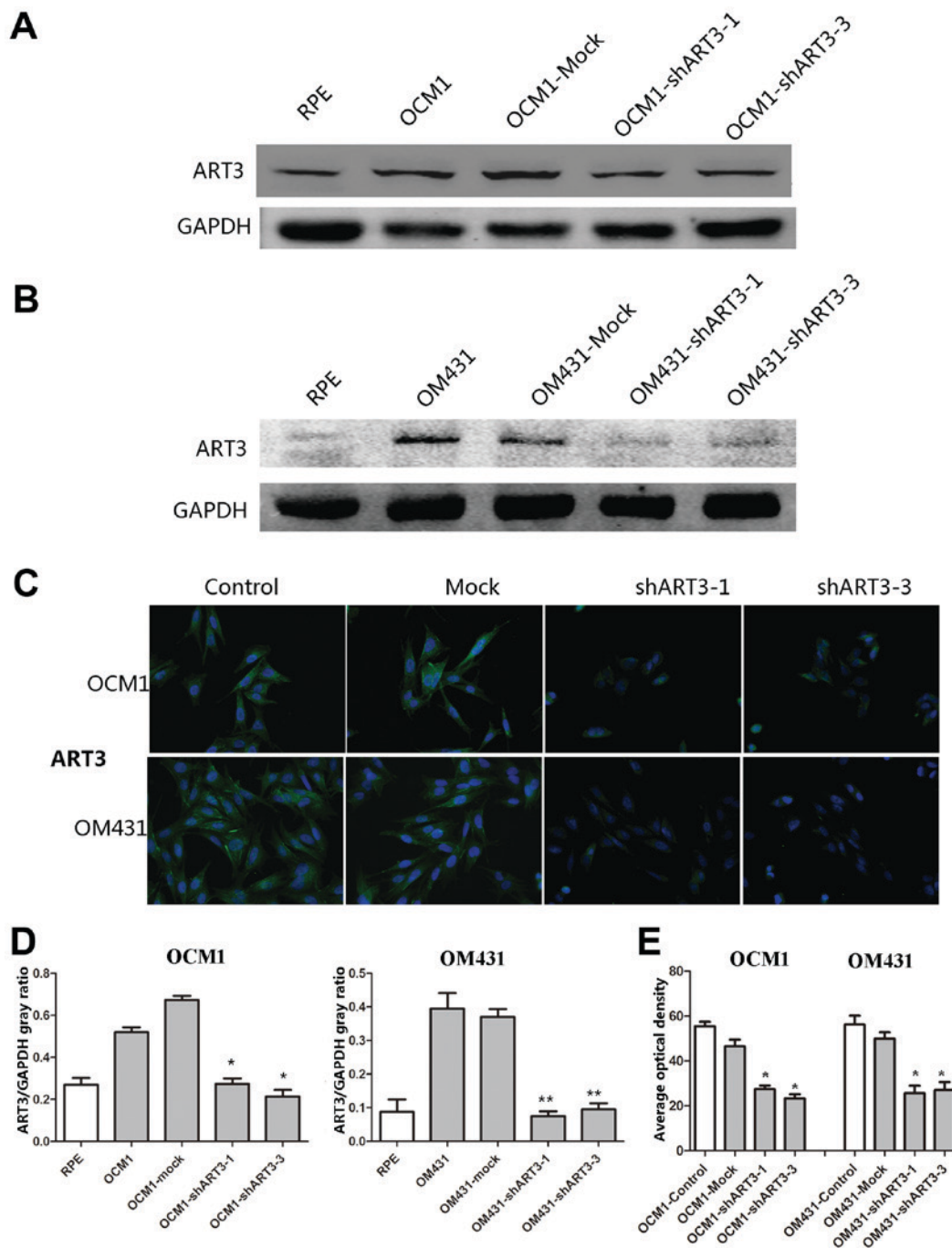


Figure 3. Detecting the knockdown of ART3 in melanoma cells by western blotting and immunofluorescence staining. Western blotting of the ART3 protein in (A) OCM1 cells and (B) OM431 cells that stably expressed sh-ART3-1 and sh-ART3-3 and an empty vector (mock). (C) Immunofluorescence staining analysis of ART3 protein in OCM1 and OM431 cells that stably expressed sh-ART3-1 and sh-ART3-3 and an empty vector (mock). Magnification: x20. (D) Quantitative analysis of western blotting results performed with Image J, and the results were calculated from three independent repeated experiments. The ratio of ART3/GAPDH was analyzed using a one-way ANOVA with the Tukey's HSDd1 post-hoc test. * $P < 0.05$ and ** $P < 0.01$ vs. mock. (E) Intensity quantitative analysis of immunofluorescence results performed using Image J, and the results were calculated from 50 different cells. Statistic intensity in each group were analyzed using one-way ANOVA with the Tukey HSD post-hoc test. * $P < 0.05$ vs. mock. ART3, ADP-ribosyltransferase 3; shRNA, short hairpin RNA; ANOVA, analysis of variance, HSD, honest significant difference.

melanoma cells is being explored clinically, but evidence of antitumor activity in pre-clinical models remains limited (24). The combination of si-B-cell lymphoma 2 and H101 recombinant oncolytic adenovirus has proven to be effective in the treatment of melanoma (25). Hu *et al* (26) revealed that the pharmacological nuclear factor- κ B inhibitor, BAY11-7082, induced cell apoptosis and inhibited the migration of human melanoma cells. Therefore, identifying effective targets that

inhibit melanoma metastasis is necessary and ART3 may be a potential therapeutic target in the future.

The mechanism of the involvement of ART3 in melanoma cell migration requires further study, as the ADP-ribose moiety from NAD⁺ was transferred to the cysteine residues of the target protein (11), suggesting that ART3 may be a cysteine-specific enzyme. Paraoan *et al* (27) revealed that the shift in the balance between cathepsin S and cystatin C may

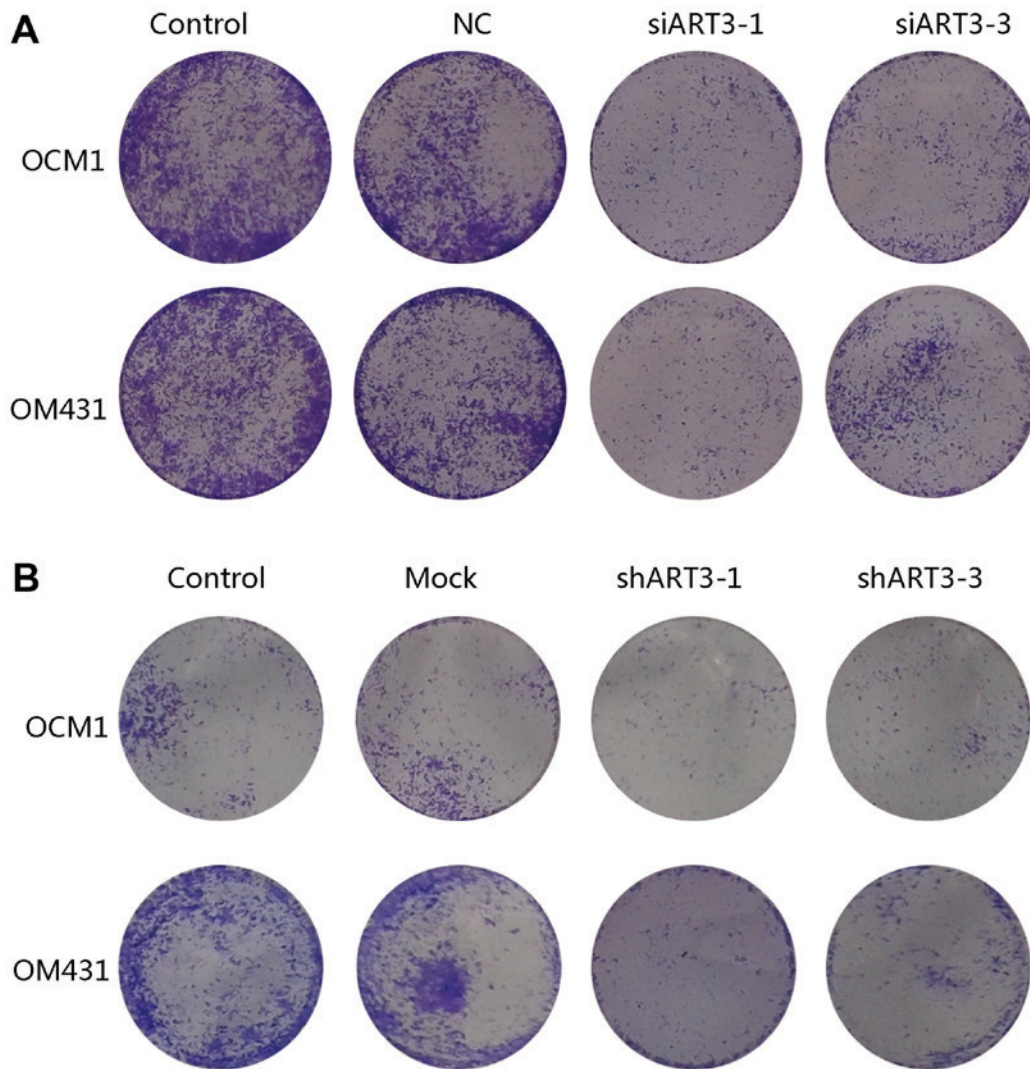


Figure 4. Knockdown of ART3 inhibits the migration of melanoma cells. (A) Cell migration was assessed using a Transwell assay in siART3-1 and siART3-3 transfected OCM1 and OM431 cells. Magnification: $\times 4$. (B) Cell migration was assessed using a Transwell assay in OCM1 and OM431 cells that stably expressed shART3-1 and shART3-3 and empty vector (mock). Magnification: $\times 4$.

be part of the deregulated proteolytic pathways that contribute to the invasive phenotype of melanoma. It has been suggested that ART3 may function as a cysteine enzyme involved in melanoma metastasis. Previously, small molecular inhibitors have been studied as novel promising drugs for the treatment of melanoma (28). Bi *et al* (14) revealed that a chemical inhibitor of C-X-C chemokine receptor type 4, AMD3100, may inhibit the proliferation and migration of melanoma cells. At present, no small molecule inhibitors of ART3 have been identified, thus the development of novel ART3 inhibitors is a promising area of research.

In summary, the targeted silencing of ART3 expression inhibited the migration of melanoma cells. This suggests that ART3 may be a metastasis-associated gene in melanoma. Future research should focus on the mechanism of the ART3 anti-metastasis function and examine its potential role as a molecular target for the treatment of melanoma.

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Availability of data and materials

Not applicable.

Authors' contributions

In this report, XQF and SFG designed and directed the experiments, discussed, revised and wrote the manuscript; JH, YYL,

and YW designed and performed the experiments and drafted the manuscript; JH and HZ were responsible for sample collection and data analysis. All authors approved this manuscript.

Ethics approval and consent to participate

The ethical approval was signed by ethics committee of Shanghai Ninth People's Hospital affiliated to Shanghai Jiao Tong University School of Medicine (Shanghai, China), and written informed consent was obtained from all patients involved.

Consent for publication

Informed consent for the publication of their clinical data was obtained from all patients.

Competing interests

The authors declare that they have no competing interests.

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