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Effect of ART1 on the proliferation and migration of mouse colon carcinoma CT26 cells *in vivo*

JIAN-XIA XU*, WEI XIONG*, ZHEN ZENG*, YI TANG, YA-LAN WANG, MING XIAO, MING LI, QING SHU LI, GUANG-LIN SONG and JING KUANG

Department of Pathology, Molecular Medicine and Cancer Research Center, Chongqing Medical University, Chongqing 400016, P.R. China

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Abstract. Arginine-specific mono-ADP-ribosyltransferase 1 (ART1) is an important enzyme that catalyzes arginine-specific mono-ADP-ribosylation. There is evidence that arginine-specific mono-ADP-ribosylation may affect the proliferation of smooth muscle cells via the Rho-dependent signaling pathway. Previous studies have demonstrated that ART1 may have a role in the proliferation, invasion and apoptosis of colon carcinoma *in vitro*. However, the effect of ART1 on the proliferation and invasion of colon carcinoma *in vivo* has yet to be elucidated. In the present study, mouse colon carcinoma CT26 cells were infected with a lentivirus to produce ART1 gene silencing or overexpression, and were then subcutaneously transplanted. To observe the effect of ART1 on tumor growth or liver metastasis *in vivo*, a spleen transplant tumor model of CT26 cells in BALB/c mice was successfully constructed. Expression levels of focal adhesion kinase (FAK), Ras homolog gene family member A (RhoA) and the downstream factors, c-myc, c-fos and cyclooxygenase-2 (COX-2) proteins, were measured *in vivo*. The results demonstrated that ART1 gene silencing inhibited the growth of the spleen transplanted tumor and its ability to spread to the liver via metastasis. There was also an accompanying increase in expression of FAK, RhoA, c-myc, c-fos and COX-2, whereas CT26 cells with ART1 overexpression demonstrated the opposite effect. These results suggest a potential role for ART1 in the proliferation and invasion of CT26 cells and a possible mechanism *in vivo*.

Correspondence to: Professor Ya-Lan Wang, Department of Pathology, Molecular Medicine and Cancer Research Center, Chongqing Medical University, 1 Medical College Road, Yuzhong, Chongqing 400016, P.R. China
E-mail: wangyalan074@126.com

*Contributed equally

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Introduction

Adenosine diphosphate (ADP) ribosylation, which includes mono-ADP-ribosylation, poly-ADP-ribosylation, ADP-ribose cyclization and formation of O-acetyl-ADP-ribose, is involved in a wide range of human physiological and pathological processes and serves important roles in cell signal transduction, transcriptional regulation, genetic stability maintenance, cell proliferation and differentiation, adhesion and migration (1). Mono-ADP-ribosyltransferases (ART), the enzymes of mono-ADP-ribosylation, consist of seven members (ART1-7). ART1 catalyzes the mono-ADP-ribosylation of nicotinamide adenine dinucleotide to arginine residues in proteins, thereby releasing nicotinamide, which may alter the structure and chemical property of acceptor proteins resulting in a change in their activity and function (2). Research on ART1 is mainly concentrated on the inflammatory response and on non-neoplastic cells (3,4). In the epithelial cells of the respiratory tract and the bronchoalveolar lavage fluid of people with asthma, ART1 may catalyze the mono-ADP-ribosylation of human neutrophil peptide-1 (3), resulting in an inflammatory response. Yau *et al* (5) demonstrated that meta-iodobenzylguanidine (MIBG), a selective inhibitor of arginine-specific mono-ADP-ribosylation (6), is able to suppress the proliferation and differentiation of vascular smooth muscle cells. The researchers hypothesized that mono-ADP-ribosylation is involved in a Rho-dependent signaling pathway.

However, although ART1 is associated with the proliferation and migration of colon cancer cells *in vivo*, its molecular mechanism has yet to be fully elucidated. In the present study, mouse colon carcinoma CT26 cells were infected with a lentivirus to change the expression of ART1 in CT26 cells. To observe the effect of ART1 on the development of colon carcinoma *in vivo*, CT26 cells with ART1 silencing or overexpression were injected into BALB/c mice to construct a subcutaneously transplanted tumor model or a spleen transplant tumor model. Growth of the tumor and liver metastases were observed. In addition, the expression of focal adhesion kinase (FAK), Ras homolog gene family member A (RhoA) and their downstream factors, c-myc, c-fos and cyclooxygenase-2

(COX-2) proteins, were measured. The potential role of ART1 in the proliferation and invasion of CT26 cells and its possible mechanism *in vivo* were explored.

Materials and methods

Cell lines and animals. The mouse colon adenocarcinoma CT26 cell line was obtained from Professor Yu-Quan Wei (Sichuan University, Chengdu, Sichuan, China), Tang *et al* and Kuang *et al* (7,8), having successfully constructed ART1-short hairpin RNA (shRNA), ART1-overexpression and vector-control CT26 cells. BALB/c mice (6-8 weeks old, 18-22 g) were obtained from the animal experimental center of Chongqing Medical University (Chongqing, China) and placed in the specific pathogen-free feeding room (20-26˚C, 12 h:12 h light/dark cycle) of the animal experimental center at Chongqing Medical University.

Subcutaneously transplanted tumor model of CT26 cells in BALB/c mice. Each experimental group consisted of 12 mice. Each mouse was anesthetized by the intraperitoneal injection of 2% chloral hydrate (0.3 g/kg). CT26 cell suspension ($1x10^7$/mlx50 μl) was subcutaneously injected into the lateral skin of the right armpit of each mouse. After 14 days, six mice were randomly selected from each group for sacrifice, and the weight and volume of the subcutaneous tumor was recorded. The survival time of the rest of the mice in each group was recorded. Tumor volume was calculated according to the formula: Volume=the maximum diameter x the most trails^2 x ½ (9).

Spleen transplant tumor model of CT26 cells in BALB/c mice to observe liver metastases. A total of 48 BALB/c mice were randomly divided into four groups. Following the method described by Liu *et al* (10), each mouse was anesthetized with 2% chloral hydrate (0.015 ml/g) injected into the abdominal cavity. Subsequently, the abdominal wall was incised along with the left subcostal margin layer by layer. The spleen was identified in the abdominal cavity, and then CT26 cell suspension ($1x10^7$/ml x 50 μl) was injected under the capsule of the spleen. Finally, the abdominal wall was sutured. The entire procedure was performed under sterile conditions to ensure the survival rate of the mice. While being reared the mice were provided with standard chow and tap water *ad libitum*. After 14 days, six mice were randomly selected from each group for sacrifice and the remaining mice of each group continued to be fed until their natural death to enable the recording of the survival time and the plotting of a Kaplan-Meier survival curve.

The volume of the spleen tumors was calculated according to the formula volume=the maximum diameter x the most trails^2 x ½ (9). Nodules of liver metastases were graded as follows: Grade 0, no visible metastatic nodule in liver; grade 1, 1-5 metastatic nodules in liver; grade 2, 6-10 metastatic nodules in liver; grade 3, >10 metastatic nodules, or fused nodules difficult to count exactly (10).

Expression levels of ART1, RhoA, c-myc, c-fos and COX-2 detected with western blotting in the subcutaneously transplanted tumor. The subcutaneous tumors were cut into small pieces, weighed, homogenized, and then lysed with radio-immunoprecipitation assay (RIPA) lysis buffer (100 μl of RIPA lysis buffer/10 mg tissue; Beyotime Institute of Biotechnology, Shanghai, China) for 30 min on ice. The lysate was transferred into a 1.5 ml centrifuge tube and centrifuged at 4˚C 12,000 rpm (8,418 g) for 5 min. A bicinchoninic acid (BCA) protein assay kit (Beyotime Institute of Biotechnology) was used to measure the concentration of protein. Protein (80 μg/lane) was electrophoresed on 10% polyacrylamide gels (SDS-PAGE) and then transferred to polyvinylidene fluoride (PVDF) membranes. The membranes were blocked with 5% non-fat dried milk dissolved in Tris-buffered saline with Tween-20 (TBST) at room temperature for 2 h, and incubated respectively with primary antibodies of ART1 (cat. no. AP2311a; Abgent, Inc., San Diego, CA, USA), RhoA (cat. no. BS6470), and c-fos (cat. no. BS6433; Bioworld Technology, Inc., St. Louis, MO, USA), c-myc (cat. no. C10262; Anbo, Inc., San Francisco, CA, USA), COX-2 (cat. no. 12375-1-AP; Proteintech Group, Inc., Chicago, IL, USA) and β-actin (cat. no. BA2305; Boster Systems, Wuhan, China) overnight at 4˚C. The most effective working concentration of these primary antibodies was 1:500. The membranes were washed three times with TBST, and then incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody at a dilution of 1:1,000 (ZSGB-BIO, Beijing, China) for 1.5 h at room temperature. The membranes were washed three times with TBST, and then dipped into BeyoECL Plus (Beyotime Institute of Biotechnology, Shanghai, China) for exposure and imaging (Bio-Rad Laboratories, Inc., Hercules, CA, USA). β-actin was used as a loading control for the western blotting experiments.

Western blot analysis of expression levels of ART1, RhoA and FAK in transplanted spleen tumors. Total protein was extracted from transplanted spleen tumors. The tissue was washed with phosphate-buffered saline (PBS) and then homogenized prior to being lysed with RIPA lysis buffer (100 μl/10 mg) for 30 min on ice. The homogenate was transferred to a pre-cooled centrifuge tube, and then centrifuged at 4˚C, 12,000 rpm (8,418 g) for 10 min. The rest of the procedure was as detailed in the previous paragraph with the exception that the primary antibodies, ART1 (Abgent, Inc.), RhoA and FAK (cat. no. BS6899; Bioworld Technology, Inc.) at a dilution of 1:500, and β-actin (Boster Systems) at a dilution of 1:1,000 were used to incubate the PVDF membranes.

Statistical analysis. Data were presented as the mean ± standard deviation. Analysis of variance statistical evaluation was used and analyses were performed using SPSS software, version 17.0 (SPSS, Inc., Chicago, IL, USA). The Kruskal-Wallis and Nemenyi methods were used to analyze the level of metastatic nodules in the liver. The differences in tumor-bearing mice survival time were analyzed using the log-rank test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Effect of ART1 on the growth of subcutaneous transplanted CT26 tumors in BALB/c mice. Compared with subcutaneous

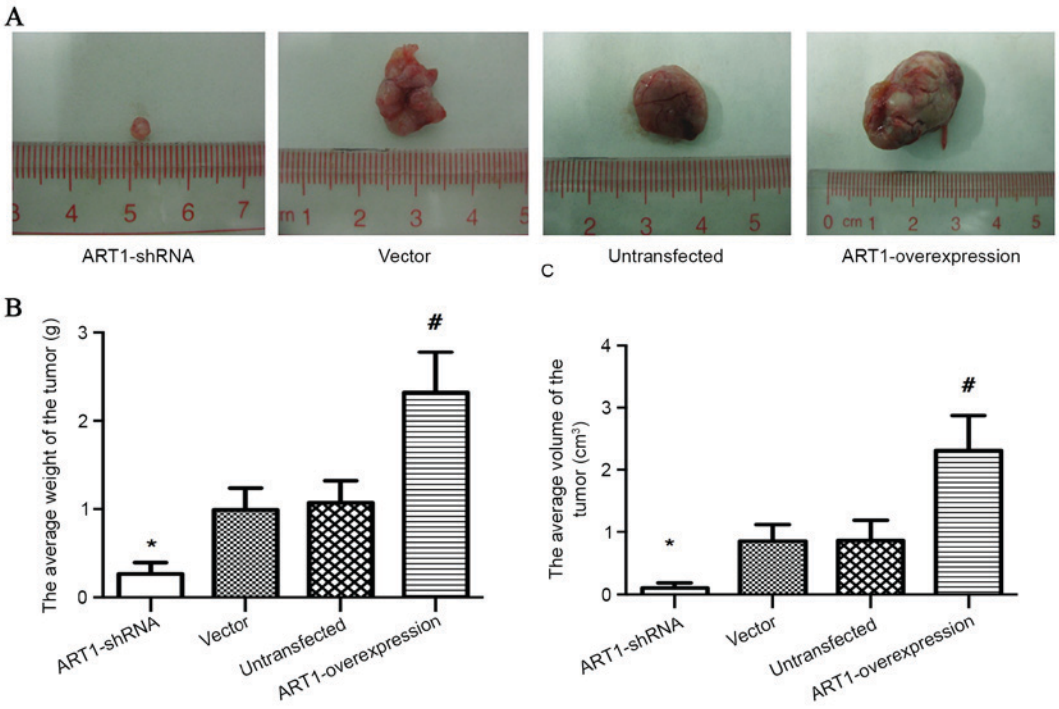

Figure 1. Subcutaneously transplanted CT26 tumors. (A) Photographic images of subcutaneously transplanted CT26 tumors in BALB/c mice are shown. The (B) average weight and (C) volume of subcutaneous transplanted tumors in the four groups. *P<0.05, ART1-shRNA group vs. vector and untransfected groups; #P<0.05, ART1-overexpression group vs. vector and untransfected groups. ART1-shRNA, ADP-ribosyltransferase 1-short hairpin RNA.

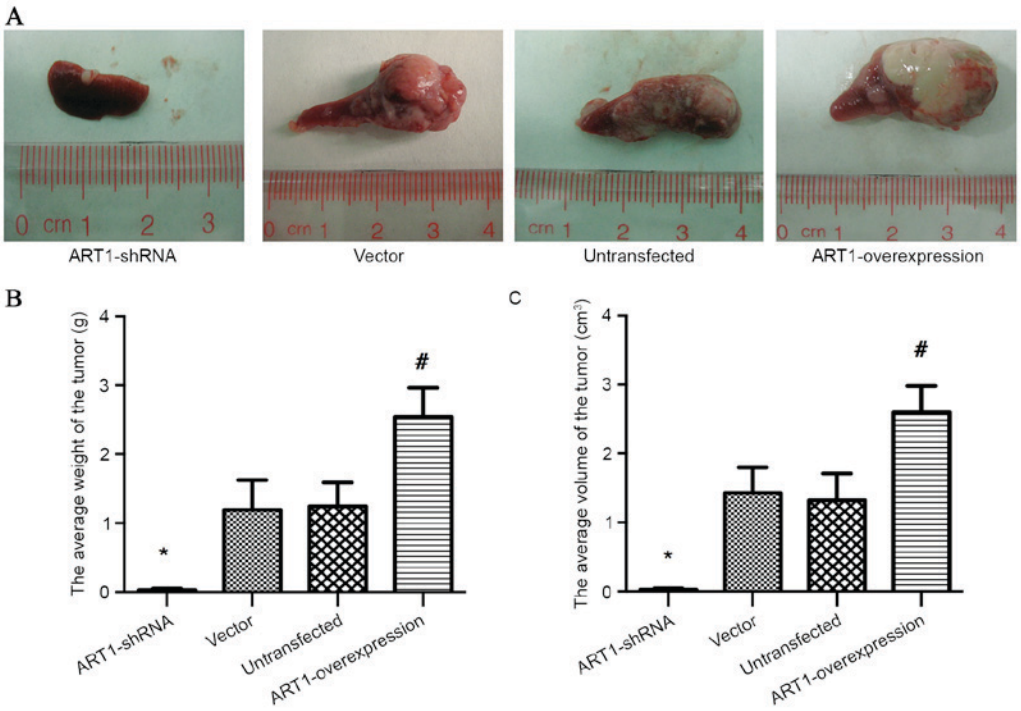

Figure 2. Spleen-transplanted CT26 tumors. (A) Spleen-transplanted CT26 tumors in BALB/c mice are shown. Comparison of the (B) weight and (C) volume of spleen-transplanted tumors in the four groups. *P<0.05, ART1-shRNA group vs. vector and untransfected groups; #P<0.05, ART1-overexpression group vs. vector and untransfected groups. ART1-shRNA, ADP-ribosyltransferase 1-short hairpin RNA.

transplanted vector-control and untransfected CT26 tumors, the volume and weight of subcutaneous transplanted tumors were decreased in the ART1-shRNA group (P<0.05) and increased in the ART1-overexpression group (P<0.05; Fig. 1A-C).

Effects of ART1 on the growth of spleen transplanted CT26 tumor in BALB/c mice. The volume and weight of spleen-transplanted ART1-shRNA CT26 tumors were significantly decreased compared with the spleen-transplanted vector-control and untransfected CT26 tumors (P<0.05).

Table I. The quantity and grading of metastases in liver (n=6, mean ± standard deviation).

Group	Quantity of metastases in the liver	Grade			
		0	1	2	3
ART1-shRNA	0.33±0.82[a]	5	1	0	0
Vector	2 2.33±12.11	0	0	1	5
Untransfected	23.83±10.340	0	0	1	5
ART1-overexpression	39.50±8.38[b]	0	0	0	6

[a]P<0.05, ART1-shRNA group vs. vector and untransfected groups; [b]P<0.05, ART1-overexpression group vs. vector and untransfected groups. ART1-shRNA, ADP-ribosyltransferase 1-short hairpin RNA.

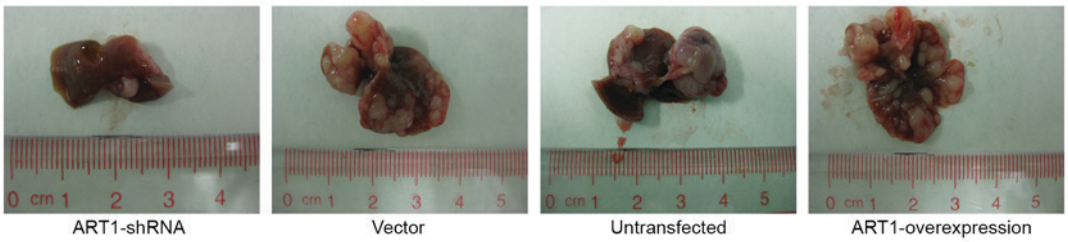

Figure 3. Liver metastasis of colon carcinoma in BALB/c mice. ART1-shRNA, ADP-ribosyltransferase 1-short hairpin RNA.

However, the volume and weight of spleen-transplanted ART1-overexpression CT26 tumors were increased (P<0.05). No significant differences were identified between the spleen-transplanted vector-control and untransfected CT26 tumors (P>0.05; Fig. 2A-C).

Effect of ART1 on liver metastasis of colon carcinoma in BALB/c mice. The number of liver metastatic tumor nodules in the spleen-transplanted CT26 tumor model were counted in each group. The number of liver metastatic tumor nodules in the ART1-shRNA group was lower than in the vector-control and untransfected groups (P<0.05). The number of liver metastatic tumor nodules in the ART1-overexpression group was higher compared with the vector-control and untransfected groups (P<0.05). No significant differences were identified in the number or the appearance of liver metastatic tumor nodules in the vector-control and untransfected groups (P>0.05; Fig. 3 and Table I).

Influence of ART1 on the survival time of BALB/c mice with subcutaneous transplanted CT26 tumor or spleen transplanted CT26 tumor. The average survival time of BALB/c mice with subcutaneously transplanted CT26 tumors was extended in the ART1-shRNA group (P<0.05), and was shortened in the ART1-overexpression group (P<0.05). However, no significant differences were identified between the vector-control and untransfected groups (P>0.05; Fig. 4A).

The average survival time of BALB/c mice with spleen-transplanted ART1-shRNA CT26 tumors was longer compared with vector-control and untransfected groups (P<0.05). The average survival time of BALB/c mice with spleen-transplanted ART1-overexpression CT26 tumors was shorter than in the control groups (P<0.05). No significant differences were identified between the vector-control and untransfected groups (P>0.05; Fig. 4B).

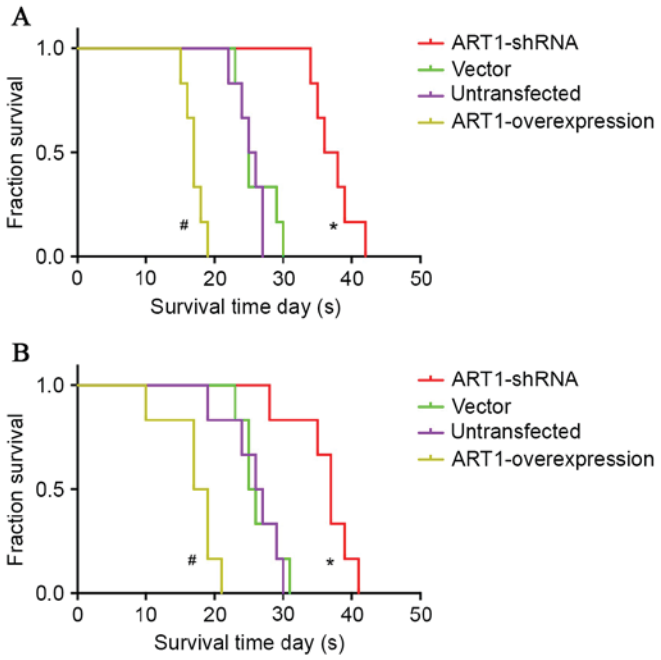

Figure 4. Kaplan-Meier survival curves of BALB/c mice with (A) subcutaneously transplanted or (B) spleen-transplanted CT26 tumors. *P<0.05, ART1-shRNA group vs. vector and untransfected groups; #P<0.05, ART1-overexpression group vs. vector and untransfected groups. ART1-shRNA, ADP-ribosyltransferase 1-short hairpin RNA.

Effect of ART1 on the expression levels of RhoA, c-myc, c-fos and COX-2 in subcutaneously transplanted CT26 tumor

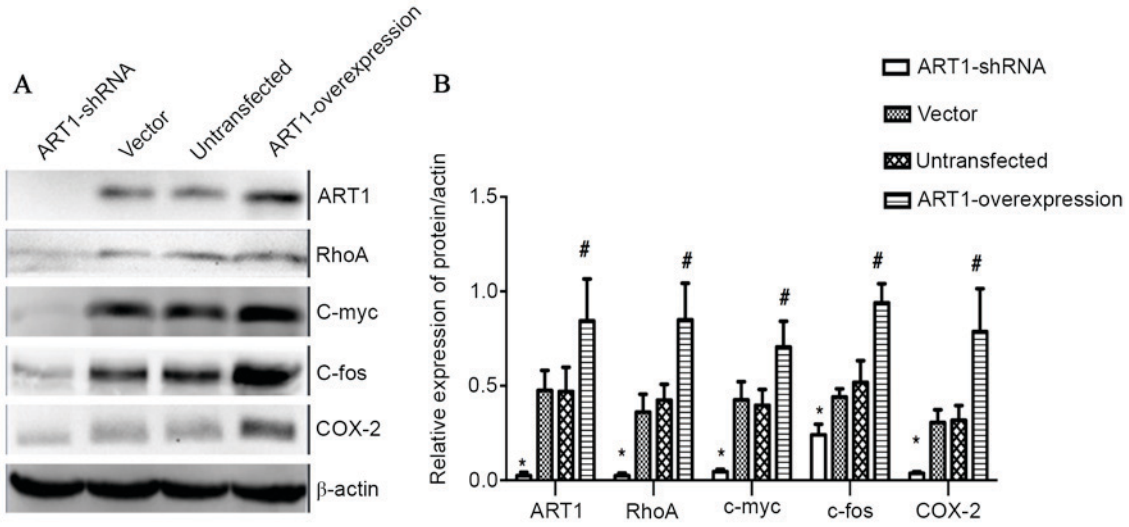

Figure 5. Effect of ART1 on the expression levels of RhoA, c-myc, c-fos and COX-2 in subcutaneously transplanted CT26 tumor tissue of BALB/c mice. (A) A representative western blot showing the expression levels of ART1, RhoA, c-myc, c-fos, COX-2 in subcutaneously transplanted CT26 tumor tissue in BALB/c mice. (B) Quantitative analysis revealed that the expression levels of ART1, RhoA, c-myc, c-fos and COX-2 in subcutaneously transplanted ART1-overexpression CT26 tumors were increased. However, the expression levels of ART1, RhoA, c-myc, c-fos and COX-2 in subcutaneously transplanted ART1-shRNA CT26 tumors were decreased. *P<0.05, ART1-shRNA group vs. vector and untransfected groups; #P<0.05, ART1-overexpression group vs. vector and untransfected groups. ART1, ADP-ribosyltransferase 1; RhoA, Ras homolog gene family member A; COX-2, cyclooxygenase-2; ART1-shRNA, ART1-short hairpin RNA.

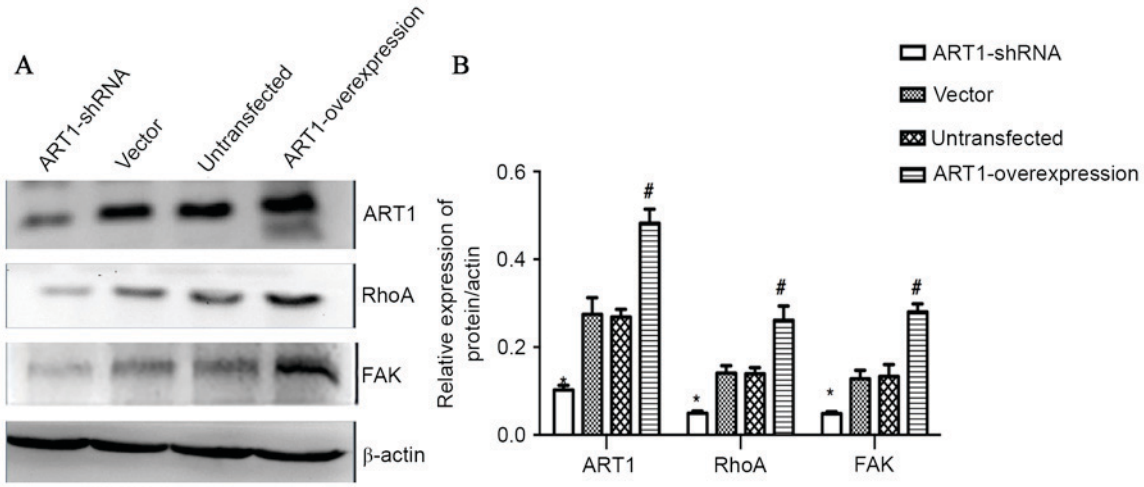

Figure 6. Effect of ART1 on the expression levels of RhoA and FAK in spleen-transplanted CT26 tumor tissue of BALB/c mice. (A) A representative western blot showing the expression levels of ART1, RhoA and FAK in spleen-transplanted CT26 tumor tissue of BALB/c mice. (B) Quantitative analysis revealed that the expression levels of ART1, RhoA and FAK in spleen-transplanted ART1-overexpression CT26 tumor were increased. However, the expression levels of ART1, RhoA, c-myc, c-fos and COX-2 in spleen-transplanted ART1-shRNA CT26 tumor were decreased. *P<0.05, ART1-shRNA group vs. vector and untransfected groups; #P<0.05, ART1-overexpression group vs. vector and untransfected groups. ART1, ADP-ribosyltransferase 1; RhoA, Ras homolog gene family member A; FAK, focal adhesion kinase; COX-2, cyclooxygenase-2; ART1-shRNA, ART1-short hairpin RNA.

tissue of BALB/c mice. The expression levels of ART1, RhoA, c-myc, c-fos and COX-2 in subcutaneously transplanted ART1-overexpression CT26 tumors were all higher than those in the control groups (P<0.05). However, the expression levels of ART1, RhoA, c-myc, c-fos and COX-2 in subcutaneously transplanted ART1-shRNA CT26 tumors were lower than in those in the control groups (P<0.05). No significant differences were identified between the vector-control and untransfected groups (P>0.05; Fig. 5A and B).

Effect of ART1 on the expression levels of RhoA and FAK in spleen-transplanted CT26 tumor tissue of BALB/c mice. Compared with the vector-control and untransfected groups, the expression levels of ART1, RhoA and FAK in spleen transplanted ART1-shRNA CT26 tumors were decreased, and the expression levels of these proteins in spleen-transplanted ART1-overexpression CT26 tumors were increased (P<0.05). No significant differences were identified between the expression levels of these proteins in the vector-control and untransfected groups (P>0.05; Fig. 6A and B).

Discussion

Yau *et al* (6) hypothesized that mono-ADP-ribosylation enzymes may be associated with the progression of gastric cancer. A previous study (11) has shown that ART1 expression

was increased in colorectal cancer and has a positive correlation with the expression of vascular endothelial growth factor (VEGF), which suggests that it may have an association with tumor angiogenesis. It has also been observed that the silencing of ART1 in CT26 cells may inhibit the proliferation of cells by restraining cell cycle at the G0/G1 phase, suppressing matrix adhesion and migration *in vitro* (12-14). However, whether the changes of ART1 in CT26 cells are able to affect the proliferation and invasion *in vivo* has yet to be fully elucidated. The present study demonstrated the reduction in the volume and weight of subcutaneously transplanted ART1-shRNA CT26 tumor tissue in BALB/c mice. However, there was an increase in the volume and weight of subcutaneously transplanted ART1-overexpression CT26 tumor tissue in BALB/c mice. The results also demonstrated that a reduction in the volume and weight of spleen-transplanted CT26 tumor tissue occurred in BALB/c mice with the silencing of ART1 in CT26 cells, and an increase with the overexpressing of ART1 in CT26 cells. The average survival time of BALB/c mice with subcutaneously transplanted CT26 tumors or spleen transplanted CT26 tumors was significantly shortened with the overexpression of ART1, and was extended with the silencing of ART1. Taken together, these data demonstrated that ART1 may affect the growth and development of transplanted CT26 tumor *in vivo*.

In skeletal muscle cells, ART1 catalyzes the modification of mono-ADP-ribosylation on integrin α7β1, which may promote the binding of integrin and laminin and lead to the activation of FAK and of Rho, resulting in the formation of stress fibers and the shrinkage of cells (15-18). Integrin β1, an important signaling molecule on the cell membrane, is able to associate with a variety of intracellular signaling molecules, including FAK, Rho and integrin-linked kinase (ILK) (19). It has been suggested that phosphorylation of the Rho effector may also be inhibited by an appropriate amount of MIBG (5). The same study also hypothesized that arginine-specific mono-ADP-ribosylation is involved in a Rho-dependent signaling pathway. In the present study, expression levels of FAK and RhoA decreased in the ART1-shRNA group, whereas they increased in the ART1=overexpression group. Therefore, the change in the levels of ART1 may exert an influence on the FAK and RhoA signaling pathways in colon carcinoma.

FAK is known as a regulator of cell migration. Schaller (20) demonstrated that enhanced FAK signaling may promote cell motility, whereas inhibited FAK signaling could suppress cell migration. Sieg *et al* (18) demonstrated that integrin β1-FAK is inactive in non-metastatic cancer cells, whereas it exhibits strong activity in metastatic cancer cells. Silencing integrin β1 could control the activity of FAK and further promote cell migration. The small G-protein, RhoA, also may mediate the RhoA/Rho-kinase (ROCK) and FAK signaling pathways, and have a marked effect on tumor cell migration (18,21). The present study demonstrated that expression levels of RhoA and FAK in spleen-transplanted CT26 tumors were decreased significantly due to ART1 gene silencing, and increased with ART1 overexpression in CT26 cells.

RhoA, a member of the Rho GTPase family, has been known to regulate the actin cytoskeleton in the formation of stress fibers (22), cytoskeletal dynamics, gene transcription, cell-cycle progression and cell transformation (23). ROCK is an important downstream effector of RhoA. It has been demonstrated that the RhoA/ROCK pathway serves an important role in various fundamental cellular functions, including proliferation (24). The c-myc proto-oncogene is an important regulator of cell proliferation, growth and differentiation (25). Kamaraju and Roberts (26) indicated that inhibition of Rho/ROCK activity is required for down-regulation of the expression levels of c-myc protein, and the subsequent suppression of the growth of breast cancer cells. The Rho-ROCK-c-myc cascade partly contributes to vascular endothelial growth factor induction by lysophosphatidic acid in ovarian cancer (27). C-myc silencing not only efficiently downregulates the expression of c-myc, but also inhibits the proliferation of HT-29 cells and suppresses the growth of colon cancer cells *in vivo* (28). Rho is also involved in the shear-stress induction of c-fos (29) and may stimulate the expression levels of c-fos (30). The inhibition of ROCK activity, and the subsequent disruption of actin filaments, may induce a decrease in c-fos activity (29). C-fos-siRNA attenuated the invasive ability of Lovo cells (31) and the growth of human colon carcinoma cells in athymic mice (32). C-fos, the dysregulation of which may lead to the development of cancer, is involved in important cellular events, including cell proliferation, differentiation and survival (33). RhoA may promote the expression of COX-2 via a mechanism dependent on the transcription factor, nuclear factor-κB (34). The inhibition of COX-2 may suppress the growth of HCA-7 and Moser-S colon cancer cells (35). Increased COX-2 activity has a positive effect on the progression of colorectal cancer (36). The present study demonstrated that the expression levels of RhoA and the downstream factors, c-myc, c-fos, and COX-2 proteins, were decreased significantly *in vivo* due to ART1 gene silencing, and increased with ART1 overexpression in CT26 cells. Thus, it has been demonstrated that the effect of ART1 on the proliferation of CT26 cells may be associated with RhoA and its downstream signal-transduction pathway.

Thus, ART1 serves a facilitatory role in the proliferation and migration of CT26 cells *in vivo*, and this effect may be associated with the factors downstream of FAK and RhoA, c-myc, c-fos, and COX-2. However, the underlying mechanisms require further investigation.

Acknowledgements

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