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Lentivirus-Mediated Silencing of *Tiam1* Gene Influences Multiple Functions of a Human Colorectal Cancer Cell Line[1]*

Li Liu, Qingling Zhang, Yanfei Zhang, Shuang Wang and Yanqing Ding

Department of Pathology, Nanfang Hospital, Southern Medical University, Guangzhou 510515, Guangdong Province, China

Abstract

T lymphoma invasion and metastasis 1 (*Tiam1*) is a metastasis-related gene of T lymphoma that is also involved in the metastasis of a variety of other cancers. In this study, we tested the hypothesis that *Tiam1* is a determinant of proliferation and metastasis in colorectal cancer, and we examined the effect of the inhibition of *Tiam1* expression on proliferation and metastasis. We succeeded in establishing the *Tiam1* knockdown colorectal cancer cell line using human immunodeficiency virus lentivirus-mediated RNA interference (RNAi) and found that the silencing of *Tiam1* resulted in the effective inhibition of *in vitro* cell growth and of the invasive ability of colorectal cancer cells. Using an orthotopic xenograft model in nude mice, we confirmed that *Tiam1* silencing could reduce tumor growth by subcutaneous injection and could suppress lung and liver metastases of colorectal cancer cells. Our results suggest that *Tiam1* truly plays a causal role in the metastasis of colorectal cancer and that RNAi-mediated silencing of *Tiam1* may provide an opportunity to develop a new treatment strategy for colorectal cancer.

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Keywords: *Tiam1* gene, RNA interference, colorectal cancer, proliferation, metastasis.

Introduction

Small guanosine triphosphate (GTP)–binding proteins of the Ras superfamily function as molecular switches in fundamental events such as signal transduction, cytoskeleton dynamics, and intracellular trafficking [1,2]. Mutations or aberrant regulation of these proteins can contribute to malignant phenotypes of human tumors. Guanine nucleotide exchange factors (GEFs), which catalyze the dissociation of guanosine diphosphate from inactive GTP-binding proteins, positively regulate these GTP-binding proteins in response to a variety of signals. Currently, many GEFs, including *Vav1*, *LARG*, *Bcr*, and T lymphoma invasion and metastasis 1 (*Tiam1*), have been identified as oncogenes [3–6].

Tiam1 was originally identified as an invasion-inducing and metastasis-inducing gene by proviral tagging, in combination with *in vitro* selection for invasiveness in T lymphoma cells [7]. The role of *Tiam1* in cellular migration, invasion, and metastasis may not be limited to T lymphoma. It has been reported to be important in promoting tumor progression in a variety of cancers, such as breast cancer, colorectal cancer, lung cancer, and Ras-induced skin tumors [8–11].

In our previous studies, to screen metastasis-associated genes, we prepared a cDNA microarray of tumor metastasis–associated genes and obtained 51 genes that were closely associated with metastases of colorectal cancer [12,13]. Mining microarray gene expression data by literature profiling, we found that *Tiam1* had potential relation to metastatic colorectal cancer [14]. To further investigate the effect of *Tiam1* on metastases of colorectal cancer, we detected *Tiam1* expression in cell lines and in different tissue specimens of colorectal cancer. We found that *Tiam1* expression was highly related to metastatic potential in colorectal cancer and that *Tiam1* was a metastasis-related gene [15]. However, it is unclear whether the knockdown of *Tiam1* is responsible for metastatic phenotypes in colorectal cancer, and its mechanism is unclear as well.

In this study, we established a stable *Tiam1* silencing colorectal cancer cell line with lentiviral vector–mediated RNA interference (RNAi) technology and determined whether *Tiam1* silencing could influence *in vitro* proliferation and invasion and *in vivo* tumor growth and metastasis.

Materials and Methods

Cell Line and Animals

The human colorectal cancer cell line SW480/EGFP was established from SW480 by transfection of pEGFP-N1 plasmid and was cultured in RPMI 1640 medium (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) and 100 U/ml penicillin/streptomycin in a 5% CO_2 humidified atmosphere at 37°C. The 293FT cell line, which stably expresses SV40 large T antigen and facilitates the optimal production

Abbreviations: *Tiam1*, T lymphoma invasion and metastasis 1; RNAi, RNA interference

Address all correspondence to: Dr. Yanqing Ding, Department of Pathology, Nanfang Hospital, Southern Medical University, Guangzhou 510515, Guangdong Province, China.
E-mail: yanqing.ding@gmail.com

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of viruses, was cultured in Dulbecco's modified Eagle's medium (Gibco).

Four- to 6-week-old female/male athymic BALB/c nu/nu mice were purchased from the Central Laboratory of Animal Science at Southern University (Guangzhou, China) and maintained in laminar-flow cabinets under specific pathogen-free conditions.

Preparation of Lentiviral Vectors

Firstly, we selected four different sequences for targeting the *Tiam1* gene using the BLOCK-It RNAi Designer (Invitrogen, Carlsbad, CA) (http://rnaidesigner.invitrogen.com/rnaiexpress/rnaiExpress.jsp) (Table 1). The preparation of lentiviral vectors expressing human *Tiam1* short hairpin RNA (shRNA) was performed using the BLOCK-It Lentiviral RNAi Expression System (catalog no. K4944-00; Invitrogen), following the manufacturer's instruction. In brief, four pLenti6/*Tiam1* expression vectors containing the human *Tiam1* shRNA-expressing cassette were constructed. Replication-incompetent lentivirus was produced by cotransfection of the pLenti6/*Tiam1* expression vector and ViraPower packaging mix (Invitrogen) containing an optimized mixture of three packaging plasmids: pLP1, pLP2, and pLP/VSVG) into 293FT cells. RNA; expression system includes ViraPower packaging mix. Viral supernatant was harvested 48 hours after transfection, filtered through a 0.45-μm cellulose acetate filter, and frozen at −70°C. The lentivirus containing the human Lamin A/C shRNA-expressing cassette (sequence 5′-CTGGACTTCCAGAAGAACA-3′) was used as a positive control for lentivirus production, and the lentivirus-only–containing pLenti6/U6 mock vector was used as negative control. Viral concentrations were determined by *in vitro* transduction and blasticidin selection.

Construction of Stable Silencing Lines

SW480/EGFP cells were transduced with specific or negative control lentiviral vectors and selected for stable integrants by culturing a complete medium containing blasticidin. After 12 days of selection, there were no viable cells in mock wells and discrete blasticidin resistance colonies.

Real-Time Reverse Transcription Polymerase Chain Reaction (PCR) Analysis for Tiam1

cDNA was synthesized by oligo dT primed reverse transcription from 2 μg of total RNA using an access reverse transcription system (Promega, Madison, WI). Real-time PCR was performed using Mx3000P Real-Time PCR System (Stratagene, La Jolla, CA) and Brilliant SYBR Green QPCR Master Mix kit (Stratagene), following the manufacturer's protocol. In brief, the reaction mixture (total volume, 25 μl) contained 500 ng of cDNA, the forward primer 5′-AAGACGTACTCAGGCCATGTCC-3′, and the reverse primer 5′-GACCCAAATGTCGCAGTCAG-3′ to amplify human *Tiam1* (GeneBank, NM_003253) at a final concentration of 250 nM and with 12.5 μl of 2× SYBR Green QPCR Master Mix kit. Thermal cycling conditions were as follows: 95°C for 5 minutes and 45 cycles at 95°C for 40 seconds, followed by 58°C for 40 seconds and 72°C for 40 seconds. Experiments were performed in triplicate in the same reaction. Human β-*actin* gene was amplified as internal control. Target genes and β-*actin* gene were amplified in the same reaction. Comparative quantification is determined using the $2^{-\Delta\Delta C_t}$ method [16].

Table 1. Targeted Sequences of *Tiam1* Gene for RNAi.

Site	Start	Target sequence
A	920	5′- GCATTCCTATACATCCAATGG-3′
B	2223	5′- GGTGAAATGCAGCTGTCTTCA-3′
C	3631	5′- GCACCTACGTGAAGGATTTAA-3′
D	4368	5′- GCTGTGGTCCTTGTGTATAAA-3′

Western Blot Analysis

Cells were washed twice with cold phosphate-buffered saline (PBS) and lysed on ice in RIPA buffer [1× PBS, 1% NP40, 0.1% sodium dodecyl sulfate (SDS), 5 mM EDTA, 0.5% sodium deoxycholate, and 1 mM sodium orthovanadate] with protease inhibitors. Protein lysates were resolved on 6% SDS polyacrylamide gel, electrotransferred to polyvinylidene fluoride membranes (Immobilon P; Millipore, Bedford, MA), and blocked in 5% nonfat dry milk in Tris-buffered saline, pH 7.5 (100 mM NaCl, 50 mM Tris, and 0.1% Tween-20). Membranes were immunoblotted overnight at 4°C with anti-*Tiam1* polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA), anti–Lamin A/C antibody (Chemicon, Temecula, CA), and anti–β-actin antibody (Santa Cruz Biotechnology), followed by their respective horseradish peroxidase–conjugated secondary antibodies. Signals were detected by enhanced chemiluminescence (Pierce, Rockford, IL).

In Vitro *Cell Growth Assay*

The cells were prepared at a concentration of 1×10^4 cells/ml, respectively. Aliquots (100 μl) were dispensed into 96-well microtiter plates. The cells were incubated for 1, 2, 3, 4, 5, 6, and 7 days, respectively, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed by adding 20 μl of MTT (5 mg/ml; Promega) for 4 hours. When MTT incubation had been completed, supernatants were removed. One hundred fifty milliliters of dimethyl sulfoxide (Sigma, St. Louis, MO) was added to each well. Fifteen minutes later, the absorbance value (OD) of each well was measured with a microplate reader set at 570 nm. All experiments were performed in triplicate.

Plate Clone Formation Assay

About 1×10^2 cells were added to each well (3 cm in diameter) of a six-well culture plate, and each cell group contained three wells. After incubation at 37°C for 12 days, the cells were washed twice with PBS and stained with Giemsa solution. The number of colonies containing ≥ 50 cells was counted under a microscope [plate clone formation efficiency = (number of colonies / number of cells inoculated) × 100%].

Flow Cytometry Analysis for Cell Cycle

Cells were harvested at an exponential growth phase, and single-cell suspensions containing 1×10^6 cells were fixed with 70% alcohol. Cell cycle was monitored using propidium

iodide (PI) staining of nuclei. The fluorescence of DNA-bound PI in cells was measured with a FACScan flow cytometer (BD Biosciences), and the results were analyzed with ModFit 3.0 software (Verity Software House, Topsham, ME).

In Vitro *Invasion Assay*

This assay was performed using the method of Albini et al. [17], with modifications. The cell invasion chamber (Chemicon) contains a polycarbonate membrane with an 8-μm pore size, over which a thin layer of ECMatrix (Chemicon) is dried. The extracellular matrix (ECM) layer occludes membrane pores, blocking noninvasive cells from migrating. To the top chamber was added warm serum-free medium to rehydrate the ECM layer for 2 hours at room temperature. Tumor cells in a serum-free medium (300 μl containing 1×10^5 cells) were added to the top chamber. The bottom chamber was prepared using 10% FBS as chemoattactant. After 24 hours of incubation, noninvasive cells were removed with a cotton swab. The cells that had migrated through the membrane and had stuck to the lower surface of the membrane were fixed with methanol and stained with hematoxylin. For quantification, the cells were counted under a microscope in five predetermined fields (original magnification, ×200).

In Vivo *Tumor Growth Assay*

To evaluate *in vivo* tumor growth, 1×10^7 *Tiam1* silencing cells and mock virus-transduced cells were each injected subcutaneously into the left flank and right flank of six nude mice. After subcutaneous injection of cells, fluorescence emitted by cells was collected and imaged through a whole-body green fluorescence protein (GFP) imaging system (Lighttools, Encinitas, CA). Using IPP5.0 software (Cybermetics Co., Silver Spring, MD), images were analyzed such that whole-body optical images visualized real-time tumor growth and tumor area was calculated.

In Vivo *Metastasis Assays*

The cells were first harvested by trypsinization, washed thrice with cold serum-free medium, and resuspended with serum-free medium. Nude mice were injected subcutaneously with 1×10^7 cells. After 10 days, the tumors were extirpated and washed twice with antibiotic-containing RPMI 1640 medium. Cancerous tissue was divided into small pieces, approximately 1 mm in diameter. Nude mice were anesthetized with Nembutal (Sinopharm Chemical Reagent Co., Shanghai, China) and sterilized with alcohol. For colon surgical orthotopic implantation (SOI) using the method described previously [18], a small midline incision was made, and the colocecal part of the intestine was exteriorized. A small area of the colonal serosa was removed, and a 1-mm^3 tumor fragment per mouse was implanted. A 7-0 nylon surgical suture was used to penetrate tumor pieces and to suture them to the intestine wall. The intestine was returned to the abdominal cavity, and the abdominal wall was closed with a 6-0 silk surgical suture. Whole-body optical images visualized real-time primary tumor growth and the formation of metastatic lesions. Two months later, all mice were killed, individual organs were excised, and metastases were checked by hematoxylin–eosin (H&E) staining.

Statistical Analysis

SPSS 12.0 software (Abbott Laboratories, North Chicago, IL) was used for statistical analysis. Assay differences between *in vitro* cell growth and *in vivo* tumor growth were tested for statistical significance using analysis of variance (ANOVA) for factorial design. Plate clone formation assay and *in vitro* invasion assay were tested using one-way ANOVA. Differences were considered statistically significant when $P < .05$.

Results

Establishment of Tiam1 *Silencing Colorectal Cancer Cell Line*

To deliver siRNA into the SW480/EGFP colorectal cancer cell line, we developed four lentiviral-based vectors that express different shRNA. After the cotransfection of pLenti6/*Tiam1* vector and ViraPower packaging mix, replication-incompetent lentivirus was released into the medium and collected 48 hours after transfection. Viral stocks were tittered on SW480/EGFP cells, and 7×10^5 transducing units (TU)/ml was obtained (Figure 1).

SW480/EGFP cells were transduced with pLenti6/U6, pLenti6/*Tiam1*, and pLenti6/Lamin A/C viral stocks and were selected by blasticidin 48 hours after transduction. Ten days posttransduction, cells were analyzed for Lamin A/C and *Tiam1* expression using real-time PCR and Western blot analysis. As shown in Figure 2, site B lentiviral vector was most effective at blocking *Tiam1* expression. There was no difference in cell morphology. Subsequently, we transduced pLenti6/*Tiam1* (site B) and pLenti6/U6 lentiviruses into SW480/EGFP cells to select blasticidin-resistant single clones. Transduced clones were expanded and examined by real-time PCR and Western blot analysis (Figure 3). Results showed that *Tiam1* knockdown clone 2 (*Tiam1*KD/clone 2), knockdown clone 8 (*Tiam1*KD/clone 8), and knockdown clone 6 (*Tiam1*KD/clone 6) exhibited 88%, 80%, and

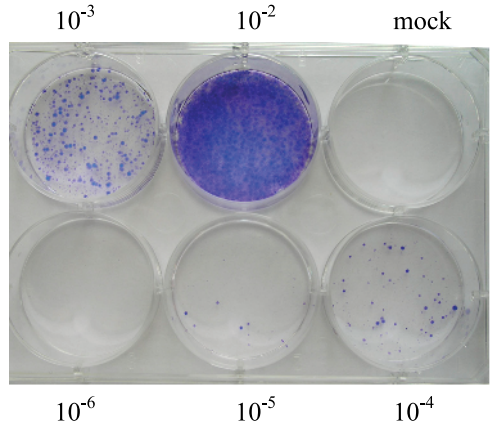

Figure 1. *Titering lentiviral stock. Cells were either transduced with 10-fold serial dilutions of lentiviral supernatant (10^{-2}–10^{-6} dilutions) or untransduced (Mock cells). Forty-eight hours posttransduction, the cells were placed under blasticidin selection. After 14 days of selection, the cells were stained with crystal violet, and colonies were counted. The virus titer was 7×10^5 TU/ml.*

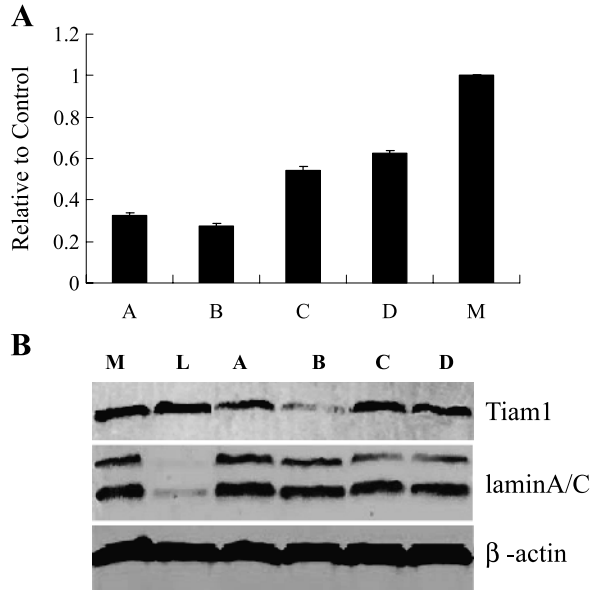

Figure 2. *Screening of the most effective targeting site for the* Tiam1 *gene by real-time PCR and Western blot analysis. (A) Quantification of* Tiam1 *mRNA expression in cells of different interference sites relative to controls (M, pLenti6/U6 Mock virus-transduced cells), as detected by real-time PCR. (B) Tiam1 protein expression in cells of different interference sites, as detected by Western blot analysis. Lane L shows that a greater degree of Lamin A/C knockdown was observed after transduction with a lentivirus containing the human Lamin A/C shRNA-expressing cassette. Lane B shows that lentiviral vectors were effective and that site B was the most effective construct.*

66% reduction in Tiam1 protein, respectively. The clone transduced by pLenti6/U6 lentivirus exhibited no change in *Tiam1* expression. For the sake of convenience, the SW480/EGFP cells and the clone transduced by pLenti6/U6 lentivirus are termed WT and Mock cells.

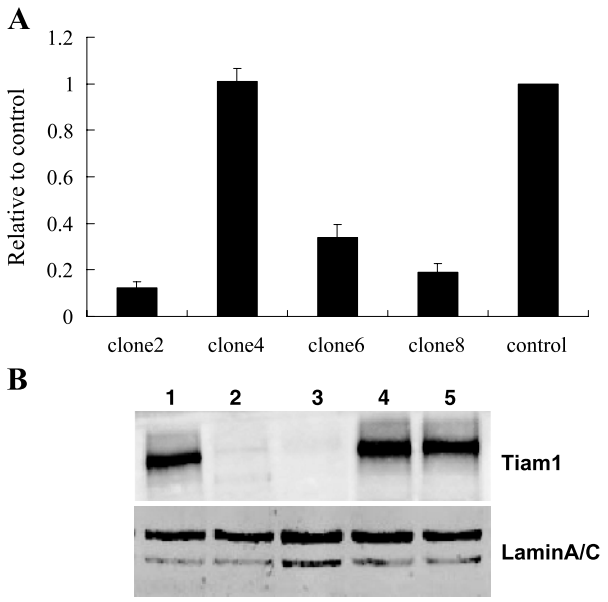

Figure 3. *Confirmation of* Tiam1 *expression in different clones by real-time PCR and Western blot analysis. (A) Quantification of* Tiam1 *mRNA expression in clones transduced by the pLenti6/*Tiam1 *(site B) lentivirus relative to controls (clone transduced by pLenti6/U6 lentivirus). (B) Western blot analysis shows marked reduction of Tiam1 protein expression in clones 2 and 8. Lane 1, SW480/EGFP cells; lane 2, pLenti6/*Tiam1 *lentivirus–transduced clone 2; lane 3, pLenti6/*Tiam1 *lentivirus–transduced clone 8; lane 4, pLenti6/*Tiam1 *lentivirus–transduced clone 4; lane 5, pLenti6/U6 lentivirus–transduced clone.*

Tiam1 *Gene Silencing Suppresses Cell Proliferation* In Vitro

The effect of Tiam1 protein reduction on the proliferation of colorectal cancer cells was determined by MTT assay and plate clone formation assay. As shown in Figure 4*A*, although Mock cells showed *in vitro* growth ability approximating that of WT cells, *Tiam1*KD/clone 6 had reduced growth ability with respect to Mock cells, *Tiam1*KD/clone 8 had reduced growth ability with respect to *Tiam1*KD/clone 6, and *Tiam1*KD/clone 2 had reduced growth ability with respect to *Tiam1*KD/clone 8. Therefore, *in vitro* cell growth ability correlates with *Tiam1* expression.

The ability of cells to form colonies in plates was examined because there exists a correlation between clonogenicity and metastatic propensity. Figure 4*B* shows that *Tiam1* knockdown cells, compared with WT and Mock cells, had a significant reduction in their ability to form colonies, and their ability to form colonies correlates with *Tiam1* expression.

Flow Cytometry Analysis for Cell Cycle

In Figure 5, cell cycle analysis by flow cytometry reveals that the proportion of cells in the G_0/G_1, S, and G_2/M phases

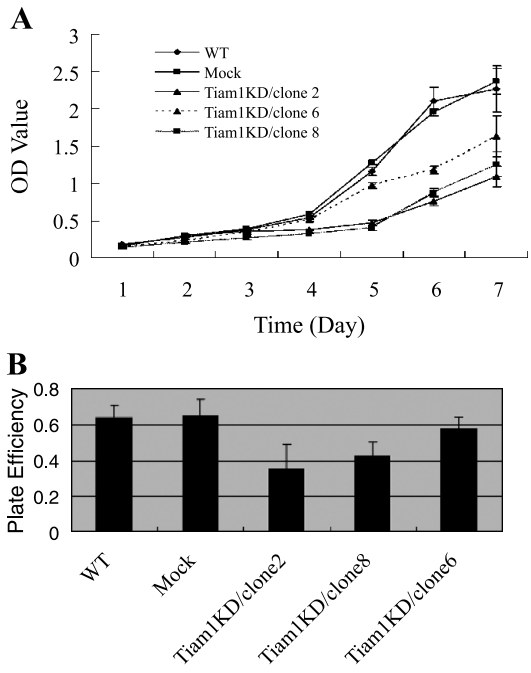

Figure 4. Tiam1 *gene silencing suppresses cell proliferation* in vitro. *(A) The* in vitro *proliferative abilities of WT cells, Mock cells,* Tiam1*KD/clone 2,* Tiam1*KD/clone 6, and* Tiam1*KD/clone 8 cells were evaluated by MTT assay. Each value represents the mean ± SD of the absorbance value (OD). Results showed that all* Tiam1 *knockdown cells grew significantly more slowly than WT and Mock cells, and Tiam1 protein expression correlated with cell proliferation. (B) The plate colony formation efficiency of WT cells, Mock cells,* Tiam1*KD/clone 2,* Tiam1*KD/clone 6, and* Tiam1*KD/clone 8 cells. Data represent the mean ± SD of triplicate dishes. Compared with WT and Mock cells,* Tiam1*KD/clone 2 had significantly reduced ability for colony formation ($P < .01$), and* Tiam1*KD/clone 8 also showed decreased clonogenicity ($P < .05$).* Tiam1*KD/clone 6, compared with WT and Mock cells, displayed no difference in clonogenicity ($P > .05$). Results showed that plate colony formation efficiency also correlated with Tiam1 protein expression.*

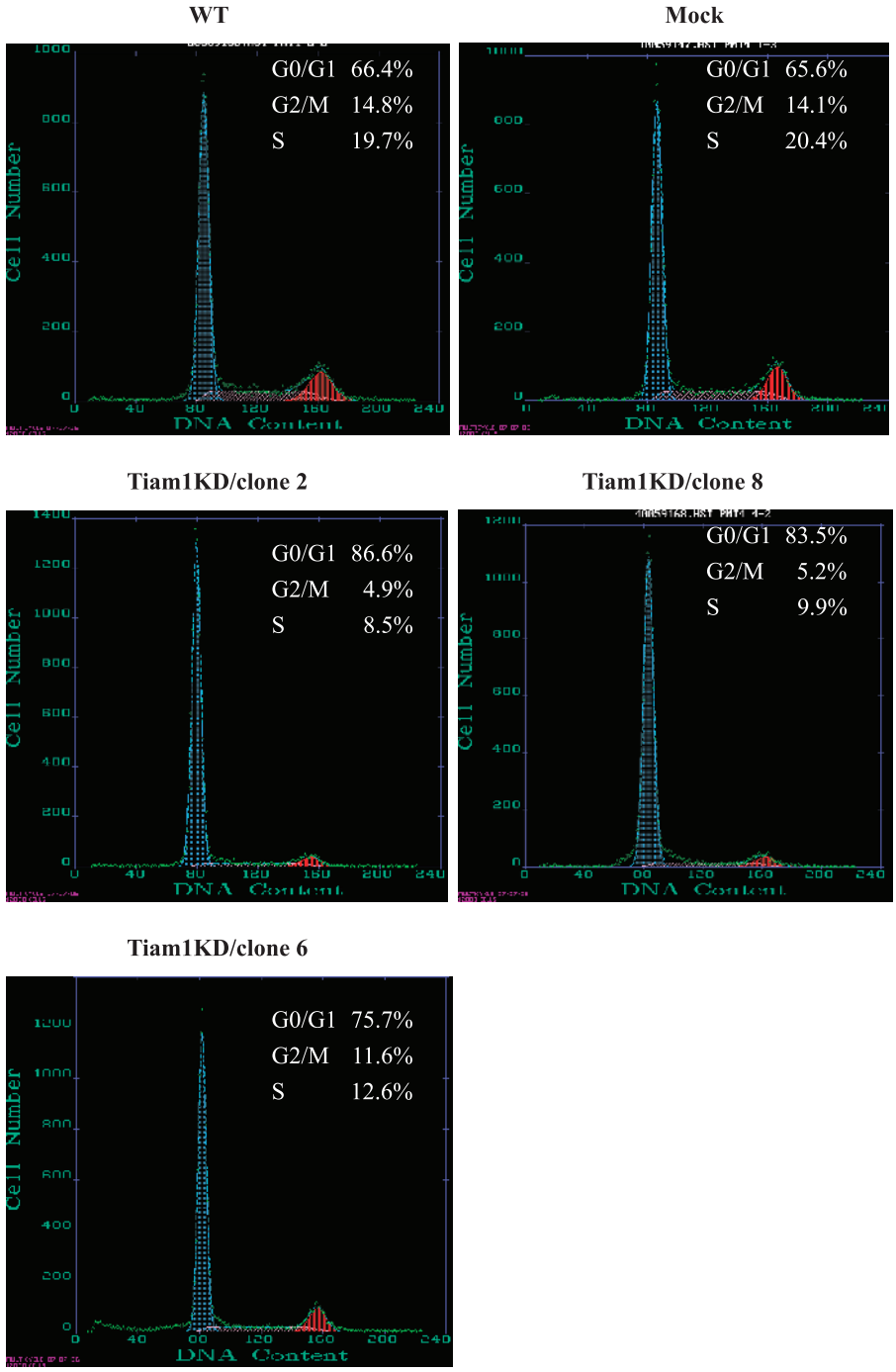

Figure 5. *Effect of* Tiam1 *knockdown on the cell cycle detected by flow cytometry analysis.* Tiam1 *silencing cells showed* G_0/G_1 *phase arrest and* G_2/M *and S phase reduction. The difference was statistically significant.*

for WT cells, Mock cells, *Tiam1*KD/clone 2, *Tiam1*KD/clone 6, and *Tiam1*KD/clone 8 cells were significantly different. The proportion of G_0 to G_1 phase was highest in *Tiam1*KD/clone 2, whereas the proportion of S phase was lowest in *Tiam1*KD/clone 2.

Tiam1 *Gene Silencing Suppresses Cell Invasion* In Vitro

Invasion through the ECM is an important step in tumor metastasis. ECMatrix serves as a reconstituted basement membrane matrix of proteins. The number of cells migrating to ECMatrix was counted. *Tiam1* knockdown cells, compared with WT and Mock cells, display a remarkable decrease in invasiveness ($P < .01$). As shown in Figure 6, *Tiam1*KD/clone 2, *Tiam1*KD/clone 8, and *Tiam1*KD/clone 6 displayed a 1.72-fold, 1.44-fold, and 1.20-fold decrease in invasive ability, respectively, compared with WT cells. These results demonstrate that the invasive ability of colorectal cancer cells correlated with *Tiam1* expression, and *Tiam1* silencing alone is sufficient to attenuate invasion in colorectal cancer cells.

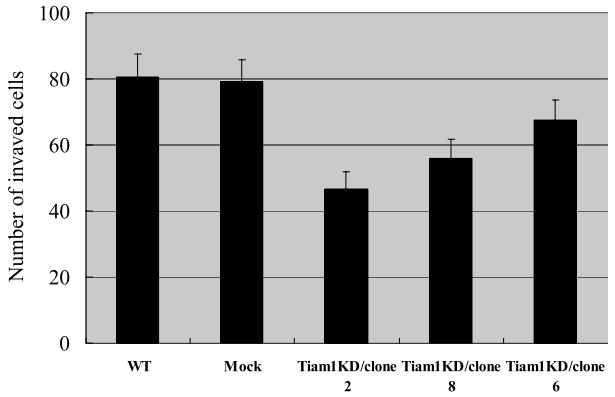

Figure 6. *Effect of* Tiam1 *knockdown on the invasive potential of colorectal cancer cells.* In vitro *invasion assay was carried out to compare and quantify the invasiveness of WT cells, Mock cells,* Tiam1*KD/clone 2,* Tiam1*KD/clone 6, and* Tiam1*KD/clone 8. Results are representative of three independent experiments, and bars represent the mean ± SD.* Tiam1 *silencing cells showed decreased invasion that correlated with Tiam1 protein expression.*

Tiam1 *Gene Silencing Suppresses Cell Proliferation* In Vivo

The effect of *Tiam1* on *in vivo* tumor growth was assessed by the subcutaneous injection of *Tiam1*KD/clone 2 and Mock cells for 30 days. External fluorescent images can provide invaluable real-time data for tracking tumor growth. As shown in Figure 7, compared with that of Mock cells, the knockdown of *Tiam1* expression progressed from a pronounced decrease in *Tiam1*KD/clone 2 cell growth by day 15 to a 1.3-fold decrease in tumor area by day 30 after cell injection.

Tiam1 *Silencing Reduces Metastatic Tumor Formation*

To unambiguously elucidate the effect of *Tiam1* on colorectal cancer metastasis, we performed *in vivo* metastasis assay by SOI. Mice were sacrificed 2 months later because the mice of the Mock group were moribund. Autopsy was performed, and the incidence of metastasis in the liver, lungs, and other organs was determined by macroscopic and histologic examinations (Figure 8). In the Mock group, 75% (six of eight) of mice developed peritoneal metastasis, which appeared as numerous bright green fluorescence on the peritoneum and on abdominal organs under a GFP imaging system. A comparison of external fluorescent images was made, and the results showed a direct image of mice and a external fluorescent image closely matching the direct image. In addition, the *Tiam1*KD/clone 2 in the orthotopical cecum of mice seldom expressed Tiam1 protein, whereas Mock cells in the orthotopical cecum of mouse stained positive for *Tiam1* (Figure W1). In the *Tiam1*KD/clone 2 group, only 37.5% (three of eight) of animals had peritoneal metastasis. The incidence of hepatic metastasis and lung lesions in mice of the Mock group was 37.5% (three of eight) and 12.5% (one of eight), respectively, and metastatic lesions were validated by pathological methods. The *Tiam1*KD/clone 2 group did not produce detectable tumors in the liver and other organs.

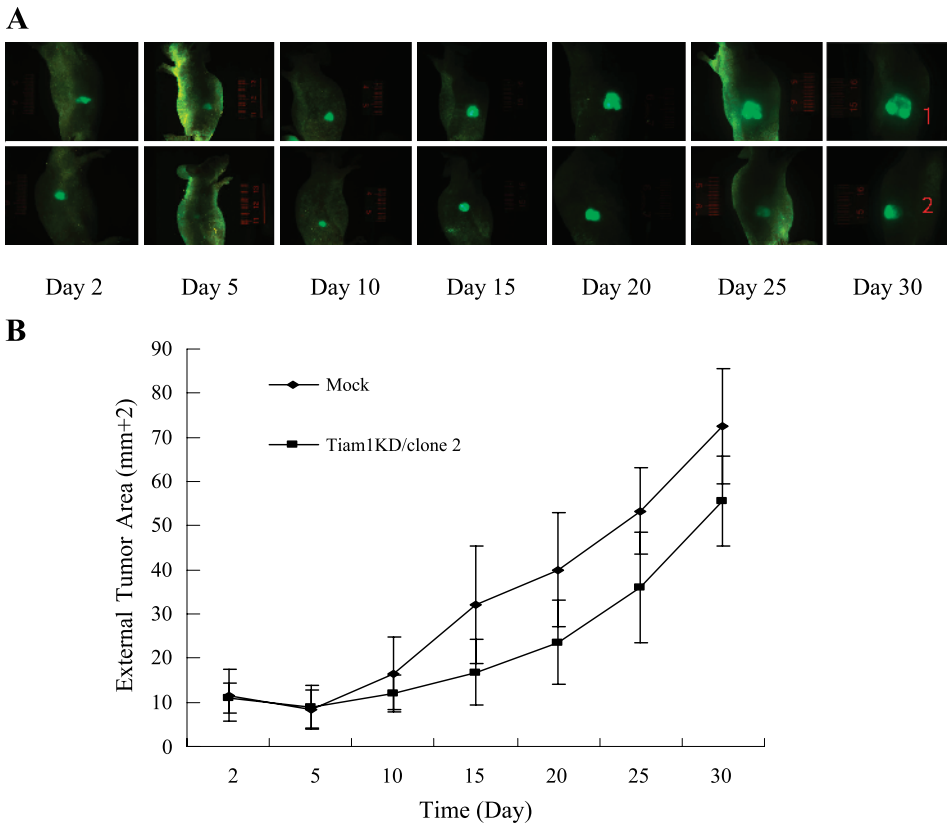

Figure 7. Tiam1 *gene silencing suppresses cell proliferation* in vivo. *(A) Consecutive external whole-body fluorescence images of Mock cells and* Tiam1*KD/clone 2 tumors were obtained from days 2 to 30 after subcutaneous injection into nude mice. (B) Tumor areas were calculated by IPP5.0 software and were indicated as the mean ± SD of six mice. Compared with Mock cells,* Tiam1*KD/clone 2 had a significantly reduced* in vivo *proliferative ability (F = 2.256, P < .05).*

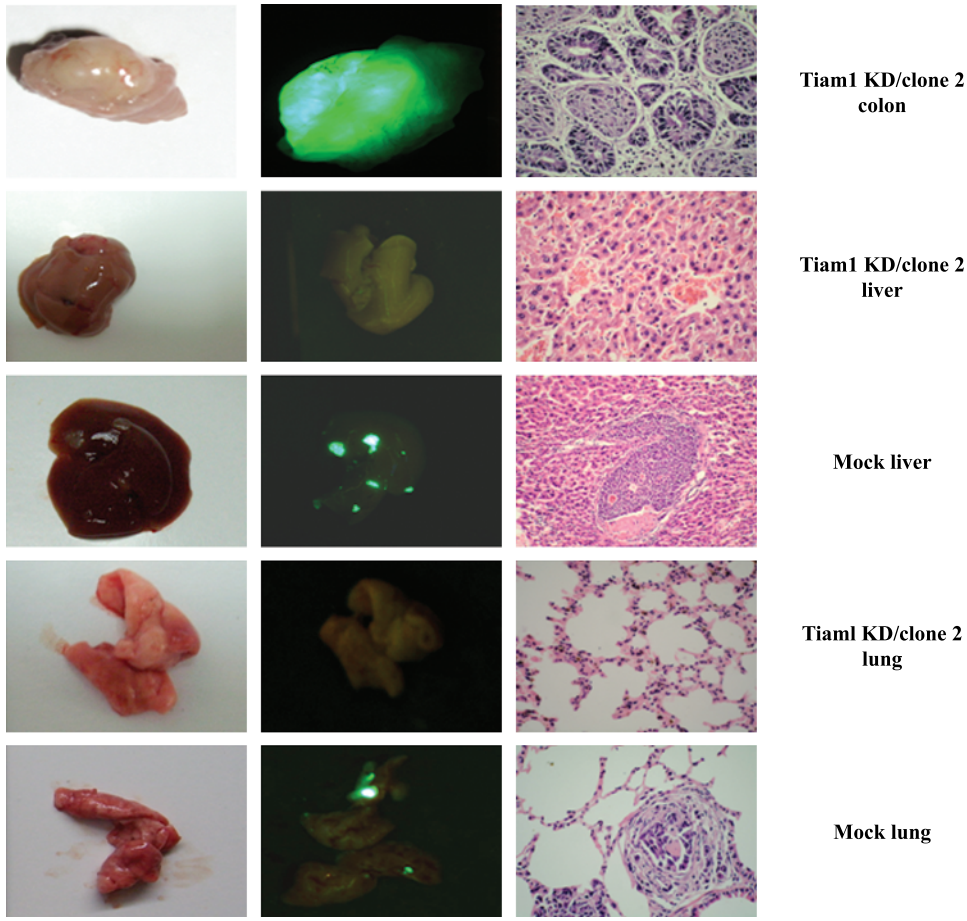

Figure 8. Tiam1 *silencing reduces metastatic lesions* in vivo. *We assessed the effect of* Tiam1 *silencing on metastasis using an orthotopic xenograft model in nude mice. After 2 months, the colon, lungs, and liver of mice were resected and analyzed for metastasis. Left panel: Direct image of colon, lungs, and liver. Middle panel: External fluorescent image of colon, lungs, and liver. Right panel: Histologic photomicrographs of colon, lung, and liver tissue sections stained with H&E (original magnification, ×400).*

These results indicate that *Tiam1* silencing was sufficient to decrease metastasis in colorectal cancer cells.

Discussion

RNAi describes the phenomenon by which double-stranded RNA induces potent and specific inhibition of eukaryotic gene expression through the degradation of complementary messenger RNA (mRNA) and is functionally similar to the processes of posttranscriptional gene silencing. In the past few years, siRNA and shRNA have been widely used by researchers to silence the expression of many target genes because of their high specificity and apparent nontoxicity [19,20]. Furthermore, systems based on lentiviral vectors have provided new solutions to achieving stable shRNA-mediated knockdown [21,22].

In the present study, we used a lentivirus-mediated RNAi method to obtain an efficient knockdown of *Tiam1* gene. Overexpression of *Tiam1* seems to play a critical role in many human cancers. Recent results from other laboratories [9] and ours have demonstrated that increased *Tiam1* expression correlates with the metastatic potential of human colorectal cancer and that *Tiam1* was upregulated in the intestines of adenomatous polyposis coli (APC) conditional mutant mice [23]. In $Tiam1^{-/-}$ mice, susceptibility to the development of Ras-induced skin tumors, following application of a chemical carcinogenesis protocol, was significantly reduced [11]. This suggests that *Tiam1* is a significant modulator of tumor development and metastasis, thus a potentially interesting therapeutic target.

In this study, we found that *Tiam1* was one of the colorectal cancer genes associated with proliferation. The proliferation ability of tumor cells always correlates with metastatic phenotype and poor outcome [24]. We found that knockdown of *Tiam1* expression strongly inhibited *in vitro* cell growth and colony formation efficiency. Moreover, through whole-body optical imaging that enables a continuous visual monitoring of malignant growth within intact animals, we found that silencing of *Tiam1* expression appeared to have an inverse correlation with tumorigenicity. All data indicated that *Tiam1* may be a positive regulator of tumor growth in colorectal cancer.

In agreement with Minard [9], our experiments demonstrated that knockdown of *Tiam1* expression in colorectal cancer cells reduced their ability to invade ECMatrix-coated membranes in an invasion chamber assay. These results support the involvement of *Tiam1* with the malignant behavior of human colorectal cancer cells and underline the relevance of a stable lentivirus-based *Tiam1* knockdown

mediated by RNAi in the impairment of the invasive ability of colorectal cancer.

Tiam1 has been extensively studied for the exchange factor's role in cellular migration, adhesion, and invasion. However, limited information on the promotion of *in vivo* metastasis is available. Our results demonstrated clearly that downregulated *Tiam1* expression in colorectal cancer cells significantly suppressed the metastatic potential in *Tiam1* silencing cells. We checked *Tiam1* expression levels in *Tiam1* knockdown clone before each experiment to ensure that *Tiam1* expression was consistently of low level. We used an orthotopic xenograft model to evaluate the effect of *Tiam1* silencing on metastasis because the models developed with SOI can exhibit patient-like metastasis. The reduced expression of *Tiam1* in SW480/EGFP cells abrogated their ability to develop lung and hepatic metastases.

Taken together, our results indicate that the silencing of *Tiam1* expression by RNAi suppressed the proliferation of colorectal cancer both *in vitro* and *in vivo*. This is the first study to demonstrate that targeting *Tiam1 in vivo* reduced the metastatic potential of colorectal cancer. *Tiam1* is an important determinant of malignant cellular behavior and is a promising target for therapeutic intervention.

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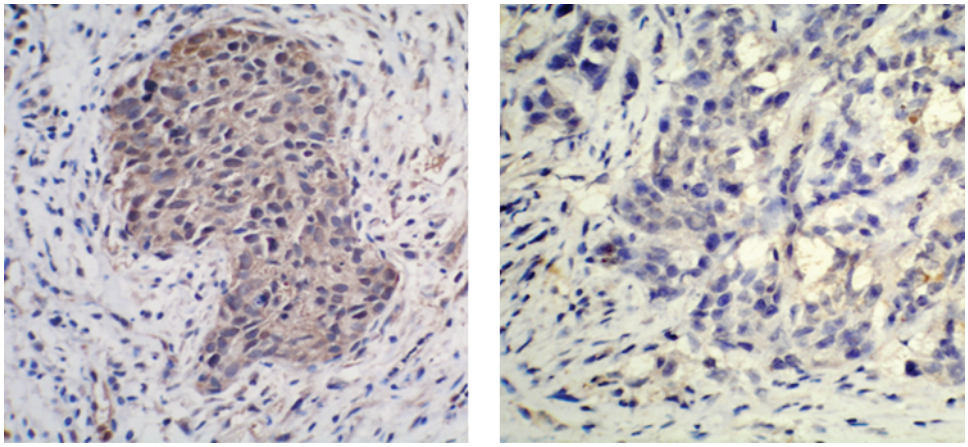

Figure W1. *Tiam1 expression in the orthotopical cecum of mouse, by immunohistochemical method. If positive signals were present, the cytoplasm stained brown. For negative controls, the antibody was replaced by PBS. The results showed that Tiam1 was positive in Mock tumor (A) and was weak positive in Tiam1KD/ clone 2 tumor (B).*