

APRIL Induces Tumorigenesis and Metastasis of Colorectal Cancer Cells via Activation of the PI3K/Akt Pathway

Guihua Wang¹, Feng Wang¹, Weifeng Ding¹, Jingchun Wang, Rongrong Jing, Haiquan Li, Xudong Wang, Yueguo Wang, Shaoqing Ju, Huimin Wang*

Department of Clinical Laboratory Center, Affiliated Hospital of Nantong University; School of Public Health, Nantong University, Nantong, Jiangsu, People's Republic of China

Abstract

A proliferation-inducing ligand (APRIL) is highly expressed in colorectal cancer (CRC) tissues and cell lines. However, the biological functions and precise signals elicited by APRIL in CRC have not been fully understood. Here, we used small interfering RNA to selectively deplete APRIL and to determine its tumorigenic effects in a CRC cell line SW480 both *in vitro* and *in vivo*. Knockdown of APRIL in SW480 cells was associated with modulation of cell proliferation as well as reduction of cell migration and invasion *in vitro*. Moreover APRIL-knockdown SW480 cells displayed markedly inhibited tumor growth and decreased metastasis to the liver in immunodeficient mice upon subcutaneous injection. Importantly, we observed that downregulation of APRIL in SW480 cells resulted in greatly decreased activity of phosphoinositide 3-kinase (PI3K)/Akt pathway. In addition, we observed that recombinant human APRIL mediated activation of the PI3K/Akt pathway in CRC cells resulting in induced expression of important cell cycle proteins and matrix metalloproteinases in a PI3K/Akt dependent manner. This was concurrent with marked cell growth viability as well as increased cell migration and invasion. Together, these compelling data suggest that APRIL-induced tumorigenesis and metastasis of CRC cells may be accomplished through activation of the PI3K/Akt pathway. These findings may lead to a better understanding of the biological effects of APRIL and may provide clues for identifying novel therapeutic and preventive molecular markers for CRC.

Citation: Wang G, Wang F, Ding W, Wang J, Jing R, et al. (2013) APRIL Induces Tumorigenesis and Metastasis of Colorectal Cancer Cells via Activation of the PI3K/Akt Pathway. PLoS ONE 8(1): e55298. doi:10.1371/journal.pone.0055298

Editor: Claudio M. Costa-Neto, University of São Paulo, Brazil

Received: September 19, 2012; **Accepted:** December 20, 2012; **Published:** January 29, 2013

Copyright: © 2013 Wang et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This work was supported by the National Natural Science Foundation of China (numbers 81201351 and 81201350). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: onehuimin@gmail.com

† These authors contributed equally to this work.

Introduction

Colorectal cancer (CRC) is a major public health problem in the United States and globally. In the United States, it is the third most common cancer and the third leading cause of cancer mortality if men and women are considered separately [1]. Occurrence of metastases due to tumor progression is the cause of the majority of cancer-related deaths. Despite much progress in diagnostic and therapeutic methodology, the prognosis for CRC patients remains poor. Recently, advances in molecular biology have led to an increased knowledge of the mechanisms underlying CRC development. Recent large-scale sequencing and other genomic analyses of human colorectal tumors indicate that there are up to 80 nonsilent mutations in each tumor and that individual tumors have different mutations. Although the mutations are many and varied, classification of common and rare mutations revealed that 38 pathways are disrupted with particular frequency, and many of these pathways intersect with phosphoinositide 3-kinase (PI3K) signaling [2]. The PI3K signaling pathway plays an essential role in cancer cell proliferation, survival, metabolism, motility and invasion. It is frequently deregulated in CRC cells, making it an attractive therapeutic target [2,3,4]. Upregulated

cytokines, growth factors, and their receptors observed in CRC cells via PI3K probably represent a principal contribution to tumorigenesis.

APRIL (a proliferation-inducing ligand, also known as TRDL-1, TALL-2, and TNFSF13), is a member of the tumor necrosis factor (TNF) superfamily, with homologous structure and function to several other cytokines in this family. It was discovered as a cytokine over-expressed by transformed cells and could stimulate cellular proliferation [5,6]. APRIL is predominantly expressed by tumor tissues such as colon carcinoma, pancreatic cancer, gastric cancer, and by normal immune cells such as B cells, monocytes, macrophages, dendritic cells, neutrophils, epithelial cells, T-cells and by some non-immune cells such as osteoclasts. Recent studies have analyzed the tumor-promoting role of APRIL in patients with hematological malignancies or with solid tumors. Aberrant expression of APRIL and the subsequent activation of pro-survival pathways may allow the survival of several hematological malignancies [6,7,8,9]. In solid tumor cells, studies have been restricted to the proliferation or survival activity implicated in the natural growth of tumors, which is consistent with the current concept that inflammatory reactions constitute an important part of tumor development [5,10,11]. There are different opinions

about the secretion of APRIL produced by tumor cells or by tumor-infiltrating cells. Mhawech-Fauceglia et al [10] demonstrated that tumor-infiltrating neutrophils present in the stroma rather than the tumor cells, were the major source of APRIL. This is in contrast to a recent study by V Lascano et al [12], who demonstrated that APRIL produced by both tumor and non-tumor cells had a clear tumor-promoting effect in colorectal tumorigenesis. However, downstream signaling cascades activated by APRIL that may be involved in tumor formation have yet to be characterized. We recently confirmed that APRIL is overexpressed in digestive system carcinomas, specifically in the colon and pancreas [13,14,15]. APRIL is more highly expressed in tumors than in normal colon, and APRIL-knockdown inhibited the migration and invasion of colorectal cancer cells *in vitro* [16]. Furthermore, serum APRIL levels measured by ELISA in patients with pancreatic cancer suggested that APRIL has a positive diagnosis and prognosis value for pancreatic cancer [15]. These preliminary studies indicate that APRIL may have an important role in carcinogenesis and tumor progression.

The present study investigated the role of APRIL in colorectal carcinogenesis and characterized APRIL-mediated signal transduction. Human CRC cell lines and immunodeficient mice were used to evaluate the effects of knockdown of APRIL on key features of the carcinogenic and metastatic process. We assessed the role of APRIL in cell proliferation, cell cycle, migration and invasion in CRC cell lines and identified the related molecular and cellular components. Using these CRC cells and xenograft model tissues, we suggest that APRIL could be related to tumorigenesis and metastasis. Moreover, we observed that APRIL stimulation mediated PI3K/Akt pathway signals involved in the regulation of cell cycle and invasion of carcinoma cells.

Materials and Methods

Ethics statement

The study protocols were approved by the Animal Care and Research Ethical Committee, College of Medicine, Nantong University. All animal work was conducted according to relevant guidelines (Contract 2010-0089). All the patients gave their written informed consent to participate in the study.

Patients and tissue specimens

CRC tissues were collected from surgical resection specimens of eight patients who had not undergone radiotherapy or chemotherapy in the Affiliated Hospital of Nantong University between April 2008 and May 2009. Their ages ranged from 45 to 71 years, with a mean age of 56.9 years. The male:female ratio was 5:3. The diagnosis was confirmed histologically in all cases. Tissue specimens were immediately processed after surgical removal. Protein was analyzed in eight snap-frozen tumorous and adjacent nontumorous tissue samples that were stored at -80°C .

Cell culture and reagents

All human CRC cell lines (SW480, HT-29, Colo-205, HCT-116) were obtained from the Academy of Life Science, Shanghai, China and were maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, San Diego, CA) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, USA) and antibiotics (100 mg/ml streptomycin and 100 U/ml penicillin) at 37°C in a humidified incubator containing 5% CO_2 . Human umbilical vein endothelial cells (HUVEC) were obtained from Fngbio, Shanghai, China and were maintained in DMEM/F12 (Gibco). GM6001, LY294002 were purchased from Chemicon (Temecula, CA, USA). Rapamycin was obtained from Sigma.

Recombinant human APRIL (rhAPRIL) was purchased from PeproTech Inc. (USA).

Enzyme-linked immunosorbent assay (ELISA) for detection of secreted APRIL

CRC cell lines were cultured at 37°C in 5% CO_2 in DMEM with 0.5% FBS for 72 h. Cell culture supernatants were collected and the concentration of APRIL was measured using a human APRIL ELISA kit (R&D, MN, USA), according to the manufacturer's instructions. The concentration of APRIL was calibrated from a dose response curve based on reference standards. The experiment was repeated three times.

Transfection and generation of stable cell lines

Knockdown SW480 cells were generated using short hairpin RNA directed against human APRIL gene (shAPRIL) constructed in pGCsi/H1/Neo/GFP plasmid vector (Genechem, Shanghai, China). Two pairs of chemically-synthesized shAPRIL were denoted as sh637 and sh1750 [17]. A plasmid carrying a nontargeting control (NTC) sequence was used as a shRNA control (shNTC). Transient transfections were performed as previously described [14]. At 48 h post-transfection, cells were harvested and subjected to proliferation assay, cell cycle analysis, invasion and migration assays and APRIL mRNA levels were determined by reverse-transcription (RT)-PCR. For stable transfections, the recombinant vectors were transfected with lipofectamine 2000 (Invitrogen) and stable transfectants were selected with 600 $\mu\text{g}/\text{ml}$ G418. G418-resistant transfectants were cloned to establish cell lines and analyzed by RT-PCR for APRIL mRNA expression. Stable transfected SW480 cells were prepared for animal experiments.

Semiquantitative RT-PCR

Total RNA isolation from cultured cells at 80% confluency using TRIzol (Invitrogen), generation of cDNA using SuperScriptTMIII (Fermentas, USA) according to standard protocols, and subsequent PCR was carried out with the following primer pairs (5'-3'): APRIL, ACTCTCAGTTGCCCTCTGGTTG and GGAACCTCTGCTCCGGGAGACTC; MMP-2, GATGCCGCCTTTAACTGG and TCAGCAGCCTAGC-CAGTCC; MMP-9, GCACCACCACAACATCACCTAT and CACAACCTCGTCATCGTCGAAA; TIMP-1, CAGAAGT-CAACCAGACCACCT and ATTCTCACAGCCAACAGTG; GAPDH, ACGCATTTGGTTCGTATTGGG and TGATTTTG-GAGGGATCTCGC. GAPDH was screened as the control housekeeping gene. The PCR products were run on a 1.5% agarose gel, stained with ethidium bromide and visualized by UV illumination. Bands were quantified by densitometry using ImageJ software (<http://rsb.info.nih.gov/ij/>).

Cell proliferation assay

Cell proliferation assays *in vitro* were performed using a Cell Counting Kit-8 (CCK-8; Donjindo, Japan) according to the manufacturer's instructions. shAPRIL SW480 cells, shNTC SW480 cells and nontransfected SW480 cells were seeded in 96-well plates in DMEM with 0.5% FBS at a density of 4×10^3 cells/well. APRIL-stimulated HCT-116 cells were seeded in 96-well plates in DMEM that contained 0.5% FBS in the presence of 100 ng/ml rhAPRIL, 400 ng/ml rhAPRIL, 800 ng/ml rhAPRIL or PBS (control group). Cells in each group were plated in triplicate. At 24, 48, 72 and 96 h, the optical density at 450 nm wavelength which correlates to the number of viable cells was

measured and the results were expressed as mean of A450 \pm SEM.

Cell cycle analysis

For cell cycle analysis, APRIL-stimulated cells were cultured with rhAPRIL for 24 h, and shAPRIL cells were harvested 48 h after transfection. Cells were then washed with PBS, fixed in 70% ethanol for an hour at 4°C and then incubated with 1 mg/ml RNase A for 30 min at 37°C. Subsequently, cells were stained with propidium iodide (PI, 50 μ g/ml) (Becton Dickinson, San Jose, CA) in PBS, 0.5% Tween-20, and analyzed using a Becton Dickinson flow cytometer BD FACScan (San Jose, CA) and Cell Quest acquisition and analysis programs.

Western blot analysis

Protein lysates were separated by 10% SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were blocked with TBS containing 0.1% Triton X-100 and 5% nonfat milk incubated with primary antibodies. The primary antibodies were anti-human APRIL (BD, NJ, USA), anti-pAkt, anti-Akt, anti-mTOR, anti-p-mTOR (Cell Signaling), anti-c-myc, anti-cyclin D1, anti-CDK4, anti-p-Rb anti- β -actin (Santa Cruz Biotechnology, Santa Cruz, CA). HRP-conjugated mouse immunoglobulin was used as the secondary antibody. Signal detection was carried out with an ECL system (Amersham Pharmacia, Piscataway, NJ). Bands were quantified by densitometry using ImageJ software. Integrated density values of phosphorylated proteins were normalized to total protein for each sample. Unstimulated or untransfected cells were given a value of 1.0, and the level of phosphorylation in all other samples was normalized to this value.

Invasion and migration assays

Tumor cell invasion was measured by examining cell invasion through Matrigel (BD, USA)-coated polycarbonate filters (8- μ m pore size) using Transwell invasion chambers (Costar, VWR Albertslund, Denmark). Briefly, after 48 h of transfection, SW480 cells including shAPRIL transfected cells and shNTC transfected cells were trypsinized, and 10^5 cells suspended in 100 μ l DMEM with 0.5% FBS were plated in the upper chamber. For the APRIL-stimulated HCT-116 cell invasion assay, parallel experiments were performed in the presence of rhAPRIL at a concentration of 100 ng/ml in 0.5% FBS media in the upper chamber. The lower chamber contained 600 μ l DMEM medium with 10% FBS. After incubation at 37°C in 5% CO₂ incubator for 72 h, cells on the upper surface of the filter were removed by wiping with a cotton swab. Migration assays with 10^6 cells per well incubated for 48 h were performed using the same procedure but with uncoated polycarbonate filters. Where indicated, cells were plated onto the inserts with GM6001, or the same volume of DMSO (Sigma). GM6001 at a concentration of 10 μ M in 0.5% FBS media dissolved in DMSO was added to the media to inhibit MMPs in cell invasion assays. Cells attached to the bottom surface of the membrane were stained with 0.1% crystal violet and then counted by microscope. Cells were examined, counted and photographed at 100 \times magnification under an inverted microscope. Five different fields were counted per filter in each group and each experiment was repeated in triplicate. The means of five individual fields selected at random were obtained for each well.

Measurement of MMP-2 and MMP-9 production

MMP-2 and MMP-9 activities were determined using a quantitative sandwich enzyme immunoassay technique. CRC cells were seeded into 96-well plates at a concentration of 4×10^3

cells per well in DMEM with 0.5% FBS for three days. The levels of secreted MMP-2 and MMP-9 in the culture supernatants were determined by ELISA. The assay was conducted using the Quantikine MMP-2 and MMP-9 kits (R&D, MN, USA) according to the instruction manuals. The levels of MMP-2 and MMP-9 were quantified from standard curves. The assay allows quantification of the active as well as the total protein. Each assay was performed in triplicate. This method has been validated against zymography by R&D Systems.

Animal experiments

All animal experiments were performed with eight-week-old athymic female BALB/c nu/nu mice. Animals were divided into three groups, n = 10 per group. Untransfected SW480 cells, shNTC stably transfected SW480 cells and shAPRIL (sh637) stably transfected SW480 cells (2×10^6 in 100 μ l of PBS) were injected subcutaneously into the right flank of each mouse. Tumor growth was monitored externally using a vernier caliper every five days, until 40 days post-injection. Tumor volume was calculated using the following formula: $\pi/6 \times \text{length} \times \text{width}^2$. Seven weeks after injection, mice were sacrificed by cervical dislocation. Tumors were removed and APRIL expression of tumor tissues was analyzed by western blot and immunohistochemistry. Livers and lungs were excised and fixed in 4% paraformaldehyde for 24 h. Metastatic foci on the hepatic surface were counted macroscopically aided by a surgical telescope and were confirmed microscopically. Then tumor tissues and metastatic tissues were paraformaldehyde fixed, paraffin embedded and cut into slices. The sections were stained with hematoxylin and eosin or by immunohistochemical staining. Primary antibodies were against human APRIL, MMP-2, MMP-9 (BD, NJ, USA), Ki-67, cyclin D1, CDK4, p-Rb (Santa Cruz Biotechnology), p-Akt, p-mTOR (Cell Signaling) and Ki-67 (Abcam, Cambridge, UK) for immunohistochemistry studies.

Statistical analysis

Results were expressed as mean \pm SEM. Statistical analysis was performed by analysis of variance (ANOVA). Liver metastasis data analysis was performed using Poisson distribution events test. Differences were considered to be significant when $P < 0.05$.

Results

Expression of APRIL is upregulated in CRC tissues and cell lines

We investigated whether APRIL expression was upregulated in tissues by examining specimens from eight CRC cancer patients using western blot analysis. APRIL expression at the protein level was highly elevated in all CRC tissues tested compared with adjacent normal tissues (Fig. 1A). This suggests a possible role for APRIL in cancer development or progression in the colon. APRIL mRNA was detected at relatively higher levels in a variety of human CRC cell lines compared with human umbilical vein endothelial cells, although the levels of APRIL differed. APRIL was detected at relatively higher levels in SW480 and HT-29 cells and was intermediately expressed in Colo-205 cells. APRIL expression was weakly detected in HCT-116 cells (Fig. 1B). The human colon cancer cell lines HCT-116 and SW480 were chosen as a model system for our research. To reduce APRIL expression, we developed two short hairpin RNAs (shRNA) termed sh637 and sh1750 [17] which were directed against the human APRIL gene (shAPRIL) and caused significant reduction of APRIL expression compared with nontargeting control transfected (shNTC) and nontransfected cells. Following shRNA transfection, substantial

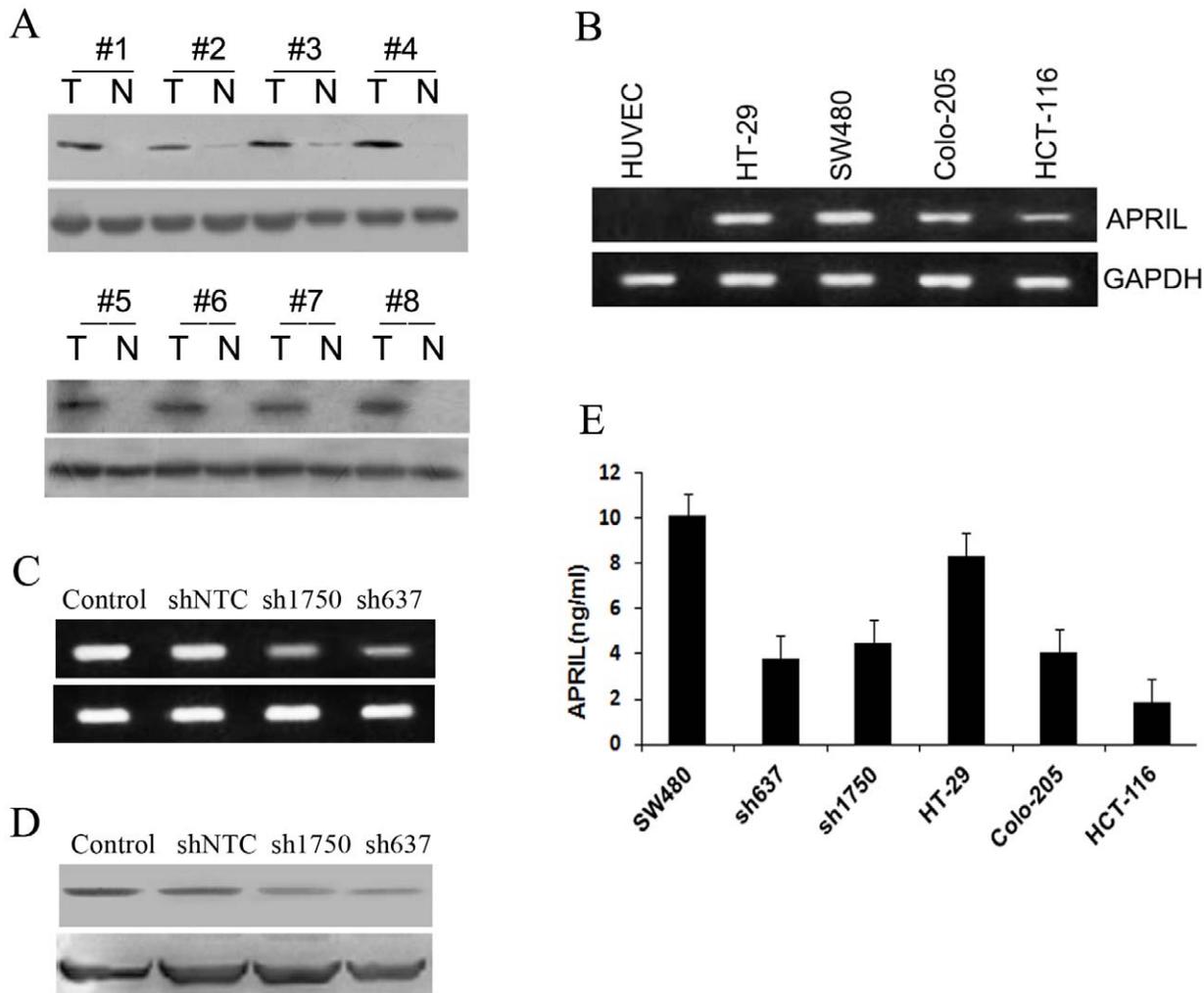


Figure 1. APRIL expression is upregulated in CRC tissues and human CRC cell lines. (A) Western blot analysis of APRIL in eight pairs of colorectal tumors (T) and adjacent normal tissues (N). #1–8 indicates patients' numbers. β -actin was used as an internal control. (B) Semiquantitative RT-PCR analysis of APRIL expression in four human CRC cell lines (SW480, HT-29, Colo-205 and HCT-116). HUVECs were used as a negative control and GAPDH was an internal control. (C) SW480 cells were transfected with shRNA directed against nontargeting control (shNTC) or human APRIL gene (sh637, sh1750), and after 48 h, APRIL mRNA level was determined by semiquantitative RT-PCR. GAPDH was an internal control. (D) SW480 cells were transfected with shNTC or shAPRIL, and after 48 h, APRIL protein levels were determined by western blot. β -actin was an internal control. (E) CRC cell lines and shAPRIL-transfected SW480 cells were cultured for 72 h, and supernatants were collected and assessed for secreted APRIL by ELISA. doi:10.1371/journal.pone.0055298.g001

knockdown of the APRIL transcript was observed in SW480 cells at both the mRNA (Fig. 1C) and protein (Fig. 1D) levels. APRIL expression measured by ELISA in CRC cell culture supernatants demonstrated that the cell lines secreted different levels of soluble APRIL, which confirmed that APRIL is a secreted protein (Fig. 1E).

The biological impact of APRIL on colorectal cancer cells

To analyze the activities of APRIL on CRC, we examined the effects of APRIL knockdown and APRIL stimulation on cell proliferation, migration and invasion in the human CRC cell lines HCT-116 and SW480. CCK-8 assay cell growth viability was determined 24, 48, 72 and 96 h after transfection and APRIL stimulation. We observed a significant decrease in cell growth viability in sh637 transfected SW480 cells ($P < 0.05$; Fig. 2A), and a significantly increased cell growth viability in rhAPRIL (400 ng/ml, 800 ng/ml) stimulated HCT-116 cells ($P < 0.05$; Fig. 2B) compared with respective controls. To explore the role of APRIL

in CRC cell invasion and metastasis, we performed cell migration and invasion assays, which were considered important *in vitro* properties associated with the malignancy of cells. CRC cells transfected with sh637 or stimulated with rhAPRIL (100 ng/ml) significantly affected the number of migrating and invading cells attached to the bottom surface of the membrane, compared with control cells ($P < 0.05$; Figs. 2D and E). Knockdown of APRIL resulted in significant suppression of cell migration and cell invasion of a reconstituted basement membrane (Matrigel), while rhAPRIL (100 ng/ml) treatment dramatically increased CRC cell migration and invasion. Our MTT assays showed that treatment with 100 ng/ml rhAPRIL did not noticeably increase the proliferation of HCT-116 cells (Fig. 2C), suggesting that APRIL induced cell migration was not associated with increased cell proliferation. Proliferation and invasion assays were also performed in sh1750 transfected SW480 cells, and the results were consistent with sh637 transfected cells (data not shown). Taken

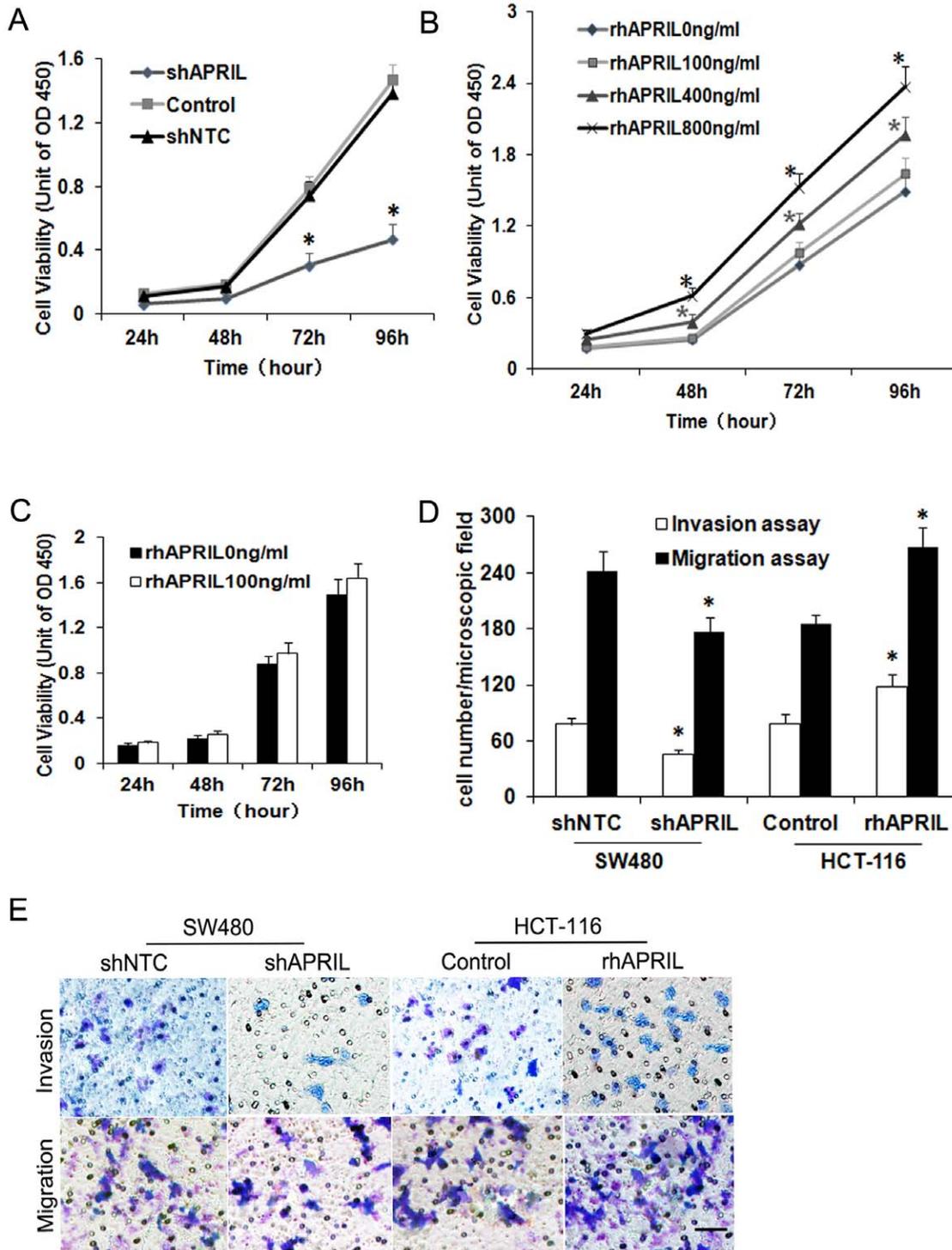


Figure 2. APRIL knockdown or rhAPRIL stimulation regulates cell proliferation, migration and invasion in the CRC cell lines. (A) shAPRIL transfection significantly reduced cell viability assayed by CCK-8 in SW480 cells as compared with the nontransfected and shNTC transfected controls. The data represent means \pm SEM; $*P < 0.05$ compared with shNTC. (B) rhAPRIL (400 ng/ml or 800 ng/ml) stimulation significantly enhanced cell viability in HCT-116 cells compared with nonstimulated cells; $*P < 0.05$. (C) Treatment with 100 ng/ml rhAPRIL did not significantly increase the proliferation of HCT-116 cells. (D and E) Cell motility through uncoated filters (migration) and through Matrigel-coated filters (invasion) in the shAPRIL-transfected SW480 cells, rhAPRIL-stimulated HCT-116 cells and their respective controls. Cells were stained with crystal violet, visualized with microscopy, and counted. (D) The number of cells that had invaded was counted in five representative low power ($\times 100$) fields (LPFs) per Transwell insert. All determinations were performed at least in triplicate in three independent experiments. Numbers represent means \pm SEM. $*P < 0.05$ versus controls. (E) Representative example of D. Scale bar, 40 μ m. doi:10.1371/journal.pone.0055298.g002

together, these results strongly suggest that APRIL plays an important role in tumorigenesis and metastasis of CRC cells.

APRIL-knockdown inhibits tumor growth and metastasis of SW480 cells *in vivo*

As APRIL affected tumor cell growth and invasion *in vitro*, we next examined whether APRIL influenced the behavior of tumors *in vivo* in nude mice BALB/c, which are immunodeficient and susceptible to tumor formation and metastasis. Mice were injected subcutaneously in the right flank with non transfected SW480, shAPRIL (sh637) transfected SW480 or shNTC transfected SW480 cells. Tumor growth was significantly reduced in mice injected with APRIL-knockdown (shAPRIL) SW480 cells ($P < 0.05$) compared with mice injected with siRNA control (shNTC) and non-transfected (control) cells (Fig. 3A, B and C). Total numbers of metastatic liver nodules (>0.5 mm) in individual mice were counted under a surgical telescope. Total numbers of metastatic nodules are shown in Table 1. Metastatic liver nodules were confirmed histologically and representative hematoxylin and eosin stained images are shown in Fig. 3D. No lung metastases were found in any of the three groups. When compared with nontransfected and shNTC transfected SW480 cells, APRIL-knockdown SW480 cells showed reduced metastasis to the liver in nude mice (Table 1, Fig. 3D). Liver metastatic nodules were observed in the nontransfected SW480 group ($n = 89$) and shNTC SW480 group ($n = 78$), whereas three nodules were found in the APRIL-knockdown SW480 group ($P < 0.05$) (Table 1). Though the shNTC group formed fewer metastatic nodules than nontransfected group, this was not statistically significant ($P > 0.05$). These results suggest that APRIL plays a key role in the carcinogenic and metastatic process of SW480 cells *in vivo*.

APRIL stimulates the PI3K/Akt pathway in CRC cells

The PI3K/Akt pathway has been reported to play a critical role in cell proliferation, migration and invasion of various cancer types. Moreover, the TNF superfamily members APRIL and B-cell-activating factor (BAFF), upon engagement with their receptors, have recently been shown to activate the PI3K/Akt pathway in malignant B cells [18,19]. Thus, we determined whether the PI3K/Akt pathway was involved in the APRIL-mediated cellular response in solid tumor cells. Akt is a substrate for PI3K, and the mammalian target of rapamycin (mTOR) is a major downstream effector of Akt. We evaluated the effect of APRIL-knockdown on the PI3K/Akt pathway in SW480 cells by measuring the phosphorylation profile of Akt and mTOR. APRIL-knockdown reduced the phosphorylation of Akt and mTOR in SW480 cells by western blot (Fig. 4A). We also examined whether APRIL influenced Akt and mTOR phosphorylation *in vivo*. In mouse tumor tissues with APRIL knock down, decreased p-Akt and p-mTOR protein content was observed by immunohistochemistry (Fig. 3F, upper panel). We next investigated the effects of APRIL stimulation on the PI3K/Akt pathway in HCT-116 cells. Increasing concentrations of rhAPRIL in HCT-116 cells stimulated the phosphorylation of Akt and mTOR, detected 3 h after APRIL stimulation in a dose-dependent manner (Fig. 4B). Subsequently, pretreatment with LY294002, a synthetic inhibitor of the p110 catalytic subunit of PI3K, was used to investigate the possible involvement of PI3K in APRIL-mediated Akt and mTOR phosphorylation in CRC cells. Pretreatment of HCT-116 cells with LY294002 significantly inhibited APRIL-induced Akt and mTOR activity, suggesting that Akt and mTOR phosphorylation in response to APRIL was PI3K dependent (Fig. 4C).

APRIL mediated cell proliferation of CRC cells by inducing Akt and mTOR activation through the PI3K/Akt pathway

Because proliferation of cancer cells is regulated by the cell cycle, we next determined which phase of the cell cycle was altered in APRIL-knockdown cells by flow cytometry with PI staining. As shown in Fig. 5A, APRIL-knockdown SW480 cells were arrested in the G1 phase 48 h after transfection and the percent of G0/G1 phase cells significantly increased, suggesting that APRIL-knockdown prevented entry of cells from G1 to S phase of the cell cycle. To further understand how APRIL-knockdown prevented S-phase entry of cells, we examined the effect of shAPRIL transfection on the expression of c-myc, cyclin D1, CDK4 (critical regulators of the G1/S transition) and p-Rb. We observed a reduction of c-myc, cyclin D1, CDK4 and p-Rb by knockdown of APRIL (Fig. 5C), which suggested that these factors could be involved in APRIL regulation of cell proliferation. In mouse tumors with knock down of APRIL, we observed decreased APRIL protein content by immunohistochemistry and western blot (Fig. 3E), and decreased cell proliferation (cyclin D1, CDK4, p-Rb and Ki-67) (Fig. 3F, lower panel), suggesting that reduced tumor growth observed by APRIL knockdown could be the result of APRIL regulation of cell proliferation. Because PI3K/Akt activation in G1 phase is required for c-myc stabilization and S-phase entry of cells [20], we determined whether APRIL-mediated regulation of cell-cycle regulatory proteins was PI3K and Akt dependent. We confirmed rhAPRIL could induce cell cycle progression by significantly increasing the proportion of cells in the S, G2M phase and the expression of c-myc, cyclin D1, CDK4, p-Rb proteins in comparison with untreated cells (Fig. 5B and D). Simultaneously, we investigated the effect of PI3K/Akt inhibition on APRIL-induced cell cycle progression of SW480 cells, which were pretreated with the PI3K inhibitor, LY294002, before APRIL stimulation. We found that activation of p-Akt and p-mTOR by APRIL was completely blocked by the PI3K inhibitor LY294002, accompanied with significant inhibition of c-myc, cyclin D1, CDK4 and p-Rb expression (Fig. 5E). This suggests that APRIL-induced Akt and mTOR activity is directly regulated through the PI3K/Akt pathway. To determine whether APRIL-mediated cell-cycle regulatory protein expression was mTOR dependent, APRIL-stimulated cells were treated with the mTOR inhibitor, rapamycin, and mTOR inhibition prevented the up-regulation of cell-cycle regulatory proteins stimulated by APRIL (Fig. 5F). Collectively, these results suggest that APRIL-induced cell proliferation is dependent on both the PI3K and Akt pathways.

APRIL knockdown inhibited the invasiveness of SW480 cells by decreasing the expression and activity of MMPs

Invasion is a key process of cancer cell metastasis. Secreting proteolytic enzymes, including matrix metalloproteinases (MMPs) degrade extracellular matrix (ECM) and basement membranes. MMPs have recently been shown to be related to colorectal tumorigenicity and metastagenicity. To explore whether the suppressed invasiveness of shAPRIL cells was mediated by MMPs, we examined the requirement of MMP activity for APRIL-mediated invasion. shNTC and shAPRIL cells were treated with the MMP inhibitor GM6001 and analyzed for invasion ability with transwell chambers. Fig. 6A showed that GM6001 significantly reduced APRIL-induced invasion in shNTC ($P < 0.05$), but not in shAPRIL treated cells, supporting a functional link between APRIL and MMPs activity. To dissect the mechanism of MMPs in APRIL mediated cell invasion, two key members of the MMP family, secreted MMP-2 and MMP-9, were measured by ELISA. We also evaluated the mRNA expression of MMP-2, MMP-9 and

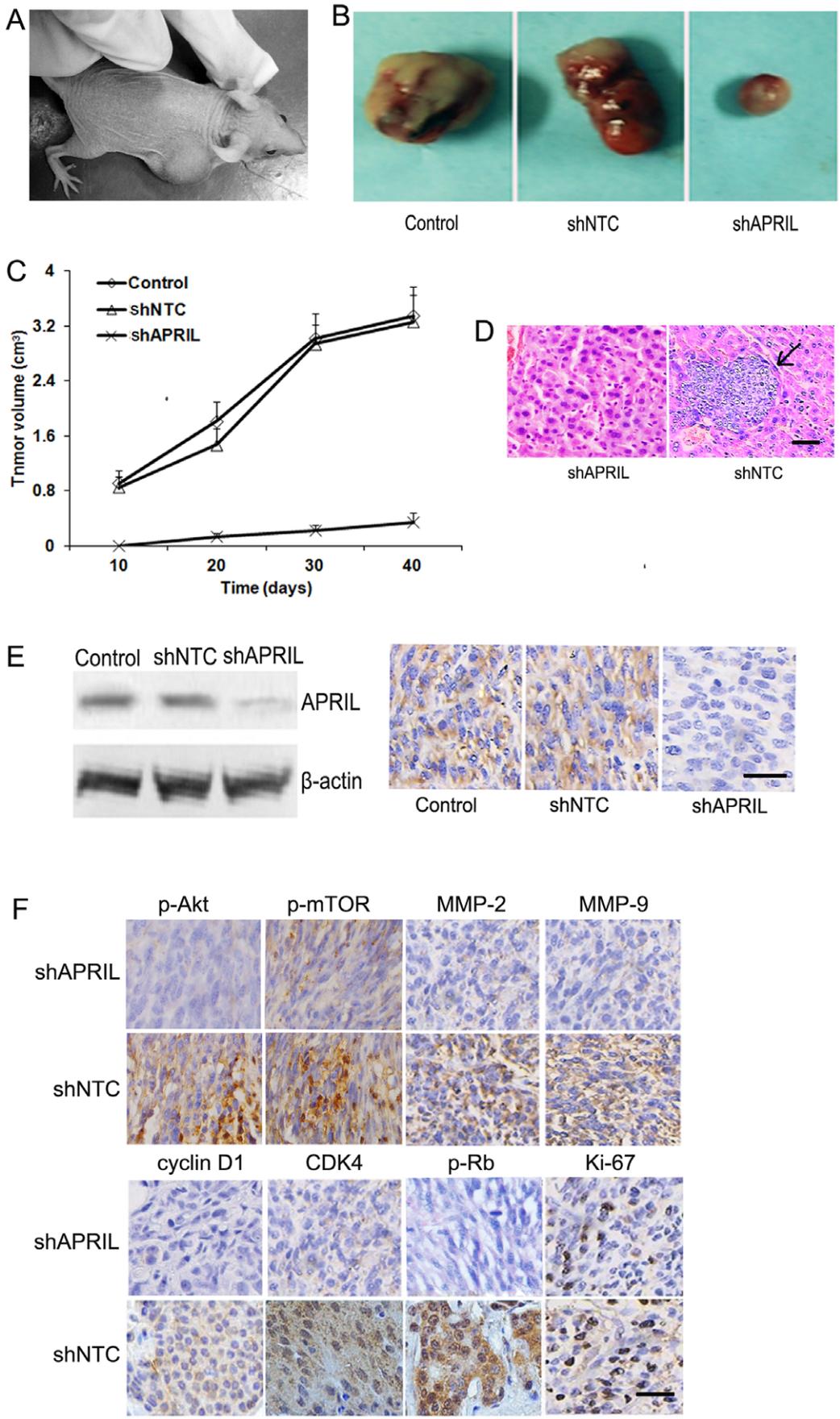


Figure 3. APRIL knockdown increases tumor growth and promotes metastasis *in vivo*. (A) Nude mice were subcutaneously injected in the right flank with control cells, shNTC transfected cells and shAPRIL (sh637) transfected cells. (B) A sample tumor from each group is shown. (C) Tendency of tumor growth after injection in nude mice in different groups. Tumor volume was measured by vernier caliper in cm^3 . (D) Total numbers of metastatic liver nodules ($>0.5 \text{ mm}$) in individual mice were counted under a surgical telescope. Representative hematoxylin and eosin staining of 4- μm sections of livers from shAPRIL and shNTC groups are shown. Arrow indicates tumor nodules. Scale bar, 100 μm . (E) Western blot and immunohistochemistry of APRIL in tumors of nude mice from each group. (F) Immunohistochemical analysis of p-Akt, p-mTOR, Ki-67, cyclin D1, CDK4, p-Rb, MMP-2 and MMP-9 in tumors from nude mice injected with shNTC or shAPRIL SW480 cells. Scale bar, 40 μm . doi:10.1371/journal.pone.0055298.g003

TIMP-1 by RT-PCR. As shown in Fig. 6B and Fig. 6C, shAPRIL transfection decreased MMP-2 and MMP-9 secreted protein and mRNA expression in SW480 cells, compared with control cells. MMPs are regulated by their specific inhibitors, called tissue inhibitors of MMPs (TIMPs). The balance between the levels of activated enzymes and free TIMPs determines the overall MMP activity. Maintenance of this equilibrium is essential, and any disturbance in the balance is a critical determinant of proteolysis and tissue invasion. TIMP-1 expression was upregulated in APRIL knockdown SW480 cells (Fig. 6C). Therefore, APRIL might alter the balance between MMPs and TIMP-1 to facilitate CRC cell invasion. Xenograft model tissues specimens stained for MMP-2 and MMP-9 demonstrated decreased expression in the APRIL-knockdown group (Fig. 3F, upper panel). Taken together, the decrease in MMP activity may account for the inhibited invasiveness of SW480 cells with APRIL-knockdown.

Down-regulation of PI3K/Akt mediated MMP expression by APRIL knockdown in CRC cells

PI3K/Akt is strongly associated with tumor progression, and PI3K/Akt-mediated cell signals induce the expression of MMPs [21,22,23,24]. In this experiment, we focused on whether Akt functioned as an essential kinase in APRIL induced MMP expression in SW480 cells. The gelatinolytic activity in APRIL knockdown cells and rhAPRIL stimulated cells was determined by ELISA. As shown in Fig. 6B and Fig. 7A, the expression of MMP-2 and MMP-9 was decreased in APRIL-knockdown SW480 cells, and after treatment with various concentrations of rhAPRIL, the expression levels of MMPs were increased in SW480 cells in a dose-dependent manner. To determine whether APRIL controls MMP expression in a PI3K-dependent or mTOR-dependent manner, we tested the effects of LY294002 or rapamycin treatment on MMPs expression. The expression of MMPs was examined in either SW480 cells treated with rhAPRIL+LY294002 or rhAPRIL+rapamycin. Treatment of cells with LY294002 concomitantly for 24 h dramatically decreased the expression of MMP-2 and MMP-9, indicating that APRIL-mediated induction of gelatinolytic activity was strongly associated with Akt activation (Fig. 7B). To determine whether modulation of MMP production depended on mTOR activation, we treated cells with rapamycin and measured MMPs by ELISA. As shown in Fig. 7C, rapamycin

had a slight effect on the APRIL-mediated induction of MMP-9 activity. Taken together, these results demonstrate that Akt phosphorylation is involved in the APRIL-mediated modulation of MMPs production.

Discussion

APRIL, initially identified in several cell types derived from solid tumors or solid tumors themselves, provides a proliferation/survival signal to solid tumor cells [5]. However, whether APRIL expression has a role in malignancy and prognosis is not yet clear. Here, we assessed the role of APRIL in the biological behavior of CRC and characterized the signaling mechanisms by which the specific biological process occurred. Knockdown of APRIL in colorectal cancer cells *in vitro* and in BALB/c nude mice *in vivo* inhibited malignancy, tumor growth and metastasis in the liver. We also provide mechanistic insight into how APRIL, via activation of the PI3K/Akt pathway, mediates these processes by demonstrating that: (i) APRIL stimulates the PI3K/Akt pathway in CRC cells; (ii) APRIL-mediated regulation of cell-cycle regulatory proteins is PI3K and Akt dependent; (iii) PI3K/Akt has a role in mediating the effects of APRIL on invasiveness, potentially by increasing MMP-2 and MMP-9 expression (Fig. 8).

Several studies have shown that the dysregulation of APRIL enhances tumor cell survival. APRIL mediates a survival/proliferation signal to lymphoma cells, and was substantiated clinically as patients harboring high levels of APRIL expression in chronic lymphocytic leukaemia and diffuse large B-cell lymphoma had a worse prognosis [7,25]. Reports on APRIL expression in solid tumor lesions are controversial. Our findings demonstrated that APRIL was upregulated in CRC tissues compared with normal tissues and various CRC cell lines express APRIL at various levels. As the SW480 cell line has high expression of the APRIL gene and RNA interference (RNAi) has been widely employed as an experimental tool in studying gene function, RNAi targeting APRIL was used to silence APRIL gene expression in SW480 cells. Alterations of CRC cell biology were analyzed from different aspects. Our study showed that APRIL knockdown inhibited cell proliferation and induced G0/G1 phase arrest. We observed that APRIL knockdown decreased c-myc, cyclin D1, CDK4 and p-Rb expression. Cyclin D1 is a key regulator governing normal cell cycle progression and its cell cycle-dependent activity is mainly mediated through binding and activating CDK4. Activation of CDK4 leads to hyperphosphorylation of the Rb protein. Phosphorylated Rb protein releases bound E2F transcription factor and allows the cell cycle to progress. C-myc is an important transcription factor that regulates the expression of various cell cycle proteins such as cyclins, cdks, and the E2F family of proteins [26]. The deregulated cell cycle control of normal epithelial cells leading to uncontrolled proliferation is one of the major features of tumor progression. In SW480 cells, APRIL knockdown causes G0/G1 phase arrest at least partially through the down-regulation of c-myc, cyclin D1, CDK4 and p-Rb expression. Therefore, the elevated expression of APRIL in CRC may cause deregulated cell cycle control leading to

Table 1. Liver Metastasis of SW480 Cells in Nude Mice BALB/C.

Group	Total number of mice	Number of mice with metastasis	Number of metastatic foci
Control	10	9	89
shNTC	10	8	78
shAPRIL	10	2	3 *

* $P < 0.05$ when compared to shNTC and Control groups. Statistical analysis was performed with Poisson distribution events test. doi:10.1371/journal.pone.0055298.t001

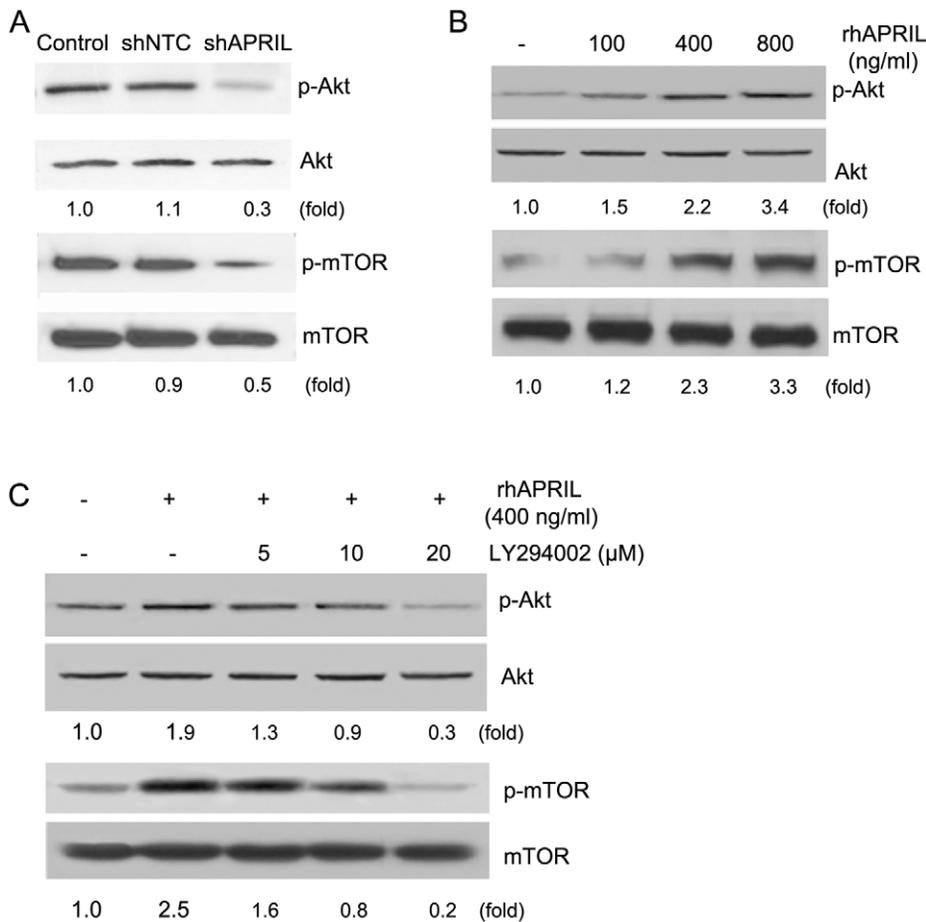


Figure 4. APRIL stimulates the PI3K/Akt pathway in CRC cells. (A) SW480 cells were transfected with shAPRIL (sh637) and shNTC for 48 h and analyzed for Akt and m-TOR phosphorylation by western blot. (B) HCT-116 cells were treated with the indicated concentrations of rhAPRIL for three hours and were analyzed for expression of phosphorylation of Akt and mTOR. (C) HCT-116 cells were treated with the indicated doses of LY294002 for 24 h, and then stimulated with rhAPRIL (400 ng/ml) for three hours, and analyzed for Akt and mTOR phosphorylation. The phosphorylation of Akt and mTOR was determined by SDS-PAGE and western blotting. Densitometric values are listed below each blot. doi:10.1371/journal.pone.0055298.g004

uncontrolled proliferation, which might be a possible cause of CRC carcinogenesis.

Given the involvement of APRIL in CRC cell proliferation, a key process in cancer, our next objective was to evaluate the function of APRIL in other steps of oncogenesis. Here we showed that APRIL clearly modulates cell migration, invasion and the expression of MMPs by use of RNAi. MMPs, a family of zinc-dependent endopeptidases, are crucial in ECM degradation associated with tissue repair, cancer cell invasion, metastasis and angiogenesis. Among MMPs, the type IV collagenases, such as MMP-2 and MMP-9, are considered to be associated with tumor cell invasion and migration during carcinogenesis [27]. Increased MMP-9 expression is associated with advanced Dukes stage and distant metastasis in colorectal cancer [28]. Here, we demonstrated that APRIL knockdown suppressed MMP-2 and MMP-9 gene expression and secretions, and increased the gene expression of TIMP-1. In addition, we found that APRIL induced cancer cell invasion in a MMP(s)-dependent manner, since the general MMP inhibitor GM6001 appeared to significantly inhibit the invasion of nontargetted sequence transfected cells but not the APRIL knock down cells. Our data demonstrated that MMPs regulated by APRIL could be involved in CRC cell invasion.

Our *in vivo* results strongly supported the *in vitro* data. The evaluation of CRC tumorigenesis and metastasis *in vivo* using our xenograft model seems to be a physiological approach to generate “primary tumors” and lead to spontaneous metastasis to other organs. After injection of APRIL knockdown SW480 cells in nude mice, a significant decrease in tumor growth was observed with respect to controls. Immunohistochemistry analysis of tumors strongly indicated that APRIL was involved in tumor growth and tumorigenesis, as shown by the decreased cyclin D1, CDK4, p-Rb and Ki-67 expression with APRIL knockdown cells. To determine whether APRIL contributed to tumor cell invasion and metastasis, we examined several related markers in tumor tissues and counted metastatic nodules on the surfaces of livers in the BALB/c xenograft model. APRIL knockdown significantly reduced the tumor invasion capabilities, as shown by decreased MMP-2 and MMP-9 expression. We also found that APRIL knockdown in SW480 cells inhibited the formation of metastatic tumor nodules in nude mice liver. Given that MMP expression is associated with increased invasion in human CRC, APRIL might elicit metastatic properties by enhancing MMP-2 and MMP-9 production. Thus, reduced liver metastasis with APRIL knockdown could be due, in part, to APRIL regulation of MMPs expression.

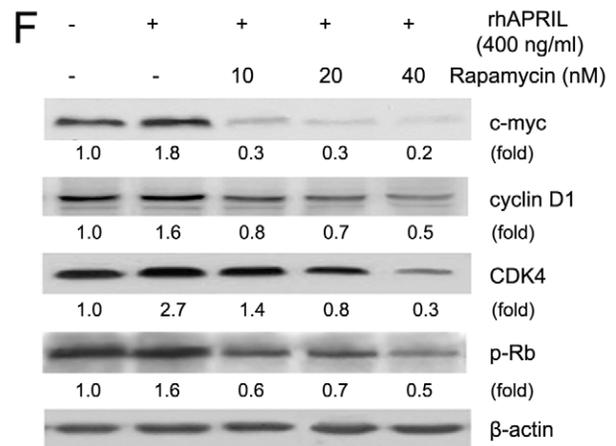
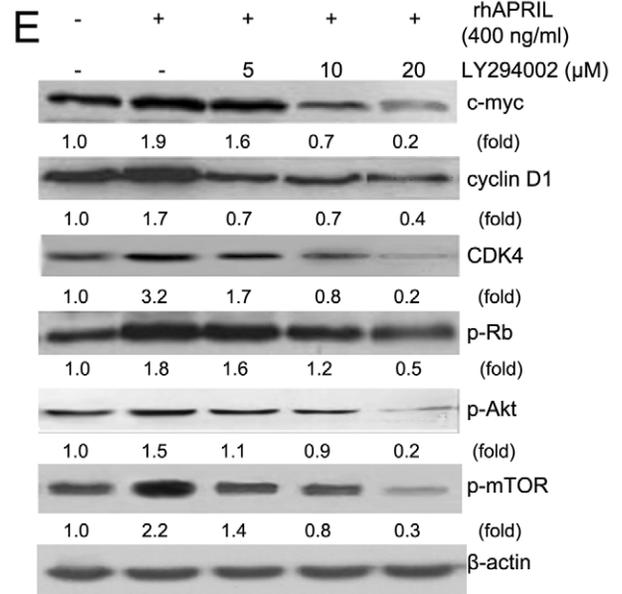
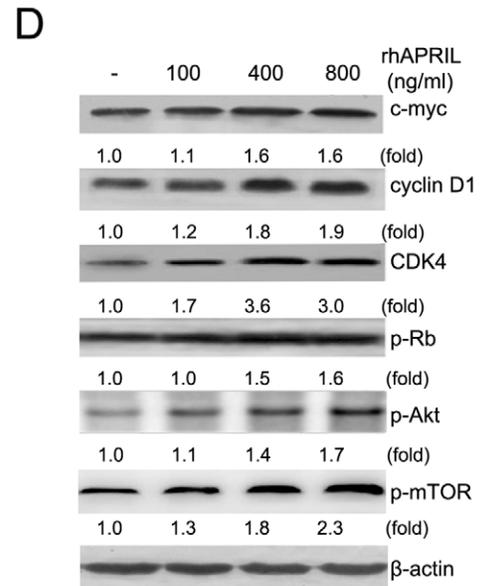
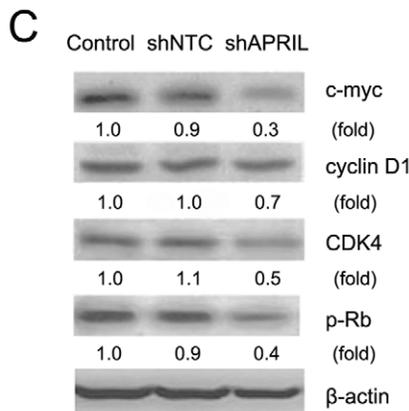
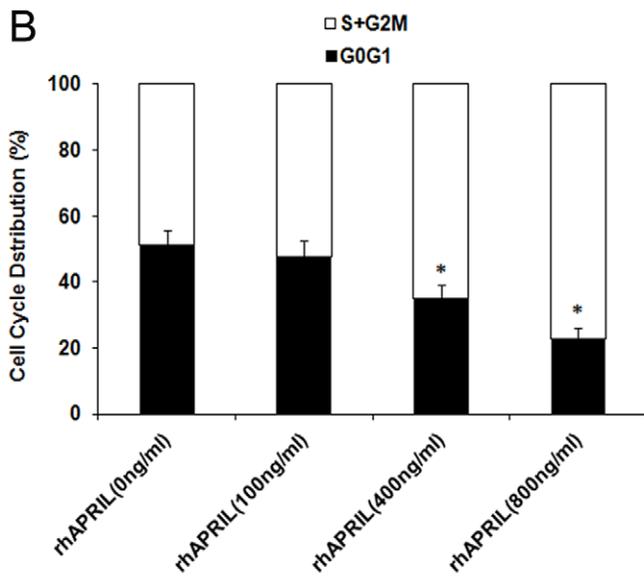
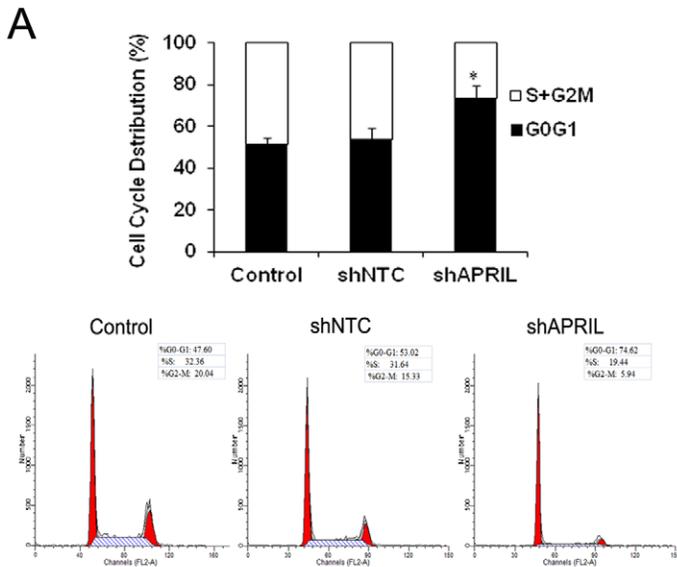


Figure 5. APRIL regulates the cell cycle and cell cycle regulatory proteins of SW480 cells through PI3K/Akt pathway. Cell cycle profiles of shAPRIL (sh637) transfected SW480 cells (A), or rhAPRIL stimulated SW480 cells (B) were examined by flow cytometry with propidium iodide staining and cell numbers were counted according to DNA content of G0/G1, S and G2/M phases. The cell cycle distribution was from three independent experiments, and shows the percentage of G0/G1 and S+G2/M cells after shAPRIL transfection or rhAPRIL stimulation. Western blot of c-myc, cyclin D1, CDK4, p-Rb, p-Akt, p-mTOR and β -actin in SW480 cells 48 h after shAPRIL transfection (C) or rhAPRIL stimulation (D). SW480 cells were treated with the indicated doses of LY294002 (E) or rapamycin (F) for 24 h, and then stimulated with rhAPRIL (400 ng/ml) for 12 h, and analyzed for expression of c-myc, cyclin D1, CDK4, p-Rb. Densitometric values are listed below each blot. doi:10.1371/journal.pone.0055298.g005

The underlying mechanism that connects APRIL stimulation to tumor-promoting activity in CRC cell lines is an active area of investigation. CRC is believed to arise and progress as a result of cumulative genetic and epigenetic changes in tumor cells, where certain mutations appear at consistently high frequencies and at particular stages of disease progression. Growing evidence shows that PI3K, Akt, and their upstream and downstream molecules are commonly altered in human cancers and are important for tumorigenesis and metastasis. Constitutive genomic studies indicate that PI3K/Akt signaling is one of the most frequently deregulated pathways in CRC [2]. Recent studies have shown that the active form of PI3K is an oncogene, and that amplifications and mutations of PI3K are commonly found in many kinds of human cancers. PI3K may transmit oncogenic signals to Akt to regulate tumorigenesis through several downstream targets. Overexpression and activation of Akt regulates cell proliferation

and tumor growth by increasing cell-cycle progression, which plays an important role in carcinogenesis. Akt blocks FOXO-mediated transcription of cell-cycle inhibitors, promotes G1 to S phase transition and stabilizes c-myc and cyclin D1 through the activation of NF- κ B pathway and GSK-3 β / β -catenin-signaling axis [29,30]. In addition, it has also been demonstrated that Akt can regulate mTOR to control protein synthesis and cell proliferation, which is associated with carcinogenesis. One of the critical downstream gene targets of the PI3K/Akt/mTOR pathway is cyclin D1, which associates with CDK4 and CDK5, leading to Rb phosphorylation and subsequent progression of the cell into S phase. Abnormalities of PI3K/Akt upstream molecules are common in cancer and this cascade has a role in tumorigenesis and metastasis. Our studies suggest that APRIL stimulation up-regulates cyclin D1, c-myc, CDK4 and Rb phosphorylation in a PI3K/Akt-dependent manner. The ability of APRIL or BAFF to

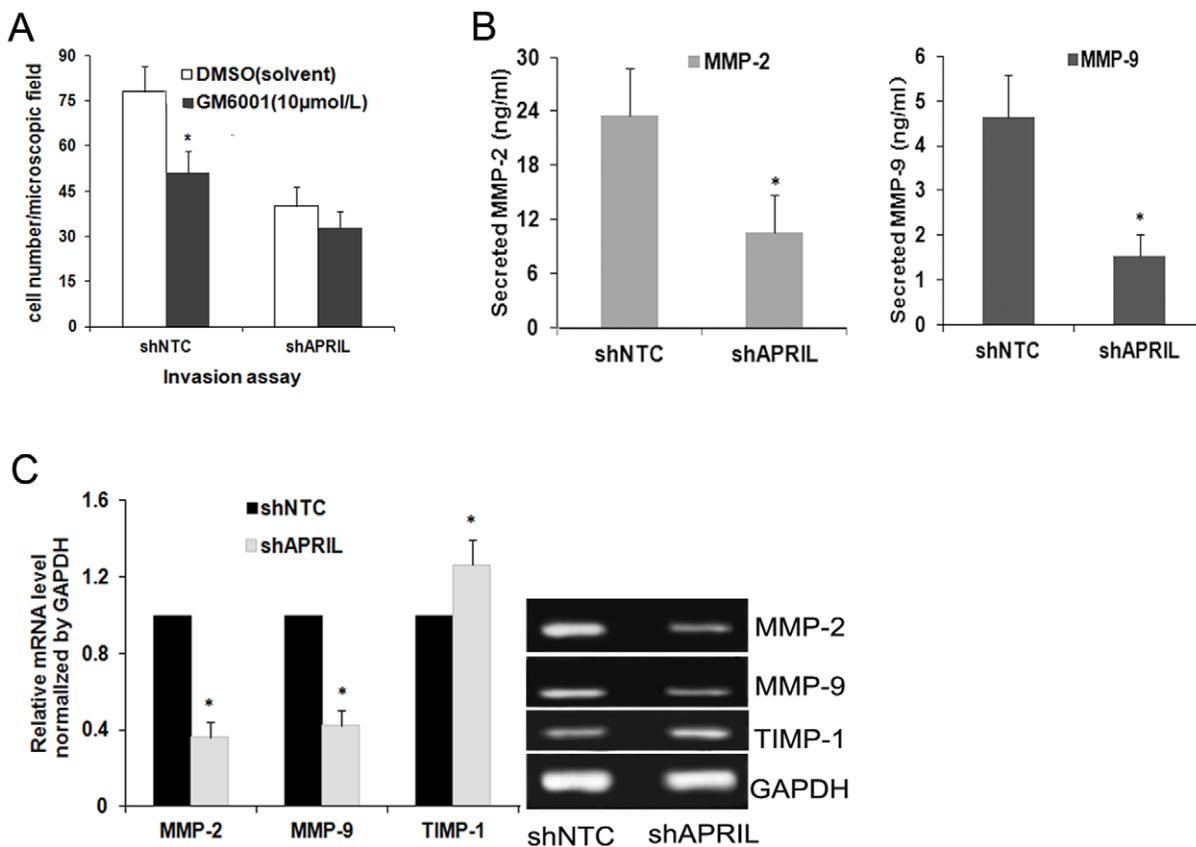


Figure 6. shAPRIL (sh637) inhibited the invasiveness of SW480 by decreasing the expression and activity of MMPs. (A) shNTC or shAPRIL cells were treated with 10 μ M GM6001 or DMSO (control), and analyzed for their invasion ability with Matrigel coated transwell chambers. The invasion assay was performed three times independently. (B) At 48 h post-transfection, secreted MMP-2 and MMP-9 in the culture supernatants were determined by ELISA. (C) At 48 h post-transfection, cells were harvested and MMP-2, MMP-9 and TIMP-1 mRNA levels were determined by semiquantitative RT-PCR 48 h post-transfection. GAPDH was an internal control. Bars represent means \pm SEM from three independent experiments; * P <0.05 compared with shNTC. doi:10.1371/journal.pone.0055298.g006

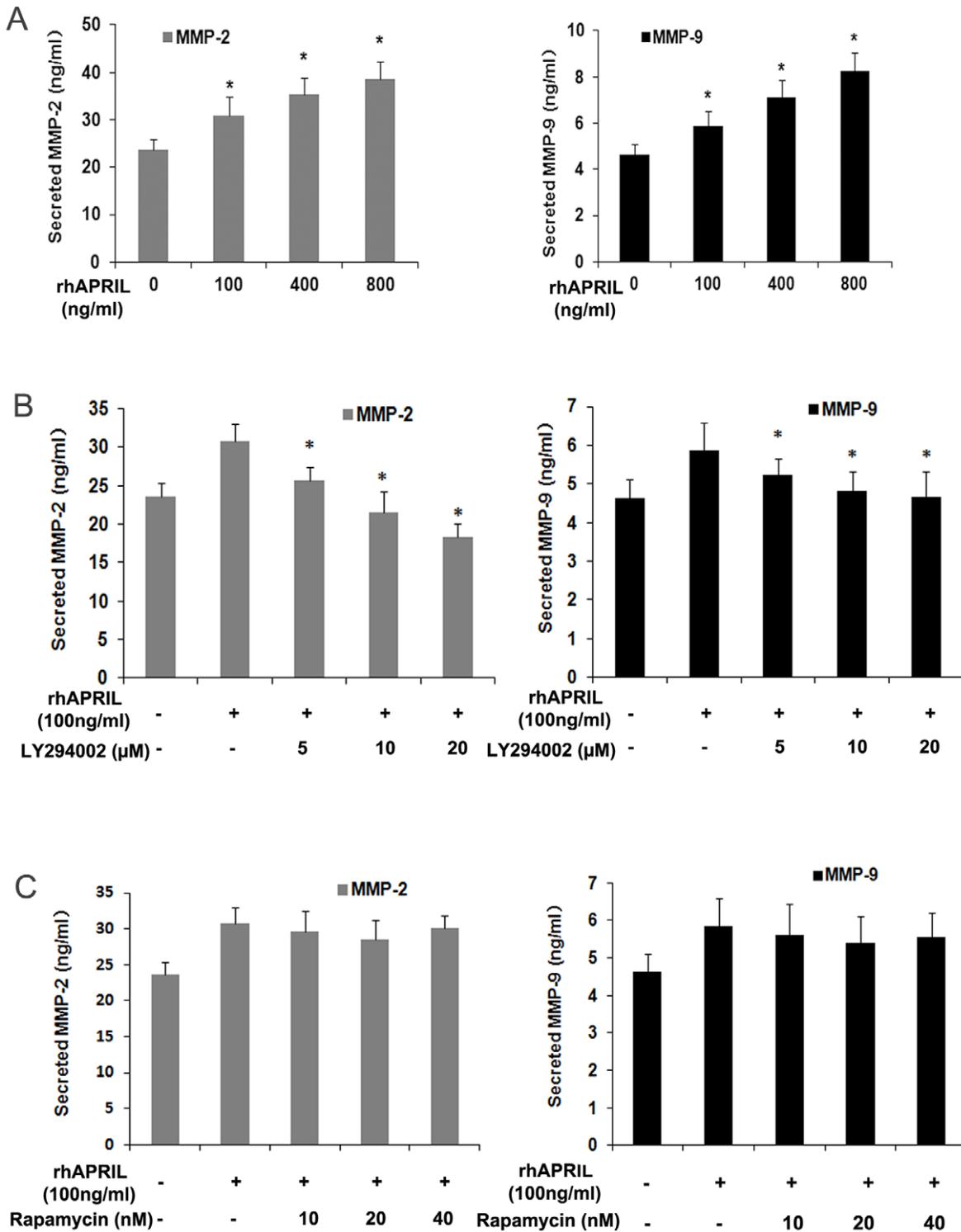


Figure 7. APRIL controls MMP-2 and MMP-9 expression in a PI3K-dependent manner. (A) SW480 cells were treated with indicated concentrations of rhAPRIL for 48 h and secreted MMP-2 and MMP-9 in the culture supernatants were determined by ELISA. SW480 cells were stimulated with APRIL (100 ng/ml) for 48 h, followed by incubation with the indicated doses of LY294002 (B) or rapamycin (C) for another 24 h, and analysis of secreted MMP-2 and MMP-9 was performed. Bars represent means \pm SEM from three independent experiments. * P <0.05 compared with cells treated with rhAPRIL.

doi:10.1371/journal.pone.0055298.g007

modulate cell cycle protein expression has been documented in hematological malignancies, where stimulation of cells with APRIL or BAFF resulted in upregulation of cyclin-D1 and Rb

[9,18]. Our observations in CRCs are also consistent with these previous studies, and this may be one mechanism by which APRIL overexpression results in solid tumor development. Previously,

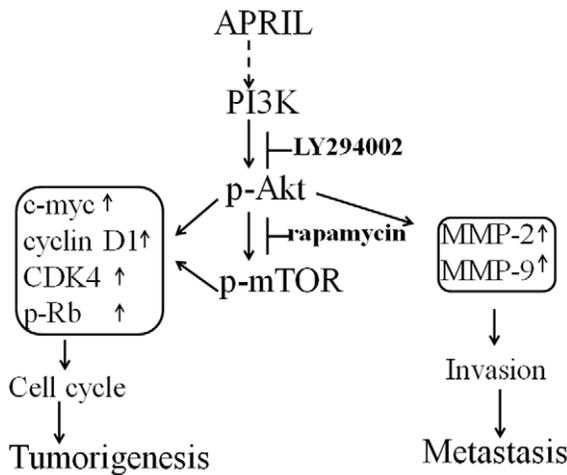


Figure 8. Schematic representation of the PI3K/Akt pathway involved in APRIL-mediated regulation of tumorigenesis and metastasis of CRC cells.
doi:10.1371/journal.pone.0055298.g008

PI3K was shown to be essential for MMP production in several cell lines [22,23,24,30], indicating a significant role for PI3K in tumor progression. Epidemiological studies and animal studies revealed that PI3K activates MMP-2, MMP-9, and Urokinase-type plasminogen activator (uPA), leading to destruction of the ECM [31,32] PI3K activity was higher in metastatic cells compared to non-metastatic cancer cells. Increased levels of MMPs are also due to the activation of the Akt/IKK/NF-κB pathway and Akt/GSK-3β/β-catenin axis [30,33]. However, it remains unknown as to whether APRIL stimulates PI3K pathway and the activation of PI3K is involved in APRIL-mediated MMPs expression in CRC. In our study, we demonstrated that APRIL stimulation via PI3K/Akt pathway played a critical role in CRC

cell invasion. The invasion-promoting action of APRIL in CRC cells was mediated by a cellular pathway involving PI3K, Akt, MMP-2, MMP-9 and TIMP-1. The precise mechanism by which APRIL activates PI3K is a matter for further investigation, but may be through its interaction with its receptor, transmembrane activator and cyclophilin ligand interactor (TACI) or B-cell maturation antigen (BCMA), which is associated with TNFR-associated factor (TRAF) proteins. TRAF has recently been shown to be important in TNF-mediated PI3K activation [9,18]. The tumor-promoting ability of APRIL can be abrogated in APRIL-expressing carcinoma cell lines in immune-deficient mice with soluble BCMA-Fc, which blocks APRIL binding to the high affinity receptors BCMA [34]. Previously we showed that APRIL binding to heparin sulphate proteoglycans (HSPGs) was essential for APRIL mediated tumor-promoting ability in SW480 cells, and heparin could prevent this effect [17]. HSPG-APRIL interactions in CRC indicated that HSPGs may act as a direct receptor or alternatively that HSPGs provide a platform for a yet to be identified receptor [12]. Further work is required to address how APRIL-receptor interactions convey effective signaling for the promotion of cell proliferation and migration.

The *in vitro* data obtained from the experimental manipulation of CRC cell line SW480 and the *in vivo* data from the nude mouse studies indicate that APRIL, via activation of the PI3K/Akt signaling pathway, induces significant changes in cellular behavior, such as tumor cell proliferation, invasion and metastasis. Therefore, APRIL may be a potential novel molecular target for human CRC, and combined therapies that target both the APRIL and PI3K/Akt pathway may have clinical significance for the treatment of CRC.

Author Contributions

Conceived and designed the experiments: HW GW. Performed the experiments: FW GW WD. Analyzed the data: HL RJ XW. Contributed reagents/materials/analysis tools: JW YW SJ. Wrote the paper: GW FW.

References

- Siegel R, Naishadham D, Jemal A (2012) Cancer statistics, 2012. *CA Cancer J Clin* 62: 10–29.
- Wood LD, Parsons DW, Jones S, Lin J, Sjoblom T, et al. (2007) The genomic landscapes of human breast and colorectal cancers. *Science* 318: 1108–1113.
- Gulhati P, Bowen KA, Liu JY, Stevens PD, Rychahou PG, et al. (2011) mTORC1 and mTORC2 Regulate EMT, Motility, and Metastasis of Colorectal Cancer via RhoA and Rac1 Signaling Pathways. *Cancer Research* 71: 3246–3256.
- Zhang HH, Walker F, Kifleariam S, Whitehead RH, Williams D, et al. (2009) Selective inhibition of proliferation in colorectal carcinoma cell lines expressing mutant APC or activated B-Raf. *Int J Cancer* 125: 297–307.
- Hahne M, Kataoka T, Schroter M, Hofmann K, Irmeler M, et al. (1998) APRIL, a new ligand of the tumor necrosis factor family, stimulates tumor cell growth. *J Exp Med* 188: 1185–1190.
- Yu G, Boone T, Delaney J, Hawkins N, Kelley M, et al. (2000) APRIL and TALL-I and receptors BCMA and TACI: system for regulating humoral immunity. *Nat Immunol* 1: 252–256.
- He B, Chadburn A, Jou E, Schattner EJ, Knowles DM, et al. (2004) Lymphoma B cells evade apoptosis through the TNF family members BAFF/BlyS and APRIL. *J Immunol* 172: 3268–3279.
- Belhoue E, Pihlgren M, McGaha TL, Tougne C, Rochat AF, et al. (2008) APRIL is critical for plasmablast survival in the bone marrow and poorly expressed by early-life bone marrow stromal cells. *Blood* 111: 2755–2764.
- Gupta M, Dillon SR, Ziesmer SC, Feldman AL, Witzig TE, et al. (2009) A proliferation-inducing ligand mediates follicular lymphoma B-cell proliferation and cyclin D1 expression through phosphatidylinositol 3-kinase-regulated mammalian target of rapamycin activation. *Blood* 113: 5206–5216.
- Mhawech-Fauceglia P, Kaya G, Sauter G, McKee T, Donze O, et al. (2006) The source of APRIL up-regulation in human solid tumor lesions. *J Leukoc Biol* 80: 697–704.
- Hendriks J, Planelles L, de Jong-Odding J, Hardenberg G, Pals ST, et al. (2005) Heparan sulfate proteoglycan binding promotes APRIL-induced tumor cell proliferation. *Cell Death Differ* 12: 637–648.
- Lascano V, Zabalegui LF, Cameron K, Guadagnoli M, Jansen M, et al. (2012) The TNF family member APRIL promotes colorectal tumorigenesis. *Cell Death Differ* 19: 1826–1835.
- Wang F, Chen L, Mao ZB, Shao JG, Tan C, et al. (2008) Lentivirus-mediated short hairpin RNA targeting the APRIL gene suppresses the growth of pancreatic cancer cells in vitro and in vivo. *Oncol Rep* 20: 135–139.
- Wang F, Ding W, Wang J, Jing R, Wang X, et al. (2011) Identification of microRNA-target interaction in APRIL-knockdown colorectal cancer cells. *Cancer Gene Ther* 18: 500–509.
- Wang F, Chen L, Ding W, Wang G, Wu Y, et al. (2011) Serum APRIL, a potential tumor marker in pancreatic cancer. *Clin Chem Lab Med* 49: 1715–1719.
- Ding W, Wang J, Sun B, Ju S, Yuan H, et al. (2009) APRIL knockdown suppresses migration and invasion of human colon carcinoma cells. *Clin Biochem* 42: 1694–1698.
- Ding WF, Ju SQ, Jiang SY, Zhu L, Wang YG, et al. (2009) Reduced APRIL Expression Induces Cellular Senescence via a HSPG-Dependent Pathway. *Pathology & Oncology Research* 15: 693–701.
- Woodland RT, Fox CJ, Schmidt MR, Hammerman PS, Opferman JT, et al. (2008) Multiple signaling pathways promote B lymphocyte stimulator dependent B-cell growth and survival. *Blood* 111: 750–760.
- Quinn J, Glassford J, Percy L, Munson P, Marafioti T, et al. (2011) APRIL promotes cell-cycle progression in primary multiple myeloma cells: influence of D-type cyclin group and translocation status. *Blood* 117: 890–901.
- Kumar A, Marques M, Carrera AC (2006) Phosphoinositide 3-kinase activation in late G1 is required for c-Myc stabilization and S phase entry. *Mol Cell Biol* 26: 9116–9125.
- Chen X, Liao J, Lu Y, Duan X, Sun W (2011) Activation of the PI3K/Akt pathway mediates bone morphogenetic protein 2-induced invasion of pancreatic cancer cells Panc-1. *Pathol Oncol Res* 17: 257–261.
- Hwang MK, Song NR, Kang NJ, Lee KW, Lee HJ (2009) Activation of phosphatidylinositol 3-kinase is required for tumor necrosis factor-alpha-induced

- upregulation of matrix metalloproteinase-9: its direct inhibition by quercetin. *Int J Biochem Cell Biol* 41: 1592–1600.
23. Bae IH, Park MJ, Yoon SH, Kang SW, Lee SS, et al. (2006) Bcl-w promotes gastric cancer cell invasion by inducing matrix metalloproteinase-2 expression via phosphoinositide 3-kinase, Akt, and Sp1. *Cancer Res* 66: 4991–4995.
 24. Galli A, Svegliati-Baroni G, Ceni E, Milani S, Ridolfi F, et al. (2005) Oxidative stress stimulates proliferation and invasiveness of hepatic stellate cells via a MMP2-mediated mechanism. *Hepatology* 41: 1074–1084.
 25. Planelles L, Castillo-Gutierrez S, Medema JP, Morales-Luque A, Merle-Beral H, et al. (2007) APRIL but not BLyS serum levels are increased in chronic lymphocytic leukemia: prognostic relevance of APRIL for survival. *Haematologica* 92: 1284–1285.
 26. Wang C, Lisanti MP, Liao DJ (2011) Reviewing once more the c-myc and Ras collaboration: converging at the cyclin D1-CDK4 complex and challenging basic concepts of cancer biology. *Cell Cycle* 10: 57–67.
 27. Dragutinovic VV, Radonjic NV, Petronijevic ND, Tatic SB, Dimitrijevic IB, et al. (2011) Matrix metalloproteinase-2 (MMP-2) and -9 (MMP-9) in preoperative serum as independent prognostic markers in patients with colorectal cancer. *Mol Cell Biochem* 355: 173–178.
 28. Chu D, Zhao Z, Zhou Y, Li Y, Li J, et al. (2012) Matrix metalloproteinase-9 is associated with relapse and prognosis of patients with colorectal cancer. *Ann Surg Oncol* 19: 318–325.
 29. Tanaka H, Fujita N, Tsuruo T (2005) 3-Phosphoinositide-dependent protein kinase-1-mediated I κ B kinase beta (I κ B) phosphorylation activates NF- κ B signaling. *J Biol Chem* 280: 40965–40973.
 30. Lu Y, Wahl LM (2005) Production of matrix metalloproteinase-9 by activated human monocytes involves a phosphatidylinositol-3 kinase/Akt/IKK α /NF- κ B pathway. *J Leukoc Biol* 78: 259–265.
 31. Ispanovic E, Haas TL (2006) JNK and PI3K differentially regulate MMP-2 and MT1-MMP mRNA and protein in response to actin cytoskeleton reorganization in endothelial cells. *Am J Physiol Cell Physiol* 291: C579–588.
 32. Shukla S, MacLennan GT, Hartman DJ, Fu P, Resnick MI, et al. (2007) Activation of PI3K-Akt signaling pathway promotes prostate cancer cell invasion. *Int J Cancer* 121: 1424–1432.
 33. Agarwal A, Das K, Lerner N, Sathe S, Cicek M, et al. (2005) The AKT/I κ B kinase pathway promotes angiogenic/metastatic gene expression in colorectal cancer by activating nuclear factor- κ B and beta-catenin. *Oncogene* 24: 1021–1031.
 34. Rennett P, Schneider P, Cachero TG, Thompson J, Trabach L, et al. (2000) A soluble form of B cell maturation antigen, a receptor for the tumor necrosis factor family member APRIL, inhibits tumor cell growth. *J Exp Med* 192: 1677–1684.