

## Original Article

# Downregulation of ARG2 inhibits growth of colorectal cancer cells and increases expression of the CD3 $\zeta$ chain in co-cultured T-cells

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**Abstract:** Arginase (ARG) 2 is upregulated in some malignancies and has been associated with poor prognosis in patients with these malignancies. The present study aimed to explore expression and function of ARG2 in colorectal cancer (CRC). Expression of ARG2 in CRC tumor tissues was analyzed using the public database Cancer RNA-seq Nexus (CRN). Using Western blotting and RT-qPCR, HT29 cells were selected as target cells for ex vivo experiments. Small interfering RNAs (siRNAs) were then used to silence ARG2 in HT29 cells. The siRNA with the highest interfering efficiency was selected. Cell Counting Kit-8, wound healing assays, and Transwell assays were subsequently performed, aiming to elucidate the proliferation and invasion of CRC cells. The impact of ARG2 on cell cycle/apoptosis was also investigated. In addition, HT29 cells were co-cultured with Jurkat T-cells in a Transwell system to mimic the tumor microenvironment. Expression of the CD3 $\zeta$  chain in T-cells was examined via Western blotting. Results showed that expression of ARG2 was significantly higher in CRC tumor tissues than in adjacent normal mucosae. Knockdown of the ARG2 gene significantly attenuated proliferation, migration, and invasion abilities of CRC cells. It also affected the cell cycle and apoptosis of these cells. Expression of the CD3 $\zeta$  chain in co-cultured T-cells was markedly increased. The current study highlights the important role that ARG2 plays in CRC, providing a potential novel target for diagnosis and treatment of CRC.

**Keywords:** Arginase 2, colorectal cancer, CD3 $\zeta$

## Introduction

Colorectal cancer (CRC) is one of the most common and lethal malignancies worldwide, posing a huge threat to human health [1]. Despite great advancements made in the treatment of CRC in recent decades, surgery and radio-chemotherapy remain the primary therapeutic strategies for treatment of CRC. A large proportion of patients ultimately experience recurrence or metastasis as the major causes of tumor-related deaths. An important recent development in CRC treatment is the application of targeted drugs, such as cetuximab and bevacizumab. They exert anti-tumor effects by blocking epidermal growth factor receptors and vascular endothelial growth factor (VEGF) signaling pathways. Several large-scale clinical tri-

als have shown that targeted therapy can benefit CRC patients [2, 3]. However, gene mutations and drug resistance have limited the therapeutic effects of these drugs [4, 5]. Therefore, a comprehensive exploration of the pathogenesis of CRC is of primary concern for the identification of additional biomarkers and therapeutic targets that may facilitate prognostic prediction and personalized treatment, ultimately improving survival rates of CRC patients.

Arginase (ARG), an enzyme associated with tumorigenesis, partly due to its contribution to the immunosuppressive tumor microenvironment [6, 7], is overexpressed in several malignancies. It has been associated with poor prognosis in these patients [8-10]. ARG is expressed as two subtypes, ARG1 and ARG2, which show

different cellular and histological localization under physiological conditions. The former is localized in the cytoplasm and is produced mainly in the liver, while the latter exists in the mitochondria and is mainly expressed by the kidneys. *Ex vivo* experiments have demonstrated the pro-tumorigenic features of ARG in some cancers. In the context of prostate cancer, androgens can upregulate expression of ARG1 and ARG2 in human LNCaP cells. This inhibits the activation of tumor-specific T-cells and ultimately promotes tumor growth. In contrast, androgen-deprivation therapy can increase the infiltration of immune cells within tumor tissues through the downregulation of ARG1/ARG2. In addition, knockdown of ARG1/ARG2 by small interfering (si) RNAs has been shown to limit the growth of LNCaP cells. A conditioned medium from these LNCaP cells activates peripheral blood mononuclear cells [11]. ARG2 is also highly expressed in most human thyroid carcinoma cases. However, it is seldom expressed in normal thyroid tissues and thyroid adenoma cells. Silencing of the ARG2 gene in thyroid WRO cells by siRNAs promotes apoptosis and decreases expression of proliferation markers Ki67 and PCNA [8]. Tate et al. [12] reported that three murine cell lines, SIRCC-1.2 (CL-2), SIRCC 1.19 (CL-19), and Renca, express ARG2 and that the addition of the ARG inhibitor nor-NOHA (NG-hydroxy-L-arginine) into the culture medium of these cells significantly suppresses proliferation. Similarly, nor-NOHA exerts inhibitory effects on the growth of the human CRC cell line Caco2, which displays robust ARG activity. Additionally, because nor-NOHA is an intermediate product of nitric oxide synthase (NOS), the growth of Caco2 cells is markedly reduced after co-culturing with rat aortic endothelial cells (rich in NOS). Furthermore, these effects may be blocked by the NOS inhibitor S-ethylisothiourea (EITU) [13].

Previous studies have revealed that ARG activity in the serum and/or tumor tissues of CRC patients is significantly increased, compared with that in healthy individuals and/or normal mucosae [14-17]. Moreover, ARG1 is seldom expressed in CRC [18-20]. Therefore, it would be interesting to determine whether ARG2 is overexpressed in CRC, assessing its impact on proliferation, migration, and invasion of CRC cells, as well as its effects on cell cycle and apoptosis of these cells. Additionally, the cur-

rent study investigated the possible relationship between ARG2 and tumor immunity in CRC. It has been considered one of the most important features of ARG2.

### Materials and methods

#### *Public database*

The public database 'Cancer RNA-seq Nexus (CRN, <http://syslab4.nchu.edu.tw/index.jsp>)' was used to analyze differences in ARG2 expression levels between CRC tumor tissues and adjacent normal mucosae. The keyword 'ARG2' was used to search corresponding datasets.

#### *Cell culturing and co-culturing*

Human CRC cell lines Caco2, HT29, and HCT116, as well as the leukemia cell line Jurkat, were obtained from the ATCC (Manassas, VA, USA). Caco2 and HCT116 cells were cultured in Dulbecco's Modified Eagle's medium (Thermo Fisher Scientific, Inc., Waltham, MA, USA). HT-29 cells were cultured in McCoy's 5A medium (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). Jurkat T-cells were cultured in RPMI 1640 medium (Gibco; Thermo Fisher Scientific, Inc.). All media were supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc) and 100 IU/mL penicillin/streptomycin (Invitrogen; Thermo Fisher Scientific, Inc). The cells were cultured at 37°C with 5% CO<sub>2</sub>.

Co-culturing of CRC cells and T-cells was performed using a Transwell system (BD Biosciences, Franklin Lakes, NJ, USA). Briefly, 6×10<sup>5</sup> CRC cells were plated in the lower chamber and 5×10<sup>5</sup> Jurkat T-cells were seeded in the upper chamber. Seventy-two hours after seeding, the T-cells were harvested and analyzed via Western blotting, determining expression levels of the CD3ζ chain. This experiment was repeated with L-arginine supplementation. In this experiment, 50 μL of L-arginine (Beyotime Institute of Biotechnology, Shanghai, China, 10 mM) was added to the lower chamber of the co-culture system every 24 hours.

#### *Cellular transfection*

Three small interfering (si) RNAs (siRNA\_1, 2, 3) targeting ARG2 and one scrambled negative control siRNA (siRNA\_NC) were obtained from

Shanghai GenePharma Co., Ltd (Shanghai, China). Sequences were as follows: siRNA\_1, 5'-CTGAAGAAATCCGTCCACT-3'; siRNA\_2, 5'-GGCAATCGGTACCATTAGT-3'; siRNA\_3, 5'-CATGGAACGAACATTGAT-3'; and siRNA\_NC, 5'-TTCTCCGAACGTGTCACGTTT-3'. Lipofectamine 2000 Reagent (Invitrogen; Thermo Fisher Scientific, Inc.) was used for cell transfection, according to manufacturer protocol. CRC cells were divided into three groups, including blank (cells treated with Lipofectamine 2000 only), NC (cells treated with negative control siRNA and Lipofectamine 2000), and siRNA (cells treated with ARG2 siRNA and Lipofectamine 2000) groups. All the experiments were repeated at least three times.

## RT-qPCR

Total RNA from the cells was extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA), according to manufacturer instructions. Next, cDNA was synthesized using a RevertAid First Strand cDNA Synthesis kit (Fermentas). RT-qPCR was performed using a SYBR Green PCR kit (Thermo Fisher Scientific, Inc.) and an ABI7300 Real-Time PCR system (Applied Biosystems, Carlsbad, CA, USA). Primer sequences were as follows: ARG2, 5'-ACAGCCAGTTTCATTTCTC-3' (sense) and 5'-TCTTTGTCTCTTGCCAATC-3' (anti-sense); GAPDH, 5'-CACCCACTCCTCCACCTTTG-3' (sense) and 5'-CCACCACCCTGTTGCTGTAG-3' (anti-sense). Conditions for PCR amplification were 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 45 seconds, along with a dissociation cycle consisting of 95°C for 15 seconds, 60°C for 1 minutes, 95°C for 15 seconds, and 60°C for 15 seconds. The  $2^{-\Delta\Delta Ct}$  method was employed for data analysis. GAPDH was used as an internal control.

## Cell counting kit (CCK)-8 assays

CRC cells were inoculated in 96-well plates at a density of  $5 \times 10^3$  cells/well. Moreover, 10  $\mu$ L of CCK-8 reagent (Dojindo, Molecular Technologies, Inc., Kumamoto, Japan) was added to the cells at 0, 12, 24, 48, 72, or 96 hours. The cells were then incubated for 2 hours at 37°C. Absorbance values at a wavelength of 450 nm were measured using an automated plate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

## Flow cytometry

Regarding cell cycle analysis, the cells were centrifuged at 1,000 g for 5 minutes. They were washed with cold PBS and re-centrifuged. After suspension in 300  $\mu$ L of PBS containing 10% FBS, the cells were fixed with 70% ethanol at 4°C for 24 hours. They were centrifuged, washed twice with cold PBS, and incubated with 100  $\mu$ L of RNaseA (1 mg/mL) for 30 minutes at 37°C. The cells were subsequently stained with 400  $\mu$ L of propidium iodide (PI, 50  $\mu$ g/mL) (Becton-Dickinson, San Jose, CA, USA) for 10 minutes and detected using a FACSCalibur flow cytometer (BD Bioscience, USA). Data was assessed using FLOWJO v10 software. For cell apoptosis analysis, the cells were centrifuged and washed twice with cold PBS. They were then stained with 5  $\mu$ L of Annexin V-APC (BD Biosciences) for 15 minutes and 5  $\mu$ L of propidium iodide (PI, 50  $\mu$ g/mL) (Becton-Dickinson) for 5 minutes (both staining steps were performed at 4°C). Finally, detection was conducted using a flow cytometer (BD Bioscience, USA).

## Wound healing assays

The cells were seeded in six-well plates ( $5 \times 10^5$ /well) and incubated until reaching 90% confluence. Linear wounds were then created by scratching the plate using sterile 200- $\mu$ L pipette tips. The cells were subsequently washed with PBS and cultured in serum-free medium for 24 hours at 37°C. The width of each wound was observed and measured under a microscope at 0 hours and 24 hours.

## Transwell assays

Cell invasion was determined via Transwell assays (BD Biosciences) using polycarbonate membranes with 8- $\mu$ m pores, coated with Matrigel (BD Biosciences). Approximately  $1 \times 10^5$  transfected CRC cells were suspended in 200  $\mu$ L of serum-free medium and added to the upper chamber of the Transwell plates. The lower chamber was filled with 600  $\mu$ L of medium supplemented with 10% FBS. The cells were cultured at 37°C with 5% CO<sub>2</sub> for 24 hours. At the end of the incubation period, the upper chamber was removed. Cells on the upper surface of the membranes were wiped with a cotton bud. Cells that had penetrated the lower

**Table 1.** Comparison of arginase 2 expression between CRC tumor tissues stratified by tumor node metastasis stage and adjacent normal mucosae in the dataset obtained from CRN

CRC tumor tissues (label: 1)		vs.	Adjacent normal mucosae (label: 2)	P value	Total
Stage	Number				
I	74		40	0.002	479
II	29			0.007	
IIA	137			<0.001	
IIB	9			0.006	
III	21			0.016	
IIIA	12			<0.001	
IIIB	56			0.004	
IIIC	39			<0.001	
IV	62			0.002	

CRC, colorectal cancer.

surface of the membranes were fixed for 15 minutes using 4% paraformaldehyde. They were washed three times with PBS, stained with crystal violet (0.5%, Biosharp, Hefei, China) for 20 minutes, and observed under a microscope. Five microscopic fields were randomly selected for counting the number of penetrated cells.

#### Western blotting

Expression levels of ARG2 in CRC cells and expression levels of the CD3 $\zeta$  chain in Jurkat T-cells were determined by Western blotting. GAPDH was used as an internal control. Total protein from the cells was extracted using radioimmunoprecipitation assay cell lysis buffer (Beyotime Institute of Biotechnology, Beijing, China). Protein concentrations were determined using the bicinchoninic acid assay method (Thermo Fisher Scientific, Inc.). Subsequently, 60  $\mu$ g of protein was separated by 10% SDS-PAGE (Sigma-Aldrich; Merck KGaA). Separated proteins were then transferred to polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). The membranes were blocked for 2 hours with 5% skimmed milk in Tris-buffered saline-Tween-20. They were incubated with a primary antibody overnight at 4°C, washed three times, incubated with horseradish peroxidase (HRP)-conjugated secondary antibody at room temperature for 2 hours, and visualized using the electrochemiluminescence method (Pierce; Thermo Fisher Scientific, Inc.). The relative quantity of the target protein was determined with Image-Pro Plus software (ver-

sion 6.0; Media Cybernetics, Inc., Rockville, MD, USA). The following primary antibodies were used: Rabbit anti-ARG2 (ab137069, 1:4,000, Abcam, Cambridge, UK), rabbit anti-CD3 $\zeta$  (ab226475, 1:2,000, Abcam), and mouse anti-GAPDH (#97166, 1:1,000, Cell Signaling Technology Inc., Danvers, MA, USA). HRP-labeled goat anti-mouse immunoglobulin G (IgG; ab-6789, 1:2,000, Abcam) and goat anti-rabbit IgG (ab6721, 1:2,000, Abcam) were used as secondary antibodies.

#### Statistical analysis

Statistical analyses were performed using GraphPad Prism (version 6.0; GraphPad Software, Inc., La Jolla, CA, USA). Data are presented as mean  $\pm$  standard deviation from at least three independent experiments. Analysis of variance was used to determine differences among groups. Bonferroni's correction was applied as a post-hoc test. Two-tailed *P* values <0.05 indicate statistical significance.

## Results

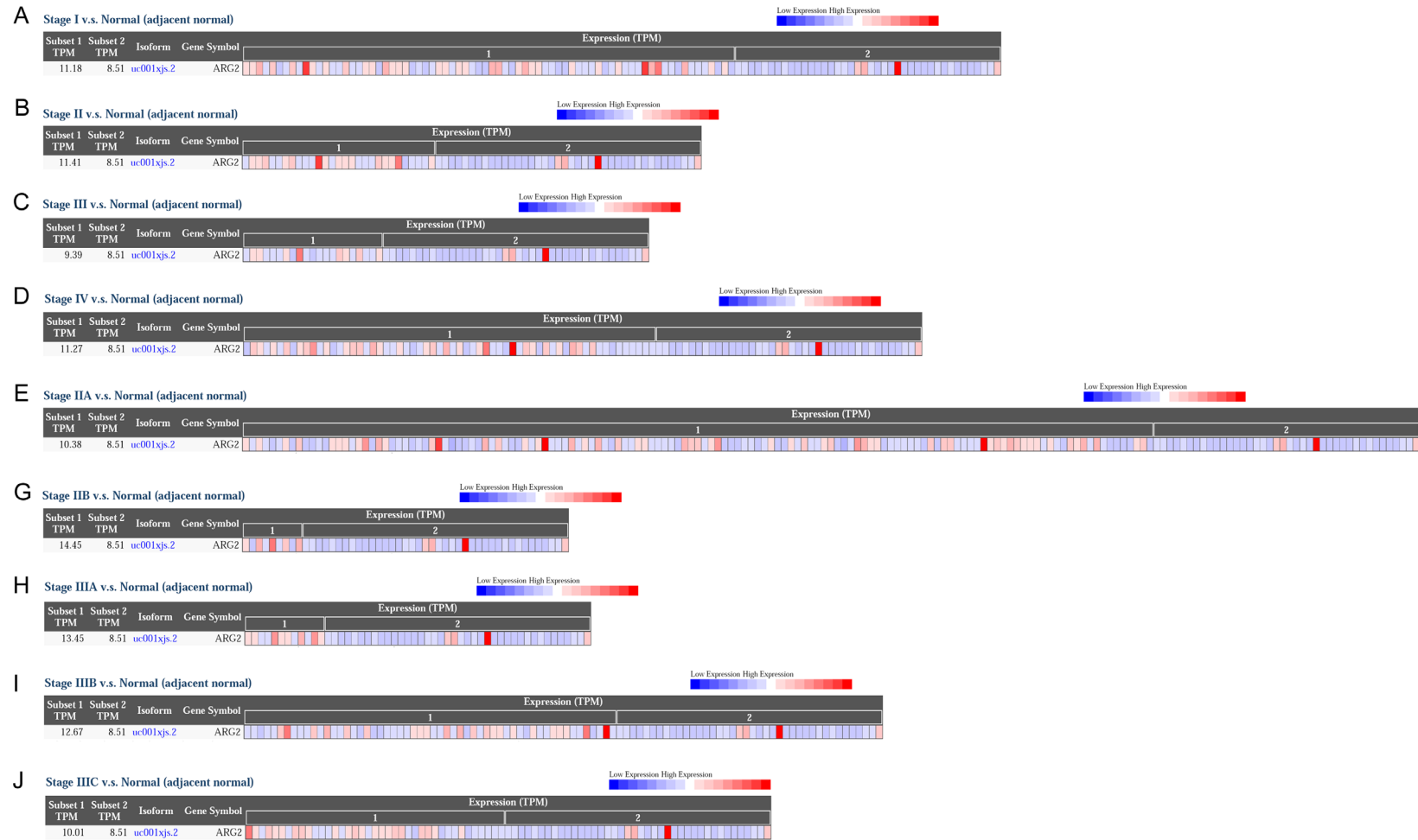
### ARG2 is overexpressed in CRC

A search of the public database CRN yielded data from 479 colorectal tissue samples, including 439 CRC tumor tissues and 40 adjacent normal mucosae. Distribution of patient tumor node metastasis stages among the CRC cases was as follows: 74 stage I, 29 stage II, 137 stage IIA, 9 stage IIB, 21 stage III, 12 stage IIIA, 56 stage IIIB, 39 stage IIIC, and 62 stage IV (**Table 1**). Comparison analysis showed that expression of ARG2 was significantly higher in CRC tumor tissues, compared with adjacent normal mucosae (**Figure 1**). Therefore, ARG2 was upregulated in CRC.

### ARG2 promotes proliferation of CRC cells

To determine the biological function of ARG2 in CRC, the present study investigated expression of ARG2 at protein and mRNA levels in three CRC cell lines, Caco2, HT29, and HCT116, via Western blotting and RT-qPCR. Results showed that HT29 and Caco2 cells expressed higher levels of ARG2 than HCT116 cells (**Figure 2A**,

# Downregulation of ARG2 inhibits CRC cell growth



**Figure 1.** Expression of arginase 2 mRNA significantly increased in the tumor tissues of CRC patients, compared with adjacent normal mucosa.



**2B**). Thus, HT29 cells were selected for subsequent experiments. HT29 cells were divided into three groups (blank, NC, and siRNA). ARG2 expression was silenced by siRNAs. Silencing efficiency was assessed by PCR. ARG2 expression was significantly lower in cells treated with ARG2 siRNAs, compared with levels in control cells. Moreover, siRNA\_2 exhibited the highest interfering efficiency (**Figure 2C**). Thus, it was used for subsequent experiments. Effects of ARG2 on proliferation of CRC cells were then determined by CCK-8 assays. Knockdown of ARG2 significantly impaired proliferation (**Figure 2D**) of CRC cells, relative to that observed in the two control groups. Results suggest that ARG2 promotes growth of CRC and plays a role during the formation and development of CRC.

### *ARG2 impacts cell cycle and apoptosis in CRC*

Cell cycle and apoptosis levels of CRC cells were assessed via flow cytometry. Results showed that silencing of ARG2 increased the ratio of HT29 cells in the G1 phase and decreased the ratio of these cells in the S or G2 phase (**Figure 2E**). In addition, the ratio of apoptotic cells (**Figure 2F**) was increased significantly after ARG2 was silenced. Current findings indicate that ARG2 can accelerate the cell cycle and promote cell apoptosis in CRC.

### *ARG2 promotes migration and invasion of CRC cells*

*In vitro* experiments have revealed that ARG2 promotes aggressive biological behaviors in certain tumor types [7]. Therefore, to validate the effects of ARG2 on migration and invasion of CRC cells, wound healing assays and Transwell assays with Matrigel were performed. Results showed that migration (**Figure 3A**) and invasion (**Figure 3B**) abilities of CRC cells were significantly inhibited after silencing of ARG2.

### *ARG2 reduces expression of the CD3ζ chain in co-cultured T-cells*

It has been reported that ARG is associated with immunity and impairs expression levels of the CD3ζ chain in tumor-infiltrating T-cells, leading to T-cell dysfunction through the depletion of L-arginine within the tumor microenvironment [10, 21]. To elucidate the effects of ARG2 in CRC cells on tumor immunity, HT29

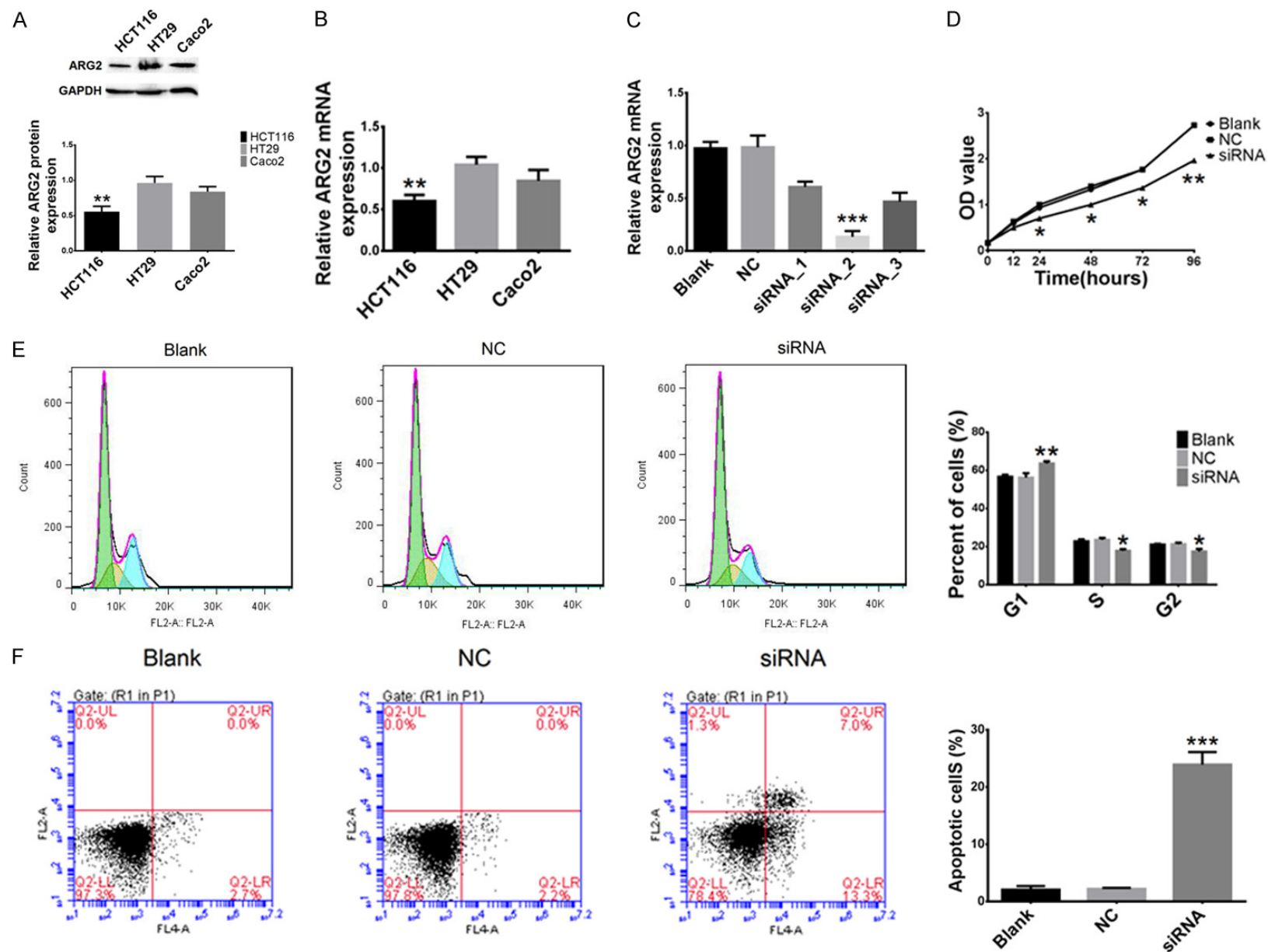
cells were cocultured with Jurkat T-cells in a Transwell system to mimic the tumor microenvironment. After 72 hours of co-culturing, expression of the CD3ζ chain was detected. T-cells co-cultured with CRC cells in which ARG2 was knocked down expressed higher levels of the CD3ζ chain, demonstrated by Western blotting (**Figure 4A**). To exclude the possible impact of delayed growth of HT29 cells after the silencing of ARG2, the media of both control groups was periodically supplemented with L-arginine. A completely different result was obtained. Expression of the CD3ζ chain in the siRNA-treated group was not higher than levels observed in the other two groups (**Figure 4B**), while cell proliferation results obtained after 72 hours of culturing, determined through CCK8 assays, were consistent with those obtained in the absence of L-arginine supplementation (**Figure 4C**).

## Discussion

ARG, or L-arginine amidinohydrolase, is a type of binuclear manganese enzyme (enzyme code 3.5.3.1). Its physiological function is to catalyze the conversion of L-arginine to L-ornithine and urea. Subsequently, L-ornithine can be transformed to polyamines (putrescine, spermidine, and spermine) by a series of enzymes, including ornithine decarboxylase (ODC), spermidine synthetase (SDS), and spermine synthetase (SS). Furthermore, L-arginine can be catalyzed into L-citrulline and NO (nitric oxide) by NOS. These two pathways cooperatively regulate L-arginine metabolism within the human body (**Figure 5**).

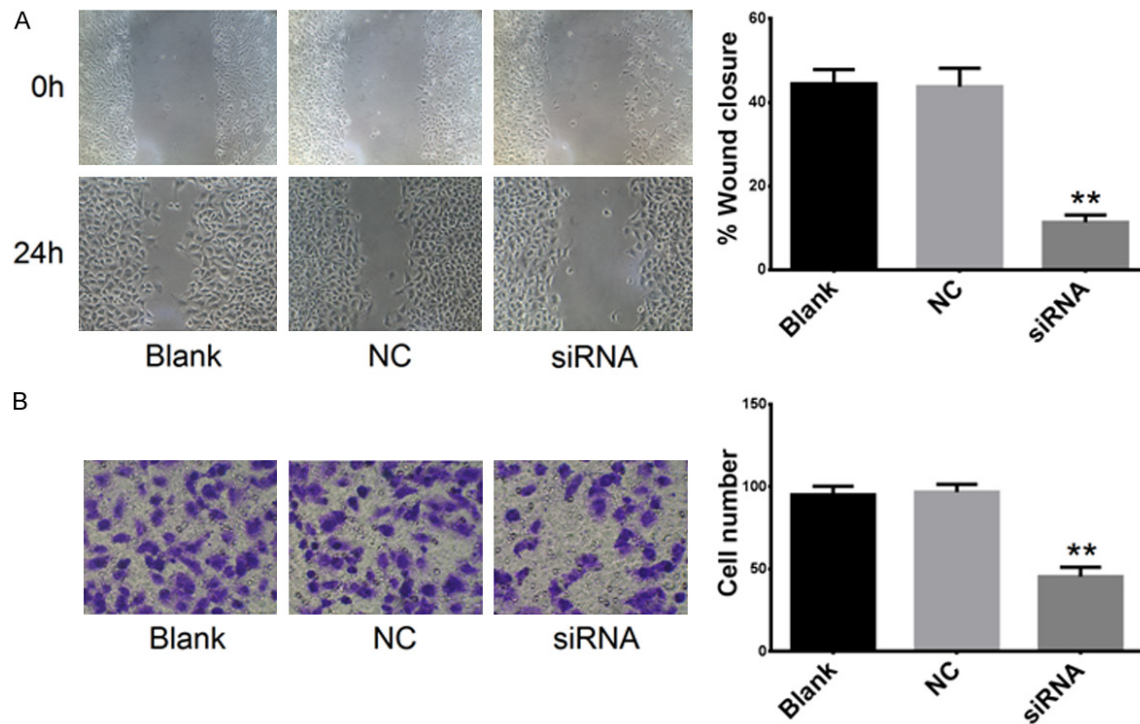
Previous studies have emphasized a close relationship between ARG and various malignancies, including CRC. Using enzyme immunoassays, Leu et al. detected serum ARG levels in 31 CRC patients and 115 nontumor controls. They found that these equaled  $18.96 \pm 4.83$  and  $3.09 \pm 0.22$  ng/mL, respectively ( $P < 0.005$ ). Levels of ARG in tumor tissue homogenates from 15 CRC patients were assayed using the same method. Results showed that levels were two-fold greater than those found in normal mucosae ( $1.74 \pm 0.31$  pg/g vs.  $0.77 \pm 0.09$  pg/g,  $P < 0.005$ ) [14]. A study conducted by del Ara et al. [15] demonstrated that the activity of ARG in CRC tumor tissues was significantly increased, compared with that in normal mucosae ( $87 \pm 7.7$

# Downregulation of ARG2 inhibits CRC cell growth



## Downregulation of ARG2 inhibits CRC cell growth

**Figure 2.** Biological function of ARG2 in CRC cell lines. A. Expression of ARG2 protein in three CRC cell lines (Caco2, HT29, and HCT116), determined by Western blotting. HT29 and Caco2 cells expressed higher levels of ARG2 than HCT116 cells. HT29 cells were selected for subsequent experiments; B. Expression of ARG2 mRNA in the above three CRC cell lines conformed to the expression of ARG2 protein; C. Cells were divided into five groups and the transfection efficacy of three siRNAs was determined by RT-qPCR. Moreover, siRNA\_2 exhibited the highest interfering efficiency; D. Proliferation of HT29 cells was significantly suppressed by the silencing of ARG2, as demonstrated by Cell Counting Kit-8 assays; E. Cell cycle analysis: siRNA-mediated silencing of ARG2 increased the ratio of HT29 cells in G1 phase and significantly decreased the ratio of HT29 cells in S or G2 phase; F. Knockdown of ARG2 significantly increased the ratio of apoptotic cells, as shown by flow cytometry. \*,  $P < 0.05$ , \*\*,  $P < 0.01$ , \*\*\*,  $P < 0.001$ . ARG, arginase.



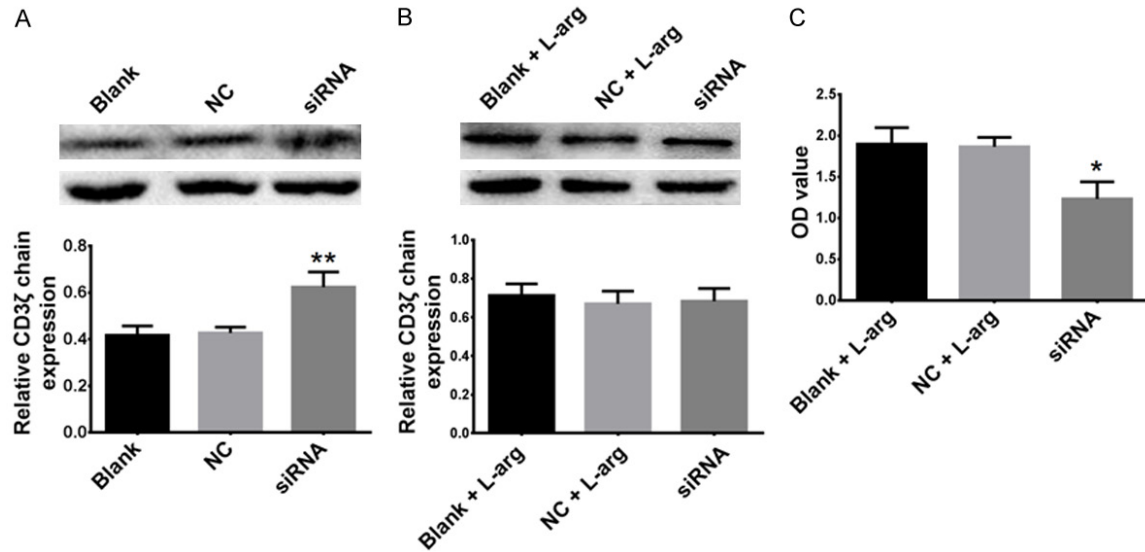
**Figure 3.** Impact of ARG2 on cell migration and invasion. A. Cell migration was assessed through wound healing assays. Silencing of ARG2 significantly attenuated the migration ability of HT29 cells; B. Cell invasion was assessed using a Matrigel-coated Transwell system. The number of cells that penetrated the membrane was significantly decreased after silencing of ARG2. \*\*,  $P < 0.01$ .

U/g vs.  $40.7 \pm 3.3$  U/g). However, no differences in serum ARG activity were found between CRC patients and healthy controls. Additionally, Porembaska et al. [16] prospectively studied serum ARG activity in 40 CRC and 100 CRCLM patients, before and after surgery. They followed-up these patients for 3 years. Results showed that preoperative serum ARG activity was higher in CRC and CRCLM patients, compared with healthy controls, and returned to a normal level following tumor resection. Furthermore, serum ARG activity was also low in patients free of tumor recurrence and was significantly elevated in patients with recurrent tumors. These results are consistent with find-

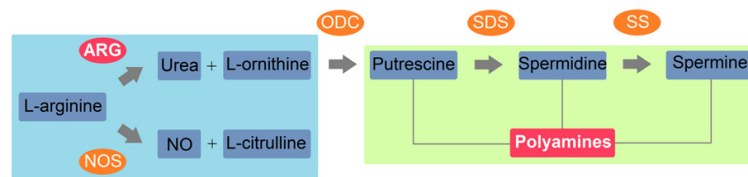
ings reported by Graboń et al. [17] in 2009. In addition, elevated ARG levels in serum and/or tumor tissues have been detected in patients with gallbladder cancer [22], prostate cancer [23] and non-small-cell lung cancer [24, 25], compared with healthy controls and/or normal tissues. These results indicate the important roles of ARG during tumor development.

However, the abovementioned studies either did not clarify the exact ARG subtype or only examined the total enzyme activity of ARG. Thus, whether ARG1 or ARG2 is expressed in CRC tumor tissues remains unknown. Based on a literature review of IHC staining results, it was





**Figure 4.** Effects of ARG2 on expression of the CD3ζ chain in tumor-infiltrating T-cells. HT29 cells were cocultured with Jurkat T-cells in a Transwell system to mimic the tumor microenvironment. A. Silencing of ARG2 significantly increased expression of the CD3ζ chain in the co-cultured Jurkat T-cells; B. L-arginine was added to the two control groups (Blank, NC) and expression of the CD3ζ chain showed no differences among the groups; C. After 72 hours of co-culturing with L-arginine supplementation, the control groups showed higher cell proliferation than the siRNA groups. \*,  $P < 0.05$ , \*\*,  $P < 0.01$ . ARG, arginase.



**Figure 5.** The metabolism of L-arginine was catalyzed by a series of enzymes, including ARG. ARG, arginase; ODC, ornithine decarboxylase; SDS, spermidine synthetase; SS, spermine synthetase; NO, nitric oxide; NOS, nitric oxide synthase.

found that ARG1 is not expressed in primary or metastatic CRC [18-20]. Further analysis of the public database CRN showed that expression of ARG2 mRNA was significantly higher in CRC tumor tissues, compared with normal mucosae. Hence, ARG2 might be a novel oncogenic target in CRC.

*In vitro* experiments have revealed that ARG2 enhances proliferation and invasion abilities of some tumor cells [8-10]. Hence, the biological function of ARG2 in CRC was investigated in the present study. First, expression of ARG2 in three CRC cell lines, HCT116, Caco2, and HT29, which have been reported to exhibit high ARG activity [13, 26, 27], was assessed by Western blotting and RT-qPCR. HT29 was selected as

the target cell line for subsequent experiments and siRNAs were employed to block ARG2 expression. The proliferation ability of HT29 cells, as shown by CCK8 assays, was clearly attenuated after knockdown of ARG2. This effect might be partially due to alterations in the cell cycle and apoptosis, as indicated by the finding that silencing of

the ARG2 gene in HT29 cells significantly increased the ratio of cells in the G1 phase and apoptotic cells. However, it reduced the ratio of cells in the S or G2 phase. Moreover, results showed that ARG2 promoted migration and invasion abilities of HT29 cells. Results indicate that ARG2 is a pro-tumor factor in CRC and might play a vital role during tumor development. However, the mechanisms through which ARG2 promotes tumor growth have not been fully highlighted. However, the following two aspects are considered pertinent: (1) The main product of ARG2, L-ornithine, is a precursor of polyamines, including putrescine, spermidine, and spermine. They actively participate in cell proliferation, differentiation, and malignant transformation, possessing strong tumor-pro-

moting abilities [8, 9, 13]; and (2) ARG2 competes with NOS for co-substrate L-arginine, which leads to a reduction in the generation of NO. This is a two-sided small molecule that can induce cytotoxicity through reactive oxygen species/reactive nitrogen species at high concentrations (mM) and mediate angiogenesis and immunosuppression at low concentrations ( $\mu$ M) [8, 28, 29].

Components of the host immune system act as key driving factors during tumor formation and development. Immune therapy via checkpoint blockades, including anti-PDL1/PD1 (programmed death ligand 1/programmed death 1) and anti-CTLA-4 (cytotoxic T lymphocyte-associated antigen) agents and chimeric antigen receptor-T cells (CAR-T), have achieved exciting success in recent years. They have been acknowledged as noteworthy in the domain of tumor therapy. As primary immune cell populations within the tumor microenvironment, tumor-associated macrophages, regulatory dendritic cells, and myeloid-derived suppressor cells can secrete high levels of ARG1. This has been identified as one of their basic features [21, 30, 31]. ARG1 depletes extracellular L-arginine and lowers its concentrations within the tumor microenvironment. This ultimately leads to a reduction in expression of the CD3 $\zeta$  chain in tumor-infiltrating T-cells. The CD3 $\zeta$  chain is an indispensable component of the T-cell receptor complex. Disruption of CD3 $\zeta$  expression might impact T-cell functions, which could manifest through reduced T-cell proliferation and cytokine production rates and weakened cytotoxicity. These effects may eventually impair the anti-tumor effects of the host immune system [32-34]. However, the roles and significance of ARG2 in tumor immunity have not been investigated as frequently as those of ARG1. In the present study, CRC cells were co-cultured with Jurkat T-cells to mimic the tumor microenvironment. Expression of the CD3 $\zeta$  chain in the T-cells was detected. Results demonstrated that T-cells co-cultured with CRC cells in which ARG2 was knocked-down exhibited significantly increased expression of the CD3 $\zeta$  chain. Furthermore, this effect was reversed by external supplementation with L-arginine. It did not depend on alterations in the proliferation of HT29 cells, indicating the possibility of ARG2-induced immune dysfunction in CRC. Therefore, inhibition of this pathway might have therapeutic effects, in

part, by rescuing the host anti-tumor immune functions.

Despite the pro-tumorigenic characteristics of ARG2 identified in most prior studies, its association with the prognosis of patients with different tumor types has not been consistently established. A study of 120 lung cancer patients conducted by Rotondo et al. [35] demonstrated that, despite its overexpression in lung cancer cells, ARG2 neither induced immune evasion or disease progression nor influenced patient survival. Several factors might account for these discrepancies. First, although positive results have been obtained in *ex vivo* experiments, it is difficult to deplete L-arginine within the tumor microenvironment *in vivo* because L-arginine can be continuously provided by the peripheral circulation or produced through protein metabolism. Rotondo et al. [35] cultured tumor cells isolated from tumor tissues, together with activated autologous T-cells. They found that proliferation and function levels of T-cells were not impacted. In another study, Trad et al. [36] demonstrated that, despite a 40% reduction in the amount of L-arginine in the culture medium, its concentration remained higher than 50  $\mu$ M. This was greater than the minimum concentration (40  $\mu$ M) required for suppressing T-cells. Second, NOS is considered indispensable for ARG2-induced T-cell apoptosis because NOS catalyzes the production of superoxide ions under low-L-arginine conditions. Superoxide ions then interact with NO to generate peroxynitrite. This inhibits the phosphorylation of protein tyrosine kinases induced by activated T-cells through the nitration of tyrosine residues, thereby initiating T-cell apoptosis. However, as shown by IHC, the examined lung cancer cells did not express NOS and nitrated tyrosine was not detected within tumor tissues. Therefore, under these conditions, T-cells were likely not affected by ARG2 alone. Furthermore, the transformation from L-arginine to polyamines is mediated by a series of enzymes, including ARG2, as well as downstream ODC, SDH, and SS. Variations in expression patterns (yes/no, strong/weak) of these enzymes in different tumors and patients might also contribute to the ultimate effects exerted by ARG2 *in vivo*. Further in-depth studies are necessary to clarify the exact clinical relevance of ARG2.

In conclusion, ARG2 was upregulated in CRC. It promoted the proliferation and invasion of CRC

cells and decreased expression of CD3 $\zeta$  in co-cultured T-cells. Therefore, ARG2 is a potential novel target for the prognostic evaluation and personalized treatment of CRC patients. The present study provides a basis for further research on this topic.

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## Disclosure of conflict of interest

None.

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