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Upregulated STAT3 and RhoA signaling in colorectal cancer (CRC) regulate the invasion and migration of CRC cells

G.-Y. ZHANG, W.-H. YANG, Z. CHEN

Department of General Surgery, Zhengzhou Central Hospital Affiliated to Zhengzhou University, Zhengzhou, China

Abstract. – OBJECTIVE: We aimed to reveal the expression and activation of signal transducers and activators of transcription 3 (STAT3) and RhoA/Rho-associated coiled-coil forming kinase 1 (ROCK1) signaling in CRC tissues, and to investigate the regulatory role of STAT3 and RhoA signaling in the invasion and migration of colorectal cancer cells.

MATERIALS AND METHODS: We examined the expression of STAT3, RhoA and ROCK1 in CRC tissues with real-time PCR and Western blotting methods. And then we examined the interaction between STAT3 and RhoA/ROCK1 signaling in CRC HT-29 cells with gain-of-function and loss-of-function strategies. In addition, we determined the regulation by STAT3 and RhoA/ROCK1 on the invasion and migration of CRC HT-29 cells.

RESULTS: Our study demonstrated a significant upregulation of RhoA and ROCK1 expression and STAT3-Y705 phosphorylation in 32 CRC specimens, compared to the 17 normal CRC tissues. Further study demonstrated there was a coordination between STAT3 and RhoA/Rock signaling in the HT-29 cells. Moreover, STAT3 knockdown or RhoA knockdown significantly repressed the migration and invasion in HT-29 cells and vice versa.

CONCLUSIONS: STAT3 and RhoA signaling regulate the invasion and migration of CRC cells, implying the orchestrated and oncogenic roles of STAT3 and RhoA/ROCK1 signaling in CRC.

Key Words:
STAT3, RhoA, ROCK1, Colorectal cancer, Metastasis.

Introduction

Colorectal cancer (CRC) is one of the most lethal cancer, and the third diagnosed cancer. CRC causes about 1.2 million new cases and 608, 000 deaths every year worldwide[1,2] and also leads to huge economic and social costs[3,4]. Accumulating prognostic variables have been found to correlate with CRC[5-8]. The majority of studies about the CRC pathogenesis have been focused on the inflammatory microenvironment where the normal, premalignant intestinal epithelial and tumor cells lived in. The pro- or anti-tumor effects of immune factors have been indicated to play central roles in cancer research including CRC[9].

Multiple immune markers linking to tumor cells are regulated by cytokines which make the oncogenic factors activating. Signal Transducers and Activators of Transcription 3 (STAT3) is such kind of important mediator[10]. It is consist of N-terminal domain, coiled-coil domain, DNA-binding domain, Src homology 2 and transactivation domain[11,12] and plays versatile roles in cell survival, apoptosis, migration, and differentiation[12]. In terms of cancer inflammation and regulators of the tumor microenvironment, STAT3 takes intrinsic activator effects[13,14]. STAT3 activation also is induced by many cytokines and growth factors in the tumor microenvironment, including IL-6, IL-10, IL-22, EGF, IL-23, as well as STAT3-dependently inducted of cell surface growth factor and cytokine receptors or cytoplasmic proto-ontogenesis markers[15,16]. Moreover, the activated STAT3 could induce the expression of many oncogenic genes such as *Bcl-XL, Bcl-2,* and *Mcl-1* as well as *cyclinD1* and *PCNA* to play roles in cell cycle progression and suppression of apoptosis[13,17]. Therefore, on the one hand, STAT3 could response to immune regulators; on the other hand, it is responsible to activate downstream oncogenic genes.

RhoA belongs to Rho GTPases superfamily and carries out various oncogenic effects, by binding to its immediate downstream target, Rho-associated coiled-coil forming kinase 1

Corresponding Author: Wenhui Yang, MD; e-mail: wenhuizzchyang@sina.com

(ROCK1), in response to an activator[18-20]. Dysregulation of RhoA and ROCK1 has been reported to promote the cancer progression, metastasis and prognosis [20]. In terms of CRC, upregulated RhoA and ROCK1 have been indicated to stimulate the proliferation, migration, invasion and metastasis[21,22]. Furthermore, the RhoA/ROCK signaling has been shown to response to a variety of stimulators. Recent investigations[23] have clearly revealed the hypoxia-inducible factor induced by intratumoral hypoxia triggered the RhoA/ROCK1 signaling in breast cancer. Notwithstanding findings revealed the common functions of these molecular on cell migration and apoptosis[24,25]; little is known about the interactions of STAT3 and RhoA/ROCK1 signaling in cancers, particularly in CRC.

In the present study, we focused on the expression of STAT3, RhoA and ROCK1 in CRC, and then investigated the interactions of these molecules in CRC cells. We also monitored the effects on association of STAT3 and RhoA/ROCK1 signaling and the effects of these molecular on invasion and migration of CRC cells. All our findings demonstrated that both STAT3 and RhoA signaling regulated the invasion and migration in CRC cancer cells. Firstly, it has been reported the positive regulatory association of STAT3 and RhoA/ROCK signaling in CRC.

Materials and Methods

Human CRC Tissues

32 colorectal cancer tissues were obtained from patients after undergoing endoscopy and surgical resection. And 17 normal colon tissues were acquired from patients with non-tumor diseases taking long colon and vascular malformation. Human beings study has been approved by the institutional Ethics Committee of our hospital. Each human CRC specimen in the present study was allowed by its donator for scientific research. All the human tissues were formaldehyde-fixed paraffin-embedded (FFPE) for pathological study or stored at -80 °C immediately for protein or mRNA isolation.

Cells, Reagents and Cell Treatments

The human colorectal adenocarcinoma cancer cells (HT-29) were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). HT-29 cells were cultured in McCoy's 5A (Modified) Medium (Thermo Scientific, Rockford, IL, USA) containing 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA), 100 units/ml penicillin and 100 mg/ml streptomycin (CSPC Pharmaceutical Group Limited, Shijiazhuang, China). FHC cells were grown in the DMEM (Invitrogen, Carlsbad, CA, USA), which was supplemented with 25 mM N'-2-Hydroxyethylpiperazine-N'-2 ethanesulfonic acid (HEPES) (JRH Biosciences, KS, USA), 5 mg/ml insulin (Sigma-Aldrich, St. Louis, MO, USA), and 100 ng/ml hydrocortisone (Sigma-Aldrich, St. Louis, MO, USA). Both types of cells were cultured in a humidified incubator containing 5% CO_2 at 37 °C. pRC/CMV-STAT3-FLAG (STAT3 up), and control pcRC/CMV (Control up) plasmids were purchased from Promega (Promega, Madison, WI, USA). Plasmids encoding wild-type RhoA (pcDNA3-His-RhoA) was purchased from SIGMA (Sigma-Aldrich, St. Louis, MO, USA). Cell Line Nucleofector Kit T (Lonza Walkersville Inc., Walkersville, MD, USA) was conducted to transfect vectors into HT-29 cells. RhoA knockdown and STAT3 knockdown were conducted by using RNAi (5'- CAG AUA CCG AUG UUA UAC U-3') (RhoA KD) and shRNA STAT3/Puro (Sigma-Aldrich, St. Louis, MO, USA) (STAT3 KD) lentiviral vectors respectively. shRNAi MISSION Non-Target shRNA Control/Puro (Sigma-Aldrich, St. Louis, MO, USA) was adopted as a negative control of gene knockdown (control KD). Y-27632 was purchased from Tocris Bioscience (Tocris Bioscience, Bristol, UK) to inhibit the ROCK1[26].

RNA extraction and Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNA of tissue samples was extracted by using the RecoverAll Total Nucleic Acid Isolation Kit (Ambion, Austin, TX, USA). The RNeasy kit (Qiagen, Valencia, CA, USA) was utilized to extract total mRNA from cells. qRT-PCR was performed on ABI 7500 with SYBR Premix Ex Taq™ Kit (Takara, Tokyo, Japan). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was denoted as the internal control. Data were normalized by using $2^{-\Delta\Delta Ct}$ method[27] as relative quantification. The used primers were as following: Forward primer for STAT3: 5'-GGG TGG AGA AGG ACA TCA GCG GTA A-3', Reverse primer for STAT3: 5'-GCC GAC

AAT ACT TTC CGA ATC C-3'. Forward primer for RhoA: 5'-CCT ATC CTA CAG GCT GCT GAA-3', Reverse primer for RhoA: 5'-TAA GCC CAC CAG CTT AAT GG -3'. Forward primer for ROCK1: 5'-CCC TCT TAC ACC GGG CGT-3', Reverse primer for ROCK1: 5'-CTG GGT TGA AGC AAT TCC CC-3'. Forward primer for GAPDH: 5'-GGA GTC AAC GGA TTT GGT C-3', Reverse primer for GAPDH: 5'-GGA ATC ATT GGA ACA TGT AAA C-3'.

Western Blotting Assay

Cellular protein samples were extracted with Nuclear/Cytosol Fractionation Kit (BioVision, San Diego, CA, USA) and were supplemented with Protease Inhibitor Cocktail (Abcam, Cambridge, UK) (ab65621). Then, the protein samples were subject to 10% SDS-PAGE electrophoresis, to the transfer onto nitrocellulose membranes (Millipore, Bedford, MA, USA) after the electrophoresis. Immunoblotting were performed by using the polyclone rabbit antibody against human STAT3 (Cell Signaling Technology Inc., Danvers, MA, USA), against phosphorylated STAT3 (Tyr705) (Cell Signaling Technology Inc., Danvers, MA, USA), against RhoA (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or against -actin antibody (Sigma-Aldrich, St. Louis, MO, USA). Horseradish peroxidase-linked goat anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA, USA) and ECL detection systems (Thermo Scientific, Rockford, IL, USA) were utilized to visualize the specific binding.

Migration and Invasion Assay

Migration assay of HT-29 cells was performed by transwell migration assays. In brief, HT-29 cells were grown to 1×10^4 cells in Transwell migration chambers (Costar; Corning, Lowell, MA, USA) which contains upper chamber with noncoated membrane (8 μm pore size; Millipore, Bedford, MA, USA) and lower chamber contained media with 20% FBS as a chemoattractant. Cells in the upper chamber were discarded After 24 h culture and migration cells were detected by microscope in the lower chamber. Invasion assay was the same as the Transwell migration assays, expect for 2×10^4 cells on the upper chamber coated with Matrigel (Sigma-Aldrich, St. Louis, MO, USA).

Statistical Analysis

The data are normalized as mean ± standard deviation. Student *t*-test was performed to compare differences in two groups. Comparisons among multiple samples were made by ANOVA. Statistical analyses were conducted by SPSS 17.0 software (IBM SPSS, Armonk, NY, USA). Statistically significant difference was considered when $p < 0.05$ or less.

Results

Overexpression of RhoA and ROCK1 and the promoted STAT3 phosphorylation in colorectal cancer (CRC) tissues

The essential functions of STAT3 and RhoA signaling on the regulation in cancers have been reported in previous studies[13,14,18]. However, such regulatory roles by these markers were not clear on CRC. The expression of STAT3, RhoA and ROCK1 was assayed by qRT-PCR in 32 CRC tissues, compared to that in 17 normal colorectal tissues. It was showed the expression of RhoA was increased significantly in CRC tissues, by contrast to that in normal colon tissues (1.653 ± 0.1311 *vs.* 1.000 ± 0.1085, $p = 0.0018$) (Figure 1A). And the relative mRNA level of ROCK1 was 1.879 ± 0.1707, also markedly higher than 1.000 ± 0.1239 ($p = 0.0011$, Figure 1B). However, the mRNA level was not significantly different between the CRC and normal groups ($p = 0.500$, Figure 1C). Moreover, there was a significant correlation between the mRNA levels of RhoA and ROCK1 in human CRC tissues ($R^2 = 0.3900$, $p < 0.0001$, Figure 1D).

We further examined the protein levels of RhoA, ROCK1 and STAT3 and the level of STAT3 phosphorylation in these CRC specimens. Western blotting results (Figure 2A) demonstrated that both RhoA and ROCK1 were markedly higher in protein level in CRC specimens (N = 15) than in the control group (N = 8) ($p < 0.01$ respectively, Figure 2B). However, the STAT3 in protein level and the phosphorylated STAT3 at Ser 727 were not significantly different between the two groups (Figure 2A and Figure 2C). However, the phosphorylated STAT3 at Tyr 705 was markedly higher in the CRC group than in the normal group ($p < 0.01$, Figure 2A and Figure 2D). All these data revealed that the overexpression of RhoA and ROCK1 and the promotion of STAT3-Tyr 705 phosphorylation in CRC cancer tissues.

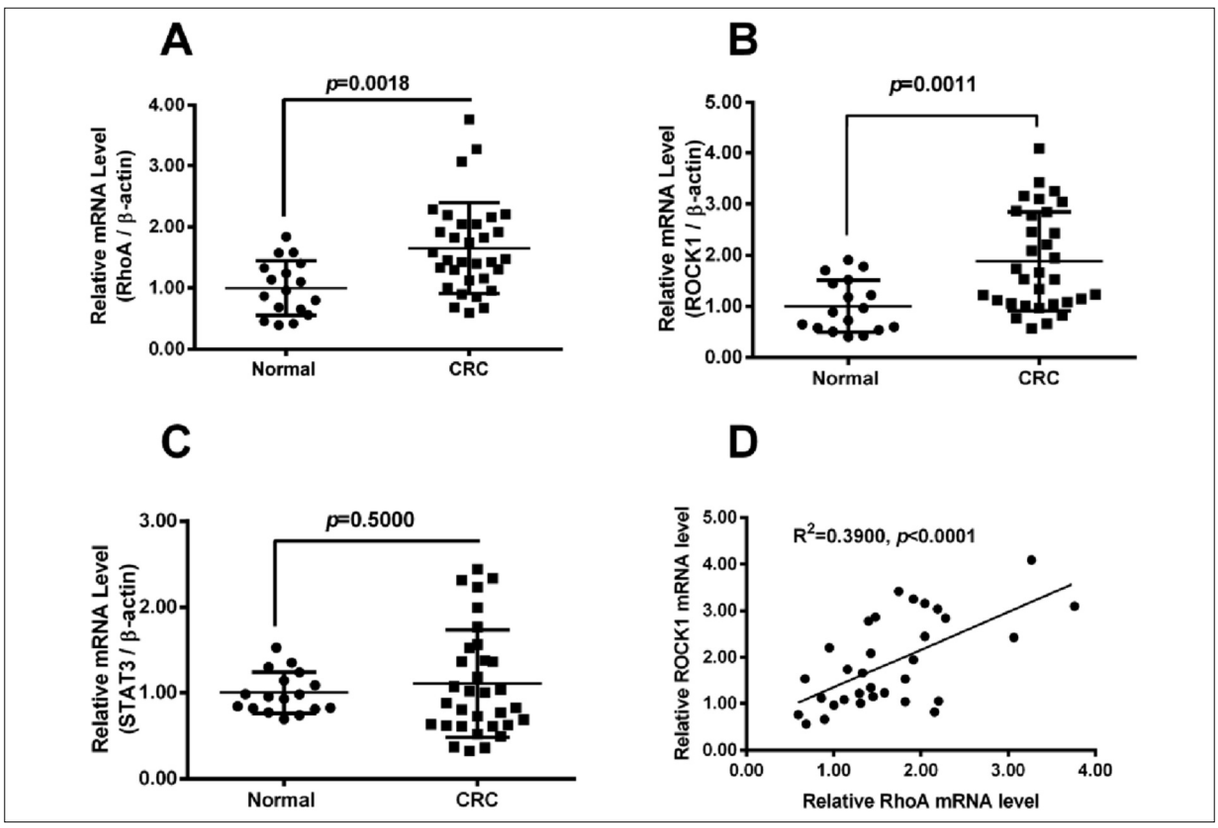

Figure 1. mRNA levels of RhoA, ROCK1 and STAT3 in colorectal cancer (CRC) or normal colorectal tissues. The expression of RhoA, ROCK1 and STAT3 in mRNA levels in CRC tissues (N = 32) or normal colon tissues (N = 17) by relative qRT-PCR. The mRNA levels of RhoA ***(A)***, ROCK1 ***(B)*** and STAT3 ***(C)*** were presented as relative levels to β-actin. ***D,*** The correlation of RhoA mRNA level with ROCK1 mRNA level in CRC tissues. Statistical significance was considered when $p < 0.05$ or less.

STAT3 Overexpression Upregulates the Expression of RhoA and ROCK1 in Human Colorectal Adenocarcinoma Cancer Cells (HT-29)

In order to reveal if dysregulation of STAT3 correlated with the RhoA/ROCK1 signaling in CRC cells, the regulation of STAT3 on the expression of RhoA/ROCK1was examined with gain-of-function and loss-of-function approaches. Firstly, the STAT3 knockdown and overexpression were performed in HT-29 cells. As shown in Figure 3A, the overexpression and the knockdown of STAT3 was confirmed in mRNA level ($p < 0.05$, $p < 0.01$ or $p < 0.0001$). And the western blotting results also confirmed the upregulation of downregulation of STAT3 in HT-29 cells ($p < 0.01$ or $p < 0.0001$, Figure 3B and 3C). Also, though the relative level of phosphorylated STAT3 to STAT3 was not markedly promoted by the STAT3 knockdown or overexpression, the relative level of phosphorylated STAT3 to -actin was significantly up- or down-regulated by the STAT3 overexpression or knockdown ($p < 0.01$ respectively, Figure 3B and 3D).

Subsequently, the expression of RhoA and ROCK1 was investigated in the STAT3-overexpressed of -knocked HT-29 cells. Figure 3E demonstrated that both RhoA and ROCK1 were markedly upregulated by the STAT3 overexpression in the STAT3-overexpressed HT-29 cells than that in the control HT-29 cells ($p < 0.01$ respectively). All these findings indicated the positive promotion of STAT3 to the expression of RhoA and ROCK1 in CRC cells.

RhoA Promotes the STAT3 Phosphorylation in HT-29 Cells

In a reciprocal experiment, we investigated the regulation by RhoA overexpression or knockdown on the expression or phosphorylation of STAT3 in HT-29 cells. As shown in Figure 4A, there was no significant difference in the mRNA

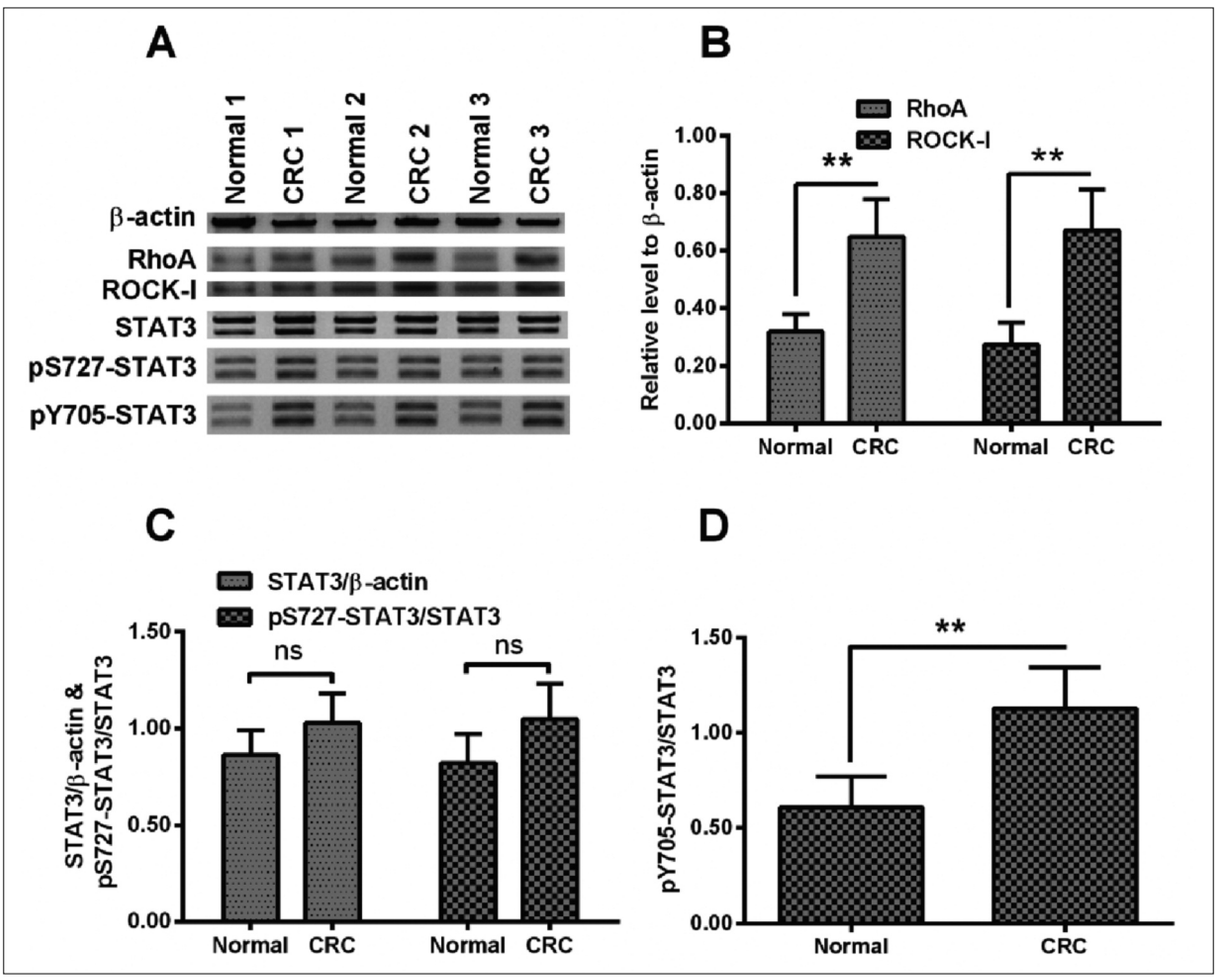

Figure 2. Protein levels of RhoA, ROCK1 and STAT3 (with or without phosphorylation) in colorectal cancer (CRC) or normal colorectal tissues. The expression of RhoA, ROCK1 and STAT3 (with or without phosphorylation) in protein levels in CRC tissues (N = 15) or normal colon tissues (N = 8) by western blotting assay ***(A)***. The protein levels of RhoA and ROCK1 ***(B)*** and STAT3 (with or without Ser 727 phosphorylation) ***(C)***, STAT3 (with Tyr 705 phosphorylation) were presented as relative levels to β-actin or to STAT3. Statistical significance was considered when $p < 0.05$ or less. $^{**}p < 0.01$, ns: no significance.

level of STAT3 among the groups of HT-29 cells, post RhoA overexpression or not (RhoA Up and Mock), post RhoA knockdown or not (RhoA KD and Ctrl KD), though the western blotting results confirmed the overexpression or knockdown of RhoA in protein level ($p < 01$ or $p < 0001$, Figure 4B and 4C). And the non-significantly different protein level of STAT3 reconfirmed the non-regulation by RhoA on the STAT3 expression. However, we found the overexpression of RhoA promoted the STAT3 phosphorylation via the western blot analysis ($p < 0.05$ or $p < 0.01$, Figure 4E). Taken together, RhoA promotes the STAT3 phosphorylation in HT-29 cells. Our data illustrated the coordinated effects of STAT3 and RhoA/Rock signaling in the HT-29 cells.

STAT3 and RhoA/ROCK1 Signaling Regulated the Migration and Invasion of CRC Cells

To investigate the effect of STAT3 and the interactions of STAT3 and RhoA/ROCK1 signaling on tumor metastasis, we observed the migration and invasion of the HT-29 cells, post the overexpression or knockdown of STAT3 or RhoA, by Transwell migration/invasion assays. The differences in migration among cells with a STAT3 knockdown, STAT3 overexpression and without treatment were monitored. As shown in Figure 5A, the upregulated STAT3 promoted the HT-29 cells migration significantly ($p < 0.05$, STAT3 Up *vs.* Control Up). Moreover, there was dramatically lower migration in

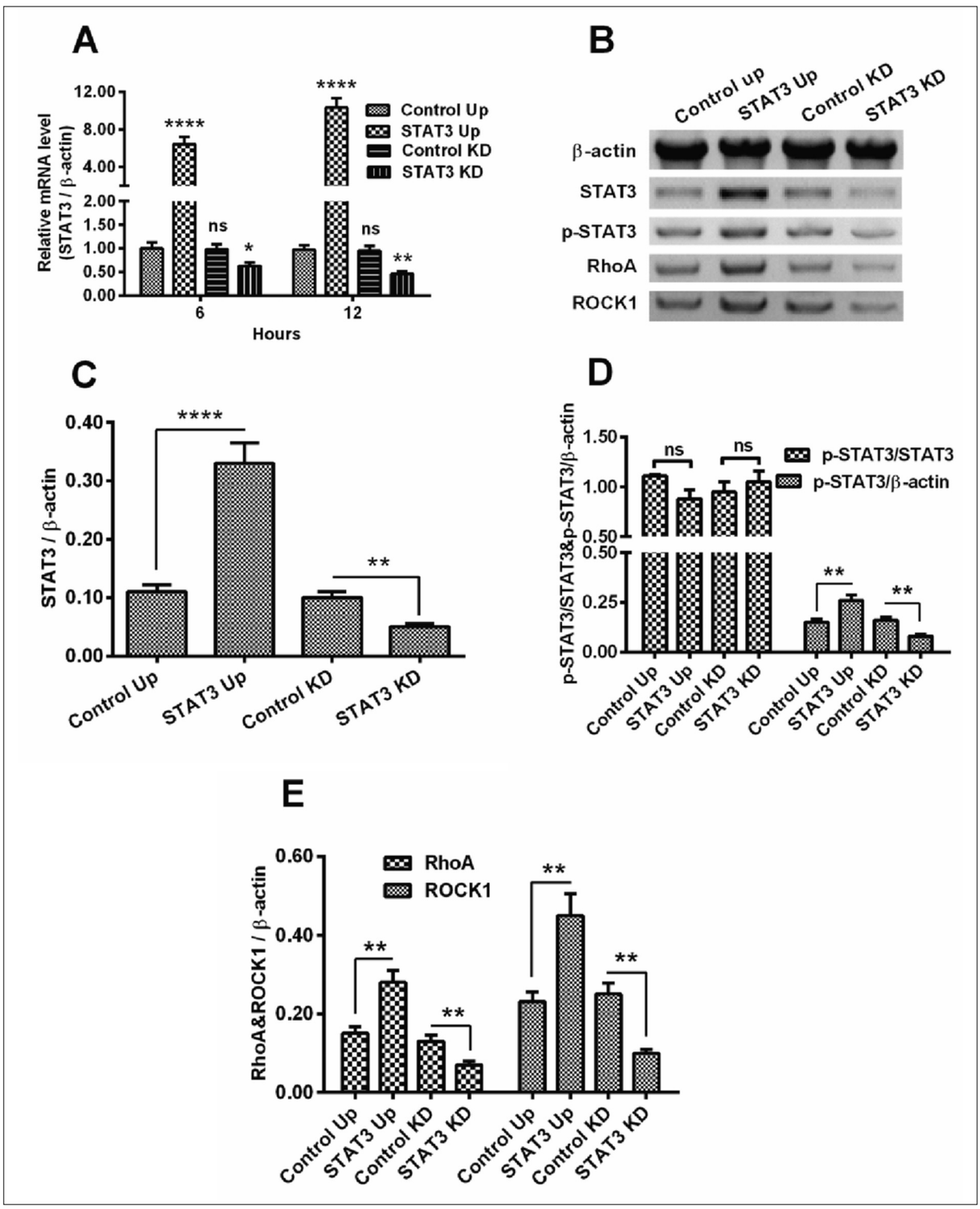

Figure 3. STAT3 positively modulated the RhoA signaling in HT-29 cells. ***A,*** The mRNA level of STAT3 in HT-29 cells, in which STAT3 was overexpressed (STAT3 Up) or not (Control Up), STAT3 was knocked down (STAT3 KD) or not (Control KD) at 6 or 12 hour post treatment; ***B,*** Western blot analysis of STAT3 with or without Tyr705 phosphorylation, RhoA and ROCK1 in protein levels in the above-mentioned groups of HT-29 cells at 24 hour post treatment; ***C*** to ***E,*** Relative levels of STAT3 to β-actin ***(C)***, of STAT3 with phosphorylation to STAT3 without phosphorylation or to β-actin ***(D)*** or of RhoA or ROCK1 to β-actin ***(E)*** were presented. * represented $p < 0.05$, ** represented $p < 0.01$ or **** represented $p < 0.0001$, ns: no significance.

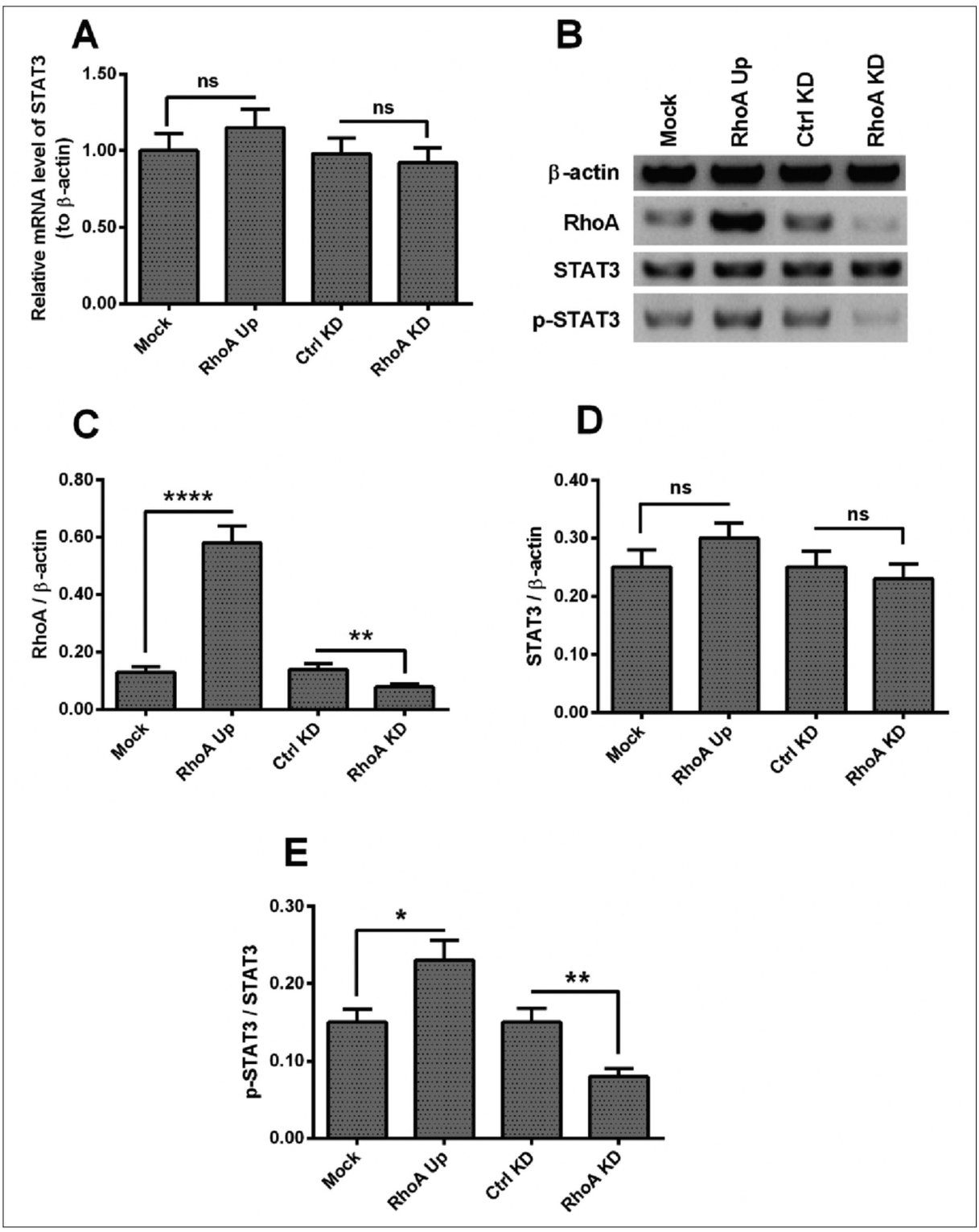

Figure 4. Positive regulation of RhoA overexpression in STAT3 phosphorylation in HT-29 cells. ***A,*** The mRNA level of STAT3 in HT-29 cells, in which RhoA was overexpressed (RhoA Up) or not (Mock), RhoA was knocked down (RhoA KD) or not (Control KD) at 12 hour post treatment; ***B,*** Western blot analysis of RhoA, STAT3 with or without Tyr705 phosphorylation, in protein levels in the above-mentioned groups of HT-29 cells at 24 hour post treatment; ***C*** to ***E,*** Relative levels of RhoA to β-actin ***(C)***, of STAT3 to β-actin STAT3 ***(D)***, or of STAT3 with phosphorylation to STAT3 without phosphorylation ***(E)*** were presented. * represented $p < 0.05$, ** represented $p < 0.01$ or **** represented $p < 0.0001$, ns: no significance.

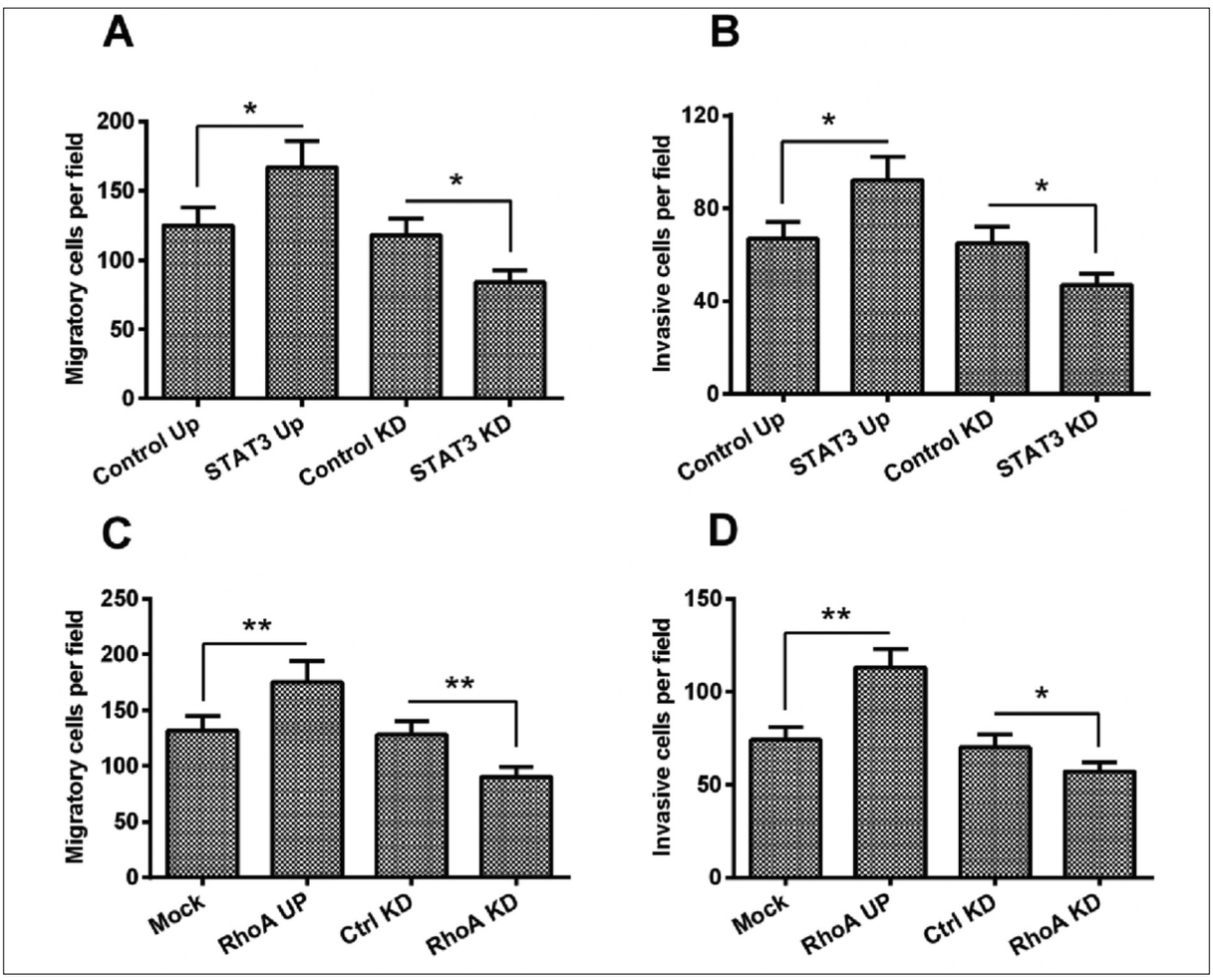

Figure 5. STAT3 and RhoA/ROCK1 signaling regulated the migration and invasion of CRC cells. The number of tumor cells was calculated to compare the differences in migration and invasion of HT-29 cells with indicated treatments by using transwell migration/invasion assays. The effects of STAT3 on cells migration ***(A)*** and invasion ***(B)***, the effects of RhoA on cells migration ***(C)*** and invasion ***(D)*** were presented respectively. The three individual experiments were performed. Statistical significance was showed as $*p < 0.05$ and $**p < 0.01$.

STAT3 knockdown HT-29 cells than that in HT-29 without treatment ($p < 0.05$, STAT3 KD *vs.* Control KD). In addition, the effect of STAT3 on invasion was consistent with that on migration, as shown in Figure 5B ($p < 0.05$, STAT3 KD *vs.* Control KD, or STAT3 KD *vs.* Control KD). The data demonstrated that the up- or down-regulation of STAT3 could promote or inhibit the metastasis of HT-29 cells, in which RhoA and ROCK1 were up- or down-regulated. Moreover, we assessed the effect of RhoA up- or down-regulation on migration and invasion in HT-29 cells. As showed in Figure 5C and 5D, the RhoA overexpression stimulated, whereas the RhoA knockdown could markedly attenuate the metastasis ability of HT-29 cells. Owing to above study on the highly positive connection of STAT3 and RhoA/ROCK1 signaling, it declared STAT3 coordinated RhoA signaling to regulate the invasion and migration of CRC cells.

Discussion

Dysregulation of RhoA and ROCK1 has been reported to regulate the cancer progression, metastasis and prognosis[20]. STAT3 takes intrinsic activation on cancer inflammation and regulators of the tumor microenvironment[13,14]. In this research, we found the upregulated expression of RhoA and ROCK1 and the upregulated phospho-

rylation of STAT3 Tyr 705 in CRC tissues, compared to that in normal CRC tissues. However, the STAT3 in protein level and the phosphorylation of STAT3 Ser 727 were not markedly different between the CRC and normal groups. These data illustrated the hyperregulation of STAT3 phosphorylation and RhoA/ROCK1 expression presented in CRC. It also meant these molecules might take functions on tumorigenesis or cancer inflammation.

Our study *in vitro* in HA-29 cells found the feedback regulation of RhoA/ROCK1 expression and STAT3 phosphorylation. The overexpression/hyperphosphorylation of STAT3 promoted the expression of RhoA and ROCK1, and the upregulated RhoA expression also upregulated the STAT3 Tyr 705 phosphorylation in HT-29 cells. It implies the connection of RhoA/Rock activation and the STAT3 phosphorylation, as is also indicated in hepatoma cells[25]. The fact that RhoA activation led to phosphorylation and activation of STAT3 has also been confirmed by other study[26]. In addition, Lin et al[12] found the IL-6, one of the main regulators of STAT3 activation, induced AGS gastric cancer cell invasion via activation of the c-Src/RhoA/ROCK signaling pathway[28]. Here, we found the STAT3 positively regulated the expression of RhoA and ROCK1 in CRC cells by using gain-of-function and loss-of-function approaches. It supplied the evidence supporting the relationships between STAT3 and /RhoA/ROCK signaling. The current work firstly revealed the coordinated effects of STAT3 and RhoA/Rock signaling in CRC.

Invasion and metastasis lead to dissemination of cancer cells that cytoskeletal reprogramming played a critical role in the members of the Rho family acts as molecular switches to control morphogenesis and movement of cells[23,29]. Clinical features and data showed the high associations between increased ROCK1 and RhoA with poor prognosis in breast cancers[30,31]. Moreover, other markers such as PAI-1 also played a role in the cell behavior needed for amoeboid migration by maintaining the RhoA/ROCK1/MLC-P pathway activation in SW620 human colorectal cancer cells[32]. In addition, STAT3 is constitutively activated in many cancers and plays a pivotal role in tumor growth and metastasis[33]. In this report, upregulated STAT3 and RhoA could promote metastasis, while inhibiting STAT3 and RhoA could repress the metastasis. Our findings were consistent with the previous paper on roles of RhoA/ROCK1 signaling and STAT3 in cancer invasion and metastasis. There was a high association between RhoA/ROCK1 signaling and STAT3 to modulate the invasion and metastasis in CRC HT-29 cells.

Conclusions

In overall, STAT3 and RhoA signaling regulated the invasion and migration in CRC cells. It firstly revealed the coordinated effects of STAT3 and RhoA/Rock signaling and high associations between RhoA/ROCK1 signaling with STAT3 to modulate the invasion and metastasis in CRC.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

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