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MiR-198 affects the proliferation and apoptosis of colorectal cancer through regulation of ADAM28/JAK-STAT signaling pathway

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Abstract. – OBJECTIVE: Colorectal cancer (CRC) is a leading cause of cancer-related death worldwide. microRNA-198 (miR-198) was reported to be a tumor suppressive miRNA but its role in CRC is largely unknown. Thus, we aimed to investigate the role of miR-198 and its downstream signaling pathway in CRC.

PATIENTS AND METHODS: Quantitative Real-time PCR was conducted to measure miR-198 expression in human CRC cell lines (SW620, SW480 and HT29) and normal colon cell line (FHC). Using MTT, colony formation and flow cytometry assay, we investigated the effects of miR-198 on cell proliferation, colony formation and apoptosis. Luciferase activity reporter assay and Western blot assay were performed to validate the target of miR-198. Using Western blot assay, we detected the protein levels of Janus kinase (JAK)/signal transducer and activator of transcription (STAT) signaling pathway.

RESULTS: The results showed that miR-198 expression was significantly reduced in CRC cell lines compared with FHC. Overexpression of miR-198 inhibits CRC cell proliferation and colony formation but promotes apoptosis. Further study revealed ADAM metallopeptidase domain 28 (ADAM28) was a direct target of miR-198, and the overexpression of ADAM28 reversed the effects of miR-198 on cell behaviors. Besides that, miR-198 blocks the JAK/STAT pathway through regulating ADAM28.

CONCLUSIONS: These results collectively revealed miR-198 inhibited cell proliferation but promoted apoptosis through targeting ADAM28 and blocking JAK/STAT pathway in CRC cells.

Key Words:
MiR-198, Colorectal cancer, ADAM28, JAK, STAT.

Introduction

Colorectal cancer (CRC) ranks as the third leading cause of cancer incidence and the second leading cause of cancer death[1]. It was estimated over 1.8 million new CRC cases and 881,000 deaths will occur in 2018. Half of the CRC patients were diagnosed at advanced stage, and therefore results in the 5-year overall survival ranging from 12.5% to 70.4%[2]. Therefore, it is urgent to develop biomarkers that can help early diagnosis or cancer treatment. microRNAs (miRNAs) are non-coding RNAs able to target multiple protein-coding transcript and lead to translation inhibition[3]. The abnormal expression of miRNAs was reported to be closely associated with human diseases, especially cancer, because its role in regulating gene expression[4]. miRNAs were reported to have crucial roles in the progression of CRC and were found to have dual functions in CRC, either tumor suppressor or oncogene[5,6]. The tumor suppressor miRNA was frequently found downregulated in tumor and inhibited tumor progression through down-regulating oncogenes. miR-382 was revealed to be reduced expression in CRC tissues and cell lines, and its overexpression could inhibit CRC cell proliferation and migration through targeting the expression of specificity protein 1[7]. In addition, oncogenic miRNA mainly inhibits the expression of tumor suppressor genes to promote carcinogenesis. For instance, high miR-32 expression was associated with lymphatic invasion, metastasis, and correlated with patients' poor survival[8]. Moreover, bone morphogenetic protein 5 was iden-

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tified as a target of miR-32 and as a mediator for the stimulation effects of miR-32 on proliferation and migration[8]. ADAM metallopeptidase domain 28 (ADAM28) was reported to overexpressed in multiple human cancers including acute myeloid leukemia, breast cancer and lung carcinoma to function as oncogene[9-11]. With the antibody specific targeting ADAM28, it was found the usage of antibody could suppresses lung carcinoma cell growth and metastasis[11]. Moreover, ADAM28 could be downregulated by miR-552 to regulate CRC cell proliferation, migration, and clonogenicity[12]. Previous studies found miR-198 has crucial roles in human cancers[13-15]. However, its expression and role in CRC remain not fully understood. In this study, we investigated the expression pattern of miR-198 in CRC. The association between miR-198 and ADAM28 was validated by luciferase activity reporter assay and Western blot assay. Moreover, the effects of miR-198 on cell behaviors were investigated by cell counting kit-8 assay, colony formation assay and flow cytometry with the aim to clarify the role of miR-198 on the progression of CRC.

Materials and Methods

Cell Lines

Three CRC tumor cell lines (HT29, SW480, and SW620) and one normal colon epithelium cell line (FHC) were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). These cells were kept in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS, Invitrogen, Carlsbad, CA, USA) in a 37°C incubator containing 5% CO_2 humidified air.

Cell Transfection

miR-198 mimic (5'-GGUCCAGAGGGGAGAUAGGUUC-3') and negative control sequence (miR-NC, 5'-AUCAAUUGGUCUCAGUCGUCC-3') were purchased from Genechem (Shanghai, China). An ADAM28 expression vector with its open reading frame inserted into pcDNA3.1 plasmid (ADAM28-vector) and a NC-vector was purchased from GenScript (Nanjing, China). Cell transfection was conducted using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions with the following concentrations: miRNAs (50 pmol/ml), ADAM28-vector (2 mg/ml), or NC-vector (2 mg/ml).

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted with TRIzol reagent (Beyotime, Shanghai, China). Subsequently, complementary DNA was synthesized from extracted RNA using One Step PrimeScript miRNA cDNA Synthesis Kit (TaKaRa, Dalian, Liaoning, China). qRT-PCR was conducted at ABI Prism 7500 sequence detection system (Applied Biosystems, Foster City, CA, USA) using SYBR Premix Ex Taq (TaKaRa, Dalian, Liaoning, China). The relative expression of miR-198 was analyzed by the $2-\Delta\Delta Ct$ method and normalized to U6 small nuclear RNA (U6 snRNA). The primers used were as follows: miR-198: Forward 5'-GGTCCAGAGGGGAGAT-3', Reverse 5'-GAATACCTCGGACCCTGC-3'; U6 snRNA: Forward 5'-CTCGCTTCGGCAGCACATA-3', Reverse 5'-AACGATTCACGAATTTGCGT-3'.

Western Blot

Total protein was extracted by radio immunoprecipitation assay (RIPA) lysis buffer (Beyotime, Shanghai, China). Equal amounts samples were separated at 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membrane (Beyotime, Shanghai, China). Followingly, membranes were blocked with 5% fat-free milk before incubated with primary antibodies (anti-ADAM28: ab28292, anti-pJAK1: an203784; anti-JAK1: ab133666; anti-pSTAT3: ab76315; anti-STAT3: ab68153; anti-GAPDH: ab181602; all from Abcam, Cambridge, MA, USA) at 4°C for overnight. Next, the membranes were incubated with horseradish peroxidase(HRP)-conjugated secondary antibody (ab6721; Abcam, Cambridge, MA, USA) at room temperature for 2 h. BeyoECL kit (Beyotime, Shanghai, China) was used to visualize the protein bands. The protein signals were analyzed with ImageJ 1.42 software (NIH, Bethesda, MD, USA).

Cell Viability Assay

Cells were seeded into 96-well plates at the density of 5.0×103 cells/well and cultured at 37°C to plate adherence. At indicated time points (0, 24, 48 and 72 h) after seeding, 20 µl MTT reagent (5 mg/mL; Beyotime, Shanghai, China) was added to the medium and further incubated for 4 h at 37°C. Then, 150 µl dimethyl sulfoxide (DMSO) was added to the medium followed by detection optical density at 570 nm using enzyme-linked immunosorbent assay microplate reader (Thermo Fisher Scientific, Inc., Waltham, MA, USA).

Colony Formation Assay

1,000 cells were seeded to the 6-well plate and incubated at the above-mentioned conditions for 2 weeks until the appearance of visible colonies. Colonies were washed with PBS and fixed with 4% paraformaldehyde for 15 min. Then, colonies were stained with 0.5 % crystal violet for 30 min and observed under light microscope.

Cell Apoptosis Assay

Cell apoptosis was analyzed with the annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis kit (Beyotime, Shanghai, China). Then, cells were washed and resuspended in binding buffer followed by incubation with Annexin V-FITC/PI at dark for 15 min. Finally, cell apoptosis was analyzed using flow cytometer (Beckman Coulter, Brea, CA, USA).

Luciferase Assay

Both the wild-type (wt) and mutant (mut) 3'-untranslated region (3'-UTR) of ADAM28 were cloned into the pmirGLO (Promega, Madison, WI, USA) and named as ADAM28-wt and ADAM28-mut. For luciferase reporter assays, cells were co-transfected with miR-198 mimic or miR-NC and reporter plasmids. Firefly and Renilla luciferase activity was measured using a Dual-Luciferase Reporter System Kit (Promega, Madison, WI, USA) according to the manufacturer's protocol after 48 h of transfection. Relative luciferase activity was expressed as a firefly/Renilla luciferase ratio.

Statistical Analysis

Data were presented as mean ±standard deviation (SD). Differences in groups were analyzed using Student's t-test or one-way ANOVA following Tukey post-hoc test at GraphPad Prism 5 software (GraphPad Software Inc., La Jolla, CA, USA). Differences were considered statistically significant for $p < 0.05$.

Results

Dysregulation of miR-198 in CRC

qRT-PCR analysis was used to investigate miR-198 expression in three CRC cell lines. It was found that miR-198 expression in CRC tumor cell lines (HT29, SW480, and SW620) and one normal colon epithelium cell line (FHC). We found miR-198 expression was significantly downregulated in all the three investigated CRC cell lines compared with FHC cell line (Figure 1).

3'-UTR of ADAM28 was a Direct Target of miR-198

To identify the target of miR-198, we performed bioinformatic analysis using TargetScan. ADAM28 was found contain a binding site for miR-198 in its 3'-UTR (Figure 2A). Luciferase activity reporter assay showed that miR-198 mimic significantly decreased luciferase activity of cells transfected with ADAM28-wt but not ADAM28-mut (Figure 2B).

MiR-198 Overexpression Inhibits CRC Cells Proliferation and Colony Formation but Promotes Apoptosis

SW480 and SW620 cell lines were selected to investigate the biological roles of miR-198 in CRC. It was revealed that miR-198 mimic transfection remarkedly increased the levels of miR-198 in both cell lines compared with miR-NC transfection (Figure 3A). MTT assay showed that overexpression of miR-198 inhibits cell proliferation rate in both cell lines (Figure 3B). Colony formation assay showed that miR-198 mimic transfection inhibited colony formation ability in both cell lines (Figure 3C). Furthermore, flow cytometry analysis showed that cell apoptosis was stimulated by miR-198 mimic compared with miR-NC (Figure 3D). Western blot analysis showed that ADAM28, p-JAK1, and p-STAT3 expression could be repressed by miR-198 mimic (Figure 3E). However, the introduction of miR-198 mimic did not influence the expression of JAK1 and STAT3 (Figure 3E).

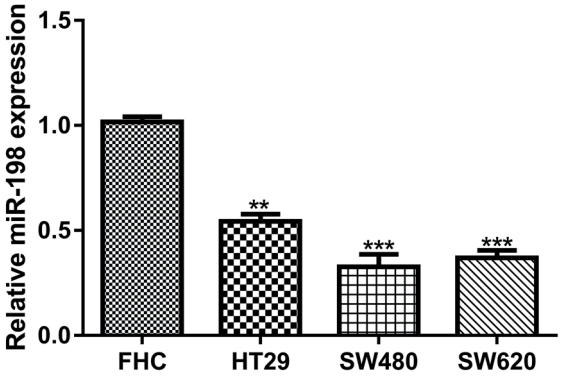

Figure 1. miR-198 was downregulated in CRC cell lines as analyzed by qRT-PCR. (**p<0.01, ***p<0.001) miR-198: microRNA-198; CRC: colorectal cancer; qRT-PCR: quantitative Real-time polymerase chain reaction.

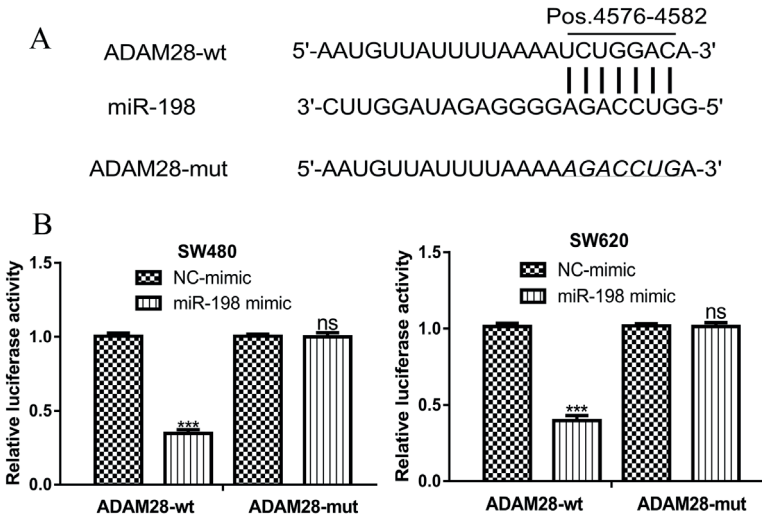

Figure 2. ADAM28 was a direct target of miR-198. ***(A)*** Predicted binding site between miR-198 and 3'-UTR of ADAM28. ***(B)*** miR-198 mimic inhibited luciferase activity of cells transfected with ADAM28-wt but not ADAM28-mut. (ns not significant, ***p<0.001) miR-198: microRNA-198; ADAM28: ADAM metallopeptidase domain 28; UTR: untranslated region; wt: wild-type; mut: mutant; NC-mimic: negative control.

Restoration of ADAM28 Counteracts the Effects of miR-198

Then, we investigated whether ADAM28 was an effector for the roles of miR-198. Functional analysis results showed that overexpression of ADAM28 significantly increased cell proliferation (Figure 4A), colony formation (Figure 4B), but decreased cell apoptosis (Figure 4C). It was found that ADAM28-vector transfection significantly increased the expression of ADAM28 (Figure 4D). Meanwhile, it was found p-JAK1, and p-STAT3 expression could be upregulated by ADAM28-vector (Figure 4D). However, the suppressive effect of miR-198 on CRC cell behaviors was reversed by targeting ADAM28.

Discussion

Cancer is a genetic disease caused by mutational or epigenetic alterations in DNA and results in abnormal status of cell behaviors[16]. miRNAs have been increasing recognized as crucial regulator for cancer cell hallmarks although no miRNAs have been put to clinical to date[16]. Cancer related molecular and cellular markers can be classified in four groups: diagnostic markers, prognostic markers, predictive markers, and surveillance markers[17,18]. The increased cancer-associated morbidity and mortality is partially due to the lack of efficient biomarkers[17]. Therefore, it is still urgent to discover the mechanisms related to cancer progression to identify novel markers. miR-198 was previously reported to be downregulated in cancers including gastric cancer, non-small-cell lung cancer, and lung adenocarcinoma[13-15]. In this work, we measured the expression of miR-198 in CRC cell lines using qRT-PCR. We found miR-198 expression was significantly downregulated in CRC cell lines compared with the normal cell line, which indicated miR-198 might function as tumor suppressor in CRC. It has been well recognized that loss or gain of function is a powerful approach to study the gene function. Therefore, miR-198 mimic was transfected to CRC cell lines and miR-198 levels were found significantly elevated by miR-198 mimic. Functional assays showed that overexpression of miR-198 inhibits CRC cell proliferation and colony formation, but promotes apoptosis. Targets of miR-198 including Toll-like receptor 4 and Livin have been identified in previous studies[13-15]. In this study, bioinformatics analysis showed that ADAM28 contains a binding site for miR-198. Experiments showed that overexpression of ADAM28 reversed the effects of miR-198 on CRC cell behaviors. Collectively, our results showed that miR-198 inhibits CRC cell behaviors through targeting the expression of ADAM28. JAK/STAT pathway is involved in cell proliferation, migration, and invasion of human cancers 19-23. Therefore, we also detected whether JAK/STAT pathway was involved in

miR-198/ADAM28 mediated CRC cell behaviors. We found p-JAK1 and p-STAT3 expression could be repressed by miR-198 but increased by ADAM28. Therefore, our results indicated that miR-198 regulates CRC progression through the ADAM28/JAK-STAT signaling pathway.

Conclusions

We demonstrated that downregulated of miR-198 expression promoted cell proliferation, colony formation but inhibited cell apoptosis via targeting ADAM28 and JAK-STAT pathway. Therefore, miR-198 and ADAM28 may be potential therapeutic targets for CRC.

Conflict of Interest

The Authors declare that they have no conflict of interest.

Acknowledgement

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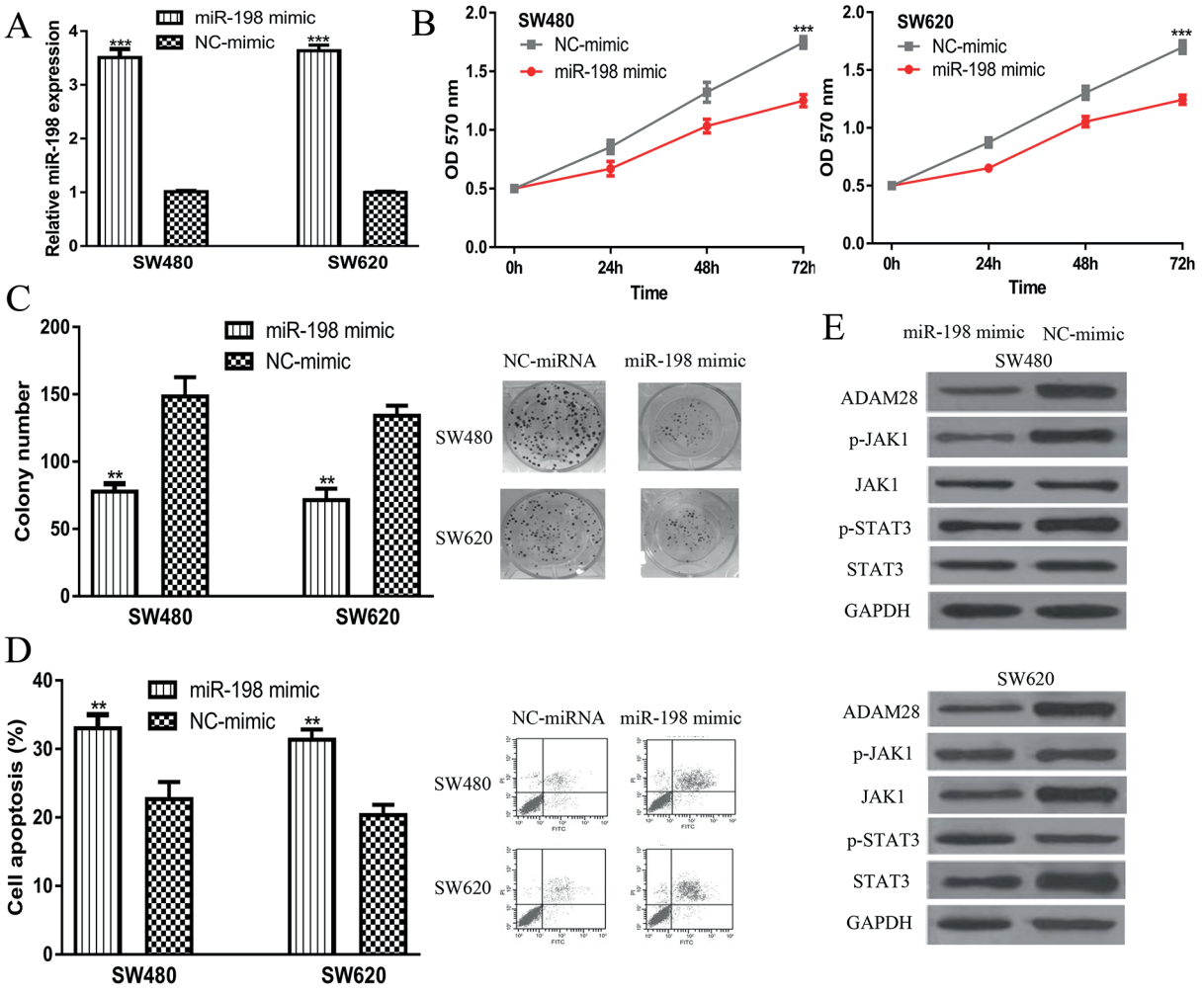

Figure 3. Overexpression of miR-198 inhibits CRC cell proliferation, colony formation but promotes cell apoptosis. ***(A)*** miR-198 expression, ***(B)*** Cell proliferation, ***(C)*** Colony formation, ***(D)*** Cell apoptosis, ***(E)*** Protein levels of ADAM28 and JAK/STAT pathway in cells transfected with miR-198 mimic or NC-mimic. (**p<0.01, ***p<0.001) miR-198: microRNA-198; CRC: colorectal cancer; ADAM28: ADAM metallopeptidase domain 28; JAK: Janus kinase; STAT: signal transducer and activator of transcription; NC-mimic: negative control.

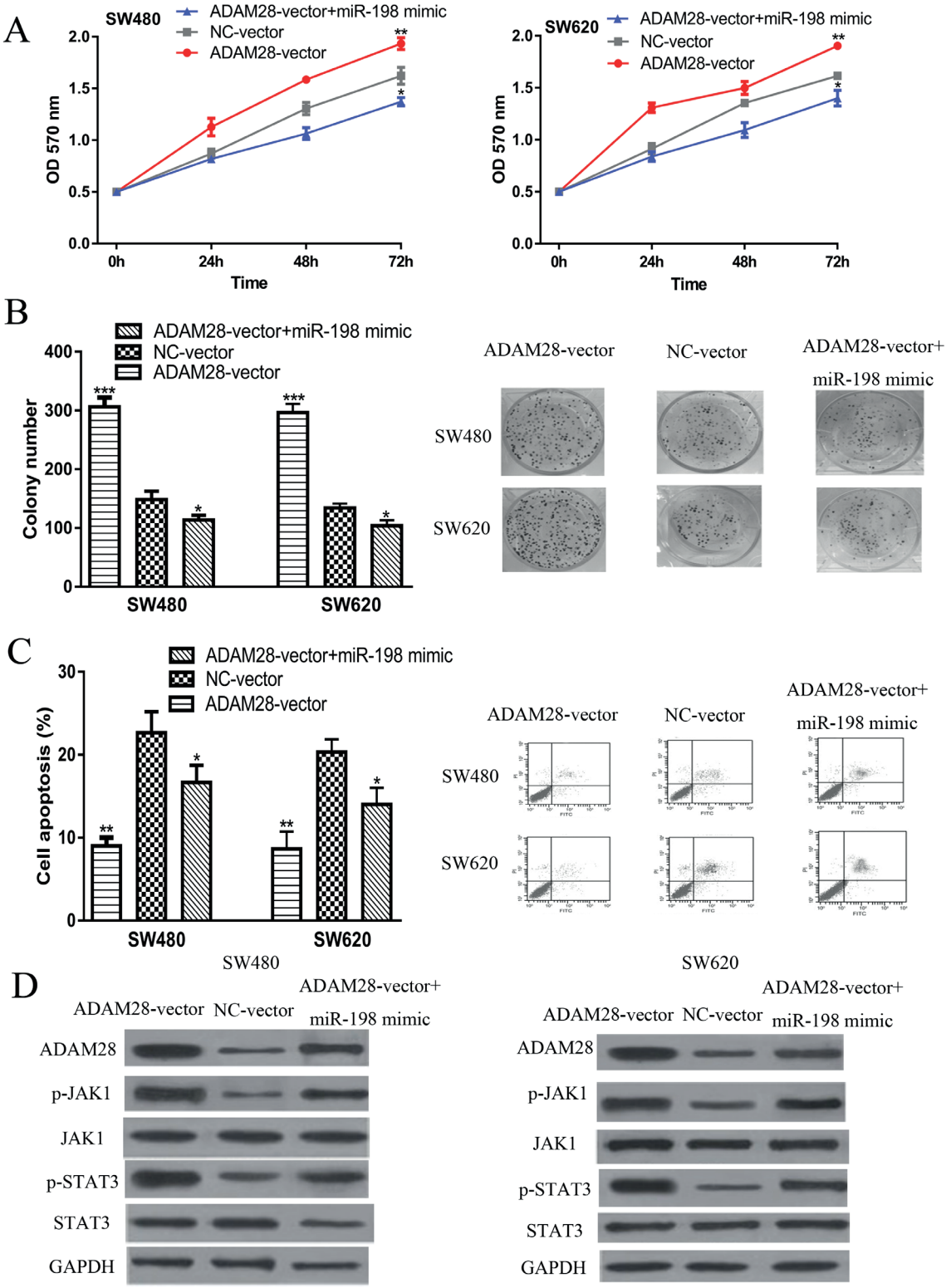

Figure 4. miR-198 inhibited CRC cell behaviors through targeting ADAM28. ***(A)*** Cell proliferation, ***(B)*** Colony formation, ***(C)*** Cell apoptosis, ***(D)*** Protein levels of ADAM28 and JAK/STAT pathway in cells transfected with ADAM28-vector, NC-vector, or ADAM28-vector and miR-198 mimic. (*p<0.05, **p<0.01, ***p<0.001) miR-198: microRNA-198; CRC: colorectal cancer; ADAM28: ADAM metallopeptidase domain 28; JAK: Janus kinase; STAT: signal transducer and activator of transcription; NC-vector: negative control.

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