

SRF Potentiates Colon Cancer Metastasis and Progression in a microRNA-214/PTK6-Dependent Manner

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Objective: Serum response factor (SRF), a sequence-specific transcription factor, is closely related to metastasis of gastric cancer, a digestive tract cancer. Herein, we probed the effect of SRF on metastasis and progression of colon cancer (CC), another digestive tract disorder, and the detailed mechanism.

Methods: Microarray analysis was conducted on tumor and adjacent tissues to filter differentially expressed miRNA, followed by RT-qPCR validation in CC cell lines. The transcription factor and the target gene of microRNA-214 (miR-214) were predicted, and their binding relationships were tested by luciferase reporter assays and ChIP assays. Subsequently, SRF and protein tyrosine kinase 6 (PTK6) expression in CC patients and cells was evaluated by RT-qPCR, while JAK2 and STAT3 expression in cells by Western blot analysis. To further explore functions of miR-214, PTK6 and SRF on CC, CC cells were delivered with si-PTK6, miR-214 mimic and/or SRF overexpression.

Results: miR-214 expressed poorly in CC tissues and cell lines, which related to advanced TNM staging and survival. miR-214 mimic inhibited proliferation, migration, invasion, xenograft tumor growth and metastasis of CC cells. SRF, overexpressed in CC samples and cells, suppressed the transcription of miR-214. Meanwhile, SRF upregulation counteracted the inhibitory role of miR-214 mimic in CC cell growth. miR-214 negatively regulated PTK6 expression to impair the JAK2/STAT3 pathway activation, thereby halting CC cell proliferation, migration, invasion, xenograft tumor growth and metastasis.

Conclusion: Altogether, miR-214 may perform as a tumor suppressor in CC, and the SRF/miR-214/PTK6/JAK2/STAT3 axis could be applied as a biomarker and potential therapeutic target.

Keywords: SRF, microRNA-214, PTK6, Colon cancer, The JAK2/STAT3 pathway

Introduction

As for the cause of cancer-related death, the ranking of colon cancer (CC), also named as colorectal cancer, showed a downward trend (the third place) in the United States by 2014, partly as a consequence of historical changes in risk factors, the use and spread of early detection tests, as well as improvements in therapeutic interventions.¹ Nevertheless, incidence is still higher in men than women and strongly associated with age, and the median age at diagnosis is around 70 years old in developed countries.² Even though surgery can cure patients with CC at early stage, the 5-year survival rate is dismal for patients with metastatic CC (only 10%).³ Metastasis is a complicated event that involves a series of procedures

where tumor cells proliferate, detach from the primary tumor sites, and migrate to a distant organ.⁴ Therefore, clarification of the mechanisms underlying the metastasis of CC may be beneficial to the discovery for new diagnostic biomarkers and effective treatment methods.⁵

MicroRNAs (miRNAs) are short non-coding RNAs with 19–25 nucleotides in length that function importantly, practically in cancer-related processes involving proliferation, cell cycle control, apoptosis in addition to migration.^{6–8} Among them, miR-214 has been reported to play a tumor suppressor role in multiple cancers, including gastric cancer,⁹ pancreatic cancer,¹⁰ non-small lung cancer¹¹ as well as osteosarcoma.¹² Also, miR-214, notably reduced in CC, was modulated by a transcription factor FOXD3 and tightly correlated with lymphatic metastasis.¹³ Also, the proliferation and invasion of CC SW620 cells were weakened by miR-214, yet the apoptosis rate was enhanced.¹⁴ Moreover, serum response factor (SRF), another transcription factor regulating the expression pattern of over 200 genes, was observed to be hypermethylated in gastric carcinoma metastasis, signifying its function as an attractive biomarker for predicting gastric carcinoma metastasis and prognosis.¹⁵ Therefore, we postulated that SRF may mediate the expression of miR-214 to influence the metastasis of CC. Protein tyrosine kinase 6 (PTK6), an intracellular tyrosine kinase that proposed to facilitate apoptosis in CC,¹⁶ has been validated as a target gene of miR-214 in CC cells in the present work. In addition, endogenous PTK6 enhanced cell survival and oncogenic signaling following DNA damage in CC.¹⁷ Taken together, our study speculated that miR-214 regulated the metastasis and progression of CC by directly targeting 3'UTR of the PTK6 gene in an SRF-dependent manner. Gain- and loss-of-function assays were carried out in CC LOVO and SW620 cells as well as in nude mice to verify our hypothesis.

Materials and Methods

After the determination of miR-214 using microarray analysis, RT-qPCR was carried out to assess its expression. For the purpose of examining the effects of SRF, miR-214 and PTK6 on CC, EdU, Transwell, tumor growth and metastasis assays were performed. Dual-luciferase reporter assays were used to detect targeting relationships between genes. Afterwards, CHIP was utilized to detect the binding relationship between SRF and miR-214. Western blot was finally applied to detect the activity of the JAK2/STAT3 signaling.

Clinical Data

From August 2014 to August 2016, 80 patients with CC (48 males and 32 females) treated in China-Japan Union Hospital of Jilin University were enrolled. The average age of the patients was 59.06 ± 9.08 years. The clinical stage of patients was 17 in stage I, 28 in stage II, 23 in stage III and 12 in stage IV. Adjacent tissues were extract from 5–10 cm away from tumor tissue. The patients with radiotherapy, chemotherapy or any immunotherapy were excluded from this study. All patients with complete clinical data received blood routine, urine routine, liver and kidney function, electrocardiogram and other examinations to exclude the existence of chronic diseases or complications. All patient cases were independently reviewed histologically as primary CC. The clinicopathological features of the enrolled patients are summarized in Table 1. The present study was reviewed and approved by the Ethics Committee of China-Japan Union Hospital of Jilin University, and we obtained informed consent from all patients.

Microarray Analysis

The total miRNA molecules of CC tissues and adjacent tissues from three patients were extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). RNA purity and concentration were determined by NanoDrop (Thermo Fisher Scientific Inc., Waltham, MA, USA). Following cDNA synthesis using cDNA Reverse Transcription kits (Takara Biotechnology, Co., Ltd., Dalian, Liaoning, China), cDNA was labeled with a miRNA Complete Labeling and Hyb Kit (Agilent Technologies, Santa Clara, CA, USA). A total of 15 μg cDNAs was dissolved in 3 μL ddH₂O and incubated with 1 μL calf intestinal phosphatase at 37°C for 30 min. The denatured RNA was inactivated at 95°C for 15 s and incubated with the mixture of 3 μL marker buffer, 1.5 μL Hy3 marker, 2 μL dimethylsulfoxide and 2 marker enzymes at 16°C in the dark for 1 h. Totally 125 μL labeled RNA samples were incubated with 65 μL hybridization buffer (2 \times) at 95°C for 2 min in the dark. The 200 μL mixture containing 15 μg cRNA was then hybridized with Human miRNA Microarray Release 14.0,8x 15K (Agilent) in a hybridization oven (hybridization oven) overnight, then scanned with Agilent SureScan Dx (Agilent). A quality control evaluation of the analysis results was carried out. Background correction and normalization of raw data was performed using Robust Multi-Array Average. miRNA was used analyzed and identified using *t*-test. miRNAs with $p < 0.05$ and |Fold change| > 1.5

Table I Demographic Characteristics of the Enrolled Patients

Number	Age	Gender	Classification
1	63	Female	I
2	77	Male	I
3	48	Female	I
4	63	Male	I
5	59	Female	I
6	31	Female	I
7	51	Male	I
8	49	Male	I
9	58	Male	I
10	64	Female	I
11	74	Male	I
12	66	Female	I
13	58	Male	I
14	57	Female	I
15	59	Male	I
16	57	Female	I
17	86	Male	I
18	55	Male	II
19	63	Female	II
20	65	Male	II
21	54	Female	II
22	63	Male	II
23	50	Female	II
24	64	Male	II
25	66	Female	II
26	49	Male	II
27	56	Female	II
28	50	Female	II
29	53	Male	II
30	45	Male	II
31	71	Male	II
32	69	Male	II
33	69	Male	II
34	68	Female	II
35	56	Female	II
36	62	Male	II
37	65	Female	II
38	63	Male	II
39	64	Female	II
40	54	Male	II
41	60	Female	II
42	73	Male	II
43	69	Female	II
44	67	Male	II
45	52	Male	II
46	52	Male	III
47	53	Female	III
48	64	Male	III
49	49	Female	III
50	52	Male	III
51	52	Male	III

(Continued)

Table I (Continued).

Number	Age	Gender	Classification
52	59	Male	III
53	69	Male	III
54	59	Male	III
55	54	Female	III
56	54	Male	III
57	53	Female	III
58	58	Female	III
59	66	Male	III
60	65	Male	III
61	72	Female	III
62	69	Male	III
63	62	Male	III
64	55	Female	III
65	64	Male	III
66	55	Female	III
67	66	Male	III
68	58	Female	III
69	66	Male	IV
70	42	Male	IV
71	46	Female	IV
72	63	Female	IV
73	47	Male	IV
74	51	Male	IV
75	47	Female	IV
76	59	Male	IV
77	56	Male	IV
78	55	Male	IV
79	74	Male	IV
80	42	Male	IV

was defined as differentially expressed miRNAs and plotted as a heat map by hierarchical clustering.

RNA Isolation, cDNA Synthesis and Quantitative Polymerase Chain Reaction (qPCR)

The total RNA in tissues was extracted with the help of the TRIzol Reagent (Invitrogen). After the removal of the genomic DNA contamination, the template RNA was subjected to enzyme digestion. The purity and concentration of RNA was determined using a spectrophotometer. RNA integrity was detected by 1.5% agarose gel electrophoresis, and the RNA concentration was adjusted to 500 ng/ μ L. RNA samples were transcribed into cDNA using a cDNA Reverse Transcription kit (Takara Biotechnology). The SYBR RT-qPCR kit (Thermo Fisher Scientific) was used for amplification. Primers for this experiment were designed by Primer

Premier 5.0 (Premier Biosoft International, Palo Alto, CA, USA) and synthesized in Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. (Shanghai, China). Glyceraldehyde 3-phosphatedehydrogenase (GAPDH) and U6 were treated as internal controls for SRF, PTK6 and miR-214, respectively. All primer sequences are listed in Table 2. The $2^{-\Delta\Delta Ct}$ method was applied to measure the relative expression of mRNA and miRNA.

Cell Culture and Treatment

CC cell lines SW620, SW480, LOVO, HCT 116, HT 29, LS174T, normal human colon fibroblast cells (CCD-18Co) and human embryonic kidney epithelial cells (HEK293T) were purchased from Keygen Biotech Co., Ltd. (Nanjing, Jiangsu, China). SW620 and SW480 were grown in a Leibovitz L-15 medium supplemented with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) and LOVO, HCT 116, HT 29, LS174T and HEK293T cells were exposed to Roswell Park Memorial Institute 1640 (RPMI-1640) medium containing 10% FBS. All aforementioned cells were maintained in a 37°C cell incubator containing 5% CO₂. Routine examination of mycoplasma in all cell lines showed negative results.

SRF fragments, miR-214 mimic and small interfering RNA (siRNA) targeting PTK6 (System Biosciences, Palo Alto, CA, USA) were cloned into a pmirGLO vector (Promega Corporation, Madison, WI, USA), and the constructed vectors were transfected into cells to establish cells that stably overexpressing SRF, miR-214 or PTK6 deficit. SRF-OE vectors were delivered into cells overexpressing miR-214 in the rescue experiment. Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) was applied for transient

transfections of vectors and siRNA. Detection of transfection efficiency was carried out using RT-qPCR.

5-Ethynyl-2'-Deoxyuridine (EdU) Incorporation Assay

EdU assays were conducted to assess the viability of CC cells. LOVO and SW620 cells in good growth condition were seeded in a 24-well plate. The culture medium was added with the reagent from an EdU Staining Proliferation Kit (iFluor 488, Abcam Inc., Cambridge, UK) to a final concentration of 10 μmol/L, and incubated in the incubator for 2 h. The cells were then fixed with phosphate buffered saline (PBS) solution containing 4% paraformaldehyde at room temperature for 15 min and incubated with PBS containing 0.5% Triton-100 at room temperature for 20 min. Afterwards, the cells in each well were incubated for 30 min with 100 μL Apollo 567 (Guangzhou RiboBio Co., Ltd., Guangzhou, Guangdong, China) void of light and stained for 5 min with 4',6-diamidino-2-phenylindole. Five visual fields were randomly taken under a TCS SP8 confocal microscope (Leica, Bannockburn, IL, USA). The blue fluorescence indicates all cells, whereas the red fluorescence is the replicating cells infiltrated by EdU. The rate of EdU-positive cells was calculated.

Transwell Assays

Transwell assays were carried out for cell migration and invasion evaluation. Matrigel (BD Biosciences) was placed into the apical chamber under sterile conditions and cultured for 30 min. The apical chamber was then incubated with RPMI-1640 medium in a CO₂ incubator for later use. After detachment and centrifugation, the cells were resuspended in serum-free medium and diluted into cell suspension (5×10^5 cell/mL). A total of 500 μL RPMI 1640 containing 10% FBS was supplemented to the basolateral chamber, and the diluted cell suspension (200 μL) was supplemented to the apical chamber for a 48-h culture in an incubator at 37°C with 5% CO₂. Afterwards, cells that did not migrate or invade were removed by wiping them with a cotton swab. Cells left on the lower surface of the membrane were stained for 10 min with crystal violet. Five visual fields were arbitrarily chosen under a microscope (Leica DM500) to photograph the invading cells. Migration test was carried out without Matrigel precoating on the apical chamber, and the rest of the operation was consistent with invasion detection.¹⁸

Tumor Growth Assay

Forty-eight specific-pathogen-free (SPF) female BALB/c nude mice (4–6 weeks old, 20 ± 2 g) were from Beijing Vital River Laboratory Animal Technology Co., Ltd.

Table 2 List of Primers Used in This Study

Targets	Primer Sequence (5'-3')
U6	Forward: CTCGCTTCGGCAGCACA Reverse: AACGCTTCACGAATTTGCGT
GAPDH	Forward: GGGAGCCAAAAGGGTCATCA Reverse: TGATGGCATGGACTGTGGTC
SRF	Forward: TGCTGAATGCCTTCTCCA Reverse: GCCTGCTGCCCTATCACA
miR-214	Forward: CTGGGGGCTGGATTCGGGGGTGGG Reverse: ACCCCCACCCCGAATCCAGCCCCCA
PTK6	Forward: TACTTTGGGGAGGTCTTCGAG Reverse: TGCCCGAGCTTCTTCATG

Abbreviations: GAPDH, glyceraldehyde 3-phosphatedehydrogenase; SRF, serum response factor; miR-214, microRNA-214; PTK6, protein tyrosine kinase 6.

(Beijing, China). The nude mice were injected with LOVO or SW620 cells stably transfected with miR-214 mimic, miR-214 control, SRF-overexpression (OE), SRF-negative control (NC), miR-214 mimic + SRF-OE, miR-214 mimic + SRF-NC, si-PTK6 or PTK6-NC ($n = 3$). Totally 4×10^6 LOVO and SW620 cells dispersed in 2 mL saline was injected subcutaneously into the nude mice. Mouse tumor volume was measured at an interval of 7 days after injection by formula: $L \times W^2/2$ where L indicates length and W indicates width. After 28 days, mice were euthanized with 1% pentobarbital sodium at 120 mg/kg, and the tumor was weighed for histological experiments. All animal experiments were implemented as per the principles and procedures permitted by the Committee on the Ethics of Animal Experiments of China-Japan Union Hospital of Jilin University (Approval number: 20140216CJD).

Immunohistochemical Staining

The extracted mouse tumor tissues were embedded in paraffin, deparaffinized and hydrated. Five sections were obtained from each xenograft tumor. Afterwards, the sections were allowed to stand with 3% H_2O_2 at room temperature for 15 min. After being blocked with normal goat serum for 15 min at room temperature, the sections were probed with 50 μ L primary antibody against KI67 (1:500, ab197234, Abcam) at 4°C overnight. Subsequently, the secondary antibody (1:500, ab199091, Abcam) was applied and incubated for 15 min at 37°C. Following treatments with 40 μ L horseradish peroxidase at 37°C for 15 min and diaminobenzidine, the sections were counter-stained with hematoxylin for 30 s, dehydrated and sealed with neutral gum. Under a microscope (Leica DM500), five non-overlapping fields were chosen for each section. The cells presenting brown or brown granules in the nucleus were defined as KI67 positive.¹⁹

In vivo Metastasis Assays

Another 48 SPF female BALB/c nude mice (4–6 weeks old, 20 ± 2 g) were used for in vivo metastasis assay. The nude mice were injected with LOVO or SW620 cells stably transfected with miR-214 mimic, miR-214 control, SRF-OE, SRF-NC, miR-214 mimic + SRF-OE, miR-214 mimic + SRF-NC, si-PTK6 or PTK6-NC ($n = 3$). A total of 4×10^6 LOVO and SW620 cells were inoculated into the nude mice through tail vein. After 45 days, the nude mice were euthanized with 1% pentobarbital sodium. The lung tissues of nude mice were paraffin-embedded and subjected to a hematoxylin-eosin (HE) staining (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) to observe

the formation of metastatic lesions. The sections were dewaxed with xylene for 8 min, soaked in gradient alcohol, immersed into hematoxylin for 15 min. The sections were differentiated in 1% hydrochloric acid alcohol for 30 s, immersed into eosin solution for 5 min, dehydrated with gradient alcohol, cleared for 10 min with xylene and sealed with gum. The lung sections were then visualized under a microscope (Leica DM500), and 5 fields were arbitrarily chosen for photographs.

Dual-Luciferase Assay

TransmiR v2.0 (<http://www.cuilab.cn/transmir>) and ALGGEN (<http://alggen.lsi.upc.es/>) were used to predict the binding sequences between SRF and miR-214 as well as between SRF and PTK6 promoter. The binding sequences between miR-214 and PTK6 3'untranslated region (3'UTR) were predicted by StarBase (<http://starbase.sysu.edu.cn/>). PTK6 3'UTR sequence and miR-214 vector, PTK6 promoter sequence and SRF vector, miR-214 promoter sequence and SRF vector were inserted into pMIR-REPORTTM (Thermo Fisher Scientific).²⁰ These vectors were co-transfected into HEK293T cells using Lipofectamine 3000 (Invitrogen). After 24 h, the cells were lysed, and the luciferase activity intensity (intensity = RLU1/RLU2, RLU1 was firefly luciferase intensity, RLU2 was renilla luciferase intensity) was determined by a Dual-Luciferase Reporter Assay System (Promega Corporation).

Chromatin Immunoprecipitation (ChIP) Assay

The ChIP was performed to validate the binding relationship between SRF and miR-214 using a Pierce Agarose ChIP Kit (Thermo Fisher Scientific). LOVO and SW620 cells were diluted with 37% formaldehyde to 1% concentration and incubated in a 37°C incubator for 10 min. After the addition of glycine, the cells at concentration of 0.125 M were allowed to stand at room temperature for 5 min. Following a 5-min centrifugation at 2000 rpm, the cells were lysed using sodium dodecyl sulfate (SDS) lysis buffer and proteinase inhibitor, and DNA was sheared by a sonicator (Shunma Tech., Nanjing, Jiangsu, China). After a 10,000 g centrifugation at 4°C for 10 min to remove insoluble substances, the samples were added with 900 μ L ChIP dilution buffer and 20 μ L 50 \times protease inhibitor cocktail, and mixed with 60 μ L ProteinA Agarose at 4°C for 1 h. Following a 10-min standing and a 700-rpm centrifugation, the supernatants were incubated with antibodies against SRF or IgG (ab2410, Abcam) at 4°C

overnight. The immunoprecipitates were eluted using 250 μ L elution buffer, and the precipitated DNA was dissolved in 100 μ L ddH₂O for the following PCR analysis.²¹

Western Blot

The cells were lysed using radio immunoprecipitation assay buffer (Amresco, Radnor, PA, USA) and centrifuged at 800 g at 4°C for 5 min. Next, the cells were ice-bathed with 5 \times lysis buffer for 10 min and centrifuged at 12,000 g for 10 min at 4°C to obtain the supernatant. Proteins were subjected to an SDS-polyacrylamide gel electrophoresis. Separated proteins were blotted onto polyvinylidene fluoride membranes (Millipore, Merck, Darmstadt, Germany) and sealed with 5% skim milk. The membranes were then immunoblotted with the specific primary antibodies against janus kinase 2 (JAK, 1:5000, ab108596, Abcam), p-JAK2 (1:2000, ab108596, Abcam), signal transducer and activator of transcription 3 (STAT3, 1:5000, ab68153, Abcam), p-STAT3 (1:1000, ab76315, Abcam) and β -actin (1:2000, ab32101, Abcam). After 16 h, the membranes were probed with the secondary antibody (1:3000, ab205718, Abcam). The optical density (OD) analysis of immunoblot images was performed using ImageJ software (version 1.8.0; National Institutes of Health, Bethesda, MD, USA).²²

Statistics

All the statistical analyses were implemented using the SPSS 22.0 software (IBM Corp. Armonk, NY, USA). For all assays, at least 3 independent trials were carried out. Results are shown as the means \pm standard deviation (SD) and compared using a two-tailed *t*-test for two-group comparison or one-way or two-way analysis of variance (ANOVA) followed by Tukey's multiple comparison post-hoc test for comparing different groups. The Kaplan-Meier and Log rank tests were applied for evaluating survival comparisons. A two tailed *p*-value of less than 0.05 was considered as significant.

Results

miR-214 expression was poorly expressed in CC patients and cells which was not only negatively correlated with tumor, node, metastases (TNM) stage, but also showed good prognosis. The proliferation, invasion and migration of CC cells were weakened by miR-214 mimic. Also, the tumorigenic and metastatic potentials of CC cells were decreased in vivo after miR-214 overexpression. SRF bound to the miR-214 promoter and inhibited its transcription. SRF restored the activity of CC cells,

while upregulation of SRF in cells overexpressing miR-214 also promoted the activity of CC cells. miR-214 targeted PTK6 and inhibited its expression, and depletion of PTK6 reduced the activity of CC cells. SRF/miR-214/PTK6 axis mediated the activity of the JAK2/STAT3 pathway, and SRF and PTK6 shared positive correlations with the pathway, while miR-214 had a negative correlation with the JAK2/STAT3 pathway.

miR-214 is Significantly Reduced in CC Tissues and Cells

We first performed a miRNA microarray analysis of tumor tissues and adjacent tissues in clinically obtained CC patients. Among all differentially expressed miRNAs, we found that miR-214 was the most downregulated one (Figure 1A). RT-qPCR tests were performed on tumor tissues and adjacent tissues in all CC patients, which displayed that miR-214 was reduced in tumor tissues (Figure 1B). We then analyzed the correlation between TNM staging and miR-214 expression in CC patients. It was demonstrated that miR-214 expression was negatively correlated with TNM staging in CC (Figure 1C). Survival analysis of CC patients displayed that high expression of miR-214 was more favorable for postoperative survival (Figure 1D). Meanwhile, we examined the miR-214 expression in LOVO, SW620, SW480, HCT116, HT-29, LS174T and CCD-18Co cells, and the results showed that miR-214 was also significantly lower in CC cell lines, and the downregulation was more pronounced in LOVO and SW620 cells (Figure 1E). LOVO and SW620 cells were then transfected with miR-214 mimic for subsequent experiments (Figure 1F).

Overexpression of miR-214 Inhibits CC Cell Viability

We subsequently examined the involvement of miR-214 in cell growth. It was showed that the number of EdU-positive cells in cells overexpressing miR-214 was decreased significantly compared with the cell transfected with miR-214 control (Figure 2A). After 48 h, miR-214 mimic led to significantly reduced cell migration distance and invasive cell number (Figure 2B and C). Also, cells overexpressing miR-214 were subcutaneously injected into three nude mice, and the volume of subcutaneous tumor in mice with miR-214 mimic was reduced compared to those with miR-214 control (Figure 2D). The positive rate of surface marker KI67 was significantly decreased following miR-214 overexpression (Figure 2E). Even though mice in both groups

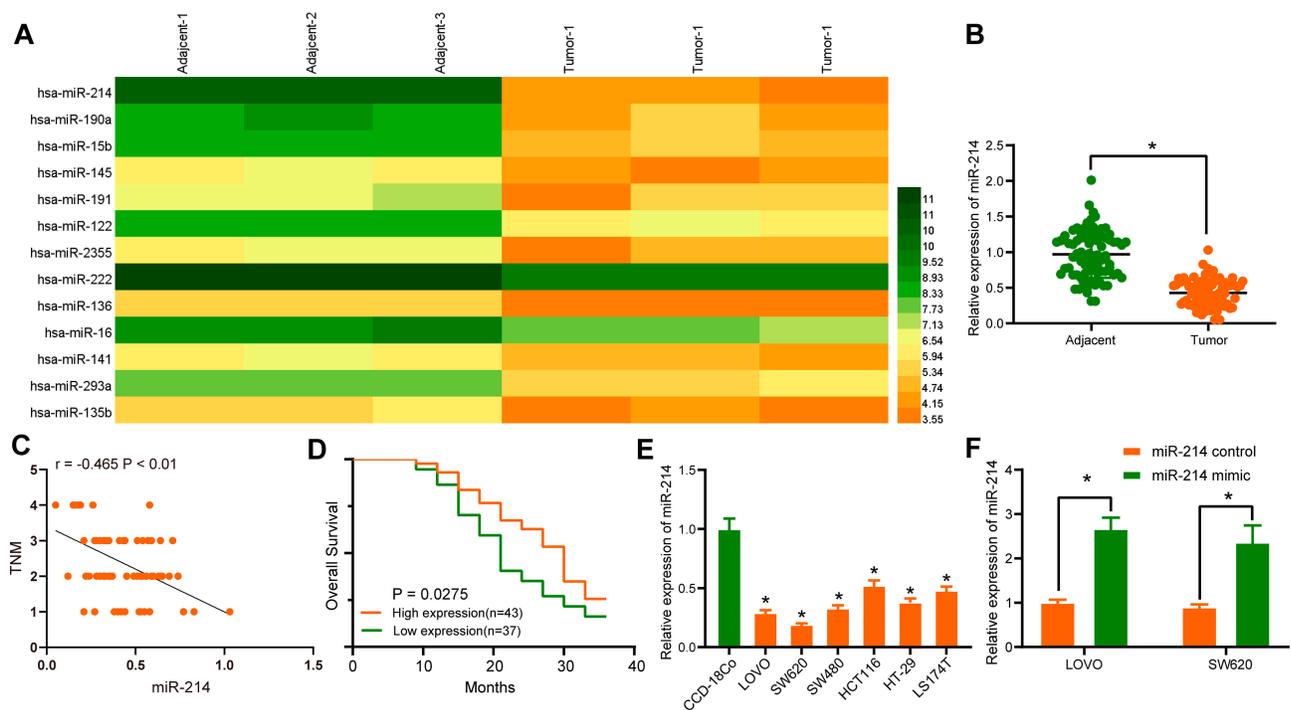


Figure 1 The analysis of differentially expressed miRNAs in CC tissues. **(A)** differentially expressed miRNAs in tumor and adjacent tissues screened out by microarray analysis; **(B)** miR-214 expression in CC tumor and adjacent tissues evaluated by RT-qPCR (* $p < 0.05$ according to the two-way ANOVA); **(C)** the correlation analysis between miR-214 expression and TNM stage of patients with CC; **(D)** survival analysis of CC patients; **(E)** miR-214 expression in CC cell lines assessed RT-qPCR analysis (* $p < 0.05$ according to the one-way ANOVA); **(F)** miR-214 mimic was transfected into LOVO and SW620 cells (* $p < 0.05$ according to the two-way ANOVA).

displayed metastasis dissemination. The pulmonary nodules were notably diminished after overexpression of miR-214, and the area of individual pulmonary nodules was also significantly reduced (Figure 2F).

SRF Interacts with the miR-214 Promoter

To examine the molecular mechanism of miR-214 in CC, we predicted the binding sites between the transcription factor SRF to its promoter by TransmiR and ALGGEN (Figure 3A). The luciferase reporter assays demonstrated that SRF effectively increased the luciferase signals of miR-214-wild type (WT) (Figure 3B). We then performed ChIP analysis to further test the combination of the two, and the results exhibited that SRF could directly bind to miR-214 promoter region (Figure 3C). RT-qPCR detection of tumor tissues and adjacent tissues in CC patients illustrated that SRF expression in CC tissues was promoted (Figure 3D), which was also validated in CC cell lines as well (Figure 3E). These results indicated that SRF bound to specific promoters of miR-214 (Figure 3F). Therefore, LOVO and SW620 cells were infected with lentiviral vectors overexpressing SRF to construct SRF-OE cells for the subsequent experiments (Figure 3G).

Upregulation of SRF Enhances CC Cell Viability

The viability of SRF-OE cells was detected, and the number of EdU-positive cells was elevated, indicating the viability of cells was enhanced (Figure 4A). Meanwhile, the cell migration distance (Figure 4B) and the number of invaded cells (Figure 4C) were elevated significantly after 48 h. Moreover, the results of in vivo assays suggested that upregulation of SRF in three nude mice resulted in larger tumor volume (Figure 4D), higher KI67 positive rate (Figure 4E) as well as more and larger pulmonary nodules (Figure 4F) relative to the counterparts.

SRF Inhibits the Transcription of miR-214

SRF-OE was then delivered into cells overexpressing miR-214 to construct miR-214 mimic + SRF-OE cells (Figure 5A). The viability of cells transfected with miR-214 mimic + SRF-OE was significantly increased, suggested by increased number of EdU-positive cells (Figure 5B). In support, the cell migration distance (Figure 5C) and the invasive cell number (Figure 5D) were upregulated after the administration of the miR-214 mimic + SRF-OE cell after 48 h. Three nude mice

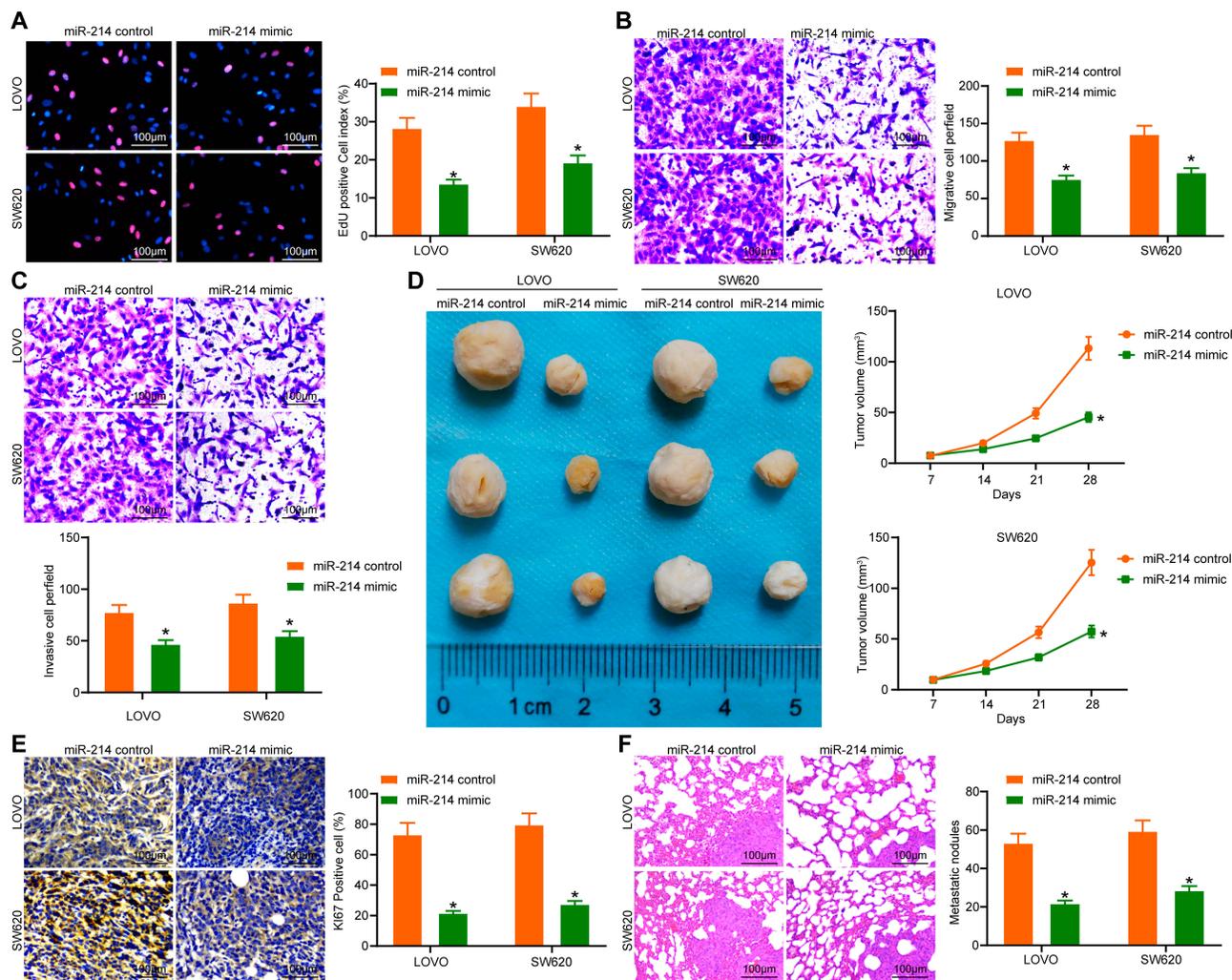


Figure 2 Increased miR-214 is associated with decreased CC cell proliferative, migratory, invasive, tumorigenic and metastatic capacities. (A), CC cell proliferation evaluated by EdU staining; (B), CC cell migration evaluated by Transwell assays; (C), CC cell invasion evaluated by Transwell assays; (D), representative tumor images and tumor volume from mice injected with CC cells overexpressing miR-214; (E), Ki67 positive rate of tumors detected by immunohistochemistry; (F), changes of pulmonary nodules detected by HE staining. * $p < 0.05$ according to the two-way ANOVA. Data represent averages of three independent experiments.

that received miR-214 mimic + SRF-OE cell delivery appeared to exhibit a heavier tumor burden (Figure 5E), greater Ki67 positive rate (Figure 5F) and aggravated metastasis (Figure 5G) versus nude mice received miR-214 mimic + SRF-NC cell delivery.

miR-214 Negatively Regulates PTK6 Expression by Directly Binding

To identify downstream targets of miR-214, we used StarBase to predict PTK6 as a potential target. We cloned PTK6 3'UTR into a luciferase plasmid. In HEK293T cells, miR-214 mimic suppressed the luciferase activity of PTK6 3'UTR (Figure 6A). To determine whether PTK6 was regulated by SRF through

a direct transcription, we overexpressed SRF to find out its impact on the dual luciferase activity of PTK6-WT and PTK6-MT. It turned out that SRF-OE had no notable effect on of the PTK6 promoter relative to the SRF-NC (Figure 6B). RT-qPCR detection of CC patients showed that PTK6 expression in CC tissues was significantly enhanced (Figure 6C). We identified a similar trend in CC cell line (Figure 6D). Moreover, the PTK6 expression decreased remarkably in the cells overexpressing miR-214 and increased significantly in the cells overexpressing SRF (Figure 6E). We may conclude that SRF modulated the PTK6 expression in a miR-214-dependent manner. As a result, cells with PTK6 knockdown were constructed for subsequent experiments (Figure 6F).

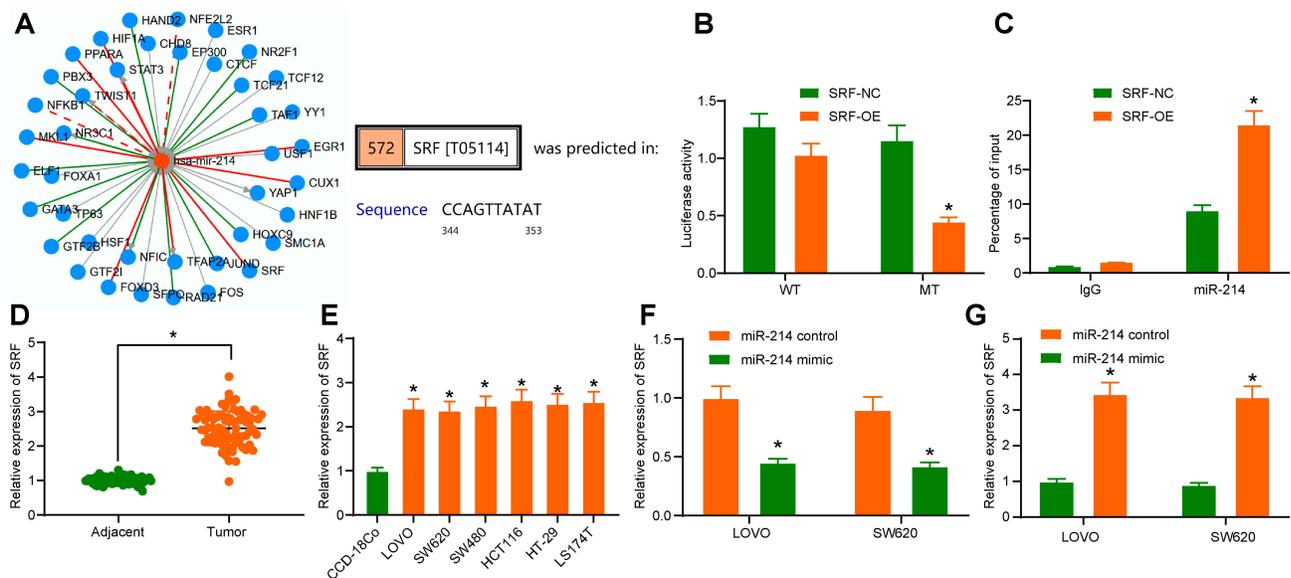


Figure 3 The binding relationship between miR-214 and SRF is identified. **(A)** the transcription factor that regulate miR-214 and the binding promoter sequences predicted by TransmiR and ALGGEN; **(B)** the targeting relationship between SRF and miR-214 verified by a dual-luciferase reporting assay; **(C)** the effect of SRF-NC and SRF-OE on miR-214 promoter validated by ChIP; **(D)** SRF expression in CC and adjacent tissues evaluated by RT-qPCR detection; **(E)** SRF expression in CC cell lines evaluated by RT-qPCR detection; **(F)** SRF expression in CC cell lines overexpressing miR-214 evaluated by RT-qPCR detection; **(G)** SRF expression in CC cell lines overexpressing miR-214 evaluated by RT-qPCR detection. * $p < 0.05$ according to one-way (panel **E**) or two-way ANOVA (panel **BD**, **F** and **G**). Data represent averages of three independent experiments.

Knockdown of PTK6 Decreased CC Cell Viability

The viability detection of cells with PTK6 knockdown found that the number of EdU-positive cells was notably reduced (Figure 7A). Furthermore, reduced PTK6 expression was linked to decreased ability of migration (Figure 7B) and invasion (Figure 7C). More importantly, CC cells with PTK6 knockdown illustrated hampered tumorigenic and metastatic potentials, supporting by a lighter tumor burden (Figure 7D), lower KI67 positive rate (Figure 7E) and smaller and fewer pulmonary nodules (Figure 7F).

SRF/miR-214/PTK6 Axis Mediates the JAK2/STAT3 Pathway

Finally, we examined the protein expression of JAK2 and STAT3 in cells transfected with SRF-NC, SRF-OE, miR-214 control, miR-214 mimic, miR-214 mimic + SRF-NC, miR-214 mimic + SRF-OE, PTK6-NC or si-PTK6. Overexpression of SRF was found to expedite the JAK2/STAT3 signaling pathway, while miR-214 restoration and PTK6 knockdown diminished their phosphorylation. Besides, SRF flattened the inhibitory role of miR-214 mimic on the JAK2/STAT3 pathway induction (Figure 8A and B).

Discussion

CC arises as a result of genomic instability with a gathering of genetic errors because of dysregulation of molecular signaling pathways governing cell migration, apoptosis and proliferation.²³ miRNAs have been indicated to play vital parts in the metastasis in CC. In the present investigation, miR-214 regulated by SRF was verified to inhibit metastasis and progression of CC by directly targeting the PTK6/JAK2/STAT3 pathway.

Previously, miR-214 was acknowledged as a tumor suppressor in the progression of cell migration, invasion and drug sensitivity of cervical cancer, implying its role as a promising diagnostic and therapeutic option for cancers.²⁴ The similar role of miR-214 has also been indicated in gastric cancer.²⁵ Moreover, circulating miR-214 has exhibited diagnostic potentials in breast cancer as an indicator of metastatic spread to lymph nodes.²⁶ As for its relevance in CC, miR-214 was notably diminished in the tissues from CC patients and cell lines, which was tightly linked to promoted cell proliferation.²⁷ Besides, miR-214 sensitized CC cells to irradiation by suppression of ATG12-regulated autophagy.²⁸ Even though miR-214 was corroborated to inhibit CC tumor growth in nude mice,²⁹ its connection with metastasis in vivo remains unclear. Our microarray analysis revealed that miR-214

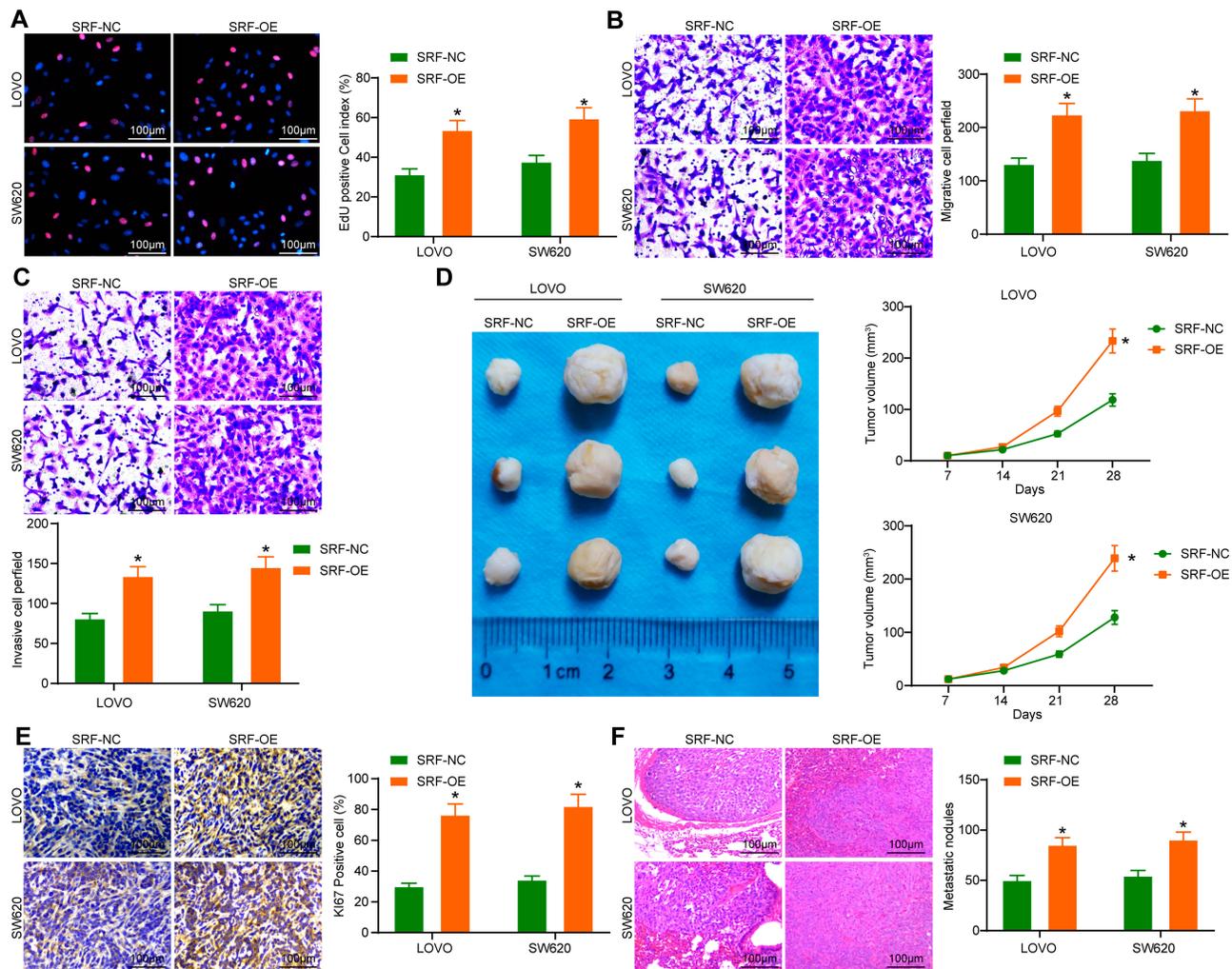


Figure 4 Increased SRF is associated with elevated CC cell proliferative, migratory, invasive, tumorigenic and metastatic capacities. SRF-OE or SRF-NC was delivered into CC cell lines. **(A)** CC cell proliferation determined by EdU staining; **(B)** CC cell migration evaluated by Transwell assays; **(C)** CC cell invasion evaluated by Transwell assays; **(D)** representative tumor images and tumor volume from mice injected with CC cells overexpressing SRF; **(E)** Ki67 positive rate of tumors detected by immunohistochemistry; **(F)** changes of pulmonary nodules detected by HE staining. * $p < 0.05$ according to the two-way ANOVA. Data represent averages of three independent experiments.

was one of the most significantly downregulated miRNAs in CC. We further validated miR-214 expression profile in CC tissue samples and cells. Our data suggested that miR-214 expression was diminished in both CC tissues and cell lines. Additionally, this study exposed that miR-214 restoration hampered the proliferative, migratory and invasive capacities of CC cells, and most significantly, metastasis and tumor growth in vivo. Even though the biological functions of miR-214 knockdown have not been investigated in the current work, both Chandrasekaran et al and Zhou et al demonstrated that suppression of miR-214 increased proliferation, migration and invasion in CC cells and metastasis in vivo.^{30,31}

To disclose the molecular mechanism of miR-214 in CC, we explored both the upstream and downstream

genes of miR-214. The bioinformatic tools revealed that the transcription factor SRF could bind to miR-214, and their binding relationship was substantiated by the following dual-luciferase and ChIP assays. SRF has been proposed to regulate the hepatic stellate cells.^{32,33} Moreover, SRF and myocyte enhancer factor-2 worked collaboratively to modulate the expression of muscle-specific genes, such as miR-133a.³⁴ The myocardin-related transcription factors A and B-SRF pathway is of great significance for cell proliferation, motility, and adhesion, which were all major processes underlying the progression of cancers.³⁵ For instance, SRF expedited gastric cancer metastasis by accelerating myofibroblast-cancer cell crosstalk.³⁶ More specifically, SRF was monitored to transactivate miR-199a-5p and miR-

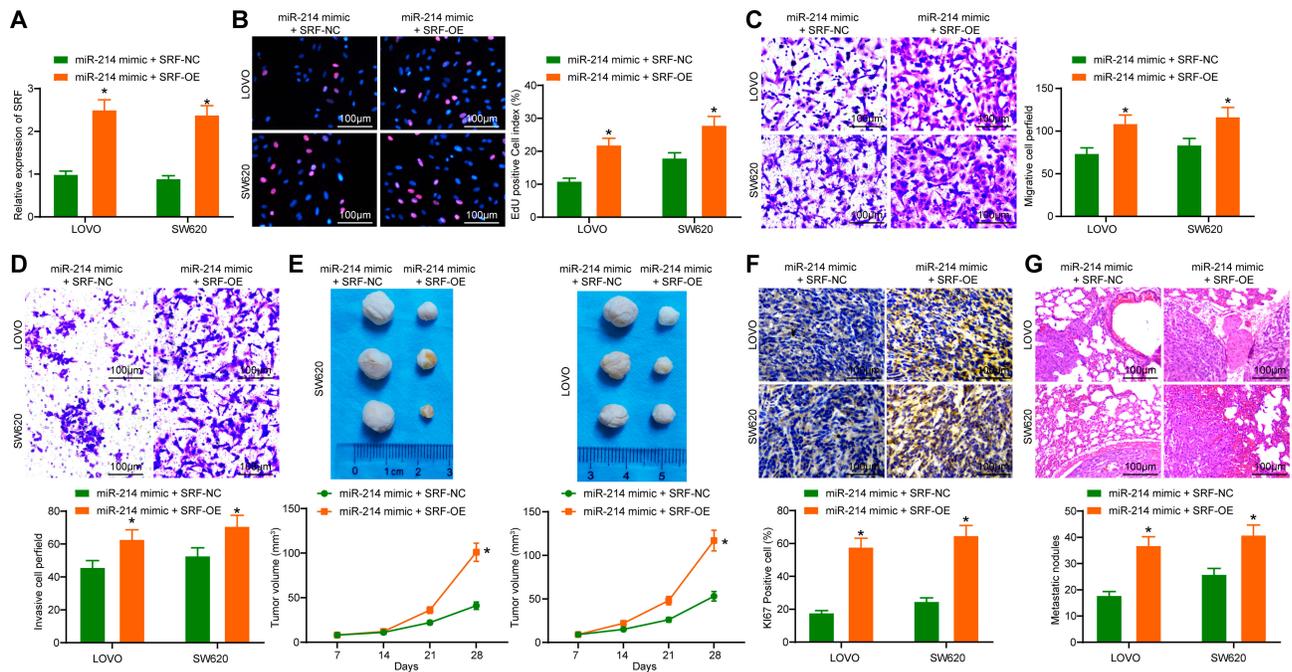


Figure 5 SRF overexpression rescues the repressive role of miR-214 mimic on CC cell viability. CC cell lines were transfected with SRF-OE or SRF-NC in the presence of miR-214 mimic. (A) SRF expression in CC cell lines co-transfected with miR-214 mimic and SRF-OE evaluated by RT-qPCR detection; (B) CC cell proliferation determined by EdU staining; (C) CC cell migration evaluated by Transwell assays; (D) CC cell invasion evaluated by Transwell assays; (E) representative tumor images and tumor volume from mice injected with CC cells overexpressing SRF; (F) KI67 positive rate of tumors detected by immunohistochemistry; (G) changes of pulmonary nodules detected by HE staining. **p* < 0.05 according to the two-way ANOVA. Data represent averages of three independent experiments.

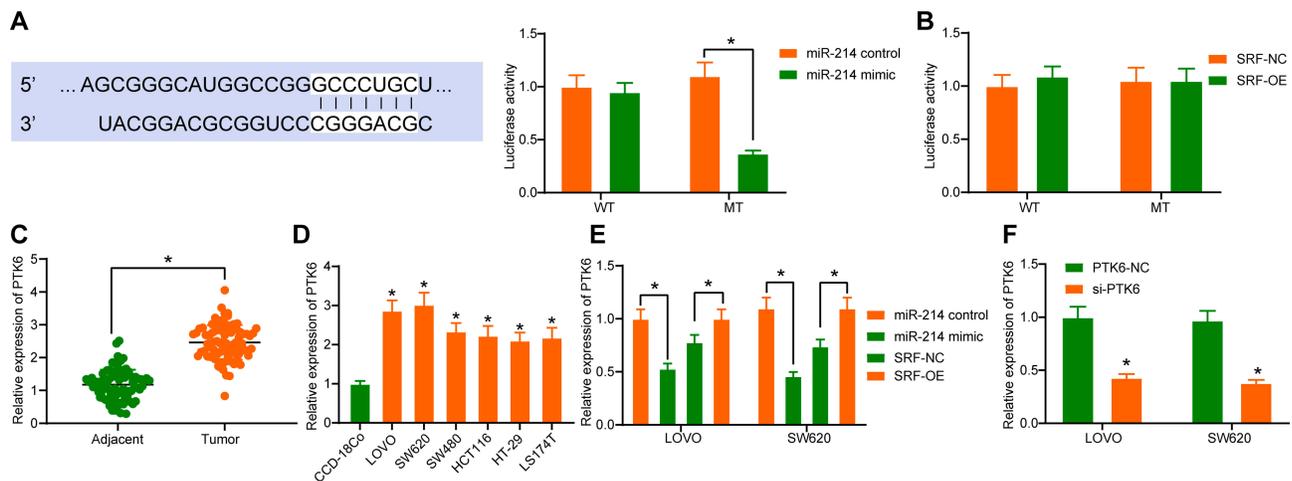


Figure 6 The binding relationship between miR-214 and PTK6 is identified. (A) the targeting relationship between PTK6 and miR-214 predicted by bioinformatic analysis and verified by a dual-luciferase reporting assay; (B) no direct targeting relationship between PTK6 and SRF verified by a dual-luciferase reporting assay; (C) PTK6 expression in CC and adjacent tissues evaluated by RT-qPCR detection; (D) PTK6 expression in CC cell lines evaluated by RT-qPCR detection; (E) PTK6 expression in CC cell lines overexpressing miR-214 or SRF evaluated by RT-qPCR detection; (F) PTK6 expression in CC cell lines with PTK6 knockdown evaluated by RT-qPCR detection. **p* < 0.05 according to one-way (panel D) or two-way ANOVA (panel A, C, E and F). Data represent averages of three independent experiments.

199a-3p by interacting with their promoters in a direct manner to modulate epithelial-mesenchymal-transition and pulmonary metastases.³⁷ Similarly, we found that SRF overexpression enhanced CC cell metastatic and tumorigenic potentials by impeding the transcription of miR-214.

A miRNA prediction database revealed PTK6 as a candidate target of miR-214; we next carried out a dual-luciferase reporter assay and RT-qPCR to verify this hypothesis. Consistently, Cagle et al validated that miR-214 overexpression induced prostate cancer cell apoptosis, while repressed cell proliferation and colony

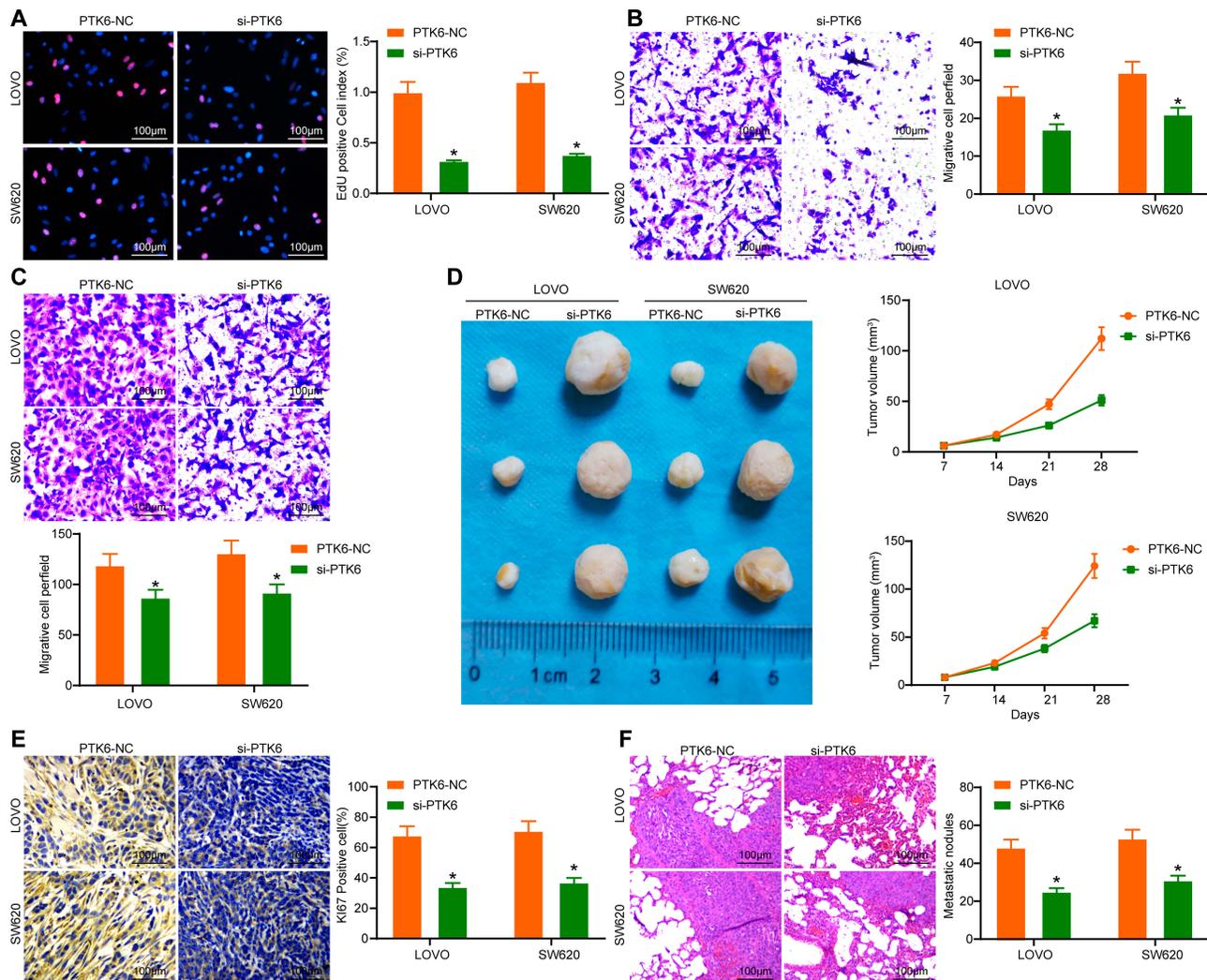


Figure 7 PTK6 knockdown is associated with reduced CC cell proliferative, migratory, invasive, tumorigenic and metastatic capacities. Si-PTK6 or PTK6-NC was delivered into CC cell lines. (A) CC cell proliferation determined by EdU staining; (B) CC cell migration evaluated by Transwell assays; (C) CC cell invasion evaluated by Transwell assays; (D) representative tumor images and tumor volume from mice injected with CC cells with PTK6 knockdown; (E) Ki67 positive rate of tumors detected by immunohistochemistry; (F) changes of pulmonary nodules detected by HE staining. * $p < 0.05$ according to the two-way ANOVA. Data represent averages of three independent experiments.

forming capacities by targeting PTK6.³⁸ PTK6 overexpression strongly correlated to the grade, recurrence as well as poor prognosis of bladder cancer patients, and PTK6 knockdown contributed to considerably suppressed cell proliferation and migration.³⁹ Meanwhile, the oncogenic role of PTK6 was also revealed by Wozniak et al in prostate cancer.⁴⁰ Furthermore, sustained induction of the JAK2/STAT3 pathway is highly correlated to tumorigenesis, proliferation, apoptosis and metastasis of tumors, including CC.⁴¹ While PTK6 overexpression might induce the expression of STAT3 and proliferation of breast cancer cells.⁴² In addition, miR-214 modulated CC procession through c-Met sialylation via the JAK2/STAT3 pathway.⁴³ In the current

work, our Western blot results revealed that miR-214 overexpression or PTK6 knockdown reduced the extent of JAK2 and STAT3 phosphorylation, whereas SRF upregulation reversed the function of miR-214 on JAK2/STAT3 pathway activation.

All in all, the present study established that miR-214 downregulation, at least partially due to the upregulation of SRF, may promote CC growth and metastasis via activation of the PTK6/JAK2/STAT3 pathway, and miR-214 is probably a biomarker for predicting the prognosis of CC (Figure 9). Therefore, concentrating on the SRF/miR-214/PTK6/JAK2/STAT3 regulatory axis may be an attractive strategic option for the treatment of CC. Further examination is necessary for the validation of JAK2/STAT3 pathway in CC.

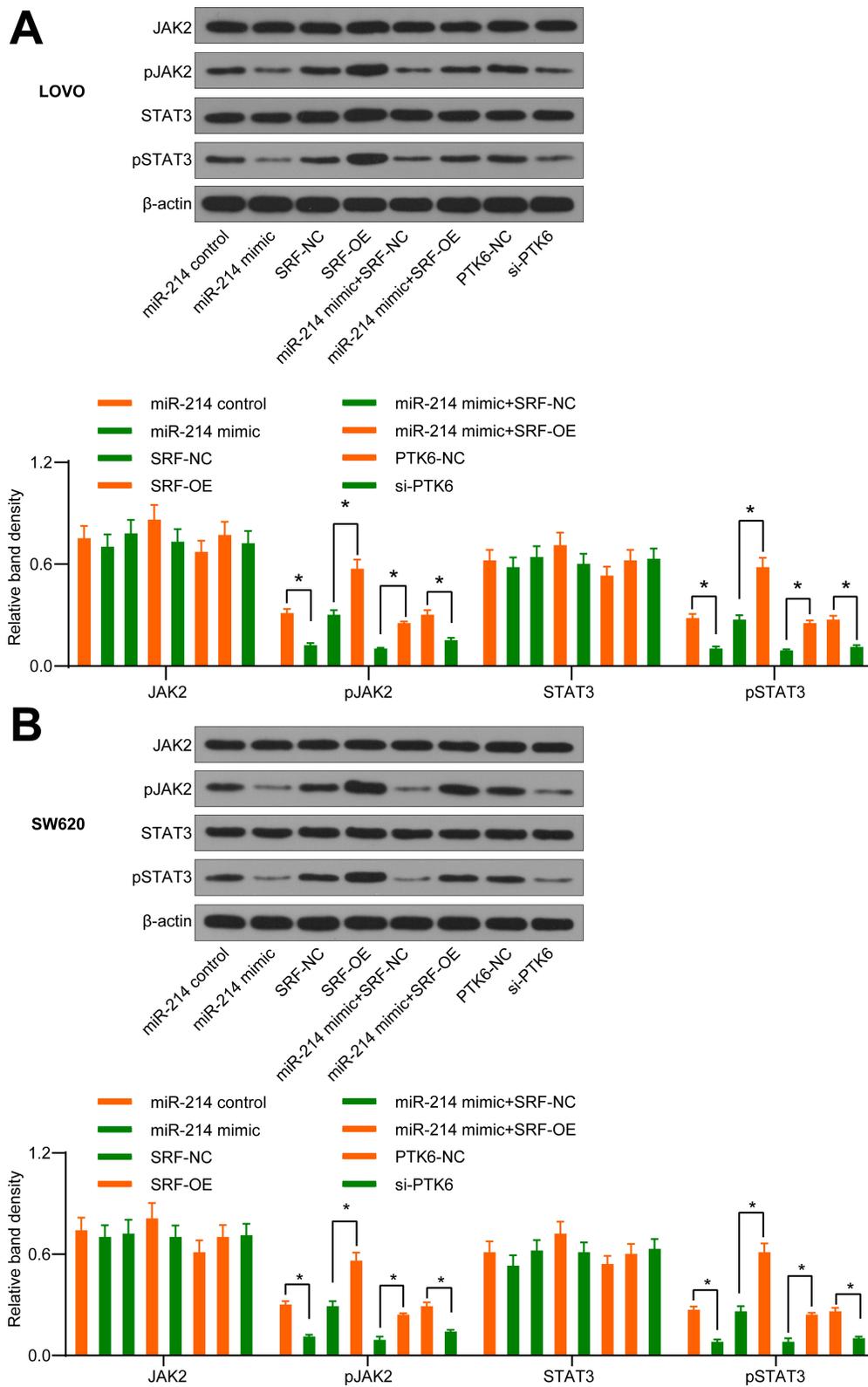


Figure 8 The JAK2/STAT3 pathway is regulated by the SRF/miR-214/PTK6 axis. The JAK2 and STAT3 protein expression and phosphorylation in LOVO (**A**) and SW620 (**B**) cells transfected with SRF-NC, SRF-OE, miR-214 control, miR-214 mimic, miR-214 mimic + SRF-NC, miR-214 mimic + SRF-OE, PTK6-NC or si-PTK6. * $p < 0.05$ according to the two-way ANOVA. Data represent averages of three independent experiments.

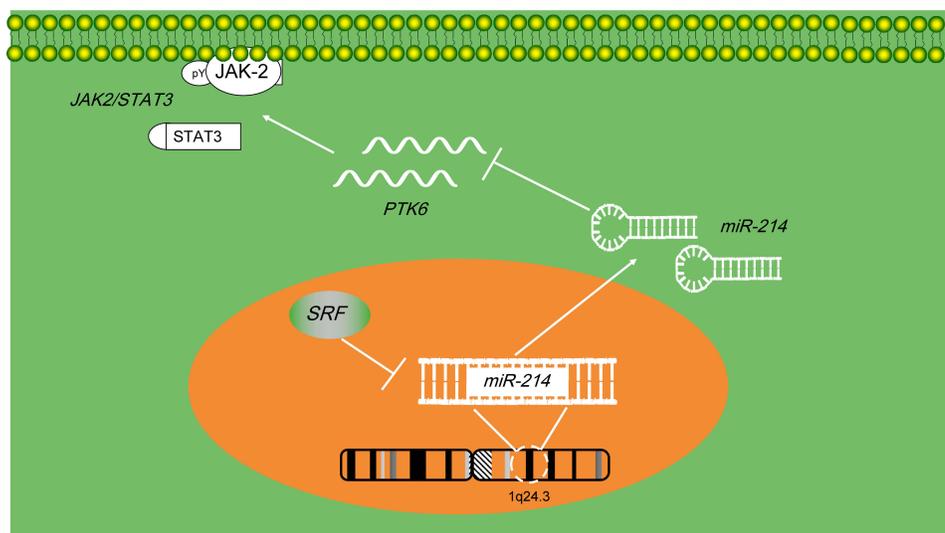


Figure 9 The model for miR-214 regulation upon CC cell progression. SRF could bind to the miR-214 promoter region, located on chromosome I q24.3, and repress its expression. The restoration of miR-214 lowers the expression PTK6, thereby inhibiting the activity of the JAK2/STAT3 pathway, which in turn inhibits CC cell proliferation, migration, invasion, tumorigenesis, and metastasis.

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Author Contributions

TL contributed to the conception of the study. YCW and ZYS designed the study. JYL provided advice on statistical methods and the analyses of the data. MNH prepared the draft of the manuscript, tables and figures. TL and CYZ provided comments on the final draft of the manuscript. All authors contributed to data analysis, drafting or revising the article, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

Disclosure

The authors declare no conflict of interest.

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