ORIGINAL RESEARCH

IRF1 inhibits the proliferation and metastasis of colorectal cancer by suppressing the RAS-RAC1 pathway

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Background: Interferon regulatory factor 1 (IRF1) plays a role in the immune response, cellular necrosis, DNA damage, and DNA repair, offering an attractive target for anticancer treatment. However, little is known about the role of IRF1 in the regulation of CRC progression.
Methods: Quantitative reverse transcription-PCR, Western blot, and immunohistochemistry were used to examine the expression level of IRF1; Cell Counting Kit-8, migration assay, and xenograft mouse models were used to examine the function of IRF1 in CRC cell lines; a ChIP assay was used to examine the binding between IRF1 and Ras association domain-containing protein 5 (RASSF5).
Results: IRF1 expression was lower in colorectal cancer (CRC) than in normal mucosa and the IRF1 expression level was inversely associated with CRC metastasis. In addition, IRF1 could inhibit CRC cell proliferation, migration, and metastasis in vivo and in vitro; IRF1 also induced cell cycle arrest but had no effect on cell apoptosis. IRF1 enhanced the expression of RASSF5 by increasing its promoter activity. Moreover, this study revealed a novel mechanism for inhibiting the RAS-RAC1 pathway by overexpression of RASSF5.
Conclusion: Altogether, the results indicate that IRF1, which promotes RASSF5 expression, suppresses CRC metastasis and proliferation possibly through downregulation of the RAS-RAC1 pathway.
Keywords: IRF1, RASSF5, Rac1, Ras, colorectal cancer

Background

Metastasis is one of the most important reasons for the treatment of colorectal cancer (CRC).[1] Metastasis in patients is associated with a poor prognosis and a high mortality rate. Although there have been many therapeutic advances in CRC treatment, the 5-year survival rate is only 10% in metastatic cases.[2] Therefore, clarifying the molecular mechanisms of CRC and searching for effective targets for clinical therapy are urgently needed.

IRF1, also called interferon regulatory factor 1, is encoded by the *IRF1* gene in humans.[3] As a transcription factor, IRF1 is the first member of the interferon regulatory factor family.[4] IRF1 was originally thought to be a transcription factor that activates the expression of β-interferon and was subsequently confirmed to have a role in the transcriptional activation and repression of its target genes.[5] IRF1 binds to interferon-stimulated response elements (ISREs) in the target gene promoter region to regulate the expression of target genes. IRF1 binds to ISREs through a DNA-binding domain, which is a helix-turn-helix in the N-terminus of IRF1 protein, and the DNA-binding

domain is highly conserved in all members of the IRF family.[6,7] In addition to its function as a transcription factor, IRF1 can also bind with its cofactor P300 and transactivate the tumor suppressor p53 and plays a role in the regulation of the immune response, cell necrosis, DNA damage response, and inhibition of tumor progression.[8]

It has been reported that Ras association domain-containing protein 5 (RASSF5) is one of the target genes of IRF1, but there have been no experiments confirming this.[9] RASSF5 is recognized as a tumor suppressor gene.[10] RASSF5 represses tumor proliferation and induces cell apoptosis in lung cancer by inhibiting extracellular signal-regulated protein kinase (ERK) pathway activity.[11] Moreover, RASSF5 is a downstream protein of the Ras pathway. RASSF5 interacts with Ras and Rap1 in an activated GTPase form to regulate the adhesion of tumor cells and to inhibit cell cycle progression.[12]

At present, it has been confirmed that IRF1 functions as a tumor suppressor gene in breast cancer, gastric cancer, esophageal cancer, renal cell carcinoma, and other malignant tumors.[13–15] IRF1 promotes the apoptosis of tumor cells, increases tumor cell sensitivity to chemotherapeutic drugs, and has other effects, but the relationships between IRF1 and CRC are rarely reported. In this study, we confirmed that IRF1, a tumor suppressor gene that promotes RASSF5 expression, suppresses CRC metastasis and proliferation potentially through downregulation of the RAS-RAC1 pathway.

Methods
Clinical specimens and cell lines
A total of 39 fresh-frozen CRC tissues and paired normal colorectal mucosa were collected from patients at NanFang Hospital of Southern Medical University (Guangzhou, China). Written informed consent was obtained from all the patients, and this study was approved by the Ethics Committee of Southern Medical University. All the tissues were frozen in liquid nitrogen and then stored at −80°C for further use. The patients included in this study had not received any preoperative therapies. The study was conducted in accordance with the Declaration of Helsinki.

All the cell lines were obtained from American Type Culture Collection (ATCC), and the cells were cultured and stored according to ATCC recommendations.

Plasmid construction and RNA interference
The full-length sequence of IRF1 was amplified by PCR using cDNA from normal colorectal mucosa tissues, and then cloned into a pcDNA3.0 plasmid. The primer sequences used were as follows: IRF1 F, 5′- CCCAAGCTTATGCCCAT-CACTCGGATGC-3′ and R, 5′- CCGCTCGAGCTACG-GTGCACAGGGAATGG-3′. An shRNA targeting IRF1 (5′-GTGTCTTCACAGATCTGAA-3′) and an shRNA targeting an NC sequence (5′-AACCTTACAATGAATCTAC-3′) were purchased from GeneChem, Shanghai, China.

Quantitative reverse transcription-PCR (RT-qPCR), all RNA isolation, and RT-qPCR experiments were performed according the assay instructions and previously described methods.[16,17] Bestar™ Real-time PCR Master Mix was purchased from DBI, (Hazleton, PA, USA), RNAisoTM Plus and a PrimeScript® first Strand cDNA Synthesis Kit were purchased from TaKaRa Company (Kusatsu, Japan), and an antibody against IRF1 was purchased from Proteintech (Rosemont, IL, USA).

Western blotting
Proteins were extracted and separated in SDS-PAGE gels, then transferred onto polyvinyl difluoride membranes, and blotted according to standard methods using anti-IRF1, anti-RASSF5, anti-Rac1, anti-Ras, anti-p53, anti-p21, and anti-pTubulin, followed by their respective horseradish peroxidase-conjugated secondary antibodies. Signals were detected using an enhanced chemiluminescence reaction performed according to the manufacturer's instructions (Alpha Innotech, San Leandro, CA, USA).

Cell Counting Kit-8 (CCK8), plate colony formation, cell migration, and flow cytometry assays
The CCK8, plate colony formation, cell migration, and flow cytometry assays were performed as previously described.[16,17]

Chromatin immunoprecipitation (ChIP)
ChIP was performed using a ChIP assay kit (Millipore, Billerica, MA, USA, catalog EZ17-371) according to the manufacturer's instructions. Briefly, 1×10^6 cells were cultured, fixed with 1% formaldehyde, washed, and then lysed by sodium dodecyl sulfate lysis buffer, followed by sonication to produce chromatin of primarily mononucleosomal size. IRF1 or IgG antibody was incubated overnight with the DNA to form protein-DNA complexes; then, the DNA was eluted from the antibody. The immunoprecipitated DNA sequences containing IRF1 binding sites were amplified by PCR. The primers used for ChIP were as follows: RASSF5 promoter F, 5'-CAGAGGATATGTTACCGTGA-3', and R, 5'-AACACAGGTTCGGTTTGG-3'.

Statistical analysis

The results of all the experiments are presented as the mean ± SD of ≥3 independent experiments. One-way ANOVA or independent Student's t tests were used for comparing independent experimental groups. All statistical analyses were performed using SPSS version 19 software, and $P<0.05$ was considered statistically significant.

Ethics approval and consent to participate

This study was approved by the Committees for Ethical Review of Research at Southern Medical University.

Results

IRF1 downregulation in CRC correlates with tumor metastasis

To assess the expression pattern of IRF1 in CRC, RT-qPCR was used to analyze IRF1 expression in 39 CRC paired samples. The results showed that the level of IRF1 was significantly decreased in 33 of 39 CRC specimens ($P<0.002$) compared with their normal counterparts (Figure 1A, B). In addition, in seven paired samples, IRF1 was downregulated in six CRC tissues (Figure 1C) as determined by Western blotting. Immunohistochemistry was also used to examine IRF1 expression, and we found that IRF1 was highly expressed in 36 of 61 normal mucosa samples, while it was highly expressed in only 20 CRC samples (Figure 1D, E).

We also examined the relationship between the IRF1 expression level and the clinicopathological characteristics of the tumor specimens. As shown in Table S1, the IRF1 expression level was inversely associated with CRC serosal invasion ($P=0.002$), lymph metastasis ($P=0.002$), and TNM classification ($P=0.043$).

In addition, we also assessed the expression of IRF1 in six CRC cell lines and found that compared with SW620 cells, IRF1 was more highly expressed in SW480, HT29, and HCT116 cells. Notably, IRF1 was upregulated in SW480 cells, which have low metastatic capacity, compared with SW620 cells, which possess strong metastatic abilities (Figure 1F, G).

These results indicated that IRF1 was downregulated in CRC tissues and may serve a critical role in CRC metastasis.

IRF1 inhibits CRC cell proliferation, migration, and metastasis

To study the function of IRF1 in CRC progression, IRF1 was overexpressed or silenced in CRC cell lines (Figure S1A, B).

The results of the CCK8 assay (Figure 2A) demonstrated that overexpression of IRF1 resulted in inhibition of cell growth in SW480 cells. In contrast, knockdown of IRF1 in HT29 cells (the IRF1 level was higher in these cells than in SW480 cells) had the opposite effect. Plate clone formation assays showed that the cell proliferation activity was significantly inhibited in overexpression-IRF1 (oe-IRF1) SW480 and SW620 cells (Figure 2B). The results of cell migration assays also showed that overexpression of IRF1 significantly reduced the migratory abilities of SW480 cells compared with the vector group, while IRF1 silencing promoted HT29 cell migration (Figure 2C).

Consistent with the in vitro observations, xenograft tumors that formed in the oe-IRF1 group were generally smaller and slower growing than those that formed in the control group (Figure 2D, E). Moreover, the tumors that developed in the oe-IRF1 group displayed a lower Ki67 proliferation index (Figure 2F). We also found that overexpression of IRF1 inhibited the metastasis of SW620 cells to the lung (2/5) and liver (0/5) (Figure 2G).

IRF1 induces cell cycle arrest but has no effect on cell apoptosis

Flow cytometry was used to examine whether the decreased cell proliferation might be a result of cell cycle arrest or induction of cell apoptosis. As shown in Figure 3A, SW480 cells transfected with the oe-IRF1 plasmid had a significantly increased proportion of cells in G0/G1 phase, from 47.08% to 74.84% compared with the vector group. HT29 cells transfected with the sh-IRF1 plasmid had a significantly increased proportion of cells in G2/M phase, from 76.45% to 55.7% (Figure 3B). In addition, the proportion of apoptotic cells was detected. The results showed that transfection of IRF1 plasmid or sh-IRF1 into SW480 and HT29 cells had no obvious effect on cell apoptosis compared with transfection of the vector alone (Figure 3C, D).

IRF1 activates RASSF5 expression and inhibits the RAS-RAC1 pathway

As a transcription factor, IRF1 binds to many gene promoter areas and regulates their expression. Using bioinformatics analysis, we found that IRF1 could bind to the *RASSF5* promoter (Figure 4A). ChIP assays result shown that RASSF5 promoter sequence were obviously enriched in the anti-IRF1 groups in SW480 and SW620 cells, and this result was similar to the bioinformatics analysis result (Figure 4B).

The *RASSF5* gene is a RAS-associated gene. As a tumor suppressor gene, *RASSF5* is inactivated in many tumors. We
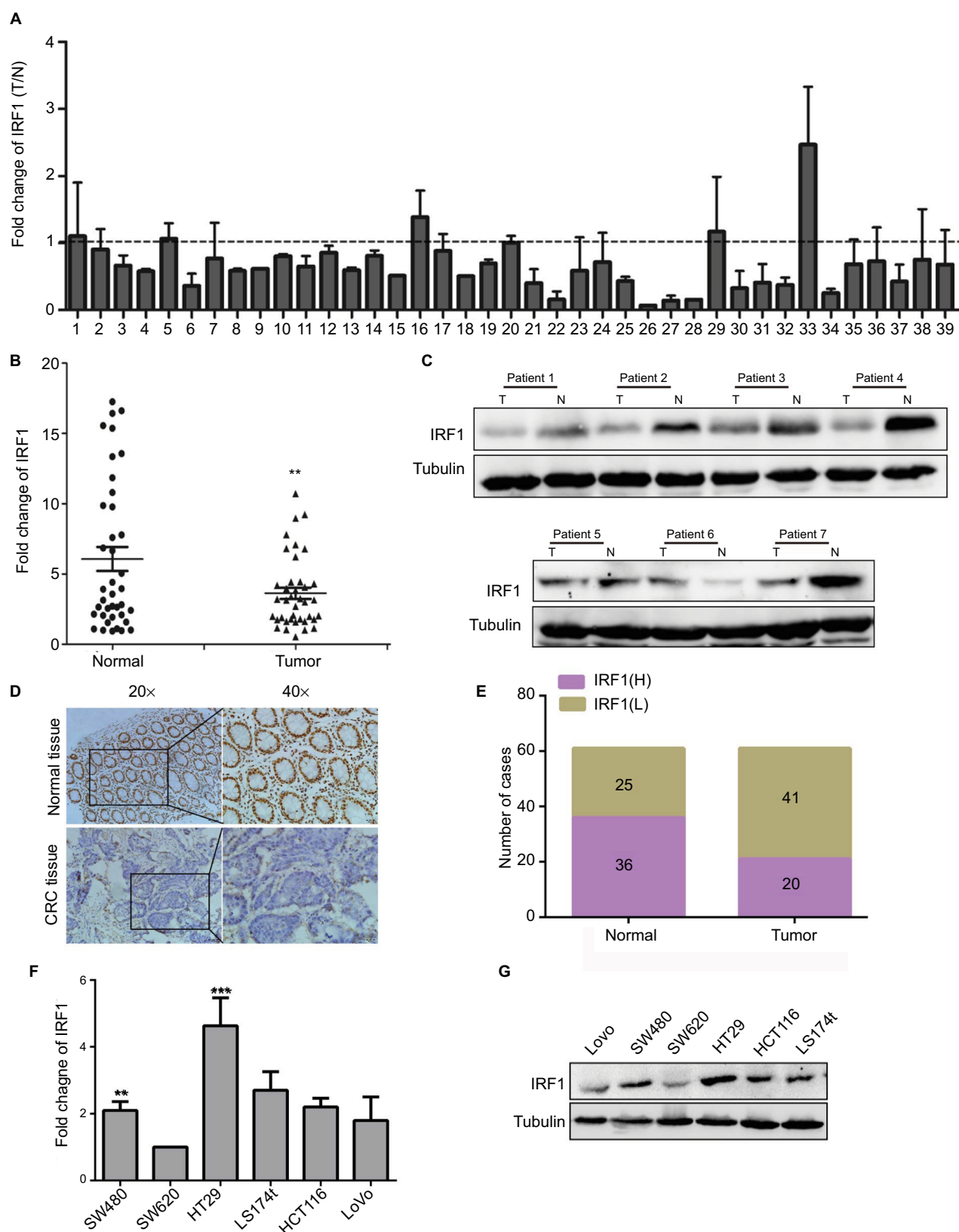
Figure 1 Expression of IRF1 in CRC tumors and cell lines.
Notes: (**A**) RT-qPCR analysis of IRF1 expression in 39 paired CRC tissues. Data are represented as the mean ± SD of three independent experiments. (**B**) Comparison of IRF1 expression in 39 primary CRC tissues (T) with paired adjacent normal tissues (N). Data are represented as the mean ± SD of three independent experiments. (**C**) The expression of IRF1 in CRC tissues and paired normal tissues as examined by Western blotting. (**D**, **E**) Immunohistochemical staining of IRF1 in CRC tissues and paired normal tissues. (**F**, **G**) The expression of IRF1 in CRC cell lines as examined by Western blotting and RT-qPCR. **$P<0.01$, ***$P<0.001$.
Abbreviations: CRC, colorectal cancer; IRF1, interferon regulatory factor 1; N, normal tissue; RT-qPCR, quantitative reverse transcription-PCR; T, colorectal tissue.
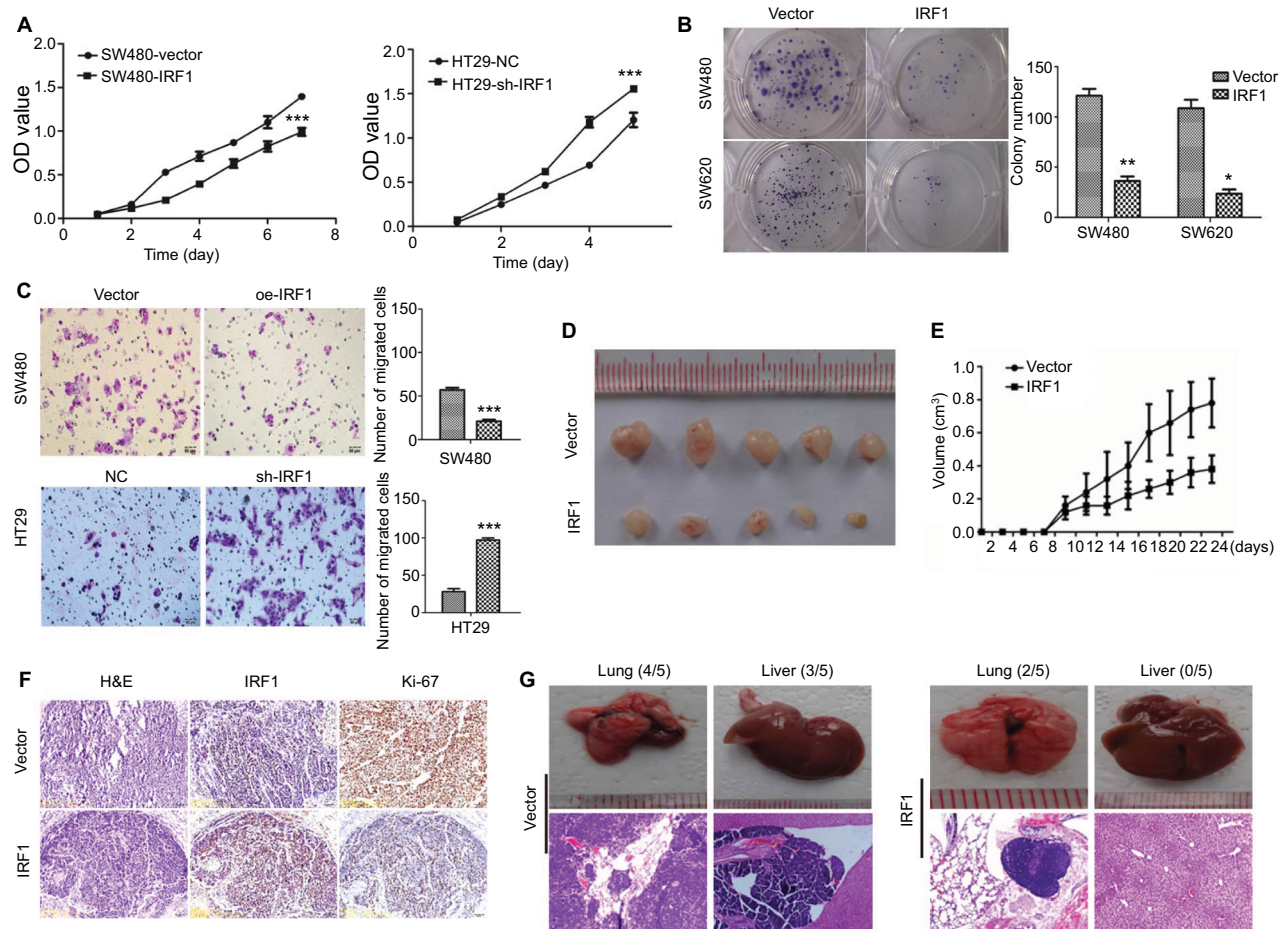
Figure 2 IRF1 inhibits CRC cell proliferation, migration, and metastasis.

Notes: (**A**) The effects of IRF1 overexpression and knockdown on cell proliferation as determined by CCK8 assays. Data are represented as the mean ± SD of three independent experiments. (**B**) The effects of IRF1 overexpression and knockdown on cell proliferation by plate colony formations assays. Data are represented as the mean ± SD of three independent experiments. (**C**) The effects of IRF1 overexpression and knockdown on cell proliferation as measured by Transwell migration assays. Data are represented as the mean ± SD of three independent experiments. (**D**) Effects of oe-IRF1 transfection on the subcutaneous tumor generation capacity of SW620 cells (n=5). (**E**) Tumor sizes were measured on the indicated days. Data are represented as the tumor volume mean ± SD. (**F**) Ki-67 index was analyzed to evaluate the proliferative capacity of subcutaneous tumor cells. (**G**) Gross images and H&E stained sections of liver and lung metastases from xenotransplanted mice who were injected via the tail vein (n=5 per group). *P<0.05, **P<0.01, ***P<0.001.

Abbreviations: CCK8, Cell Counting Kit-8; CRC, colorectal cancer; IRF1, interferon regulatory factor 1; OD, optical density.

examined the expression of RASSF5 and the RAS-RAC1 pathway-associated proteins RAS, Rac1, P21, and P53 in SW480 and SW620 cell lines that were transfected with the IRF1 plasmid. The results showed that overexpression of IRF1 significantly increased the expression of RASSF5, P53, and P21, while RAC1 and RAS protein levels were notably decreased (Figure 4C).

All these results indicated that IRF1 activates RASSF5 expression by increasing the transcriptional activity of its promoter. Thus, RASSF5 expression may inhibit the RAS-RAC1 pathway.

Discussion

Members of the IRF family are able to bind to the interferon (IFN) promoter and induce the expression of IFN when tissues are infected by viral and microbial agents.[18] With more in-depth research, additional functions of IRF1 will slowly be revealed. In ovarian cancer cells, the chemotherapy drug cisplatin increased IRF1 expression levels, which reduced the effectiveness of chemotherapeutic drugs.[19] In invasive neuroblastoma, IRF1 and nuclear factor-kappa B are responsible for repairing the major histocompatibility complex I and processing antigens for presentation by cytotoxic T cells, both of which play a role in tumor suppression.[20] In this study, we found that the level of IRF1 was significantly decreased in 33 of 39 CRC specimens (P<0.002) compared with their normal counterparts and that the IRF1 expression level was inversely correlated with CRC serosal invasion (P=0.002), lymph metastasis (P=0.002), and TNM classification.
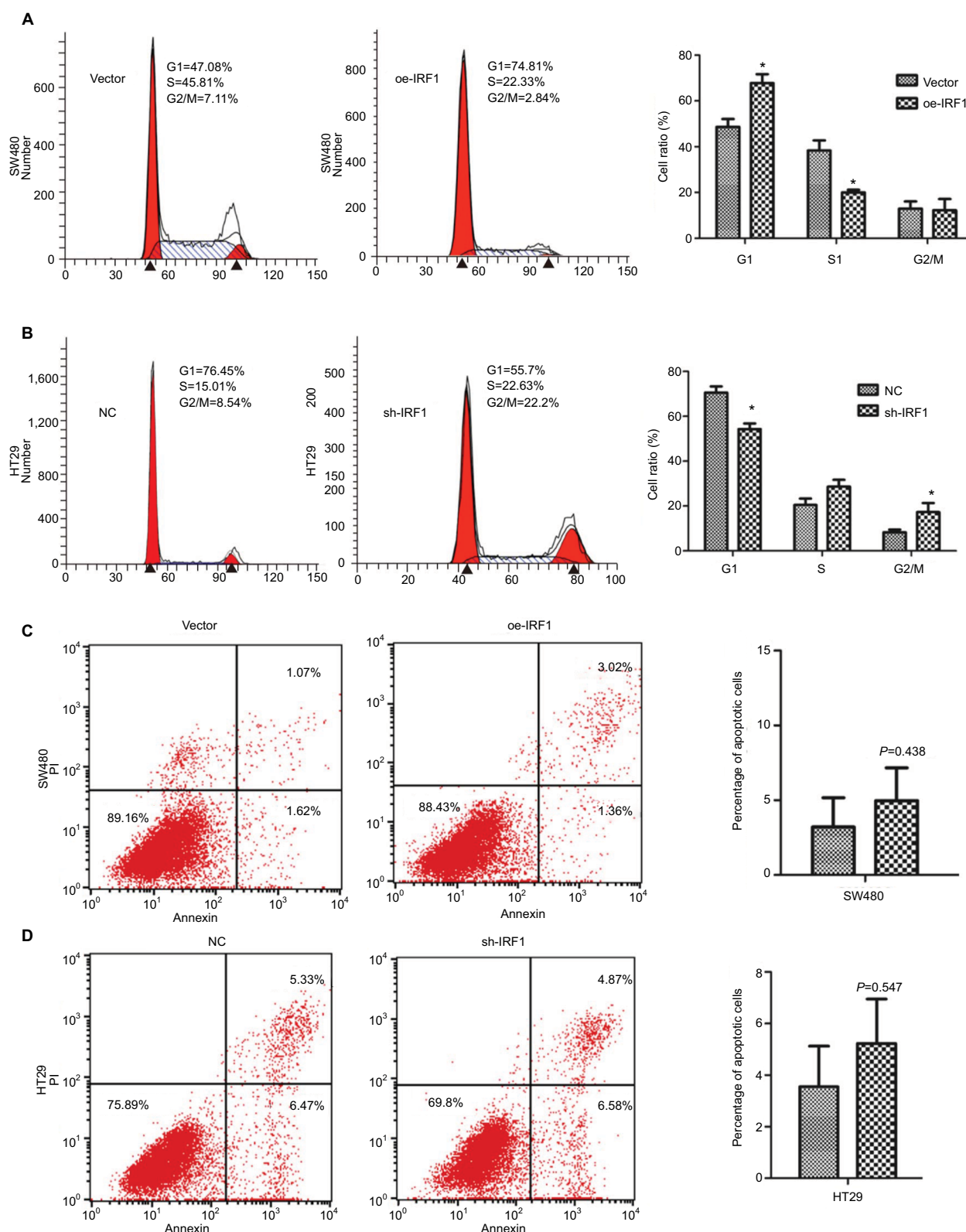
Figure 3 IRF1 induces cell cycle arrest but has no effect on cell apoptosis.
Notes: (**A**) The effect of IRF1 overexpression on the cell cycle in SW480 cells as examined by FCM. (**B**) The effect of IRF1 knockdown on the cell cycle in HT29 cells as examined by FCM. (**C**) The effect of IRF1 overexpression on cell apoptosis in SW480 cells as examined by FCM. (**D**) The effect of IRF1 knockdown on apoptosis in HT29 cells as examined by FCM. All data are represented as the mean ± SD of three independent experiments. *$P<0.05$.
Abbreviations: FCM, flow cytometry; IRF1, interferon regulatory factor 1; PI, propidine iodide.

A

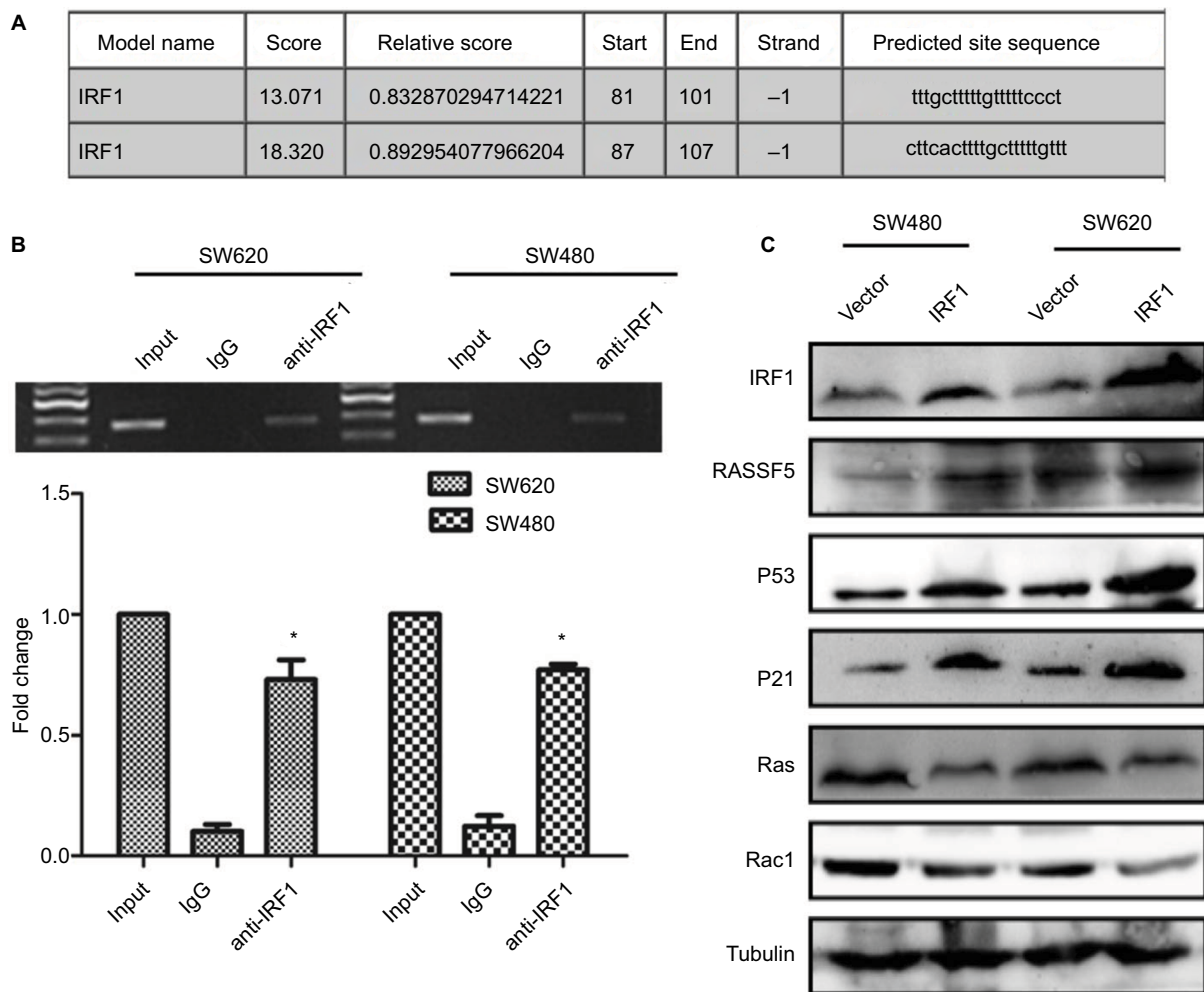| Model name	Score	Relative score	Start	End	Strand	Predicted site sequence
IRF1	13.071	0.832870294714221	81	101	−1	tttgctttttgtttttccct
IRF1	18.320	0.892954077966204	87	107	−1	cttcacttttgctttttgttt

B

C

Figure 4 IRF1 activates RASSF5 expression and inhibits RAS-RAC1 pathway.
Notes: (**A**) Predicted IRF1 binding sites on the RASSF5 promoter as determined by bioinformatics methods. (**B**) ChIP assay antibodies against IRF1 in CRC cells. RT-PCR and PCR show that the promoter sequence of RASSF5 could be enriched in the anti-IRF1 group compared with the IgG group. (**C**) Western blot analyses of RASSF5, RAS, P21, P53, and Rac1 in IRF1-transfected cells. *$P<0.05$.
Abbreviations: CRC, colorectal cancer; ChIP, chromatin immunoprecipitation; IRF1, interferon regulatory factor 1; RASSF5, Ras association domain-containing protein 5; RT, reverse transcription.

In the present study, the potential role of IRF1 was investigated in CRC cell lines through gain- and loss-of-function assays. The results demonstrated that IRF1 overexpression inhibited cell proliferation and migration in vitro and that overexpressed IRF1 suppressed CRC cell metastasis in vivo. IRF1 modulated the cell cycle distribution of CRC cells; however, IRF1 had no obvious effects on cell apoptosis. These results indicate that IRF1 serves a suppressive role in CRC progression, which is consistent with the expression patterns of IRF1 in gastric cancer and prostate carcinoma.[21,22]

It has been reported that IRF1 generally exerts its biological function by binding to the promoters of its specific target genes.[23] Using bioinformatics analysis, we found that IRF1 could bind to the promoter of the RASSF5 gene, which is a Ras-associated gene, and we confirmed this finding by

ChIP assays. RASSF5 is expressed in most normal tissues and is downregulated in many tumor tissues and cell lines, including in lung cancer.[24] Ras/rac1 proteins are effectors of RASSF5 because RASSF5 inhibited the activity of Ras/rac1 proteins by inducing the expression of other tumor suppressor genes, such as P53 and P21. Our results indicated that IRF1 could enhance the expression of RASSF5 in CRC cell lines. Meanwhile, the Ras and Rac1 proteins were suppressed, and p53 and p21 protein levels were notably increased. A large number of studies have reported that inhibition of the RAS-RAC1 pathway suppresses tumor progression, which can be seen in CRC.[25,26] Altogether, our study confirmed that IRF1, a tumor suppression gene that promotes RASSF5 expression, suppresses CRC metastasis and proliferation possibly through downregulation of the RAS-RAC1 pathway.

Conclusion

In summary, we found that IRF1, which promotes RASSF5 expression, suppresses CRC metastasis and proliferation possibly through downregulation of the RAS-RAC1 pathway.

Availability of data and materials

Literature collection was performed through PubMed. All statistical analyses were performed using SPSS 19.0. Data are stored by the corresponding author of this article and are available upon request.

Acknowledgments

Author contributions

XNL, MH, and ZZ participated in the design of the study, drafted the manuscript, and performed the experiments. JZ, XML, and QC analyzed the data and performed the statistical analysis. WJZ and WZ prepared the figures. CL and FZ conceived and designed the study. 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 report no conflicts of interest in this work.

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Supplementary materials

Table S1 Clinicopathologic characteristics of IRF1 expression in CRC patients

Clinicopathological variables	N	Low expression[c]	High expression	
All case	39	19	20	*P*
Age (years)[a]				
≤58	19	9	10	0.869
>58	20	10	10	
Gender				
Male	23	11	12	0.894
Female	16	8	8	
Tumor size (cm)[b]				
≤4.53	16	7	9	0.605
>4.53	23	12	11	
Differentiation				
Well	15	5	10	0.129
Middle/poor	24	14	10	
Serosal invasion				
Yes	16	12	4	0.006
No	23	7	16	
Lymph metastasis				
Yes	18	13	5	0.007
No	21	6	15	
TNM classification				
I–II	21	4	17	0.001
III–IV	18	13	5	

Notes: [a]Grouping of age was performed according to median. [b]Tumor size was grouped according to median. [c]IRF1 expression level was divided according to median.
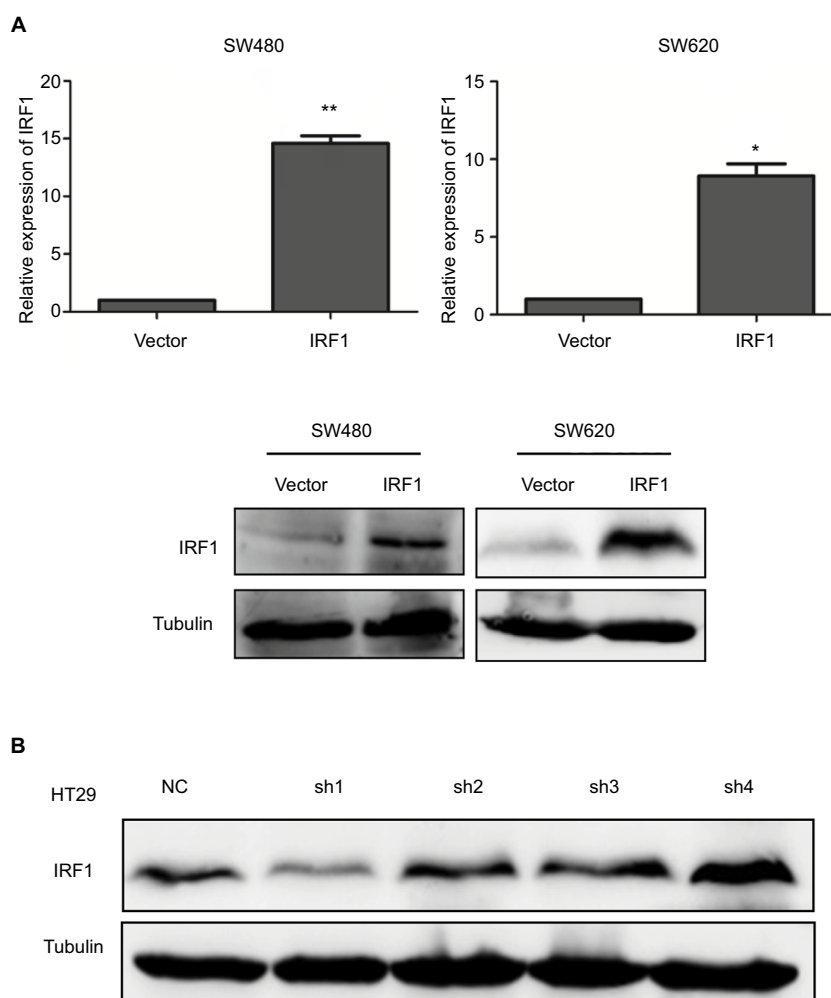**Abbreviations:** CRC, colorectal cancer; IRF1, interferon regulatory factor 1.

Figure S1 Construction of oe-IRF1 and knockdown IRF1 transfected cell lines.
Notes: (**A**) Ectopic expression of IRF1 in SW480 and SW620 cell lines was analyzed by qRT-PCR (upper) and Western blotting (lower). (**B**) Verification of the knockdown efficiency of shRNA-IRF1 in HT29 cells. *$P<0.05$, **$P<0.01$.
Abbreviations: CRC, colorectal cancer; IRF1, interferon regulatory factor 1; qRT, quantitative reverse transcription.