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Original Research

miR-330-5p Suppress Cell Growth and Invasion via Disrupting HSF4-mediated MACC1/STAT3 Pathway in Colorectal Cancer

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Abstract

Background: Recently, miRNAs are demonstrated to restrain mRNA translation through novel pattern with bind complementary sites in the coding sequence (CDS). Heat Shock Transcription Factor 4 (HSF4) has been newly described as a tumor-associated transcription factor. Therefore, the present study intends to explore miRNAs that bind CDS region of HSF4, and identify the function of their interactions in the malignant biological behavior of colorectal cancer (CRC). **Methods**: Prognostic value of HSF4 and correlation between HSF4 and MACC1 expression were estimated via bioinformatics with the Cancer Genome Atlas (TCGA) data. HSF4 and downstream MACC1/STAT3 signaling cascade was characterized by immunoblotting. To characterize the effects of miR-330-5p and HSF4 on the malignant phenotype of CRC cells by functional experiments. The binding activity of miR-330-5p to coding sequence (CDS) of HSF4 was identified using DIANA-microT-CDS algorithm and dual-luciferase reporter assay. **Results**: HSF4 was aberrantly overexpressed and associated with poor outcomes of CRC patients. Overexpression of HSF4 was correlated with Tumor Node Metastasis stage, and positively regulated malignant behaviors such as growth, migration, invasion of CRC cells. Moreover, miR-330-5p suppressed CRC cell growth, colony formation, migration and invasive. Interestingly, miR-330-5p recognized complementary sites within the HSF4 CDS region to reduce HSF4 expression. In rescue experiments, restoration of HSF4 expression functionally alleviated miR-330-5p-induced inhibition of cell growth, colon formation, invasion, and wound healing of CRC cells. HSF4 was associated positively with the well-known oncogenic factor MACC1 in TCGA cohort CRC samples, and knockdown of HSF4 resulted in downregulation of MACC1. In mechanism, MACC1 was suppressed upon miR-330-5p-induced downregulation of HSF4, leading to inactivation of phosphorylation of downstream STAT3. **Conclusion**: miR-330-5p suppresses tumors by directly inhibiting HSF4 to negatively modify activity of MACC1/STAT3 pathway.

Keywords: colorectal cancer; prognosis; bioinformatics; miR-330-5p; Heat Shock Transcription Factor 4

1. Introduction

Colorectal cancer (CRC) is a prevalent malignant tumors of gastrointestinal system worldwide, often occurring in the sigmoid colon and rectum [1]. CRC is the 3rd highest ranked cancer in terms of prevalence and mortality in both men and women [2], and five-year survival rate is 65% [3]. Due to the inconspicuous early symptoms and limitations of screening methods, many CRC patients are advanced stage when they are diagnosed [4,5]. Therefore, in-depth studies on the molecular mechanisms of CRC are necessary to develop therapeutic strategies.

miRNAs have been demonstrated as crucial regulators to mediate cellular process in tumor development and progression. In the past decade, a lot of miRNAs have been characterized oncogenic or tumor-suppressive depending on their target mRNA. The most common epigenetic mechanism of miRNAs is the negative regulation of target expression through base complementary binding to the mRNA 3′UTR. In recent years, a novel pattern of binding to coding sequence (CDS) complementary sites has been identified in the epigenetic inheritance of miRNAs [6,7]. Moreover, this sort of miRNA-gene interaction and annotation is now predictive bioinformatically through specific algorithms such as DIANA- microT-CDS and miRactDB [8,9].

Some tumor-related miRNAs have been experimentally proposed to target CDS to regulate gene expression in tumors. Specifically, miR-646 promotes tumorigenesis of pancreatic cancer by targeting CDS to destabilize MIIP mRNA and inhibit its expression [10]. Moreover, miR-3140 mitigated BRD4-NUT fusion oncoprotein through interaction with CDS of BRD4, thereby suppressing cell growth of tumor cells [11]. A recent study revealed that

a group of miRNAs was able to bind to the 3′UTR or CDS, thereby decreasing the surface expression of CD274/PD-L1 while increasing T-cell recognition, and that the inverse expression of these miRNAs and CD274 has furtherly been identified in melanoma samples [12]. Nevertheless, miRNA-related CDS-targeted regulation has not been identified in CRC so far.

Heat Shock Transcription Factor 4 (HSF4) is a class of molecules concerned with response to adverse stimuli, including heat, oxidation, oxygen deficit, free radicals, *etc.* [13]. Recent evidence suggests a role for HSF4 in tumorigenesis. Initially, HSF4 was different expression across a variety of cancer in the Cancer Genome Atlas (TCGA) dataset, whereas HSF4 was rare mutation in cancers with an average frequency of 3%–3.5% [14,15]. Specifically, overexpression of HSF4 predicts poor outcomes of hepatocellular carcinoma (HCC) patients, and enhanced epithelial-mesenchymal transition via a HIF1α-dependent manner activate AKT pathway [16]. Moreover, our previous study also disclosed that HSF4 contribute to tumor growth in CRC via transactivation of c-MET [17]. However, miRNAs targeting the CDS of the *HSF4* gene are yet to be further explored in CRC.

Based on these previous findings, this study proposed to identify the differences in HSF4 expression and its correlation with MACC1 expression and CRC prognosis by bioinformatics. Notably, miR-330-5p is demonstrated to have reduced expression and as a tumor suppressor, including CRC [18–20]. Therefore, the potential sequence of miR-330-5p to bind to the CDS region of HSF4 was estimated using DIANA-microT-CDS algorithm, and function of miR-330-5p and HSF4 on proliferation, invasion and migration were explored by *in vitro* experiments in CRC cells. It is designed to delineate molecular mechanisms of CRC, and to provide a theoretical basis and new perspectives for miR-330-5p and HSF4 as biomarkers for CRC therapy.

2. Materials and Methods

2.1 HSF4-related Bioinformatics Analyses

Differential expression of HSF4 in CRC was identified by GEPIA [21] with COAD and READ cohort in TCGA data. R2 analysis platform (AUMC, CEMM) was utilized to evaluate HSF4 expression in CRC datasets, including GSE14333 (Sieber, n = 290), GSE4554 (Watanabe, n = 84), GSE17538 (Smith, n = 232), GSE2109 (EXPO, n = 315) of CRC, and GSE8671 (Mara, n =32) of adjacent colon.

2.2 Cell Culture and Antibodies

HCT116, LoVo, SW480, DLD1, HT29 and SW620 (Human CRC cells) were obtained from NCACC (Shanghai, China). FHC cells was kindly donated by Dr. Liang Peng (Guangzhou Medical University). All cell lines were identified by NCACC using the CLAIdentiFiler™Plus PCR Kit (Applied Biosystems, Foster, CA, USA) based on short tandem repeat analysis. In addition, all cell lines have been tested by the MycoProbe® Kit (R&D Systems, Minneapolis, MN, USA) and determined to be free of mycoplasma contamination. CRC and FHC cell lines were cultured in RPMI-1640 medium (HyClone, South Logan, UT, USA; 10% FBS+1% penicillin-streptomycin solution), and grew in a 37 °C humidified atmosphere containing 5% CO_2.

Antibodies used in this study included anti-STAT3 (#9139S/Cell signaling, Danvers, MA, USA), anti-HSF4 (#18797-1-AP/Proteintech, Chicago, IL, USA; #PA5-68416/Invitrogen, Carlsbad, CA, USA), anti-p-STAT3 (#9145S/Cell signaling, Danvers, MA, USA), anti-MACC1 (ab226803/Abcam, Cambridge, MA, USA), anti-β-actin (#20536-1-AP/Proteintech, Chicago, IL, USA). All antibodies were diluted with reference to the instructions.

2.3 Immunohistochemical Staining

Three paired CRC tissues and matched adjacent tissue (2–5 cm from cancer tissue) were collected from CRC patients aged from 56 to 71. Immunohistochemical (IHC) staining as previously described [16]. Cytoplasm of tumor cell was probed with anti-HSF4 (#PA5-68416, Invitrogen, Carlsbad, CA, USA) antibody and was usually positive for HSF4. This study strictly adhered to the guidelines of the Declaration of Helsinki, and was approved by the ethics committee of the First People's Hospital of Yunnan Province (Protocol No. KHLL2021-KY109).

2.4 qPCR Assay

RNA from FHC and six CRC cells was extracted using TRIzol® reagent (Invitrogen, Carlsbad, CA, USA). RNA from each cell was taken as 1 µL to determine the RNA concentration using an ultra-micro spectrophotometer (Blue ray, Chengdu, Sichuan, China). Referring to the instructions of Hairpinit ™ miRNAs qPCR kit (Genepharma, Suzhou, Jiangsu, China), 3 µg of RNA was taken to perform reverse transcription and amplification of miR-330-5p. The reaction program for reverse transcription was performed at 25 °C for 30 min, 42 °C for 30 min and 85 °C for 5 min. The reaction program for PCR amplification was predenaturation at 95 °C for 3 min (1 cycle), amplification at 95 °C for 12 s and 62 °C for 40 min (40 cycles). RNU6B as an endogenous control. The sequence of primers are listed in **Supplementary Table 1**.

2.5 Oligonucleotide and DNA Constructs

hsa-miR-330-5p mimics, HSF4-targeted shRNA and their negative scramble controls were synthesized by Genepharma (Shanghai, China). The targeting sequences of HSF4-targeted shRNAs are shown in **Supplementary Table 1**. OmicsLink™ ORF cDNA clone of HSF4 (#Z4249) was purchased from FulenGen (Guangzhou, China). CRC cells were transfected with HSF4 cDNA clone or miR-330-5p mimics using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) according to manual.

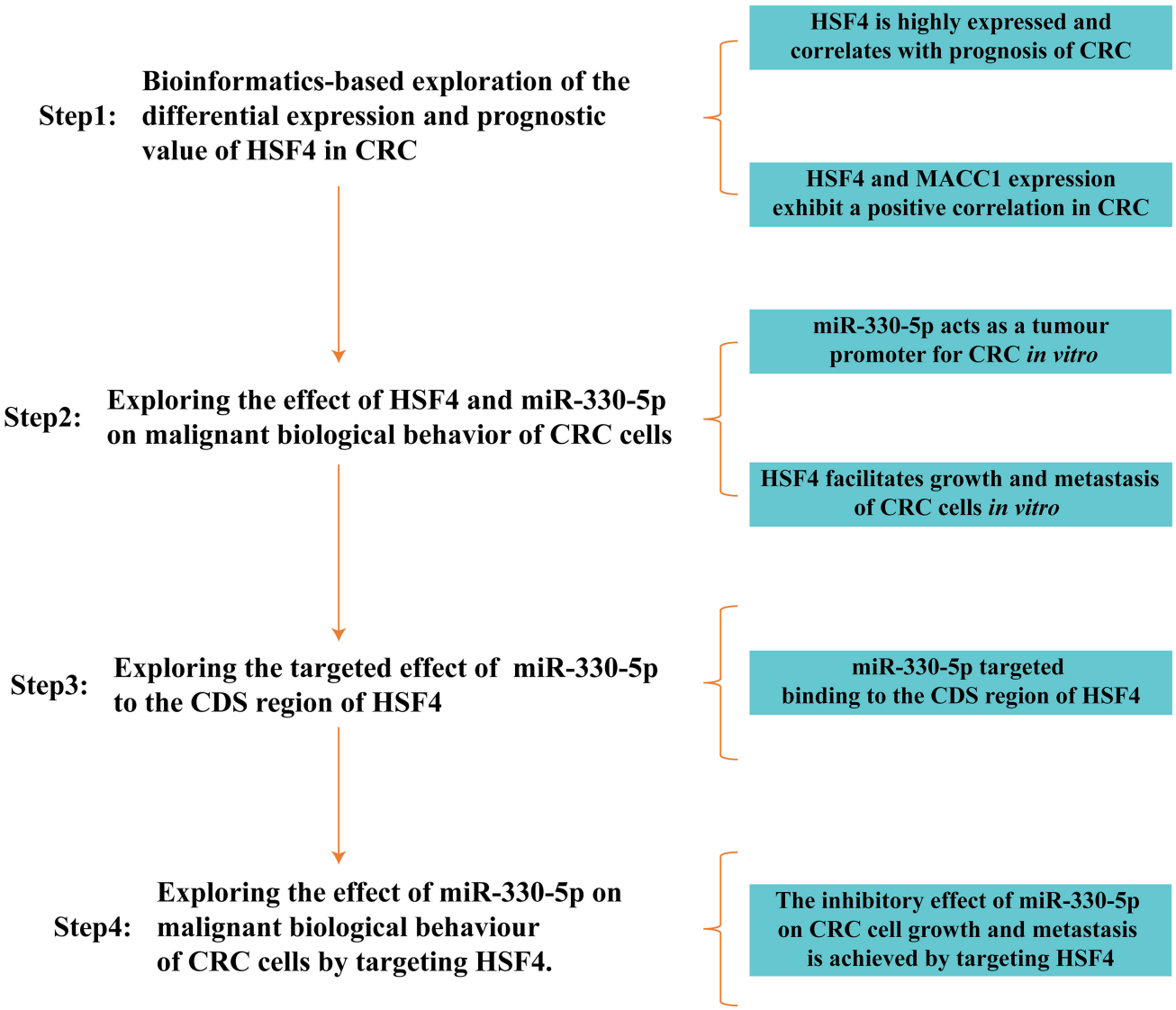

Fig. 1. Flowchart of this study regarding the analysis and experiments. HSF4: Heat Shock Transcription Factor 4; CRC: Colorectal Cancer; CDS: Coding Sequences.

2.6 Cell Function Assays

Cell viability was accessed using the Cell Counting Kit-8 (CCK-8) method (MCE, Monmouth Junction, NJ, USA) following manual. In colony formation assay, Lovo and HT29 cells were seed into 6-well plates with 300 per well. Then, cells were cultured at 37 °C for 10 d. Lovo and HT29 cells were subsequently subjected to crystal violet staining for 5 min. Colonies consisting of more than 50 cells per colony were observed and counted. In the wound healing assay, a sterile pipette tip was applied to create a scratch on the cell layer. Photomicrographs were captured at 0 h and 48 h after scratching, using a microscope. As previously described [22], Transwell with 8 µm filter (Corning, Corning, NY, USA) was applied cell invasion assay.

2.7 Dual-luciferase Reporter Assay

The miRNA with target binding to the CDS region of HSF4 was predicted by the DIANA-microT-CDS algorithm and was finalized as miR-330-5p. A fragment (485 bp) containing three potential bindings of miR-330-5p and HSF4 was amplified. Subsequently, the fragment was subcloned downstream of the luciferase gene sequence of the GV272 luciferase vector. GV272 luciferase vector was transfected into 293T cells and assayed for luciferase activity.

2.8 Immunoblotting Assay

Treated Lovo and HT29 cells were harvested and lysed using RAPI (Cell signaling, Danvers, MA, USA). Protein concentration of the lysates was determined using BCA protein assay kit (Beyotime, Shanghai China). Cell lysates with 30 µg was loaded in SDS-PAGE for electrophoresis. Subsequently, proteins of each group were transferred to polyvinylidene fluoride (PVDF) membranes. Color development was performed on the PVDF membrane using chemiluminescent substrate (Invitrogen, Carlsbad, CA, USA). β-actin served as an internal protein control.

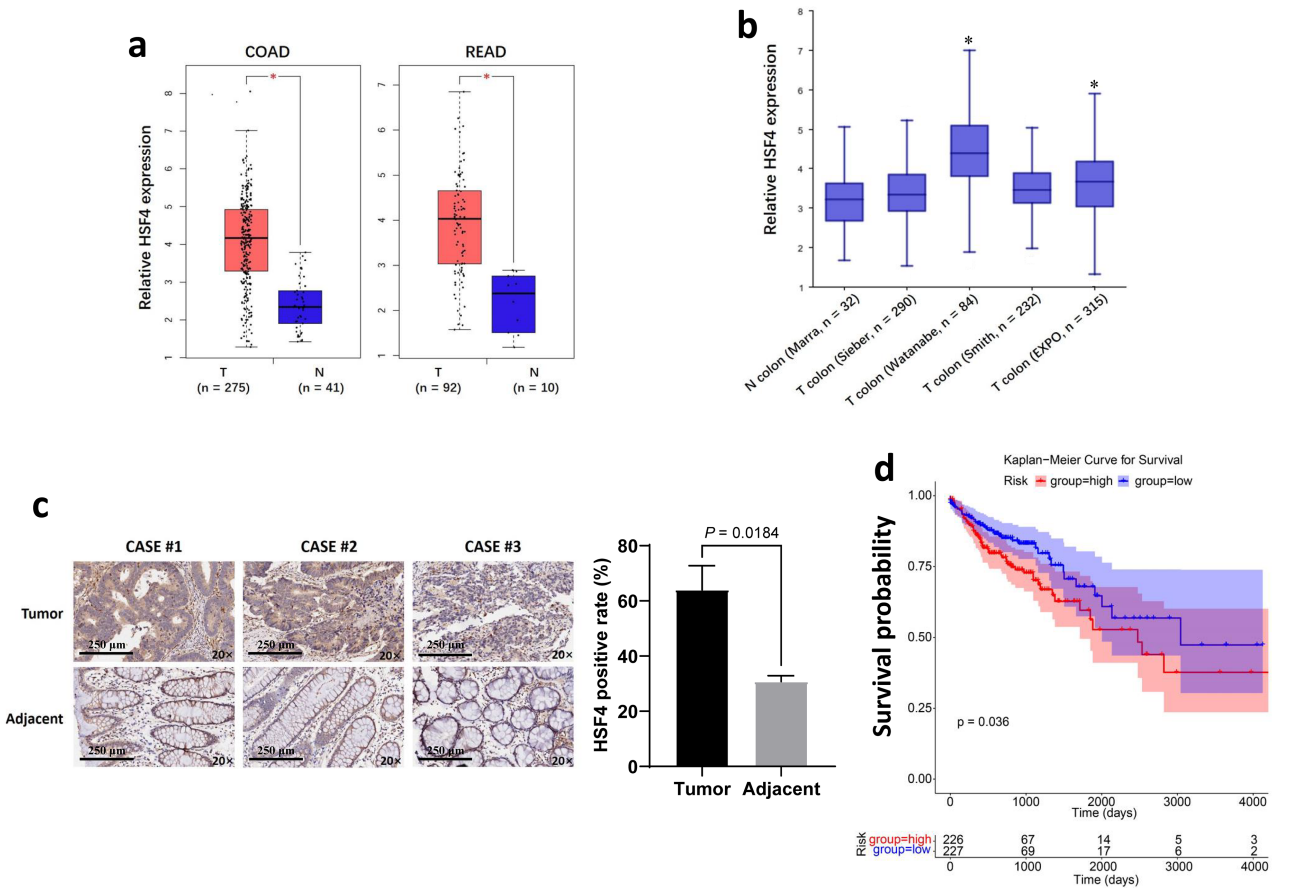

Fig. 2. HSF4 is overexpressed in CRC and associated with poor prognosis. (a) HSF4 expression is significantly increased in tumors both in colon adenocarcinoma (COAD) and rectum adenocarcinoma (READ) cohorts. (b) HSF4 expression is up-regulated in CRC cohorts than the cohort of normal colonic tissues [N colon (Mara, n = 32)] in an integrative analysis of multiple Gene Expression Omnibus (GEO) database. T and N stand for COAD/READ and adjacent tissues, respectively. (c) Immunohistochemistry (IHC) analysis on 3 pairs of CRC samples reveals that HSF4 is strongly detectable in CRC tissues but weakly in adjacent colonic tissues. (d) Overexpressed HSF4 exhibits significant association with short survival term of patients by analyzing survival information of CRC patients in The Cancer Genome Atlas (TCGA) cohort ($p = 0.036$). * stands for $p < 0.05$.

2.9 Statistical Analysis

In this study, GraphPad Prism 8.0.1 (GraphPad Software, Santiago, CA, USA) was used as the statistical analysis software. All experiments were repeated at least three times and the corresponding data were expressed as mean ± SD. To compare differences in function assays between two groups or among multiple groups, *T* tests and one-way analysis of variance were conducted. $p < 0.05$ was considered statistically significant.

3. Results

3.1 HSF4 is Overexpressed and Related to Poor Prognosis in CRC

The design process of this research is depicted in Fig. 1. In this study, we first analyzed HSF4 expression in the COAD and READ cohorts from the TCGA database, respectively, by using the online GEPIA tool. The results displayed that HSF4 was commonly overexpressed in tumor tissues compared to adjacent tissues (Fig. 2a). Moreover, HSF4 was also at a lower level in primary colon cancer than adjacent colon tissues [N colon (Mara, n = 32)] in an integrative analysis of multiple Gene Expression Omnibus (GEO) database (Fig. 2b). This study probed HSF4 protein in human CRC using IHC. As illustrated in Fig. 2c, HSF4 protein expression was enhanced significantly in CRC tissues, but not in adjacent tissues. To evaluate the predictive potential of HSF4 expression in CRC, we performed COX regressions. HSF4 expression (HR = 1.572/$p < 0.001$), tumor stage (HR = 2.061/$p < 0.001$), T (HR = 2.444/$p < 0.001$), N (HR = 1.975/$p < 0.001$), M staging (HR = 4.19/$p < 0.001$), as well as age (HR = 1.028/$p = 0.002$) were related to overall survival (OS) of CRC patients in the TCGA cohort (Table 1). In the multivariate analysis, HSF4 expression remained to be associated with poor survival of CRC (HR = 1.297/$p = 0.046$, Table 1). OS was lower in high HSF4-expressing patients than in low HSF4-expressing subgroups ($p = 0.036$, Fig. 2d).

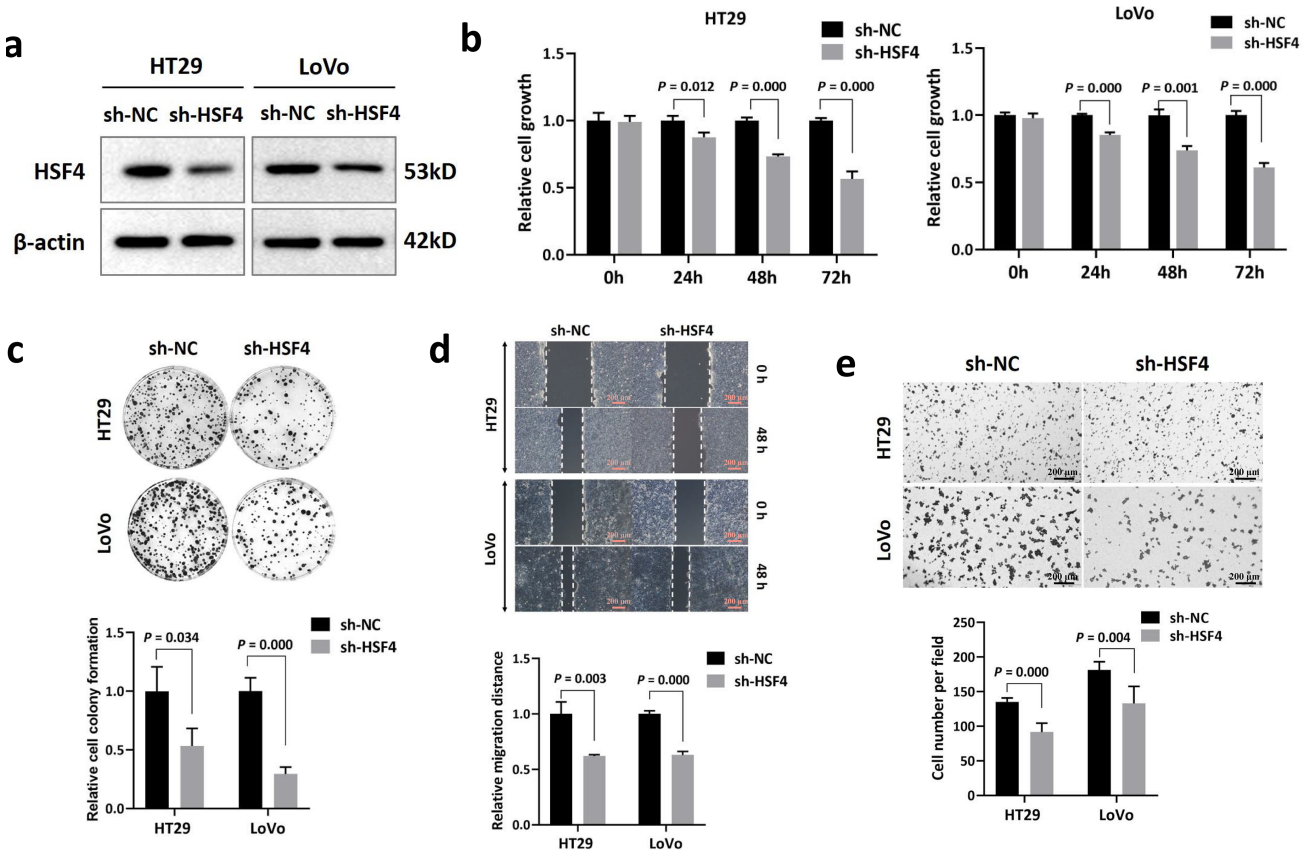

Fig. 3. Knockdown of HSF4 induces inhibition of cell growth, migration and invasion of CRC cells. (a) A specific short hairpin RNA (shRNA) targeting HSF4 is validated to effectively downregulate HSF4 expression in HT29 and LoVo cells. Down-regulation of HSF4 contribute to restriction of cell growth (b) and colony formation (c). Downregulation of HSF4 significantly postpones cell invasion (d) and migration (e) of HT29 and LoVo cells.

Table 1. Prognostic value of Heat Shock Transcription Factor 4 (HSF4) on overall survival (OS) of colorectal cancer (CRC) patients in the the Cancer Genome Atlas (TCGA) cohort.

Clinical characters	Univariate analysis		Multivariate analysis	
	HR	*p*-value	HR	*p*-value
Stage	2.061	<0.001	1.629	0.208
T classification	2.444	<0.001	1.934	0.017
N classification	1.975	<0.001	1.241	0.34
M classification	4.19	<0.001	1.516	0.425
Gender	0.934	0.732		
Age	1.028	0.002	1.035	0.001
Race category	1.074	0.768		
HSF4	1.572	<0.001	1.297	0.046

Note: Prognostic value of HSF4 and other clinical characters was recognized using Univariate and multivariate COX regression analyses in CRC patients. *p*-value < 0.05 indicates a statistically significant correlation between the corresponding clinical features and prognosis of patients with CRC. HR: Hazard ratio.

Table 2. Relationship between HSF4 and clinical characters of CRC patients in TCGA cohort.

Clinical characters	n	HSF4 OR (95% CI)	*p*-value
Age (>65 *vs.* ≤65)	452	1.161 (0.898–1.506)	0.257
Gender	452	1.094 (0.854–1.404)	0.478
Stage			
IV *vs.* (I+II+III)	441	1.498 (1.056–2.130)	0.023
T classification			
T4 *vs.* (T1+T2+T3)	451	1.613 (1.117–2.336)	0.011
M classification			
M1 *vs.* M0	395	1.532 (1.068–2.205)	0.021
N classification			
(N1+N2) *vs.* N0	452	1.535 (1.188–1.999)	0.001

Note: The correlation of HSF4 and other clinical features in the survival of CRC patients using logistic regression analysis. *p*-value < 0.05 indicates statistical significance. n: Number of samples; CI: Confidence interval.

3.2 Overexpression of HSF4 is Correlated with Tumor Stage and TNM Classification of CRC

Further, logistic regression analysis was performed in this study to calculate the relationship between HSF4 expression and clinical characters of CRC patients. As

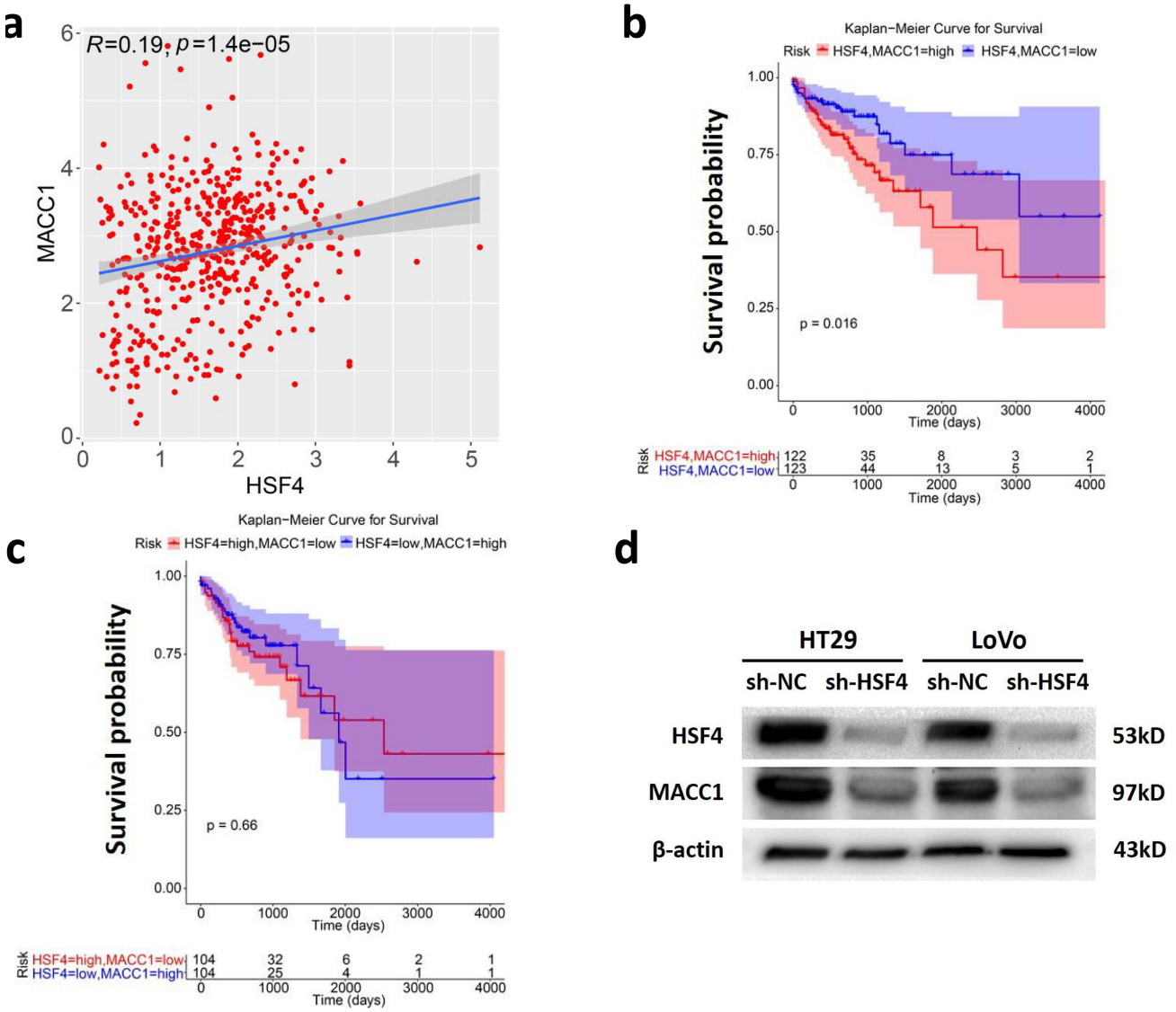

Fig. 4. HSF4 positively correlates with MACC1 expression in CRC. (a) HSF4 and MACC1 exhibited positive relationship in CRC samples is demonstrated by analyzing TCGA dataset of CRC cohort ($R = 0.19$, $p = 1.4 \times 10^{-5}$). Concurrent expression of HSF4 and MACC1 has a significant efficiency to predict prognosis of CRC patients ($p = 0.016$) (b), however there is no difference of survival between $HSF4^{high}/MACC2^{low}$ and $HSF4^{low}/MACC2^{high}$ cohorts ($p = 0.66$) (c) in survival analysis of TCGA CRC dataset. (d) shRNA-induced HSF4 expression leads to a decrease of MACC1 expression level by immunoblotting.

shown in Table 2, overexpression of HSF4 was significantly correlated to tumor stage [OR = 1.498 for IV *vs.* (I+II+III), $p = 0.023$], T classification [OR = 1.613 for T4 *vs.* (T1+T2+T3), $p = 0.011$], N classification [OR = 1.535 for N0 *vs.* (N1+N2), $p = 0.001$], M classification [OR = 1.532 for M1 *vs.* M0, $p = 0.021$].

3.3 Knockdown of HSF4 Induces Inhibition of Cell Growth, Migration and Invasion of CRC Cells

Since clinical specimens demonstrated that HSF4 expression was closely associated with tumor development and survival, we performed *in vitro* cell assays to validate the effect of HSF4 on modulating cell behaviors of CRC. An HSF4-specific shRNA plasmid was constructed and introduced into HT29 and LoVo cells, respectively. The expression of HSF4 protein was reduced in HT29 and LoVo cells after 48 h of transfection (Fig. 3a). Functionally, it was shown that decrease of HSF4 mitigated cell growth (Fig. 3b), and inhibited cell colony formation (Fig. 3c). Similarly, the migratory and invasive capacities of HT29 and LoVo cells were impaired (Fig. 3d,e). This suggests that HSF4 is a facilitator for malignant biological behavior in CRC.

3.4 HSF4 Positively Correlates with MACC1 Expression in CRC

MACC1 was firstly reported as a promotive gene involved in CRC metastasis, and subsequently has been veri-

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miRNA	Cancer	T-test *P*-value	T-test FDR	Upregulated in	Tumor log2 mean expression	Normal log2 mean expression
miR-330-5p	COAD	1.50e-03	4.61e-03	Normal	3.29	5.38
miR-330-5p	THCA	4.10e-02	7.84e-02	Normal	4.21	4.36
miR-330-5p	BLCA	4.44e-03	1.29e-02	Tumor	5.19	3.93
miR-330-5p	BRCA	2.99e-12	1.71e-11	Tumor	4.95	4.20
miR-330-5p	ESCA	5.45e-03	2.89e-02	Tumor	4.43	3.81
miR-330-5p	STAD	2.86e-07	2.16e-06	Tumor	5.20	3.85

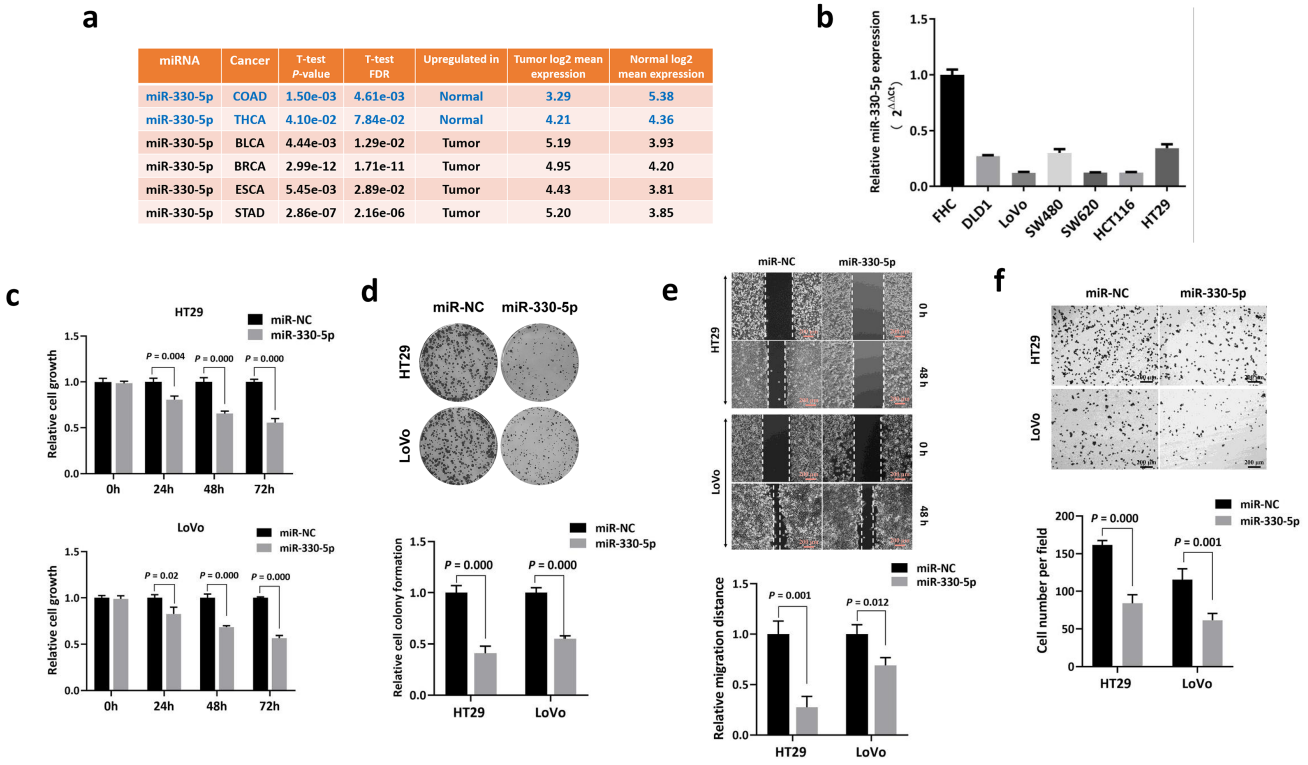

Fig. 5. miR-330-5p is a tumor suppressive miRNA in CRC. (a) The expression pattern of miR-330-5p is bifacial in a variety of tumor types. Notably, miR-330-5p expression is decreased in COAD (colon cancer) by analyzing data extracted from TCGA using OncomiR tool. (b) qPCR assay shows that miR-330-5p is commonly decreased in six CRC cell lines in comparison with normal colonic FHC cells. (c) Restoration of miR-330-5p in HT29 and LoVo cells inhibits cell growth rate by a CCK-8 assay. (d) Restoration of miR-330-5p represses colony formation of HT29 and LoVo cells. Restoration of miR-330-5p apparently postpones cell migration and invasion of HT29 and LoVo cells by wound healing (e) and transwell assays (f). COAD: Colon adenocarcinoma; THCA: Thyroid carcinoma; BLCA: Bladder Urothelial Carcinoma; BRCA: Breast invasive carcinoma; ESCA: Esophageal carcinoma; STAD: Stomach adenocarcinoma; FDR: False discovery rate.

fied to regulate multi-aspects of tumor progression across cancer entities [19]. We associated HSF4 with MACC1 by analyzing TCGA dataset of CRC cohort, as demonstrated by a positive correlation between HSF4 and MACC1 in CRC samples (Fig. 4a). HSF4 had a significant efficiency to predict prognosis in combination with MACC1. As displayed in Fig. 4b, low expression of HSF4 and MACC1 exhibited better OS than high expression of HSF4 and MACC1, while it is no difference of survival between HSF4 ^{high}/MACC2 ^{low} and HSF4 ^{low}/MACC2 ^{high} cohorts (Fig. 4c). This study evaluated the potential regulatory role between HSF4 and MACC1. In immunoblotting assay, the expression of MACC1 protein was down-regulated in HT29 and LoVo cells after 48 h of HSF4 shRNA transfection (Fig. 4d). This suggests that the promotional effect of HSF4 on CRC growth and metastasis is achieved by promoting MACC1 expression.

3.5 miR-330-5p is a Tumor Suppressive miRNA in CRC

miR-330-5p is expressed in different patterns in multiple cancer entities by using the OncomiR tool based on TCGA data. This implies the regulative role of miR-330-5p is dependent on tumor environment. Notably, miR-330-5p was declined in the COAD cohort than normal colon tissues (Fig. 5a). Moreover, qPCR assay showed that miR-330-5p was generally expressed at a decreased level commonly across CRC cells compared to normal colon FHC cells (Fig. 5b). Therefore, we exogenously regulated miR-330-5p in HT29 and LoVo cells to explain miR-330-5p effect on malignant phenotype of CRC. The results indicated that overexpression of miR-330-5p repressed cell growth during an observation of 72 hours and colony formation (Fig. 5c,d). In addition, it also reduced migration and invasion ability of CRC cells (Fig. 5e,f).

3.6 miR-330-5p Inhibits HSF4 Expression by Recognizing Its CDS Region

It has been recently emerged that miRNAs can target CDS of mRNA in addition to targeting 3′UTR, to regulate translation of mRNA in mammalian cells [6,7]. Interestingly, we predicted three putative miRNA-recognition elements (MREs) of miR-330-5p within CDS region of HSF4 by using mircoT-CDS algorithm, while there was none of putative binding sites of miR-330-5p in 3′UTR of

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No.	Binding type	Transcript position	Sequence
MRE 1	8mer	469 to 488	CTACTTCATCCAGTCGCCTCT
MRE 2	8mer+wobble	793 to 816	CAACAGCCTGAACCAGGGGAT
MRE 3	9mer	841 to 869	CCTGGAAAGCGGGGACAGGA

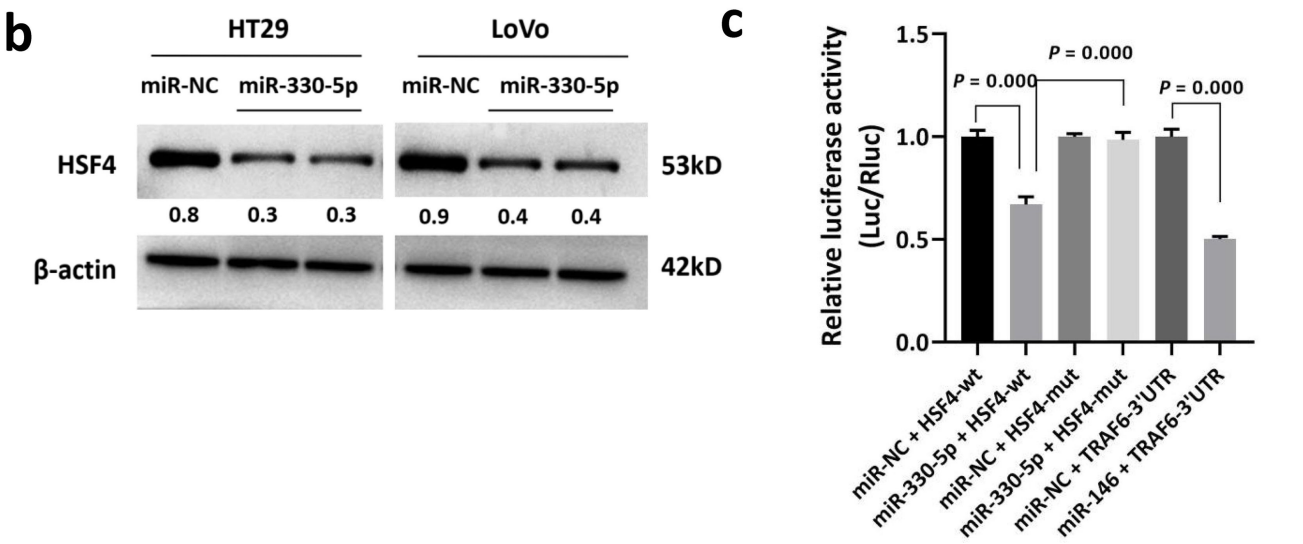

Fig. 6. miR-330-5p inhibits HSF4 expression by recognizing its coding sequences (CDS) region. (a) By online prediction using microT-CDS software, three MREs are found within CDS region of HSF4 mRNA. (b) Restoration of miR-330-5p significantly inhibits HSF4 expression by immunoblotting in both HT29 and LoVo cells. (c) miR-330-5p is observed to weaken luciferase activity of wild-type CDS of HSF4 harboring the three predicted MREs, whereas the mutant CDS of HSF4 remedies this inhibition of luciferase activity.

HSF4 mRNA. In accordance to the inhibitive cellular effects of miR-330-5p on CRC, we assumed that HSF4 may be negatively regulated by miR-330-5p. The three predicted MREs of miR-330-5p within CDS of HSF4 is as shown in Fig. 6a. By immunoblotting, overexpression of miR-330-5p markedly inhibited HSF4 expression in LoVo and HT29 cells (Fig. 6b). Dual-luciferase reporter assay was performed to investigate if miR-330-5p targets CDS of HSF4 mRNA. Assay results expectedly showed that miR-330-5p attenuated the luciferase activity of wild-type CDS sequence of HSF4 harboring all the three putative MREs but not the mutant one (Fig. 6c).

3.7 miR-330-5p Attenuates Activity of MACC1/STAT3 Signaling via Suppressing HSF4 Expression

To determine the suppressive effect of miR-330-5p on CRC via targeting HSF4, we performed function rescue experiment by introducing HSF4-expressing vector into LoVo and HT29 cells under ectopic miR-330-5p expression. In mechanism, as previously described, MACC1 was closely involved in augmenting HGF/c-MET signaling. Considering MACC1 expression was associated with HSF4 in CRC and downregulated along with HSF4 knockdown, we anticipated miR-330-5p may indirectly influence downstream cascades of HGF/c-MET signaling pathway. In immunoblotting assay, restoration of HSF4 expression in miR-330-5p expressing cells was validated (Fig. 7a). Moreover, we expectedly found overexpression of miR-330-5p apparently declined MACC1 expression, and more importantly, impaired the activity of phosphorylation of STAT3 which represents a crucial branch downstream HGF/c-MET pathway (Fig. 7a). In contrast, restoration of HSF4 expression in the presence of miR-330-5p partially abolished MACC1 inhibition and p-STAT3 inactivation (Fig. 7a). As expected, restoration of HSF4 expression partially mitigated miR-330-5p-induced cell growth inhibition in CRC cells (Fig. 7b). Similarly, HSF4 alleviated miR-330-5p-induced repression of colony formation, migration, and invasion (Fig. 7c–e) of CRC cells. These findings of rescue assays suggest miR-330-5p plays inhibitory role in reg-

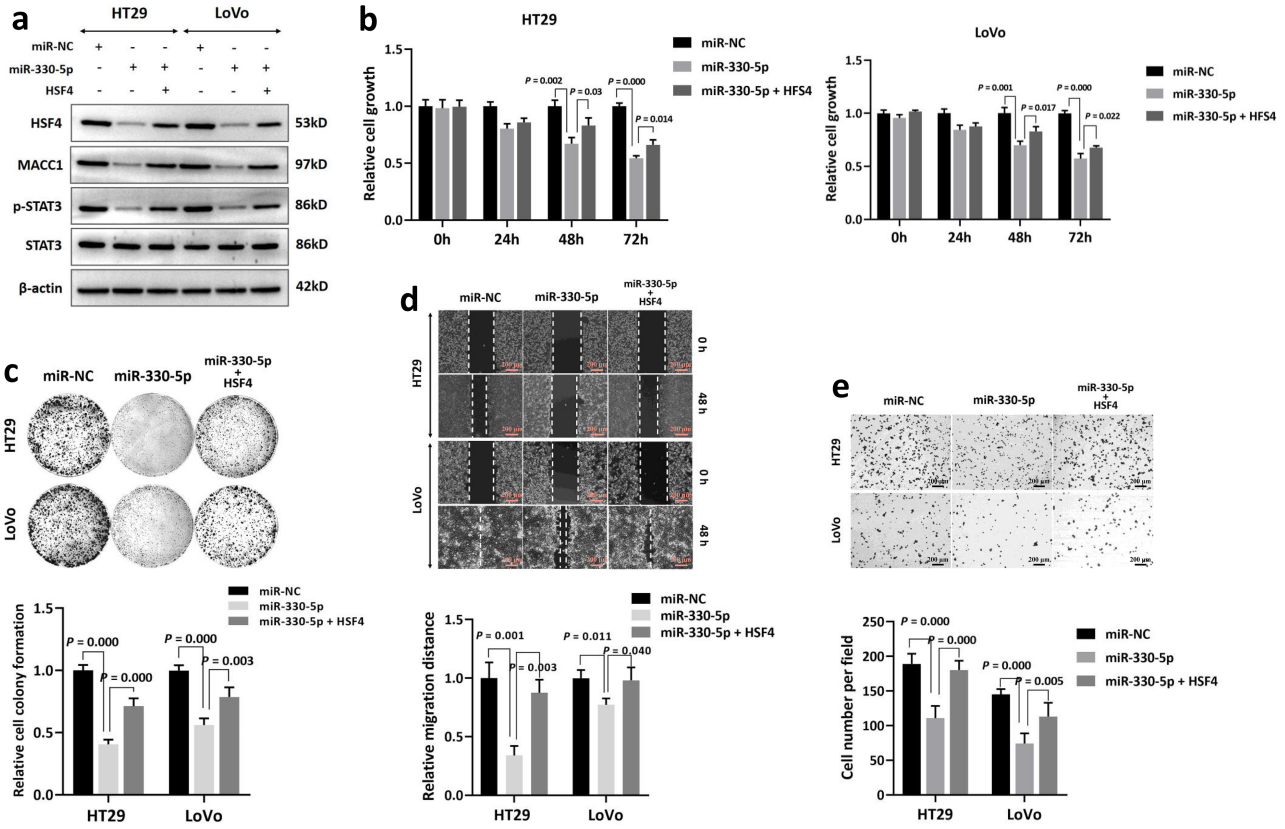

Fig. 7. miR-330-5p attenuates activity of MACC1/STAT3 signaling via suppressing HSF4 expression. HSF4 expression in the context of miR-330-5p expression is restored and validated via immunoblotting. In a series of function assays, restoration of HSF4 expression abolishes miR-330-5p-induced inhibition of cell growth (a), colony formation (b), migration (c) and invasion ability (d) of HT29 and LoVo cells. (e) An immunoblotting assay shows that, upon ectopic miR-330-5p expression, expression of MACC1 is inhibited as well as phosphorylation activity of STAT3. Reversely, restoration of HSF4 expression can partly alleviates the inhibition of MACC1/STAT3 signaling pathway led by miR-330-5p.

ulating cell behaviors of CRC by downregulating HSF4-mediated the activity of the MACC1/STAT3 pathway.

4. Discussion

Dysregulation of transcription factors (TFs) is a key event involved in tumorigenesis, which commonly results in alteration of dramatic gene profile, breaking the equilibrium of downstream signaling transduction. Studies have demonstrated that miRNAs are a common approach for inducing dysregulation of TFs as well as other epigenetic modification such as methylation and acetylation. For example, miR-222 and miR-221 bind to the CDS of RelA mRNA, to maintain constitutive activation of NF-κB [23]. Loss of tumor suppressors miR-214 and miR-302c contributed to cell growth, epithelial-mesenchymal transition (EMT) and metastasis of CRC by releasing mitochondrial transcription factor A (TFAM) and AP-4 [24,25]. However, the mechanisms involved in CRC are yet to be further explored.

Tumor-associated miRNAs exert effects tumorigenesis and progression by activating or inhibiting target genes. It is widely recognized that miRNAs bind to 3′UTR of target mRNA to induce degeneration or inhibition of mRNA translation. However, studies recently found the RNA-Induced Silencing Complex had genome-wide targeting sites by UV crosslinking Immunoprecipitation sequencing, revealing broadly similar distribution of Ago peaks in 3′UTR and CDS in two mammalian cells [26,27]. This indicates that the CDS region may also be a binding region for miRNAs. In this study, miR-330-5p acted as a tumor suppressor, and alleviated the proliferation and metastasis of CRC cells. This result is consistent with previous findings [19,20,28]. In contrast to these findings, we found that miR-330-5p can target the CDS region of HSF4 to inhibit HSF4 expression, which is one of the reasons why miR-330-5p alleviates the malignant phenotype of CRC cells.

Unlike the homologs of HSF1 or HSF2, which are well-known for tumor associated activity, HSF4 has been newly focused in tumor research. Aberrant overexpression of HSF4 intensely associated with poor clinical outcomes, and mechanistically contributes to EMT and metastasis of HCC [16], as well as tumor growth of CRC [17]. This suggests that HSF4 has an oncogenic TF role in tumors.

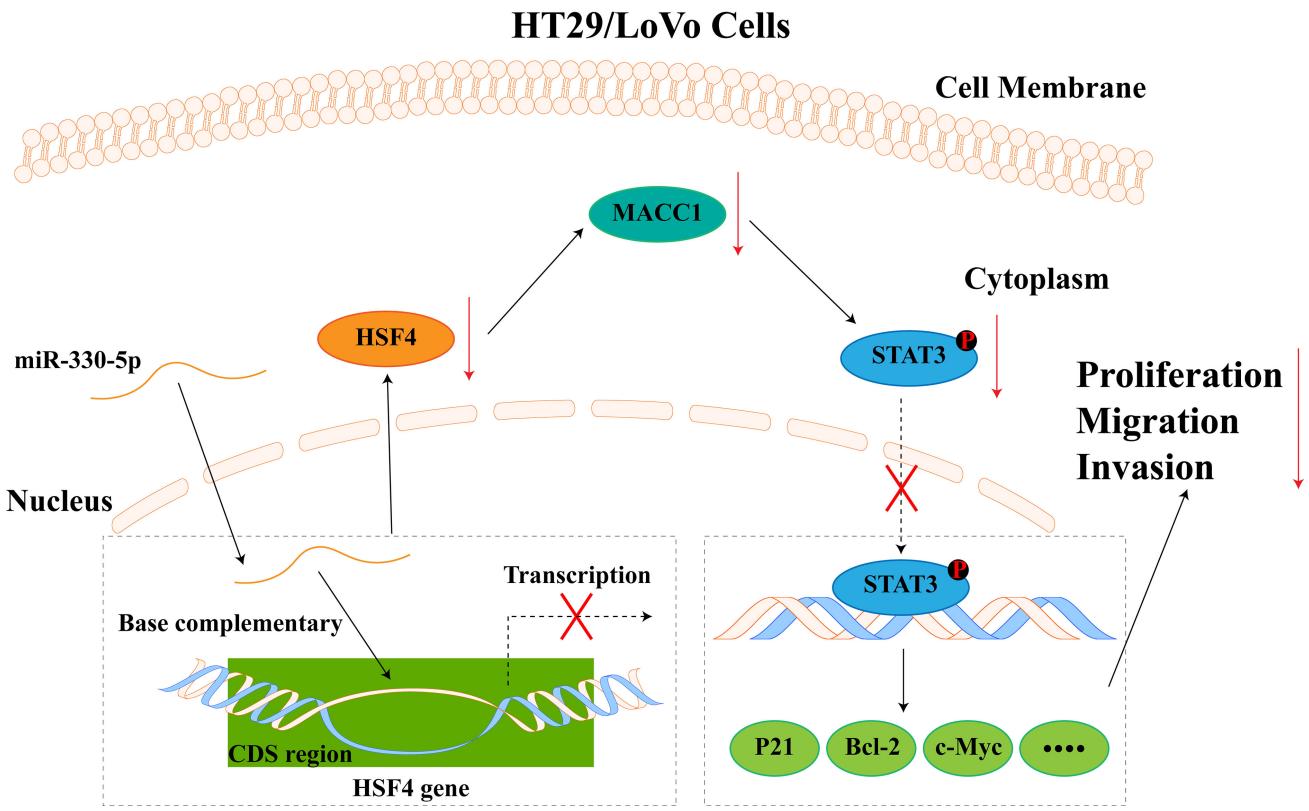

Fig. 8. Proof of concept figure in this study. miR-330-5p inactivated the MACC1/STAT3 pathway by binding to CDS region of HSF4, which alleviated the proliferationand metastasis of CRC cells. P: phosphorylation.

Nevertheless, current advances lack research to elucidate the potential regulation of HSF4 dysregulation in tumors. Our previous study has described that HSF4 overexpression plays a crucial role in CRC by promoting cell growth, invasion and tumor growth *in vivo* [17]. In this study, tumor-suppressive miR-330-5p is, at least partly, contribute to HSF4 expression in CRC, as demonstrated by the binding activity of miR-330-5p to the CDS region of HSF4, which led to inhibition of HSF4 expression in CRC cells. In addition, this study found that the promoting effect of HSF4 on CRC was mitigated by miR-330-5p. This fleshes out the molecular mechanism by which HSF4 acts as an oncogene in CRC.

MACC1 gene was initially demonstrated to be a promising biomarker for CRC [29], and then its oncogenic role has been widely demonstrated in various tumor entities [30]. Notably, this study also revealed that HSF4 and MACC1 expression showed a positive correlation in cohort of CRC patients. In immunoblotting assay, downregulation of HSF4 apparently induced suppression of MACC1. Moreover, Recent studies evidenced that MACC1 modification of the STAT3 pathway attracts solid tumor cell metastasis, stemness, and inhibition of apoptosis [31,32]. It is well known that the STAT3 pathway is activated in a variety of tumors and contributes to tumor growth, metastasis and immunosuppression [33–35]. Similarly, we found that inhibition of MACC1 expression by HSF4 knockdown also inactivated downstream STAT3 signaling transduction in CRC cells. This suggests that the promotional effect of HSF4 on the malignant phenotype of tumours is achieved through activation of the MACC1/STAT3 signalling pathway.

Although the findings provide novel molecular mechanisms for CRC, however, there are still some concerns to be investigated in this study. This study only demonstrated the effect of miR-330-3p targeting HSF4 on CRC at the cellular level, which requires our further validation at the animal level in the future. In addition, the downstream STAT3 pathway regulated by miR-330-3p and HSF4 was discovered to contribute to immune escape and immunosuppression of tumors [35,36]. However, the function of HSF4 and miR-330-3p in the tumor immune microenvironment has not been reported in relevant studies. This deserves to be explored *in vivo* and *in vitro* experiments in future.

5. Conclusion

As shown in Fig. 8, this study newly proposes that miR-330-5p induces inhibition of HSF4 by hitting CDS region, which contribute to inactivation of MACC1/STAT3 pathway, thereby alleviating proliferation, invasion and migration of CRC cells.

Availability of Data and Materials

The datasets used and/or analyzed in the current study are available from the corresponding author upon reasonable request.

Author Contributions

WZ, JL and YZ designed the study. KY, JY and CB performed most of the assays in the research. JL performed the bioinformatic analysis. YZ and KY analyzed the data statistically and wrote the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

This study strictly adhered to the guidelines of the Declaration of Helsinki, and was approved by the ethics Committee of the First People's Hospital of Yunnan Province (Protocol No. KHLL2021-KY109).

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Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/j.fbl2902053.

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