

Original Article

LncRNA RUSC1-AS1 contributes to the progression of hepatocellular carcinoma cells by modulating miR-340-5p/CREB1 axis

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Abstract: Background: Recent studies have proven that there is a relationship between long non-coding RNAs (lncRNAs) and malignant tumor hepatocellular carcinoma (HCC). However, the function of RUSC1-AS1 and its relative regulators in HCC remains unknown. Methods: *In vitro* studies, CCK-8 assays, colony formation assays, transwell assays, and wound healing tests were carried out to evaluate the proliferation, migration, and invasion of HCC cells. The correlation between RUSC1-AS1 expression with tumor size or weight was studied in nude mice. Bioinformatics analysis, dual luciferase, quantitative Real-Time PCR (qRT-PCR), and Western blot analysis aimed to discover the relevance between miR-340-5p and RUSC1-AS1 or cAMP responsive element binding protein 1 (CREB1). Results: When compared with normal groups, RUSC1-AS1 expression in HCC tissues and HCC cell lines was higher. We also found that knockdown of RUSC1-AS1 inhibited HCC cell progression, including proliferation, migration, and invasion, and suppressed tumorigenesis *in vivo*. Further studies demonstrated that the expression of RUSC1-AS1 negatively correlated with miR-340-5p expression in HCC cells. In addition, miR-340-5p was identified as a direct target of RUSC1-AS1 and tightly associated with the prevention of tumor progression. Moreover, miR-340-5p bound directly to CREB1. CREB1 overexpression reversed the impact of miR-340-5p on HCC cells. Together, lncRNA RUSC1-AS1 plays a regulatory role in the PI3K/AKT signaling pathway in HCC cells. Conclusion: We demonstrated that lncRNA RUSC1-AS1 influenced HCC cell progression by modulating its downstream target miR-340-5p/CREB1 axis via the PI3K/AKT signaling pathway, which may be a potential prognostic and therapeutic target for treating HCC.

Keywords: LncRNA RUSC1-AS1, hepatocellular carcinoma, miR-340-5p, CREB1, invasion

Introduction

According to the latest statistics of the World Health Organization (WHO), the sixth most common type of cancer in the world and the third cause of cancer-related deaths is liver cancer. Liver cancer accounts for 7% of all cancers with a high fatality rate [1], and every year, nearly half of the deaths occur in China. The characteristics of hepatocellular carcinoma (HCC) are a strong invasiveness and a high incidence of metastasis, with a high mortality and poor prognosis. Even if the tumor is surgically removed, there is still a significant risk of recurrence, and the overall 5-year survival rate is less than 10% [2]. When curative therapy is not possible, most HCC patients are diagnosed at

an advanced stage after clinical deterioration has occurred. Although HCC has made significant progress in surgical treatment, radiotherapy, chemotherapy, and molecular targeted therapy, effective therapy methods suffer from several major drawbacks, including high invasiveness, high metastasis, and high recurrence. The poor prognosis of HCC patients has not changed in recent decades [3], and the main reason is that the molecular mechanisms of liver cancer occurrence and development are poorly understood. Therefore, exploring the underlying mechanism of liver cancer invasion and metastasis, and identifying effective diagnostic molecular markers and therapeutic targets have become important directions of current HCC research.

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Until now, many studies involving genes focused on protein-coding genes, the classical central rule of DNA-mRNA-protein was well accepted by mainstream. In fact, according to the 2012 ENCODE study, only less than 2% of genes play a role in encoding proteins in the human genome. Even though over 80% of the gene sequences do not encode protein, they can still be transcribed to produce functionally active RNA. Most lncRNAs are transcribed by RNA polymerase II, and their transcription abundance is low [4, 5]. In general, lncRNA is longer than 200 nt, located in the nucleus or in the cytoplasm, and does not participate or rarely participates in protein coding [6]. Accumulating evidence has shown that lncRNA was closely related to tumor occurrence and development by an aberrant expression profile [7], including over-proliferation of tumor cells, inactivation of tumor suppressor genes, immortalizing tumor cells, tumor metastasis and invasion, and inhibition of apoptosis. In previous studies [8], it was shown that the main biological mechanisms of lncRNAs function included gene imprinting, cell cycle regulation, splicing regulation, messenger RNA degradation, and translation regulation, which could be potential novel biomarkers and novel targets of tumor diseases.

lncRNAs participate in biological events through diverse mechanisms while interacting with other biological molecules, including RNA, DNA, and protein [9]. miRNAs are small non-coding RNA molecules that can negatively control gene expression by binding to target mRNAs, thereby leading to degrade or block the translation of these transcripts. Traditional findings regarding miRNA demonstrated that they control the expression of over 30% of human genes [16]. Recent theoretical and experiments have suggested a unifying hypothesis that through miRNA response elements (MRE), lncRNAs could function as competing endogenous RNAs (ceRNAs), binding to competitively target miRNAs, and further regulate the expression of target RNAs [10]. This process has been studied to explain tumor development and to identify the reason of accelerating growth and metastasis of cancer cells, especially in gastric cancer, thyroid carcinoma [11], and hepatoblastoma [12]. In a previous study, Li et al. confirmed that lncRNA HOTAIR expedites the growth of malignant liver cancer stem cells

through down-regulating SETD2 expression [13]. Moreover, Pu et al. [14] found that lncRNA CUDR combined with CyclinD1 or the decrease of PTEN can accelerate the progression of liver cancer stem cells and malignant transformation both *in vivo* and *in vitro*. Patients with high expression of CUDR have a more severe prognosis when compared to patients with a low expression, therefore, CUDR was considered a special marker of HCC.

lncRNA RUSC1-AS1 is located on human chromatin 1q22. It has not yet been elucidated if RUSC1-AS1 plays a role in vicious cancer. Only few studies showed that higher RUSC1-AS1 expression in cervical cancer [15] and breast cancer [16]. RUSC1-AS1 was acted as a ceRNA, which bound to miR-744 and consequently increased the expression levels of B-cell lymphoma 2 in cervical cancer cells [15].

Recent studies have revealed that decreased expression of miRNA-340 (miR-340) was observed in several cancer types compared to normal tissues, demonstrating that miR-340 may play a role in cell differentiation, proliferation, and apoptosis by acting as a tumor suppressor [17-20]. miR-340-5p has been proven a modulator regarding tumor progression in melanoma [21] and thyroid cancer [22]. Furthermore, cAMP responsive element binding protein 1 (CREB1) plays a role in a variety of cellular biological activities involving survival, proliferation, and glucose metabolism [23, 24]. CREB1 also serves as an oncogene, which has been confirmed a direct and functional target of many miRNAs, such as miR-133a-3p in retinoblastoma [25], miR-122 in gastric cancer [26], and miR-506 in esophageal cancer [27]. However, the relationship between CREB1 and HCC is still lacking.

No studies have investigated RUSC1-AS1 and its relative regulator in HCC, which encouraged us to explore the identification and validation of potent therapeutic targets in HCC, and to identify a clear explanation of their interaction.

Materials and methods

Tissue samples

HCC tissues and para-carcinoma tissues were collected from HCC patients in the Shaoxing People's Hospital and the Affiliated BenQ

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Hospital of Nanjing Medical University. Extracted tissue samples were frozen in liquid nitrogen. All human participants participating in this study met the ethical standards of the Ethics Committee of the Shaoxing People's Hospital and BenQ Hospital affiliated to Nanjing Medical University, as well as the 1964 Helsinki Declaration. All patients signed the informed consent file.

Cell culture and cell transfection

Seven human cell lines, including human normal hepatic cell line L02, and human hepatocellular carcinoma cell lines (Hep3B, HCCLM3, MHCC-97H, MHCC-97L, Focus, and YY-8103) were maintained at 37 °C in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS) and 5% CO₂.

Specific small RNAs against RUSC1-AS1 (si-RUSC1-AS1) and small RNA negative control (si-NC), full length of RUSC1-AS1 (RUSC1-AS1 ov), full length of CREB1 overexpression (CREB1 ov), the negative control (NC), miR-340-5p inhibitor, miR negative control (miR-NC) miR-340-5p mimics, and mimics NC were purchased from Sangon Biotech Co., Ltd. (Shanghai, China). Following the manufacturer's instructions, si-RUSC1-AS1 and si-NC were transfected into HCCLM3 cells to establish a knock-down model of RUSC1-AS1 and NC, respectively. RUSC1-AS1 ov was transfected into YY-8103 cells to overexpress RUSC1-AS1. Furthermore, HCCLM3 cells were transfected with the miR-340-5p inhibitor, miR-NC, miR-340-5p mimics, and CREB1 ov.

Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)

Cells in the logarithmic phase or total RNA of fresh tissue samples were collected and mixed with 1 mL TRIzol Reagent. After 15 min, chloroform was added at room temperature, isopropyl alcohol was added, and the mixture was centrifuged and the RNA sediment was collected. Next, the RNA extraction was dried and stored at -80 °C until experiments. The expression of RUSC1-AS1, miR-340-5p, and CREB1 was analyzed by qRT-PCR using a PrimeScript RT reagent kit and SYBR Prime Script RT-PCR kit as per the manufacturer's guidelines. The results were calculated using the 2^{-ΔΔCt} method. U6 and GAPDH served as internal controls.

Western blot analysis

Proteins were extracted from tissues or used cells using lysis buffer at 0 °C for 30 min. A BCA kit was used to quantify proteins. Loading buffer was added to the protein solution, then samples were boiled for 5 min. Subsequently, the proteins were separated on SDS-PAGE, later target proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad Laboratories USA). Next, membranes were incubated with primary antibodies (CREB1, p-PI3K, t-PI3K, p-AKT, t-AKT, E-cadherin, N-cadherin, Vimentin, cyclin-D1, cyclin-E1, Bcl-2, BAX and GAPDH) purchased from Abcam, overnight at 4 °C. Horseradish peroxidase (HRP)-conjugated secondary antibodies (GE Healthcare Life Sciences, China) were applied, and membranes were incubated for 1 h at room temperature. Protein bands were visualized using an enhanced chemiluminescence reagent (Thermo Scientific, Waltham, MA, USA).

Dual-luciferase assay

To establish reporter plasmids, the pGL3 vector (Promega Corporation, Madison, WI, USA) and wild-type (WT) or mutant (Mut) vector of RUSC1-AS1 or CREB1 (Sangon, Shanghai, China) were used. MiR-340-5p mimics and Lipofectamine 2000 were co-transfected into cells. The control group was created by using mimics NC. The Dual-Luciferase Reporter Gene Assay was carried out after 24 h to measure the Renilla and Firefly luciferase activities.

Colony-forming assay

Wright-Giemsa stain (Baso, China) was used to perform the colony-forming assay according to the manufacturer's guidelines. In brief, cells were transferred into 6-well plates at a density of 500 cells per well. After 2 weeks, cells were fixed with 4% paraformaldehyde, and stained with 1% crystal violet. Finally, the number of colonies was calculated to evaluate the cell proliferation ability.

Transwell chamber assay

A total of 1×10⁵ cells was collected and transferred to the upper compartment of a Transwell chamber with an 8-μm pore size and a 24-well insert. In migration tests, 250 μL DMEM containing 0.2% FBS was added to the upper

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chamber, along with 2×10^4 cells. Then, 10% FBS was added to each lower chamber until the well was full. In invasion tests, under sterile conditions, pre-coated with 50- μ L of 1:5 mixture of BD Matrigel (BD Biosciences, San Jose, CA, USA) and DMEM in chamber overnight. Then, 1×10^5 cells were inoculated in the superior compartment. 24 h for migration assay and 72 h for invasion assay, cells at the top of each insert were scratched, the cells were fixed with 4% phosphate buffered neutral formalin, pH 7.4, and stained with crystal violet.

CCK-8 assay for cell proliferation determination

Cells (1×10^3 cells per well) were seeded in 96-well plates. After incubation for 24, 48, 72 and 96 h, 10 μ l CCK-8 reagent was added to each well. Then, cells were incubated at 37°C for 2 h in dark, and the absorbance at 450 nm was measured using a Gel Documentation & Analysis System set.

Wound healing assay

The monolayer of transfected cells was scratched with a 200- μ l micropipette tip. Images were recorded by an inverted microscope (Olympus, Tokyo, Japan) at 0 h and 48 h after the scratch. The wound healing was estimated by ImageJ software at three independent experiments.

Animal studies

A HCC model was established in BALB/c nude mice to verify the biological function of RUSC1-AS1. In brief, nude mice were placed in a pathogen-free environment at the Nanjing Medical University (Nanjing, China). Animal experiments were approved by the ethics committee of Nanjing Medical University and performed confirm the National Institutes of Health guide for the care and use of laboratory animals. Mice were randomly divided into the following groups: si-NC group, si-RUSC1-AS1 group, NC group, and RUSC1-AS1 ov group. In brief, a total of 5×10^6 HCCLM3 cells suspended in 100 μ L serum free-DMEM were transfected with si-control or si-RUSC1-AS1 and 5×10^6 YY-8103 cells (with NC or RUSC1-AS1 ov transfection) were suspended in 100 μ L serum free-DMEM. Cells were injected into flanks of mice to conduct a stable model. Three weeks after the

injection until tumor diameter grew to roughly 5 mm. All mice tumors were monitored once every 3 days and handled the mice to extract tumor for record after 3 weeks. Next, the weights of tumor were recorded, and the tumor volume was calculated using following the equation: Volume (mm^3) = $0.5 \times \text{Length (mm)} \times \text{Width}^2 (\text{mm}^2)$.

Statistical analyses

SPSS 20.0 (IBM Corp., Armonk, NY, USA), and GraphPad 7 (GraphPad Software, San Diego, CA, USA) were utilized to analyze experimental data. The Student's t test was employed to compare the statistical differences between-group, and multiple groups were compared using one-way analyses of variance. Kaplan-Meier and log-rank analysis were used for survival analysis. $P < 0.05$ was considered statistically significant.

Results

RUSC1-AS1 is up-regulated in HCC tumor tissues and cell lines, and closely correlates with HCC progression

Through analyzing the TCGA database, we identified a higher expression of RUSC1-AS1 in HCC tissues compared to the normal tissues (**Figure 1A, 1B**). A total of 60 pairs of HCC tissues and adjacent tissues were collected to determine the expression of RUSC1-AS1 by qRT-PCR, and the data revealed an increased expression of RUSC1-AS1 in HCC tissues compared to adjacent normal tissues ($P < 0.001$, **Figure 1C**). As shown in **Figure 1D**, the RUSC1-AS1 level in HCC cell lines (Hep3B, HCCLM3, MHCC-97H, MHCC-97L, Focus, and YY-8103) was higher compared to that in the nonmalignant hepatic cell line LO2 ($*P < 0.05$, $**P < 0.01$). Overall survival analysis showed that high expression of RUSC1-AS1 associated with a low survival rate in HCC (**Figure 1E**). Therefore, the data above suggested that overexpression of RUSC1-AS1 could be a potential marker for HCC diagnosis.

RUSC1-AS1 promotes HCC growth, metastasis, and invasion in vitro and tumorigenesis in vivo

To further explore the function of RUSC1-AS1 in HCC, we transfected HCCLM3 cells, which had highest RUSC1-AS1 expression level, with a specific si-RNA ($P < 0.01$, **Figure 2A**) and trans-

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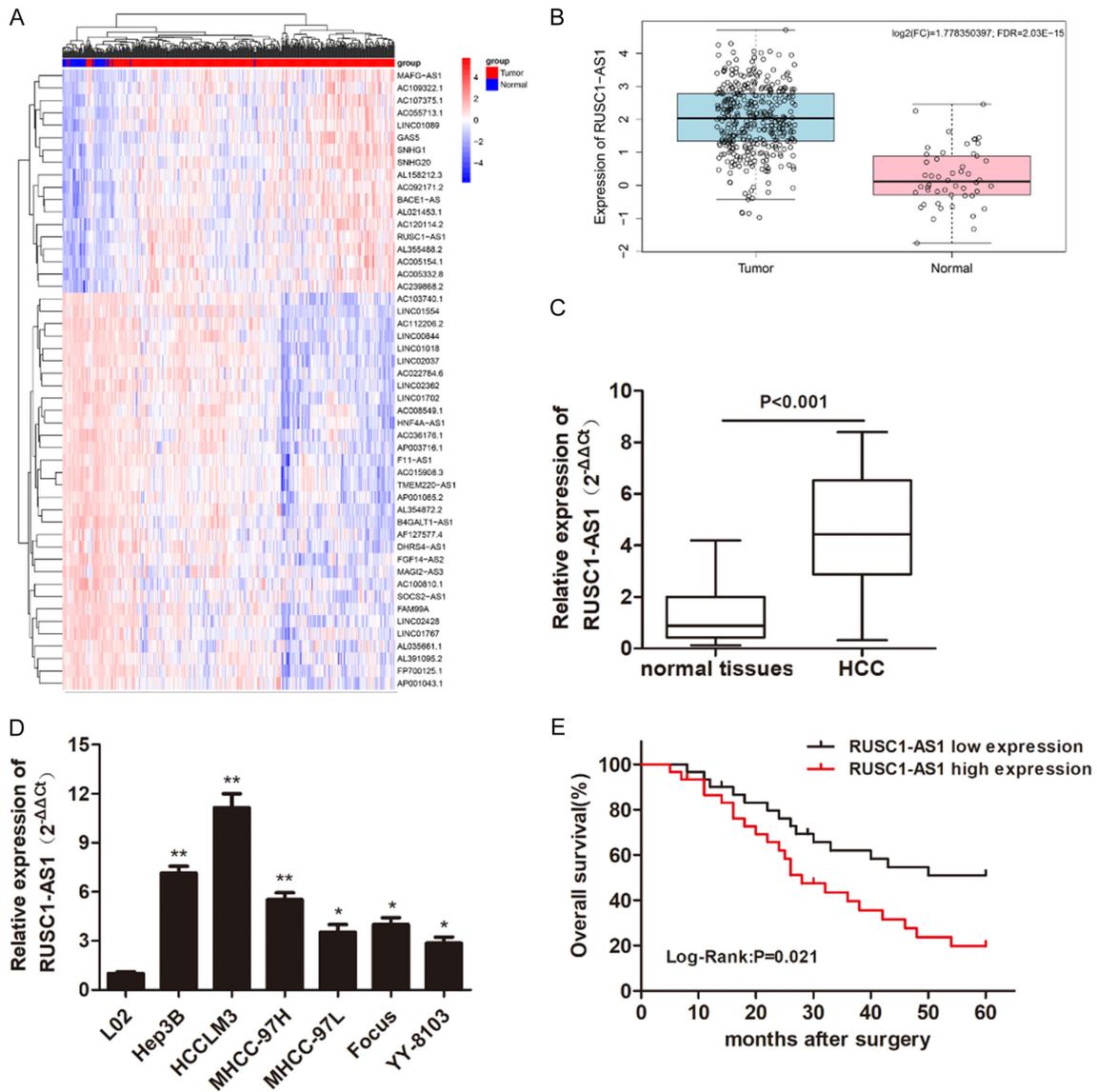


Figure 1. RUSC1-AS1 overexpression in hepatocellular carcinoma tumor tissues and cell lines. A: High expression of RUSC1-AS1 in hepatocellular carcinoma (HCC) is found through bioinformatics analysis in the TCGA database. B: Higher expression of RUSC1-AS1 in HCC tissues compared to normal tissues was observed in the TCGA database; C: Expression of RUSC1-AS1 in tumor and paracancer tissues of 60 liver cancer patients; D: Higher level of RUSC1-AS1 expression was observed in HCC cell lines compared with normal hepatocytes (LO2) (* $P < 0.05$, ** $P < 0.01$); E: Overall survival analysis analyzed by Kaplan-Meier survival analysis, according to RUSC1-AS1 expression levels, mean \pm SD, unpaired Student's t-test or Kaplan Meier analysis.

ected YY-8103 cells, which had the lowest expression level of RUSC1-AS1, with RUSC1-AS1 ov ($P < 0.01$, **Figure 3A**). In RUSC1-AS1 knockdown cells, the colony formation assay and CCK-8 test showed decreased HCC cell colony formation and proliferation (**Figure 2B, 2C**). In addition, transwell assays and wound healing tests indicated that both migration and invasion of HCC cells were restrained by RUSC1-AS1 knockdown ($P < 0.001$, $P < 0.05$, **Figure 2D-F**). On the contrary, the colony formation

assay and CCK-8 assays collectively revealed that up-regulation of RUSC1-AS1 promoted HCC cell growth and colony formation in YY-8103 cells (**Figure 3B, 3C**). Moreover, transwell assays and wound healing tests were conducted to evaluate the abilities of the cells to migrate, move, and repair. Our findings showed that when RUSC1-AS1 was overexpressed, the HCC cell migration and invasion capacity were increased (**Figure 3D-F**). Collectively, the data above suggested that RUSC1-AS1 promoted

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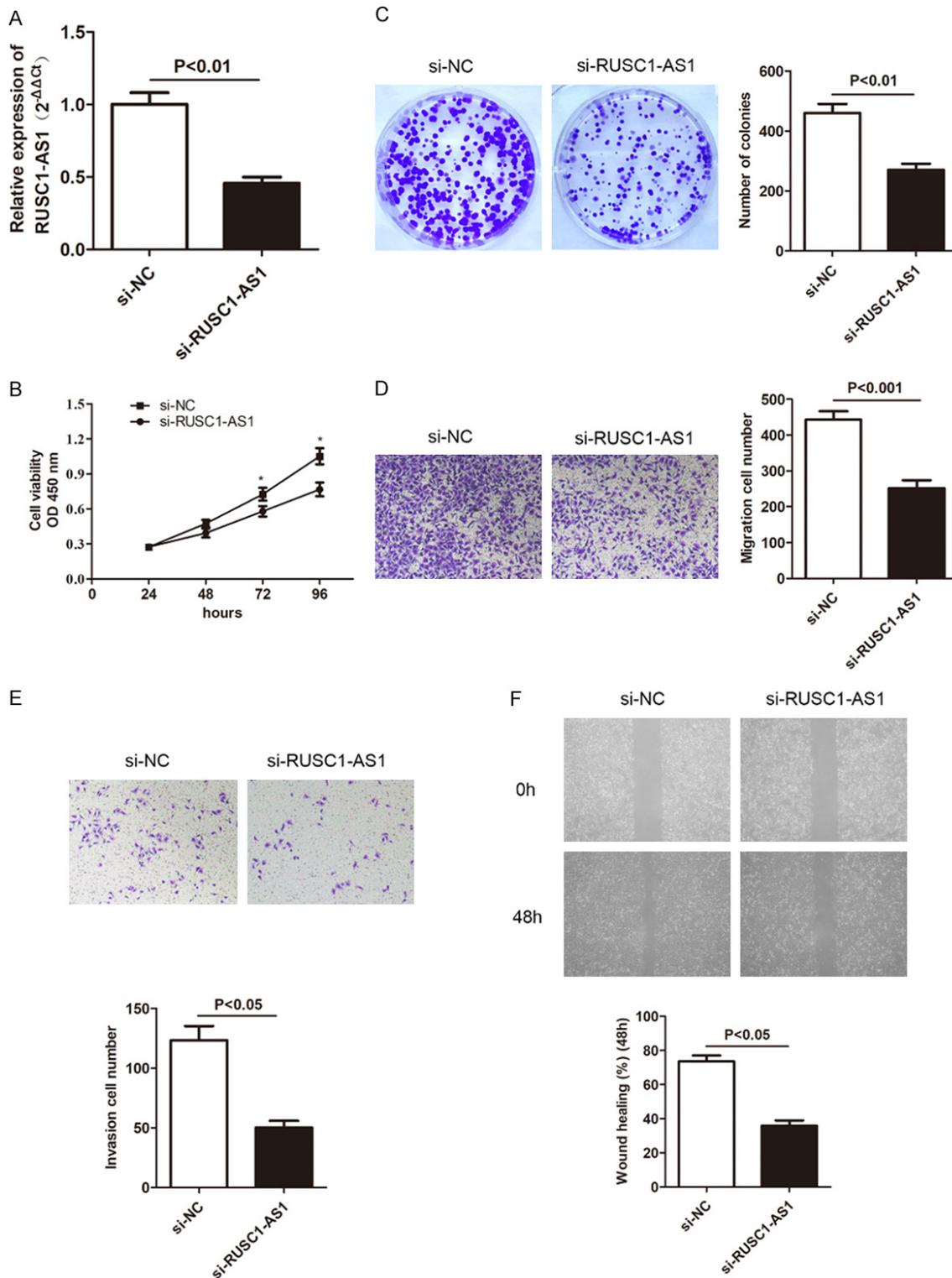


Figure 2. Knockdown RUSC1-AS1 impedes the development of HCCLM3 cells. A: RUSC1-AS1 knockdown was conducted in HCCLM3 cells transfected with si-RUSC1-AS1. The expression of RUSC1-AS1 was lower than that in si-NC as determined by qRT-PCR; B-F: CCK-8 assay, colony formation assay, transwell assays, and the wound healing test, which collectively suggested that knockdown of RUSC1-AS1 inhibited hepatocellular carcinoma cell proliferation, invasion, and migration.

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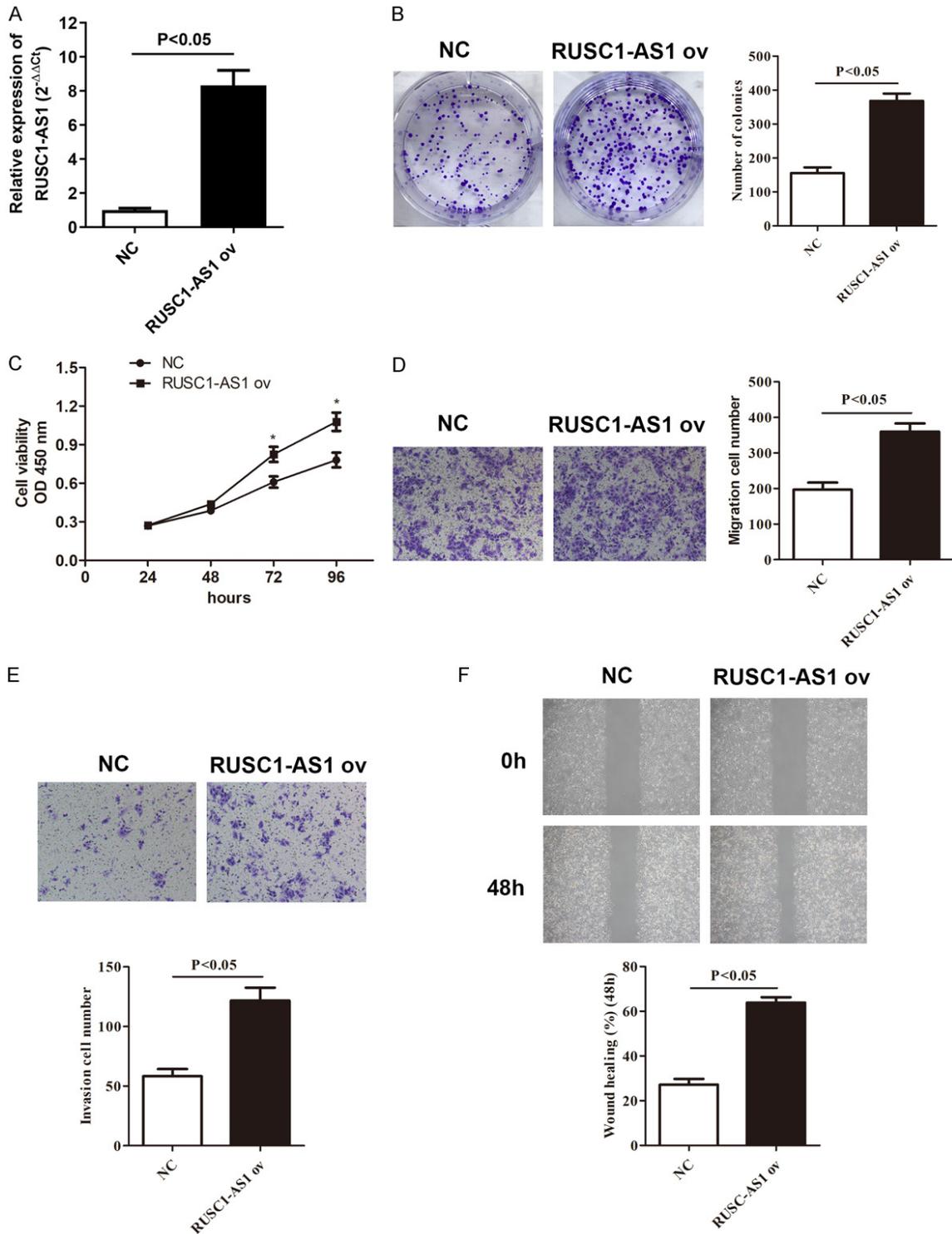


Figure 3. Overexpression of RUSC1-AS1 promotes YY-8103A cell progression. A: RUSC1-AS1 overexpression was conducted in YY-8103A cell lines transfected with RUSC1-AS1 ov. Elevated RUSC1-AS1 expression was found in the RUSC1-AS1 ov group; B-F: CCK-8 assay, colony formation assay, transwell assay, and wound healing test collectively suggested that overexpression of RUSC1-AS1 contributed to HCC cell proliferation, invasion, and migration.

HCC cell proliferation, migration, and invasion *in vitro*.

The influence of RUSC1-AS1 on HCC cell behavior was significant. To explore the effect of

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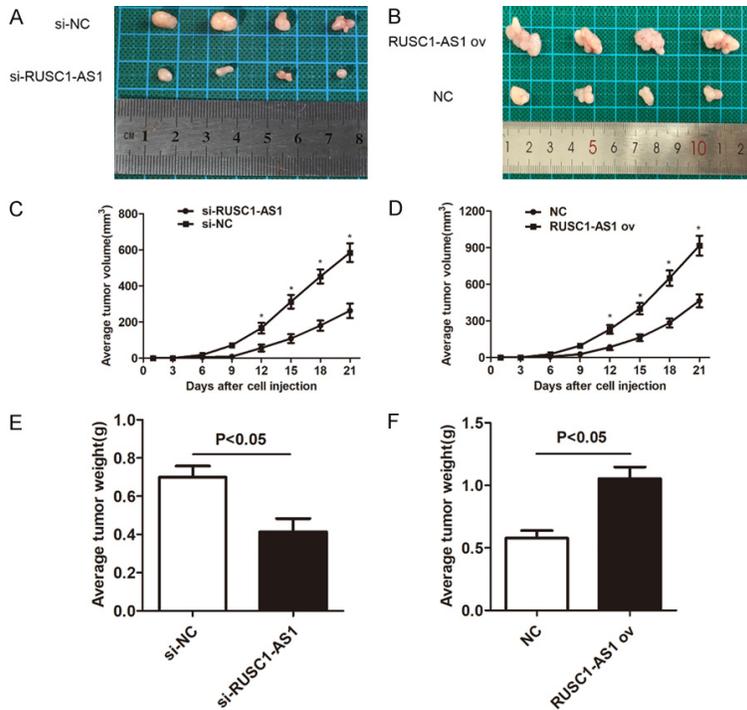


Figure 4. RUSC1-AS1 promotes hepatocellular carcinoma tumor growth *in vivo*. A: Tumor records 3-weeks after injection with HCCLM3 cells transfected with si-RUSC1-AS1. C, E: Knockdown of RUSC1-AS1 slows down tumor growth *in vivo*. B: Tumor records after 3-week injection of YY-8103A cells transfected with RUSC1-AS1 ov. D, F: Overexpression of RUSC1-AS1 induced faster tumor growth.

RUSC1-AS1 *in vivo*, we established an HCC animal model by injecting HCCLM3 cells and YY-8103 cells into nude mice, respectively. The mouse tumor volumes were monitored every 3 days after establishing the xenografts. Our data showed that the tumor volume and tumor weight of mice in the si-RUSC1-AS1 transfected group were lower than that in the control group ($P < 0.05$, **Figure 4A, 4C** and **4E**). For the RUSC1-AS1 ov transfected group, both tumor volume and tumor weight were increased compared to the control group ($P < 0.05$, **Figure 4B, 4D** and **4F**), which further indicated that up-regulation of RUSC1-AS1 promoted HCC progression. Consistent with the previous findings *in vitro*, RUSC1-AS1 was associated with cancer cell promotion.

miR-340-5p directly interacts with RUSC1-AS1 and serves as a negative regulator of RUSC1-AS1

As mentioned before, in several studies, it was shown that lncRNAs could competitively bind to

miRNAs by competing with endogenous RNAs (ceRNAs). This theory implied that miRNAs could bind directly with the corresponding lncRNAs. To investigate the target miRNA of RUSC1-AS1 and to predict the potential target of RUSC1-AS1, we carried out bioinformatics analysis from three databases, including starBase v3.0, mi-Randa, and LncBase. The results presented in **Figure 5A** show that miR-340-5p had putative binding sites with RUSC1-AS1. The luciferase activity of wt-RUSC1-AS1-transfected cells was suppressed by overexpression of miR-340-5p, however, no changes in mut-RUSC1-AS1 expression were observed ($P < 0.05$, **Figure 5B**), which suggested that miR-340-5p was negatively regulated by RUSC1-AS1 in HCC.

To more specifically illustrate miR-340-5p performance in HCC, qRT-PCR was performed to examine miR-340-5p expression in HCC tumor tissues and normal tissues. Our data showed a lower expression of miR-340-5p in HCC tumor tissues compared to normal tissues ($P < 0.001$, **Figure 5C**). Moreover, we also examined miR-340-5p expression in various HCC cell lines (Hep3B, HCCLM3, MHCC-97H, MHCC-97L, Focus, and YY-8103) with normal human hepatocyte LO2 cells as controls. In contrast to RUSC1-AS1 expression in HCC cell lines, miR-340-5p was down-regulated in HCC cells ($P < 0.01$, **Figure 5D**). Taken together, these results demonstrated that miR-340-5p served as a target of RUSC1-AS1 and was negatively influenced by RUSC1-AS1 expression.

miR-340-5p reverses RUSC1-AS1 alteration-mediated promotion of growth, metastasis and invasion in HCC

To increase our knowledge of the function of miR-340-5p in HCC, 3 groups of cells were established, including HCCLM3 cells transfected with si-NC, si-RUSC1-AS1 and si-RUSC1-AS1+miR-340-5p inhibitor, respectively. RT-

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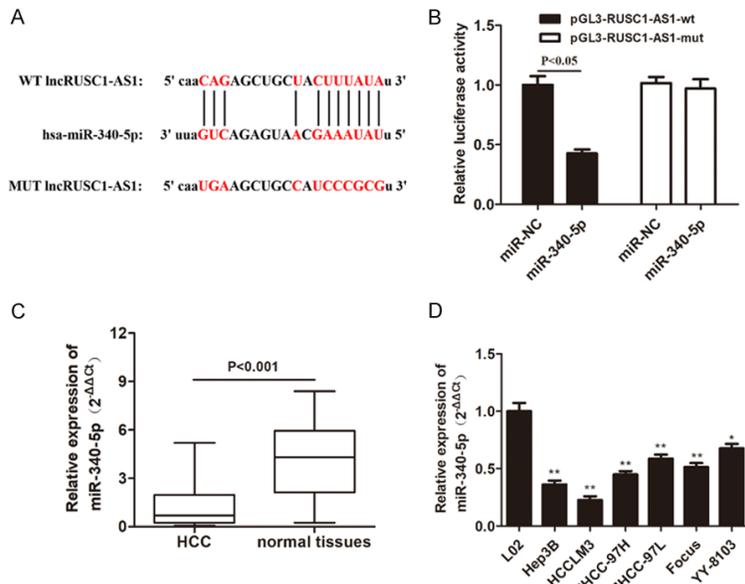


Figure 5. miR-340-5p interacts with RUSC1-AS1 by directly targeting. **A:** Through bioinformatics assay, the putative binding sites between miR340-5p and RUSC1-AS1 were found. **B:** Dual luciferase assay demonstrating that miR-340-5p and RUSC1-AS1 directly interact. **C, D:** miR-340-5p is down-regulated in HCC tumor tissues and hepatocellular carcinoma cell lines compared with normal groups.

qPCR results showed that miR-340-5p expression among these groups was as follows: miR-340-5p was increased in the si-RUSC1-AS1 group compared to the si-NC group, while miR-340-5p expression in the si-RUSC1-AS1+miR-340-5p inhibitor group was decreased compared with the si-RUSC1-AS1 group ($P < 0.01$, **Figure 6A**), which confirmed that miR-340-5p negatively related with RUSC1-AS1 expression. The colony formation assay demonstrated that the miR-340-5p inhibitor decreased the anti-proliferation activity of si-RUSC1-AS1 (**Figure 6B**). Transwell experiments depicted that down-expression of miR-340-5p contributed to cell metastasis and invasive capacity in HCC caused by si-RUSC1-AS1 ($P < 0.01$, **Figure 6C, 6D**). Furthermore, the wound healing test showed that reduced expression of miR-340-5p strengthened the ability of HCC cells to migrate when compared with the si-RUSC1-AS1 group ($P < 0.01$, **Figure 6E**). Taken together, knockdown of RUSC1-AS1 inhibited HCC cell growth and metastatic and invasive capacity, while miR-340-5p down-regulation reversed this progression, and functioned as a tumor suppressor.

CREB1 interacts with miR-340-5p via direct binding and is negatively modulated by miR-340-5p

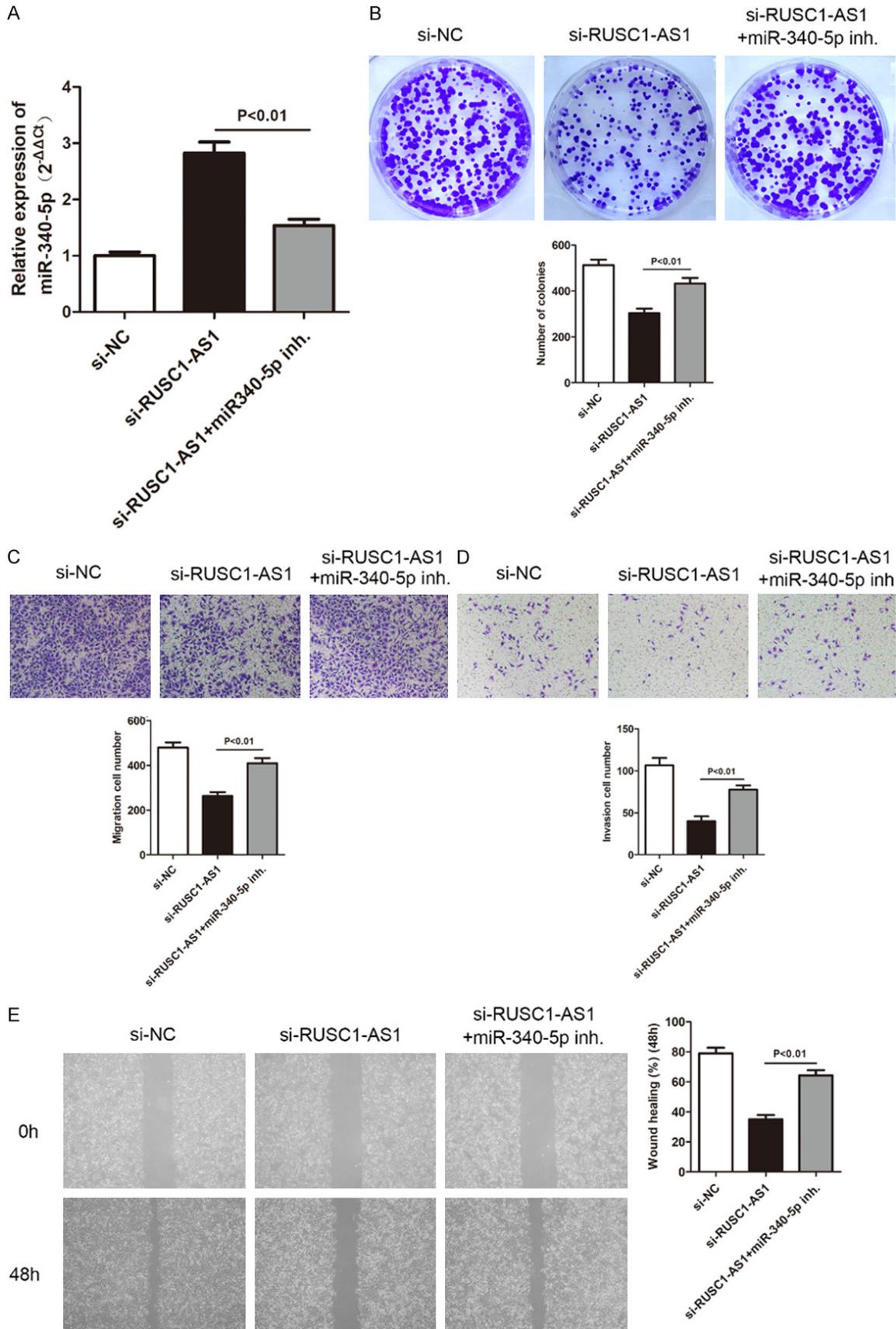
Here, we identified the relationship between RUSC1-AS1 and miR-340-5p and their corresponding impact on HCC cells. To further investigate the potential target of miR-340-5p, we utilized the online bioinformatics tools TargetScan and starBase v3.0 to discover possible targets of miR-340-5p. Intriguingly, CREB1 was identified to have conjectural binding sites with miR-340-5p (**Figure 7A**). The dual luciferase assay indicated that miR-340-5p overexpression inhibited luciferase activity of wild type (wt) CREB1 3'UTR but not mutant (mut) CREB1 3'UTR. These findings supported immediate binding between miR-

340-5p and CERB1 (**Figure 7B**). To detect whether CERB1 was abnormally expressed in HCC, we selected 6 pairs of tumor tissues and normal tissues to examine CREB1 quantity by Western blot analysis. **Figure 7C** presents that increasing CERB1 was observed in tumor tissues, which indicated that overexpression of CERB1 could be an indicator of HCC.

Alteration of CERB1 expression reverses the biological effects of miR-340-5p on HCC cells and promotes the proliferation of tumor cells

Previous studies were conducted to investigate the biological function of CERB1 in HCC. We utilized mimics NC, miR-340-5p mimics, and miR-340-5p mimics+CREB1 ov to transfect HCCLM3 cells. Using the three groups of cells, we aimed to express various CERB1 levels for further studies. Lower CERB1 mRNA and protein expression was found in the miR-340-5p mimics group compared to the mimics NC group, while CREB1 ov transfection significantly improved the level of CERB1 compared with the miR-340-5p mimics group (**Figure 8A**). As shown in **Figure 8B-E**, miR-340-5p mimics significantly suppressed the ability of colony for-

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Figure 6. miR-340-5p reverses RUSC1-AS1-mediated promotion of growth, metastasis and invasion in HCCLM3 cells. A: miR-340-5p levels in different groups of cells. B: Colony formation assay. C: Transwell migration assay. D: Transwell invasion assay. E: Wound healing assay.

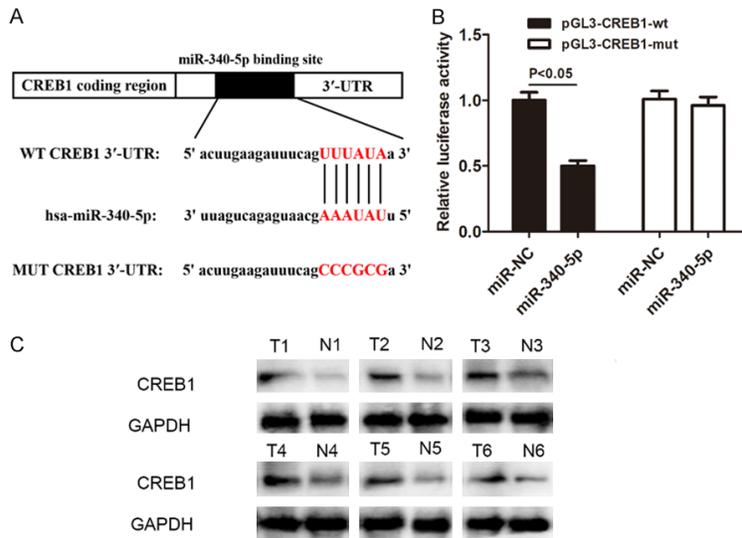


Figure 7. CREB1 is identified as a direct target of miR-340-5p and negatively related with miR340-5p. A: Binding sites between CREB1 and miR-340-5p. B: Dual luciferase assay demonstrating that ectopic miR-340-5p expression suppresses the expression of CERB1 in HCC cells. C: From 6 pairs of HCC tissues, western blot result showed that CREB1 was up regulated in hepatocellular carcinoma tumor tissues compared with normal tissues.

mation, migration, and invasion in HCCLM3 cells. CREB1 ov transfection demonstrated that CREB1 significantly but partially reversed the inhibitory functions induced by miR-340-5p mimics. Considering all the results, CERB1 plays a critical role in HCC cell progression, which is opposite to that of miR-340-5p.

LncRNA RUSC1-AS1 plays a regulatory role in the PI3K/AKT signaling pathway in HCC cells

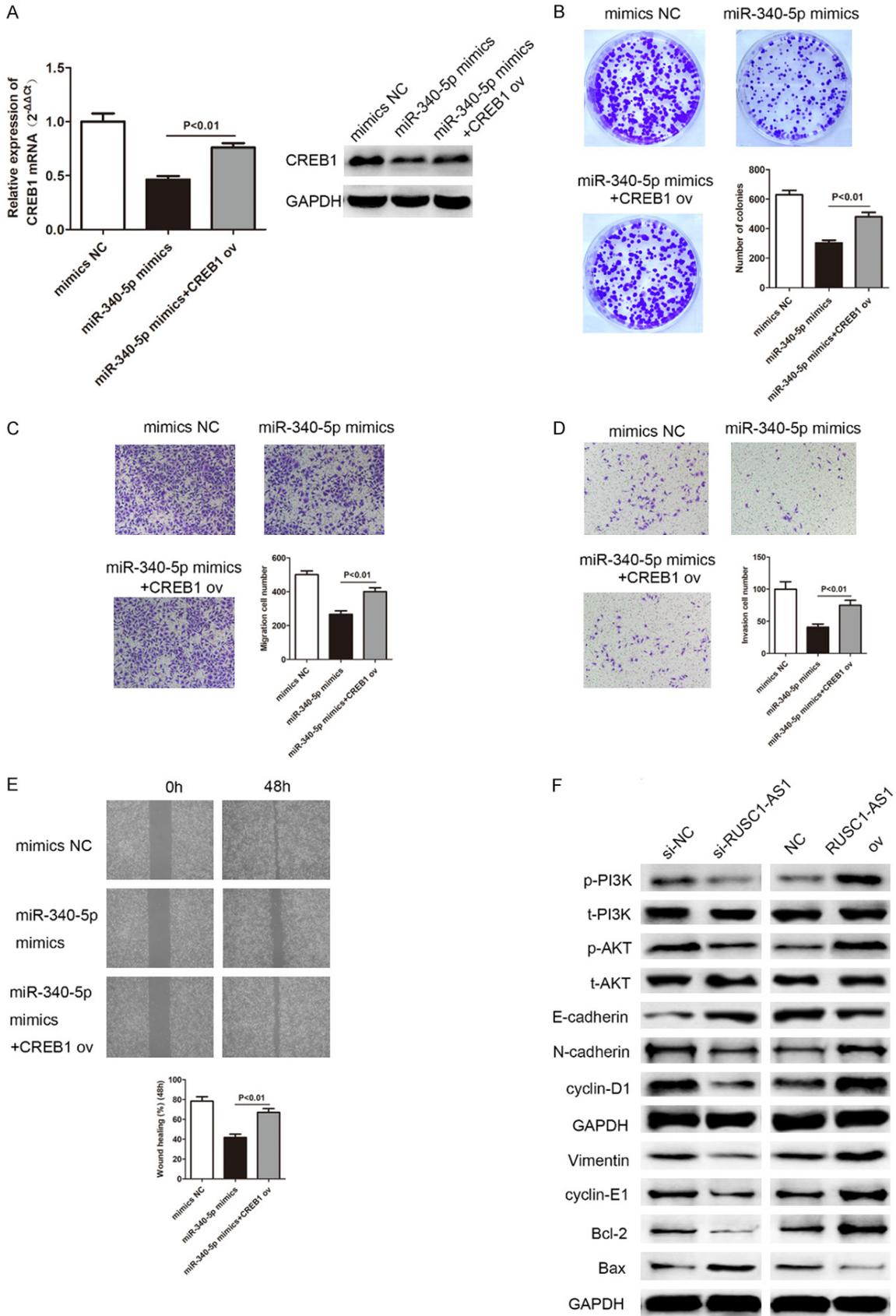
As previously reported, CREB1 was associated with PI3K/AKT signaling [28]. In this study, we explored whether RUSC1-AS1 could influence PI3K/AKT signaling via CREB1. As shown in **Figure 8F**, the phosphorylation levels of PI3K and AKT were significantly decreased by down-regulation of RUSC-AS1 in HCCLM3 cells, thereby indicating that the PI3K/AKT pathway was significantly inhibited by si-RUSC1-AS1. In contrast, higher expression levels of p-PI3K and p-AKT were observed in YY-8103 cells that showed upregulated RUSC-AS1 expression. Furthermore, in HCCLM3 cells, low expression of RUSC-AS1 triggered changes in expression

of migration-related proteins as follows: E-cadherin's expression was increased, whereas the expression of N-cadherin' and Vimentin' was decreased. Down-regulated RUSC-AS1 also decreased the expression of cell progression-related proteins, including cyclinD1 and cyclinE1. Regarding apoptosis-related proteins, knockdown RUSC-AS1 resulted in reduced Bcl-2 expression and elevated Bax expression. Conversely, high expression of RUSC-AS1 produced opposite effects in YY-8103 cells. In summary, RUSC1-AS1 plays a regulatory role in the PI3K/AKT signaling pathway in HCC cells, which influenced the expression of proteins involved in migration, cell progression, and apoptosis.

Discussion

HCC is a highly malignant tumor with a poor prognosis. In this study, research focused on the relationship between lncRNA RUSC1-AS1 and HCC cells. Furthermore, we identified RUSC1-AS1 downstream modulators and the possible molecular mechanism involved. Our results showed that RUSC1-AS1 expression was up-regulated in HCC tumor tissues and cell lines through bioinformatics analysis. Increased RUSC1-AS1 expression was associated with a reduced postoperative survival rate. Moreover, RUSC1-AS1 overexpression induced cell proliferation, invasion, and migration in HCC cells. Similarly, RUSC1-AS1 overexpression increased the growth rate of tumors *in vivo*. Furthermore, our data showed that lncRNA RUSC1-AS1 plays a regulatory role in the PI3K/AKT signaling pathway in HCC cells. We showed that RUSC1-AS1 directly interacted with miR-340-5p and suppressed miR-340-5p expression through bioinformatics analysis and dual luciferase assay. By employing qRT-PCR, our results suggested that miR-340-5p expression was nega-

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Figure 8. CREB1 reverses hepatocellular carcinoma proliferation, migration, and invasion that was induced by miR-340-5p, and lncRNA RUSC-AS1 regulates the PI3K/AKT signaling pathway. A: Expression of CREB1 mRNA and protein in HCCLM3 cells transfected with mimics-NC, miR-340-5p mimics, and miR-340-5p mimics+CREB1 ov. B: Colony formation assay. C: Transwell migration assay. D: Transwell invasion assay. E: Wound healing assay. F: Downregulation of RUSC-AS1 suppresses the PI3K/AKT signaling pathway, and regulates EMT-related proteins cell cycle-related proteins and apoptosis-related proteins in HCCLM3 cells and upregulation of RUSC-AS1 activates PI3K/AKT signaling pathway, and regulates EMT-related proteins, cell cycle-related proteins and apoptosis-related proteins in YJ-8103 cells.

tively related with RUSC1-AS1 expression in HCC cells. We also discovered that the level of miR-340-5p in HCC tumors samples was down-regulated compared with that in non-cancerous tissues. Furthermore, we demonstrated that miR-340-5p exerted suppressive effects on proliferation, invasion, and migration in HCC cells. Collectively, we demonstrated for the first time that RUSC1-AS1 contributed to HCC cell progression by negatively modulating miR-340-5p.

CREB1 was identified as tumor oncogene, and is involved in the oncogenesis in many organs. In this study, we first confirmed that CREB1 was a direct downstream target of miR-340-5p, and mediated the biological function of miR-340-5p and RUSC1-AS1 in HCC. In addition, miR-340-5p negatively regulated CREB1. Decreased CREB1 expression reversed the biological function of miR-340-5p by inhibiting HCC cell proliferation, invasion, and migration.

RUSC1-AS1 has rarely been reported in tumorigenesis. For example, Hui et al. reported that non-methylation-mediated RUSC1-AS1 may act as ceRNAs interacting with some type of miRNA that is responsible for the induction of laryngeal squamous cell carcinoma [29]. Hu et al. demonstrated that RUSC1-AS1 was highly expressed in breast cancer, which promoted the progression of BCa through mediating CDKN1A and KLF2 [16]. Our findings revealed for the first time that RUSC1-AS1 was up-regulated in HCC and promoted HCC cell proliferation, invasion, and migration. It has been widely shown that lncRNAs can influence various tumorigenesis processes by targeting miRNAs in diverse mechanisms. Wu et al. reported that lncRNA NRAL mediated cisplatin resistance in HCC via miR-340-5p [30]. Furthermore, Xiong et al. discovered that a direct functional target of miR-340-5p was a signal transducer and activator of transcription (STAT)3 [31]. In this study, we confirmed that miR-340-5p was a direct target of RUSC1-AS1 and served as a potential tumor

suppressor. To fully understand the downstream modulators involved, we showed that CREB1 directly bound to miR-340-5p, and its abnormal expression had effects that were similar to that of RUSC1-AS1 in HCC.

Conclusions

Taken together, we demonstrated that overexpression RUSC1-AS1 was observed in HCC cells and tumor tissues. RUSC1-AS1 contributed to the progression of HCC cells by affecting their proliferation, invasion and migration ability through modulation of the miR-340-5p/CREB1 axis. Together, these findings may provide a novel treatment strategy in HCC.

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Disclosure of conflict of interest

None.

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