# Circular RNA hsa_circ_0006401 promotes proliferation and metastasis in colorectal carcinoma

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**Research**

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# Abstract

**Background:** Dysregulation of circular RNA (circRNA) expression is involved in the progression of cancer. Here, we aimed to study the potential function of hsa_circ_0006401 in colorectal cancer (CRC).

**Methods:** CircRNA hsa_circ_0006401 expression levels in CRC and adjacent nontumor tissues were analyzed by real-time quantitative PCR (qRT-PCR) and circRNA in situ hybridization (RNA-ISH). Two siRNAs were designed according to the splice junction of hsa_circ_0006401, and both effectively downregulated the hsa_circ_0006401 expression level. Then, CRC cell proliferation was assessed by cell counting. Wound-healing and transwell assays were utilized to detect the effect of hsa_circ_0006401 on CRC migration. A circRNA-ORF-GFP construct was created, and a specific antibody against the splice junction of hsa_circ_0006401 was prepared.

**Results:** Hsa_circ_0006401 was closely related to CRC metastasis and exhibited upregulated expression in metastatic CRC tissue samples. Proliferation and migration were inhibited *in vitro* when hsa_circ_0006401 expression was silenced. Downregulation of hsa_circ_0006401 expression decreased CRC proliferation and liver metastasis *in vivo*. A 198-aa peptide was encoded by sequences of the splice junction absent from *col6a3.* Hsa_circ_0006401 promoted CRC proliferation and migration by encoding the hsa_circ_0006401 peptide. Hsa_circ_0006401 peptides decreased the protein level of the host gene *col6a3* but did not alter its gene expression level.

**Conclusion:** Our study revealed that circRNAs generated from *col6a3* that contain an open reading frame (ORF) encode a novel 198-aa functional peptide and hsa_circ_0006401 peptides transcriptionally regulates the host gene col6a3 to promote CRC proliferation and metastasis.


# Background

Globally, the morbidity and mortality of colorectal cancer (CRC) rank third and fourth among those of malignant tumors, respectively[1]. In recent years, the incidence of CRC has gradually increased, and the age of onset has become younger. Although great improvements in the diagnosis and treatment of CRC have been made, the prognosis is still not promising. Therefore, the development of new therapeutic strategies is urgently needed.

Circular RNA (circRNA) is one of the largest classes of noncoding RNA. CircRNAs are formed by a back-splicing mechanism and are abundantly present in eukaryotic transcriptomes [2]. Compared with linear noncoding RNAs, circRNA molecules have a closed circular structure that is not affected by RNA exoenzymes and is more stable [3]. Although circRNAs are usually expressed at low levels, they play important roles in regulating various physiological and pathological processes in the human body. Recent studies have found that aberrant circRNA expression is involved in tumorigenesis and progression [4–6]. For example, circRNA_102171 promotes the progression of thyroid cancer by interacting with CTNNBIP1 to activate the Wnt/β-catenin pathway [7], high expression of circ-Foxo3 blocks the progression of the cell cycle by interacting with CDK2 through the action of promoting cell division [8], and the circRNA

cSMARCA5 promotes the expression of the tumor suppressor TIMP3 by acting as a "sponge" for miR-17-3p and miR-181b-5p, which inhibits the proliferation and migration of liver cancer cells [9]. Although an increasing number of studies suggest that circRNAs are involved in the development of tumors, circRNA research in colorectal cancer is still in its infancy. The identification of tumor-related circRNAs and the study of functional mechanisms are of great significance for the development of new diagnostic methods.

Because of the lack of the 5' cap structure, which is considered necessary for RNA translation, circRNAs have long been considered noncoding RNAs. In recent years, studies have found that circRNAs can be used as a protein synthesis template to encode and translate proteins under certain conditions [10]. Emerging evidence has also shown that circRNA-derived proteins play important biological roles in the cell stress response, myogenesis control and tumor progression [6, 10, 11].

Here, we revealed that the expression of hsa_circ_0006401 in metastatic CRC was significantly increased compared with that in nonmetastatic CRC. Further studies revealed that silencing hsa_circ_0006401 expression with siRNA could significantly inhibit the proliferation and migration of CRC cells and promote their apoptosis and that inoculation of hsa_circ_0006401-silenced colorectal cancer cells into nude mice significantly reduced the size of subcutaneous tumors and the number of liver metastases. These results strongly suggest that hsa_circ_0006401 may play an important regulatory role in the development of CRC. In this project, we also found a novel 198-aa peptide produced from hsa_circ_0006401 that regulates the aggressive phenotype of CRC cells.

# Materials And Methods

### Cell culture and transfection

The CRC cell lines SW480 and SW620 were purchased from the Shanghai Institute of Biochemistry and Cell Biology and cultured in DMEM (Gibco, Invitrogen, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, Invitrogen, USA) and 0.5% penicillin/streptomycin (Gibco, Invitrogen, USA). Cells were maintained at 37 °C in a humidified atmosphere containing 5% CO2. Lipofectamine® 2000 reagent (Invitrogen, USA) was used for cell transfection with small interfering RNAs (siRNAs) (RiboBio, Guangzhou, China) or constructed plasmids (GenePharma, Shanghai) according to the manufacturer's instructions. The siRNA sequences were as follows:

siRNA#1 5' ACAGAAAUGUUCCGAAUAA dTdT 3',

siRNA#2 5' CUCUCACUGAAACAGAAAU dTdT 3'.

### Patient samples

This work was approved by the Research Ethics Committee of Zhejiang Provincial People's Hospital, Hangzhou Medical College (code: 2020QT084). Twelve samples from CRC patients were collected from

Zhejiang Provincial People's Hospital between May 2017 and July 2020. Patient information is shown in Table 1. Tissue samples were collected and frozen in liquid nitrogen immediately after surgical resection.

## RNA extraction and purification

Total RNA was extracted from tissue and cell samples with TRIzol (Invitrogen, USA), and the RNA concentration and purity were checked by the Agilent 2100 Bioanalyzer according to the manufacturer's protocol (Agilent Technologies, USA). RNA was then reverse transcribed into cDNA using the SuperScript$^{TM}$ IV First-Strand Synthesis System (Invitrogen, USA) according to the instructions provided by the manufacturer.

## qRT-PCR

Hsa_circRNA_0006401 was detected by qRT-PCR utilizing TB Green$^{TM}$ Premix DimerEraser$^{TM}$ (Perfect Real Time) on the ABI 7900 Real-Time PCR System (Thermo Fisher Scientific, USA). The reaction conditions were set according to the manufacturer's protocol (Takara, Beijing). The human GAPDH reference gene was used as an internal control. All assays were conducted in triplicate. The PCR products were sent to GENESEED (Guangzhou, China) for sequencing to ensure the accuracy of circRNA detection. Primer information is as follows:

hsa_circ_0006401 Forward, 5′-TGGCTCTCACTGAAACAGAAATG-3′;

Reverse, 5′-GTCGTCAC TGGGTTGGATGTAG-3′

TGFβ1 Forward, 5′-CGGCTGC TGCTGAAAGCCGACCA-3′;

Reverse, 5′-GGTCGGGGCCAAAAGCGTGT-3′

GAPDH Forward, 5′- AGTCAGCATT TCACAAGACCTC-3′;

Reverse, 5′- CAGGCGAAGATGTTCTGGC-3′.

## CircRNA in situ hybridization (RNA-ISH)

Tissue slides were deparaffinized and rehydrated. Cells were inoculated into a Petri dish with a preloaded glass coverslip, and the coverslip was removed after the cells had grown into a single layer and washed twice with phosphate-buffered saline (PBS). The cells were fixed with paraformaldehyde and washed with PBS 3 times for 5 min each time. Then, the cells were permeabilized and washed with PBS 3 times for 5 min each time. After blocking, the cells were incubated with a primary antibody at 4°C overnight. The samples were rinsed 3 times with PBST for 5 min each time. A secondary antibody was added and incubated for 1 h at room temperature in the dark. The samples were rinsed 3 times with PBST and then rinsed with distilled water; each wash was 5 min long. A drop of mounting medium was used to mount slides, and the slides were evaluated with a fluorescence microscope.

## Flow Cytometry

Adherent CRC sw480 cells were harvested and washed with PBS supplemented with 0.5% bovine serum albumin (BSA). After fixation with 70% ethanol at -20 °C overnight, the sw480 cells were resuspended in PBS supplemented with 40 μg/ml PI at 37 °C for 30 min, and 100 μg/ml RNase A was subsequently added to the cells and incubated in a 4 °C dark room for 30 min. Cell apoptosis was determined with a flow cytometer (BD Biosciences).

## Xenograft assay

SW620 cells ($2 \times 10^6$) in 150 μl of PBS were subcutaneously injected into nude mice. Seven weeks after cancer cell inoculation, the tumors were isolated, and tumor volume was determined. The tumor tissue and liver of nude mice were collected and fixed in a 10% buffered formaldehyde solution, and hematoxylin and eosin (H&E) staining was applied to assess tumor invasion and metastasis.

## Hematoxylin and eosin staining

Tissue sections were formalin fixed, paraffin embedded, and stained with hematoxylin and eosin (H&E). Images of tumors were acquired with a light microscope. Ten sections were randomly chosen to analyze local invasion and liver metastasis. The liver lesion number was quantified by ImageJ software (version 2.1.4).

## Immunohistochemistry

Fresh CRC and paratumor tissues were washed with PBS and processed into tissue blocks. Then, the tissue blocks were fixed, embedded, sectioned at a thickness of 5 μm, attached to a polylysine slide overnight at 60°C and dewaxed. An antigen retrieval solution and a blocking solution were added to the sections. Then, primary antibodies were applied to the sections and incubated at 4°C overnight. Next, the sections were incubated with a biotinylated secondary antibody for 20 min at room temperature. The staining intensity of the hsa_circ_0006401 peptide was scored independently by two physicians.

## Western Blot

Proteins were isolated from CRC cells and incubated with primary antibody detecting hsa_circ_0006401 peptide (1: 1000 dilution, HuaAn Biotechnology Co., Ltd, China) and GAPDH (1:1000 dilution, Abcam) was used as a control. Amino acid sequence for hsa_circ_0006401 peptide antibodies were as follows: 1. HAPL0559 147-161aa CSFSTKKSQPPPPQPA□2. HAPM0617 splice junction TEMFRITLLQVLHPTQC. The anti- rabbit secondary antibody was then applied (1:1000 dilution, Abcam). Finally, enhanced chemiluminescence was utilized to observe immunoreactive proteins.

## Statistical analysis

Statistical analysis was carried out by using SPSS 21.0 software (SPSS, USA). Differences between individual groups were compared by Student's t-test. A p-value < 0.05 was considered significant.

# Results

### Hsa_circ_0006401 expression was upregulated in metastatic CRC tissue

Hsa_circ_0006401 is back-spliced from three exons (exons 4, 5, and 6) of *col6a3* on Chr2(q37.3) (Fig. 1A). To investigate the potential function of hsa_circ_0006401 in CRC, the expression level of hsa_circ_0006401 was analyzed by qRT-PCR and compared between 12 pairs of CRC tissue and corresponding paratumor tissue specimens. We found that the hsa_circ_0006401 expression level was higher in most of the CRC tissue samples and significantly related to lymph node metastasis (Table 2). Moreover, in regard to the expression of hsa_circ_0006401 in metastatic CRC, qRT-PCR was performed to compare metastatic and nonmetastatic CRC tissue samples. As shown in Fig. 1B, hsa_circRNA_0006401 expression was upregulated in CRC patients with lymphatic metastasis. Primers are shown in Fig. 1C. An RNA-ISH assay was also applied to assess the hsa_circ_0006401 expression level. We found that hsa_circ_0006401 was barely expressed in normal colorectal tissue specimens and was expressed at higher levels in metastatic CRC tissue samples than in nonmetastatic CRC tissue samples (Fig. 1D). To evaluate the diagnostic potential of hsa_circ_0006401 for CRC, receiver operating characteristic (ROC) curve analysis was carried out, and we found that the area under the curve (AUC) of the selected circRNAs was 0.770 (95% CI 0.549-0.991, P=0.041) (Fig. 1E). Moreover, to further validate our results, the sequences of the PCR products were identified and confirmed to be the indicated circRNAs with a back-spliced junction (Fig. 1F).

### Hsa_circ_0006401 promotes an aggressive phenotype in CRC cells *in vitro*

To further confirm the function of hsa_circRNA_0006401, the location and expression of hsa_circRNA_0006401 were explored by circRNA in situ hybridization. As shown in Fig. 2A, hsa_circRNA_0006401 mainly existed in the cytoplasm of SW480 and SW620 cells. Next, two siRNAs were designed according to the splice junction of hsa_circRNA_0006401. As shown in Fig. 2B, hsa_circRNA_0006401 was perfectly downregulated in SW480 cells by both siRNAs (Fig. 2B). Then, cell proliferation was analyzed, and we found that compared to negative control treatment, downregulation of hsa_circRNA_0006401 by both siRNAs decreased CRC cell counts (Fig. 2C). Moreover, wound-healing and transwell assays were utilized to investigate CRC cell migration. As shown in Fig. 2D and 2E, the numbers of migrated CRC cells were significantly decreased by silencing hsa_circRNA_0006401. Finally, cell apoptosis was also evaluated by flow cytometry, and we found that hsa_circRNA_0006401 knockdown increased apoptotic CRC cell numbers (Fig. 2F). Overall, hsa_circRNA_0006401 promotes an aggressive phenotype in CRC cells *in vitro*.

### Hsa_circ_0006401 increased the proliferation and metastasis of colorectal cancer *in vivo*

To evaluate the function of hsa_circRNA_0006401 *in vivo*, control CRC cells and hsa_circRNA_0006401-silenced CRC cells were subcutaneously injected into the right and left subaxillary regions, respectively, of nude mice. Two weeks after cancer cell inoculation, the mice were sacrificed, and the tumors were isolated. As shown in Fig. 3A, the tumor size of the hsa_circRNA_0006401-silenced tumors was much smaller than that of the control tumors. As a negative control group, PBS-injected mice did not form tumors. Tumors in situ and the surrounding tissues as well as the liver were fixed and subjected to H&E staining. The results showed that the deep tumor margins of the hsa_circRNA_0006401-silenced group were distinct and invaded nearby structures, such as skeletal muscles. However, the margins in control CRC cell-formed tumors were well encapsulated with a noninvasive nature (Fig. 3B). The numbers of liver metastases were also assessed, and we found that hsa_circRNA_0006401 knockdown significantly decreased the number of liver metastasis lesions in nude mice (Fig. 3C). H&E staining results for nude mouse livers are shown in Fig. 3D.

## Hsa_circ_0006401 encodes a small peptide

Hsa_circ_0006401 originates from the circularization of the fourth to sixth exons of the host gene *col6a3*. A 594-nt open reading frame (ORF) is present in hsa_circ_0006401, spanning across the splice junction, which has the potential to encode a 198-aa peptide (Fig.4A). To determine whether this ORF has peptide-encoding potential, an expression vector with a circular transcript-producing capacity was adopted. P-Circ contained the fourth to sixth exons of the *col6a3* gene and was able to express hsa_circ_0006401 circRNA at high levels. The sequence produced from this construct was perfectly consistent with that of hsa_circ_0006401 (data not shown). A construct was derived from p-Circ-GFP that contained a GFP sequence without an AUG initiation codon immediately upstream of the STOP codon, such that a GFP fusion protein could be produced when a circular template formed. We observed GFP expression in P-Circ-GFP-transfected 293T cells. However, a construct with a mutation in the ORF start codon (the start codon ATG was mutated to TTG) did not exhibit GFP fusion protein expression after transfection (Fig.4B).

To confirm the peptide encoded by the hsa_circ_0006401 ORF in CRC, we produced two antibodies (HAPL0559, HAPM0617) against the hsa_circ_0006401 peptides, one of which was against unique reads of the circular junction (HAPM0617). As shown in Fig. 4C, the hsa_circ_0006401 peptides were highly expressed in the P-Circ-transfected CRC group compared to the control group. However, there was barely any hsa_circ_0006401 peptide expression when the hsa_circ_0006401 ORF start codon was mutated or hsa_circ_0006401 RNA expression was silenced. Taken together, these data indicate that the hsa_circ_0006401 peptides are expressed in CRC. Moreover, IHC staining was performed to assess hsa_circ_0006401 peptide expression in CRC and paratumor tissue samples from twelve CRC patients. We found that the IHC scores for the hsa_circ_0006401 peptides were higher in the CRC tissue samples than in the paratumor tissue specimens and closely related to lymphatic metastasis (Fig.4D).

## Hsa_circ_0006401 peptide promotes CRC Growth and Migration

To investigate whether hsa_circ_0006401 regulates CRC growth, migration and metastasis by encoding the hsa_circ_0006401 peptides, the p-Circ and p-ORFmut constructs were transfected into CRC cells. As

shown in Fig. 5, transfection of p-Circ, which encoded the hsa_circ_0006401 peptides, enhanced CRC growth and migration and decreased CRC apoptosis. However, transfection of p-ORFmut, which contained a mutated start codon for the hsa_circ_0006401 ORF and did not encode the hsa_circ_0006401 peptides, did not alter CRC growth, migration or apoptosis. Together, the results indicate that hsa_circ_0006401 promotes an aggressive phenotype in CRC cells by encoding the hsa_circ_0006401 peptides.

### Hsa_circ_0006401 peptides transcriptionally regulated COL6A3 expression

The collagen alpha-3 (VI) chain (COL6A3) is a key component of the extracellular matrix and highly expressed in multiple malignants [12]. Endotrophin, the cleaved C5 domain fragment of COL6A3, can directly regulate the malignancy of cancer cells via TGFβ-dependent mechanisms [12]. Data from TCGA showed that COL6A3 is widely expressed in various cancer tissues, including colorectal carcinoma (Fig.6A). Compared with normal tissues, COL6A3 expression level is higher in colon cancer tissues (Fig.6B). Moreover, high COL6A3 gene expression level predicts unfavorable prognosis (Fig.6C). To further study whether the hsa_circ_0006401 peptide contributes to modulating or controlling the activity of COL6A3. We found that silencing hsa_circ_0006401 circRNA level decreased the gene expression level of the host gene *col6a3* (Fig.6D). Moreover, COL6A3 expression was also downregulated by silence of hsa_circ_0006401 circRNA level. In p-Circ transfected cells, which encoded high level of hsa_circ_0006401 peptides, mutation of start codon of hsa_circ_0006401 ORF inhibted expression level of COL6A3 (Fig.6E). Down-regulation of hsa_circ_0006401 also decreased TGFβ1 gene expression (Fig.6F). Taken together, the results indicated hsa_circ_0006401 peptides transcriptionally regulated COL6A3 expression.

## Discussion

Circular RNA (circRNA) is a novel type of endogenous RNA that is widely and stably present in eukaryotic cells. Due to the lack of a cap structure and polyA tail, circRNAs form a covalently closed loop, are highly resistant to RNase activity and are conserved across species. With the rapid development of RNA deep-sequencing technology, a few cancer-related circRNAs have been identified. However, the biological functions and mechanisms of most circRNAs are largely unexplored. Accumulating studies also suggest that circRNAs participate in the tumorigenesis of human cancer and hold promise to become new diagnostic and prognostic biomarkers for various cancers.

Hsa_circ_0006401 is derived from its host gene *col6a3* and has unknown molecular structures in CRC. In this study, we found that the expression of hsa_circ_0006401 was closely related to lymph node metastasis. Further investigation suggested that compared to CRC tissue samples from nonmetastatic patients, cancer tissue samples from metastatic patients showed hsa_circ_0006401 upregulation. *In vitro* and *in vivo* studies showed that hsa_circ_0006401 promoted CRC growth, migration, and metastasis and inhibited CRC apoptosis. Together, these results indicate that hsa_circ_0006401 may have a potential function in the tumorigenesis of CRC. The potential diagnostic capacity of hsa_circ_0006401 was also

evaluated in our study, and we revealed that hsa_circ_0006401 might serve as a novel biomarker for metastatic CRC patients. To the best of our knowledge, this is the first report to reveal the functional and diagnostic value of hsa_circ_0006401 in CRC. Our results indicate that hsa_circ_0006401 may serve as a potential biomarker of CRC and is involved in the regulation of CRC tumorigenesis, which provide the new insight that the circularization of the three exons spliced from the pre-mRNA col6a3 may maintain functions consistent with those of the host gene.

The mechanisms involved in the regulatory function of circRNAs are more complex. Some circRNAs may be sponges for microRNAs (miRNAs) [13] or interact with RNA-binding proteins (RBPs) [14] to moderate gene expression. Recently, several studies have suggested that circRNAs can be translated and encode functional proteins in a cap-independent manner through the internal ribosomal entry site (IRES) [15, 16]. Nevertheless, genome-wide studies have demonstrated that translation of circRNAs, which is driven by short sequences containing N6-methyladenosine (m6A) as the IRES, is widespread in human cells [17]. Emerging evidence also suggests that circRNA-derived proteins play important biological roles in cellular responses to environmental stress, myoblast proliferation and tumorigenesis.

Hsa_circ_0006401 is produced by 4–6 exons of its host gene *col6a3* and is localized in the cytoplasm. In our study, we found open reading frames (ORFs) across the back-spliced junction of hsa_circ_0006401. To study whether hsa_circ_0006401 has protein coding potential, a construct producing a high hsa_circ_0006401 circRNA expression level was generated, and a GFP sequence without an AUG initiation codon was inserted immediately upstream of the ORF termination codon. The GFP-fusion protein was detected by both fluorescence microscopy and Western blotting in 293T cells. However, most GFP expression was blocked when the AUG codon of the hsa_circ_0006401 ORF was mutated. With comparison to the host gene, we found that a novel 198-aa peptide with an additional amino acid might be produced from this ORF, which was absent in the host gene *col6a3* mRNA transcript.

To further confirm peptide expression, the peptide sequence spanning the back-splice junction, unambiguously identified as hsa_circ_0006401-encoded products, was detected by IHC. The peptide was found to be expressed in both the nucleus and cytoplasm in human colon cancer and paratumor tissue specimens. Therefore, the hsa_circ_0006401 peptide was considered to be naturally endogenously produced in human colon cancer tissues. Hsa_circ_0006401 peptide expression was also confirmed in SW480 colon cancer cells. Moreover, mutation of the hsa_circ_0006401 ORF prevented the increased proliferation and migration of CRC cells induced by overexpression of hsa_circ_0006401, suggesting that hsa_circ_0006401 may promote an aggressive phenotype in CRC cells by encoding the hsa_circ_0006401 peptide. Recently, a few studies also reported that circRNAs might encode functional peptides or proteins. For example, the circular RNA Circ-ZNF609 is translated into a protein and regulates myogenesis. Furthermore, the circRNA-derived protein SHPRH-146aa suppresses glioma tumorigenesis by protecting full-length SHPRH from degradation by the ubiquitin proteasome. Taken together, these results emphasize the potentially important roles of peptides encoded by circRNAs.

To date, we have not determined the molecular activity of the peptide derived from hsa_circ_0006401. We found that hsa_circ_0006401 could promote an aggressive CRC phenotype by encoding hsa_circ_0006401 peptides. Many circRNA-derived proteins have sequence overlap with proteins conventionally generated from linear mRNA. Therefore, it is possible that hsa_circ_0006401-encoded proteins could interfere with the function of counterparts derived from linear mRNA COL6A3. COL6A3, encoded by the host gene *col6a3*, is an essential component of the extracellular matrix and structurally has a short triple-helical domain and two large globule-like N-terminal and C-terminal non-collagenous domains [18]. The cleaved C5 domain fragment, called endotrophin, can directly regulate the cancer phenotype by activating TGFβ-dependent pathway [12]. Previous studies showed COL6A3 is a potential prognosis marker of colorectal carcinoma [19] and alternatively spliced COL6A3 transcripts are associated with the progression of colon cancer [18, 20]. In our study, we found COL6A3 is localized in the nucleus and cytoplasm and highly expressed in colon cancer and its expression to correlate with poor survival outcomes, which further emphasized the crucial role of COL6A3 as a tumor promoter in colorectal carcinoma. Moreover, hsa_circ_0006401 peptides decreased the mRNA and protein level of the host gene *col6a3*, suggesting hsa_circ_0006401 peptides may promote proliferation and metastasis of CRC by transcriptionally regulating COL6A3 expression and its downstream TGFβ1 signaling pathway. However, the underlying mechanism by which hsa_circ_0006401 peptides interfere with the function of COL6A3 is still unclear, which needs to be investigated in future studies.

In conclusion, we revealed that hsa_circ_0006401 promotes proliferation and metastasis *in vivo* and *in vitro* by encoding a novel peptide. However, the function of the hsa_circ_0006401 peptide requires further investigation.

# Abbreviations

CRC: colorectal cancer; FBS:Fetal Bovine Serum; DMEM:Dulbecco's modified Eagle's medium; NC:Negative control; IHC:Immunohistochemistry; OS:Overall survival; BSA:bovine serum albumin; H&E:hematoxylin and eosin; ORF:open reading frame; COL6A3:The collagen alpha-3 (VI) chain.

# Declarations

### Ethics approval and consent to participate:

This study was approved by t Research Ethics Committee of Zhejiang Provincial People's Hospital, Hangzhou Medical College (code: 2020QT084).

### Consent for publication:

Informed consent was obtained from all individual participants included in the study.

### Availability of data and materials:

All data generated or analyzed during this study are included in this published article.

**Competing interests:**

The authors declare that they have no conflicts of interest.

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**Authors' contributions:**

CJZ, XLZ, XGG, JYW and WSP were involved in the experimental design, acquisition of data, analysis and interpretation of data, and drafting of the manuscript. CJZ, XGG and JYW were involved in the experimental design and acquisition of xenografts. JYJ and WSP were involved in the study conception and design, analysis and interpretation of data, revision of the manuscript, and study supervision. The authors read and approved the final manuscript.

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# Tables

**Table 1.** Clinical characteristics of the patients

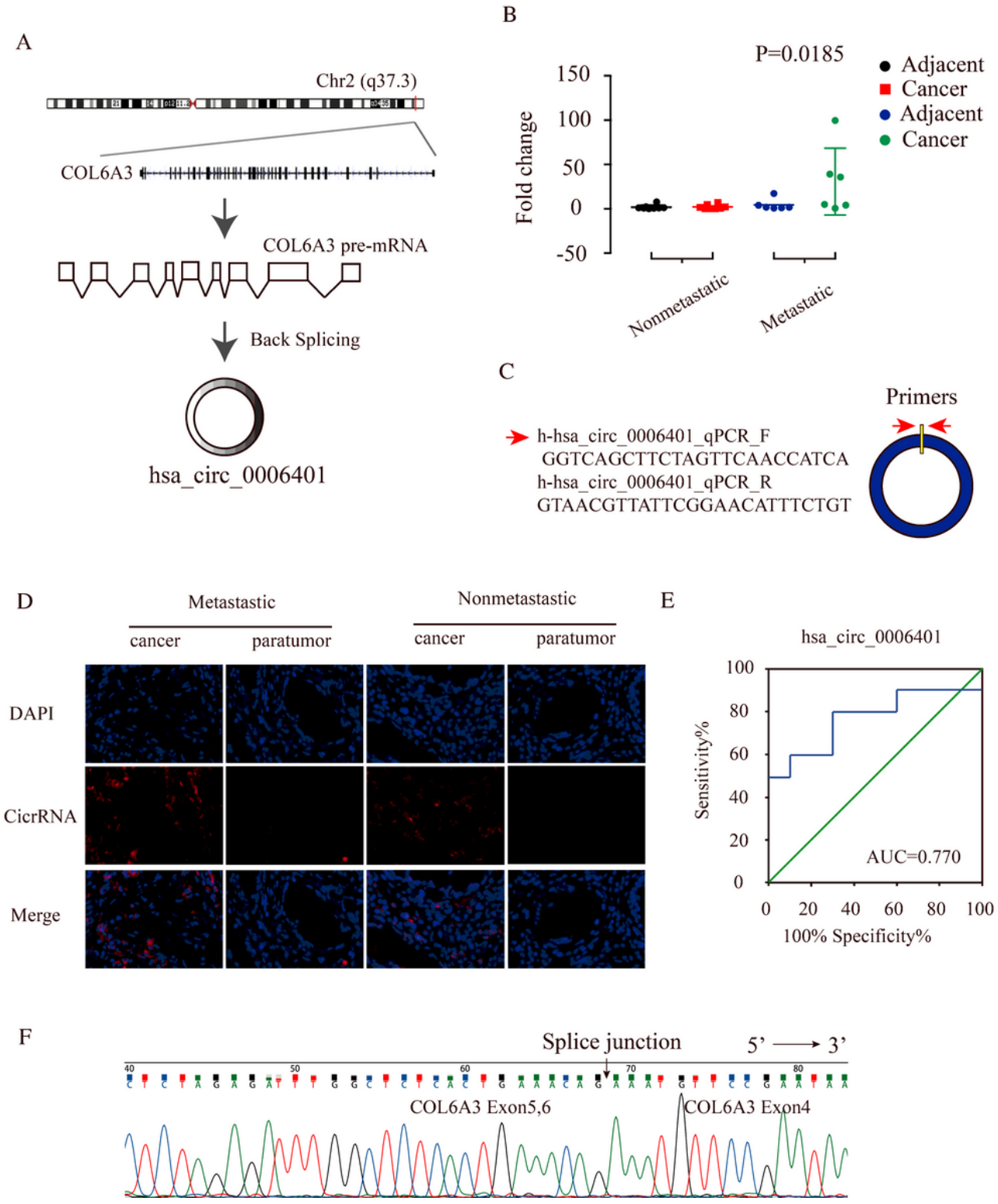
| Characteristics | qRT-PCR | FISH | IHC |
|---|---|---|---|
| Total number | 12 | 3 | 12 |
| Gender (male/female) | 2/1 | 31/10 | 3/2 |
| Age (year, mean) | 55.3 ± 3.5 | 65.1 ± 11.2 | 62.2± 11.4 |
| Adenocarcinoma (yes/no) | 3/0 | 41/0 | 5/0 |
| Pathology stage | | | |
| pTa-pT1 | 1 | 13 | 1 |
| pT2-T4 | 2 | 28 | 4 |
| Histologic differentiation | | | |
| Well differentiated | 0 | 6 | 0 |
| Moderately differentiated | 0 | 17 | 0 |
| Poorly differentiated | 3 | 18 | 5 |
| Lymphatic metastasis (yes/no) | 2/1 | 20/21 | 4/1 |
| Vascular invasion (yes/no) | 2/1 | 20/21 | 4/1 |

**Table 2** Correlation between hsa_circ_0006401 expression and clinical features in CRC

| Characteristics | | hsa-circ-0006401 expression | | |
|---|---|---|---|---|
| | No.(%) | Low(%) | High(%) | P-value |
| Gender | | | | |
| Male | 8(67) | 4(67) | 4(67) | 0.264 |
| Female | 4(33) | 2(33) | 2(33) | |
| Age | | | | |
| <60 | 7(58) | 4(67) | 3(40) | 0.149 |
| ≥60 | 5(42) | 2(33) | 3(60) | |
| Tumor size | | | | |
| <5cm | 2(50) | 3(50) | 3(50) | 0.319 |
| ≥5cm | 10(50) | 3(50) | 3(50) | |
| Pathology stage | | | | |
| pTa-pT1 | 0(0) | 0(0) | 0(0) | 0.899 |
| pT2-T4 | 12(100) | 6(100) | 6(100) | |
| Grade | | | | |
| Low | 11(92) | 6(100) | 5(83) | 0.781 |
| High | 1(8) | 0(0) | 1(17) | |
| Lymphatic metastasis | | | | |
| Yes | 6(50) | 1(17) | 5(83) | 0.040* |
| No | 6(50) | 5(83) | 1(17) | |
| Vascular invasion | | | | |
| Yes | 4(33) | 2(33) | 2(33) | 0.548 |
| No | 8(67) | 4(67) | 4(67) | |
| Total | 12 | 6 | 6 | |

Chi-square test. *$P < 0.05$

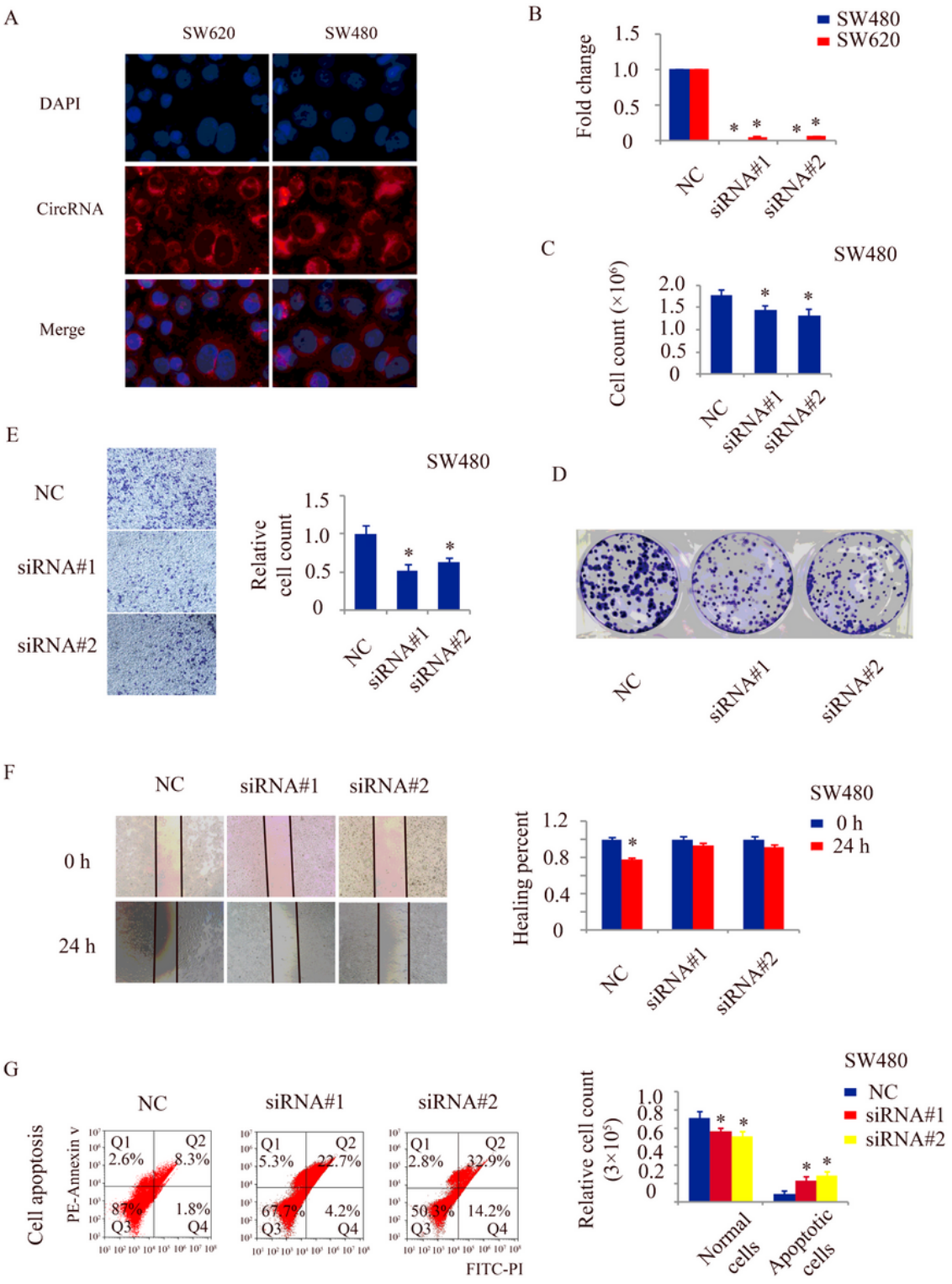
# Figures




## Figure 1

Analysis of the expression level of hsa_circ_0006401 in CRC and corresponding normal tissue specimens. (A) Schematic representation of the back splicing of hsa_circ_0006401. (B) Primers for hsa_circ_0006401. (C) Gene expression level of hsa_circ_0006401 in CRC patients in either the metastasis or control group. (D) Reprehensive images of hsa_circ_0006401 in metastatic and

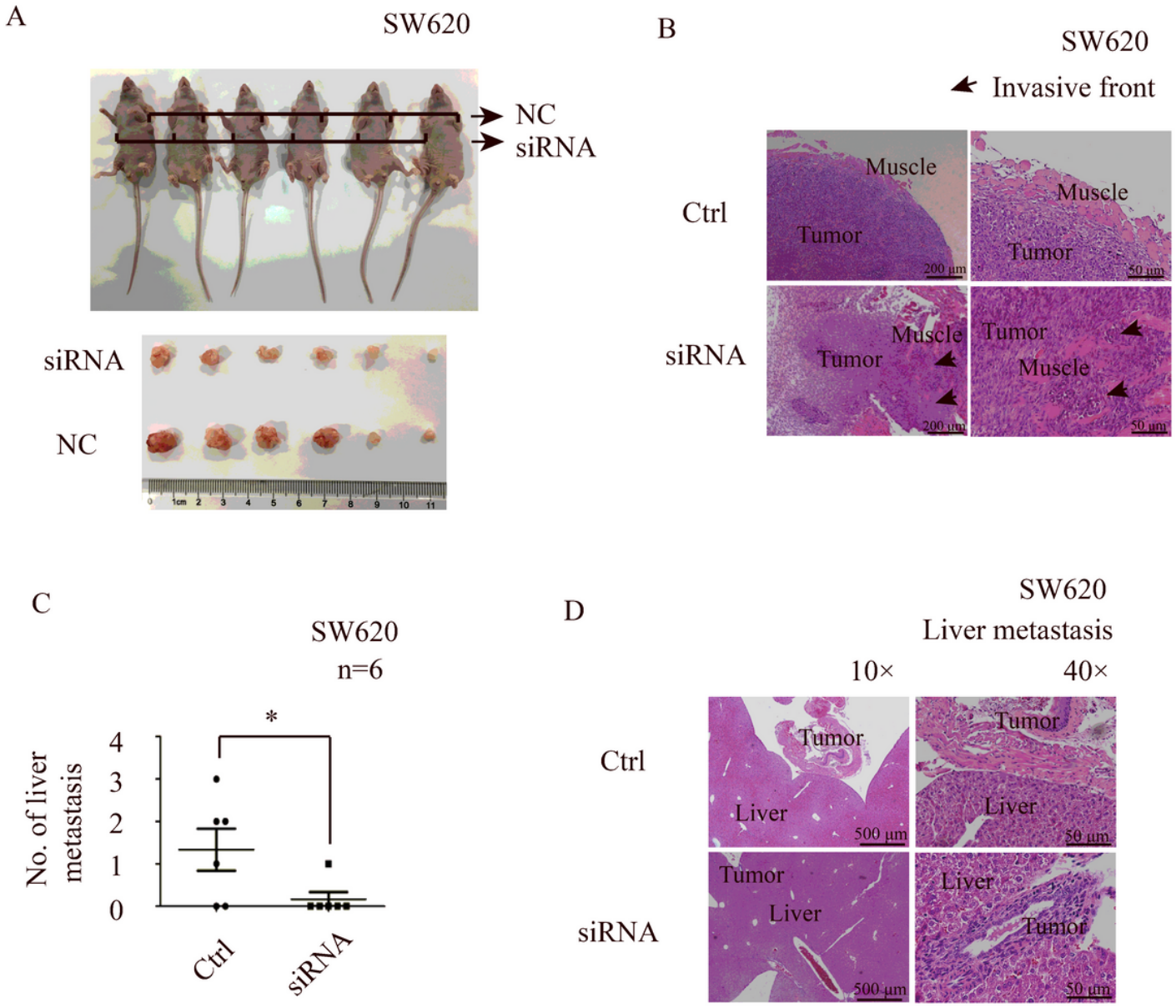
nonmetastatic CRC tissue samples. ROC curve analysis of hsa_circ_0006401 in CRC patients. (E) Sequencing of PCR products with a splice junction.




## Figure 2

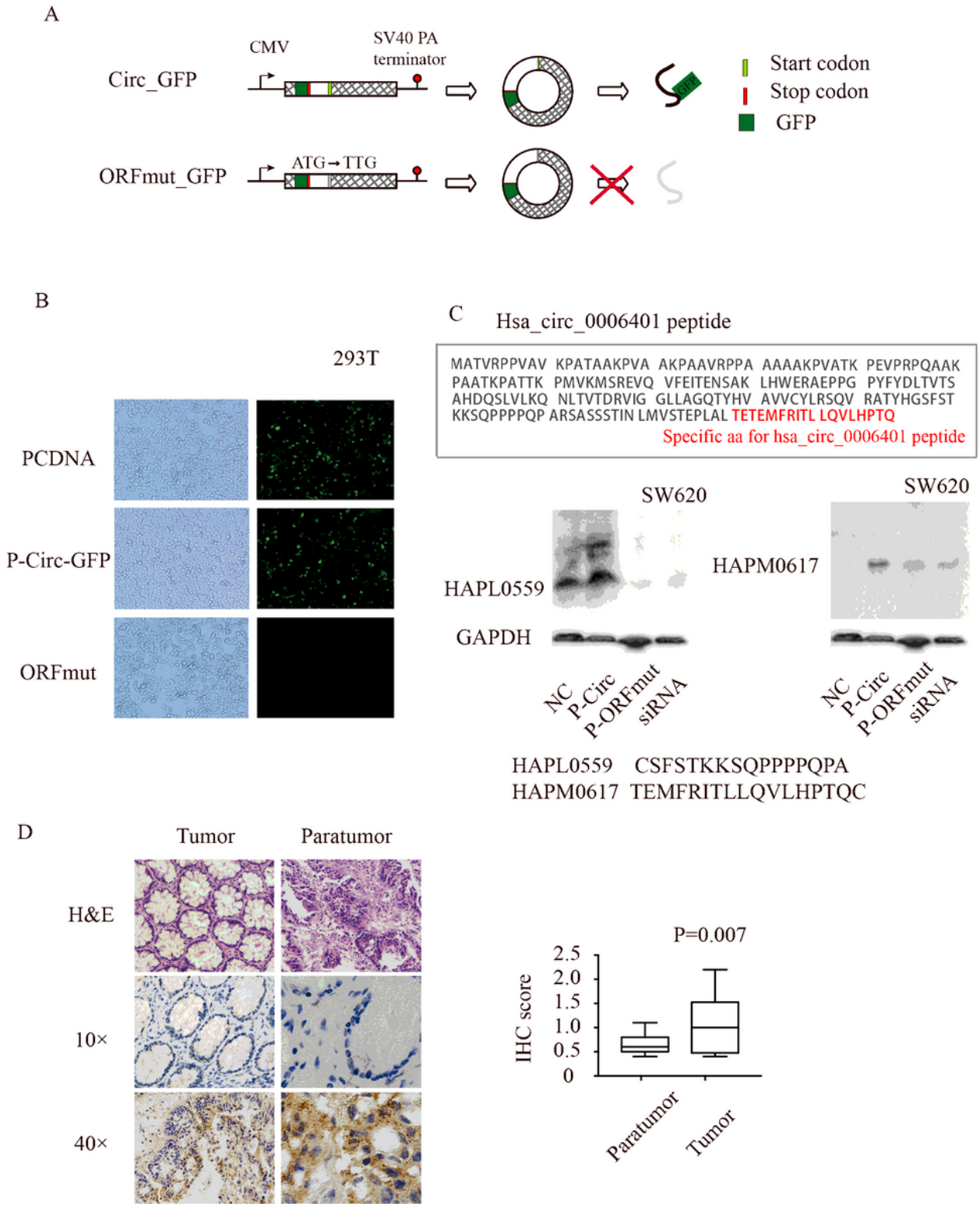
Hsa_circ_0006401 promoted the proliferation and migration of CRC cells in vitro. (A) Representative images of the expression and location of hsa_circ_0006401 in SW480 and SW620 cells. (B) Silencing efficiency of two siRNAs in the CRC cell lines SW480 and SW620 by qRT-PCR. (C) Cell counts of CRC cells

in the control group and hsa_circ_0006401 downregulation group. (D) Transwell assay to detect the migratory ability of CRC cells in the control group and hsa_circ_0006401 downregulation group. (E) Wound-healing assay to detect the migratory ability of CRC cells in the control group and hsa_circ_0006401 downregulation group. (F) Flow cytometry to assess cell apoptosis in the control group and hsa_circ_0006401 downregulation group.




## Figure 3

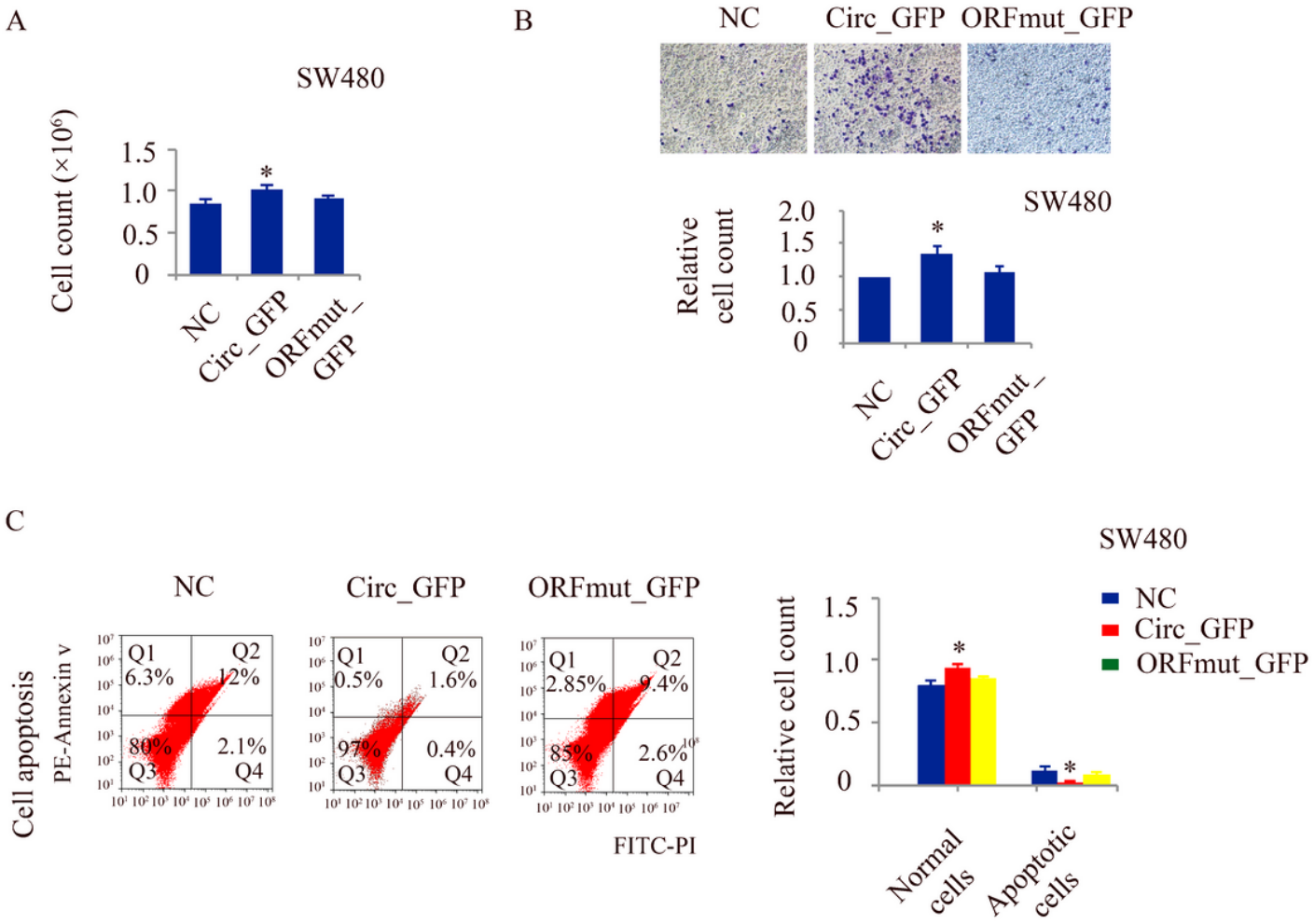
Hsa_circ_0006401 promoted the proliferation and metastasis of CRC in vivo. (A) Cells were subcutaneously injected into nude mice. All injected mice formed tumors. Tumor were isolated, and tumor volume was measured. (B) Representative images of hematoxylin and eosin staining of the tumors of mice from different groups are shown. (C) Tumors and the surrounding tissues from mice in the control and hsa_circ_0006401-silenced groups were fixed and subjected to hematoxylin and eosin staining. (D) The number of liver metastases and the metastatic burden were determined and graphed.




## Figure 4

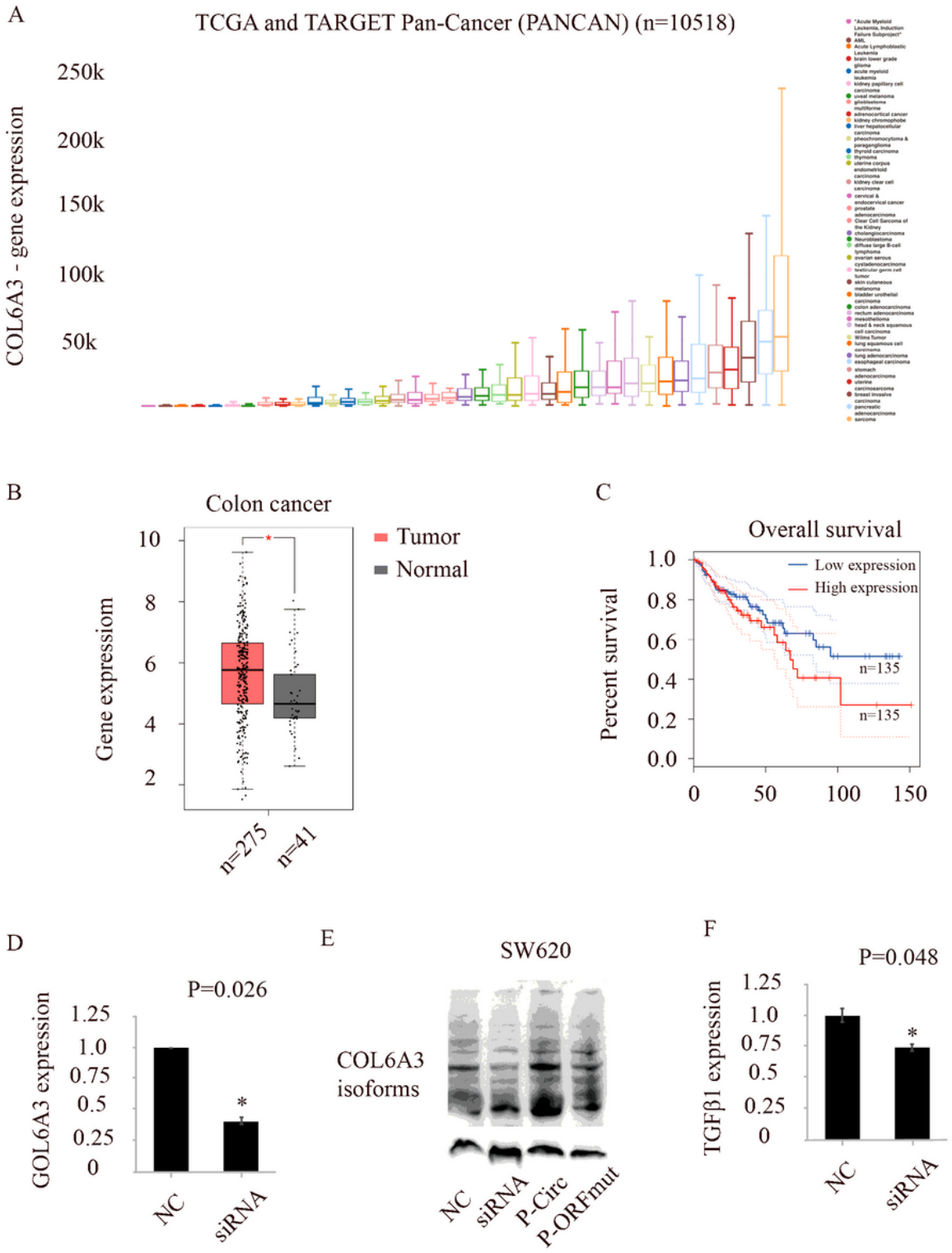
Hsa_circ_0006401 encoded a novel peptide. (A) Schematic representation of plasmid construction. (B) Detection of GFP fluorescence. The indicated constructs were transfected into HeLa cells for 24 h. (C) Western blot analysis with anti- hsa_circ_0006401 antibodies to evaluate proteins from SW620 cells transfected with different constructs. NC (negative control), p-Circ (hsa_circ_0006401 circRNA level was highly expressed), p-ORF-GFP (ATG start codon of p-Circ was mutated to TTG), siRNA (hsa_circ_0006401

circRNA level was silenced by siRNA). GAPDH was used as a loading control. (D) Reprehensive images of IHC analysis with an antibody to evaluate proteins from colon cancer tissues and normal colon tissues (left panel). IHC scores were calculated (right panel).




## Figure 5

Function of the ORF in CRC. (A) Cell count of CRC cells in the control group and hsa_circ_0006401 downregulation group. (B) Transwell assay to detect the migratory ability of CRC cells in the control group and hsa_circ_0006401 downregulation group. (C) Flow cytometry assay to assess cell apoptosis in the control group and hsa_circ_0006401 downregulation group.




## Figure 6

Hsa_circ_0006401 peptides transcriptionally regulated COL6A3 expression. (A) Gene set analysis of COL6A3 expression in various cancer tissues using TCGA cancer browser. (B) Gene set analysis of COL6A3 expression in normal tissue and colon cancer using TCGA cancer browser. The number of patients from each subtype is indicated below the box plot. (C) COL6A3 expression was assessed by Kaplan-Meier survival analysis for 5-year overall survival outcome in 270 colon cancer patients. (D)

COL6A3 mRNA expression level in negative control SW620 cancer cells (NC) and hsa_circ_0006401 silencing SW620 cancer cells (siRNA). (E) COL6A3 protein expression level in SW620 cells transfected with different constructs. NC (negative control), p-Circ (hsa_circ_0006401 circRNA level was highly expressed), p-ORF-GFP (ATG start codon of p-Circ was mutated to TTG), siRNA (hsa_circ_0006401 circRNA level was silenced by siRNA). (F) TGFβ1 mRNA expression level in negative control SW620 cancer cells (NC) and hsa_circ_0006401 silencing SW620 cancer cells (siRNA).