

# FOXCUT Promotes the Proliferation and Invasion by Activating FOXC1/PI3K/AKT Pathway in Colorectal Cancer

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**Introduction:** Colorectal cancer (CRC) is the third most commonly diagnosed world cancer. Long noncoding RNAs (lncRNAs) serve important regulatory roles in tumorigenesis. However, the contributions of lncRNAs to human CRC remain largely unknown.

**Material and Methods:** FOXC1 and FOXCUT lncRNA expression levels were detected in a panel of paired specimens obtained from 48 patients' tissues and cell lines with CRC using RT-qPCR. RNA interference was used to investigate potential correlations between FOXC1 and FOXCUT expression in HT29. Cell proliferation was assessed by MTT assay and EdU incorporation assay. The migration and invasion of CRC cells were detected by transwell assay. Western blot was applied to assess the protein expression and PI3K/AKT signaling pathway.

**Results:** In this study, a novel long noncoding RNA (FOXCUT) was frequently over-expressed in CRC tissues and cell lines. In addition, the expressions of FOXCUT and FOXC1 were positively correlated. When the expression of FOXCUT was downregulated by small interfering RNA (siRNA), the expression of FOXC1 was also decreased. Moreover, knockdown of FOXCUT significantly inhibited proliferation and invasion of CRC cell lines and resulted in downregulated expression of the matrix metalloproteinase 1 (MMP-1). Mechanistically, FOXCUT promotes the expression of FOXC1 to activate PI3K/AKT signaling pathway for its regulation of cell growth and proliferation.

**Conclusion:** In summary, our findings indicate that FOXCUT plays an important oncogenic role and may serve as a novel biomarker and therapeutic target in CRC progression.

**Keywords:** colorectal cancer, FOXCUT, FOXC1, proliferation, invasion

## Introduction

Colorectal cancer (CRC), which third in morbidity and second-highest mortality, is one of the most common malignancies.<sup>1,2</sup> The incidence of CRC is increasing year by year, which seriously threatens the life and health of the public in China.<sup>3</sup> Due to the lack of biomarkers for early diagnosis, prognostic indicators and effective treatment targets, the treatment of CRC has always been a bottleneck. Therefore, it is of great clinical and social significance to find new molecular markers and therapeutic targets to improve the survival rate of patients.

FOXC1 is a member of the transcription factor forkhead box (FOX) protein superfamily.<sup>4</sup> FOXC1 regulates tumor-associated genes and is regulated by several mechanisms that control its gene expression and protein activity. Additionally, it affects the cell cycle and proliferation of tumor cells by epithelial-to-mesenchymal

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transition (EMT), cellular signal transduction, migration of vascular endothelial cell.<sup>5</sup> It has been reported that FOXC1 gene is highly expressed in many kinds of tumors,<sup>4,6-8</sup> such as pancreatic ductal adenocarcinoma (PDA), basal-like breast cancer (BLBC), esophageal cancer and gastric cancer (ESCC). Importantly, FOXC1 may act as a subtype-specific biomarker for breast cancer and acute myeloid leukemia (AML). It was reported that FOXC1 expression is significantly higher in BLBC than in luminal and HER2 breast cancer.<sup>4</sup> High levels of FOXC1 in AML (FAB-M2) exhibit essential relevance with morphological classifications associated with granulocyte differentiation compared with other AML subtypes.<sup>9</sup> Furthermore, in most cancer types, high FOXC1 expression may predict clinical features such as malignant clinical manifestations, increased pathological grade, and poor prognosis of patients<sup>10,11</sup> and it may be considered as a novel target for therapy. However, the relationship between FOXC1 gene and CRC remains largely unknown.

Long noncoding RNAs (lncRNAs) are now defined as the long RNA transcripts with >200 nucleotides lacking protein encoding potential or with limited peptide coding potential.<sup>12-14</sup> Extensive evidence suggests that lncRNAs have vital roles in regulating gene expression at levels of chromatin modification, transcriptional and posttranscriptional regulation. Many lncRNAs have been identified to affect tumorigenesis.<sup>13,15-17</sup> A novel lncRNA named FOXC1 promoter upstream transcript (FOXCUT) has been identified to functionally contact its adjacent FOXC1 mRNA and take on the form of 'lncRNA-mRNA pairs' in ESCC, oral squamous cell carcinoma (OSCC), BLBC, and nasopharyngeal carcinoma (NPC).<sup>18-21</sup> Nevertheless, whether lncRNA plays a role in CRC is not clear yet. In this study, we first explored the expression correlation and clinicopathological significance of FOXCUT and FOXC1 in CRC, so as to provide new clues and research targets for the pathogenesis, prognosis and treatment of CRC.

## Materials and Methods

The colon cancer cell lines (Caco-2, HCT116, HT29 and DLD-1) and the human colonic epithelial cells (NCM-460) used for experiments were obtained from Type Culture Collection (CCTCC, China). All cells were regularly authenticated by short tandem repeat (STR) analysis and tested for the absence of Mycoplasma contamination. Human colon cancer tissues and the corresponding adjacent mucosa were obtained from biopsy in a total of 48 consecutive colon cancer cases admitted in our

Zhengzhou central hospital, China, from April 2018 to April 2019. CRC diagnosis was histopathologically confirmed, and none of the patients received treatment prior to the biopsy. The utilization of tumor material for research was approved by the ethical committee of Zhengzhou central hospital, which was in accordance with the Declaration of Helsinki. Written informed consent was obtained from the patients. The tissues from each subject were immersed overnight in RNAlater reagent (Thermo Scientific, USA) at 4°C and stored at -80°C.

## Cell Culture and Transfection

Colon cancer cell lines and NCM460 were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen, Carlsbad, CA, USA), whereas Caco-2, HCT116, HT29 and DLD-1 cells were cultivated in RPMI 1640 (21875091 Thermo Fisher Scientific) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco, Life Technologies), 100 U/mL of penicillin, and 100 µg/mL of streptomycin (P4333, Sigma). Cells in this medium were placed in a humidified atmosphere containing 5% CO<sub>2</sub> at 37°C.

The sequence of short-hairpin RNA (shRNA) directed against FOXCUT (5'- CACCGGCATTGATGCAAGGAT ATCC CGAA GGATATCCTTGCATCAATGCC -3') was ligated into the pLKO.1-Puro vector (TaKaRa Bio, Tokyo, Japan). We packaged lentivirus into human embryonic kidney 293T (HEK293T) cells using Lipofectamine 2000 (Life Technologies, Carlsbad, CA, USA) and the supernatant was collected following the manufacturer's instructions. Lentiviral particles were used to infect HT29 cells. We established stable cell lines via puromycin selection and then used for subsequent in vitro and in vivo experiments. The PCR product of the FOXC1 ORF was recovered and isolated by gel extraction, then inserted into the pcDNA3.1 Vector (TaKaRa Bio, Tokyo, Japan), and sequenced (Sangon Biotech, Shanghai, China). FOXC1 ORF forward primer containing a BamHI site and the antisense primer containing an EcoRI site to obtain the ORF fragment. For transient transfection assays small interfering RNA (siRNA) duplexes (si-FOXCUT #1 and si-FOXCUT #2), and negative control (NC) RNA duplexes were synthesized (GenePharma, Shanghai, China) by using the primer pairs (Table 1). For transfection of siRNA or pcDNA3.1-FOXC1, HT29 cells were seeded at 2x10<sup>5</sup> cells/well in 6-well plates (Eppendorf, Hamburg, Germany) 24 h before transfection. When the cells were grown to 80% confluence, cells were transfected a transfection complex consisting of experimental siRNA, pcDNA3.1-FOXC1, complex

**Table 1** Sequences for Small Interfering RNA Analysis and Vector Construction

Gene Name	Sense Strand	Antisense Strand
si-FOXCUT#1	GAAUGGAGAACUAAGACAAUUAUCT	AGAUAUUUGUCUUAAGUUCUCCAUUCGG
si-FOXCUT#2	CAGCCUCCUCCUGUGUGUGCAGAG	CUCUGCACACACAGGAGGGAGGCUGCA
Negative control	UUCUUCGAACGUGUCACGUTT	ACGUGACACGUUCGGAGAATT
FOXC1	CGGATCCATGCAGGCGCGCTACTCCGTGT	GGAATTCTCAAAACTTGCTACAGTCGTA

of siRNA and pcDNA3.1-FOXC1, or control and Lipofectamine® 2000 Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions, and cultured at 37°C and 5% CO<sub>2</sub>. For insulin-like growth factor 1 (IGF-1) treatment, IGF-1 was reconstituted at 200 ng/mL in sterile phosphate-buffered saline (PBS), cells were incubated with 200 ng/mL.<sup>22,23</sup> Then, supernatant was replaced with serum containing growth medium. Cells were lysed and harvested for RNA and protein after 48 h transfection. Recombinant human IGF-1 was purchased from R&D Systems (Minneapolis, USA).

### Quantitative PCR (qPCR)

To quantitatively determine the mRNA expression level of FOXCUT and FOXC1 in colon cancer cells, tissues and colonic epithelial cells, real-time PCR was performed, primers of qPCR were synthesized (Sangon Biotech, Shanghai, China) and primer information are showed in Table 2. Total RNA was extracted from each sample using TRIzol Total RNA reagent (Invitrogen Life Technologies, USA) according to the manufacturer's protocol. The concentration of RNA samples was measured using a NanoDrop 2000 (Thermo Scientific, USA) and the quality confirmed by agarose gel electrophoresis. Total RNA (1 µg) was reverse transcribed into cDNA with FastKing RT Kit including DNase treatment (Takara, Shiga, Japan). cDNA amplification was performed using the SYBR-Green master mix kit (Tiangen, China) following the manufacturer's instructions. The reaction mixtures were incubated at 94°C for 5 min, followed by 40 cycles of 94°C for 5 sec, 60°C for 34 sec and 72°C for 20 sec. Data were analyzed using the comparative threshold cycle (Ct) method ( $2^{-\Delta\Delta Ct}$ ).<sup>24</sup> The

*ACTB* gene was used as an endogenous control. Triplicate wells were performed per sample.

### Cell Proliferation Assay

Cell proliferation was analyzed using the MTT assay. Briefly, cells in a 96-well plate were (Eppendorf, Hamburg, Germany) incubated in a humidified 5% CO<sub>2</sub> chamber after transfection, followed by addition of 20 µL of MTT dye (2.5 mg/mL) at 0, 24, 48 and 72 h, and subsequently incubated with 150 µL of Dimethyl sulfoxide for 5 min. The absorbance at 570 nm was recorded using an Enzyme Immunoassay Analyzer (Bio-Rad, Hercules, USA).

### EdU Incorporation Assay

The cell proliferation was also examined by 5-ethynyl-2'-deoxyuridine (EdU) incorporation assay.<sup>25</sup> Briefly, indicated cells were incubated with 50 µM EdU for 2 h at 37°C after 24 h transfection. Cells were then washed with PBS for three times, and fixed with 4% formaldehyde for 30 min at room temperature (RT) according to the manufacturer's protocol (RiboBio, China). Then, 0.5% Triton X-100 was applied for 10 min at RT to permeabilize cells. Cells were incubated with 1 × Apollo reaction cocktail for 30 min after washing with PBS for three times. DNA was stained with 4',6-diamidino-2-phenylindole (DAPI) for 30 min and results were visualized with a Zeiss confocal microscope (LSM 700, USA).

### Transwell Invasion Assay

Cell invasion was examined using the transwell system chambers with 8 µm pore size polycarbonate membrane (BD Biosciences, San Jose, CA, USA) following the manufacturer's instructions. In brief, HT-29 cells (5 × 10<sup>5</sup> cells/

**Table 2** Primer Sequences for Quantitative Real-Time PCR

Gene Name	Forward	Reverse
FOXC1	AAGATCACCCCTGAACGGCATC	GGCACCTTGACGAAGCACTC
FOXCUT	TCCGATCATCTATCCCTTACGA	CCCGGCTTCAAAAGACTCA
ACTB	CCACTGGCATCGTGATGGA	CGCTCGGTGAGGATCTTCAT

well) were inoculated to an upper chamber and the inserts were coated with Matrigel (BD Biosciences, USA). Six hundred  $\mu\text{L}$  of complete medium was present in that bottom chamber. The cells were allowed to invade for 24 h at  $37^{\circ}\text{C}$  with 5%  $\text{CO}_2$ , the medium was removed from the upper chamber. The non-invaded cells were removed from on the upper side of the chamber, whereas the cells on the bottom surface of the surface were fixed with 0.05% crystal violet for 2 h. Finally, the number of invaded cells was counted by using an inverted microscope and the relative number was calculated.

## Western Blot Assay

CRC lines were harvested and extracted using RIPA lysis buffer (Thermo Scientific, MA). Antibody and dilutions used in the studies: polyclonal antibodies against PI3K, AKT, p-PI3K, p-AKT (1: 2000, 1: 1000, Cell Signaling, Danvers, MA), monoclonal MMP-1 and FOXC1 (1:2000 Bio-technie, Minnesota, USA), and anti-GAPDH antibody (Sigma-Aldrich, St. Louis, MO) was used at a 1:3000 dilution. Protein bands were quantified using Quantity One 4.5.0 software (Bio-Rad, Hercules, CA, USA). Signals were visualized using ECL Substrates (Thermo Scientific, MA). The relative protein levels in different cell lines were normalized to the concentration of GAPDH. The experiment was done in triplicate.

## Animal Studies

All animal experiments were approved by the Animal Care Committee of Zhengzhou University and performed following the guidelines and regulations of the Ethics Committee of Zhengzhou University for the welfare of animals in cancer research. Male BALB/c nude mice (4–5 weeks old) were purchased from Vital River Laboratory Animal Technologies Co. Ltd (Beijing, China). For xenograft experiments,  $1 \times 10^7$  cells were subcutaneously injected into the flanks of animals ( $n = 5$  per group). Tumor growth was examined every 4 days for 4 weeks. Tumor volumes were calculated by the equation  $V(\text{mm}^3) = a \times b \times c/2$ , where  $a$  is the length,  $b$  is the width, and  $c$  is the height. Tumors were harvested for RNA and protein assays.

## Statistical Analysis

Data were presented as means  $\pm$  standard deviation (SD), and GraphPad Prism version 8.0 (GraphPad Software, San Diego, CA, USA) software was used for data statistical analysis. Independent samples  $t$ -test (two groups) or one-

way ANOVA (no less than three groups) was used to analyse the statistical significance at a significance. \*  $P < 0.05$ .

## Results

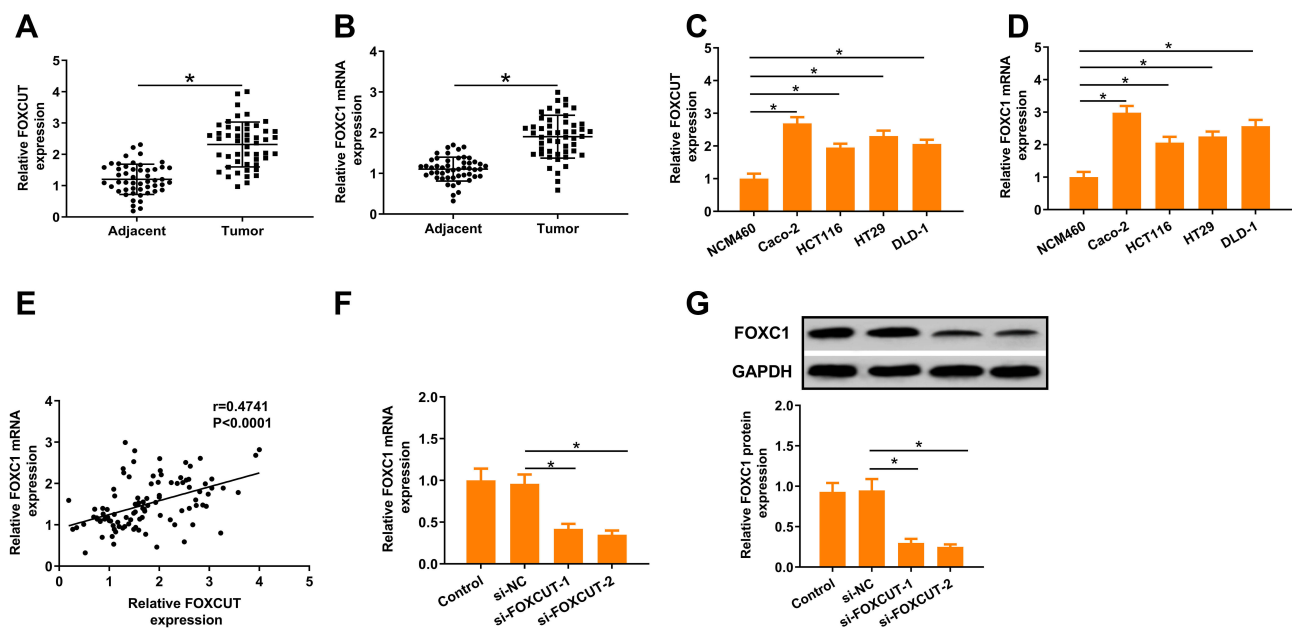
### FOXCUT and FOXC1 are Overexpressed in CRC Tissues and Cell Lines

FOXCUT and FOXC1 expression levels were detected in a panel of paired specimens obtained from 48 patient's tissues and cell lines with CRC using RT-qPCR. The results showed that, compared with the adjacent mucosa, the FOXCUT and FOXC1 mRNA level were significantly increased in human colon cancer tissues (Figure 1A and B). Similarly, the expressions of FOXCUT and FOXC1 were significantly higher in four colon cancer cell lines (Caco-2, HCT116, HT29 and DLD-1), compared with those NCM460 (Figure 1C and D). Because FOXCUT and FOXC1 are highly expressed in HT29 cells, these cell lines were exploited for further study. In addition, the relative expression of FOXCUT was positively correlated with that of FOXC1 in the CRC tissue samples. These results suggested that FOXCUT and FOXC1 were highly expressed in CRC in association with cancer progression.

To further investigate potential correlations between FOXCUT and FOXC1 expression, we used RNA interference to silence FOXCUT expression in HT29, the expression of FOXCUT was detected by RT-qPCR after transfection. The results showed that FOXCUT gene was successfully silenced in HT29 cells after transfection with FOXCUT siRNA (Supplementary Figure S1). Our results also showed that FOXC1 expression was inhibited by si-FOXCUT both in mRNA and protein levels (Figure 1E–G). These results indicate that FOXCUT promoted FOXC1 expression in HT29 cells.

### Knockdown of FOXCUT Inhibited the Cell Proliferation and Invasion Ability in CRC

We further investigated the roles of FOXCUT on CRC development. To clarify whether FOXCUT has a functional role in facilitating CRC cell progression, we examined cell proliferative activities and invasive abilities by MTT assays, EdU incorporation assay and transwell invasion assay. The results showed that inhibiting FOXCUT markedly diminished the proliferative activities of HT29 in a time-dependent manner compared to the control group (Figure 2A), the amount of cancer cells was markedly lower in FOXCUT siRNA (Figure 2C).



**Figure 1** FOXCUT and FOXC1 expression is frequently upregulated in CRC cell lines and tissues. **(A, B)** Expression of FOXCUT and FOXC1 was detected by qPCR in adjacent mucosa and colon cancer tissues (n = 48). **(C, D)** Abundance of FOXCUT and FOXC1 in CRC cell lines relative to that in the colonic epithelial cell line NCM460. The expression of FOXCUT and FOXC1 was normalized to that in NCM460. The statistical differences between groups were analyzed using independent samples t-test. **(E)** Linear regression analysis was performed on FOXC1 and lncRNA-FOXCUT expression levels in 48 CRC tissue samples (n = 48).  $r=0.4741$ ,  $P<0.0001$ . **(F, G)** FOXC1 expressions were significantly attenuated following FOXCUT siRNA transfection both in mRNA and protein levels. Relative expression of the target genes was quantified for each transcript and were normalized with the expression of  $\beta$ -actin by  $2^{-\Delta\Delta Ct}$  method. Data show the mean  $\pm$  SD. \* indicates significant differences compared with the control group ( $P < 0.05$ ).

Correspondingly, we found that the invasive potential of HT29 was apparently decreased in FOXCUT siRNA group (Figure 2E). Furthermore, the MMP-1 (matrix metalloproteinase-1) have been identified as important indicators in CRC.<sup>26–28</sup> We also found that the expression level of MMP-1 protein was significantly downregulated in FOXCUT siRNA group (Figure 2H). On the contrary, inhibiting FOXCUT markedly diminished the proliferative activities and invasive abilities of HT29 cells when overexpression of FOXC1 largely rescued these defects (Figure 2B, D, F and G; Supplementary Figures S2). Based on the findings above, we suggested that FOXC1 expression is regulated by the FOXCUT, and FOXCUT expression is necessary during the process of proliferation and invasion of HT29 cells.

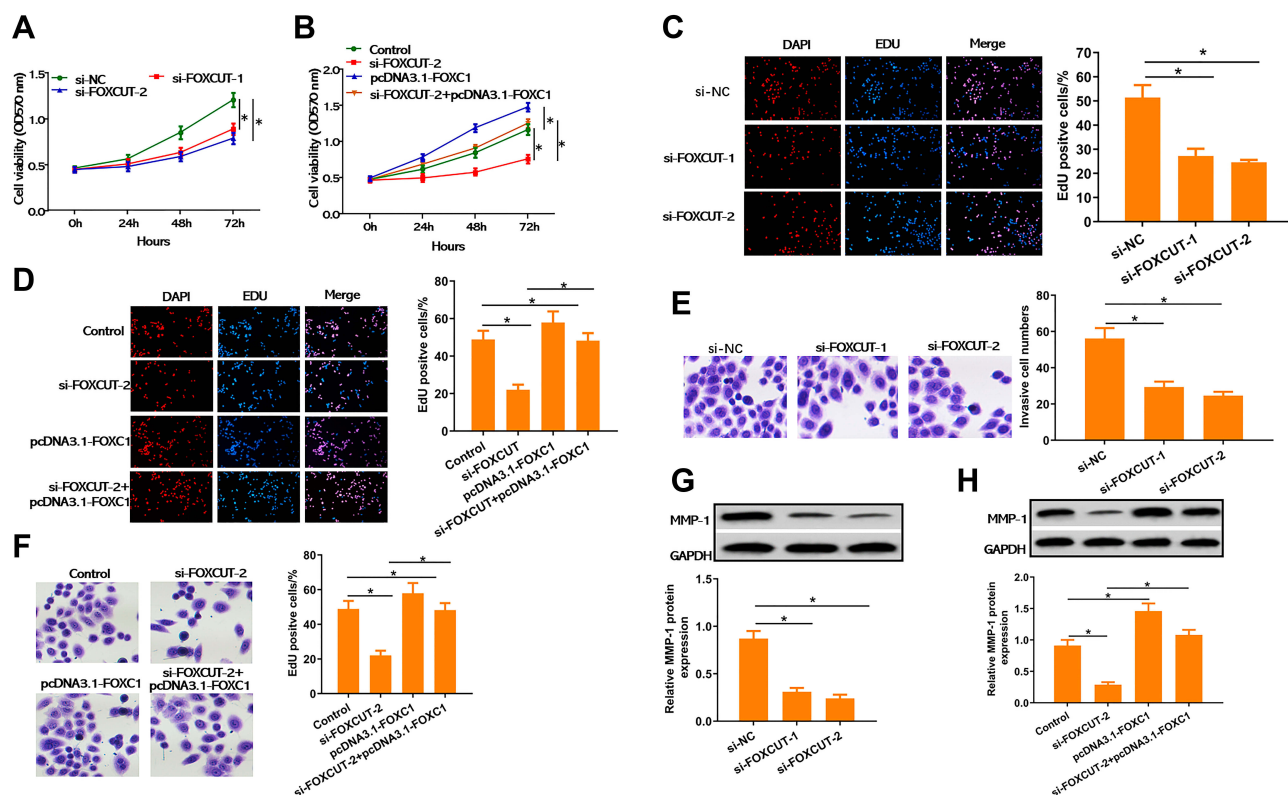
### Activated of PI3K/AKT Signaling Reversed FOXCUT Silencing Resulted in CRC Development Inhibition

Several studies reported that FOXCUT and FOXC1 through activated PI3K/AKT pathway to regulate proliferation and migration in cancer cells.<sup>5,29–31</sup> IGF-1 is known to bind to the IGF-1 receptor (IGF-1R), which is one of the tyrosine kinase receptors that activate the PI3K/AKT signaling

pathway upon stimulation. Thus, to further confirm the role of PI3K/AKT signaling during FOXCUT-mediated CRC development in HT29 cells, we measured the activation of key signal proteins, including PI3K and AKT. We found that FOXCUT silencing resulted in blockage of PI3K and AKT protein phosphorylation in HT-29 cells, while the inhibiting effect of FOXCUT silencing significantly diminished of HT29 when overexpression of FOXC1 or treatment with IGF-1 largely rescued these defects (Figure 3A). Consistent with this, MTT proliferation and transwell invasion assays showed that the IGF-1 treatment activated of PI3K/AKT signaling reversed FOXCUT silencing resulted in HT29 cells growth inhibition (Figure 3B and C). Taken together, these results suggest that the PI3K/AKT pathway is involved in FOXCUT-mediated CRC development in HT29 cells.

### FOXCUT Expression Correlates with CRC Progression in vivo

Towards further defining FOXCUT is in relation to the progression of CRC, we confirmed by in vivo tumor growth assays. Consistent with in vitro results, xenograft tumors grown from FOXCUT silenced HT29 cells had smaller mean volumes and formed more slowly than



**Figure 2** Knockdown of FOXCUT inhibited cell proliferation and invasion abilities in HT29. **(A, B)** Inhibition of FOXCUT reduces growth rate of cervical HT29 cells after treatment with or without si-FOXCUT or pcDNA3.1-FOXC1, determined by MTT assay. **(C, D)** Representative micrographs of the DAPI/EdU dyeing assay in HT29 cell after treatment with or without si-FOXCUT or pcDNA3.1-FOXC1. **(E, F)** Representative micrographs (left) and quantification (right) of transwell assay in HT29 cell after treatment with or without si-FOXCUT or pcDNA3.1-FOXC1. **(G, H)** Western blot and quantification analysis of MMP-1 after treatment with or without si-FOXCUT or pcDNA3.1-FOXC1, normalized to GAPDH. Data show the mean  $\pm$  SD. \* indicates significant differences compared with the control group ( $P < 0.05$ ).

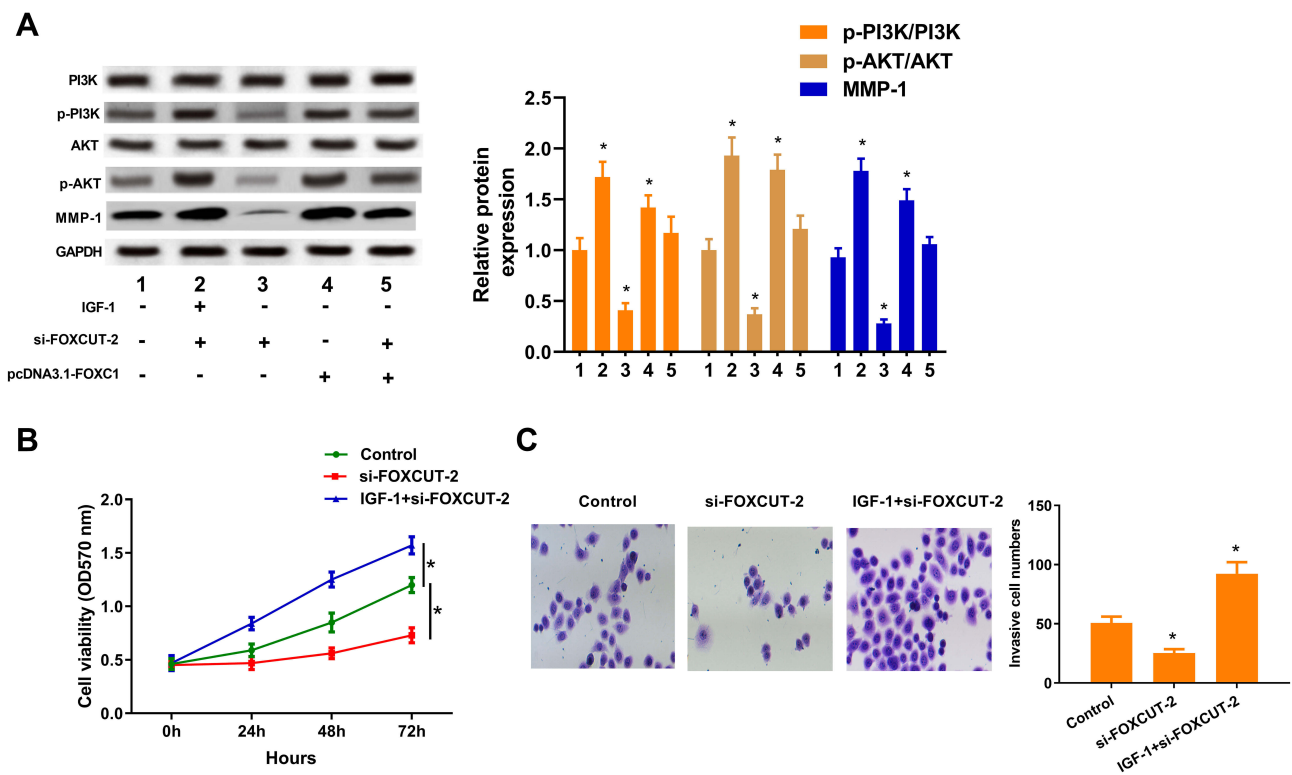
xenograft tumors grown from control cells (Figure 4A–C). In addition, the protein expression of MMP-1 and FOXC1, the ratio of p-PI3K/PI3K, p-AKT/AKT both significantly decreased in sh-FOXCUT group. Altogether these results indicate that suppression of FOXCUT expression contributed to CRC cell growth inhibition.

## Discussion

lncRNAs are considered to be the basis for eukaryotes and essential for cancer progression.<sup>12,13,32</sup> Although many lincRNAs have been identified recently, the functional characterization of lincRNAs has just begun. In addition, with the characteristic of non-invasive and humoral stability, lincRNA has become a sensitive biomarker for tumor diagnosis and prognosis. FOXCUT is a novel lincRNA located on upstream of the FOXC1 promoter. Previous studies indicated that the FOXCUT–FOXC1 regulatory network is related to tumorigenesis and cancer progression in breast cancer, oral squamous cell carcinoma and nasopharyngeal carcinoma.<sup>18,19,21</sup> Nevertheless, the function of the FOXCUT and FOXC1 in CRC has not been

determined. In our study, we explored the expression levels and clinicopathological features of the FOXCUT and FOXC1 in CRC and revealed the effects of FOXCUT in cell proliferation and migration associated with CRC to evaluate its feasibility as a potential therapeutic target.

FOXC1 initially was shown to be a key transcription factor that regulates the development of neural crest embryos.<sup>33</sup> Recent studies indicated that FOXC1 was overexpressed in a wide variety of malignant cancers and it can promote tumorigenesis, and tumor progression.<sup>4,6–8</sup> FOXC1 expression can be regulated at many levels, including modulation of DNA transcription, post-transcriptional regulation, and posttranslational modifications. lncRNAs exist as novel RNA transcripts, which function as high-level regulators that participated in post-transcriptional regulation.<sup>34</sup> FOXCUT is a FOXC1 adjacent lincRNA that belongs to a class of ncRNAs called promoter upstream transcripts (PROMPTs).<sup>35</sup> The expression and function of PROMPTs are often related to the adjacent protein-coding transcripts.<sup>21</sup> Several studies

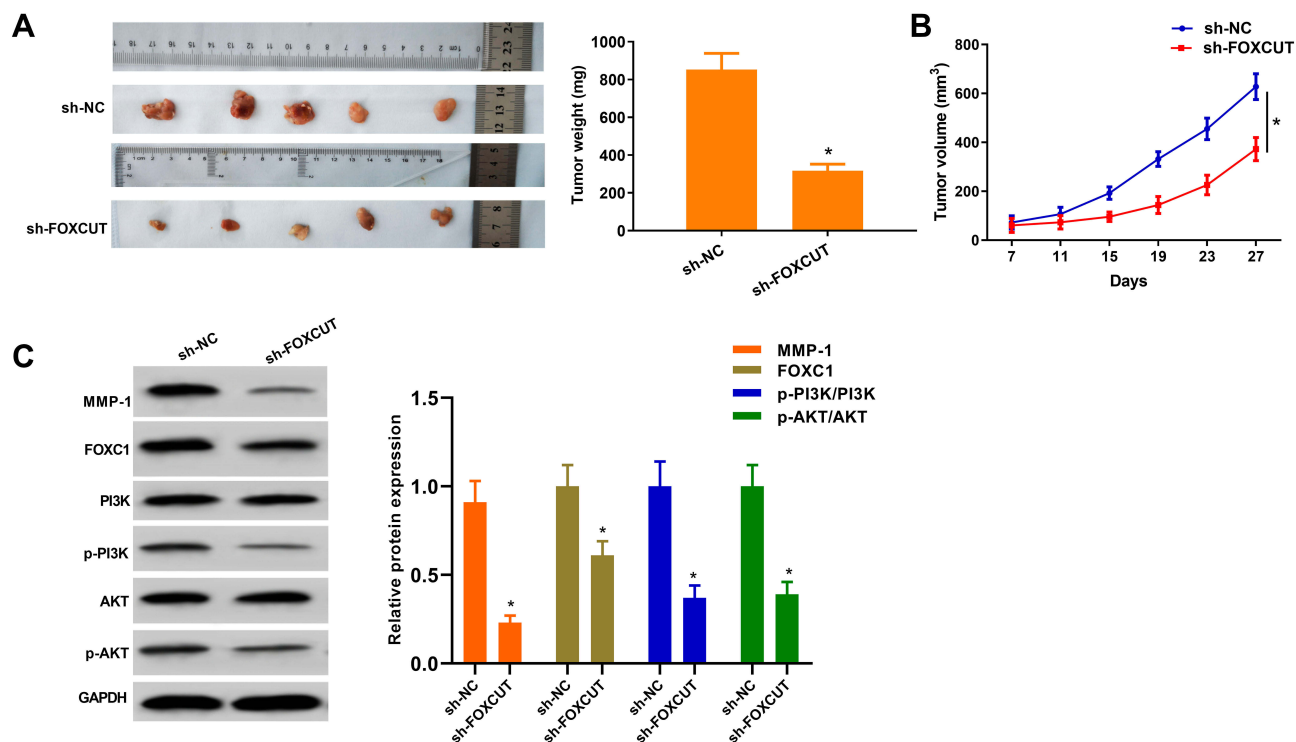


**Figure 3** Activated PI3K/AKT signaling reversed FOXCUT silencing resulted in CRC development inhibition. **(A)** Western blot analysis of PI3K, AKT and MMP-1 expression in HT29 cells after treatment with or without IGF-1 (200 ng/mL), si-FOXCUT or pcDNA3.1-FOXC1 for 48 h. GAPDH was used as a loading control. Right: quantification of p-PI3K/PI3K, p-AKT/AKT and MMP-1 protein levels, normalized to GAPDH. **(B)** Growth curves of HT29 cells after treatment with or without IGF-1 (200 ng/mL) or si-FOXCUT were determined via MTT assays. **(C)** Representative micrographs (left) and quantification (right) of transwell assay in HT29 cell after treatment with or without IGF-1 (200 ng/mL) or si-FOXCUT. Data show the mean  $\pm$  SD. \* indicates significant differences compared with the control group ( $P < 0.05$ ).

reported that FOXCUT may be associated with the regulation of proliferation and invasion in CRC cell lines by increasing FOXC1 gene expression.<sup>18,19,21</sup> In this study, we first investigated highly expression of FOXCUT and FOXC1 in clinical CRC tissues and CRC cell lines. In addition, the relative expression of FOXCUT was positively correlated with FOXC1 in the CRC cell lines and tissue samples. Besides, FOXCUT silencing reduced FOXC1 expression and abrogated CRC proliferation and invasion in vitro and in vivo. In contrast, overexpression of FOXC1 reversed FOXCUT silencing resulted in CRC development inhibition. These studies have shown that FOXCUT intensify cancer cell proliferation and invasion by promoting FOXC1 expression.

The PI3K/AKT signaling pathway plays important roles in the proliferation and their epithelial–mesenchymal transition (EMT) of CRC cells.<sup>36–39</sup> In melanoma, FOXC1 promoted melanoma by activating MST1R/PI3K/AKT pathway, which is related to poor prognosis.<sup>30</sup> It is also reported that FOXC1 promoted proliferation and epithelial–mesenchymal transition through the PI3K-AKT signal pathway in cervical

carcinoma.<sup>29</sup> However, FOXC1 inhibited migration and invasion in vitro and reduced the pulmonary metastasis in vivo.<sup>40</sup> Besides, wang reported that lncRNA AB073614 regulated proliferation and metastasis via the PI3K/AKT signaling pathway in colorectal cancer cells.<sup>31</sup> Our further study showed that FOXCUT up-regulated FOXC1 expression to activate PI3K/AKT signaling pathway. We observed that depleting FOXCUT expression decreased PI3K and AKT protein phosphorylation in vitro and in vivo, while the protein level of PI3K and AKT showed no change. In addition, EGF potently increases FOXC1 expression and phosphorylation in BLBC cells through Ras/ERK and phosphatidylinositol PI3K/AKT pathways.<sup>41</sup> FOXC1 has also been involved in inflammation-related tumor metastasis. Proinflammatory cytokine interleukin-8 (IL-8) can activate FOXC1 via activation of hypoxia-inducible factor 1 $\alpha$  (HIF-1 $\alpha$ ) by the PI3K/AKT pathway. These results also imply the feedback regulation between FOXC1 and PI3K/AKT pathway cells.<sup>42</sup> Considering the complexity of the interaction between FOXC1 and PI3K/AKT, the roles of FOXCUT–FOXC1 in the cell proliferation and invasion deserve further study.



**Figure 4** FOXCUT expression correlates with CRC progression in vivo. **(A)** Effects of FOXCUT knockdown on tumor growth after 4 weeks in vivo ( $n = 5$  per group). Upper: negative control cells. Lower: representative images of tumors formed in nude mice subcutaneously injected with FOXCUT-silenced HT29 cells. **(B)** Growth curves of xenograft tumors after subcutaneous injection of mice with FOXCUT-silenced HT29 or negative control cells. The tumor volumes were measured every 4 days after inoculation. ( $n = 5$ ). **(C)** Western blot analysis of MMP-1, FOXC1, PI3K and AKT expression in xenograft tumors after subcutaneous injection of mice with FOXCUT-silenced HT29. GAPDH was used as a loading control. The p-PI3K/PI3K, p-AKT/AKT, MMP-1/GAPDH and FOXC1/GAPDH ratios were calculated. \* $P < 0.05$  compared with the sh-NC. All data are expressed as the mean  $\pm$  SD.

In summary, the present study demonstrated that high expressions of FOXCUT and FOXC1 in CRC. Our results also indicated that FOXCUT positively regulates the expression of FOXC1 and activates PI3K/AKT pathway to regulate cell proliferation and invasion in CRC, which is the first report of FOXCUT promotes CRC progression in vivo. We have identified that FOXCUT and FOXC1 might be acted as tumor promoter that provides potential diagnostic markers and therapeutic targets for CRC.

## Abbreviations

BLBC, basal-like breast cancer; CRC, colorectal cancer; EMT, epithelial-to-mesenchymal transition; ESCC, esophageal cancer and gastric cancer; FOX, forkhead box; FOXCUT, FOXC1 promoter upstream transcript; HIF-1 $\alpha$ , hypoxia-inducible factor 1 $\alpha$ ; lncRNA, long noncoding RNA; NPC, nasopharyngeal carcinoma; OSCC, oral squamous cell carcinoma; PDA, pancreatic ductal adenocarcinoma; PVDF, polyvinylidene fluoride; siRNA, small interfering RNA; MMP-1, matrix metalloproteinase 1.

## Ethical Statement

Our study was approved by the Ethics Review Board of Zhengzhou university. All experiments were performed following the guidelines and regulations of the Ethics Committee of the Zhengzhou University.

## Author Contributions

(I) Conception and design: Lei Zhang, Xiaojie Zhang, and Shanyong Yi. (II) Administrative support: Lei Zhang, Xiaojie Zhang; (III) Provision of study materials or patients: All authors; (IV) Collection and assembly of data: Xiaojie Zhang and Shanyong Yi; (V) All authors contributed to data analysis, drafting or revising the article, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

## Disclosure

The authors have no conflicts of interest to declare.



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