The novel long intergenic noncoding RNA \textit{UCC} promotes colorectal cancer progression by sponging miR-143

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The human genome contains thousands of long intergenic noncoding RNAs (lincRNAs). However, the functional roles of these transcripts and the mechanisms responsible for their deregulation in colorectal cancer (CRC) remain elusive. A novel lincRNA termed upregulated in CRC (UCC) was found to be highly expressed in human CRC tissues and cell lines. UCC levels correlated with lymph node metastasis, Dukes' stage, and patient outcomes. In SW480 and SW620 cells, knockdown of UCC inhibited proliferation, invasion, and cell cycle progression and induced apoptosis \textit{in vitro}. Xenograft tumors grown from UCC-silenced SW620 cells had smaller mean volumes and formed more slowly than xenograft tumors grown from control cells. Inversely, overexpression of UCC in HCT116 promoted cell growth and invasion \textit{in vitro}. Bioinformatics analysis, dual-luciferase reporter assays, and RNA immunoprecipitation assays showed that miR-143 can interact with UCC, and we found that UCC expression inversely correlates with miR-143 expression in CRC specimens. Moreover, mechanistic investigations showed that UCC may act as an endogenous sponge by competing for miR-143, thereby regulating the targets of this miRNA. Our results suggest that UCC and miR-143 may be promising molecular targets for CRC therapy.

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Worldwide, colorectal cancer (CRC) is the third most commonly diagnosed cancer in males and the second most commonly diagnosed cancer in females, and an estimated 1.4 million CRC cases and 693,900 CRC-related deaths occurred in 2012\textsuperscript{1}. Understanding the molecular mechanisms that govern tumor growth and metastasis is imperative for establishing early detection strategies as well as individualized treatment. Molecular analysis has enabled the development of diagnostic and therapeutic tools facilitating precision medicine that has previously been unavailable\textsuperscript{2,3}. Although previous studies have documented that alterations in many oncogenes and tumor-suppressor genes are associated with CRC, the molecular and genetic bases of colorectal carcinogenesis remain largely unknown\textsuperscript{4}.

The human transcriptome contains not only many protein-coding messenger RNAs (mRNAs) but also a large set of non-protein-coding transcripts that have structural, regulatory, or unknown functions. Recent studies have revealed that the human genome encodes many noncoding RNAs ranging from small regulatory RNAs such as microRNAs and Piwi-associated RNAs to long noncoding RNAs (lncRNAs, longer than 200 nucleotides). The exact number of lncRNAs encoded by the human genome is a matter of debate, but most estimates place the number in the tens of thousands\textsuperscript{5,6}. Long intergenic noncoding RNAs (lincRNAs), a type of lncRNAs, are transcript units that discretely intervening between known protein-coding loci. Although the functions of a few lincRNAs, such as XIST and HOTAIR, have been characterized in some important cellular processes, such as X chromosome inactivation, genomic imprinting, pluripotency maintenance, and transcriptional regulation\textsuperscript{7,8}, the functions of most annotated lincRNAs remain unexplored. However, several studies have implicated lincRNAs in a variety of disease states, including cancers\textsuperscript{9–11}. Recent studies have demonstrated that several lincRNAs are involved in the tumorigenesis and development of CRC\textsuperscript{12,13}. However, an enormous number of lincRNAs remain to be elucidated and characterized.

In this study, differences in the lincRNA expression profiles between CRC and tumor-adjacent nontumor tissues were assessed via lincRNA expression microarray analysis, and we
observed 124 dysregulated lincRNAs and 1583 dysregulated mRNAs in CRC samples. Among the upregulated lncRNAs, we characterized the pathologic relevance of lincRNA ENST00000602992 (which we termed upregulated in colorectal cancer, UCC) in CRC growth and progression. First, we measured the levels of UCC transcripts in CRC tissues and cell lines and confirmed the upregulation of UCC in CRC. The expression of UCC closely correlated with lymph node metastasis, Dukes’ stage and overall survival. Furthermore, we identified a role of UCC in CRC cell growth and metastasis based on in vitro and in vivo functional experiments. Finally, mechanistic investigations revealed that UCC can promote CRC progression by acting as a sponge for miR-143, which is known to have a key role in diverse physiological and pathological processes.14–16 Taken together, these results suggest that UCC and miR-143 may be promising molecular targets for CRC therapy.

Results

The novel lincRNA UCC is upregulated in CRC. To identify lincRNAs that are dysregulated in CRC, we employed a lincRNA microarray analysis covering 27 958 protein-coding transcripts and 7419 annotated and/or known lincRNAs (Agilent). Filtered by P-value and fold change (P < 0.01 and fold change ≥ 2 or fold change < 0.5 for lincRNAs/ mRNAs), a total of 124 lincRNAs and 1583 mRNAs were differentially expressed between four paired CRC and non-tumor tissues. Hierarchical clustering showed systematic variations in transcript expression levels between the paired tumor and non-tumor tissues (Supplementary Figures S1A and B). Gene Ontology (GO) and pathway analyses indicated that most differentially expressed genes were involved in cell proliferation as well as cell death control (Supplementary Figure S2). The top 30 differentially expressed lincRNAs are

![Figure 1](https://example.com/figure1.png)

**Figure 1** UCC expression correlates with CRC progression. (a) UCC expression in CRC tissues from 78 cases based on qRT-PCR analysis. The high value of UCC was defined as fold change ≥ 2 (n = 50), the rest including downregulation or no evident difference in expression in CRC tissues compared with UCC expression in the paired non-tumor tissue, was defined as low values (n = 28). (b) Kaplan–Meier curves of the survival of 78 patients were evaluated using the log-rank test. (c) UCC expression in the lymph node metastasis-negative group (n = 46) and the lymph node metastasis-positive group (n = 32). (d) UCC expression in CRC tissues from different Dukes’ stages: stage A+B (n = 43) and stage C+D (n = 35). Mann–Whitney test was used to analyzed the differences between groups in c and d, data were presented as the median with range. (e) Abundance of UCC in CRC cell lines relative to that in the colonic epithelial cell line CCD841. The expression of UCC was normalized to that in CCD841. The statistical differences between groups were analyzed using independent samples t-test. Error bars represent the mean ± S.D. of triplicate experiments. *P < 0.05. (f) Cellular localization of UCC in CRC cells. GAPDH and U6 serve as a cytoplasmic and nuclear localization marker, respectively.
provided in Supplementary Table S1. The microarray data mentioned in this article are available in the National Center for Biotechnology Information Gene Expression Omnibus under accession number GSE75970.

We primarily focused on upregulated lincRNAs because this set of lincRNAs can be used more readily than downregulated lincRNAs as early diagnostic markers or therapeutic targets. We chose four overexpressed lincRNAs with fold changes in expression >2 based on microarray analysis and validated the expression results in an additional eight pairs of CRC and non-tumor tissues. UCC was the most highly upregulated lincRNA in CRC tissues compared to non-tumor tissues (Supplementary Figures S1C, S3 and Supplementary Table S1). Information from the UCSC Genome Browser shows that UCC is a 747-bp transcript with one exon and localizes in human chromosome 7p15.2 (Supplementary Figure S1D).

**UCC expression correlates with CRC progression.** Then, we examined levels of UCC in CRC tissues obtained from 78 independent patients at Sun Yat-sen Memorial Hospital of Sun Yat-sen University (Guangzhou, China) using quantitative real-time PCR (qRT-PCR). UCC expression in CRC tissues was increased in 50 cases (64%), whereas 28 cases (36%) showed downregulation or no evident difference in expression in CRC tissues compared with UCC expression in the paired non-tumor tissue (Figure 1a). Kaplan–Meier analysis suggested a positive correlation between tumoral UCC expression and a significantly reduced overall survival time among CRC patients with upregulated UCC expression compared to CRC patients without upregulated UCC expression (P<0.05, Figure 1b). High levels of UCC were also found in patients with lymph node metastasis and advanced Dukes’ stage (Figures 1c, d and Table 1). Consistently, UCC was upregulated in CRC cell lines (Figure 1e) and preferentially localized to the nucleus (Figure 1f). Taken together, these data show that UCC is indeed highly expressed in CRC in association with cancer progression.

**Knockdown of UCC inhibits CRC cell growth and invasion.** To evaluate the possible role of UCC in CRC, we transfected SW620 and SW480 cells with three different siRNAs against UCC (designated si-UCC#1-3), all of which efficiently knocked down the endogenous UCC level (Figure 2a). To avoid off-target effects, we chose si-UCC#2 and si-UCC#3 for subsequent experiments. The results of the CellTiter 96 AQueous One Solution Cell Proliferation Assay (MTS) indicated that silencing UCC reduced the viability of SW620 and SW480 cells (Figure 2b). Moreover, colony formation assays and EdU incorporation assays showed that UCC knockdown significantly inhibited the proliferative capacity of SW620 and SW480 cells (Figures 2c and d). These suppressive effects were confirmed by in vivo tumor growth assays. Xenograft tumors grown from UCC-silenced SW620 cells had smaller mean volumes and formed more slowly than xenograft tumors grown from control cells (Figures 2e and f). In addition, positive staining for the proliferation marker Ki-67 was significantly decreased in UCC-silenced cells compared to control cells (Figure 2g). Collectively, these data implied that suppression of UCC expression contributed to CRC cell growth inhibition.

To explore the potential mechanisms by which UCC enhances CRC cell growth in vitro, we analyzed differences in apoptosis and cell cycle distributions among SW620 and SW480 cells between UCC-depleted and control conditions via flow cytometry analysis. The percentage of early apoptotic cells was significantly increased in the si-UCC groups compared to the control groups (Figure 3a). In addition, significant G1/S arrest was observed in UCC-silenced cells (Figure 3b). In addition, UCC knockdown induced apoptosis of xenograft tumor cells in vivo, as determined by TUNEL assays (Figure 3c). These data demonstrated that induction of apoptosis and G1/S cell cycle arrest may contribute to UCC knockdown-mediated growth inhibition.

To further determine whether UCC is associated with the progression of CRC, we analyzed the effect of UCC knockdown on invasion of SW620 and SW480 cells. The results of the wound-healing assay showed that knockdown of UCC

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**Table 1 Characteristics of 78 pancreatic ductal adenocarcinoma patients**

| Characteristics          | Patients frequency (%) | UCC         | P-value |
|-------------------------|------------------------|-------------|---------|
|                         |                        | Low | High |                 |
| Gender                  |                        |     |      |                  |
| Male                    | 78 (50%)               | 28  | 50   | 0.637            |
| Female                  | 39 (50%)               | 13  | 26   |                  |
| Age (year)              |                        |     |      |                  |
|                         |                        | 15  | 24   | 0.271            |
| Lymph node metastasis   |                        |     |      |                  |
| Absent                  | 46 (59%)               | 25  | 21   | <0.001**         |
| Present                 | 32 (41%)               | 3   | 29   |                  |
| Distant metastasis      |                        |     |      |                  |
| Absent                  | 71 (91%)               | 26  | 45   | 0.672            |
| Present                 | 7 (9%)                 | 2   | 5    |                  |
| Dukes’ stage            |                        |     |      |                  |
| A/B                     | 43 (55%)               | 24  | 19   | <0.001**         |
| C/D                     | 35 (45%)               | 4   | 31   |                  |

Chi-square test. **P<0.001.
inhibited cell mobility compared with the control treatment (Figure 3d). In addition, Transwell assays indicated that the invasive capacity of the cells was significantly decreased by \textit{UCC} knockdown (Figure 3e).

\textbf{Overexpression of UCC abrogates CRC proliferation and invasion.} We further assessed the biological function of \textit{UCC} by upregulation its expression using pcDNA3.1-\textit{UCC} plasmid vector, focusing on CRC cell line (HCT116) with moderate \textit{UCC} level. \textit{UCC} expression level was significantly elevated after transfection with pcDNA3.1-\textit{UCC} vector (Supplementary Figure S4A). It was implied that overexpression of \textit{UCC} increased the viability of HCT116 cells by MTS assay (Supplementary Figure S4B). Also, colony formation assays and EdU incorporation assays showed that \textit{UCC} upregulation enhanced the proliferative potential of HCT116.

\begin{figure}[h]
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\caption{UCC knockdown inhibits CRC cell growth in vitro and in vivo. (a) UCC expression levels were suppressed by specific siRNAs in CRC cells. (b) Growth curves of SW620 and SW480 cells after transfection with si-\textit{UCC} or si-NC were determined via MTS assays. (c) The anchorage-independent growth of SW620 and SW480 cells was assessed via colony formation assays. (d) Cell proliferation was evaluated using EdU incorporation assays. Proliferating cells were labeled with EdU. Scale bar: 200 \textmu m. (e) Effects of \textit{UCC} 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 \textit{UCC}-silenced SW620 or negative control cells. The tumor volumes were measured every 5 days after inoculation. (n = 5). (g) Immunohistochemical staining showed that \textit{UCC} knockdown decreased the Ki-67 proliferation index. The data were represented as the mean ± S.D. of three independent experiments \textit{in vitro} or five independent experiments \textit{in vivo}. *P < 0.05, **P < 0.01 by Student’s \textit{t}-test.}
\end{figure}
cell (Supplementary Figures S4C and D). Taken together, these data indicated that overexpression of UCC promoted CRC cell growth.

In addition, we performed flow cytometry to analyze the differences in apoptosis and cell cycle distributions in HCT116 between UCC-overexpressed and control group. As expected, the percentage of early apoptotic cells was significantly decreased in the UCC-overexpressed groups compared to the control (Supplementary Figure S4E), and the proportion of G0/G1 was markedly declined after upregulation of UCC expression (Supplementary Figure S4F). Collectively, these results suggested that overexpression of UCC led to suppression of apoptosis and the proportion of G0/G1.

Furthermore, the wound healing and Transwell assays were conducted to investigate the biological role of UCC in cell invasion. Interestingly, the invasive potential of cells was enhanced in cells transfected with pcDNA3.1-UCC plasmid vector (Supplementary Figures S5A and B).

**Inversely correlated expression of UCC and miR-143 in CRC.** Accumulating evidence has shown that miRNAs are able to interact with lincRNAs and regulate their expression levels.17,18 Thus, potential miRNA candidates targeting UCC were predicted using miRCode and DIANA-LncBase software.19,20 The predicted sites of miR-143 binding to the UCC sequence are illustrated in Figure 4a. The level of UCC was upregulated in CRC tissues based on qRT-PCR,
miR-143 mimics and the UCC decrease in luciferase activities after co-transfecting cells with (referred to as UCC binding site into the psiCHECK dual luciferase reporter vector harboring a site-directed mutation in the miR-143-UCC RNA.

To examine the potential lincRNA acts as a competing endogenous RNA by directly binding to miR-143. Functional studies have indicated that some lincRNAs may act as endogenous sponges by binding to miR-143, thus abolishing the miRNA-induced repression of its target genes. Overexpressing miR-143 significantly inhibited UCC expression, whereas silencing UCC did not affect miR-143 expression (Figure 6c). Inversely, suppressing miR-143 enhanced UCC expression. Interestingly, attenuation of UCC expression was observed after co-transfection miR-143 inhibitor and si-UCC#2 when compared to the NC control group (Figure 6d). These results suggest that UCC is targeted by miR-143.

Previous studies have demonstrated that miRNAs are present in the form of miRNA ribonucleoprotein complexes that contain Ago2, the key component of the RNA-induced silencing complex (RISC).

miR-143 suppresses UCC function. We transfected SW620 and SW480 cells with the miR-143 inhibitor or with si-UCC#2 to study the UCC-mediated effects of miR-143 on cell proliferation and invasion. MTS proliferation assays revealed that the miR-143 inhibitor abrogated the effect of si-UCC#2 in reducing cell viability (Figure 5a). Consistently, the colony formation and EdU incorporation assays confirmed these findings (Figures 5b and c). Transwell invasion assays showed that the miR-143 inhibitor enhanced CRC cell invasion but that si-UCC#2 mediated miR-143 expression significantly decreased CRC cell growth, UCC expression significantly decreased CRC cell growth, induced apoptosis and G1/S arrest, and inhibited invasion, whereas overexpression of this lincRNA had the opposite effects.

Discussion

Although thousands of lincRNAs were identified recently, functional characterization of lincRNAs has just begun. Functional studies have indicated that some lincRNAs participate human cancer pathogenesis by acting as oncogenes or tumor suppressors. In the current study, we showed that the novel lincRNA UCC is frequently over-expressed in advanced CRC tissues and that UCC upregulation correlates with lymph node metastasis and patient outcomes, suggesting a pro-oncogenic activity of UCC. This observation is further supported by the results of loss-of-function and gain-of-function approaches. Suppression of UCC expression significantly decreased CRC cell growth, induced apoptosis and G1/S arrest, and inhibited invasion, whereas overexpression of this lincRNA had the opposite effects.

miRNAs, which are ~22-nucleotide RNAs with sequence complementarity to the 3′-UTR of miRNAs of target genes, play an important role in gene regulation via translational repression and/or mRNA degradation. IncRNAs are generally
more readily accessible to miRNAs because no proteins are translated from the lncRNA sequence. Several lncRNAs, such as HULC, HOTAIR, HOTTIP, GAS5, and HOST2, have been identified as miRNA targets in various cancers, and these findings provide further understanding of lncRNA regulation during tumorigenesis.

Using online software, we identified UCC as a possible target of miR-143. Generally, miR-143 is downregulated in a variety of tumors, including lung cancer, pancreatic cancer, and melanoma. As a putative tumor suppressor, miR-143 participates in CRC development and progression by targeting KRAS, IGF1R, Bcl-2, and HK2. In addition, miR-143 is a plasma miRNA that provides high diagnostic accuracy for early-stage HCC and is a predictive factor for the response to fluoropyrimidine-based chemotherapy in patients with metastatic CRC, indicating the clinical
relevance of miR-143. Although miR-143 has been experimentally shown to target many protein-coding genes, our data show that miR-143 also targets UCC. First, we found a negative correlation between UCC and miR-143 expression in clinical CRC specimens. Overexpressing miR-143 reduced UCC expression in CRC cells. In addition, we provide evidence that miR-143 targets UCC by directly binding to miRNA-binding sites in the UCC sequence.

Essentially, a miRNA is bound by a member of the Argonaute family of proteins and confers sequence specificity to a large protein complex. In the cytoplasm, the RNAi machinery uses Watson–Crick base pairing to target the RISC to a specific mRNA and facilitate its degradation. Alternatively, the Argonaute protein family has been shown to mediate functional RNAs within the nucleus. A related process is well established in the nucleus of S. pombe, where instead of targeting cytoplasmic mRNAs for destruction, a small RNA targets the RNA-induced transcriptional silencing complex to the pericentromeric regions of each chromosome and facilitates the generation of heterochromatin. Ago2 and the RNAi factors Dicer and TRBP were also detected in the human nucleus and can mediate functional RNAi in nucleus. Moreover, mature miRNAs can be transported from the cytoplasm to the nucleus by importin β. That is, there is a primary machinery for Ago2-miRNA-mediated RNA silencing in cell nuclei in humans, which explains why UCC primarily localized to the nucleus can physically interact with Ago2. Similar miRNA regulation mechanisms were observed for

Figure 6  UCC is a direct target of miR-143. (a) Schematic of the wild-type and mutant psiCHECK-UCC constructs. (b) Dual-luciferase assays showed a decrease in reporter activity after co-transfection of psiCHECK-UCC-WT and miR-143 compared with transfection of miR-143 alone, whereas no significant difference in reporter activity was observed between transfection of miR-143 alone and co-transfection of psiCHECK-UCC-MUT and miR-143 in SW620 cells. (c) Left: decreased UCC expression in cells after transfection of miR-143 mimics. Right: miR-143 expression levels in cells after UCC knockdown. (d) Relative UCC level was investigated in SW620 cells after transfection miR-143 inhibitor and/or si-UCC#2. (e) Associations of miR-143 and UCC with Ago2. SW620 and SW480 cell lysates were collected for RIP using an anti-Ago2 antibody. Detection of miR-143 and UCC was performed via qRT-PCR. (f) Effect of transfecting SW620 cells with miR-143 mimics on the expression of miR-143 target genes based on western blot. (g) Effect of transfecting SW620 cells with si-UCC and the miR-143 inhibitor on the expression of miR-143 target genes based on western blot. The bars indicate mean ± S.D. (n = 3). *P < 0.05, **P < 0.01 by Student’s t-test.
other nuclear lncRNAs. For instance, MALAT1 is a well-known nuclear lncRNA that can be directly regulated by several miRNAs.\(^{48,49}\)

In summary, we have identified that a novel lncRNA, termed UCC, is upregulated in human CRC tissues and serves as a negative prognostic factor in CRC patients. Silencing UCC inhibits CRC cell proliferation and invasion and induces apoptosis. UCC functions as an oncogene in CRC, mechanistically acting by upregulating KRAS and other target genes in part through sponging miR-143.\(^{46}\)

Materials and Methods

Clinical specimens and cell culture. The use of human specimens in this study was sanctioned by the local ethics committee at Sun Yat-sen Memorial Hospital of Sun Yat-sen University (Guangzhou, China). None of the patients received preoperative chemotherapy or radiotherapy. The data collected included age, gender, overall survival, and tumor features such as tumor size, clinical stage, tumor invasion depth, tumor location, and occurrence of distant metastasis. Tumor and adjacent non-tumor tissues were snap-frozen in liquid nitrogen immediately after extraction and stored at \(-80^\circ\text{C}\) until total RNA was extracted. The human CRC cell lines SW480, SW620, HCT116, Caco-2, DLD-1, and HT29 and the colonoscopic epithelial cell line CCK841 were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA) and maintained in RPMI-1640 medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin sodium and 100 mg/ml streptomycin sulfate in a humidified atmosphere (37 °C and 5% CO\(_2\)).

RNA isolation and qRT-PCR. Total RNA was extracted and purified from tissues and cell lines with Trizol reagent (Life Technologies, Carlsbad, CA, USA) using a standard procedure. After the quality and quantity of the extracted total RNA were confirmed using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Rockford, IL, USA), complementary DNA (cDNA) was synthesized using a reverse transcription kit (TakaRa, Dalian, China) according to the manufacturer’s protocol. In brief, a master mixture containing 1 μl of CDNA sample, 10 μl of SYBR Green qRT-PCR Master Mix (Qiagen, Hilden, Germany) and 1 μl of primers was prepared on ice. The final volume was then adjusted to 20 μl with RNase-free water. All reactions were performed in a Roche LightCycler system (Roche, Basel, Switzerland). Relative expression was calculated using the 2\(^{-\Delta\Delta CT}\) method. Each PCR amplification was performed in triplicate to verify the results. The primer sequences used for PCR are listed as follows: UCC forward: 5'-GAAAGACCTTTGGAAGCCACTG-3' and reverse: 5'-GAAGACCTACAAAAGCACAATCTC-3'; GAPDH forward: 5'-GACACGCTCAAGGCTGAGAAC-3' and reverse: 5'-TTGGTAAGACCCGATGAG-3'; LINC01558 forward: 5'-AGCTGGAGATGTGCTGCAACG-3' and reverse: 5'-ATGGACGCTCCATCTGTTGC-3'; LIN020558 forward: 5'-GTGTTGAAGAAGGGAGGGAGCT-3' and reverse: 5'-GGGTTGCTCATTCTTGCAATG-3'; HNF1A-AS1 forward: 5'-ACATGACGACCCACTTCTC-3' and reverse: 5'-TTGACTGCTGTCATGCCCTTG-3'.

LncRNA profiling. For lncRNA microarray, RNA purity and integrity was analyzed by Agilent Bioanalyzer 2100 (Agilent, Santa Clara, CA, USA). Qualified total RNA was further purified by RNaseasy mini kit (Qiagen) and RNaseasy-free DNase set (Qiagen). Total RNA was then amplified and labeled by Low Input Quick Amp Labeling Kit, One-Color (Agilent), following the manufacturer’s instructions. Labeled cRNA were purified by RNasey mini kit (Qiagen). Each Slide was hybridized with 600 ng Cy3-labeled cRNA using Gene Expression Hybridization Kit (Agilent) in Hybridization Oven (Agilent), according to the manufacturer’s instructions. After 17 h hybridization, slides were washed in staining dishes (Thermo Scientific) with Gene Expression Wash Buffer Kit (Agilent), following the manufacturer’s instructions. Slides were scanned by Agilent Microarray Scanner (Agilent) with default settings, Dye channel: Green, Scan resolution = 3 μm, 20 bit. Data were extracted with Feature Extraction Software 10.7 (Agilent). Raw data were normalized by Quantile algorithm, Gene Spring Software 11.0 (Agilent).

Subcellular fractionation. The nuclear and cytosolic fractions of SW620 or SW480 cells were separated using the PARIS Kit (Life Technologies) according to the manufacturer’s instructions. RNA was extracted from both fractions. Then, qRT-PCR was performed to assess the expression ratios of specific RNA molecules between the nuclear and cytoplasmic fractions. GAPDH served as the cytosolic control, and U6 served as the nuclear control.

Cell transfection. The sequence of short-hairpin RNA (shRNA) directed against UCC (5'-GGAGACCCTGTGAAGATTCAAGATAGAATTCTTCAAGGGC TTCC-3') was ligated into the pLKO.1-Puro vector (TakaRa). Lentivirus was packaged into HEK 293 cells using Lipofectamine 2000 (Life Technologies) and collected from the supernatant in accordance with the manufacturer’s instructions. Lentiviral particles were used to infect SW620 cells. The synthesized and purified UCC gene fragment was inserted into the expression vector pcDNA3.1 (+) (Invitrogen, Carlsbad, CA, USA) for overexpression this lncRNA in HCT116 cell line. Stable cell lines were established via puromycin selection and then used for subsequent in vitro and in vivo experiments. For transient transfection assays, miR-143 mimics, a miR-143 inhibitor, small interfering RNA (siRNA) duplexes (si-UCCL1, si-UCCL2 and si-UCCL3), and negative control (NC) RNA duplexes for miRNA mimics, the miR-143 inhibitor or the siRNAs were synthesized (Ribobio, Guangzhou, China). The siRNA sequence for si-UCCL were 5'-GGAGAGACUGCCUCUCUGAU-3', si-UCCL2, 5'-GGAGCCUCUUGGUAAGAA-3' and si-UCCL3, 5'-GCUUGAUGULUGAACUUA-3'. These oligonucleotides were transfected into SW620 and SW480 cells using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer’s protocol.

Cell growth assay. For cell proliferation assay, the MTS assay from Promega (Madison, WI, USA) (CellTiter 96 AQueous One Solution Cell Proliferation Assay) was used following manufacturer’s instruction. Briefly, cells in a 96-well plate were incubated in a humidified 5% CO\(_2\) chamber after transfection with indicated siRNAs or vector, followed by addition of 20 μl CellTiter 96 AQueous One Solution and 1–4 h incubation in humidified 5% CO\(_2\) chamber. The absorbance at 492 nm was recorded. The assay was performed using six replicates. For colony formation assay, the cells transfected with indicated oligonucleotides for 24 h or the stable HCT116 cells, and were seeded in six-well plates. After 14 day incubation, the number of clones were counted and analyzed.

Wound healing assay. Cells were incubated with normal cell growth medium in six-well plates. Once cultures reached 85% confluency, the cell layer was scratched with a 10 μl sterile pipette tip and washed with culture medium, then exchanged with medium containing 1% FBS cultured for 48 h. To prevent cell proliferation, which could confound the analysis of cell migration into the wound, the cells were preincubated with mitomycin C (10 μg/ml) for 1 h at 37 °C. At different time points (0, 48 h), images of the plates were acquired using a microscope.

Transwell assays. Cell invasion assays were carried out using 24-well Transwell chambers with 8 μm pore size polycarbonate membrane (Costar, Corning, NY, USA). Briefly, the lower chamber was filled with 600 μl RPMI 1640 containing 20% FBS. Cells were trypsinized, counted and re-suspended in serum-free RPMI 1640. Cells (2 × 10\(^5\)) in 200 μl serum-free RPMI were added to the upper chamber. The cells were allowed to invade for 24 h at 37 °C before fixing. The non-invaded cells were removed from the upper surface of the membrane by scraping with a cotton swab. Cells on the bottom surface of the membrane were fixed with 95% ethanol and then stained with 1% crystal violet in methanol/PBS. Invasion was assessed by counting the number of stained cell nuclei from five randomly fields per filter in each group at ×200 magnification using a Zeiss (Melville, NY, USA) microscope system.

Cell cycle distribution and apoptosis analysis. To detect the effect of downregulation of UCC on cell cycle distribution and apoptosis, flow cytometry assay was performed. For cell cycle distribution analysis, SW620 and SW480 cells collected at 72 h after transfection with si-UCCL, together with stable transfectected HCT116 cells, were trypsinized and fixed with ice-cold 70% ethanol for 1 h at 4 °C. The fixed cells were stained with 50% 500 ml Propidium iodide (PI) (BD Pharmingen, San Diego, CA, USA) and 50 mg/ml RNase and then analyzed using a flow cytometer (BD Pharmingen). For apoptosis analysis, si-UCCL transfected SW620 and SW480 cells harvested at 72 h after transfection, as well as stable transfected HCT116 cells, were stained with FITC-Annexin V and PI then analyzed using a flow cytometer. Triplicate experiments with triplicate samples were performed.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and western blot assays. Total cell lysate was prepared with a buffer containing...
immunocytochemistry and immunohistochemistry analyses. At 48 h after transfection, the 5-ethyl-2′-deoxyuridine (EdU) incorporation assay was performed using the Cell-Light EdU Apollo567 In Vitro Imaging Kit (Ribobio) according to the manufacturer’s instructions. The xenograft tumor tissues were harvested in 4% formaldehyde buffered with phosphate-buffered saline, embedded in paraffin and then sectioned. An antibody against Ki-67 (#9448, Cell Signaling Technology, Danvers, MA, USA) was applied. The slides were then counterstained with hematoxylin, dehydrated and mounted. Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) (Roche) assays were carried out according to the manufacturer’s protocol.

The subcutaneous xenotransplantation model. Animal experiments were approved by the Sun Yat-sen University Institutional Animal Care and Use Committee and were conducted following the animal treatment policies of Sun Yat-sen University in accordance with the National Institutes of Health guidelines. Animals were housed under specific pathogen-free conditions and provided with food and water ad libitum. All procedures involving animals were performed according to institutional guidelines. Animals were randomly assigned to different groups and incubated at 37°C with 5% CO2 for 24 h. According to previous studies, the experimental group was set up to be 10 mice per group. The animal experiment protocol was approved by the Institutional Animal Care and Use Committee of Sun Yat-sen University, and the experiments were conducted following the Guidelines for the Care and Use of Laboratory Animals of Sun Yat-sen University. All efforts were made to minimize animal discomfort and to reduce the number of animals used in the study. After 4 weeks, the tumor volume was calculated according to the following equation: $V = length \times width^2 \times 0.5$.

Dual-luciferase reporter assay. The human UCC 3′-UTR luciferase reporter construct (UCC-WT) was generated by cloning UCC mRNA 3′-UTR sequence into downstream of psiCHECK luciferase reporter vector (Promega). The miR-143 target site-mutation UCC 3′-UTR luciferase reporter(UCC-MUT) construct was generated by employing direct-site mutagenesis using mutation primers that mutate the miR-143-binding site. Nucleotide sequence of the constructs were confirmed by DNA sequencing. SW620 cells were seeded at 3 × 10⁴ cells per well in 24-well plates and allowed to settle overnight. Next day, cells were co-transfected with wild-type or mutant reporter plasmids and miR-143 mimics. Twenty-four hours after co-transfection, the relative luciferase activity was measured into 24-well plates and analyzed via the log-rank test. A P-value of 0.05 or less was considered significant.

Conflict of Interest
The authors declare no conflict of interest.

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