ORIGINAL RESEARCH

ENST00000430471 Promotes Development and Metastasis of Colorectal Cancer by Regulating the Expression of YBX-1

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Background Information: Colorectal cancer (CRC) is a common malignant tumor of the digestive system. Long non-coding (lnc) RNA *ENST00000430471* has been reported to be involved in CRC development and metastasis because of its cancer-promoting ability. However, the detailed molecular mechanisms of *ENST00000430471* in CRC remain largely unknown.

Methods: The cell proliferation assay and Xenograft experiment were performed to examine the proliferation rate of the tumor cells. Invasiveness and migration capability of the cells were evaluated using the invasion and wound healing assays, respectively. In addition, the RNA pull-down assay and subsequent mass spectrometry techniques were performed to identify the proteins interacting with *ENST00000430471*.

Results: Herein, small specific inhibiting (si) RNA-si0471#2 was used to silence *ENST00000430471* in HCT116 and SW620 cell lines. This led to a significant reduction in cell proliferation, migration, and invasion. The RNA pull-down assay and mass spectrometry further revealed that *ENST00000430471* interacted with several proteins such as the Y-box-binding protein 1 (YBX-1). On one hand, silencing of *ENST00000430471* decreased the mRNA and protein expression levels of YBX-1. On the other hand, overexpression of *YBX1* partially attenuated the suppression of cell proliferation, invasion, and migration induced by *ENST00000430471* silencing.

Conclusion: Silencing of *ENST00000430471* inhibits proliferation, migration, and invasion of CRC cells by regulating YBX-1 expression. These results provide baseline information that is essential in the identification of effective therapeutic targets for CRC therapy.

Keywords: lncRNA, *ENST00000430471*, progress, colorectal cancer, YBX-1

Introduction

Colorectal cancer (CRC) is a common malignant neoplasm characterized by high morbidity and mortality rates.[1] Although there has been progress in CRC treatment, more than 190,000 patients succumb to CRC every year in China because of the large population base.[2] Metastasis is one of the primary causes of CRC treatment failures that leads to death.[3] Lymphatic metastasis is the most common cause of CRC metastasis. It can significantly reduce the 5-year survival rate of CRC patients.[4] Cognizant to this, it is important to explore the underlying molecular mechanisms involved in the development of CRC.

Long non-coding (lnc) RNAs (length, >200 nucleotides) are a group of functional RNA molecules with limited protein-coding potential.[5] They can regulate multiple

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biological processes such as proliferation, differentiation, apoptosis, invasion and metastasis.[6] Previous studies have revealed that several lncRNAs such as *CCAT1, CCAT2, HOTAIR* and *H19* play significant roles in the development of CRC. However, the mechanism of action of lncRNAs in lymphatic metastasis is not yet clear.[7–10]

In a recent study, *ENST00000430471* was found to be significantly upregulated in the metastatic lymph node tissues than in paired tumor tissues and normal tissues. Moreover, overexpression of *ENST00000430471* promoted CRC cell proliferation and metastasis.[11] Herein, silencing of *ENST00000430471* inhibited cell proliferation, migration, and invasion in HCT116 and SW620 cells. RNA pull-down assay further revealed that *ENST00000430471* interacted with the Y-box-binding protein 1 (YBX-1). Overexpression of *YBX-1* partially restored the tumor inhibitory effects induced by *ENST00000430471* silencing.

Materials and Methods
Cell Culture
Human HCT116 and SW620 CRC cell lines were sourced from the Cell Bank of the Chinese Academy of Medical Sciences. They were then cultured in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum, penicillin, and streptomycin (Thermo Fisher Scientific, Inc.) and incubated in a humidified incubator with 5% CO_2 at 37°C.

RNA Extraction and Reverse Transcription-Quantitative PCR (RT-qPCR) Analysis
Total RNA was extracted from cultured cells using the TRIzol® reagent (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. It was then reverse transcribed into cDNA using the PrimeScript™RT reagent kit (Takara Biotechnology Co., Ltd.) and used for qPCR analysis. qPCR was performed using SYBR Green fluorescent DNA binding dye (Takara Biotechnology Co., Ltd.) according to the manufacturer's instructions. qPCR results were normalized to GAPDH. Primers used are displayed in Table 1.

Western Blot Analysis
Protein lysates were extracted from the cells during their logarithmic growth phase using the radioimmunoprecipitation assay (RIPA) lysis buffer. They were then separated using 10% Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and the resultant bands transferred onto polyvinylidene difluoride membranes. The membranes were then blocked with 5% bovine serum albumin for 1 hour at 37°C and then incubated with primary antibodies at 4°C for 12 hours. The membranes were then washed with TBST to remove the excess antibodies and subsequently incubated with the secondary antibody, and the proteins were visualized using enhanced chemiluminescence. The following primary antibodies were used: anti-YBX1 (Abcam), and anti-β-ACTIN (Abcam).

Cell Transfection
Small interfering (si) RNAs against *ENST00000430471* (si0471#1, 2 and 3), negative control (NC) siRNA (siNC), short hairpin (sh) RNA against *ENST00000430471* (sh0471) and control shRNA (shNC) were purchased from Shanghai GenePharma Co. Limited. Similarly, YBX-1 expression vector (pcDNA-YBX-1) was constructed by Shanghai GenePharma Co. Limited. Transfection of the aforementioned siRNA and pcDNA was done using Lipofectamine™ 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Two mL serum-free medium and an appropriate amount of lentivirus containing either sh0471 or shNC were added to each well of a 6-well plate containing the cells. The medium was replaced with fresh medium after 4–6 hours. The cells were transfected for 24–48 hours and then collected for determination.

Cell Proliferation Assay
The proliferation rate of HCT116 and SW620 cells was assessed using the Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Inc.) assay reagent in accordance with the manufacturer's instructions. Cell proliferation rate was measured at 12, 24, 48, 60 and 72 hours after transfection. The absorbance of cells in each well was

Table 1 Primers Used in Reverse Transcription-Quantitative PCR (RT-qPCR) Analysis

Gene of Interest	Forward Primer (5′ to 3′)	Reverse Primer (5′ to 3′)
ENST00000430471	TCTGGAGGGAGCAAGGAG	TGGAAGGCGAGAGAAATC
GAPDH	AGAAGGCTGGGGCTCATTTG	AGGGGCCATCCACAGTCTTC
YBX-1	GGGGACAAGAAGGTCATCGC	CGAAGGTACTTCCTGGGGTTA

measured at a wavelength of 450 nm using a microplate reader. The assay was done in triplicate.

Xenograft Experiment

Four weeks old BALB/c nude mice were used for these experiments. They were purchased from the Model Animal Research Center and maintained under special pathogen-free conditions prior to the experiment. Their beddings and food were changed every 2 days. Moreover, their health condition was evaluated by keeping track of their appetite, reaction and breathing. Two groups of mice each comprised of 10 randomly selected mice were subcutaneously injected with 150 μL of *ENST00000430471* knockdown and control HCT116 cells suspensions in the right flanks. The suspensions were at a concentration of $2x10^6$cells/mL. Tumor sizes were measured using a slide caliper after every 3 days. Their volumes were calculated using the following formula: volume (mm^3) = 1/2 x length x $width^2$. Ten mice were sacrificed by cervical dislocation after 21 days. The xenografts were subsequently removed after cardiorespiratory arrest.

Cell Invasion Assay

The cell invasion assay was performed in 6.5 mm Transwell chambers (8-μm pore size; EMD Millipore; Merck KGaA). 2 $x10^5$ cells were seeded onto the upper chamber coated with Matrigel. The upper chamber contained 200 μL of serum-free medium, while the lower one contained 800 μL of medium supplemented with 20% FBS. The cells were incubated for 24 hours followed by fixing and staining of those on the filter surface. The stained cells were then visualized using a phase-contrast inverted microscope at a magnification of X400. The assay was done in triplicate.

Wound Healing Assay

The wound-healing assay was performed to detect the migration ability of the cells. Cells in the logarithmic growth phase were seeded in 24-well plates and cultured for 24 hours. A 200 μL micropipette tip was then used to create a wound within the cell monolayer. The wound was then visualized and photographed using a Leica DMI3000B microscope after 48 hours. The Image J software was then used to analyze the micrographs of the wounded area. The assay was done in triplicate.

RNA Pull-Down Assay and Mass Spectrometry (MS)

LncRNA *ENST00000430471* sequence was cloned to the Nhe I/Sal I site of pCI-neo. The recombinant plasmid was linearized by Sal I to obtain the sense RNA strand. The antisense RNA strand was obtained by linearizing the recombinant plasmid with Nhe I. The biotin-labeled sense RNA (antisense RNA) of *ENST00000430471* was transcribed in vitro using Biotin RNA Labeling Mix (Roche Molecular Diagnostics) and *T7 (T3)* RNA polymerase (New England BioLabs). It was then purified using the *EZNA* RNA Probe Purification Kit (Omega Bio-Tek, Inc.). The protein lysate from $1x10^7$ HCT116 cells was then incubated with 2 μg of biotinylated RNA and mixed with T1 beads (Invitrogen; Thermo Fisher Scientific, Inc.). The RNA-bound proteins were retrieved from the beads after washing five times in RIPA buffer. The proteins were then heated in SDS buffer and separated using SDS-PAGE. The resultant bands were then silver stained. The bands of interest were then excised and detected using MS analysis.

Statistical Analysis

The GraphPad Prism v6.0 was used for all statistical analysis. Data were presented as mean ± standard deviation from at least three independent experiments. Differences between the two groups were analyzed using the Student's *t*-test. One-way ANOVA was used to compare the differences between more than two groups. P values less than 0.05 indicated that there were significant differences between the two groups.

Results

Silencing of *ENST00000430471* Inhibits Cell Proliferation Both in vivo and in vitro

Specific siRNAs (si0471#1, 2 and 3) and shRNA (sh0471) were transfected into HCT116 and SW620 cells to silence the expression of *ENST00000430471*. This was done to determine whether *ENST00000430471* played an important role in CRC proliferation. The transfection efficiencies were confirmed using RT-qPCR (Figure 1A and C; P<0.05). Si0471#2 was found to have the optimal interference efficiency. As such, cells transfected with si0471#2 were used for subsequent experiments. Silencing of *ENST00000430471* suppressed

cell proliferation in both HCT116 and SW620 cells (Figure 1B; P<0.05). Tumor volumes in mice injected with HCT116-sh0471 cells were significantly smaller compared to those in mice injected with HCT116-shNC cells (Figure 1D). These results strongly suggested that silencing of *ENST00000430471* inhibits CRC cell proliferation both in vitro and in vivo.
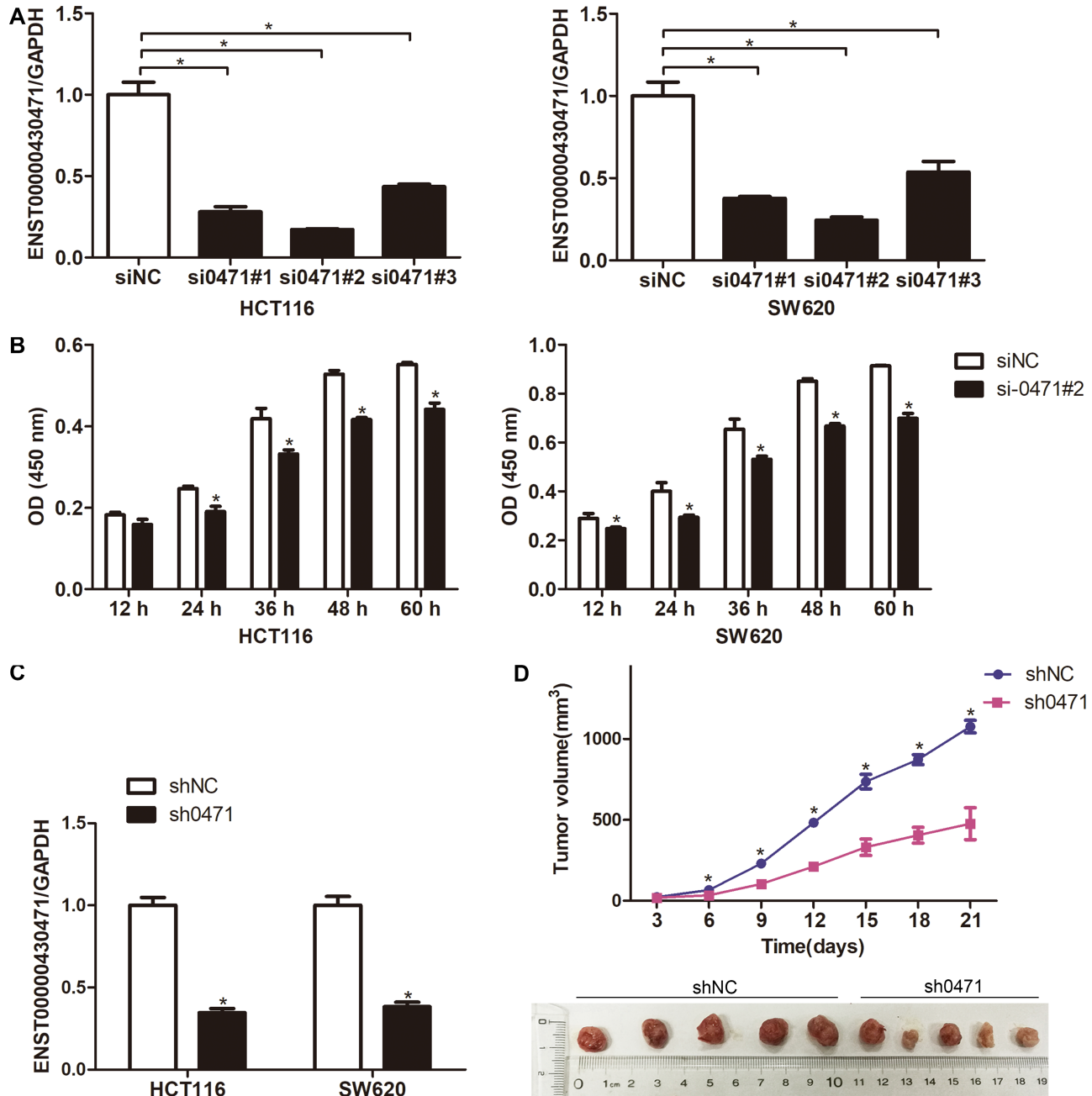

Figure 1 *ENST00000430471* knockdown inhibits HCT116 and SW620 cell proliferation. (**A** and **C**) The mRNA expression level of *ENST00000430471* was determined using reverse transcription-quantitative PCR following transfection of siNC, si0471, shNC and sh0471 into HCT116 and SW620. (**B**) Proliferation of HCT116 and SW620 cells was evaluated using Cell Counting Kit-8 assay after transfection with si0471#2 or siNC. (**D**) Subcutaneous xenografts of HCT-116 cells infected with sh0471 lentivirus or shNC (n = 5). Images of tumors from nude mice are presented (below), and the tumor volumes were measured at the indicated time points (above). Data are presented as mean ± SD. *P<0.05.
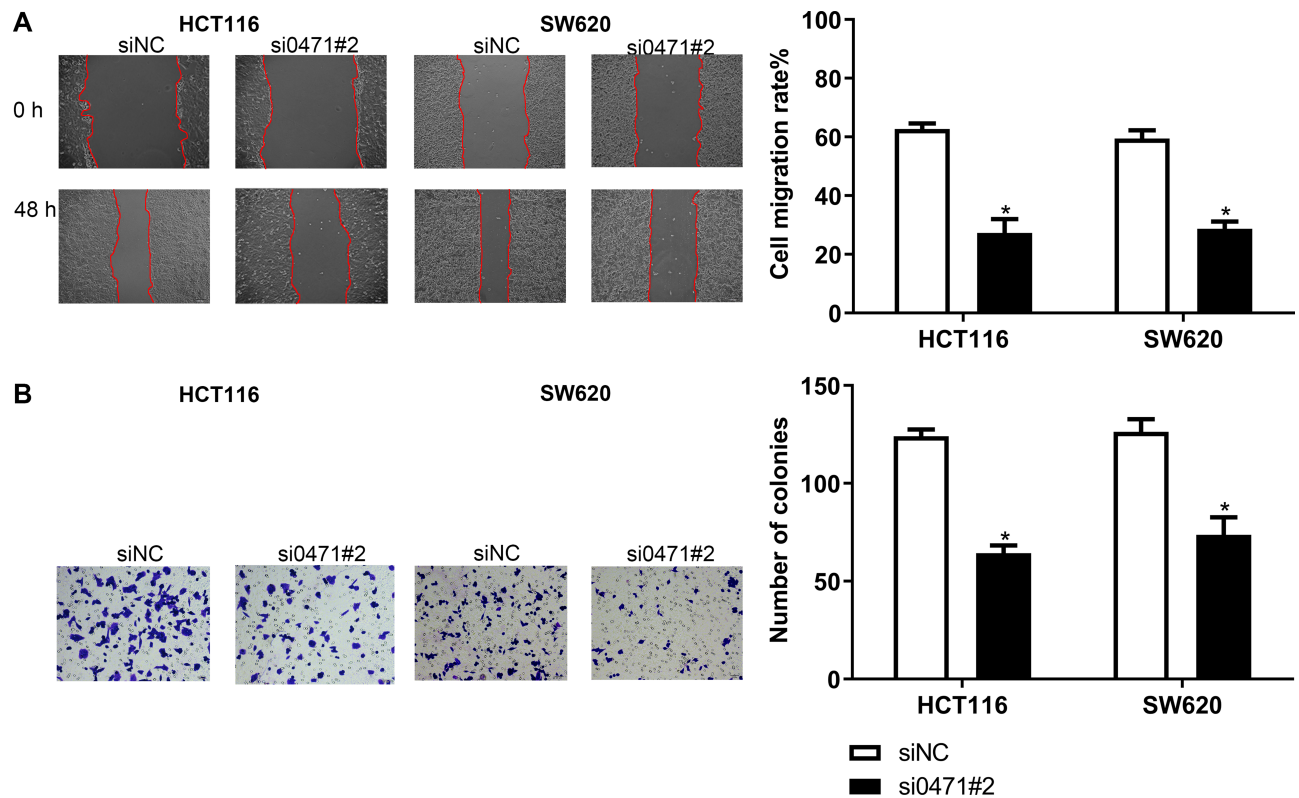**Abbreviations:** NC, negative control; si, small inhibiting; sh, short hairpin.

A

B

Figure 2 *ENST00000430471* knockdown inhibits HCT116 and SW620 cell migration and invasion. (**A** and **B**) Cell migration and invasion of HCT116 and SW480 cells after transfection with si0471#2 or siNC were evaluated using the wound healing assay and invasion assays, respectively. Data are presented as mean ± SD. *P<0.05.
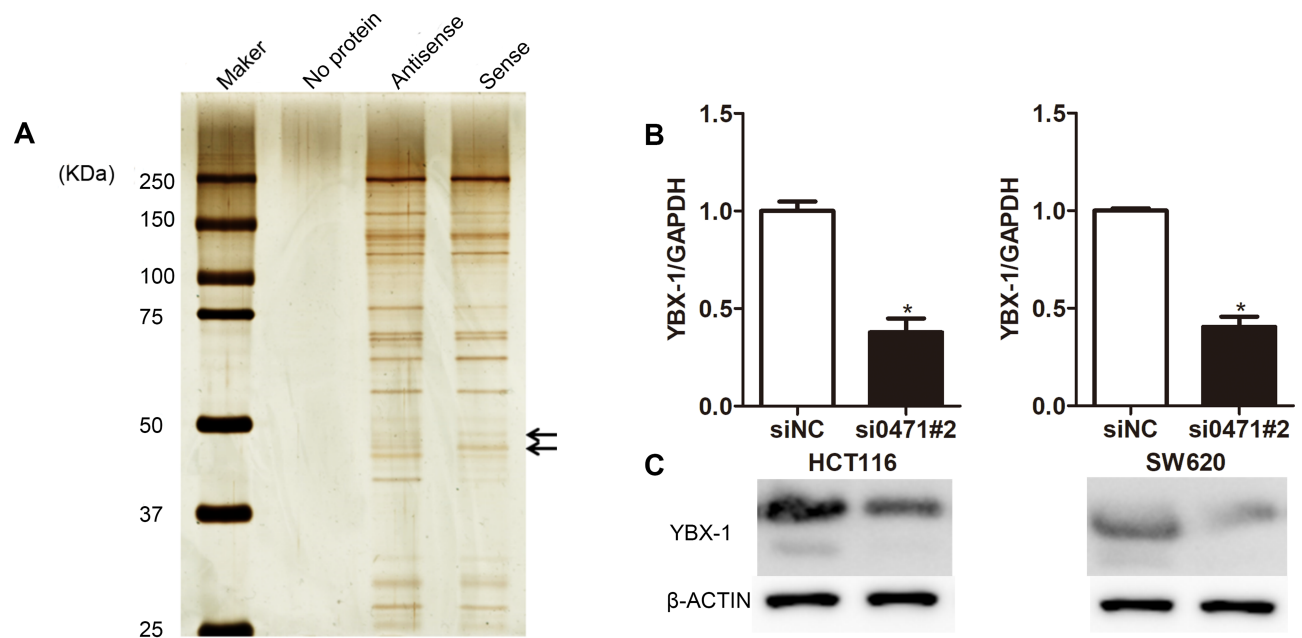**Abbreviations:** NC, negative control; si, small inhibiting; sh, short hairpin.

Figure 3 *ENST00000430471* interacts with YBX1. (**A**) *ENST00000430471* interacting proteins were separated using SDS-PAGE. The regions indicated with arrows were different protein bands between the antisense group and sense group. They were submitted for mass spectrometry analysis. (**B**) Relative YBX1 mRNA expression level was assessed using reverse transcription-quantitative PCR analysis. (**C**) Relative YBX1 protein expression level was assessed using Western blot analysis. Data are presented as mean ± SD. *P<0.05.
Abbreviation: YBX-1, Y-box-binding protein 1.

Silencing of *ENST00000430471* Suppresses CRC Cells' Migration and Invasion

The wound healing assays and Matrigel assays were performed to determine the role of *ENST00000430471* in migration and invasion of CRC cells, respectively. Silencing of *ENST00000430471* significantly suppressed the migration and invasive ability of both HCT116 and SW620 cells (Figure 2A and B; P<0.05).

ENST00000430471 Interacts with YBX-1

Several studies have postulated that lncRNAs function by binding to a partner protein. Cognizant to this, the RNA pull-down assay with biotinylated *ENST00000430471* was

performed to identify the proteins interacting with *ENST00000430471*. This was followed by MS analysis (Figure 3A). Proteins detected included YBX-1, EEF1A1P5, PA2G4 and CCT2 (Table S1). Among the primary proteins, YBX-1 had the highest score from the MS analysis. Moreover, it plays an important role in CRC development. It was therefore selected for subsequent experiments. The subsequent experiments revealed that silencing of *ENST00000430471* significantly reduced the expression levels of both YBX-1 mRNA and proteins (Figure 3B and C; P<0.05). Previous studies postulate that lncRNAs either interact with YBX-1 or stabilize the YBX1 protein thereby enabling it to perform its biological functions.[12,13] Results herein suggested that there was
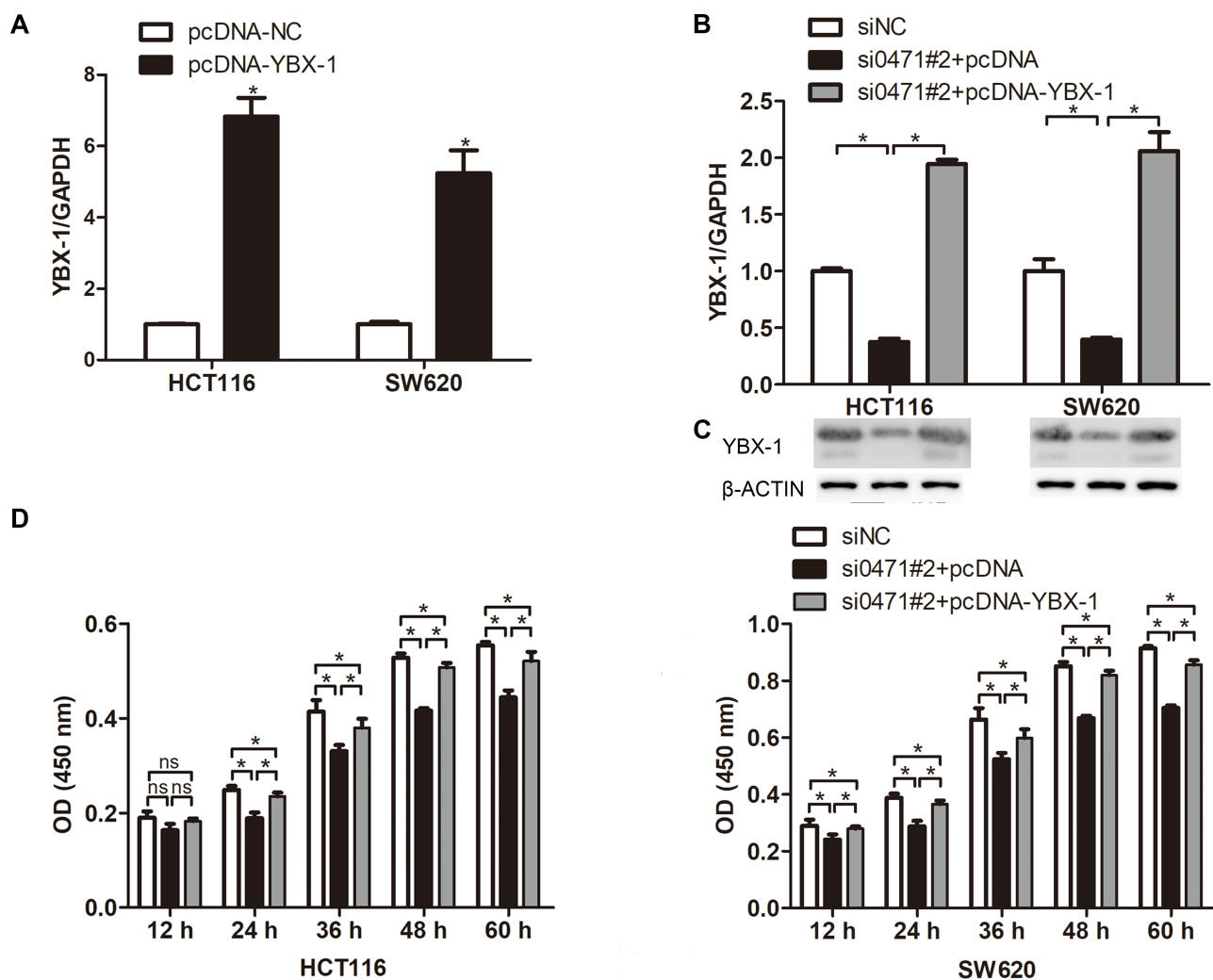
Figure 4 YBX1 overexpression partly reverses the effects of *ENST00000430471* knockdown in colorectal cancer cells. (**A**) The mRNA expression level of YBX-1 was determined using reverse transcription-quantitative PCR following transfection of pcDNA-YBX-1 and pcDNA into HCT116 and SW620. (**B**) YBX-1 mRNA expression level, (**C**) YBX-1 protein expression level, and (**D**) Proliferation ability were determined in HCT116 and SW480 cells co-transfected with si0471#2, along with pcDNA-YBX-1 or pcDNA, using transcription-quantitative PCR, Western blot, Cell Counting Kit-8, respectively. Data are presented as mean ± SD. *P<0.05.
Abbreviations: si, small inhibiting; YBX-1, Y-box-binding protein 1; ns, no significance.

a pathway that regulated YBX-1 protein transcription when it interacted with *ENST00000430471*.

Overexpression of YBX-1 Partially Reversed the Tumor Suppression Effects Caused by *ENST00000430471* Silencing

Rescue experiments were performed to validate the role of YBX-1 in *ENST00000430471*-mediated CRC progression. In the experimental setup, HCT116 and SW620 cells were infected with plasmids containing the YBX-1 expression vector (Figure 4A). The reduction in YBX-1 mRNA and protein expression was restored by pcDNA-YBX-1 in *ENST00000430471* silenced HCT116-si0471#2 and SW620-si0471#2 cells (Figure 4B and C). The CCK-8 assay further revealed that the decrease in cell proliferation caused by *ENST00000430471* silencing was partially reversed by overexpression of YBX-1 (Figure 4D; P<0.05). Furthermore, overexpression of YBX-1 partially reversed the migration and invasive inhibitory effect induced *ENST00000430471* silencing (Figure 5A and B; P<0.05). These results strongly suggested that *ENST00000430471* mediated CRC
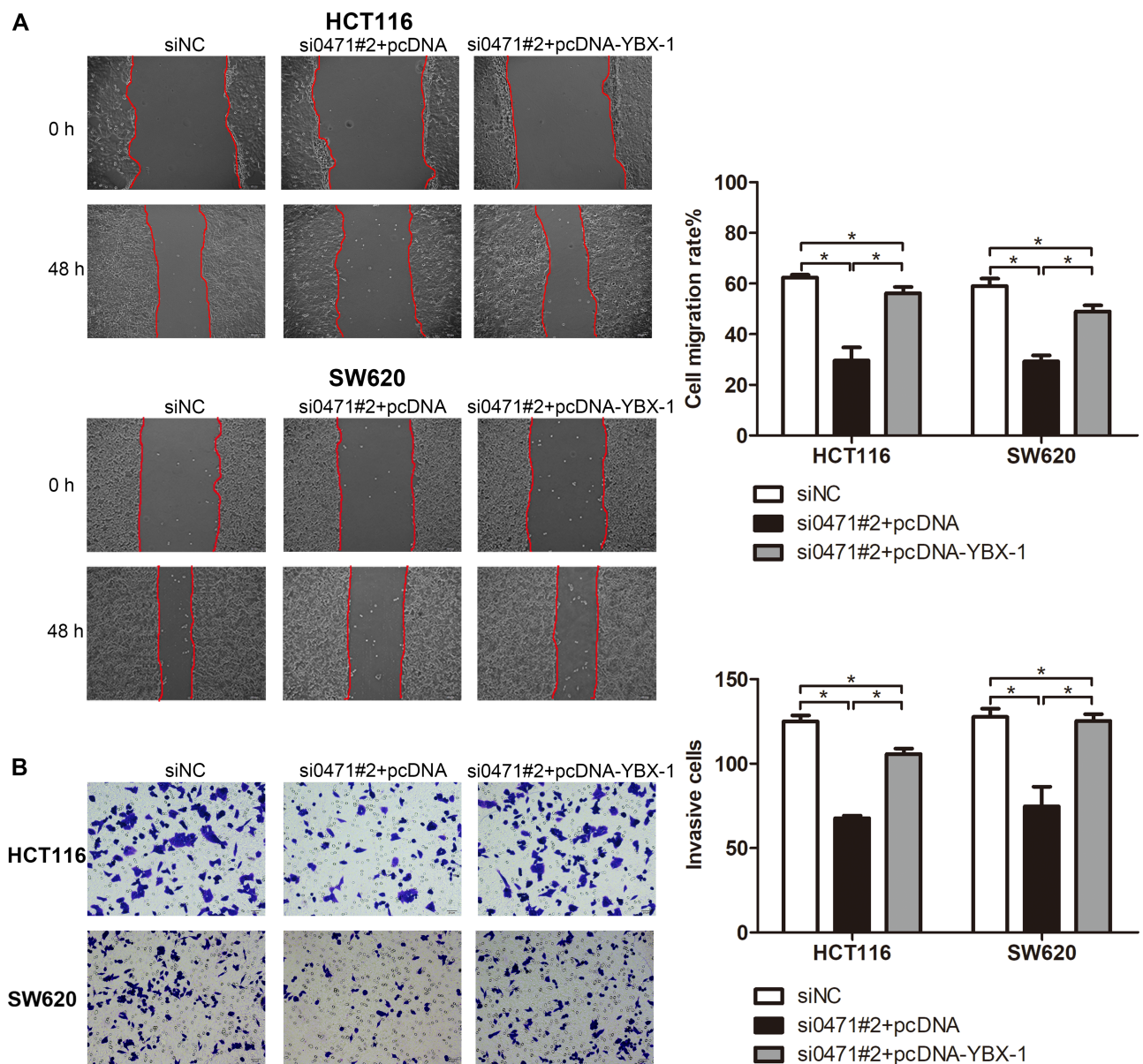

Figure 5 Overexpression of YBX1 partially reverses the inhibitory effect to migration and invasive ability of CRC caused by *ENST00000430471* silencing. (**A**) Migration, and (**B**) Invasion abilities were determined in HCT116 and SW480 cells co-transfected with si0471#2, along with pcDNA-YBX-1 or pcDNA, using wound healing and Matrigel assays, respectively. Data are presented as mean ± SD.
Abbreviations: si, small inhibiting; YBX-1, Y-box-binding protein 1.

progression partially through regulation of YBX-1 protein expression.

Discussion

Research-based evidences have revealed that dysregulation of lncRNAs promotes the development and metastasis of CRC cells. For example, high MALAT1 expression enhances CRC growth and metastasis.[14] In the same line, MIR503HG is downregulated in CRC and contributes to CRC migration and invasion.[15] However, further studies are needed to identify additional functions of lncRNAs in CRC development.

Previous studies postulate that *ENST00000430471* is highly expressed in CRC and further promotes its development.[11] Herein, silencing of *ENST00000430471* suppressed proliferation, migration and invasion of CRC cells in vitro. HCT116 was selected for xenograft experiment because its tumor volume was larger than that of SW620 in vivo.[16] Silencing of *ENST00000430471* reduced the tumor volume in vivo. These results indicated that *ENST00000430471* acted as an oncogene in CRC. Cognizant to this, further studies are required to explore the underlying mechanism of *ENST00000430471*.

LncRNAs regulate tumor biological functions through multiple pathways.[17] They exert their biological function by binding to a partner protein thus affecting the expression of target genes.[18] For instance, LncRNA OCC-1 regulates the levels of many mRNAs at the post-transcriptional level by binding to and destabilizing HuR.[19] In the same line, LncRNA SNHG1 regulates CRC cell growth by interacting with polycomb repressive complex 2 thereby modulating the histone methylation promoter of Kruppel like factor 2 and cyclin-dependent kinase inhibitor 2B.[20] Herein, the RNA pull-down assay and subsequent MS analytical technique revealed that *ENST00000430471* interacted with YBX-1, EEF1A1P5, PA2G4 and CCT2 proteins. YBX-1 had the highest score and was thus selected for subsequent experiments.

YBX-1 is an oncogene. High expression of YBX-1 is associated with poor prognosis and local recurrence in CRC.[21,22] Overexpression of YBX-1 promotes proliferation, migration and induces oxaliplatin resistance in CRC cells.[23,24] Silencing of YBX-1 inhibits the malignant progression of CRC cells by reversing epithelial-mesenchymal transition.[25] Several studies have postulated that lncRNAs such as HULC, MIR22HG, HOXC-AS3, GAS5, and TP53TG1 can bind to YBX-1. The lncRNAs regulate phosphorylation, protein turnover, stability and nuclear localization of YBX-1.[12,26-29] Herein,

ENST00000430471 silencing reduced YBX-1 expression both at the mRNA and protein level. This suggested that another pathway regulates YBX-1 mRNA expression while it interacts with *ENST00000430471*. Moreover, overexpression of YBX-1 partially attenuated the suppression of cell proliferation, invasion and migration induced by *ENST00000430471* silencing.

Conclusion

Silencing of *ENST00000430471* inhibits proliferation, migration, and invasion of CRC cells by regulating YBX-1 expression. These results provide baseline information that is essential in the identification of effective therapeutic targets for CRC therapy.

Ethics Statement

All animal-based experiments were approved by the Animal Care and Use Committee of Xishan People's Hospital (Jiangsu, China). They also followed the ARRIVE guidelines.

Disclosure

The authors report no conflicts of interest for this work.

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