LincHOXA10 Facilitates Colorectal Cancer Development By Regulating HOXA10-Mediated Epithelial-Mesenchymal Transtion

Huifang Zhu (zhf8382@163.com)

1Department of Pathology, Xinxiang Medical University, Xinxiang City 453000, Henan, People's Republic of China. 2Department of Pathology, the Third Affiliated Hospital Of Xinxiang Medical University, Xinxiang City 453000, Henan, People's Republic of China https://orcid.org/0000-0003-2374-0346

Yongzhen Li

1Department of Pathology, Xinxiang Medical University, Xinxiang City 453000, Henan, People's Republic of China. 2Department of Pathology, the Third Affiliated Hospital Of Xinxiang Medical University, Xinxiang City 453000, Henan, People's Republic of China

Yinghui Zhang

1Department of Pathology, Xinxiang Medical University, Xinxiang City 453000, Henan, People's Republic of China. 2Department of Pathology, the Third Affiliated Hospital Of Xinxiang Medical University, Xinxiang City 453000, Henan, People's Republic of China

Zheying Zhang

1Department of Pathology, Xinxiang Medical University, Xinxiang City 453000, Henan, People's Republic of China. 2Department of Pathology, the Third Affiliated Hospital Of Xinxiang Medical University, Xinxiang City 453000, Henan, People's Republic of China

Yongxia Wang

1Department of Pathology, Xinxiang Medical University, Xinxiang City 453000, Henan, People's Republic of China. 2Department of Pathology, the Third Affiliated Hospital Of Xinxiang Medical University, Xinxiang City 453000, Henan, People's Republic of China

Guoyang He

1Department of Pathology, Xinxiang Medical University, Xinxiang City 453000, Henan, People's Republic of China. 2Department of Pathology, the Third Affiliated Hospital Of Xinxiang Medical University, Xinxiang City 453000, Henan, People's Republic of China

Na Li

1Department of Pathology, Xinxiang Medical University, Xinxiang City 453000, Henan, People's Republic of China. 2Department of Pathology, the Third Affiliated Hospital Of Xinxiang Medical University, Xinxiang City 453000, Henan, People's Republic of China

Haijun Wang

1Department of Pathology, Xinxiang Medical University, Xinxiang City 453000, Henan, People's Republic of China. 2Department of Pathology, the Third Affiliated Hospital Of Xinxiang Medical University, Xinxiang

City 453000, Henan, People's Republic of China

Jiateng Zhong

1Department of Pathology, Xinxiang Medical University, Xinxiang City 453000, Henan, People's Republic of China. 2Department of Pathology, the Third Affiliated Hospital Of Xinxiang Medical University, Xinxiang City 453000, Henan, People's Republic of China

Xinlai Qian

1Department of Pathology, Xinxiang Medical University, Xinxiang City 453000, Henan, People's Republic of China. 2Department of Pathology, the Third Affiliated Hospital Of Xinxiang Medical University, Xinxiang City 453000, Henan, People's Republic of China

Yuhan Hu

1Department of Pathology, Xinxiang Medical University, Xinxiang City 453000, Henan, People's Republic of China. 2Department of Pathology, the Third Affiliated Hospital Of Xinxiang Medical University, Xinxiang City 453000, Henan, People's Republic of China

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Research

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Abstract

Background: Long non-coding RNAs (lncRNAs) have been reported to play an important role in tumorigenesis and metastasis of human colorectal cancer (CRC). However, the specific role of LincHOXA10 in CRC remains unknown.

Methods: The expression of LincHOXA10 and HOXA10 in CRC cells and tissue samples was measured by quantitative reverse transcription PCR (qRT-PCR). The protein expression of HOXA10, E-cadherin, N-cadherin, Vinmentin, p-smad2 and p-smad3 was assessed by Western blotting or immunofluorescence staining. Cell proliferation, migration, and invasion were assessed by the MTT and transwell assays. Tumor growth in vivo was carried out by subcutaneous tumor formation in nude mice.

Results: In the present study, we found that LincHOXA10 expression was significantly higher in human CRC tissues than the paired normal tissues. In fact, LincHOXA10 level correlated with the CRC tumor sizes and lymphatic metastasis. In cultured CRC cells, knockdown of LincHOXA10 inhibited cell proliferation, migration and invasion. LincHOXA10 deficiency also attenuated CRC tumor growth in vivo. Mechanistically, LincHOXA10 interacted with HOXA10 and regulated its expression. HOXA10 levels were interrelated to the LincHOXA10 level in CRC cells. Functionally, HOXA10 was essential for TGF-β1/SMADs-induced epithelial -mesenchymal transition of CRC cells, and HOXA10 played a critical role in mediating the function of LincHOXA10. Importantly, HOXA10 expression was significantly up-regulated in human CRC tissues.

Conclusions: LincHOXA10 facilitates CRC development and metastasis via regulating HOXA10-mediated epithelial-mesenchymal transition of CRC cells.

Introduction

Colorectal cancer (CRC) is the third most common cancer worldwide and the second leading cause of cancer-associated mortality[1–3]. Despite tremendous efforts for improvement in the clinical treatment of CRC, the overall survival of patients with CRC has not improved dramatically owing to cancer recurrence and metastasis. Therefore, it is an acute need to elucidate the underlying mechanism of progression in CRC, which is desirable for the development of new treatment strategies and improvement of patient prognosis.

Homeobox (HOX) genes are classified into four subgroups, namely HOX A-D. HOXA10, a member of HOX gene family, encodes a DNA-binding transcription factor that plays critical roles in gene expression, cell differentiation, and morphogenesis[4–6]. HOXA10 also takes an important part in embryo implantation[7], endometriosis [8]and hematopoietic lineage commitment[9]. Importantly, HOXA10 is involved in a wide spectrum of biological functions in human cancers, including hepatocellular carcinoma[10, 11], lung cancer[12], gastric cancer[13], bladder cancer[14], as well as glioma[15]. However, the expression and role of HOXA10 in CRC has not been previously reported.

Long noncoding RNAs (lncRNAs) are defined as RNA transcripts longer than 200 nucleotides without or with limited protein-coding potential[16–18]. They can regulate gene activity through regulation of DNA and protein modification, protein supplement, and RNA interaction[19–21]. More lncRNAs are identified and play essential roles in biological processes, including cell growth, cell differentiation, and cell cycle control[22, 23]. Therefore, lncRNAs may contribute to the development and progression of various human cancers, including CRC. A novel lncRNA, LincHOXA10, is transcribed from the antisense strand of HOXA10 gene locus at chromosome 7p15.3. The knockdown of LincHOXA10 expression was shown to induce glioma cell apoptosis[24]. Shao et al found that LincHOXA10 drives liver tumor initiating cells (TICs) self-renewal and tumorigenesis[11]. However, the function and underlying mechanism of LincHOXA10 in the development of CRC remain to be elucidated. Here we found LincHOXA10 is up-regulated in CRC, and is involved in the proliferation, migration and EMT of CRC cells. Moreover, LincHOXA10 initiates HOXA10 transcription, which mediates TGF-β2/SMADs induced EMT in CRC cells. Our study has identified a novel mechanism by which LincHOXA10 regulates CRC tumorigenesis and metastasis.

Materials And Methods

Clinical tissue samples

A total of 30 CRC tissues and 30 adjacent normal tissue samples were obtained from 30 patients aged 30–70. No patients had received chemotherapy or radiotherapy before surgery at the Third Affiliated Hospital of Xinxiang Medical University Hospital. This study was approved by Ethic Committee of Third Affiliated Hospital of Xinxiang Medical University, and the tissues were obtained after the consent of patients. All the tissue samples were immediately frozen in liquid nitrogen and stored at − 80 °C until RNA extraction.

Cell Culture And Transfection

CRC cell lines SW480, HCT116, LS174T, SW620, RKO and HT29 were obtained from the American Type Culture Collection (ATCC). Normal human fetal colonic mucosa cell line (FHC) was established at our laboratory. All cell lines were cultured in RMPI-1640 medium containing 10% fetal bovine serum (Gibco, USA) and 1% penicillin/streptomycin (Invitrogen, USA) in 5% CO_2 at 37 °C.

Rna Isolation And Real-time Quantitative Pcr (qrt-pcr)

Total RNA from tissues or cultured cells was solated using Trizol reagent (Takara, Japan) according to the manufacturer's protocols. RNA (1 μg) was reverse transcribed to cDNA with reverse transcription kit (Takara, Japan). qRT-PCR was performed on ABI 7500 RT-PCR system (Applied Biosystems, USA) with SYBR® Premix Ex Taq™ Kit (TaKaRa, Japan) and Mir-X™ miRNA qRT-PCR SYBR® Kit (Clontech Laboratories, USA) in accordance with the manufacturer's instructions. All experiments were repeated

three times. Data were calculated using comparative cycle threshold (CT) ($2^{-\Delta\Delta CT}$) method. The results were normalized to the expression of GAPDH. The primers for LincHOXA10 (F: CCC AGT AAG CCA AAG TCA AGCC, R: CTG AGG TCA ATG GTG CAA AGG).

Western Blotting

Proteins were lysed in RIPA buffer (KeyGen Biotech, China) containing 100 mmol/L phenyl methane sulfonyl fluoride (PMSF), and quantified by bicinchoninic acid (BCA) protein quantitative assay (KeyGen Biotech, China). Protein lysates were separated using 10% SDS-PAGE and transferred onto PVDF membranes (Roche, Switzerland). Then, the membranes were incubated with specific antibodies against HOXA10 (Abcam, England), E-cadherin, N-cadherin, Vimentin, p-smad2 and p-smad3 (Cell Signaling Technology, USA) followed by incubation with the appropriate second antibodies, and α-Tubulin was used as an internal control. Finally, the membranes were detected using an enhanced chemiluminescence (ECL) detection system (FDbio, China), according to the manufacturer's instructions. The results were normalized to the expression of a-tubulin (Proteintech, USA).

Lentiviral Vector Preparation And Plasmid Transfection

The siRNAs targeting LincHOXA10 (si-LincHOXA10) and siRNA negative control (si-NC) were all purchased from GenePharma (China). The full length of HOXA10 was subcloned into pcDNA3.1 to overexpress HOXA10 levels with empty pcDNA3.1 serving as control. The pcDNA3.1 vector was bought from GenePharma (Shanghai). Lipofectamine™ 2000 (Invitrogen) was used for cell transfection according to the manufacturer's instructions.

Immunohistochemistry (ihc)

According to the specifications of the S-P kit, paraffin-embedded tissues were cut into 5 μm-thick sections, dehydrated with organic solvent, retrieved with citrate buffer, incubated with primary antibody (Anti-HOXA10 antibody: Abcam, England.) and then detected with an avidin-biotin complex with 3, 3'-diaminobenzidine. The degree of staining was observed and scored independently by two pathologists. Immune staining intensity was rated as follows: 0(no staining), 1(yellow or light brown, weak staining), 2 (brown, moderate staining) and 3(darkbrown, strong staining). Immune staining quality was rated as follows: 0(no staining), 1(< 30%), 2(30%-70%) and 3(> 70%). Tumor tissue intensity was scored via summation as follows: 0–1 (-), 2–3 (+), 4 (++), and 5–6 (+++). Tissues scored 0–1 (-)/2–3 (+) were classified into the low-expression group, and tissues scored 4 (++)/5–6 (+++) were classified into the high-expression group.

Immunofluorescence Staining

For immunofluorescence staining of cultured cells, cells seeded on confocal dish were transfected with adenoviral vectors. 48 h later, cells were fixed with 4% paraformaldehyde for 30 min and permeabilized with 0.5% Triton X-100 for 10 min at room temperature. The cells were then incubated with primary antibodies at 4 °C overnight followed by washes with PBS and incubation with fluorescent secondary antibody in dark at room temperature for 1 h. After final washes with PBS, the confocal dish was mounted using an anti-fade mounting solution containing 4, 6-diamidino-2-phenylindole (DAPI). The staining was examined, and images were captured using an Olympus Confocal laser scanning microscopy FV1200.

Cell Proliferation Assay

Cell proliferation was determined with the 3-(4,5- dimethylthiazol-2 -yl)- 2,5- diphenyltetrazolium bromide (MTT) assay. In brief, 1×10^3 cells were seeded in each well of 96-well plates in 200-μL volume. At 24, 48, and 72 hours after culture, MTT (20μL, 5 mg/mL) was added into each well and incubated for 4 hour at 37 °C. After incubation, MTT was removed and 150 μl dimethyl sulphoxide (DMSO; Sigma, USA) was added to the wells. The absorbance was measured at 570 nm wavelength with a microplate reader (Bio-Rad, Hercules, CA, USA). The experiment was conducted repeatedly for three times.

Transwell Migration And Invasion Assay

Transfected cells (1×10^5) were seeded in serum-free medium in the top chamber of each transwell well (BD Biosciences, USA), which featured a pore size of 8 μm. Matrigel (BD Biosciences) was used to cover the top side of the membrane for invasion assay and Matrigelfree condition was used for migration assay. The matched lower chamber was filled with complete medium supplemented with 10% FBS. The cells were incubated at 37 °C with 5% CO_2 for 48 hours, the non-traversed cells were removed from the upper filter with a cotton swab, and the traversed cells were fixed with formaldehyde and stained with hematoxylin for 30 minutes. Then, the cells that migrated or invaded to the basal portion of the membrane in the lower compartment of the chamber were counted in 5 random visual fields using a microscope (× 200). All experiments were performed in triplicates.

In vivo **tumor growth assay**

CRC cells were harvested by trypsinization, washed twice, and then re-suspended with serum-free medium. To evaluate CRC tumor growth in *vivo*, 5×10^6 of RKO and HCT116 cells stably expressing control or LincHOXA10 shRNA via lentiviral vector were separately injected subcutaneously into the left and right back flank of nude mice (n = 3 per group). Twenty five days later, tumors were removed and measured.

Statistical analysis

All statistical analyses were performed using SPSS 19.0 (Abbott Laboratories, USA). The quantitative results of all experiments were presented as the mean ± SD. Differences among/between sample groups were analysed by one-way ANOVA or the independent-samples *t*-test. Relationships between LincHOXA10 expression and clinicopathological characteristics were tested using Pearson's χ2-test. Differences were considered significant if $P < 0.05$*; $P < 0.01$**; $P < 0.001$***.

Results

LincHOXA10 expression is upregulated in CRC tissues and correlates with clinicopathologic characteristics of patients with CRC

The expression of LincHOXA10 in 30 CRC tissues and paired adjacent non-CRC tissues was detected with qRT-PCR analysis (Fig. 1A). The expression of LincHOXA10 was significantly upregulated in CRC tissues as compared with the adjacent non-CRC tissues (Fig. 1B).

The relationship between various clinicopathological characteristics of patients with CRC and LincHOXA10 expression was analyzed (Table 1). Age and gender did not show significant correlation with LincHOXA10 expression, while tumor size and lymphatic metastasis showed significant correlation with LincHOXA10 expression.

Table 1. Clinicopathologic characteristics of patients and LincHOXA10 expression in CRC

characteristics	L	incHOXA10 Express	ion: No. of patient	s(%)	*P* value
		High	Low		
Age(y)	≥50	9(56.3)	7(43.8)	0.715	
	<50	6(42.9)	8(57.1)		
Gender	Male	8(53.3)	7(46.7)	1.000	
	Female	7(46.7)	8(53.3)		
Differentiation	High	8(61.5)	5(38.5)	0.429	
	Moderate	4(50.0)	4(50.0)		
	Low	3(33.3)	6(66.7)		
Tumor size	<5 cm	2(18.2)	9(81.8)	0.021	
	≥5 cm	13(68.4)	6(31.6)		
Lymph metastasis	Yes	2(18.2)	9(81.8)	0.021	
	No	13(68.4)	6(31.6)		

Knockdown of LincHOXA10 inhibits cell proliferation, migration, and invasion in CRC cells

In cellular level, we detected the expression of LincHOXA10 in six different CRC cells and found that RKO and HCT116 cells exhibited a much higher level of LincHOXA10 expression than other cells (Fig. 6A). Therefore, we chose RKO and HCT116 cells for the subsequent studies.

To investigate whether LincHOXA10 expression has functional impacts on CRC cells, the expression of LincHOXA10 in two CRC cell lines (RKO and HCT116) was silenced by transfection with LincHOXA10 siRNA (si-LincHOXA10). qRT-PCR results showed that the expression of LincHOXA10 in both RKO and HCT116 cells was downregulated upon transfection with si-LincHOXA10, and the silencing effect of si-LincHOXA10 $^{1\#}$ was significantly higher than that of si- LincHOXA10 $^{2\#}$ and si- LincHOXA10 $^{3\#}$ (Fig. 2A). Thus, si-LincHOXA10 $^{1\#}$ was selected for lentiviral vector mediated shRNA knockdown of LincHOXA10 in RKO and HCT116 cells (sh-LincHOXA10), which were used for further functional assays .

The effects of LincHOXA10 knockdown on RKO and HCT116 cells were investigated. MTT and transwell assay results demonstrated that LincHOXA10 knockdown significantly inhibited the proliferation (Fig. 2B and 2C), migration (Fig. 2D, 2E, 2G and 2H), and invasion (Fig. 2D and 2F) of both RKO and HCT116 cells.

Knockdown of LincHOXA10 inhibits tumor growth in vivo

To assess the effect of LincHOXA10 knockdown on tumor growth in *vivo*, we subcutaneously injected RKO and HCT116 cells that stably express scramble (sh-NC) or LincHOXA10 shRNA into nude mice, and then monitored the growth of the resultant primary tumors. As shown in Fig. 3A and 3B, the xenograft tumors developed at the injection site after 5 days. During a growing period of 25 days, primary tumors derived from LincHOXA10 deficient CRC cells grew significantly slower than that derived from control cells (Fig. 3A and 3B). Moreover, the tumor volumes of the LincHOXA10 deficient groups were significantly smaller than those of control groups (Fig. 3C and 3D).

Knockdown of LincHOXA10 inhibits epithelial to mesenchymal transition (EMT) of CRC cells

EMT is an important mechanism in malignant transformation of tumor cells[25]. We examined whether LincHOXA10 exerted its carcinogenesis through regulating EMT of CRC cells. The key characteristics of EMT are the reduction of E-cadherin along with an increased expression of neuronal cadherin (N-cadherin) and other mesenchymal markers such as vimentin. We found that knockdown of LincHOXA10 in RKO and HCT116 cells significantly increased the expression of E-cadherin while decreased N-cadherin and vimentin expression (Fig. 4A-4D), suggesting that down-regulation of LincHOXA10 inhibited the EMT of CRC cells.

Linchoxa10 Interactes With Hoxa10 And Regulates Hohxa10 Protein Expression

It's a common mechanism that lncRNAs can regulate gene expression through participating in the transcription process of nearby genes[26]. Previous studies have shown that LincHOXA10 recruits SNF2L

to HOXA10 promoter to regulate its expression[11]. Thus we hypothesize that LincHOXA10 may interact with HOXA10 protein to induce EMT. We detected the expression of HOXA10 in RKO and HCT116 cells when LincHOXA10 was blocked, and found that knockdown of LincHOXA10 significantly decreased both the mRNA and protein levels of HOXA10 in RKO (Fig. 5A, 5C and 5E) and HCT116 cells (Fig. 5B, 5C and 5F).

LincHOXA10 is required for TGF-β1/SMADs-induced EMT and HOXA10 rescues LincHOXA10 function

It is known that TGF-β/SMADs signaling plays a very important role in EMT of CRC cells[27]. To determine whether LincHOXA10 is required for TGF-β1/SMADs-induced EMT, we knocked down LincHOXA10 expression using shRNA in RKO and HCT116 cells treated with TGF-β1. As shown in Fig. 6A–D, blockade of LincHOXA10 expression restored E-cadherin expression while attenuated N-cadherin and vimentin expression in both RKO and HCT116 cells. Importantly, blockade of LincHOXA10 expression also decreased smad2/3 phosphorylation in RKO and HCT116 cells (Fig. 6A-6D). These results suggested that LincHOXA10 is required for TGF-β1/SMADs -induced EMT of RKO and HCT116 cells.

Moreover, knockdown of LincHOXA10 also blocked TGF-β1-induced HOXA10 protein expression (Fig. 6A-6D), consistent with the role of LincHOXA10 in regulating HOXA10 expression (Fig. 5A-5F). Therefore, we hypothesize that HOXA10 mediates LincHOXA10 function in TGF-β1/SMADs -induced EMT, and thus HOXA10 overexpression can rescue the function of LincHOXA10 on the EMT and smad2/3 phosphorylation. Indeed, overexpression of HOXA10 attenuated the E-cadherin expression and restored p-smad2, p-smad2, N-cadherin and vimentin expression that was altered due to LincHOXA10 knockdown in TGF-β1-treated cells (Fig. 6A-6D). These results clearly showed that LincHOXA10 mediates TGF-β1/SMADs -induced EMT of CRC cells via inducing HOXA10 expression.

HOXA10 expression is upregulated in CRC cells and human CRC tissues

To determine whether HOXA10 is expressed in different CRC cells and human CRC tissues, we detected HOXA10 protein expression in control FHC cell and six different CRC cell lines by Western blot and in 30 paired paraffin-embedded human CRC tissue samples by IHC. The results showed that HOXA10 expression was expressed in all CRC cells detected, and HOXA10 expression in CRC cells was higher than that in FHC cell (Fig. 7A-7D). However, a high level of HOXA10 expression was observed in RKO cells, consistent with LincHOXA10 expression (Fig. 7A). Indeed, HOXA10 expression correlated with LincHOXA10 in most CRC cells (Fig. 7A vs Fig. 7C- 7D). Moreover, the protein expression of HOXA10 were significantly elevated in 25 out of 30 human CRC tissues (Fig. 7B), consistent with the LincHOXA10 expression (Fig. 1).

Discussion

Many different mechanisms are implicated in tumorigenesis of different cancers. EMT is one of the mechanisms commonly believed to control the process of cancer cell invasion and metastasis[27]. It's

well known that many signaling pathways are involved in EMT regulation, such as TGF-β[28], Wnt[29], Notch[30], TNF[31], and BMPs[32]. Several transcription factors also take part in the regulation of EMT, including the Snail/Slug family, Twist, and SIP1/ZEB2, function as molecular switches for the EMT program[33–35]. Recent studies have shown that dysregulated expression of lncRNAs in some cancers may regulate EMT and affect disease progression[36]. Here we identified LincHOXA10, A novel lncRNA, drives the EMT of CRC cells though TGF-β1/SMADs pathway. Interestingly, HOXA10, a member of HOX transcription factor family, is regulated by LincHOXA10 and rescues LincHOXA10 function.

LncRNAs execute critical modulators in many physiological and pathological processes, including embryonic pluripotency, developmental transitions, differentiation and epigenetic programs of the transcriptome[22, 23]. In addition, lncRNAs may also play important roles in driving tumor suppression or in exerting oncogenic functions in a wide variety of cancer types, by promoting tumor cell proliferation, migration, invasion and metastasis[24, 37]. Recently, a lncRNA named LincHOXA10, which resides at HOXA10 promoter and coordinates the activation of multiple 5'HOXA genes, has been identified as one of 231 lncRNAs associated with the human HOX loci[24]. Furthermore, expression of LincHOXA10 has been identified as a negative prognostic factor in glioma patients[24]. However, the functional role of LincHOXA10 in CRC remains unknown. This present study offers the first insight into the effect of LincHOXA10 on proliferation, migration, invasion and EMT of CRC cells. Here, we found LincHOXA10, which is upregulated in human CRC tissues and cells compared with non-tumoral tissues and cell lines, enhances proliferation, migration and invasion of CRC cells, as well as EMT. In addition, we show that knockdown of LincHOXA10 inhibites the tumorigenesis of CRC cells both in *vitro* and in *vivo*.

The HOX family of homeobox genes encodes transcriptional regulators that play critical roles in many processes. For example, HOXA4, HOXA7, HOXA13 were dysregulated in gastric cancer, and participate in gastric carcinogenesis[38]. Here, we identified HOXA10, a member of the HOXA gene cluster, as a predominant HOX TF in CRC tumorigenesis. HOXA10 exerts an oncogenic role in several tumors, including oral squamous cell carcinoma[39], pancreatic carcinoma[40], prostate carcinoma[41], hepatocellular carcinoma[10], and leukemia[42]. Overexpression of HOXA10 leads to tumor propagation and it can serve as a good marker for prognosis of various tumors. Despite of the oncogenic role in various tumors, the role of HOXA10 in CRC tumorigenesis is unclear. Here, we detected the expression and critical role of HOXA10 in CRC cells and human tissues. we found HOXA10 is upregulated in CRC cells and tissues, along with LincHOXA10. Interestingly, HOXA10 overexpression can rescue the function of LincHOXA10 on the EMT and smad2/3 phosphorylation. That is to say, HOXA10 mediates LincHOXA10 function in TGF-β1/SMADs -induced EMT.

It’s a common mechanism that lncRNAs can regulate gene expression through participating in the transcription process of nearby genes. LincHOXA10 locates at HOXA10 promoter, we have found LincHOXA10 is upregulated in CRC, along with HOXA10. However, further studies are required to elucidate the detailed mechanism of how LincHOXA10 regulates HOXA10 transcription. The results of the present study demonstrated that LincHOXA10, which binds to HOXA10 promoter to drive HOXA10, recruits SNF2L chromatin remodeling complex to HOXA10 promoter to drive its expression[11]. Therefore, the potential

mechanism of LincHOXA10- SNF2L-HOXA10 RNA hybrid may act to stabilize the HOXA10 transcript and required for TGF-β1/SMADs-induced EMT of CRC cells.

Conclusions

Our present study demonstrates that LincHOXA10, which is significantly overexpressed in CRC, plays a significant role in CRC tumorigenesis and metastasis. LincHOXA10 contributes to the CRC progress by regulating HOXA10 expression and consequently TGF-β1/SMADs-induced EMT of CRC. Since LincHOXA10 level is associated with tumor size and lymphatic metastasis, LincHOXA10 may be used as a biomarker to monitor the progression of CRC cancer in human.

Abbreviations

CRC
colorectal cancer; qRT-PCR:quantitative reverse transcription PCR; lncRNAs:Long non-coding RNAs; HOX:Homeobox; EMT:mesenchymal transition; ECL:chemiluminescence; N-cadherin; neuronal cadherin.

Declarations

Ethics approval and consent to participate

The use of 30 pairs human colorectal carcinoma tissues for this study was approved by Ethic Committee of Third Affiliated Hospital of Xinxiang Medical University, and the tissues were obtained after the consent of patients.

All animal experiments were conducted such that the animals received ethical and humane treatment, and all procedures were approved by the Institutional Animal Care and Use Committee of Xinxiang Medical University.

Consent for publication

Not applicable.

Availability of data and material

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

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Huifang Zhu, Yuhan Hu and Yongzhen Li conceived and performed the experiments, analyzed and interpreted the data, and drafted the manuscript. Yinghui Zhang and Zheying Zhang conceived the experiments, analyzed and interpreted data. Huifang Zhu, Yongzhen Li, Yinghui Zhang, Zheying Zhang, Yongxia Wang, Guoyang He, Na Li, Haijun Wang, Jiateng Zhong, Xinlai Qian, and Yuhan Hu participated in the experiments, helped analyzed data, read and approved the final manuscript.

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Figures

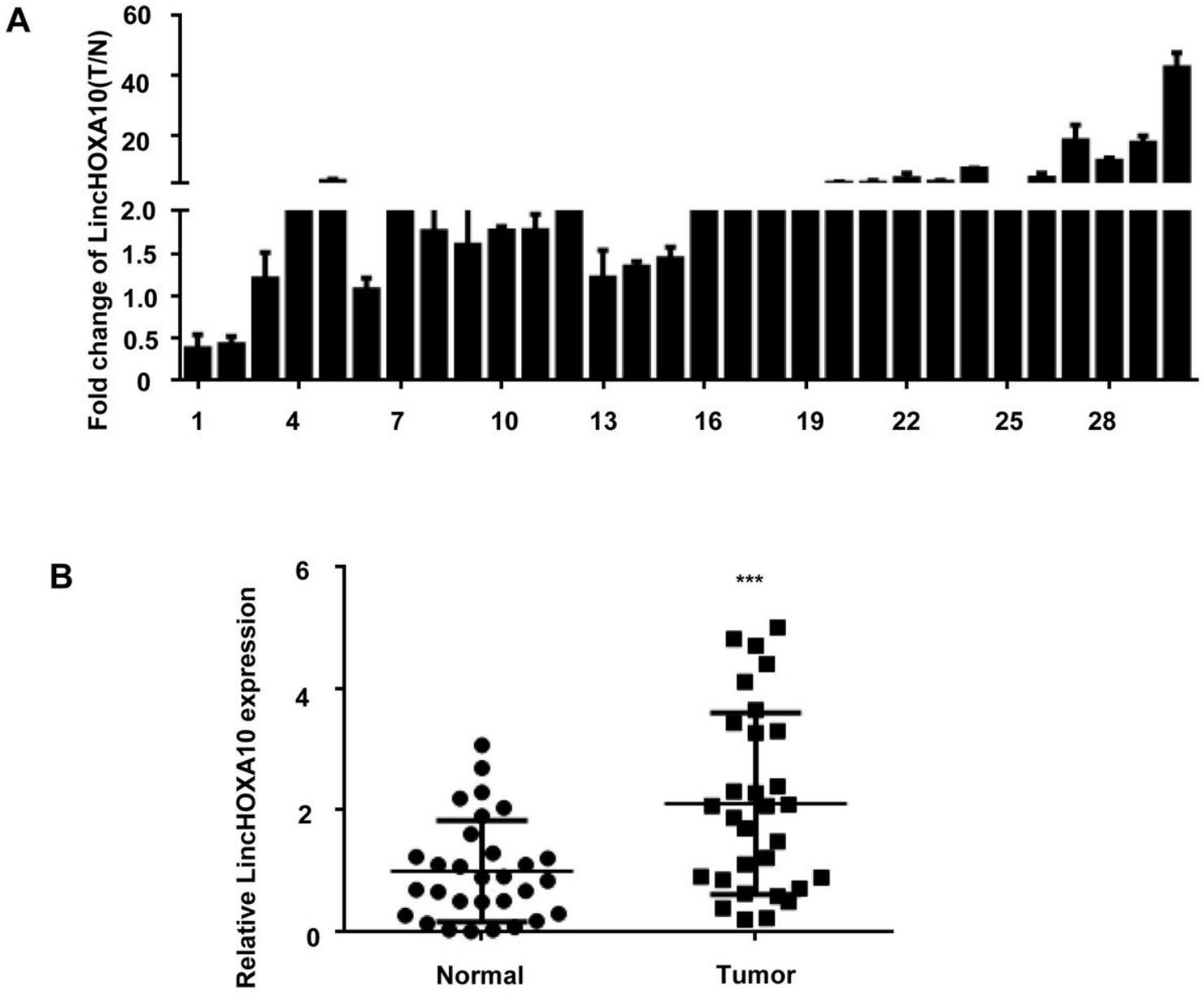

Figure 1

LincHOXA10 expression level in human CRC tissues. (A) Quantitative analysis of LincHOXA10 expression in 30 paired human CRC tissues. LincHOXA10 expression was quantified by qPCR and normalized to the matched adjacent normal tissues. (B) Comparison of LincHOXA10 abundance in 30 paired primary CRC tissues (Tumor) with paired adjacent normal tissues (Normal). ***P< 0.001.

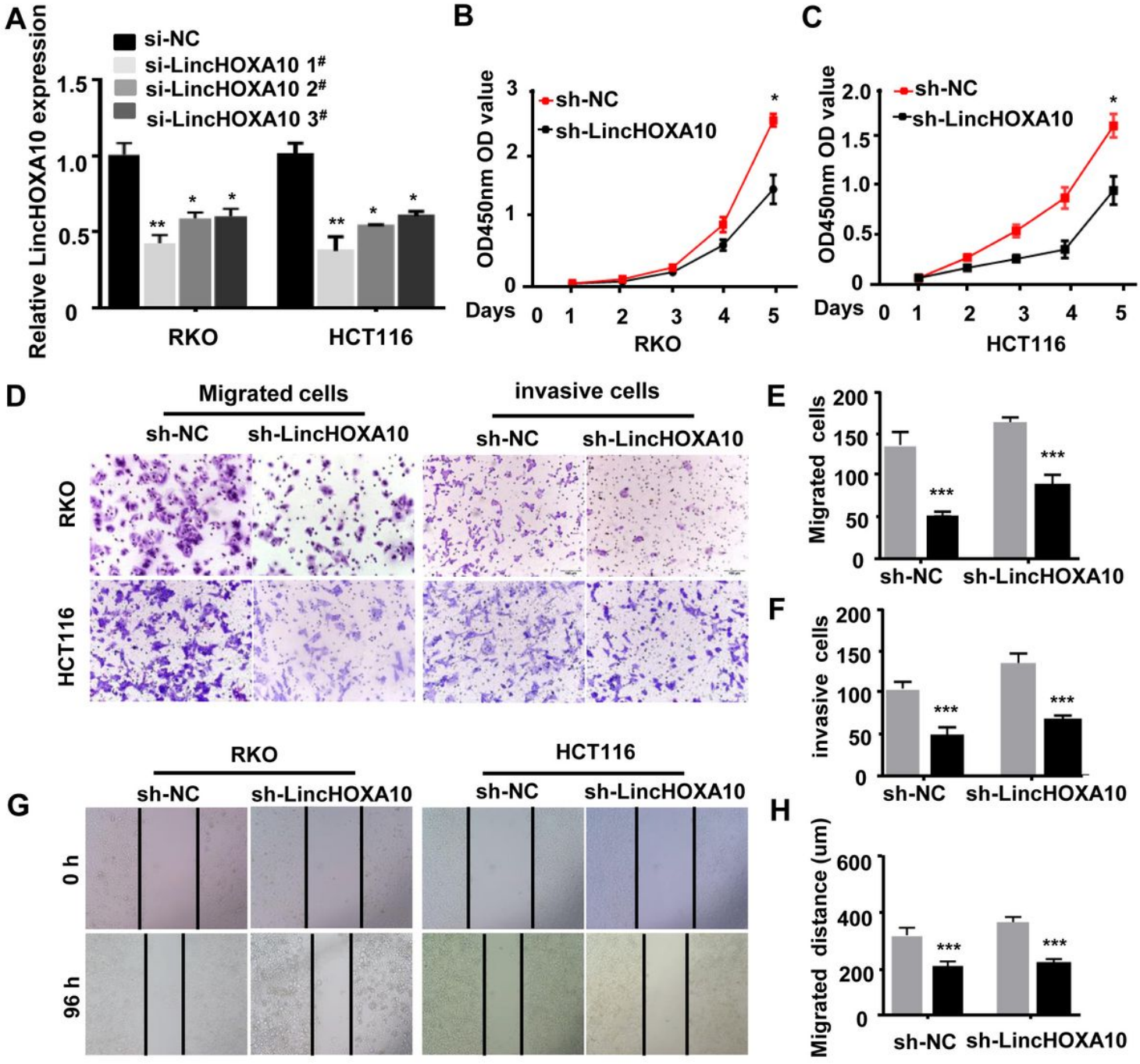

Figure 2

Knockdown of LincHOXA10 inhibits cell proliferation, migration, and invasion in CRC cells. (A) Expression level of LincHOXA10 in RKO and HCT116 cells transfected with si- LincHOXA10 1#, si- LincHOXA10 2#, and si- LincHOXA10 3#, or si-negative control (si-NC), as analyzed by qPCR . (B-C) RKO and HCT116 cells were stably transfected with vector (sh-NC) or si-LincHOXA10 1# (sh-LincHXA10). Knockdown of LincHOXA10 inhibited cell proliferation, as determined by the the MTT assay in RKO (B) and HCT116

cells (C). (D) The representative graphs of migrated cells and invasive cells stained by hematoxylin staining in transwell assay. (E) The statistical results of average migrated cells stained by hematoxylin in transwell assay. (F) The statistical results of average invasive cells stained by hematoxylin in transwell assay. (G) The migrative ability of RKO and HCT116 cells detected by the wound-healing assay. (H) The statistical results of average migrated distance of RKO and HCT116 cells. *P<0.05, **P<0.01, ***P<0.001.

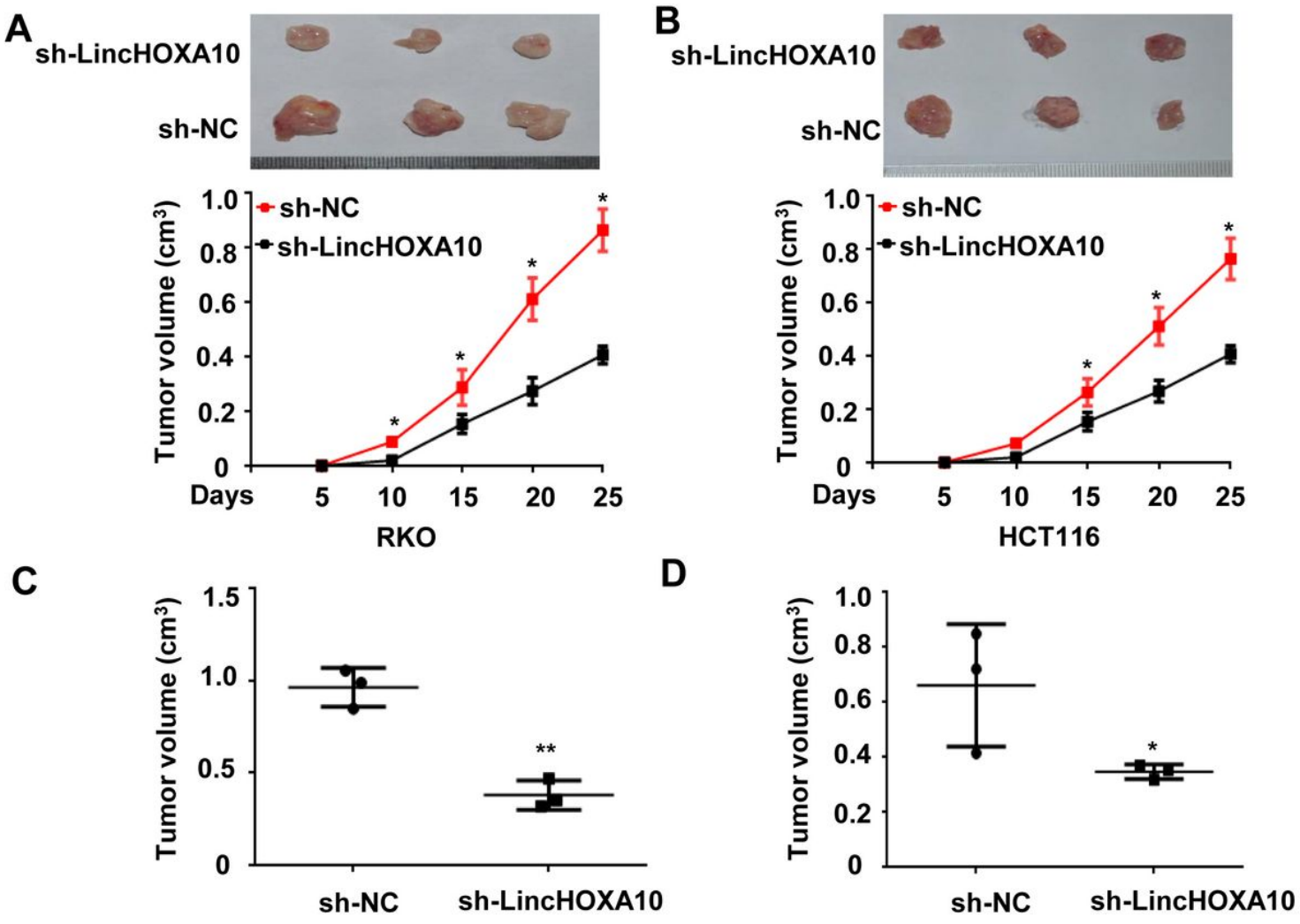

Figure 3

Knockdown of LincHOXA10 inhibited tumor growth in vivo. (A–B) GFP-labeled RKO (A) or HCT116 cells (B) with stable transfection of control (sh-NC) or LincHOXA10 shRNA (sh- LincHOXA10) were injected subcutaneously into nude mice. 25 days later, the tumors were removed and imaged (upper panels). Tumor growth curves were obtained by using a whole-body GFP imaging system during the growth of the tumors (lower panels). Tumors derived from cells expressing sh-LincHOXA10 grew significantly slower than that from cells with sh-NC. *P<0.05 compared to sh-NC group in each corresponding time point for both A and B, n = 3. (C–D) Scatter plot of the tumor sizes at 25 days post-injection. Tumors derived from RKO (C) and HCT116 (D) cells expressing sh-LincHOXA10 were significantly smaller than that from cells with sh-NC. *P<0.05, **P<0.01. n= 3.

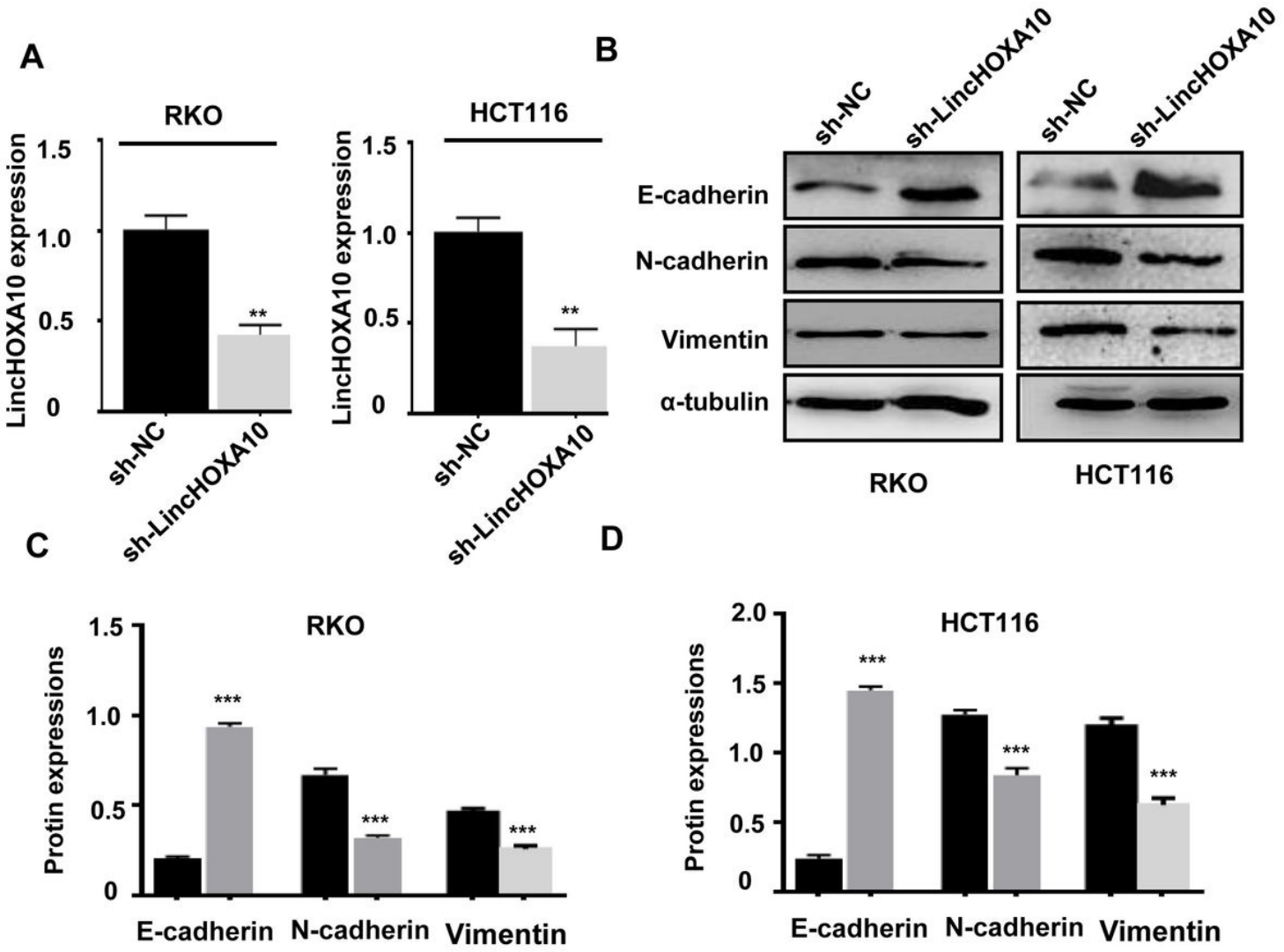

Figure 4

Knockdown of LincHOXA10 inhibits EMT of CRC cells. (A) Expression level of LincHOXA10 in RKO and HCT116 cells transfected with sh-NC or sh- LincHOXA10. (B) The expression of mesenchymal and epithelial markers in RKO and HCT116 cells transfected with sh-NC or sh- LincHOXA10 were detected by Western blotting. (C-D) Quantification of protein expression shown in B by normalized to α-Tubulin. **P<0.01, ***P<0.001.

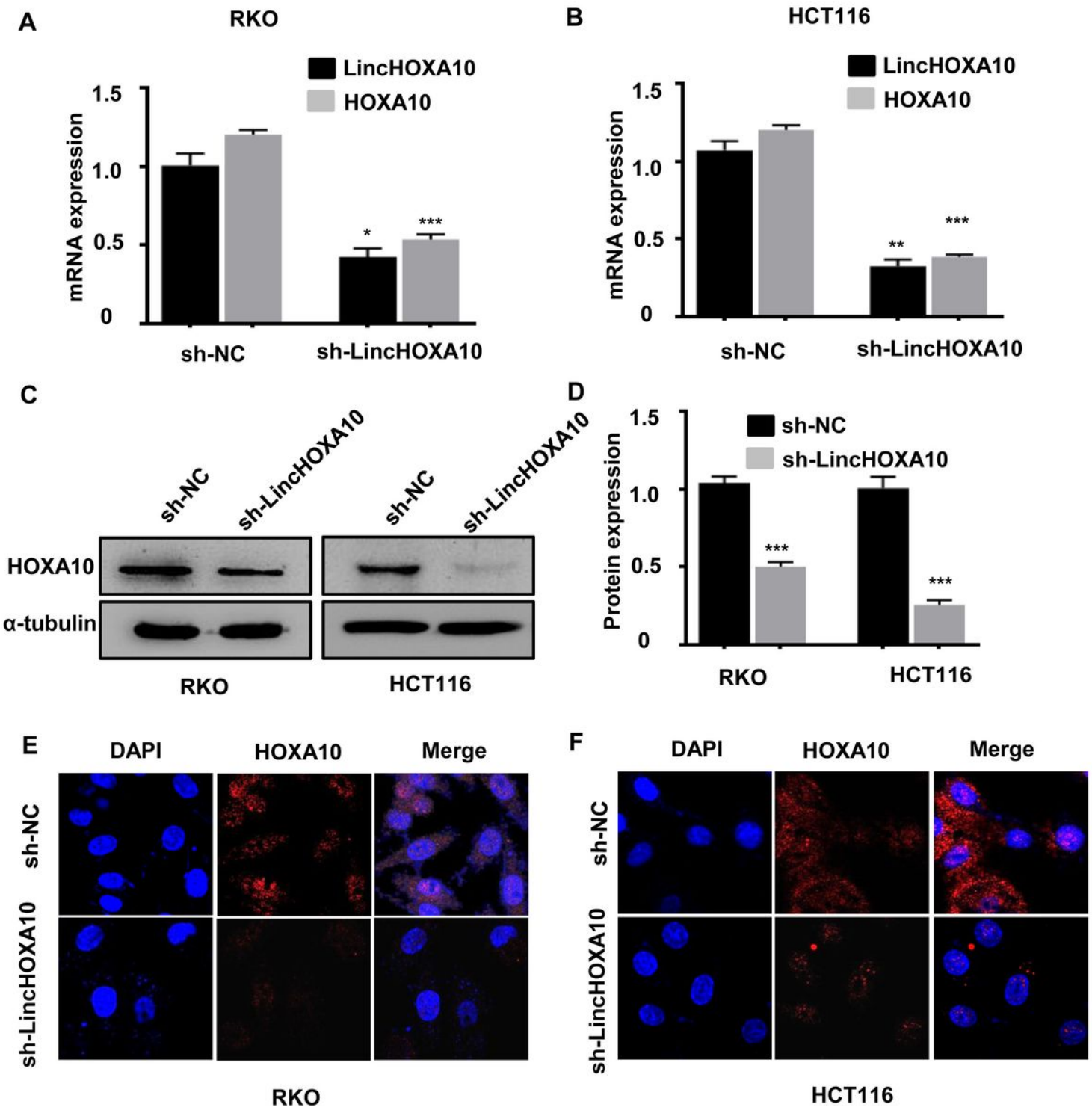

Figure 5

LincHOXA10 regulated HOXA10 expression in CRC cells. (A-B) Knockdown of LincHOXA10 attenuated HOXA10 mRNA expression in RKO (A) and HCT116 cells (B) as detected by qRT-PCR. (C) Knockdown of LincHOXA10 attenuated HOXA10 protein expression in RKO and HCT116 cells. (D) Quantification of HOXA10 protein levels shown in C by normalized to α-Tubulin. (E-F) HOXA10 expression in RKO (E) and HCT116 (F) cells was detected by immunostaining. DAPI stains nuclei. *P<0.05, **P<0.01, ***P<0.001.

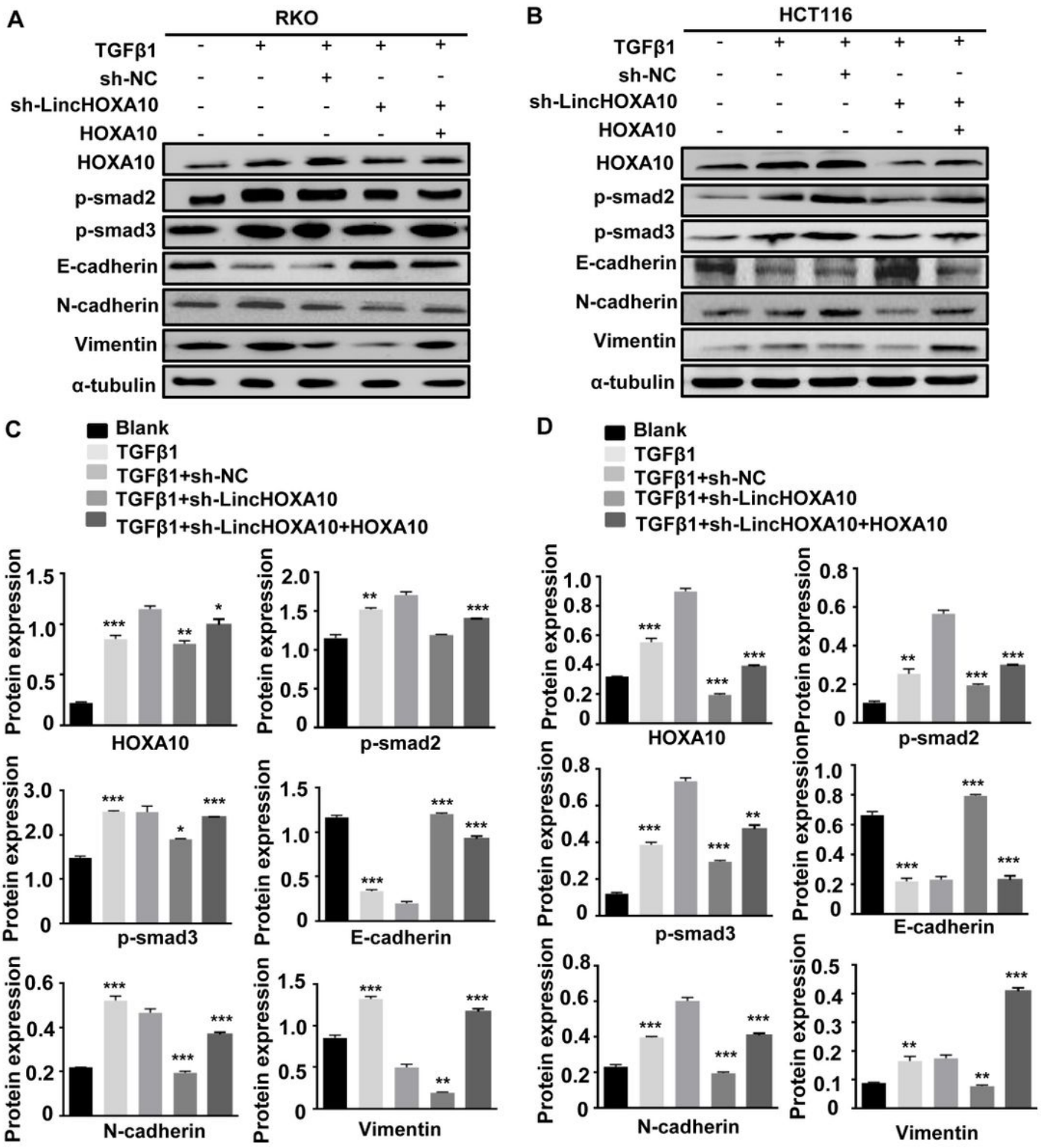

Figure 6

LincHOXA10 is required for TGF-β1/SMADs-induced EMT EMT via CIP4 in CRC cells. (A-B) TGF-β1 promoted smads signaling and HOXA10 expression and induced RKO (A) and HCT116 (B) cells EMT. HOXA10 overexpression in LincHOXA10 deficient cells rescued smads signaling and TGF-β1-induced EMT. (C-D) Quantification of protein levels shown in A and B by normalized to α-Tubulin. *P<0.05, **P<0.01, ***P<0.001.

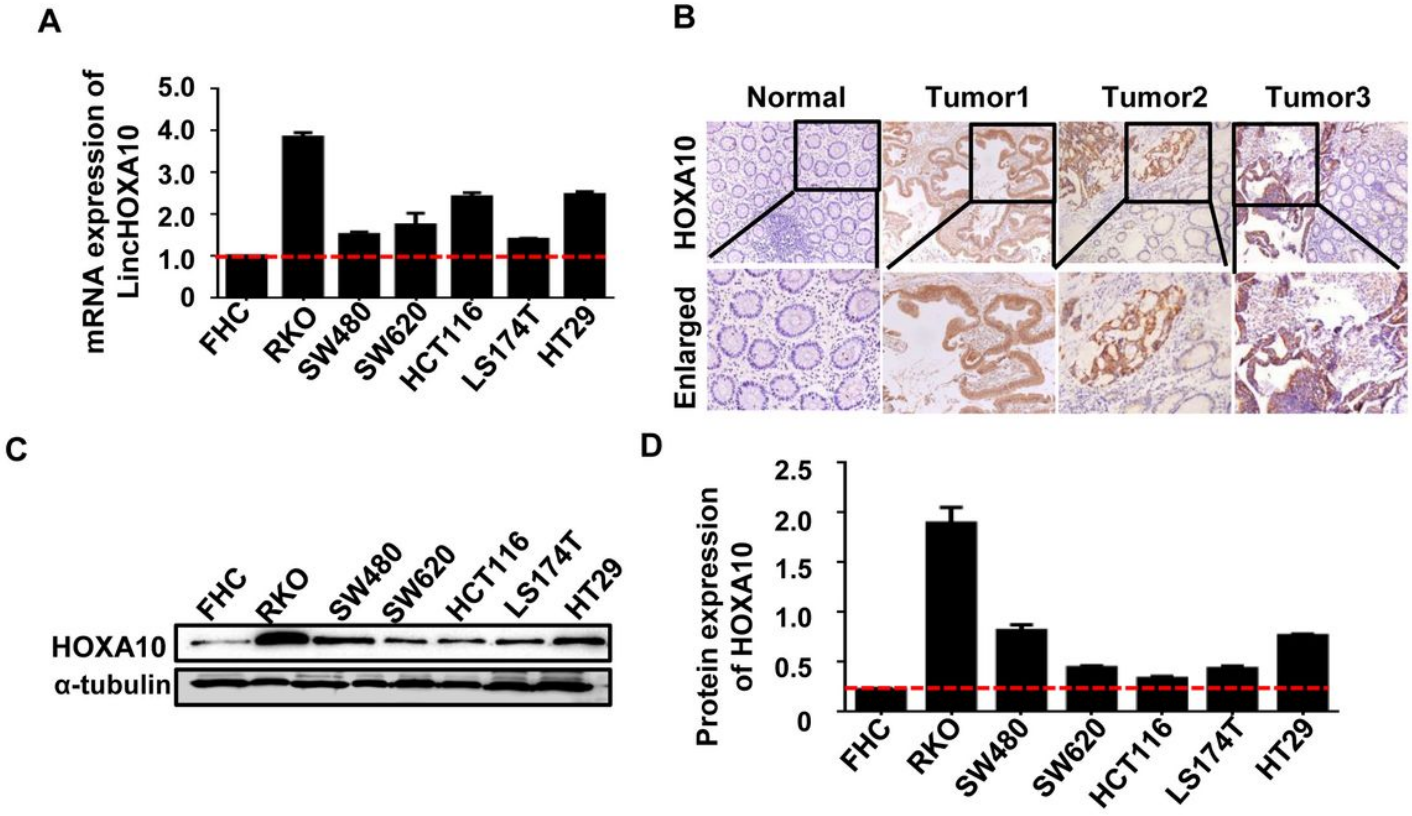

Figure 7

LincHOXA10 and HOXA10 expression is upregulated in CRC cells and tumor tissues. (A) LincHOXA10 expression in the normal fetal colonicmucosa cells (FHC) and six CRC cell lines was detected by qPCR. (B) HOXA10 expression in 30 paired paraffin-embedded CRC tissue samples were detected by immunohistochemistry staining. Representative HOXA10 immuohistochemical staining photographs of normal tissue (Normal) and tumor tissue samples (Tumor 1, Tumor 2 and Tumor 3) as indicated.(C-D) HOXA10 protein expression in FHC and eight CRC cell lines was detected by Western blotting and normalized to α-Tubulin level.