# Circular RNA circ-SMAD7 is downregulated in colorectal cancer and suppresses tumor metastasis by regulating epithelial mesenchymal transition

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**Abstract.** – **OBJECTIVE:** Recently, circular RNAs play a vital role in many diseases including tumor progression. Colorectal cancer (CRC) is one of the most ordinary malignant tumors. The purpose of our study is to detect the potential function of circ-SMAD7 in CRC.

**PATIENTS AND METHODS:** The level of circ-SMAD7 was detected by Real Time-quantitative Polymerase Chain Reaction (RT-qPCR) in CRC tissue samples. The circ-SMAD7 expressi el and the patients' overall survival ti analyzed. Functional experiments were ducted to identify the changes of the biol behaviors in CRC cells after the overexpres of circ-SMAD7. The transwell as the Ma gel assay, and the Wound h say we conducted. The Western b was performed to analyze the eff of cir AD7 on the epithelial-to-mesen tran process.

**RESULTS:** In the r rch, ession level of circ-SMAD7 w ignifican eased in CRC tissues co with that i jacent samples. Circ- ression wa positively associated pat erall survival time. The expression of cir 7 was also decreased C cell lines. regulation of circ-SM led to the inhibiti of cell migration and i ion in C. In addition, the results of fur peri ts revealed that the EMT-related p e regu d *via* overexpression of circ- in CR

**ONCL** se results suggest that MAD7 hibit cell migration and inva of CRC b uppressing the EMT process, wh offer a potential therapeutic target f

rds:
RNA, Circ-SMAD7, Colorectal cancer, Epithelia -mesenchymal transition.

## Intr on

lorectal cancer (CRC) is one of the most lent malignan umors in the world, espe in develope untries[1]. It is estimated that 1 new were diagnosed of CRC in Am and 50,260 CRC patients died in the same year which accounted for 8.4% of cancer-related deaths. Most of the patients etastasis by the time they are firstly d. Numerous studies have been carried out to find out the understanding mechanism contributing to the progression of CRC for the past decades. However, there are few improvements in the poor prognosis for the advanced cases[2]. Therefore, it is urgent to have a deeper understanding of the underling molecular mechanisms of tumorigenesis and progression in colorectal cancer and figure out new therapeutic strategies.

Circular RNAs (circRNAs) represent a novel class of endogenous noncoding RNAs with their 3′- and 5′-ends joined together to form a covalently closed loop. Due to the special structure, circRNAs are more stable than linear RNA isoforms. CircRNAs have recently attracted much research interest due to its wide expression found in many genes. Moreover, it was found that circRNAs functioned as microRNA (miRNA) sponges which were involved in the initiation and progression of different cancers. Consistently, by regulating the expression of LATS1 and sponging miR-424-5p, circ-RNA_LARP4 suppresses the proliferation and invasion of gastric cancer cells[3]. As a miR-1252 sponge, hsa_circ_0043256 inhibits cell proliferation and induces cell apoptosis in non-small cell lung cancer[4]. By competing with microRNA-150-5p, circRNA ZNF609 enhances



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tumor growth and metastasis of nasopharyngeal carcinoma[5]. The knockdown of circ_0025039 inhibits the proliferation, invasion and colony formation ability of melanoma cells[6]. However, the function of circular RNA in CRC and the potential molecular mechanism have not yet been studied so far. To uncover the role of circ-SMAD7 in CRC, its expression was detected in CRC tissues and the related mechanism was researched.

In this study, circ-SMAD7 was remarkably downregulated in CRC tissues and cell lines. Moreover, circ-SMAD7 inhibited migration and invasion of CRC *in vitro*. Our further experiments also showed that circ-SMAD7 suppressed the epithelial-to-mesenchymal transition (EMT) process of CRC.

## Patients and Methods

### Tissue Samples

The tumor samples and the adjacent tissues (≥ 5 cm away from the edge of tumor) were gathered from CRC patients (n = 52) who underwent surgery at Qingdao Chengyang District People's Hospital from June 2016 to December 2018. [illegible]ten informed consent was taken before op[illegible]. All fresh tissues were preserved at -80°[illegible]his study was approved as the Human Re[illegible] Ethics Committee from of Qingdao Cheng[illegible] District People's Hospital required.

### Cell Culture and Transf[illegible]

The human CRC cell[illegible] (HC[illegible]T29, SW620, SW480) and no[illegible]thelial cells (NCM460) were[illegible]ed from the American Type Cult[illegible]ollectio[illegible]C, Manassas, VA, USA). T[illegible]lture mediu[illegible]ted of Roswell Park M[illegible]itute-1640 (R[illegible]M-1640, Hyclone, Sout[illegible]gan, [illegible]A), 10% fetal bovine serum (FB[illegible] Invitrogen, [illegible] CA, USA), and penicilli[illegible]ptomycin. Besid[illegible] cells were cultured i[illegible] incub[illegible]or containing 5% $CO_2$ at 37°C. We [illegible]ased [illegible]ntivirus targeting circ-SMAD7 (Ge[illegible]anghai, China) for the transfection of [illegible]C cell[illegible]ng Lipofectamine 3000 [illegible]itrogen, [illegible]A, USA). The transfection [illegible]ncy was [illegible]ed by RT-qPCR.

### R[illegible]on and RT-qPCR

[illegible] h a[illegible]ransfection, TRIzol RNA isolation [illegible]nvitrogen, Carlsbad, CA, USA) was utilized to [illegible] the total mRNA from tissues and cells. The firs[illegible]nd complementary deoxyribonucleic acids (cDNAs) was synthesized using the Transcriptor first strand cDNA synthesis kit (TaKaRa Biotechnology Co., Ltd., Dalian, China) according to the ma[illegible]turer's instructions. The primer sequen[illegible] RT-qPCR were as follows: circ-SM[illegible]orwa[illegible] 3'-TGAGAAGAGAAATCTAT[illegible]AACC-5', circ-SMAD7, reverse: 3'-GGT[illegible]TCCGCTGCTTTA-5'; GAPDH primers for[illegible]CAATGACCCCTTCATTGACC-3[illegible]nd rev[illegible]TT GATTTTGGAGGGATC[illegible]-3′. The c[illegible] were as follows: 30 sec[illegible]5°C, 5[illegible] for 40 cycles at 95°C, 35 sec at 60[illegible]

### Wound Heal[illegible] Assay

After trans[illegible]on, the C[illegible]re seeded in 6-well pl[illegible] incubated in[illegible]M-1640 medium ov[illegible] the cells were scratched with a plastic tip [illegible]ltured in serum-free RPI[illegible]0. Each assa[illegible]peated in triplicate in[illegible]ndently. The relat[illegible]istance was viewed u[illegible] a light microscope (Olympus Corp., Tokyo, [illegible]) at 48 h.

### T[illegible]ll A[illegible]

A[illegible]on, $1\times10^5$ cells in 200 µL serum-free R[illegible]M-1640 were replanted in top chamber [illegible]orning, Inc., Corning, NY, USA). RPIM-1640 and [illegible]dded to the lower chamber. Then, they [illegible]ured overnight in an incubator supplemented with 5% $CO_2$ at 37°C. The top surface of the chambers was treated by methanol for 30 min after wiped by cotton swab. Next, they were stained in crystal violet for 20 min. Five fields were randomly chosen under a Leica DMI4000B microscope (Leica Microsystems, Heidelberg, Germany).

### Matrigel Assay

After transfection, $1\times10^5$ cells in 200 µL serum-free RPIM-1640 were replanted in the top chamber (Corning, Inc., Corning, NY, USA) with 50 µg Matrigel (BD, Bedford, MA, USA). RPIM-1640 and FBS were added to the lower chamber. Then, they were cultured overnight in an incubator supplemented with 5% $CO_2$ at 37°C. The top surface of the chambers was treated by methanol for 30 min after being wiped by cotton swab. Next, they were stained in crystal violet for 20 min. Five fields were randomly chosen under a Leica DMI4000B microscope (Leica Microsystems, Heidelberg, Germany).

### Western Blot Analysis

The reagent radio immunoprecipitation assay (RIPA, Beyotime, Beijing, China) was utilized to extract the protein from cells. The bicinchoninic

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acid (BCA) protein assay kit (TaKaRa, Otsu, Shiga, Japan) was chosen for quantifying the protein concentrations. The target proteins were separated by dodecyl sulfate, sodium salt-polyacrylamide gel electrophoresis (SDS-PAGE). Then, they were incubated with antibodies after that the polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA) was replaced. The Cell Signaling Technology (CST, Danvers, MA, USA) provided us the rabbit anti-GAPDH and rabbit anti-E-cadherin, rabbit anti-N-cadherin, rabbit anti-Vimentin, as well as goat anti-rabbit secondary antibody. The Image J (Media Cybernetics, Silver Springs, MD, USA) software was applied for the assessment of the protein expression.

### *Statistical Analysis*

The Statistical analysis was conducted by the Statistical Product and Service Solutions (SPSS) 17.0 (SPSS Inc., Chicago, IL, USA). GraphPad 4.0 (GraphPad Software, Inc., La Jolla, CA, USA) helped presenting these consequences. The Kaplan-Meier method and the independent-sample *t*-test was used when appropriate. The quantitative data was presented as mean ± SD (sta... deviation). Moreover, $p<0.05$ was consid... indicate a statistically significant differen...

## Results

### *The Association Betwee... irc- ...AD7 Expression and the P... osis ... CRC Patients*

Firstly, based on t... me... ression, the CRC patients were ... ed into ... ups, low ... igh circ-SMA... 7 groups. The CRC patients i... low circ-S... D7 expression group had a p... erall ... ival compared with those in the ... AD7 expression group ($p<0.05$; Figure 1).

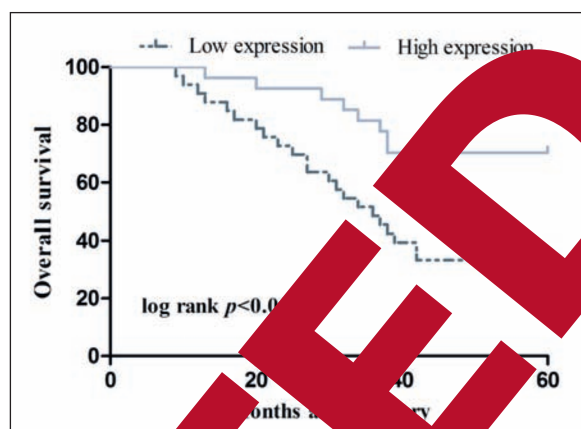
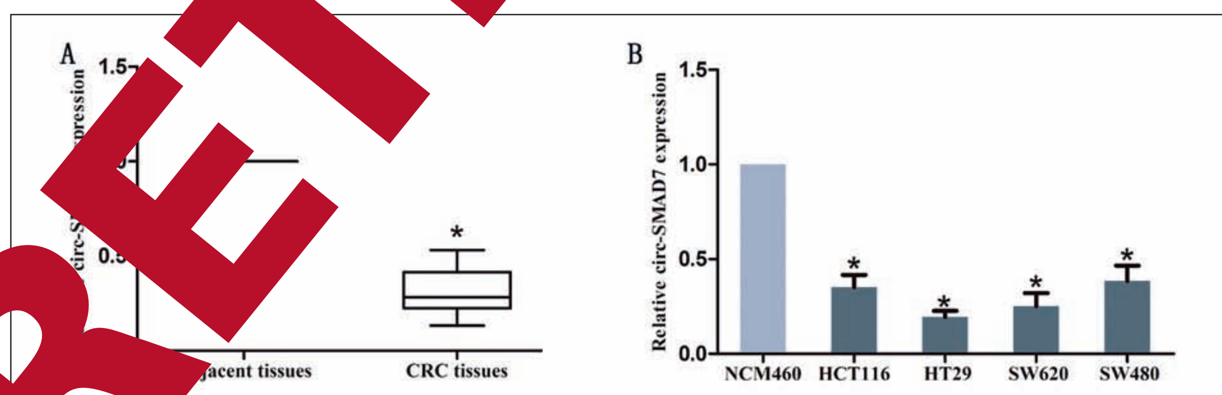



**Figure 1.** T... ion between ci... MAD7 expression level ... e ... of CRC patients. The expression levels ... circ-SM... positively associated with patien... rall survival ... 0.05.

### *...D7 Expression Level Was Lower ... Tissues and Cells*

The circ-SMAD7 expression was detected *via* RT-qPCR in 52 CRC patients' tissue samples and matched adjacent samples. The results showed that circ-SMAD7 was significantly lower-expressed in tumor tissue samples than that in the adjacent tissues (Figure 2A). The circ-SMAD7 expression in human CRC cell lines and NCM460 was also detected. The circ-SMAD7




...2. The expression levels of circ-SMAD7 were decreased in CRC tissues and cell lines. **A,** Circ-SMAD7 expression w... ...cantly decreased in the CRC tissues compared with adjacent tissues. **B,** The expression level of circ-SMAD7 rela... GAPDH was determined in the human CRC cell lines and normal human colonic epithelial cells (NCM460) by RT-qPCR. GAPDH was used as an internal control. The data are presented as the mean ± standard error of the mean. *$p<0.05$.

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expression level was lower in CRC cells than that in NCM460 (Figure 2B). The results suggested that circ-SMAD7 might function in the development of CRC.

### *Circ-SMAD7 Overexpression Inhibited Cell Migration and Invasion in CRC Cells*

In this study, we chose HT29 cells for the overexpression of circ-SMAD7 and the transfection efficiency was detected by RT-qPCR (Figure 3A). To explore how circ-SMAD7 affected the cell migration of CRC cells, the Wound healing assay and the transwell assay were performed. As shown in Figure 3B, the migrated length of the CRC cells was reduced after circ-SMAD7 was overexpressed. As shown in Figure 3C, the number of migrated cells was remarkably decreased after circ-SMAD7 was overexpressed in CRC cells. To explore how circ-SMAD7 affected cell invasion of CRC cells, the Matrigel assay was performed. As shown in Figure 3D, after circ-SMAD7 was overexpressed, the number of the invaded cells was remarkably decreased.

### *Circ-SMAD7 Overexpression Inhibite[illegible] EMT Process in CRC*

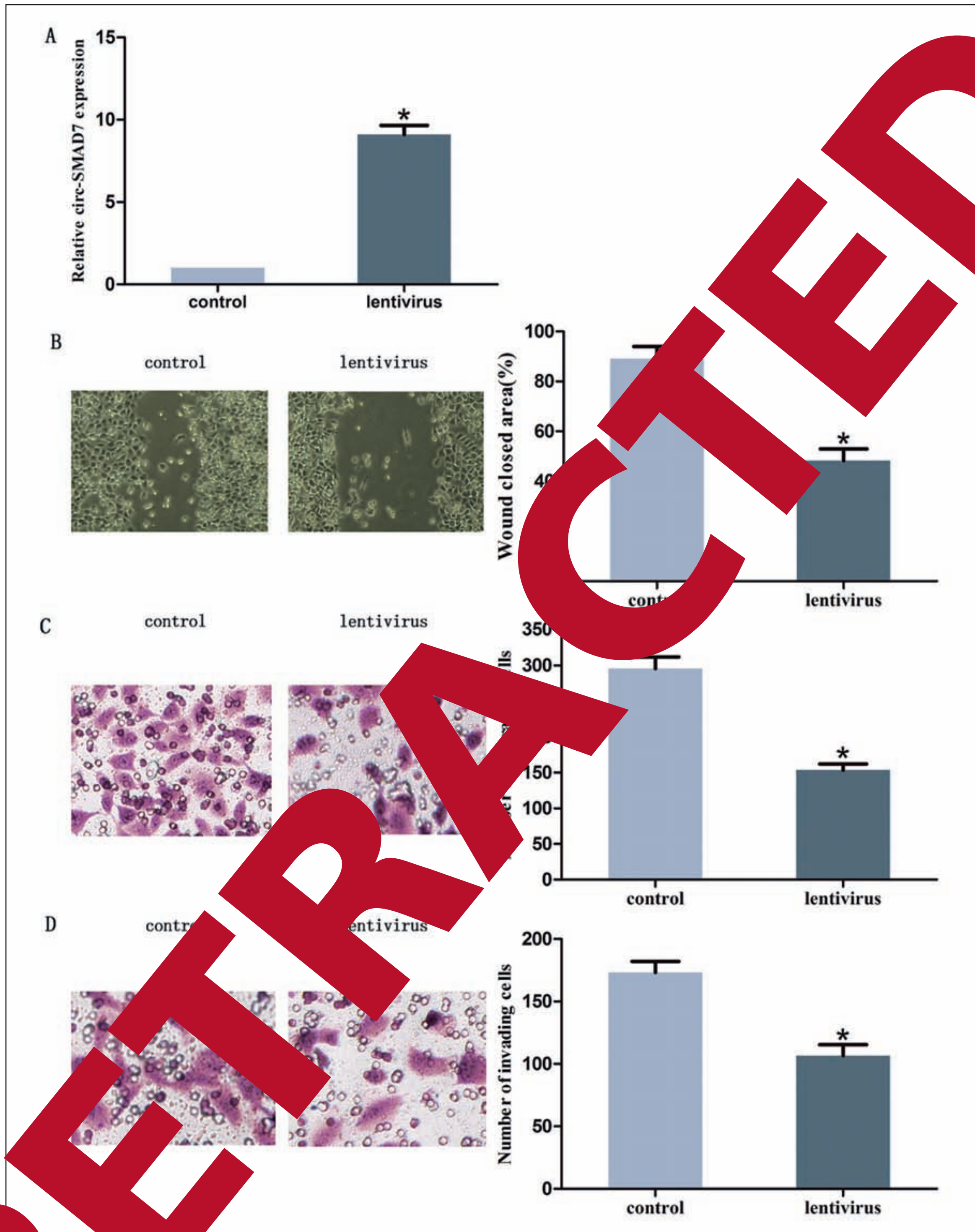
Previous studies have reported the key [illegible] of EMT process in cancer metastasis. In this [illegible] the effect of circ-SMAD7 on EMT process [illegible] further studied. The EMT-related [illegible]ins inclu[illegible] E-cadherin, N-cadherin, and [illegible] Previou[illegible] studies identified that E-cadh[illegible] was [illegible]egulated, and N-cadherin and Vimen[illegible] were d[illegible]ated when the EMT process [illegible] assay showed that the [illegible]pres[illegible]-cadherin in CRC cells was hig[illegible]n circ-S[illegible] lentivirus group than that i[illegible]ontrol group, [illegible]he expression of N-c[illegible] Vimentin in [illegible]RC cells was lower in [illegible]-SM[illegible]ntivirus group than that in the [illegible]ntrol group ([illegible]A). The Western blot assa[illegible]owed that the p[illegible] level of E-cadherin i[illegible]C cell [illegible]as upregulated in circ-SMAD7 lenti[illegible] grou[illegible]pared with that in the control grou[illegible] expr[illegible]n of N-cadherin and Vimenti[illegible]C cell[illegible]s downregulated in circ-[illegible]D7 le[illegible] compared with that in the [illegible] group ([illegible]4B).

## Discussion

[illegible] of the functions of circRNAs have been elu[illegible]ed over the past few years. The dysregulated expression of circRNAs has been reported as an important factor in the progression of CRC. For instance, the overexpression of [illegible] circ_0000069 enhances cell prolifera[illegible] migration in CRC[7]. Hsa_circ_0019[illegible] sign[illegible]cantly down regulated in CRC [illegible] may be a novel potential biomarker and [illegible]utic target for the CRC cases[8]. By promoting [illegible]ression of p16, the downregulatio[illegible] hsa_c[illegible] inhibits tumor growth in [illegible]rectal cance[illegible] circ_0071589 promot[illegible]arcino[illegible]esis *via* [illegible]e miR-600/EZH2 axi[illegible]olor[illegible] cancer[10]. By sponging miR-10[illegible] c[illegible]s over[illegible]essed in CRC which s[illegible]s as a[illegible]ene[illegible]RC[11].

Generated [illegible]n chromos[illegible] circRNA SMAD7 is [illegible] circRNA [illegible]n has been reported [illegible]ressed in the esophageal squam[illegible]s cell ca[illegible] and it inhibits tumor proli[illegible]on and mig[illegible]. To further determi[illegible]whether circ-SMA[illegible] participated in the r[illegible]ation of CRC metastasis, circ-SMAD7 was [illegible]xpressed in C[illegible]C cells. The Wound healing a[illegible]and the tra[illegible]ll assay were conducted in th[illegible]ted c[illegible]he results showed that circ-SM[illegible]pression repressed the cell migrated a[illegible] of CRC cells. Besides, the Matrigel [illegible]y was also conducted and the results showed [illegible]MAD7 overexpression repressed the [illegible]ded ability of the CRC cells. The above [illegible]ata indicated that circ-SMAD7 inhibited cell migration and invasion of CRC.

The epithelial-to-mesenchymal transition (EMT) is a crucial biological process involved in a multitude of developmental and pathological events. EMT is characterized by the progressive loss of the cell-to-cell contacts resulting in filopodia formation and mesenchymal gene expression which enable cell migration and invasion. EMT process could be regulated by circular RNAs. EMT is an important biological process in the progression of multitude cancers and characterized by the progressive loss of the cell-to-cell contacts which enables cell migration and invasion. Consistently, URG11 promotes cell proliferation and EMT in benign prostatic hyperplasia cells through RhoA/ROCK1 pathway[13]. Through the activation of ZEB1 and the interaction with miR-139-5p, lncRNA HCP5 enhances epithelial-mesenchymal transition in colorectal cancer[14]. Circ-10720 participates in the Twist1-mediated regulation of EMT and promotes the progression of hepatocellular carcinoma[15]. Moreover, circMTO1 inhibits tumor metastasis in bladder cancer by serving as miR-221 sponge and restraining EMT[16].




**Fig**[illegible] The over[illegible]pression of circ-SMAD7 inhibited CRC cell migration and invasion. **A,** Circ-SMAD7 expression in CR[illegible]ted with circ-SMAD7 lentivirus (lentivirus) or control was detected by RT-qPCR. GAPDH was used as an [illegible]**,** The Wound healing assay showed that the migrated length of the cells in circ-SMAD7 lentivirus group [illegible] significantly decreased compared with the control group in CRC cells (magnification: 40×). **C,** The transwell assay showed [illegible]erexpression of circ-SMAD7 significantly repressed cell migration in CRC cells (magnification: 40×). **D,** The Matrigel a[illegible]ved that overexpression of circ-SMAD7 significantly repressed cell invasion in CRC cells (magnification: 40×). The resu[illegible]present the average of three independent experiments (mean ± standard error of the mean). $^*p<0.05$, as compared with the control cells.

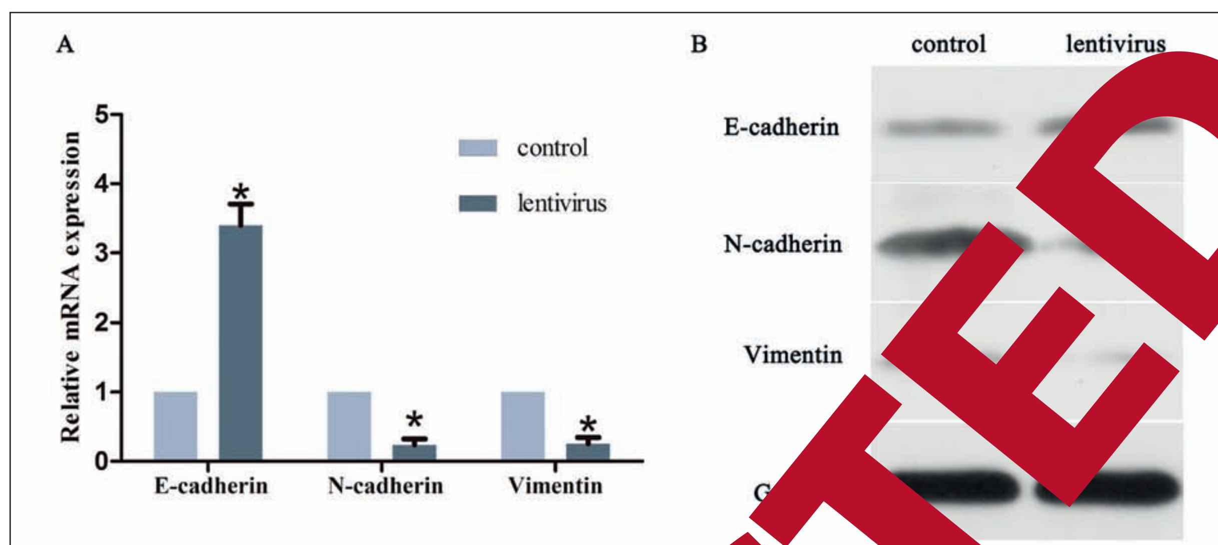



**Figure 4.** Circ-SMAD7 overexpression inhibited EMT process of C[illegible]. **A,** RT-qPCR assay [illegible]sed to detect the mRNA expression of E-cadherin, N-cadherin, and Vimentin of CRC cells i[illegible]-SMAD7 lentivirus group and control group. **B,** The Western blot assay was used to detect the protein level of E-cadher[illegible]cadherin, and Vimentin of CRC cells in circ-SMAD7 lentivirus group and control group. The data are presented as the m[illegible] standard error [illegible]e mean. $^*p<0.05$.

In the present study, the potential interaction between the EMT process and circ-SMAD[illegible] firstly studied. E-cadherin, N-cadherin, [illegible]mentin are vital proteins in the EMT proc[illegible]. By detecting the expression of those proteins i[illegible] cells, we found that those proteins could be [illegible]ulated by the overexpression of ci[illegible]-SMAD7. [illegible] these results indicated that c[illegible]D7 cou[illegible] inhibit the EMT process of C[illegible].

## Co[illegible]

The results of [illegible] study identi[illegible] circ-SMAD7 was [illegible] downregulat[illegible] in CRC tissues and wa[illegible]ocia[illegible] the prognosis of the CRC patie[illegible]. Circ-SMA[illegible]bited cell migration and [illegible]sion of CRC by [illegible]ting EMT process. T[illegible] findi[illegible] suggest that circ-SMAD7 may serv[illegible] pros[illegible]ve therapeutic target for CRC.

## [illegible]flict o[illegible]

[illegible]thors de[illegible]ey have no conflict of interests.

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