

RETRACTED ARTICLE: LncRNA HCP5 Promotes Cell Invasion and Migration by Sponging miR-29b-3p in Human Bladder Cancer

This article was published in the following Dove Press journal:
OncoTargets and Therapy

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Background: Bladder cancer (BC) is one of the most common malignant tumors in the urinary system. In this study, the roles of lncRNA HCP5 (human major histocompatibility complex p5) and miR-29b-3p in human BC were investigated. Their regulations involved in cell invasion and migration were also evaluated.

Methods: Luciferase reporter assay was performed to detect the binding between miR-29b-3p and HCP5 or high-mobility group box 1 (HMGB1). Cell viability, migration, invasion and apoptosis were assessed by CCK-8, colony formation, transwell assay and flow cytometry, respectively. Expression levels of HMGB1/toll-like receptor 4 (TLR4) proteins were measured by Western blot. Xenograft model was built, and tumor volumes and weights were calculated.

Results: The results revealed dysregulation of HCP5 and miR-29b-3p in BC samples and cells. HCP5 negatively regulated the expression of miR-29b-3p and enhanced cell viability, migration and invasion. miR-29b-3p mediated the effect of HCP5 on cell viability, proliferation, migration and invasion in RT4 cells. In addition, miR-29b-3p could regulate the expression of HMGB1 through interaction with HMGB1.

Conclusion: The findings in this study supported that lncRNA HCP5 could promote cell invasion and migration by sponging miR-29b-3p in human BC.

Keywords: lncRNA HCP5, invasions and migration, miR-29b-3p, human bladder cancer

Introduction

Bladder cancer (BC) is one of the most common malignant tumors in the urinary system.^{1,2} The incidence and recurrence rate of BC are both high but the cause is not fully clear.³ Therefore, clarification of the underlying molecular mechanisms will be beneficial to the diagnosis and treatment of BC. Recent studies have found that long non-coding RNAs (lncRNAs, >200nt) play important roles in the regulation of gene expression in normal cells and tumor cells.⁴ Abnormal expression of lncRNAs is closely related with tumorigenesis and cancer progression.^{3,5}

lncRNAs were originally thought to be “noise” or “transcriptional waste” of genomic transcription.^{6,7} With the deepening of researches and the development of sequencing technologies, more and more lncRNAs have been discovered.⁸ lncRNAs can regulate gene expression through multiple pathways, which then affect the occurrence and development of diseases including tumors.⁹ The involvement of lncRNAs in BC has also attracted more attention.¹⁰ lncRNAs can participate in the regulation of bladder tumor cell proliferation, invasion and metastasis

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through the action of oncogenes or tumor suppressor genes.¹¹ lncRNA can be used as a diagnostic and prognostic indicator for BC, and is expected to become a new target for disease treatment.^{11,12} lncRNA HCP5 (human major histocompatibility complex p5) has been reported as a novel genetic loci in clinical thyroid disease.^{13,14} lncRNA HCP5 has been verified to elicit tumor-promoting function including proliferation, migration and chemotherapy resistance in lung adenocarcinoma, colorectal cancer, pancreatic cancer and prostate cancer.^{15–19} lncRNA HCP5 has been verified to elicit tumor-promoting function including proliferation, migration and chemotherapy resistance in lung adenocarcinoma, colorectal cancer, pancreatic cancer and prostate cancer.^{15–19} However, the functions of lncRNA HCP5 in BC have not been studied. This study was to investigate the roles of lncRNA HCP5 in BC and the underlying mechanisms of cancer development and progression.

MicroRNAs (miRNAs, ~20 nt) are a class of non-coding small RNAs that are highly conserved in evolution.^{20,21} Increasing evidence has shown that miRNAs regulate gene expression at the post-transcriptional level and play important roles in cell proliferation and tumor formation.²² Down-regulation of tumor-inhibiting miRNAs or up-regulation of miRNAs in tumors can cause tumor formation.²³ MiR-29 family, including miR-29a, miR-29b and miR-29c, participated various cytological behavior in BC occurrence and development, such as cells proliferation, migration, and invasion, epigenetic modification, and epithelial-to-mesenchymal transition (EMT).^{24,25}

High-mobility group box 1 (HMGB1) is a member of the HMGB protein family, is expressed to some extent in the cytoplasm, and shuttles back and forth from the nucleus. Extracellular, all-nial HMGB1 may be oxidized to the disulfide form of HMGB1, which then binds to Toll-like receptor 4 (TLR4) to induce cytokine production. Intracellular HMGB1 is extensively bound to DNA and involved in transcriptional regulation, DNA replication and repair, telomere maintenance, and nucleosome assembly. Studies showed that the relationship between miRNAs and occurrence of tumors by targeting HMGB1.^{26,27}

In the present study, the roles of lncRNA HCP5 and miR-29b-3p in human BC were investigated. Their regulations involved in cell invasion and migration were also assessed by targeting HMGB1/TLR4.

Materials and Methods

Tissue Samples and Cells Information

A total of 28 BC patients (22 males and 6 females, with the age of 55–70 years old) admitted at Xiangya Hospital, Central South University between 2018 and 2019 were included in this study. Bladder tumor tissues and adjacent normal tissues were collected. None of the patients received chemotherapy or radiotherapy before surgery. For tumor size, 14 patients had tumors less than 3 cm, and 16 of them were diagnosed at pathology stages of pTa-Pt1, and 12 at pT2-T4. For lymphatic metastasis, 10 patients were identified to have metastasis (Supplemental Table 1). HUC4449 cells were purchased from Sciencell, USA. RT4, 5637, HT1197 and A24 cells were provided by ATCC, USA. Cells were cultured in the Dulbecco's Modified Eagle's Medium (DMEM, USA) with 10% FBS at 37°C with 5% CO₂. This study was approved by the Ethics Committee of Xiangya Hospital, Central South University. The informed consent was obtained from all the patients.

Cell Transfections

sh-HCP5 was ligated into the U6/GFP/Neo plasmid (GenePharma, China). The plasmid carrying a non-targeting sequence was used as negative control (sh-NC). The pCHCP5 construct was produced by GenePharma, China. Cells were transfected with lipofectamine 3000 (Life Tech. USA). Cells were then cultured with G418 (Sigma, USA) and harvested after 35 days. MiR-29b-3p-mimic, NC-mimic, miR-29b-3p-inhibitor and inhibitor NC were produced by GenePharma and were transfected to RT4 cells. Cells were collected after 3 days of transfection.

CCK-8 Assay for Cell Viability

Cells were plated in 96-well plates (5000 cells/well). Cell Counting Kit-8 (CCK-8) was used and 10 μ L CCK-8 solution was added for incubation at 37°C with 5% CO₂ for 1 hour. UV-vis was used to measure the absorbance at 450 nm.

Apoptotic Cells

Apoptotic cells were analyzed using Annexin V-FITC/PI (Invitrogen, USA). Cells were washed, re-suspended, and stained by 5 μ L of Annexin V-FITC and 10 μ L of PI. After 15 min of incubation in dark, cells apoptosis was measured through a FACScan (Beckman Coulter, USA) at 530 nm wavelength.

Transwell Assay for Cell Migrations and Invasion

For migration assays, 24-well plates were placed below Boyden chambers containing transwell membrane filters (Millipore Bio; Massachusetts, US). For invasion assays, a diluted Matrigel solution (356,234; BD Biosciences, San Jose, CA, USA) was used. Approximately 1×10^5 transfected cells were plated on 8 mm pore size top chambers in 200 μ L serum-free, EGF-free RPMI 1640 medium. The bottom chamber was filled with 800 μ L RPMI 1640 medium containing 10% FBS. Migration and invasion were assayed, and the cells were stained after 48 hours. The experiment was repeated 3 times.

qRT-PCR

Total RNAs were extracted using Trizol reagents (Invitrogen). Expression levels of miR-29b-3p were determined using TaqMan Kit. U6 was internal reference. Expression levels were calculated with the $2^{-\Delta\Delta Ct}$ method. Primers used are listed as following: GAPDH, forward, 5'-GAAGGTGAAGGTCGGAGTC-3', reverse, 5'-GAAGATGGTGTGGGATTTC-3'; U6, forward, 5'-CTCGCTTCGGCAGCAC-3', reverse, 5'-AACGCTTCACGAATTTGCGT-3'; miR-29b-3p, forward, 5'-ACACTCCAGCTGGGTAGCACCATTGAAATC-3', reverse, 5'-GTCGTATCCAGTGCCTGTCGTGGATTC-3'; HCP, forward, 5'-CAGCCTGAGAAAGAGGCG-3', reverse, 5'-TCAGTCGCATTTCCAGGTAAAT-3'; HMGB1, 5'-ACAAGGCCCGTTATGAAAGAG-3', reverse, 5'-CAAGAGGAAGAAGGCCGAAG-3'; TLR4, forward, 5'-GAGCCGGAAGGTTATTGTGGAC-3', reverse, 5'-TCAAGGACAATGAAGATGATGCCAGAC-3'.

Luciferase Reporter Assay

A fragment of the 3'-UTR of HCP5 or HMGB1, and muted HCP5 or HMGB1 that contains the binding site for miR-29b-3p was cloned into pMir-report vector (Ambion, USA). Cells were co-transfected with HCP5-wt/HCP5-mut or HMGB1-wt/HMGB1-mut and miR-29b-3p mimic/mimic NC using Lipofectamine 3000. A Dual GloTM Luciferase Assay System (Promega) was used to measure the luciferase activity.

PCR was performed to amplify the partial sequences of HCP5 which contained the putative binding sites of miR-29b-3p. The sequences were then cloned into the pmirGLO Dual-Luciferase miRNA Target Expression Vector (Promega Corp., Fitchburg, WI, USA). Gene ArtTM Site-Directed Mutagenesis system (Thermo Fisher Scientific, Inc.) was

used to induce site-directed mutagenesis of miR-29b-3p complementary bases in the sequences of HMGB1. Then, cells were transfected with the constructed wild-type (WT) and mutant (MUT) reporter vectors, respectively, in the presence of miR-29b-3p mimics or miR-NC. The activity of luciferase was measured using the Dual-Luciferase Assay system (Promega) and then normalized to *Renilla* luciferase activity based on the manufacturer's instructions. The assay was performed in triplicate experiments.

RNA Immunoprecipitation (RIP)

RIP was performed using GFP antibody (Abcam, CA), IgG antibody (Millipore, USA) and RIP kit (Millipore). For anti-AGO2 RIP, RT4 cells were transfected with miR-29b-3p or NC. After 72 hours, cells were lysed using an AGO2 antibody (Millipore).

Western Blotting

Cell lysates were prepared. Proteins were separated and transferred to PVDF membrane (Bio-Rad, USA). After incubation with anti-HMGB1 (1:1000, abcam, UK) and anti-TLR4 (1:1000, abcam, UK) at 4°C for overnight, the membrane was incubated with goat anti-rabbit IgG secondary antibody and protein signals were detected with chemiluminescence. GAPDH was used as an endogenous control.

Xenograft Mouse Model

Mouse experiment was performed according to Animals Usage Guideline by the Committee of Xiangya Hospital, Central South University. And the study has been approved by the Ethics committee of Xiangya Hospital, Central South University (No. XH202065433). Male athymic BALB/c nude mice (4–5 weeks old) were used. To detect the effect of HCP5 or miR-29a-3p on the growth and migration of BC cells in vivo, the HCP5 lentivirus vector or miR-29a-3p inhibitor were stably transfected to luciferase labeled RT4 cells. Transfected cells were harvested and 2×10^6 cells were injected subcutaneously into each mouse used (six to eight-week-old mice). Tumors were collected six weeks after injection. Both the weight and volume of the subcutaneously-induced tumors were measured, and the number of organic tumors formed was counted.

Statistical Analysis

All experiments were performed in triplicates. Data analysis was carried out using Graphpad Prism 6.0 (GraphPad, USA). The data were shown as mean \pm standard deviation (SD). The P-values were obtained using Student's *t*-test,

one-way analysis of variance (ANOVA), and Pearson correlation coefficient test. Newman–Keuls tests were used post hoc multiple comparison after ANOVA.

Results

Dysregulation of HCP5 and miR-29b-3p in BC Samples and Cells

Our data presented that the expression of HCP5 was elevated in bladder tumor samples compared to that in normal samples ($P < 0.001$) (Figure 1A). The relationship of HCP5 and miR-29b-3p in urinary BC samples was reversely correlated (Figure 1C). To explore the clinicopathological value of HCP5 in BC, the relationship between HCP5 expression and clinicopathological features of bladder cancer patients were analyzed by Student's *t*-test. There is no significant difference between HCP5 levels and in patients with high histological grade, tumor lymphoid node metastasis or tumor stage T2-4 (Supplementary Table 1).

Expression of HCP5 was significantly upregulated in BC cells compared to normal cells ($P < 0.05$, $P < 0.001$) (Figure 1D). The expression of miR-29b-3p was markedly inhibited in BC samples ($P < 0.001$) (Figure 1B) and cells ($P < 0.001$) (Figure 1E).

HCP5 Enhanced Cell Viability, Migration and Invasion

Expression levels of lncRNA HCP5 were detected by qRT-PCR in RT4 cells transfected with pc-NC, pcHCP5, sh-NC or shHCP5 (Figure 2A). The effect of HCP5 on cell viability of RT4 cells was detected by CCK-8 assays. As shown in Figure 2B and C, transfections of pcHCP5 markedly promoted cell viability and proliferation in RT4 cells in comparison with transfections of pcNC. Transfections of shHCP5 markedly decreased cell viability and proliferation in RT4 cells in comparison with transfections of shNC. Transwell assay demonstrated that transfections of pcHCP5 markedly promoted

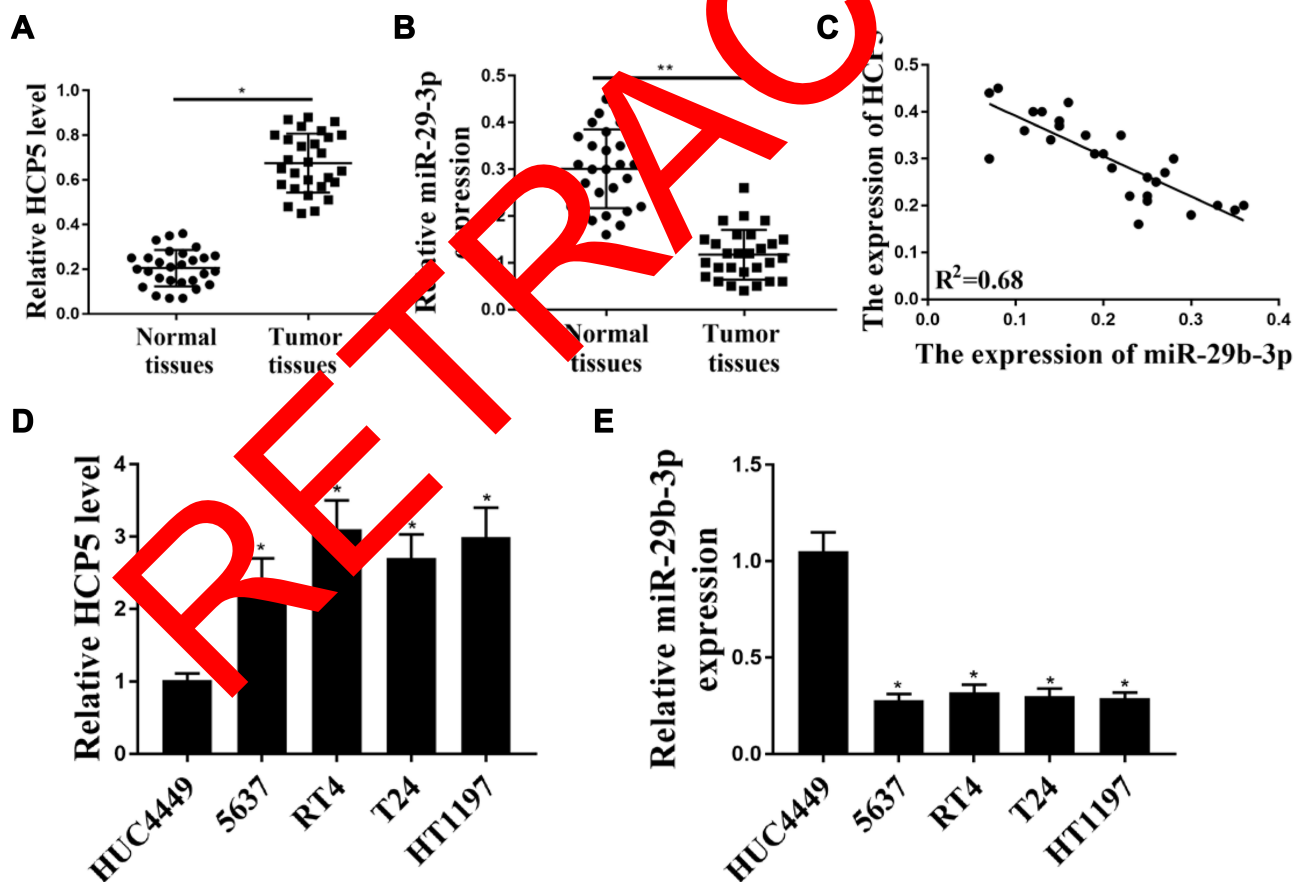


Figure 1 Dysregulated HCP5 and miR-29b-3p in BC samples and cells. (A and B) HCP5 was overexpressed and miR-29b-3p was lower expression in BC tissues compared with adjacent normal tissues; $N=28$. (C) The relationship of HCP5 and miR-29b-3p in urinary BC samples. (D and E) HCP5 and miR-29b-3p mRNA expression in BC cell lines. $N=3$; * $P < 0.001$. ** $P < 0.05$.

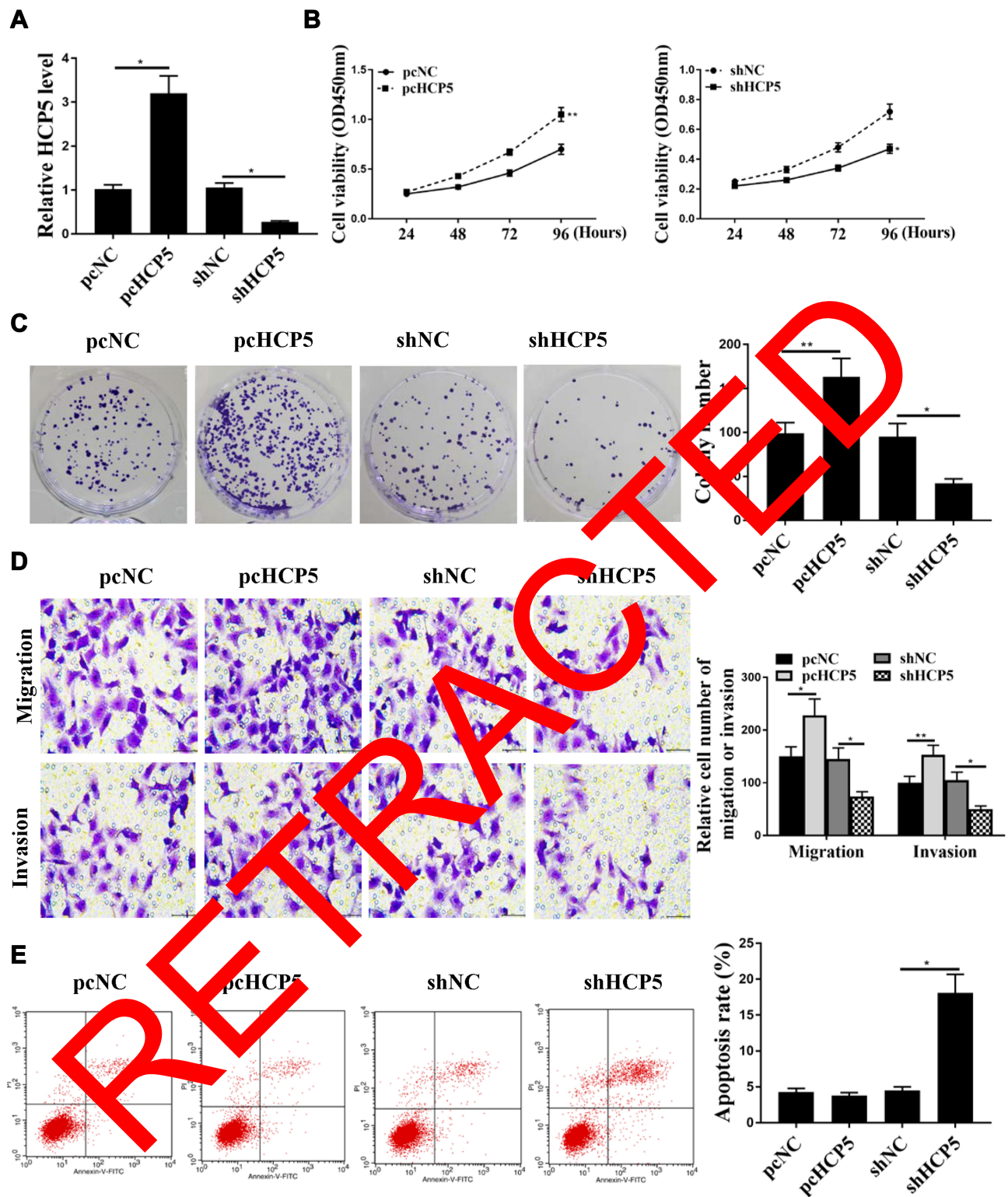


Figure 2 HCP5 enhanced viability, migration and invasion. (A) qPCR assay of HCP5 expression in RT4 cells. (B) CCK-8 assay for cell viability under pcHCP5. (C) Colony formation for the colony ability of cells under pcHCP5 or shHCP5. (D) Transwell assay for cell migration and invasion under pcHCP5 or shHCP5. (E) Flow cytometry used to determine apoptotic cells. N=3; * $P < 0.001$, ** $P < 0.05$.

migration and invasion of RT4 cells in comparison with transfections of pcNC, while transfections of sh-HCP5 had the reverse effects (Figure 2D). As shown in Figure 2E,

transfections of pc-HCP5 had no effect on cell apoptosis of RT4; however, transfections of sh-HCP5 markedly increased cell apoptosis.

HCP5 Negatively Regulated the Expression of miR-29b-3p

Bioinformatic analysis predicted that HCP5 could bind to miR-29b-3p (Figure 3A). Expressions of miR-29b-3p in RT4 cells were suppressed in miR-29b-3p inhibitor group that indicated successful transfection (Figure 3B). The

luciferase activities in HCP5-wt cells had transfection with miR-29b-3p mimic notably decreased, in contrast to that of HCP5-wt cells transfected with miR-29b-3p inhibitor increased the luciferase activities (Figure 3C). HCP5 RIP in RT4 cells was enriched under miR-29b-3p compared with Ago2 and IgG (Figure 3D). As shown in Figure 3E,

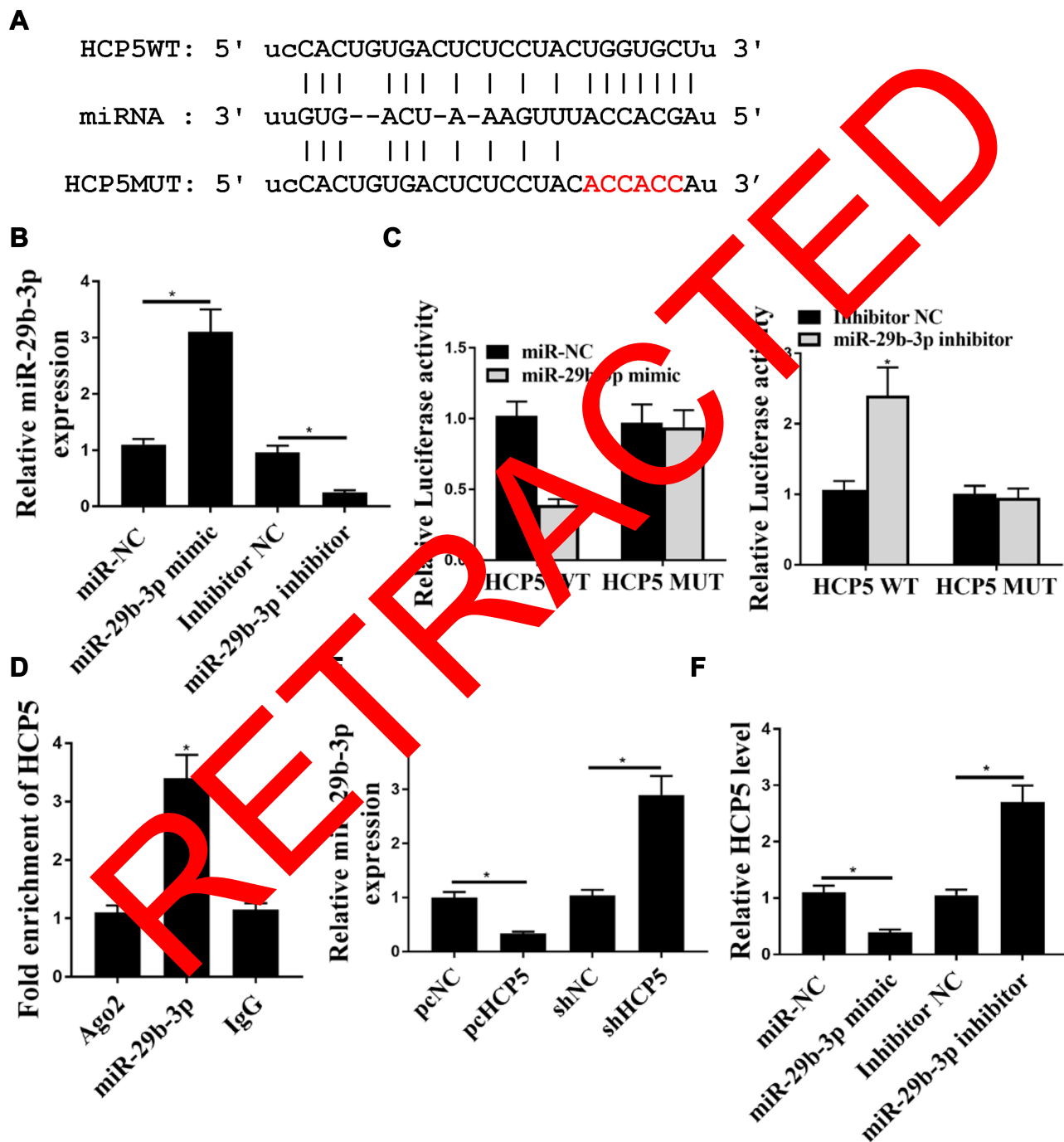


Figure 3 HCP5 negatively regulate the expression of miR-29b-3p. (A) Bioinformatic analysis was performed to identify putative binding sites between HCP5 and miR-29b-3p. (B) miR-29b-3p expression in RT-4 cells. (C) Relative luciferase activities in HCP5-WT and HCP5-MUT group. (D) HCP5 RIP in RT4 cells. (E) miR-29b-3p expression levels in RT-4 cells transfected with pcHCP5 and shHCP5. (F) HCP5 expression in RT-4 cells transfected with miR-29b-3p mimic and inhibitor. N=3; *P< 0.001.

transfections of pcHCP5 markedly reduced the expression of miR-29b-3p, while transfections of sh-HCP5 markedly increased the expression of miR-29b-3p in RT4 cells. What is more, that transfection of miR-29b-3p mimic clearly reduced the expression of HCP5, while transfection of miR-29b-3p inhibitor increased HCP5 in RT4 cells (Figure 3F). These results indicated that HCP5 could negatively regulate the expression of miR-29b-3p.

MiR-29b-3p Mediated the Effect of HCP5 on Cell Viability, Proliferation, Migration and Invasion in RT4 Cells

As shown in Figure 4A and B, shHCP5 decreased cell viability and proliferation, which were attenuated by miR-29b-3p inhibitor. Transwell assay showed the same results for cell migration and viability (Figure 4C). Furthermore, shHCP5 induced apoptotic cells and miR-29b-3p inhibitor could reduce cell apoptosis (Figure 4D).

MiR-29b-3p Negatively Regulated the Expression of HMGB1

Bioinformatic analysis was performed to identify potential binding site between HMGB1 and miR-29b-3p (Figure 5A). The binding of miR-29b-3p with HMGB1 in RT4 cells was evaluated by luciferase reporter assay. MiR-29b-3p reduced the luciferase activities of HMGB1-wt construct (Figure 5B), but luciferase activities in cells transfected with the miR-29b-3p-mimic in HMGB1-wt were similar with that of control cells. As shown in Figure 5C, overexpression of miR-29b-3p reduced the expression of HMGB1 and TLR4, while suppression of miR-29b-3p had reverse effects. And shHCP5 reduced the expression of HMGB1 and TLR4, while pcHCP5 had reverse effect (Figure 5D). Then, HMGB1 and TLR4 were up-regulated in BC tumor tissues and BC cells (Supportary Figure 1).

HCP5 Promoted the Tumorigenesis of BC in vivo

Our data indicated that shHCP5 remarkably reduced the tumor volume and weight at the beginning of the second week compared to that in the control group (Figure 6A–C). MiR-29b-3p inhibitor could blunt the effects of shHCP5 on tumor growth. Ki-67 staining showed the same results (Figure 6D). Western blot analysis of HMGB1/TLR4 expression in tumor samples (Figure 6E).

Discussion

Recent studies have confirmed that certain lncRNAs are up-regulated in tumors, while other lncRNAs are down-regulated and have cancer-promoting or tumor-suppressing effects.²⁸ As a carcinogenic or tumor suppressor gene, researchers have studied lncRNAs associated with BC, including H19,²⁹ UCA1,⁴ MALAT-1,³⁰ MEG3,⁵ and SNHG16.³¹ In our study, we demonstrated that the expression of lncRNA HCP5 was elevated in bladder tumor samples and cells.

lncRNAs play regulatory roles in tumors by affecting the expression of downstream genes through interfering with the binding of transcription factors to promoters, inducing protein modification, and promoting chromosome remodeling.³² For example, lncRNA HXY4-IT1 has been reported to sponge miR-201-3p to promote proliferation and metastasis of BC cells through up-regulating EZH2.³³ Our bioinformatic analysis predicted that HCP5 bound to miR-29b-3p. The luciferase activities revealed that miR-29b-3p mimic was notably decreased compared with HCP5-wt cells. HCP5 RIP in RT4 cells was enriched under miR-29b-3p in comparison with the control. Transfections of pcHCP5 markedly reduced the expression of miR-29b-3p. Transfections of sh-HCP5 markedly induced the expression of miR-29b-3p in RT4 cells. In consistent with previous researches, our results established that HCP5 negatively regulated the expression of miR-29b-3p as a miR-29b-3p sponge.

lncRNA H19 was reported to enhance BC metastasis by interacting with EZH2 and suppressing E-cadherin expression.³⁴ Up-regulated H19 levels promoted BC cell migration.³⁴ Our results showed that transfections of sh-HCP5 markedly decreased cell viabilities and proliferation in RT4 cells compared with transfections of sh-NC. Transfections of pcHCP5 markedly promoted migration and invasions of RT4 cells compared with transfections of pcNC, while transfections of sh-HCP5 had the reverse effect. For the first time, we found that HCP5 enhanced viability, migration and invasion of BC cells.

It is well established that lncRNA H19 could regulate the metastasis of BC by binding with miR-29b-3p.²⁴ Also, it has reported that miR-29b had critically cancer suppressor effects; however, in some certain conditions, it might also act as an oncogene.³¹ It may depend on the situation, such as the inter regulation of genes. We found that shHCP5 decreased cell viabilities and proliferation, and these effects were attenuated by miR-29b-3p inhibitor. In

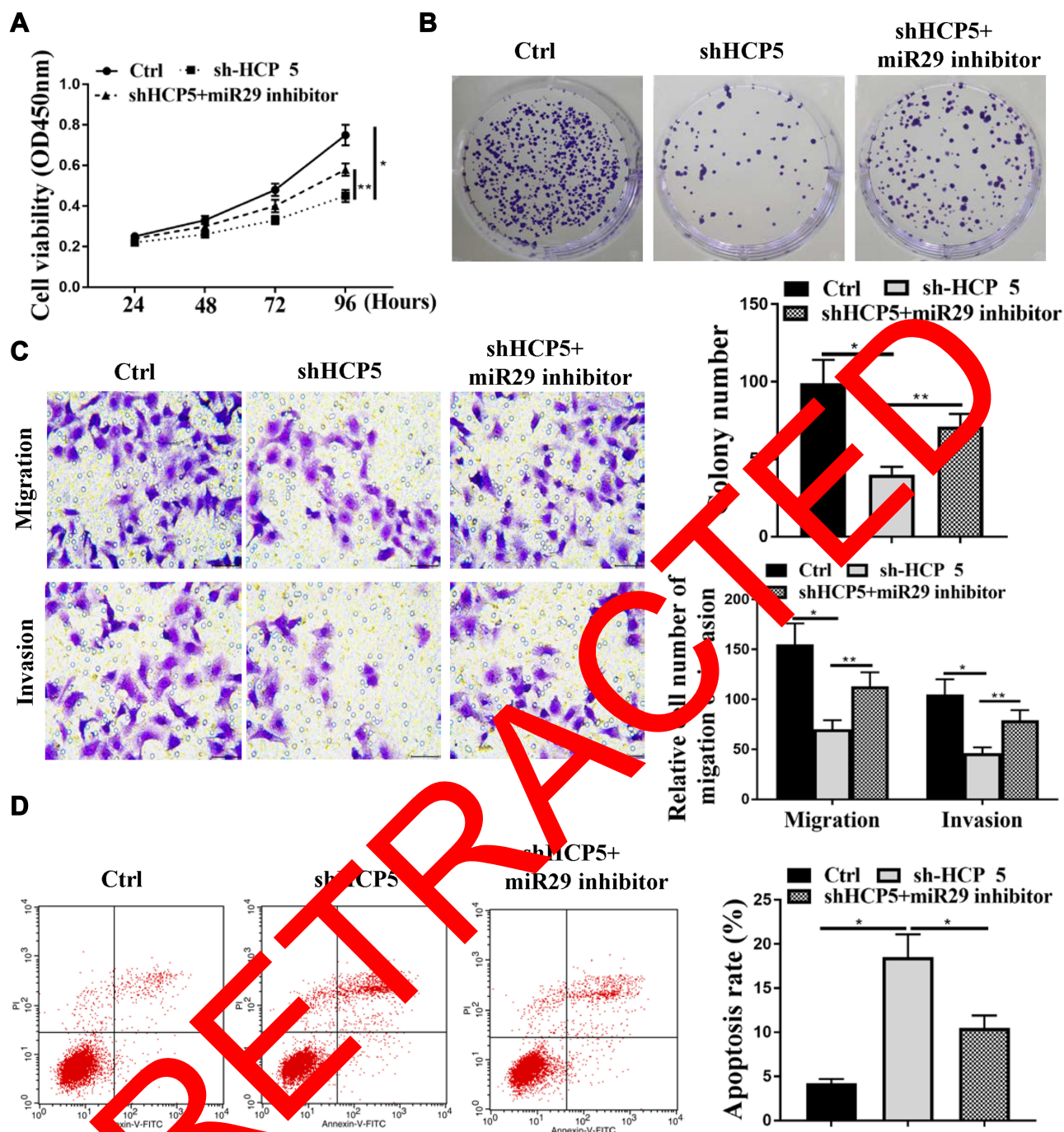


Figure 4 MiR-29b-3p mediated the effect of HCP5 on cell viability, proliferation, migration and invasion in RT4 cells. (A) CCK-8 assays used to detect cell viability. (B) Colony formation used to detect cell proliferation. (C) Transwell assay for cell migration and invasion. (D) Flow cytometry to determine apoptotic cells. N=3, *P< 0.001, **P<0.05.

addition, shHCP5 induced apoptotic cells and miR-29b-3p inhibitor could reduce apoptotic cells. Therefore, we concluded that miR-29b-3p could mediate the effect of HCP5 on cell viability, proliferation, migration and invasion in RT4 cells.

HMGB1 was demonstrated to successfully induce autophagy and facilitate liver fibrosis.³⁵ Previous studies

have indicated that miR-29b-3p targeted and inhibited the expression of HMGB1.³⁶ Our results revealed that miR-29b-3p decreased the luciferase activities of HMGB1-wt construct. Overexpression of miR-29b-3p reduced the expression of HMGB1 and TLR4. Inhibition of miR-29b-3p had an inverse effect. shHCP5 reduced the expression of HMGB1 and TLR4, while pcHCP5 had an inverse

A HMGB1WT: 5' aacauugcaaguauucGGUGCUa 3'
 |||||
 miRNA : 3' uugugacuaaaguuuaCCACGAu 5'
 HMGB1MUT: 5' aacauugcaaguauucCCACCAa 3'

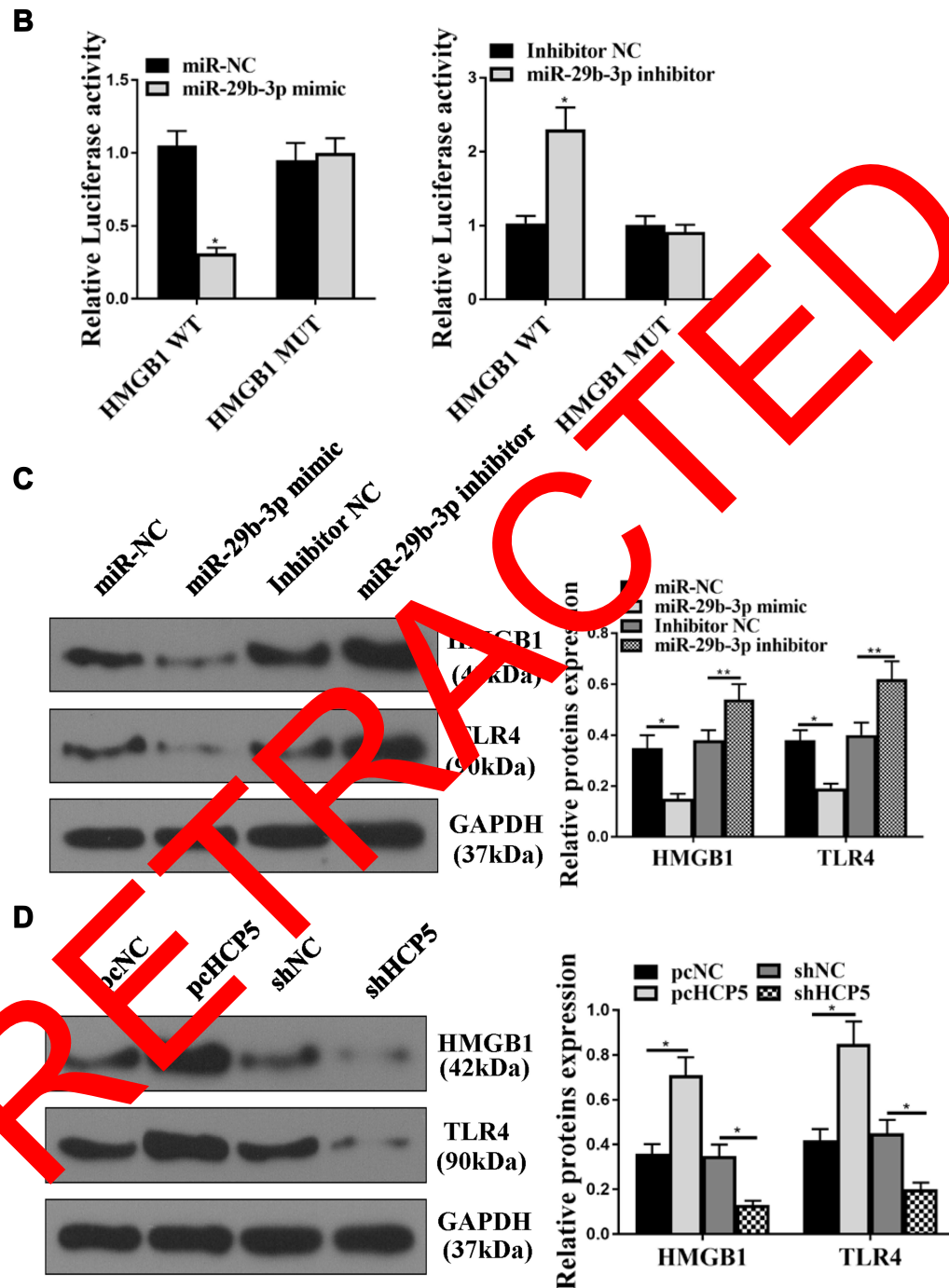


Figure 5 MiR-29b-3p negatively regulated the expression of HMGB1 by targeting HMGB1. (A) Bioinformatic analysis was performed to identify putative binding sites between HMGB1 and miR-29b-3p. (B) Relative luciferase activities in HMGB1-WT and HMGB1-MUT construct. (C and D) Western blot analysis of HMGB1 and TLR4 protein levels. N=3; *P< 0.001,** P<0.05.

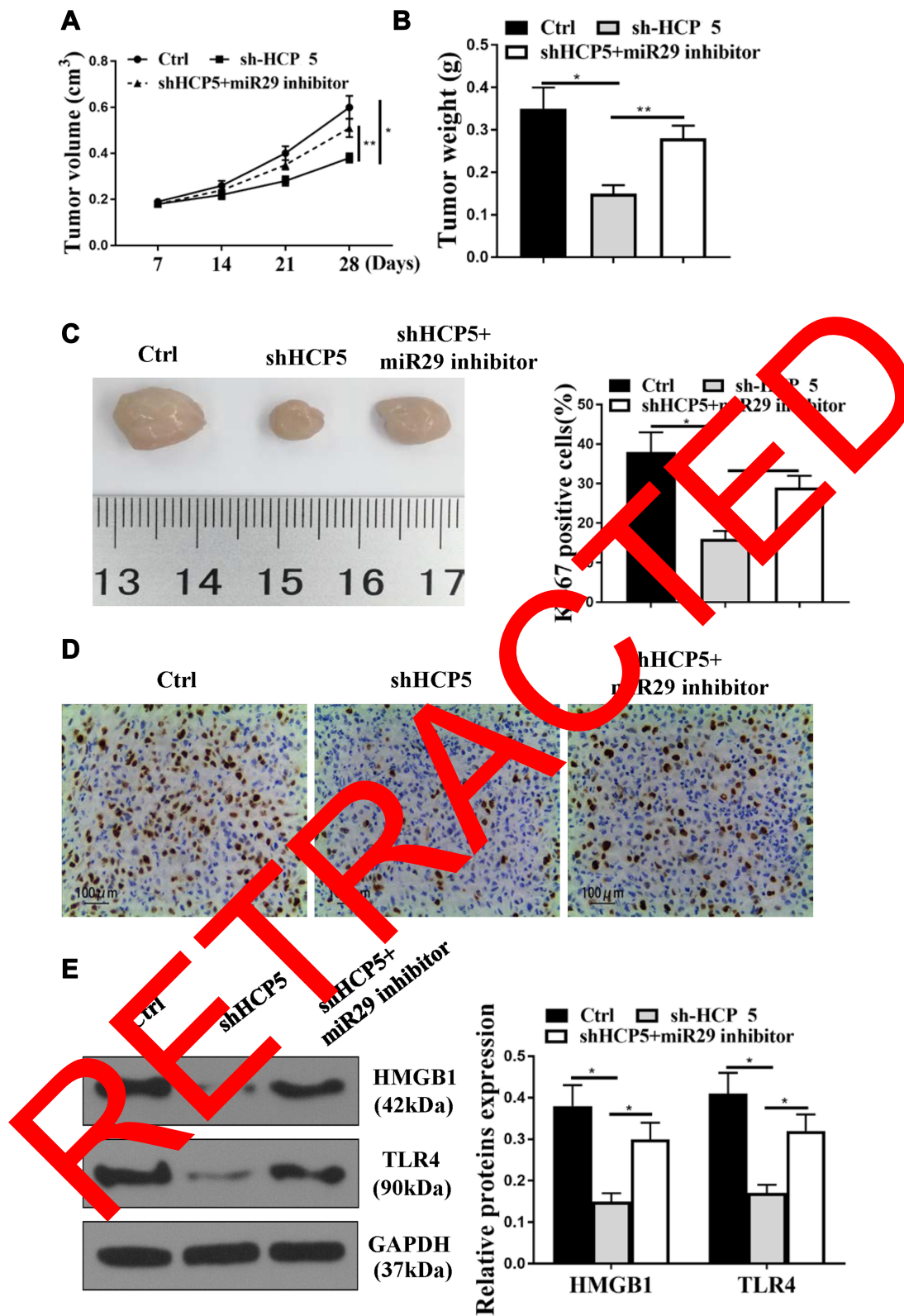


Figure 6 HCP5 promoted tumorigenesis of BC in vivo. **(A)** Tumor volume was detected every 7 days, and growth curves were drawn. **(B)** Tumor weight at the beginning of the second week. **(C)** Representative images of mice bearing tumors. **(D)** Representative images for Ki67 immunostaining of tumor samples from different groups (x200). **(E)** Western blot analysis of HMGB1 and TLR4 in tumor samples. N=5, *P<0.001, **P<0.05.

effect. Similar to what Zhang et al reported,³⁶ we found that miR-29b-3p negatively regulated the expression of HMGB1 by targeting HMGB1.

The in vivo experiments could provide a strong support to the role of the lncRNA on the development of cancer. According to our experiments, it indicated that shHCP5 reduced the tumor volume and weight. MiR-29b-3p inhibitor could blunt the effects of shHCP5 on tumor growth.

Conclusions

The findings in this study demonstrated that lncRNA HCP5 could promote cell invasion and migration by sponging miR-29b-3p in human BC.

Funding

There is no funding to report.

Disclosure

The authors declare that they have no competing interests.

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