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SP1-induced upregulation of long non-coding RNA HCP5 promotes the development of osteosarcoma

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ABSTRACT

Long non-coding RNAs (LncRNAs) are acknowledged as crucial regulators in tumorigenesis and tumor progression. In this study, we explored the mechanism and function of lncRNA HCP5 in osteosarcoma (OS). At first, five lncRNAs were chosen from GeneCard and subjected to qRT-PCR examination. The results indicated that HCP5 was significantly overexpressed in four OS cell lines. Northern blot assay further proved the higher expression of HCP5 in OS cell lines. To identify the biological role of HCP5 in OS, we silenced the expression of HCP5 in U2OS and MG-63 cells which possessed the highest level of HCP5. CCK-8 and colony formation assay revealed the inhibitory effect of HCP5 knockdown on cell proliferation. Cell apoptosis was found to be increased in cells transfected with sh-HCP5#1. Moreover, cell invasion and epithelial-mesenchymal transition (EMT) were reversed by the silencing of HCP5. The results of functional assays showed that HCP5 acted as an oncogene in osteosarcoma. Mechanically, HCP5 was found to be activated by the transcription factor SP1. Finally, rescue assays were conducted to demonstrate the function of SP1/HCP5 axis in osteosarcoma. In conclusion, we confirmed that SP1-induced upregulation of long non-coding RNA HCP5 promotes the development of osteosarcoma.

1. Introduction

Osteosarcoma (OS) is a common malignant bone tumor occurs in the metaphysis of long bones of adolescents [2]. Fast growth and metastasis are two leading causes of the poor prognosis of osteosarcoma [25]. Therefore, finding the novel molecular mechanisms correlated with the tumor proliferation and migration is compelling needed.

Non-coding RNAs (ncRNAs) are crucial modulators in various human diseases, especially malignant tumors. Based on size, ncRNAs are mainly classified into two groups: long non coding RNAs (lncRNAs) with length over 200 nt and microRNAs (miRNAs) with length approximately 22 nt. Recent years, lncRNAs have been widely reported in human cancers [1,4,10,12,28,41,42,47] including osteosarcoma [11,24,34,37]. Dysregulation of lncRNAs are correlated with various biological processes of human cancers, such as proliferation, apoptosis, invasion, migration and EMT process [7,17,39,45]. In this study, we tried to investigate the role of a certain lncRNA in osteosarcoma. At first, we chose five OS-related lncRNAs from GeneCard. Moreover, these five lncRNAs were all rarely reported in osteosarcoma. Next, these five lncRNAs subjected to qRT-PCR analysis. The significant high expression of HCP5 was found in OS cell lines. Northern blot was further used to detect the relative higher expression of HCP5 in OS cell lines. As a member of lncRNAs family, HCP5 has been studied in human malignant tumors due to its dysregulation and oncogenic role [20,31,43]. Nevertheless, it is unclear whether HCP5 can regulate cellular processes in osteosarcoma. To determine the biological function of HCP5 in osteosarcoma, we designed and conducted loss-of-function assays in U2OS and MG-63 cells which possessed the highest expression level of HCP5. MTT and colony formation assay revealed the inhibition of HCP5 knockdown on cell proliferation. Whereas, flow cytometry analysis showed the effect of HCP5 on cell apoptosis. Moreover, the effects of HCP5 on cell invasion and EMT progress were determined. Based on the results of functional assays, we identified that upregulation of HCP5 promotes OS progression.

To detect the mechanism contributed to the upregulation of HCP5 in OS cells, mechanism investigation was conducted. It is well known that lncRNAs can be upregulated by their upstream transcription activators [8,21,26]. In this study, we tried to find the transcription activator of HCP5 in OS. STAT3, SP1, E2F1 and NF-kB1 are four common transcription activators which had been reported in various human cancers

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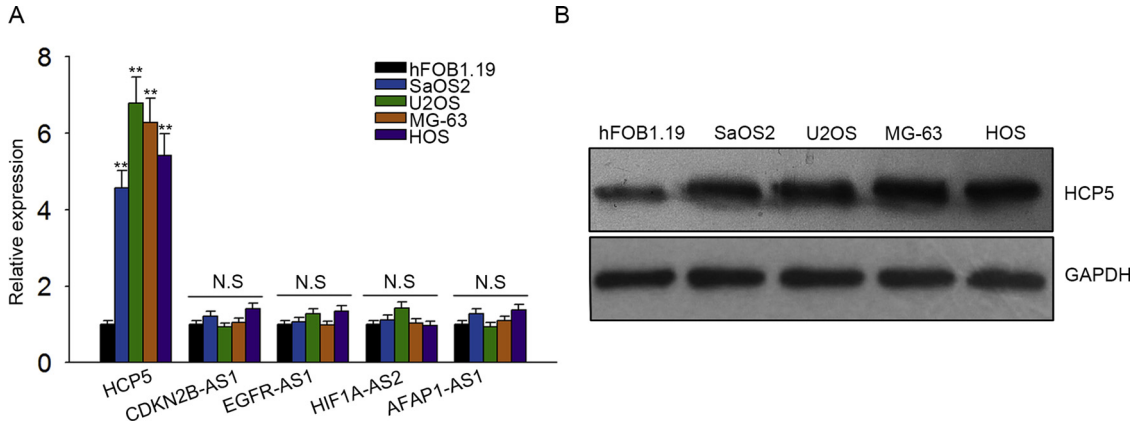

Fig. 1. HCP5 was overexpressed in OS cell lines. A. Five lncRNAs chosen from GeneCard were subjected to qRT-PCR examination in one human normal osteoblast hFOB1.19 and four OS cell lines (SaOS2, HOS and U2OS and MG-63). B. Northern blot analysis was used to detect the expression level of HCP5 in same cell lines. $^{**}P < 0.01$ vs control group. N.S: no significance.

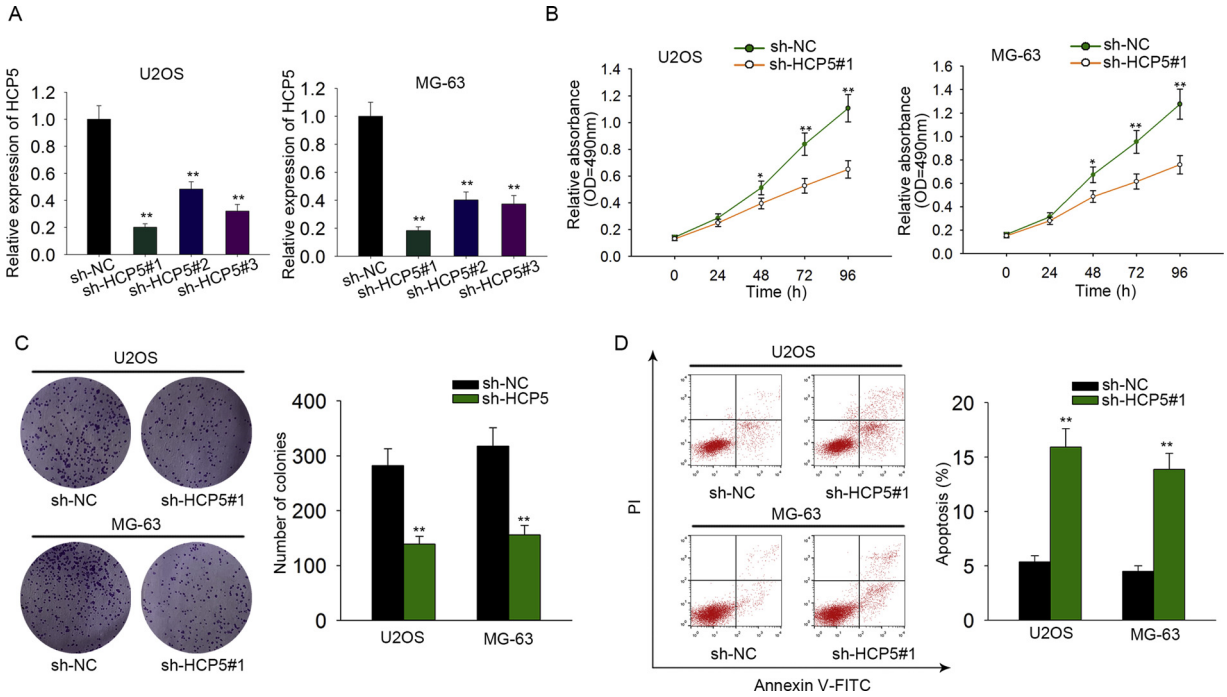

Fig. 2. Knockdown of HCP5 suppressed cell proliferation and accelerated cell apoptosis. A. HCP5 was knocked down in U2OS and MG-63 cell lines with specific shRNA (sh-HCP5#1, sh-HCP5#2, sh-HCP5#3). B-C. CCK-8 and colony formation assays were conducted in two OS cells transfected with sh-HCP5#1 and sh-NC. D. Apoptosis of OS cells transfected with sh-HCP5#1 and sh-NC was analyzed with flow cytometry analysis. $^{**}P < 0.01$ vs control group.

[3,5,8,9,22,23,27,29,32,36,38,40]. Among these four transcription factors, SP1 could positively regulate HCP5 expression. Therefore, we chose SP1 to do further study. In this study, mechanism experiments, including ChIP assay and luciferase reporter assay, were conducted to demonstrate the binding of SP1 to HCP5 promoter. Finally, rescue assays were carried out to verify the biological function of SP1/HCP5 axis in osteosarcoma.

2. Materials and methods

2.1. Cell culture

Human embryonic permanent osteogenic cell line (hFOB1.19) and four human osteosarcoma cell lines, including SaOS2, U2OS, MG-63 and HOS, were purchased from American Type Culture Collection (ATCC, Manassas, USA). OS cell lines were cultured routinely in Dulbecco's Modified Essential Medium (DMEM, Gibco, Carlsbad, USA) supplemented with 10% fetal bovine serum (FBS, Invitrogen, CA, USA) and antibiotics (1% penicillin–streptomycin) in a humidified incubator with 5% CO_2 at 37 °C. hFOB1.19 cell was cultured in DMEM F-121:1 (Gibco) supplemented with 10% fetal bovine serum (FBS, Invitrogen) and G418 (Invitrogen) in a humidified incubator with 5% CO_2 at 34 °C. DMEM medium was replaced every three days and cell passage was performed when cell attachment rate reached to approximately 80–90%.

2.2. Northern blot analysis

LncRNA HCP5 levels were tested by northern blot using an Ambion Northern Max-Gly Kit (Austin, TX, USA). Total RNA was electrophoresed and siphoned to a positively charged nylon membrane (NC). RNA was then fixed to the NC membrane using UV cross-linking. Shortly, the cross-linked membrane was then prehybridized with ULTRAhyb, and RNA was detected with an HCP5-specific

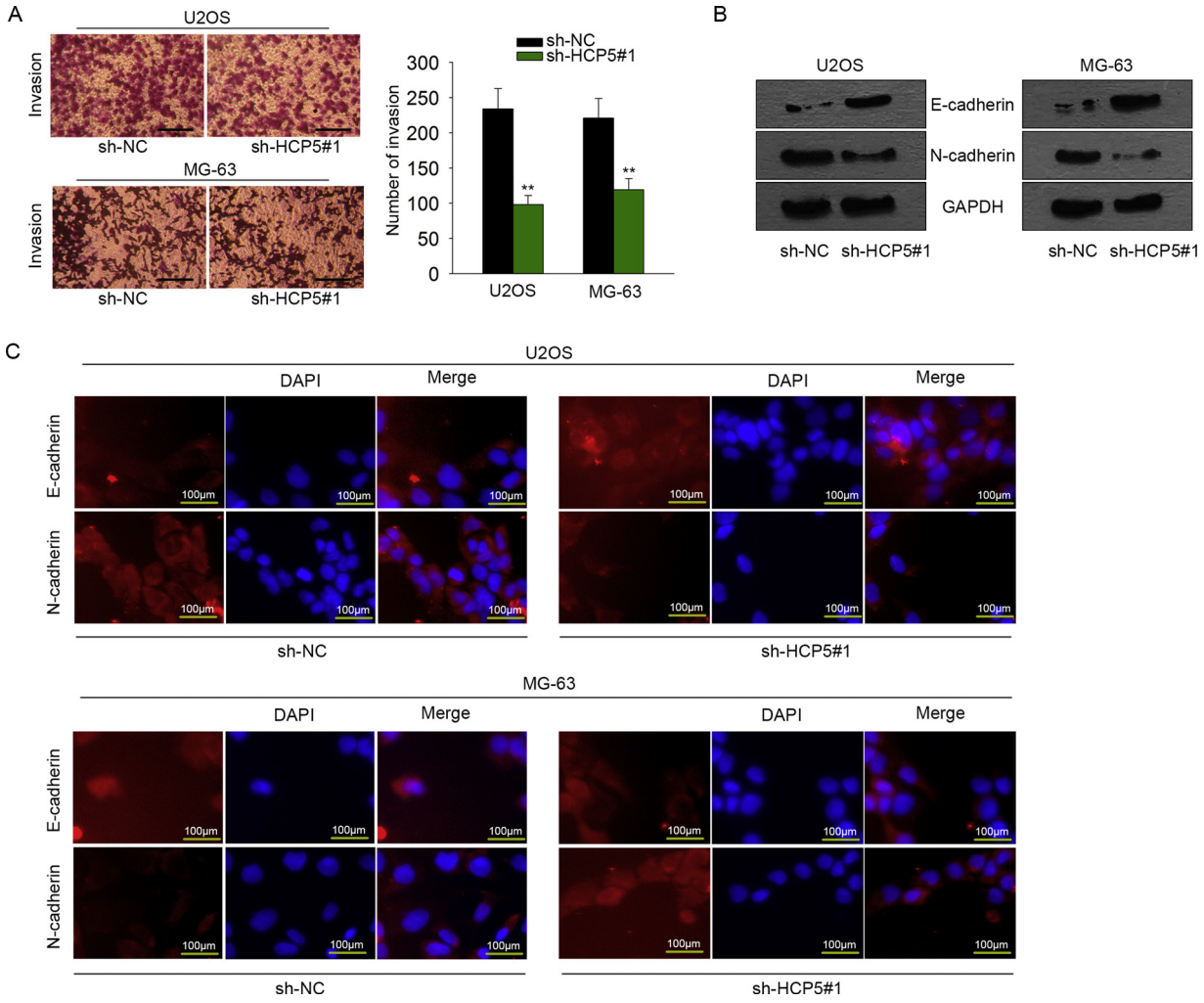

Fig. 3. HCP5 knockdown inhibited cell invasion and epithelial-mesenchymal transition. A. Transwell Matrigel assay revealed the effect of HCP5 knockdown on OS cell invasion. The images were obtained under a microscope at a magnification of × 100. B-C. The levels of EMT markers were examined with western blot and immunofluorescence in OS cells transfected with sh-HCP5#1 and sh-NC. Scale bar = 100 μm. $^{**}P < 0.01$ vs control group.

oligonucleotide probe (5′-TCAGCACTCAA TTCTTGCCAA-3′) labeled with digoxigenin-ddUTP using a DIG Oligonucleotide 3′-End Labeling Kit (Roche Diagnostics, Indianapolis, IN, USA) in roller bottles.

2.3. Transfection

Human osteosarcoma cell lines U2OS and MG-63 were cultured in six-well plates for 24 h before transfection. The short hairpin RNAs against HCP5 (sh-HCP5#1, sh-HCP5#2, sh-HCP5#3) and negative control (sh-NC), pcDNA3.1 vectors containing the whole sequence of NF-kB1, STAT3, SP1 and E2F1 and their negative control empty vectors (NC) were all synthesized and purchased from RiboBio company (Guangzhou, China). Transfections were finished using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Cells were harvested after 48 h incubation.

2.4. RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR)

TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA) was used to extract total RNA based on the standard methods provided by suppliers. Afterwards, total RNA was treated with DNase I (Thermo Scientific) and converted into cDNA with specific primers using TaKaRa Reverse Transcription Kit (TaKaRa, Dalian, China). The condition for reverse transcription was 37 °C for 15 min and 85 °C for 5 s. PCR amplification was conducted with SYBR Green Taq Mix (Takara) on a Bio-Rad Real-Time PCR System, amplification condition was shown as below: 95 °C for 5 min, 40 cycles of 95 °C for 5 s, 61 °C for 30 s. The $2^{-\Delta\Delta Ct}$ method was applied to quantify the expression levels of RNA. Data were normalized to GAPDH.

2.5. Cell proliferation assay

CCK-8 assay was used to measure the cell proliferation ability. In brief, U2OS and MG-63 cells (5×10^3) were plated in 96-well plates for 24 h in a humid atmosphere with 5% CO_2 at 37 °C. After 24 h incubation, 10 ml Cell Counting Kit-8 solution (CCK-8, Dojindo, Mashikimachi, Japan) was added into plates in accordance with the manufacturer's introduction, and incubated for another two hours. Finally, the absorbance at 490 nm was measured at 24 h, 48 h, 72 h and 96 h using FLx800 Fluorescence Microplate Reader (Biotek, USA) at room temperature.

For Colony formation assay, U2OS and MG-63 cells transfected for 48 h were seeded into six-well plates with a density of 500 cells per well and maintained in an incubator at 37 °C with 5% CO_2 for two weeks. Subsequently, cells which had been fixed with 5% paraformaldehyde for 30 min, were stained with 0.1% crystal violet solution. Finally, excessive crystal violet solution was wiped off, cells were washed with PBS until the solution was clear. The plates were photographed and visible colonies were counted using Image J software.

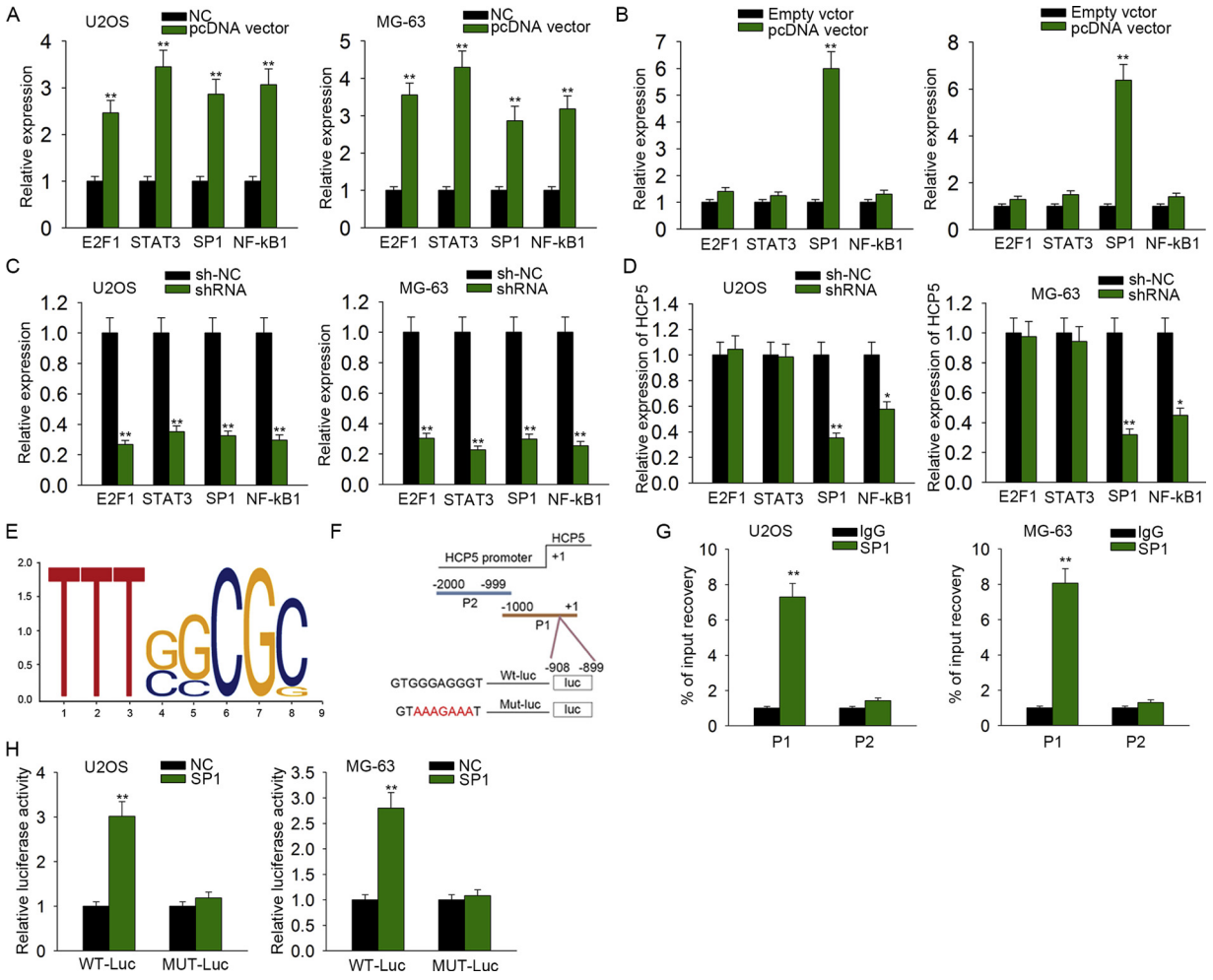

Fig. 4. Transfection factor SP1 induced upregulation of HCP5 in OS cells. A. Four transcription factors were overexpressed in both U2OS and MG-63 cells. B. The expression of HCP5 was detected in response to overexpression of SP1, STAT3, E2F1 and NF-kB1. C. Four transcription factors were silenced in both U2OS and MG-63 cells with specific shRNAs. D. The expression of HCP5 was detected in response to knockdown of SP1, STAT3, E2F1 and NF-kB1. E. The binding motif of SP1 was downloaded from JASPAR. F. Top two binding sites of SP1 to the part 1 (P1) or part 2 (P2) HCP5 promoter are shown. G. The affinity of SP1 to P1 segment of HCP5 promoter was demonstrated by ChIP assay. H. Luciferase reporter assay was carried out to prove the binding of SP1 to the P1 segment of HCP5 promoter. $^{*}P < 0.05$, $^{**}P < 0.01$ vs control group.

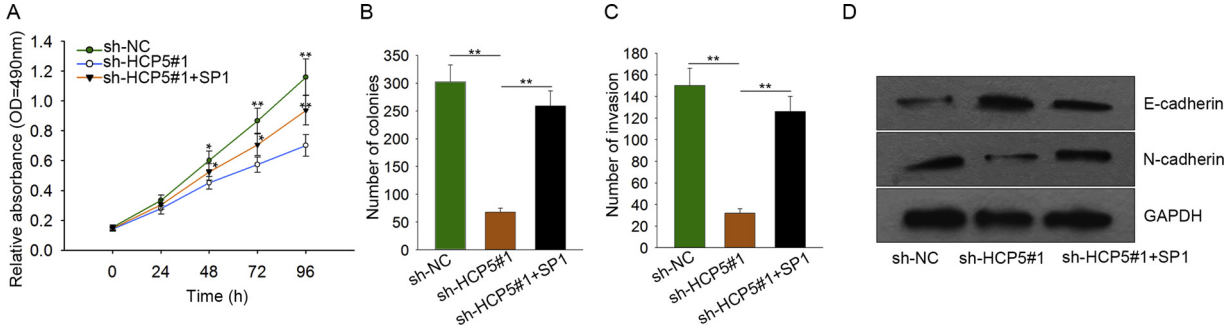

Fig. 5. SP1 reversed the inhibitory effects of HCP5 knockdown on cell proliferation, invasion and EMT progress. A–B. Cell proliferation was observed with CCK-8 and colony formation assays in U2OS cell co-transfected with sh-HCP5#1 and SP1 expression vector. C. Transwell Matrigel assay was used to analyze the effect of SP1 on sh-HCP5#1-mediated OS cell invasion. D. The levels of EMT markers were examined with western blot in OS cells transfected with sh-HCP5#1 and SP1 expression vector. $^{*}P < 0.05$, $^{**}P < 0.01$ vs control group.

2.6. Flow cytometry analysis

Fluorescein isothiocyanate (FITC) Annexin V Apoptosis Detection Kits (nvitrogen, USA) was used to detect cell apoptosis. U2OS and MG-63 cells were incubated for 48 h after transfection. After centrifugation, the supernatant was collected and trypsinized. Afterwards, cells were washed twice using ice-cold phosphate-buffered saline (PBS). Subsequently, cells were mixed with Annexin-V-FITC and continued to incubate for 15 min away from light. Finally, the apoptotic cells were then sent out by flow cytometry (BD, USA) and data analysis was performed with CELL Quest 3.0 software (BD Biosciences, USA).

2.7. Transwell invasion assay

Transwell assay was used to detect the invasive ability of indicated OS cells. Briefly, 8-mm pores in 24-well insert were placed into the transwell chamber. Thereafter, the upper chamber was pre-coated with 50 ml Matrigel (BD Biosciences, USA). After 48 h transfection, 200 ml U2OS and MG-63 cell supernatant was collected and re-suspended in serum-free DMEM, subsequently placed into upper chambers. Meanwhile, 500 ml DMEM solution mixed with 10% FBS was added into the lower chamber. Cells in the upper chamber which did not invade the membrane were removed with cotton swab after incubation for 48 h. Cells in the lower surface were fixed with 4% paraformaldehyde (PFA) and stained with 0.1% crystal violet (Beyotime Institute of Biotechnology, Haimen, China) for 15 min. Finally, a five randomly selected field was used to calculate invading cells under a microscope (Nikon, Tokyo, Japan) at a magnification of × 100.

2.8. Western blot analysis

Total protein was lysed from cells using radio-immunoprecipitation (RPPA; Sangon Biotech) and using the BCA kit (Thermo Scientific Pierce) to quantify protein concentration according to the manufacturer's introduction. Total protein was separated using 10% sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and subsequently transferred onto PVDF membranes. Tris-buffered saline (TBS) containing 5% non-fat milk was used to block the PVDF membranes for an hour. Thereafter, the membranes were incubated with primary antibodies (1:1000 dilution) against HCP5, E-cadherin, N-cadherin and GAPDH at 4 °C overnight. Subsequently, the membranes were incubated with appropriate secondary antibody conjugated with horseradish peroxidase (1:2000 dilution, Santa Cruz Co) at room temperature for one hour. Finally, band signal was visualized using the enhanced chemiluminescence (ECL) detection kit (Pierce, Rockford, IL, USA). GAPDH was considered as the internal control.

2.9. Immunofluorescence

For the immunofluorescence (IF) assay, U2OS and MG-63 cells were plated on culture slides for 48 h and rinsed three times in phosphate-buffered saline (PBS) solution. Subsequent to fixation in ice-cold methanol-acetone for 15 min, cells were permeabilized with 0.1% Triton X-100 for 10 min and sealed with 5% BSA in PBS. Cells were incubated with primary antibodies specific to E-cadherin and N-cadherin at 4 °C overnight prior to incubation with goat anti-rabbit FITC conjugated secondary antibody at room temperature in the dark. Thereafter, slides were washed with PBS and 4′, 6-Diamidino-2-phenylindole (DAPI) was used to stain the nuclei. The cell lines were imaged by fluorescence microscopy (Nikon, Tokyo, Japan). All antibodies were bought from Cell Signaling Technology (St Louis, USA).

2.10. Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) was carried out using the SimpleChIP® Enzymatic Chromatin IP Kit (CST, USA) according to the manufacturers' instructions. Cells which had been treated with 4% formaldehyde to cross-link DNA to proteins were collected for ultrasonic to shear DNA into two fragments. SP1 and negative control IgG antibodies (Santa Cruz Biotechnology, CA, USA) were added with Dynabeads Protein G (Life Technologies, CA, USA) to the DNA-protein mixture. After immunoprecipitation, the chromatin-protein-antibody complex was eluted, decrosslinked and purified to retrieve DNA. Real-time PCR was used to detect the enrichment of DNA immunoprecipitated by antibody using SYBR Green Master Mix (Takara).

2.11. Luciferase reporter assay

For the Luciferase reporter assay, U2OS and MG-63 cells were incubated in a 24-well plate before transfection. Thereafter, the wild type fragment sequences of HCP5 promoter (WT-Luc) or mutant type fragment sequences of HCP5 promoter (Mut-Luc) were co-transfected into U2OS or MG-63 cells together with SP1 expression vector or the corresponding negative control (NC). After 48 h transfection, the relative luciferase activity was assayed with the dual-luciferase reporter assay system (Promega, USA).

3. Statistical analysis

All data were shown as the mean ± SD from more than two independent experiments. Statistical analyses were carried out using the SPSS software version 17.0. Differences between different groups were analyzed by a paired two tailed Student's t-test. In addition, one-way ANOVA was used to compare the differences among more than two groups. All data were two-side. P value less than 0.05 was considered statistically significant.

4. Results

4.1. HCP5 was overexpressed in OS cell lines

At first, five lncRNAs chosen from GeneCard (https://www.genecards.org/) were subjected to qRT-PCR examination in one human normal osteoblast hFOB1.19 and four OS cell lines (SaOS2, HOS and U2OS and MG-63). As shown in Fig. 1A, only HCP5 was significantly upregulated in OS cell lines. Northern blot analysis further demonstrated the upregulation of HCP5 in OS cell lines (Fig. 1B).

4.2. Knockdown of HCP5suppressed cell proliferation and accelerated cell apoptosis

In order to examine the biological role of HCP5 in osteosarcoma, we conducted functional assays in OS cells. According to the data of Fig. 1, HCP5 was expressed highest in U2OS and MG-63 cells. Therefore, HCP5 was knocked down in these two cell lines with specific shRNAs (sh-HCP5#1, sh-HCP5#3, sh-HCP5#3). The expression level of HCP5 was decreased in two OS cell lines effectively (Fig. 2A). sh-HCP5#1 showed the best knockdown efficiency. Therefore, sh-HCP5#1 was chosen for subsequent experiments. CCK-8 and colony formation assays revealed that cell proliferation of OS cells was inhibited by silenced HCP5 effectively (Fig. 2B–C). Additionally, flow cytometry analysis showed that cell apoptosis was promoted by transfecting with sh-HCP5#1 (Fig. 2D).

4.3. HCP5 knockdown inhibited cell invasion and epithelial-mesenchymal transition

Above findings suggested the oncogenic role of HCP5 in cell proliferation and apoptosis. Here, we further detected the effect of HCP5 on cell invasion and epithelial-mesenchymal transition. Transwell invasion assay was carried out in two OS cell lines transfected with sh-HCP5#1. Unsurprisingly, cell invasion was obviously inhibited (Fig. 3A). Epithelial-mesenchymal transition was an important biological process which is closely related with cell invasion. To analyze whether HCP5 can regulated EMT progress, western blotting and immunofluorescence staining were used to test the levels of EMT Markers (N-cadherin and E-cadherin). Both results revealed that HCP5 knockdown reversed EMT process in U2OS and MG-63 cells (Fig. 3B–C).

4.4. Transfection factor SP1 induced upregulation of HCP5 in OS cells

All findings above suggested that upregulation of HCP5 promoted the malignant progress of OS cells. It is necessary to explore the

molecular mechanism which led to the upregulation of HCP5 in OS cells. Four known transcription factors were selected out for further analysis. After bioinformatics analysis, we found that these four transcription factors possessed binding sites with the promoter region of HCP5. All these four transcription factors were overexpressed or silenced in both U2OS and MG-63 cells (Fig. 4A, C). Whereas, only overexpression and knockdown of SP1 changed the expression level of HCP5 (Fig. 4B, D), indicating the positive regulation of SP1 on HCP5. The binding motif of SP1 downloaded from JASPAR (http://jaspar.genereg.net/) is shown in Fig. 4E. Top two binding sites of SP1 to the part 1 (P1) or part 2 (P2) HCP5 promoter are shown in Fig. 4F. The affinity of SP1 to P1 segment of HCP5 promoter was demonstrated by ChIP assay (Fig. 4G). Furthermore, luciferase reporter assay revealed that SP1 cloud not reduce the luciferase activity of P1 segment after the binding sequence was mutated (Fig. 4H), suggesting the effect of SP1 on HCP5 transcription.

4.5. SP1 reversed the inhibitory effects of HCP5 knockdown on cell proliferation, invasion and EMT progress

Rescue assays were conducted to prove the function of SP1/HCP5 axis in OS cell proliferation, invasion and EMT formation. As shown in Fig. 5A and B, SP1 partly attenuated the inhibitory effect of sh-HCP5#1 on cell proliferation. Moreover, cell invasion and EMT progress inhibited by sh-HCP5#1 was partially recovered by SP1 (Fig. 5C–D). Taken together, SP1/HCP5 axis promoted cell proliferation, invasion and EMT formation in osteosarcoma.

5. Discussion

In the current study, we found a novel functional lncRNA HCP5, which contributed to cell proliferation, invasion and epithelial-mesenchymal transition in osteosarcoma. At first, the high expression of HCP5 was detected in OS cell lines. Numerous studies have revealed the oncogenic function of the abnormally expressed lncRNAs in human cancers [13–15,18,30,44,46], including osteosarcoma [6,16,19,33]. In this study, we designed and conducted loss-of-function assays in two OS cell lines. Intriguingly, knockdown of HCP5 suppressed cell proliferation, invasion and epithelial-mesenchymal transition. Moreover, knockdown of HCP5 promoted cell apoptosis. All these experimental results indicated the oncogenic role of HCP5 in osteosarcoma.

LncRNA HCP5 has been demonstrated to be an oncogene in cervical cancer [43], thyroid carcinoma [20] and glioma [31]. Combining with all these findings, we confirmed that upregulation of HCP5 promoted tumor progression in all above cancers. In this study, we analyzed the oncogenic role of HCP5 in osteosarcoma. Although upregulation of HCP5 has been identified to be an oncogenic factor for tumor progression, the molecular mechanism which contributed to the upregulation of HCP5 is still a secret. Therefore, our study focused on the upstream molecular mechanism of HCP5 I osteosarcoma. In recent years, the mechanisms contributed to the dysregulation of lncRNAs are studied. It is known that lncRNAs can be activated by their upstream transcription factors [21,26,35], thereby promoting tumorigenesis and tumor progression. It has been reported that transcription factors such as SP1, STAT3, E2F1 and NF-kB1 can contribute to the upregulation of lncRNAs by promoting the transcription of lncRNAs. Here, we hypothesized that lncRNA HCP5 might be activated by a certain transcription activator. Next, these four potential transcription factors were overexpressed or silenced in two OS cell lines. The expression of HCP5 was then examined in indicated OS cells. We found that only SP1 can positively regulated the expression of HCP5. Therefore, we chose SP1 to do further study. Based on the bioinformatics analysis, we predicted the binding sites of SP1 in the HCP5 prompter. ChIP assay validated the binding of SP1 to HCP5 promoter. Luciferase activity analysis further revealed the transcription activation of SP1 on HCP5. In summary, mechanism investigation suggested that HCP5 was activated by its upstream transcription activator SP1, thereby promoting the OS cell proliferation, invasion and EMT progress.

To validate the function of SP1/HCP5 axis in osteosarcoma progression, rescue assays were carried out. The results showed that SP1 attenuated the inhibitory effects of HCP5 on cell proliferation, invasion and EMT progress, indicating the role of SP1/HCP5 axis in osteosarcoma progression. Taken together, we concluded that SP1-induced upregulation of HCP5 promotes cell proliferation, invasion and EMT progress in osteosarcoma.

Availability of data and materials

Not applicable.

Conflicts of interest

There are no conflicts of interest to disclose.

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