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Original Article

LncRNA LINC00704 drives cancer stemness and malignant properties in oral squamous cell carcinomas by sponging miR-204

Pei-Yin Chen [a,b], Lo-Lin Tsai [c,d†], Shih-Min Wang [e†], Yi-Wen Liao [e,f], Cheng-Chia Yu [a,b,e**], Shih-Chi Chao [f*]

[a] School of Dentistry, Chung Shan Medical University, Taichung, Taiwan
[b] Department of Dentistry, Chung Shan Medical University Hospital, Taichung, Taiwan
[c] Department of Medical Research and Education, Lo-Hsu Medical Foundation, Lotung Poh-Ai Hospital, Yilan, Taiwan
[d] School of Dentistry, College of Oral Medicine, Taipei Medical University, Taipei, Taiwan
[e] Institute of Oral Sciences, Chung Shan Medical University, Taichung, Taiwan
[f] Department of Medical Research, Chung Shan Medical University Hospital, Taichung, Taiwan

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Abstract *Background/purpose:* Oral squamous cell carcinoma (OSCC) remains a significant challenge with a high recurrence rate and metastasis, which are believed to be driven by cancer stem cells (CSCs). The long non-coding RNA LINC00704 has been implicated in the malignant progression of various cancers. This study aimed to investigate the critical role for LINC00704 in oral cancer stemness.

Materials and methods: The clinical significance of LINC00704 was determined in our OSCC cohort and the TCGA-HNSC dataset. Silencing of LINC00704 in OCSCs was established by lentiviral-mediated RNA interference. Cellular ALDH activity, stemness markers expression, and sphere formation ability were assessed to evaluate cancer stemness, while cell migration, colony formation, and apoptosis were measured to determine malignancy. A luciferase reporter assay and microRNA inhibitor transfection were conducted to validate the molecular function of LINC00704.

Results: The present study demonstrated that LINC00704 was significantly upregulated in OSCC tumors compared to paired normal mucosa and that its elevated expression positively correlated with advanced staging and poor survival in HNSC patients. LINC00704 knockdown in

* Corresponding author. Department of Medical Research, Chung Shan Medical University Hospital, No. 110, Sec. 1, Jianguo N. Rd., Taichung 40201, Taiwan.
** Corresponding author. Institute of Oral Sciences, Chung Shan Medical University, No. 110, Sec. 1, Jianguo N. Rd., Taichung 40201, Taiwan.
E-mail addresses: ccyu@csmu.edu.tw (C.-C. Yu), h5l4g4fu6123@gmail.com (S.-C. Chao).
† These two authors contributed equally to the results of this study.

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OCSCs significantly diminished their stemness, evidenced by reduced ALDH activity, decreased expression of stemness markers, and impaired migration and colony formation capabilities. Mechanistically, miR-204 was demonstrated to direct interact with LINC00704 3′UTR. Furthermore, inhibition of miR-204 attenuated the effects of LINC00704 knockdown on suppressing self-renewal and inducing apoptosis in OCSCs.
Conclusion: This study revealed that LINC00704 functions as a miR-204 sponge to promote the cancer stemness and malignancy of OCSCs, highlighting the LINC00704/miR-204 axis as a novel and potentially therapeutic target for OSCC.
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Introduction

Oral squamous cell carcinoma (OSCC), a prevalent type of head and neck cancer, encompasses a range of malignancies arising in various locations within the oral cavity, including the lips and tongue.[1] The International Agency for Research on Cancer (IARC) estimated approximately 377,600 new cases of oral cancer globally in 2020, with projections indicating a substantial rise to 564,700 new cases by 2040. (GLOBOCAN 2022) In Taiwan, OSCC is a significant public health challenge, ranking as the fourth most prevalent cancer among males and the seventh most prevalent across the general population.[2] The prognosis for individuals diagnosed with OSCC remains concerning, as evidenced by high recurrence rates following treatment. For instance, patients undergoing therapeutic surgery face a nodal relapse rate of approximately 78 %, while those receiving elective surgery experience a non-nodal recurrence rate of around 52 %.[3] Furthermore, the diagnosis of distant metastases is associated with a particularly poor prognosis, with a median survival time of approximately 4 months.[4] These statistics highlight the critical need for research to elucidate the mechanisms driving OSCC progression and identify novel therapeutic targets.

A small subpopulation of cells within tumors, known as cancer stem cells (CSCs), is increasingly recognized for its critical role in cancer progression.[5,6] These cells can self-renew and generate heterogeneous tumor cell populations, thereby promoting tumor growth, drug resistance, metastasis, and recurrence.[5,6] Given the significant role of CSCs in cancer progression, understanding the factors that regulate their behavior is of paramount importance. Non-coding RNAs (ncRNAs) have increasingly been recognized as key regulators of CSCs behavior.[7]

The human genome predominantly transcribes ncRNAs, with only a small fraction coding for proteins. These ncRNAs, including long ncRNAs (lncRNAs; >200 nucleotides long) and microRNAs (miRNAs; 18—22 nucleotides long), have been implicated in various types of cancers, including OSCC.[7—11] For example, the lncRNA LINC00963 has been shown to enhance cancer stemness properties, metastasis, and drug resistance in OSCCs through regulation of ABCB5,[9] while miR-1246 promotes cancer stemness and chemoresistance via targeting CCNG2.[10] Recently, the concept of competing endogenous RNA (ceRNA) has gained attention, indicating the intricate regulatory interplay between different RNA species. Specifically, lncRNAs can act as miRNA sponge, attenuating their regulatory effect on target mRNAs and consequently increasing mRNAs expression.[8,11] In OSCC, the lncRNA MEG3 has been shown to suppress cancer stemness features, such as self-renewal and invasion, by sponging miR-421.[11] However, further research is needed to fully elucidate the complex ceRNA network in regulating cancer stemness of OSCC.

LncRNA LINC00704, also known as mitotically associated long non-coding RNA (MANCR), has been found to be upregulated in various cancers, often correlating with poor prognosis.[12—16] Studies have shown that LINC00704 depletion can lead to genomic instability, cell cycle dysregulation, and apoptosis, while its overexpression can promote cell proliferation, migration, and invasion.[12—16] The potential role of LINC00704 as a miRNA sponge has recently been proposed. Specifically, LINC00704 was found to be overexpressed in mantle cell lymphoma, thereby enhancing cell proliferation by inhibiting miR-218.[17] Given the limited understanding of the role of LINC00704 in regulating cancer properties and the increasing importance of ceRNA networks in tumor progression, further investigation into the potential involvement of LINC00704 during oral carcinogenesis is warranted. This study aimed to elucidate the role of LINC00704 in OCSCs and to explore its potential interaction with miRNAs, thereby contributing to a better understanding of oral carcinogenesis and potentially identifying novel therapeutic targets.

Materials and methods

Tissue specimen collection and preparation

Tissue sample from oral squamous cell carcinoma (OSCC) tumor (T) and adjacent normal mucosa (N) were collected from patients undergoing surgical at the Department of Dentistry, Chung Shan Medical University Hospital (Taichung, Taiwan). All procedures were carried out according to the approved protocol by the Institutional Review Board of Chung Shan Medical University Hospital (CSMUH No:CS2-23010). Prior to tissue collection, all patients provided written informed consent. Following resection, the tissue sample were placed in Hanks' Balanced Salt Solution (HBSS; Thermo Fisher Scientific, Waltham, MA, USA) and either immediately processed for RNA extraction or stored in liquid nitrogen for later use.

Cell culture and enrichment of cancer stem cells

The parental OSCC cell line (SAS) was cultured in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12) supplemented with 10 % fetal bovine serum (FBS) and 1 % penicillin/streptomycin/amphotericin B (PSA) at 37 °C in a 5 % CO_2 humidified incubator. Only cells within the first 10 passages were utilized for experiments. To enrich for the oral cancer stem OSCCs (OCSCs) from the parental SAS cells, single-cell suspensions of SAS cells at a density of 2×10^3/mL were added into ultra-low attachment 6-well culture plates (Corning, Corning, NY, USA) and cultured in serum-free, chemically defined DMEM/F12 medium supplemented with 10 ng/mL basic fibroblasts growth factor (bFGF), 10 ng/mL epidermal growth factor (EGF), and 1 % N2-supplement. The medium was replenished every 2–3 days. After 14 days, tumorspheres were trypsinized into single cells, and were then transferred into 10-cm culture dishes. These cells were grown as a monolayer and maintained in serum-free chemically defined DMEM/F12 medium for further experiments. Unless otherwise stated, all reagents were sourced from Thermo Fisher Scientific.

RNA sequencing analysis

Total RNA was extracted from OSCC clinical specimens using TRIzol reagent (Sigma–Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions. RNA purity and integrity were assessed using a NanoVue Plus spectrophotometer (GE Healthcare Technologies Inc., Chicago, IL, USA) and an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA), respectively. RNA library preparation and sequencing, and the analysis of transcriptome discrepancies were conducted by Genomics Inc. (New Taipei city, Taiwan). Data were analyzed using R (version 2023.06.1 + 524) with Bioconductor packages. Differentially expressed genes were identified based on an adjusted p-value of <0.05, using the Benjamini-Hochberg method for false discovery rate (FDR) correction.

Real-time quantitative PCR

Total RNA, including miRNA, was isolated using the miRNeasy Mini Kit (Qiagen, Venlo, Netherlands) as per the manufacturer's protocol. RNA quality control methods were performed as described above. MiRNA was reverse-transcribed using the TaqMan MicroRNA Reverse Transcription Kit, with specific assays for hsa-miR-204 (Applied Biosystems, Thermo Fisher Scientific). LINC00704 and target mRNAs were reverse transcribed using the SuperScript III First-Strand Synthesis System (Invitrogen, Thermo Fisher Scientific). Real-time quantitative PCR (RT-qPCR) was carried out using an ABI StepOnePlus Real-Time PCR System (Applied Biosystems) with SYBR Green master mix (Applied Biosystems) for mRNAs/lncRNAs and TaqMan MicroRNA Assay for *hsa-miR-204*. The following primer sequences were used (5′-3′): *LINC00704*, TCCACTCACCACTCGCTCACTG (forward), CAGGATTAGCACGTTCCAGGTTCC (reverse);[18] POU5F1, *GTGGAGAGCAACTCCGATG* (forward), TGCTCCAG CTTCTCCTTCTC (reverse);[19] *SOX2*, GGGAAATGGGAGGGGTG CAAAAGAGG (forward), TTGCGTGAGTGTGGATGGGATTGG TG (reverse);[20] *GAPDH*, GGAGCGAGATCCCTC CAAAAT (forward), GGCTGTTGTCATACTTCTCATGG (reverse).[21] The mature sequence of *hsa-miR-204* (Assay ID: 000508) and the *U6 snRNA* control sequence are proprietary information provided within the respective TaqMan MicroRNA Assay product documentation. Relative gene expression levels were calculated using the 2-ΔCt method, with *LINC00704* and target mRNAs normalized to GAPDH, while the level of *hsa-miR-204* was normalized to *U6 snRNA*.

Knockdown of LINC00704

Lentiviral vector expressing short hairpin RNA (shRNA) targeting LINC00704 were obtained from BioSettia Inc. (San Diego, CA, USA). The shRNA sequences for LINC00704 were cloned into the pLV-RNAi vector, following the manufacturer's protocol. The target sequences for LINC00704 were: Sh-LINC00704-1: 5′- AAAAGGTGGCTCACGTCTATAATTTGGA TCCAAATTATAGACGTGAGCCACC-3'; Sh-LINC00704-2: 5′-AAAAGGAACCTCCTTTCTTACATTTGGATCCAAATGTAAGAAAG-GAGGTTCC-3' (https://biosettia.com/support/shrna-designer/). For lentivirus production, 293T cells (ATCC, Manassas, VA, USA) were co-transfected with pLV-shRNA vectors and helper plasmids (packaging and envelope plasmids) using Lipofectamine 2000 reagent (Invitrogen). Lentiviral particles were collected from the culture supernatants 48 h post-transfection. OCSCs were infected with LINC00704-targeting or non-targeting control (Sh-Luc.) lentivirus in the presence of polybrene, followed by selection with puromycin. (Sigma–Aldrich).

Aldehyde dehydrogenases activity assay

Aldehyde dehydrogenases (ALDH) activity was assessed using the ALDEFLUOR™ Kit (STEMCELL Technologies, Vancouver, Canada), following the manufacturer's instructions. Cells were resuspended in ALDEFLUOR assay buffer and divided into test and control groups. Both groups were incubated with the ALDEFLUOR substrate (BAAA), with the control group receiving the ALDH inhibitor diethylaminobenzaldehyde (DEAB). Cellular ALDH enzymatic activity was analyzed using Calibur flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA). The percentage of ALDH-positive cells in each group was determined by comparing the fluorescence intensity between the test and control samples.

Surface marker expression assessment

Cells (5×10^5) were incubated in 100 μL PBS comprising 0.1 % bovine serum albumin (BSA; Sigma–Aldrich) and incubated with either a FITC-conjugated anti-ABCG2 antibody (Millipore, Merck, Darmstadt, Germany), or a corresponding IgG isotype control (BD Biosciences) for 30 min on ice. After washing, stained cells were analyzed using a Calibur flow cytometer (BD Biosciences), and data were processed with FlowJo software (Version 10.1, BD Biosciences).

Western blot

Twenty μg of protein from cell lysates were separated by SDS-PAGE and transferred to PVDF membranes. Membranes were blocked with blockEBL buffer (EBL Biotechnology, Taipei, Taiwan) and incubated overnight at 4 °C with primary antibodies such as anti-OCT4 (Millipore), anti-Sox2 (Cell signaling, Danvers, MA, USA), and anti-GAPDH (Thermo Fisher Scientific). HRP-conjugated secondary antibodies (Thermo Fisher Scientific) were applied for 1 h at room temperature. Chemiluminescent signals were detected using the LAS-4000 mini analyzer (GE Healthcare) following ECL substrate incubation.

Transwell migration assay

Cells (1×10^5) were placed in the upper chamber of a Transwell insert (Corning) with serum-free medium, while medium with 10 % FBS was added in the lower chamber as a chemoattractant. After 48 h, cells that had migrated through the membrane were stained with 0.1 % crystal violet and quantified using ImageJ software (version 1.53k, NIH, Bethesda, MD, USA).

Colony formation assay

Each well of a six-well plate was coated with 1 mL of 0.525 % agar (Thermo Fisher Scientific) in DMEM/F12 with 15 % FBS. After solidification, 1 mL of 0.3 % agar containing 1×10^4 cells was added to each well. Cultures were maintained for 3 weeks, stained with 0.05 % crystal violet and colonies counted under a light microscope.

Modulation of miR-204 expression

According to the manufacturer's manuals, cells were transfected with either pre-miR-204 mimic or miR-204 inhibitor (Applied Biosystems) using Lipofectamine 2000 reagent (Invitrogen). A non-targeting miR-scrambled (miR-Scr.) was utilized as the negative control.

Luciferase reporter assays

Cloning of the wild-type (wt) or mutant (mut) 3′UTRs of LINC00704 into the downstream of firefly luciferase gene in the pmirGLO dual-luciferase miRNA target expression vector (Promega, Madison, WI, USA) were first carried out as per manufacturer's instructions. Cells were then co-transfected with the reporter construct, and pre-miR-204 mimics or a non-targeting scrambled (miR-Scr.) using Lipofectamine 2000 (Invitrogen). Firefly luciferase activity was normalized to *Renilla* luciferase activity to control.

Apoptosis detection

Apoptosis was measured using an Annexin V-FITC apoptosis detection kit (Abcam, Cambridge, UK) in accordence to the manufacturer's manuals. 1×10^5 cells were incubated in 500 μL of 1X binding buffer containing 2.5 μL of Annexin V-FITC and 2.5 μL of propidium iodide (PI) staining solution for 5 min, followed by the quantification of apoptotic cells using a Calibur™ flow cytometer (BD). Cells positive for Annexin V-FITC and negative for PI were considered to be undergoing apoptosis.

Statistical analysis

The data are expressed as the mean ± standard deviation (SD) from a minimum of 3 independent experiments. To compare groups, paired or un-paired, two-tailed Student's t-test were used. All statistical analyses were performed using GraphPad Prism (version 10, GraphPad Software, Boston, MA, USA). A *P*-value of <0.05 was deemed statistically significant.

Results

RNA sequencing analysis of tumor (T) and adjacent normal mucosa (N) tissues from two OSCC patients revealed that LINC00704 was among the most significantly upregulated lncRNAs in OSCC tumors (Fig. 1A). This overexpression was validated in an independent cohort of 30 OSCC patients using RT-qPCR (Fig. 1B). Consistent with our data, analysis of the TCGA-HNSC cohort demonstrated significantly higher LINC00704 levels in tumors (T) compared to healthy controls (N; Fig. 1C). Notably, significantly increased LINC00704 expression was restricted to a subset of tumor types compared to their healthy control (Fig. 1D), and it exhibited the third highest expression level in HNSC tumors among all tumor types (Fig. 1E). These observations suggest that LINC00704 may function as an oncogenic lncRNA in a tissue-specific manner, with potentially heightened relevance in HNSC. Furthermore, LINC00704 expression was consistently elevated across different cancer stages and tumor grades compared to healthy control (Fig. 1E and F). Importantly, patients with high LINC00704 expression exhibited significantly shorter overall survival and disease-free survival compared to those with low LINC00704 expression (Fig. 1G). These results suggest that LINC00704 may be a critical lncRNA involved in the malignant progression of HNSC, including OSCC.

To further elucidate the oncogenic mechanisms of LINC00704, the stable knockdown of LINC00704 in oral cancer stem cells (OCSCs) was achieved (Fig. 2A). LINC00704 silencing significantly reduced cancer stemness markers, including cellular ALDH enzymatic activity (Fig. 2B) and the expression of CD44, Oct4, and Sox2 (Fig. 2C and D). Furthermore, LINC00704 silencing significantly impaired cell migration (Fig. 3A), anchorage-independent growth (Fig. 3B), and self-renewal capacity (Fig. 5A), while also inducing apoptosis (Fig. 5B). These finding indicate that LINC00704 is necessary for promoting cancer stemness and metastatic properties of OCSCs.

Bioinformatic analysis identified a putative miR-204 binding site within the 3′ UTR of LINC00704 (Fig. 4A), and the direct interaction between LINC00704 and miR-204 was confirmed using a luciferase reporter assay (Fig. 4B). To investigate the role of LINC00704/miR-204 axis in OCSCs, a miR-204 inhibitor was transfected into control and LINC00704-silenced OCSCs to suppress endogenous miR-204 expression (Fig. 5). As anticipated, the effects of LINC00704

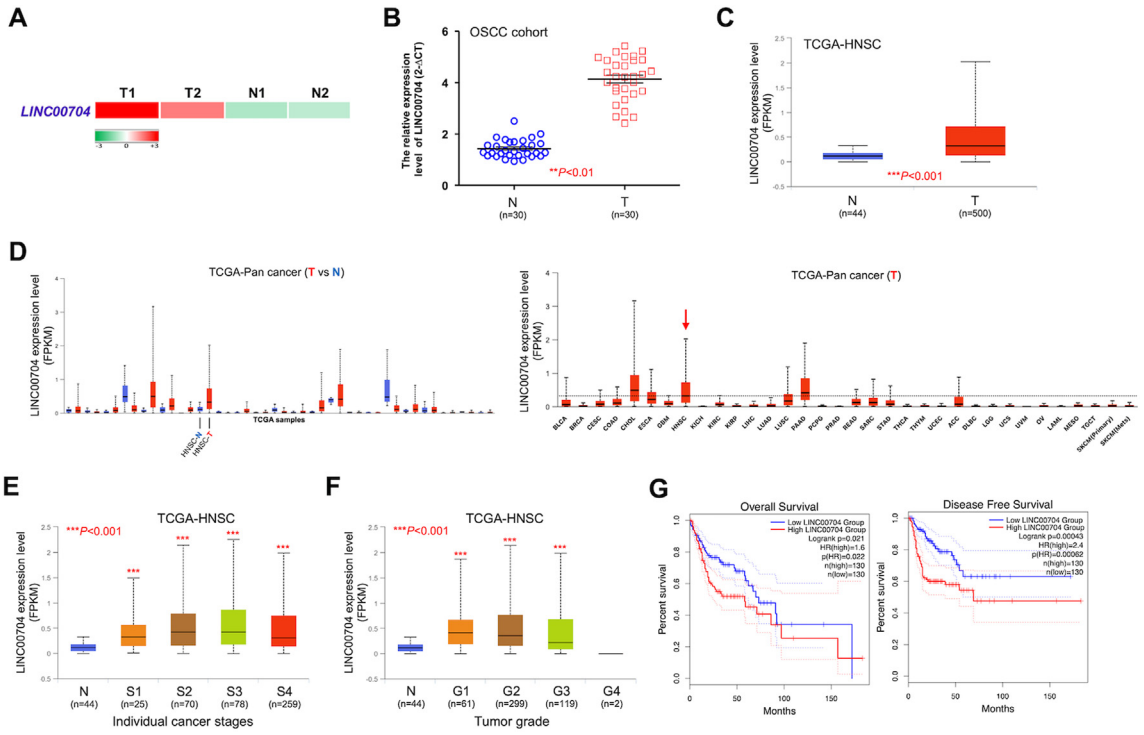

Figure 1 Elevated LINC00704 expression correlated with poor prognosis in patients. (A) RNA sequencing analysis of LINC00704 expression levels in oral squamous cell carcinomas (OSCC) tumors and paired adjacent normal mucosa samples from two OSCC patients. (B) RT-qPCR analysis of LINC00704 expression levels in tumor (T; n = 30) and the paired adjacent normal mucosa (N; n = 30) samples in the OSCC cohort. (C) LINC00704 expression levels in head and neck squamous cell carcinomas (HNSC) tumor (T; n = 500) and normal (N; n = 44) samples in TCGA-HNSC dataset. (D) LINC00704 expression levels across different cancer types in the TCGA pan-cancer dataset. The dashed line represents the median expression level of LINC00704 in HNSC tumors. LINC00704 expression levels across different (E) individual cancer stages and (F) tumor grade in the TCGA-HNSC dataset. (G) Kaplan–Meier curves showing the overall survival (left) and disease-free survival (right) in patients with high (≥75th percentile) vs. low (≤25th percentile) LINC00704 expression in the TCGA-HNSCC cohort.

silencing on inhibiting self-renewal and inducing apoptosis of OCSCs were effectively attenuated by inhibition of miR-204 (Fig. 5). Collectively, thesis data suggest that LINC00704 promotes OCSC stemness, metastatic potential, and survival by sponging miR-204, thereby antagonizing its tumor-suppressive effects.

Discussion

LINC00704, also known as mitotically-associated lncRNA (MANCR), is encoded at chromosome 10p15.1. Researchers first reported that LINC00704 played a role in regulating DNA repair, cell division, and genomic stability of thyroid and aggressive breast cancers.[13,14] Subsequent investigations have demonstrated that dysregulated LINC00704 expression correlates with malignant progression across a spectrum of tumor types and is associated with poor prognosis in patients.[15,16,22–27] In the present study, we determined overexpression of LINC00704 in tumors of TCGA-HNSC cohort (Fig. 1C) and further confirmed a significant upregulation of LINC00704 in OSCC tumors compared to paired normal mucosa in our OSCC cohort (Fig. 1A and B). Moreover, high LINC00704 expression was associated with inferior overall survival and disease-free survival in HNSCC patients (Fig. 1H). Corroborating our findings, a recent study encompassing 49 HNSCC patients, which also incorporated data from the TCGA-HNSCC dataset, not only found significantly elevated LINC00704 expression in tumors but also established a positive correlation between high LINC00704 expression and lymph node metastasis, tumor grade IV, T4 stage, clinical stage IV, smoking, and pro-inflammatory immune cell infiltration.[26] These results provide evidence of specific clinically relevant associations, thereby addressing the limitation of our analysis, which only examined LINC00704 expression across different OSCC stages (Fig. 1F and G). Notably, the aforementioned study also reported at least a 30-fold higher LINC00704 expression in HNSCC cell lines compared to normal oral keratinocytes. Furthermore, knockdown of LINC00704 in two OSCC lines (SCC9 and Cal27) significantly attenuated their migratory, invasive, and proliferative capabilities.[26] Consistent with this, another study observed minimal LINC00704 expression in non-tumorigenic breast cancer cells.[28] In our investigation, silencing of LINC00704 in OCSCs impaired cancer stemness, including a reduction in ALDH1 activity, decreased protein levels of ABCG2, OCT4, and SOX2 (Fig. 2B–D), and significantly reduced sphere-forming ability, an indicator of self-renewal capacity (Fig. 5A). These results provide the first direct evidence of a crucial role of LINC00704 in maintaining cancer stemness in OCSCs. Indeed, a recent study also found

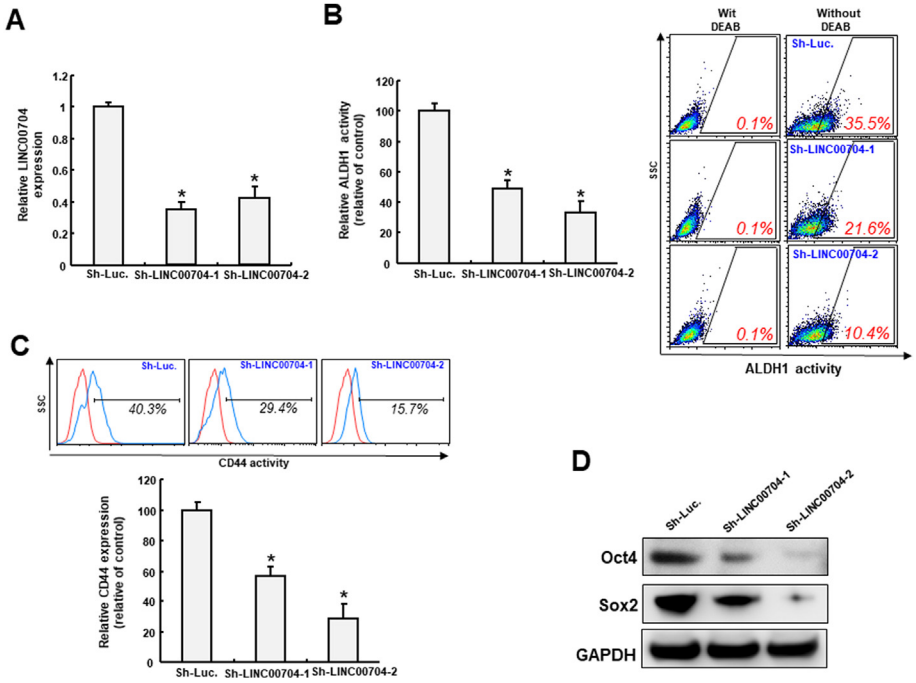

Figure 2 Knockdown of LINC00704 repressed stemness markers in oral cancer stem cells. (A) Relative expression levels of LINC00704 in oral cancer stem cells (OCSCs) expressing short-hairpin RNA targeting LINC00704 (Sh-LINC00704) or a non-targeting control (Sh-Luc.) were analyzed using RT-qPCR. Flow cytometric analysis of (B) cellular aldehyde dehydrogenase (ALDH) enzymatic activity and (C) the cell surface expression of the CD44 in OCSCs. (D) Western blot analysis of the expression of pluripotency transcription factors Oct4 and Sox2 in OCSCs. GAPDH was used as a loading control. $^*P < 0.05$ vs Sh-Luc.

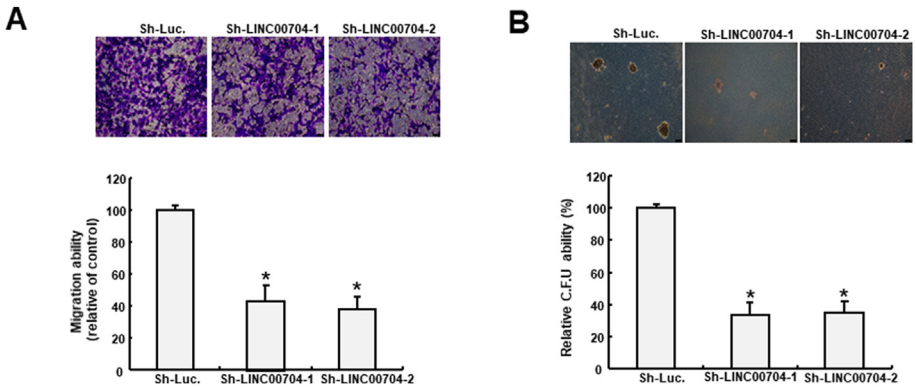

Figure 3 Knockdown of LINC00704 inhibited the malignant properties in oral cancer stem cells. The effect of LINC00704 knockdown on cell migration and anchor-independent growth abilities of oral cancer stem cells (OCSCs) expressing short-hairpin RNA targeting LINC00704 (Sh-LINC00704) or a non-targeting control (Sh-Luc.) were assessed using (A) Transwell culture system and (B) colony formation assay, respectively. $^*P < 0.05$ vs Sh-Luc.

significantly higher LINC00704 expression in the breast cancer spheroids compared to their parental cells.[28] Taken together, these findings suggest that elevated LINC00704 expression not only promotes tumorigenesis and malignant progression but is also required for the dedifferentiation of cancer cells into CSCs. Therefore, LINC00704 holds the potential as a prognostic biomarker for HNSCC patients, including those with OSCC, particularly for predicting malignant progression such as tumor recurrence and metastasis, which are putatively driven by a small CSC population.

Although the study of LINC00704 is still in its nascent stages, current research revealed that it employs multiple mechanisms to promote malignancy. For instance, LINC00704 has been shown to directly bind to PDE4D mRNA, inhibiting its translation and consequently promoting esophageal tumor growth *in vivo*.[29] LINC00704 can also exert its pro-tumorgenic effects via interactions with specific proteins.[18,28] A previous study demonstrated that LINC00704 bound to the hnRNPL protein, preventing hnRNPL-mediated suppression of NET1A mRNA. NET1A, in turn, modulated the cytoskeleton through RhoA/RhoB,

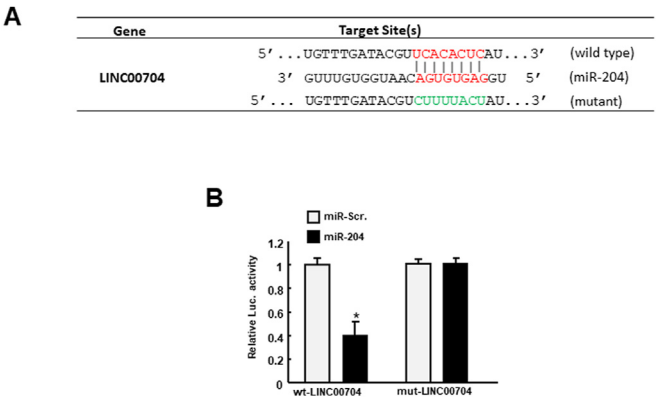

Figure 4 LINC00704 directly interacted with miR-204. (A) Schematic representation of the predicted seed sequence of miR-204 within the LINC00704 3′UTR. (B) Luciferase reporter activity in cells co-transfected with the miR-204 mimics (or scrambled control; miR-Scr.) and the pmirGLO reporter vector containing either the wild-type (wt) or mutated (mut) LINC00704 3′UTR. *$P < 0.05$ vs miR-Scr.

ultimately enhancing the metastatic potential of breast cancer cells, as evidenced by increased lung colonization *in vivo*.[28] Notably, a study reported that LINC00704 stabilized RUNX2 mRNA in lymphoma cells by modulating the miR-218/RUNX2 axis.[17] To our knowledge, this was the first report of LINC00704 acting as a ceRNA. While other studies have suggested that LINC00704 exerted its oncogenic effects by downregulating miR-122a[15] and miR-101a,[16] overexpression of these miRNAs did not affect LINC00704 levels, indicating that LINC00704 does not act by sponging these particular miRNAs. In our study, the direct interaction between LINC00704 and miR-204 was confirmed using a luciferase reporter assay (Fig. 4). We further revealed that LINC00704 functioned as a miR-204 sponge to promote OCSC stemness properties, as demonstrated by the restoration of self-renewal capacity and prevention of apoptosis in LINC00704-silenced OCSCs following miR-204 knockdown (Fig. 5). Supporting our findings, a previous study also proposed a ceRNA mechanism for LINC00704, demonstrating that the LINC00704/miR-204/HMGB1 axis regulated proliferation, migration, and invasion in papillary thyroid carcinoma cells.[27] MiR-204 has been implicated in the progression of various diseases, including diabetic nephropathy-associated fibrosis,[30] osteoarthritis,[31] and sepsis.[32] Notably, it has been identified as a key factor in suppressing cancer stemness of HNSC.[33–35] For example, miR-204 expression was significantly reduced in OSCC spheroids and their derived ALDH$^+$ population compared to parental OSCCs.[34] Similarly, miR-204 expression was found to be downregulated in the CD44$^+$ population of HNSCC patient-derived tumor cells.[33,35] Enforced the expression of miR-204 by intervention with agomiR-204 significantly inhibited HNSCC patient-derived xenograft orthotopic tumor growth and lymph node metastasis *in vivo*.[35] Mechanistically, STAT3 activated by JAK2 represses miR-204 transcription by increasing the recruitment of SNAI2,

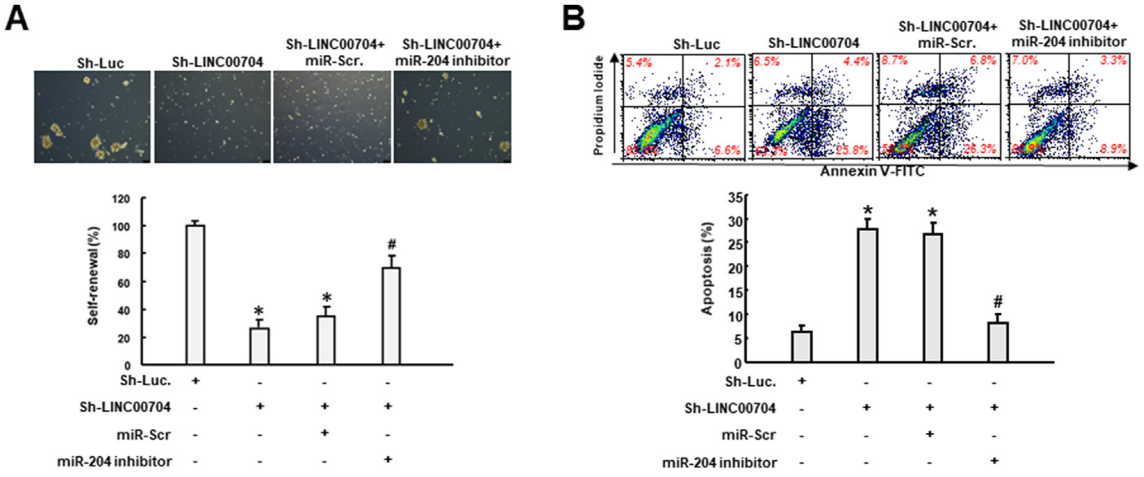

Figure 5 LINC00704 enhanced the self-renewal ability and inhibited cell apoptosis in oral cancer stem cells by inhibiting miR-204. (A) Self-renewal ability of oral cancer stem cells (OCSCs) expressing Sh-LINC00704 (or non-target control; Sh-Luc.) plus miR-204 inhibitor (or a scramble control; miR-Scr.) was assessed by quantifying of number of OCSC spheres. **(B)** Cell apoptosis of OCSCs expressing Sh-LINC704 (or Sh-Luc.) plus miR-204 inhibitor (or miR-Scr.) was evaluated by flow cytometry analysis. Annexin V-FITC positive with propidium iodide negative represents apoptotic cells. *$P < 0.05$ vs Sh-Luc., #$P < 0.05$ vs Sh-LINC00704 plus miR-Scr.

EZH2, and HDAC1 to the pri-miR-204 promoter. Reduced miR-204 expression, in turn, relieved the suppression of its target genes, including SNAI2, HDAC1, and JAK2, establishing a negative feedback loop that maintained miR-204 silencing.[35] In addition, studies have shown that HMGB1 genetic polymorphisms were associated with oral precancerous transformation, lymph node metastasis, and poorer recurrence-free survival.[36] Furthermore, OSCCs secreted HMGB1 in both paracrine and autocrine manners, which further activated the IL-6/STAT3/PD-L1 and IL-6/NFKB/MMP9 pathways in surrounding cancer cells or tumor-associated macrophages, thereby remodeling the tumor immune microenvironment to promote invasion and metastasis.[37] Based on these findings, we assume that HMGB1, stabilized by the LINC00704/miR-204 axis, may also activate STAT3 and the downstream STAT3/miR-204 negative feedback loop, thus maintaining low miR-204 expression.[35] Notably, HMBG1 has also been shown to promote self-renewal and tumorigenicity in glioma CSCs.[38] Irradiation-induced death of pancreatic cancer cells releases HMBG1, which activates stemness in neighboring $CD133^{+}$ cancer cells.[39] While these results lend further support to the role of the LINC00704/miR-204 axis in driving oral cancer stemness, a major limitation lies of our study is the lack of identifying the specific targets of miR-204 within OCSCs. Nonetheless, in the preceding literature summary, we have pointed out the multifaceted ways in which LINC00704 can reportedly regulate tumor malignancy, providing clear directions for our future investigation. Among these, the priority will be to investigate whether the LINC00704/miR-204 axis directly regulates HMGB1 expression in OCSCs. Furthermore, it is also important to explore the potential that miR-204 exerts its effects through the modulation of other target genes, such as the previously mentioned JAK2 and SNAI2. In our previous studies, the SAS cell line has been confirmed as an appropriate source for investigating OCSCs, as it consistently demonstrates stable CSCs enrichment efficiency via sphere formation assays.[9–11,40] However, we acknowledge that relying solely on the SAS cell line is a limitation of our study. To address this, we will validate our findings in additional OSCC cell lines in further research. For instance, we plan to include tongue SCC cell lines such as SCC9 and Cal27, as previous studies have demonstrated that LINC00704 knockdown impaired their malignant behaviors.[26] Furthermore, we are considering the use of OSCC cell lines derived from other oral tissue origins (e.g., the OECM-1 cell line from gingival epithelium),[40] as well as OSCC cell lines with distinct mutation profiles, to further strengthen our evidence.

In conclusion, our study elucidated the functional role of LINC00704 as a molecular sponge for miR-204, highlighting the critical significance of this interaction in maintaining OCSC stemness. These findings advance understanding of OSCC progression and suggest that the LINC00704/miR-204 axis may serve as a promising target for the development of novel therapeutic and diagnostic strategies.

Declaration of competing interest

All authors have no conflicts of interest relevant to this article.

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