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ORIGINAL ARTICLE

Effects of *long noncoding RNA H19* on cementoblast differentiation, mineralisation, and proliferation

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ABSTRACT

Objective: Cementum which is a layer of thin and bone-like mineralised tissue covering tooth root surface is deposited and mineralised by cementoblasts. Recent studies suggested *long noncoding RNA H19* (*H19*) promotes osteoblast differentiation and matrix mineralisation, however, the effect of *H19* on cementoblasts remains unknown. This study aimed to clarify the regulatory effects of *H19* on cementoblast differentiation, mineralisation, and proliferation.

Material and methods: An immortalised murine cementoblast cell line OCCM-30 was used in this study. *H19* expression was examined by real-time quantitative polymerase chain reaction (RT-qPCR) during OCCM-30 cell differentiation. OCCM-30 cells were transfected with lentivirus or siRNA to up-regulate or down-regulate *H19,* then the levels of runt-related transcription factor 2 (*Runx2*), osterix (*Sp7*), alkaline phosphatase (*Alpl*), bone sialoprotein (*Ibsp*), osteocalcin (*Bglap*) were tested by RT-qPCR or western blot. Alizarin red staining, ALP activity assay and MTS assay were performed to determine the mineralisation and proliferation ability of OCCM-30 cells.

Results: *H19* was dramatically increased during OCCM-30 cell differentiation. Overexpression of *H19* increased the levels of *Runx2*, *Sp7*, *Alpl*, *Ibsp*, and *Bglap* and enhanced ALP activity and the formation of mineral nodules. While down-regulation of *H19* suppressed the above cementoblast differentiation genes and inhibited ALP activity and mineral nodule formation. However, the proliferation of OCCM-30 cells was not affected.

Conclusions: *H19* promotes the differentiation and mineralisation of cementoblasts without affecting cell proliferation.

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KEYWORDS
long noncoding RNA H19; cementoblast; dental cementum

Introduction

The tooth cementum is a layer of thin and bone-like mineralised tissue covering the root surface. The two ends of periodontal ligament fibres insert into the alveolar bone and the cementum respectively, anchoring the tooth to the surrounding alveolar bone. Defects in the cementum weaken the attachment function and can even lead to tooth loss. Thus, the integrity of cementum is a noteworthy aspect for orthodontic treatment and is also considered to be the most critical part of successful periodontal regeneration [1]. Cementoblasts located along tooth root surfaces are responsible for cementum matrix deposition and mineralisation. Similar to osteoblasts, cementoblasts also express *Runx2* [2–4], *Sp7* [5,6] and *Ibsp* [7,8], which participate in the regulation of cementoblasts and the development of cementum. However, further studies on the regulatory mechanisms are still needed.

Long noncoding RNAs (lncRNAs) are transcripts longer than 200 nucleotides without protein coding ability [9]. Recently, critical roles of lncRNAs have been identified in skeletal biology and dental biology [10–12]. As one of the first imprinting lncRNAs identified, *H19* is highly conserved during evolution, suggesting an essential role in biological regulation [13]. Previous studies demonstrated that *H19* promotes osteogenic differentiation of human bone marrow mesenchymal stem cells (hMSC) *in vitro* through activating Wnt/β-catenin signalling pathway [14] and TGF-β1/Smad3/HDAC signalling pathway [15]. Recent studies further suggested that *H19* enhances matrix mineralisation *in vitro* through modulating the activity of miR-185-5p and IGF1 in MC3T3-E1 cells [16]. Interestingly, a study using RNA sequencing reported that the expression of *H19* in periodontitis tissues was obviously lower than that in normal tissues from healthy patients [15,17]. However, the function of *H19* in cementoblast differentiation, mineralisation and proliferation remains unclear.

This study aimed to explore the regulatory effects of *H19* on the differentiation, mineralisation and proliferation of cementoblasts.

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Materials and methods

Cell culture

An immortalised murine cementoblast cell line OCCM-30, kindly provided by Dr. Martha J. Somerman (National Institutes of Health, Bethesda, MD, USA), was cultured as previously described [18–20]. Briefly, cells were maintained and passaged in Dulbecco's Modified Eagle's Medium (DMEM; HyClone, Logan, UT, USA) containing 10% foetal bovine serum (FBS; Gibco, Waltham, MA, USA) and 1% penicillin-streptomycin at 37 °C in 5% CO_2. Cementoblast differentiation was induced at 80–90% confluence using DMEM containing 5% FBS, 50 μg/mL ascorbic acid and 10 mM Naβ-glycerophosphate. The mineral induction medium was replaced every 2–3 days.

Transient transfection

OCCM-30 cells were seeded into 6-well plates (5×10^5 cells/well). The medium was replaced by fresh DMEM plus 5% FBS without antibiotics 1 h before transfection. At 70%-80% confluence, cells were transfected with 100 nM siRNA targeting *H19* or the negative control (Ribobio Company, Guangzhou, Guangdong, China). Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) was used according to the protocols provided by the manufacturer. After transfection for 24 h, the medium was changed. Total RNA or protein was collected at the indicated times.

Establishment of a stable cell line

The stable *H19* overexpression OCCM-30 cell line was generated using a lentivirus-mediated gene delivery method. Recombinant lentivirus overexpressing *H19* or its negative control were provided by GeneChem Company (Shanghai, China). Cells were seeded into 24-well culture plates (5×10^4 cells/well). After reaching 30–40% confluence, OCCM-30 cells were transfected with the lentivirus at an optimised multiplicity of infection (MOI) of 100 and 5 μg/ml polybrene (GeneChem, Shanghai, China). 8 h later, the medium was changed to DMEM containing 10% FBS. After 72 h, 2 μg/ml puromycin was used to select the stable *H19* overexpression cells. The heterogeneous collection of puromycin-resistant cells was used for the following experiments.

RNA isolation and RT-qPCR

TRIzol reagent (Invitrogen) was used to extract total RNA at the indicated times. The quality of total RNA was detected by a NanoDrop 2000 spectrometer (NanoDrop Technology, Rockland, DE, USA), obtaining the OD260/280 and OD260/230 values. First-strand cDNA was synthesised by a reverse transcription kit (TaKaRa, Tokyo, Japan) from 1000 mg RNA. Then, RT-qPCR was performed using the SYBR Premix Kit (TaKaRa) and the Applied Biosystems QuantStudio 6 system in accordance with the manufacturer's protocols. The primers for mouse *H19*, *Bglap*, *Sp7*, *Runx2*, *Ibsp*, *Alpl* and *Gapdh* were purchased from Sango Biotech (Shanghai, China). The primer sequences are shown in Table 1.

Protein extraction and Western blot

Western blot was carried out as previously described [20]. Proteins were extracted with 180 μl total protein lysis buffer (Thermo Scientific, Waltham, MA, USA) supplemented with protease inhibitors. Then, a bicinchoninic acid kit (Appligen Company, Beijing, China) was used to detect the protein concentration. After reduction and denaturation, the proteins were separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels (Good bio, Wuhan, Hubei, China), transferred to a polyvinylidene difluoride membrane (Roche, Indianapolis, USA), blocked with 5% non-fat milk for no less than 1 h, and incubated with primary antibodies for osterix (1:1000; Abcam, Cambridge, MA, USA), Runx2 (1:1,000; Cell Signalling Technology, Beverly, MA, USA), and GAPDH (1:10,000; Good bio, Wuhan, China) overnight at 4 °C. Then,

Table 1. Sequences of the primers used for RT-qPCR.

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)
Runx2	CCCAGCCACCTTTACCTACA	TATGGAGTGCTGCTGGTCTG
Alpl	TGTGGAATACGAACTGGATGAG	ATAGTGGGAATGCTTGTGTCTG
Sp7	CCTCTCGACCCGACTGCAGATC	AGCTGCAAGCTCTCTGTAACCATGAC
H19	CCTCAAGATGAAAGAAATGGTGCTA	TCAGAACGAGACGGACTTAAAGAA
Ibsp	GAGCCTCGTGGCGACACTTA	AATTCTGACCCTCGTAGCCTTCATA
Bglap	GAGGACCATCTTTCTGCTCACT	CGGAGTCTGTTCACTACCTTATTG
Gapdh	ACCACAGTCCATGCCATCAC	TCCACCACCCTGTTGCTGTA

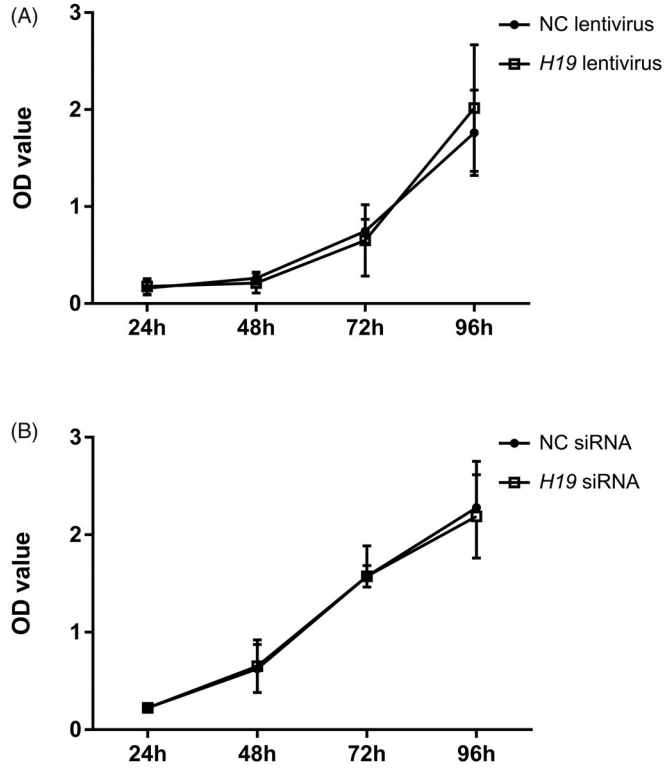

Figure 1. There was no effect of *H19* on the proliferation ability of OCCM-30 cells. (A, B) Cells without mineral induction treated with lentivirus (A) or siRNA (B) were incubated for 24 h, 48 h, 72 h and 96 h, then the proliferation ability was detected *via* the MTS assay. NC group and *H19* group were compared at each time point by *t*-test. *H19*, *lncRNA H19*; NC, negative control.

the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (Good bio, Wuhan, Hubei, China) for 1 h at room temperature. The membranes were visualised using enhanced chemiluminescent solution (Thermo Scientific, Waltham, MA, USA). Image J was used for densitometry analysis.

Alizarin red staining

Cells transfected with *H19* lentivirus, negative control lentivirus, *H19* siRNA, or negative control siRNA were incubated with mineral induction medium as indicated. After 14 days' mineral induction, cells were fixed in 4% formaldehyde for 15 min and then incubated with 0.1% alizarin red (pH 4.2) for 10 min at 25 °C. Cells were rinsed one time with distilled water to remove unbound alizarin red. Photographs were captured with a camera and light microscope. To quantitatively analysis the mineralisation degree, we used 10% cetylpyridinium chloride (Sigma-Aldrich, St Louis, MO, USA) to extract the stains and then measured the OD values at 562 nm [20,21].

Alkaline phosphatase activity

Proteins were extracted and quantified as described above. Then, the activity of ALP was measured following the instructions of the ALP activity kit (Nanjing Jiancheng Company, Nanjing, Jiangsu, China). The OD values were measured at 520 nm and then normalised by the total protein concentration [20,21].

Alkaline phosphatase staining

After 4 days' mineral induction, ALP staining was performed with a BCIP/NBT staining assay kit (Beyotime Company, Shanghai, China). Cells were fixed in 4% formaldehyde for 15 min, rinsed three times with PBS, and then stained with the BCIP/NBT substrate for 15 min at room temperature [21].

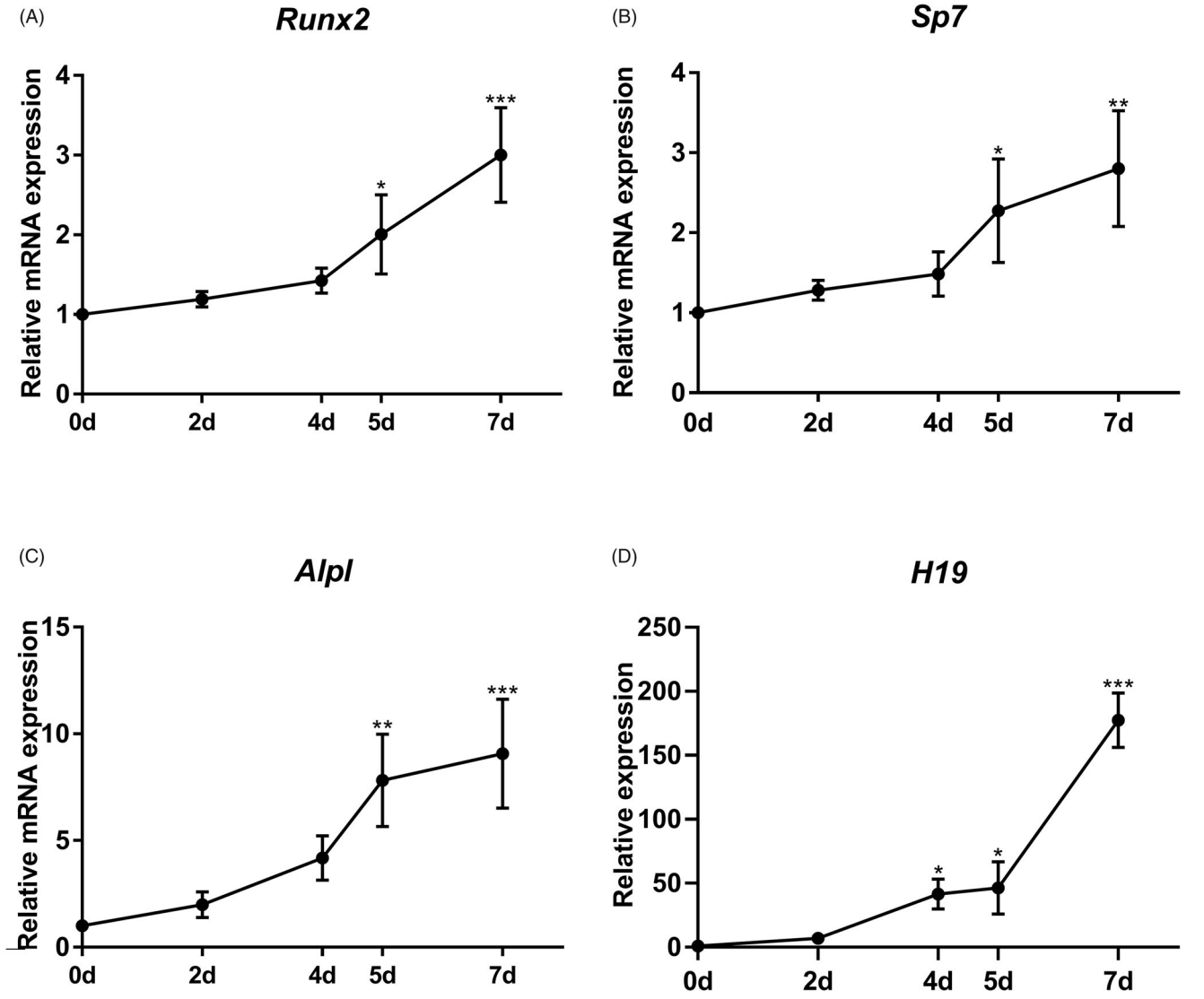

Figure 2. *H19* was increased during the differentiation of OCCM-30 cells. (A-C) OCCM-30 cells were treated with mineral induction medium, and the relative mRNA levels of *Runx2*, *Sp7* and *Alpl* were determined by RT-qPCR at days 0, 2, 4, 5 and 7. (D) RT-qPCR analysis of *H19* expression after mineral induction at the indicated time points. One-way ANOVA followed by the Bonferroni test was performed and star represented the difference compared to the 0d group. $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$.

Cell proliferation analysis

Proliferation rates of OCCM-30 cells were measured by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay. Cells were seeded in 96-well plates at a density of 5,000 cells per well overnight and then transfected with *H19* siRNA as described above. CellTiter 96 AQ_{ueous} reagent (Promega, Madison, WI, USA) was used to evaluate cell proliferation following the manufacturer's protocol, and the OD values were read at 490 nm.

Statistical analysis

All experiments were repeated at least three times, and the data are expressed as the mean ± SD. One-way analysis of variance followed by the Bonferroni test for multiple comparisons or t test was carried out as appropriate to evaluate the differences between groups at a significance level of $p < .05$.

Results

Effects of *H19* on the proliferation of OCCM-30 cells

MTS assay was performed to explore the influence of *H19* on OCCM-30 cell proliferation. The results showed that there was no significant effect of *H19* on cell proliferation in the detected time period (24 h–96h) (Figure 1(A,B)).

Expression of *H19* during the differentiation of OCCM-30 cells

To investigate the function of *H19*, we detected its expression pattern in OCCM-30 cells during differentiation. The RT-qPCR results showed that *Runx2*, *Sp7* and *Alpl* mRNA levels were up-regulated (Figure 2(A–C)) under mineral induction conditions. Further analysis revealed that *H19* expression also increased significantly, reaching >100-fold on Day 7 (Figure 2(D)).

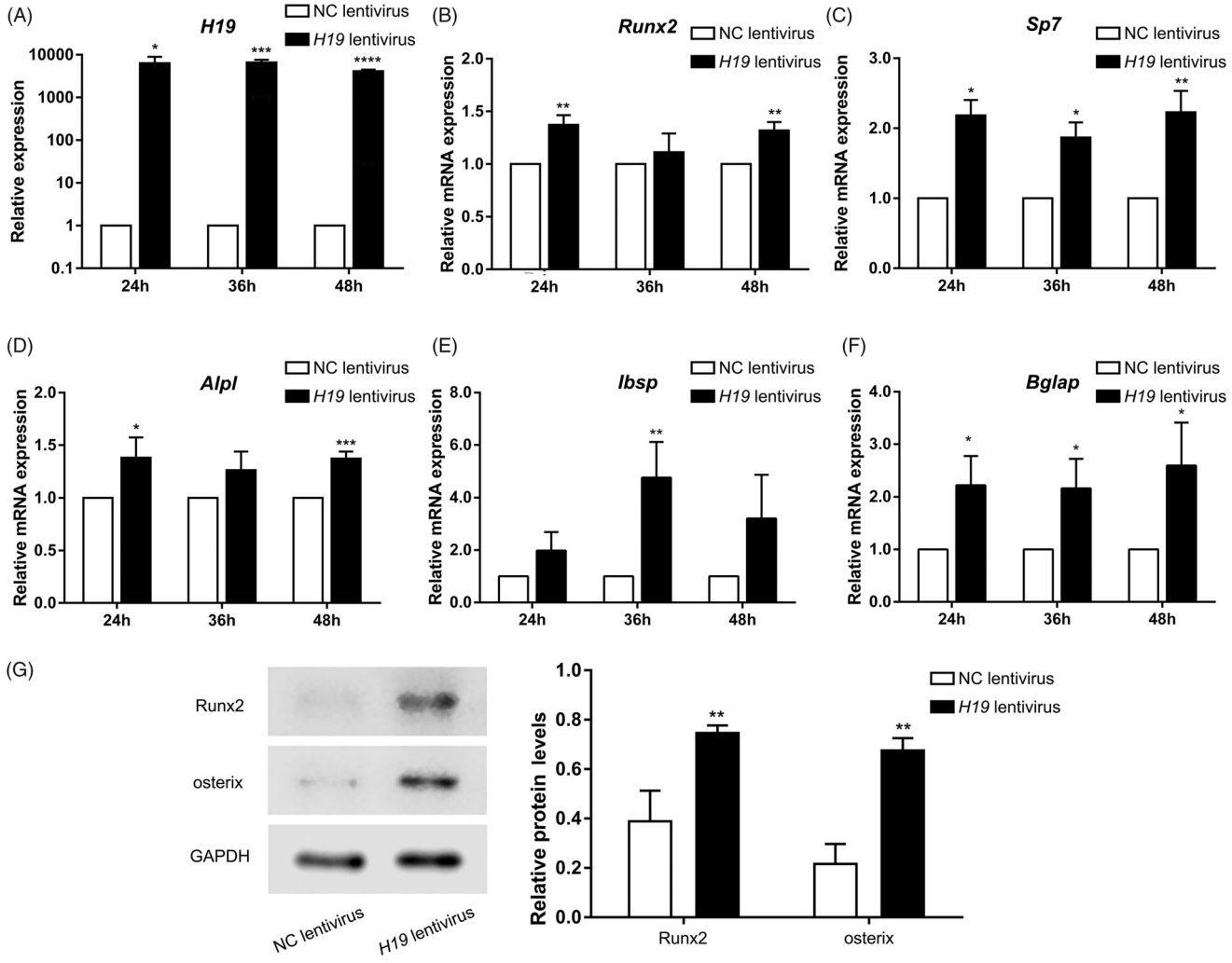

Figure 3. Overexpression of *H19* promoted the differentiation of OCCM-30 cells. Cells without mineral induction were infected with lentivirus as described above. (A) After infection for 24 h, 36 h and 48 h, the expression level of *H19* was determined by RT-qPCR. (B-G) After infection for 24 h, 36 h and 48 h, the mRNA expression levels of *Runx2*, *Sp7*, *Alpl*, *Ibsp* and *Bglap* were determined by RT-qPCR, and the protein levels of Runx2 and osterix were measured by western blot analysis. NC group and *H19* group were compared at each time point by *t*-test. * $p < .05$, ** $p < .01$, *** $p < .001$. *H19*, *lncRNA H19*; NC, negative control.

Effects of up-regulation of *H19* on the differentiation of OCCM-30 cells

To further study the influence of *H19* on cementoblast differentiation, an OCCM-30 cell line that stably overexpressed *H19* was established (Figure 3). The RT-qPCR results demonstrated markedly up-regulated *H19* expression was obtained in the *H19* overexpression group (Figure 3(A)). The mRNA levels of *Runx2*, *Sp7*, *Alpl*, *Ibsp* and *Bglap* (Figure 3(B–F)) and the protein levels of Runx2 and osterix (Figure 3(G)) were significantly increased in the *H19* overexpression group compared with those in the control group.

Effects of downregulation of *H19* on the differentiation of OCCM-30 cells

To more thoroughly understand the relationship between *H19* and cementoblast differentiation, siRNA was used to downregulate *H19*. The RT-qPCR data revealed that *H19* was obviously decreased under the treatment of *H19* siRNA (Figure 4(A)). In addition, the mRNA levels of *Runx2*, *Sp7*, *Alpl*, *Ibsp* and *Bglap* (Figure 4(B–F)) and the protein levels of Runx2 and osterix (Figure 4(G)) were decreased after *H19* knockdown.

Effects of *H19* on the ALP activity and mineralisation ability of OCCM-30 cells

To determine whether *H19* could promote the mineralisation of OCCM-30 cells, alizarin red staining was performed after 14 days' mineral induction and the results suggested that mineralised nodules significantly increased in the *H19* overexpression group and decreased in the *H19* siRNA group (Figure 5(A)). ALP activity and ALP staining assays were performed after 4 days' mineral induction. The results suggested that ALP activity was increased in *H19*-overexpression cells and decreased in *H19*-deficient cells (Figure 5(B,C)).

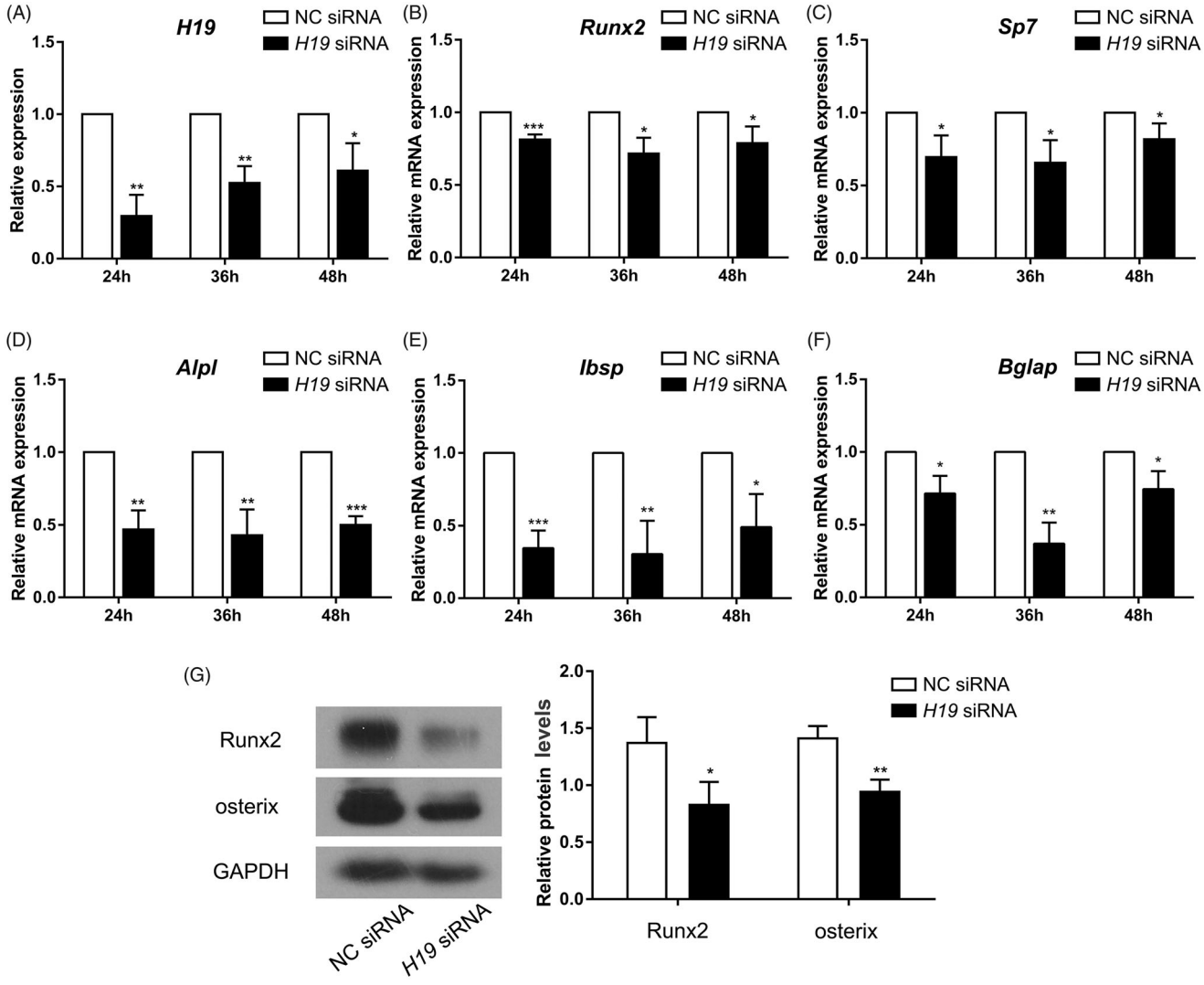

Figure 4. Knockdown of *H19* suppressed the differentiation of OCCM-30 cells. Cells without mineral induction were transfected with siRNA as described above. (A) After transfection for 24 h, 36 h and 48 h, the expression level of *H19* was determined by RT-qPCR. (B-G) After transfection for 24 h, 36 h and 48 h, the mRNA expression levels of *Runx2*, *Sp7*, *Alpl*, *Ibsp* and *Bglap* were determined by RT-qPCR, and the protein levels of Runx2 and osterix were measured by western blot analysis. NC group and *H19* group were compared at each time point by *t*-test. * $p < .05$, ** $p < .01$, *** $p < .001$. *H19*, *lncRNA H19*; NC, negative control.

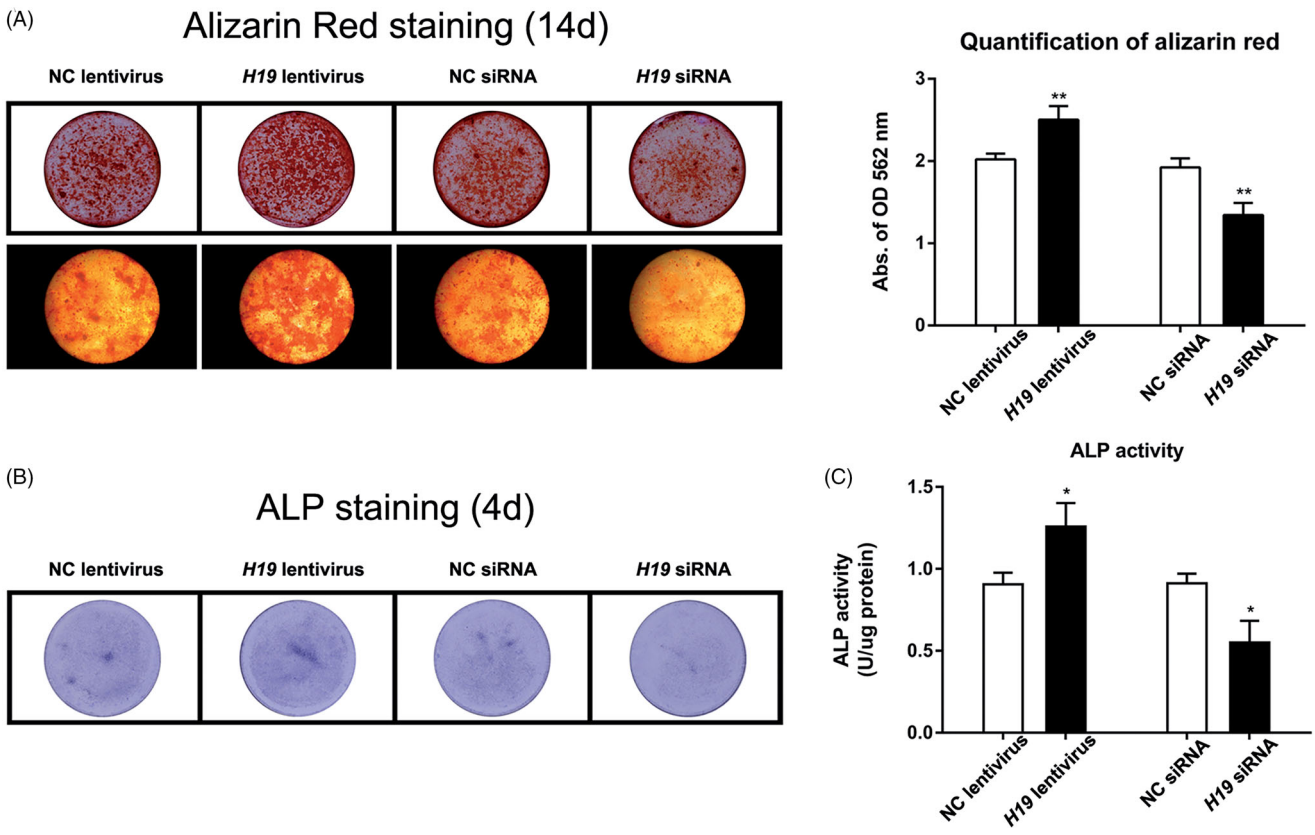

Figure 5. The mineralisation ability of OCCM-30 cells was enhanced by *H19*. (A) Cells were treated with lentivirus or siRNA as indicated. Alizarin red staining was performed after incubation with mineral induction medium for 14 d, then the mineral nodules were photographed by camera (upper row) or under microscope (lower row). The quantification of alizarin red staining was performed by using 10% cetylpyridinium chloride to dissolve the nodules. Then, the absorbance was read at 562 nm. (B) After mineral induction for 4 d, images of ALP staining were taken. (C) The ALP activity assay was also performed, and the data were normalised by the total protein concentration. NC group and *H19* group were compared at each time point by *t*-test. * $p < .05$, ** $p < .01$, *** $p < .001$. *H19*, *lncRNA H19*; NC, negative control.

Discussion

The regeneration of the cementum is considered to be the gold standard and the most important part of periodontal regeneration [1,22,23]. *H19*, which is highly conserved among different species, has an important effect on gene regulation, from cell differentiation to migration. In our experiment, in accordance with the trends of *Runx2*, *Alpl* and *Sp7*, *H19* expression was dramatically increased in OCCM-30 cells under mineral induction, suggesting a possible positive correlation between *H19* and cementoblast differentiation. This result was supported by previous studies conducted by Qinpo Zhou et al. which found that *H19* was highly expressed in mineralised MC3T3-E1 cells [16] and mouse osteoblastic osteosarcoma tissues [24,25].

Previous studies have demonstrated that *H19* was associated with cell differentiation and promoted osteoblast differentiation by mediating the transforming growth factor-β1/Smad3/histone deacetylase signalling pathway and acting as a molecular sponge to absorb microRNA-141 and microRNA-22 [14,15]. However, recent studies showed that *H19* expression was significantly decreased during the osteogenic differentiation of human adipose-derived stem cells and that the silencing of *H19* promoted the levels of Runx2 and ALP [10]. The discrepancy between the above studies raised the question how *H19* influences the differentiation of cementoblasts. In this study, we found that *H19* positively regulated the mRNA and protein levels of Runx2 and osterix (*Sp7*), both of which are essential transcription factors for the differentiation of cementoblasts [3,5]. The findings regarding the regulation of Runx2 and osterix are consistent with the results obtained in previous studies which were conducted during the osteogenesis of human bone marrow mesenchymal stem cells [14,15].

Study conducted by Hadji et al. suggested that the upregulation of *H19* in calcific aortic valve disease promoted the mineralisation of the aortic valve through preventing the recruitment of p53 to its promoter and then silencing the NOTCH1 signalling pathway [26]. Here, we further examined several other cementoblast differentiation-related genes, including *Alpl* [27], *Ibsp* [7,8] and *Bglap* [27,28], and found that the mRNA expression levels were strongly increased by *H19*. Then, ALP activity was detected and also found to be positively associated with *H19*. To further verify the effect of *H19* on cementoblast mineralisation, alizarin red staining was performed and found that the mineralisation ability of OCCM-30 cells was enhanced when the expression of *H19* was upregulated. These results were supported by studies conducted by Huang et al. [15] and Hadji et al. [26] which demonstrated that *H19* promoted the osteogenic differentiation and mineralisation of primary human bone marrow mesenchymal stem cells and primary human valve interstitial cells.

Previous studies suggested that *H19* contributed to the proliferation of osteoblasts [29,30], chondrocytes [29], and gastric cancer cells [31]. In this study, we found that *H19* did not affect the proliferation of OCCM-30 cells. Similar to our research, study conducted by Liang et al. [14] reported that neither *H19* nor its encoded microRNA-675-5p affected osteogenesis by regulating cell proliferation. The divergence between these studies may be due to the differences in cell types and treatment methods.

In summary, this study revealed a previously undefined role of *H19* and suggested that *H19* significantly promoted cementoblast differentiation and mineralisation while showed no obvious effect on cementoblast proliferation.

Disclosure statement

All authors declare that they have no conflicts of interest related to this work.

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