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Original Article

miR-141 modulates osteoblastic cell proliferation by regulating the target gene of lncRNA H19 and lncRNA H19-derived miR-675

Peiheng He*, Ziji Zhang*, Guangxin Huang, Hua Wang, Dongliang Xu, Weiming Liao, Yan Kang

Department of Joint Surgery, The First Affiliated Hospital of Sun Yat-sen University, Guangzhou 510080, China.
**Equal contributors.*

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Abstract: Increasing evidence has reported the significant roles of lncRNA or miRNAs in the biology of various diseases. This study was aimed to elucidate the potential roles of miR-141 and lncRNA H19 and H19-derived miR-675 in regulating osteoblasts proliferation and apoptosis and to explore its potential mechanism. miR-141 mimic or miR-141 inhibitor or siRNA-H19 or miR-675 inhibitor was transfected into human hFOB1.19 cells. The effects or miR-141 expression on H19 or miR-675 expression, on osteoblasts proliferation and apoptosis were analyzed. Moreover, effects of H19 and miR-675 expression on cell proliferation were also analyzed. The results showed that miR-141 was down-regulated in both hFOB1.19 cells and osteosarcoma tissues. The overexpressed miR-141 suppressed H19 and miR-675 expression in hFOB1.19 cells. Besides, miR-141 suppression significantly increased cell viability but this effect was blocked by silencing H19 or miR-675 inhibitor, which is similar to the effects on VEGF and IGF2 expression. Furthermore, miR-141 overexpression induced osteoblasts apoptosis, but decreased the levels of caspase-3 and the Bcl-2/Bax ratio. Taken together, our study revealed that tumor-suppressor miR-141 overexpression suppressed osteoblasts proliferation but induced apoptosis through down-regulating H19 or miR-675 in osteosarcoma. This study may provide theoretical basis for illustrating the interaction between lncRNA and miRNAs in osteosarcoma and for the therapeutic target of miR-141 in osteosarcoma treatment.

Keywords: Osteoblasts, cell proliferation, miR-141, lncRNA H19, lncRNA H19-derived miR-675

Introduction

Osteosarcoma is a kind of malignant bone tumors that appears mostly in children and adolescents, as well as in the aging among 70-80 years old [1]. Statistics has shown that the 5-year survival rate for osteosarcoma patients is about 53.9% [2]. Although the 5-year survival rate for osteosarcoma has been improved by the new adjuvant chemotherapy, the recurrence and metastasis make osteosarcoma treatment to be a puzzle [3]. Previous study revealed that the genetics alteration during the mesenchymal stem cells differentiated into osteoblasts resulted in osteoblasts to be variant osteosarcoma cells [4], but the molecular mechanism remains complicate. Therefore, it will be of great significance to explore the potential mechanism of genetic alteration for osteoblasts.

microRNAs are some endogenous 20- to 22-nt in length and are highly conserved non-coding RNAs that have various biological functions at the transcriptional or post-transcriptional level through targeting the 3'-UTR of genes [5]. Increasing evidence has demonstrated that various miRNAs are involved in the progression of osteosarcoma tumorigenesis and metastasis [6]. miR-141, which belongs to miR-200 family, has been proved to be a tumor suppressor for human osteosarcoma [7]. The osteoblasts differentiation is regulated by miR-141 and miR-200a through targeting Dlx5 [8].

Long noncoding RNAs (lncRNAs) are a subgroup of noncoding RNAs that are longer than 200 bp, and might play central roles in a variety of biological processes through complicated mechanisms [9]. LncRNA H19 is a 2.3 kb in length and is located in an imprinted region of chromo-

some 11 neat the insulin-like growth factor (IGF2) gene, which also contains a miR-675 in exon one [10]. It has been demonstrated that lncRNA H19 can act as either tumor suppressor or tumor contributor during the development of various cancers. For example, the up-regulated lncRNA H19 promotes the cell proliferation and metastasis bladder tumor while H19 inhibits pluripotent stem cell proliferation through regulating IGF2 [11, 12]. Previous studies have reported the pivotal roles of H19 and H19-derived miR-675 in a variety of diseases, including bladder cancer and gastric cancer [13, 14]. Research focusing on the regulatory correlation between lncRNA-miRNA functional network and diseases brings deep insight for exploring pathogen for diseases, which has become the hotspot in recent years. The lncRNA H19 can be negatively regulated by miR-141 in gastric cancer cell proliferation and migration [15]. However, few have been reported about the potential roles of miR-141 and lncRNA H19 and H19-derived miR-675 in osteoblasts proliferation.

In the current study, we investigated the potential roles of miR-141 on osteoblasts proliferation mediated by lncRNA H19 and H19-derived miR-675 using human hFOB1.19 cells and siRNA-mediated gene silencing. Various experimental methods were used to evaluate the effects of miR-141 expression on osteoblasts apoptosis and cell proliferation-related protein expression. The aim of this study was to investigate the potential roles of miR-141 on osteoblasts proliferation and to elucidate the potential mechanism of action.

Materials and methods

Cell culture and siRNA transfection

Human osteoblasts hFOB1.19 was cultured in DMEM: Ham's F-12 (1:1) medium without phenol red containing 10% fetal bovine serum (FBS) and 0.3 mg/mL of G418, and maintained at 37°C in a humidified atmosphere containing 5% CO_2.

Cells were plated onto the 60-mm dish, and after 24 h of incubation, cells were treated with Lipofectamine 2000 transfection reagent (Life Technologies, USA). siRNA-H19 or siRNA-control vectors or plasmid or miRNA mimic or miRNA inhibitor was transfected into cells. After 48 h of transfection, cells were prepared for further analysis.

MTT assay

Cell proliferation was assessed using MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide) assay as previously described [16]. Cells were plated onto 96-well plates at 5×10^3 cells/well in 0.1 mL of DMEM medium. After 24 h of incubation, cells were transfected with siRNA-H19, miR-675 inhibitor, and siRNA-control for another 24 to 96 h of incubation. Then, 20 μL of MTT were added to each well and incubated at 37°C for 4 h. Finally, 150 μL of dimethylsulfoxide (DMSO) was mixed with the cells for 10 min to stop reactions. Absorbance of cells in each well was observed at 570 nm under an absorption spectrophotometer (Olympus, Japan). All experiments were conducted independently for 3 times.

Western blotting

Protein samples (30 μg per lane) were separated onto a 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel and transferred onto polyvinylidenedifluoride (PVDF) membranes (Mippore). Then the PVDF membranes were blocked in Tris Buffered Saline Tween (TBST) containing 5% non-fat milk for 1 h at room temperature. Membranes were incubated with rabbit anti-human antibodies (caspase-3, Bcl-2, Bax, and cleaved caspase-3; 1:100 dilution, Invitrogen) and overnight at 4°C. Consequently, membrane was incubated with hoseradish peroxidase labeled goat anti-rat secondary antibody (1:1000 dilution) at room temperature for 1 h. Finally, the PVDF membranes were washed 3 times with 1× TBST buffer for 10 min each. The signals were detected after incubation with a chromogenic substrate using the enhanced chemiluminescence (ECL) method. Additionally, GAPDH served as the internal control.

Real time PCR

Total RNA was isolated from cells after 48 h of transfection using TRIzol (Invitrogen) according to the manufacturer's protocol [17]. Isolated RNA was treated with RNase-free DNase I (Promega Biotech, USA), and the concentration and purity of the isolated RNA were determined using SMA 400 UV-VIS (Merinton, Shanghai, China). cDNA synthesis was conducted using the reverse transcriptase PrimeScript 1st Strand cDNA Synthesis Kit (Invitrogen, USA). The expression levels of miRNAs were detected using the SYBR ExScript RT-qPCR Kit (Takara,

Table 1. Primers used for targets amplification in this study

Name	Primer	Sequence (5'-3')
GAPDH	Sense	TATGATGATATCAAGAGGGTAGT
	Antisense	TGTATCCAAACTCATTGTCATAC
miR-141	Sense	GGGCATCTTCCAGTACAGT
	Antisense	CAGTGCGTGTCGTGGAGT
H19	Sense	CCCACAACATGAAAGAAATGGTGC
	Antisense	CACCTTCGAGAGCCGATTCC
miR-675	Sense	GGGTGGTGCGGAGAGGGCCC
	Antisense	CAGTGCGTGTCGTGGAGT

China). Phosphoglyceraldehyde dehydrogenase (GAPDH) was chosen as the internal control. Primers used for target amplification are shown in **Table 1**.

Cell apoptosis

Cell apoptosis was detected using Annexin V-Cy5 and propidium iodide (PI) staining and analyzed by flow cytometry [18]. Briefly, after 24 h of transfection, the cells were harvested and washed with PBS 3 times. Then the cells were pelleted and resuspended in 5 μL of Annexin V-binding buffer containing Annexin V-Cy5 (1:1000) and 5 μL of PI at room temperature for 10 min. The cells were then analyzed using a FACS Calibur flow cytometer (Becton-Dickinson, CA, USA). Annexin V-positive and propidium iodide-negative cells were considered to be apoptotic cells.

Statistical analysis

All the experiments were conducted independently 3 times. Data are presented as the mean ± SD. Statistical analyses were performed using Graph Prism 5.0 software (GraphPad Prism, San Diego, CA). Significant differences in the data between two groups were analyzed using Student's t test. The $P<0.05$ was considered to indicate a significant difference.

Results

Influence of miR-141 expression on H19 and miR-675 in cells and tissues

The results showed that miR-141 expression was significantly up-regulated by miR-141 mimic transfection in hFOB1.19 cells, but was suppressed by the miR-141 inhibitor compared with the controls ($P<0.05$, **Figure 1A**). Both H19 and miR-675 levels in hFOB1.19 cells were significantly decreased by overexpression of miR-141 than that in control group, but their expression were reversed by miR-141 inhibitor ($P<0.05$, **Figure 1B** and **1C**). In addition, expression of H19 and miR-675 in adjacent tissues were both significantly higher than that in tumor tissues ($P<0.01$, **Figure 1D** and **1E**), suggesting that there may be certain correlation between miR-141 expression and H19 and miR-675 expressions in osteosarcoma.

miR-141 overexpression suppressed osteoblasts cell proliferation

When hFOB1.19 cells were transfected with miR-141 mimic, cell proliferation was significantly decreased compared to that in controls from 48 h ($P<0.05$, **Figure 2A**), but this effect was opposite to that in cells transfected with miR-141 mimic inhibitor ($P<0.05$, **Figure 2B**). These results suggested that miR-141 may play certain suppress roles in hFOB1.19 cell proliferation.

miR-141 suppression promotes osteoblasts proliferation via affecting H19 and miR-675

siRNA-H19 or miR-675 inhibitor was transfected into hFOB1.19 cells respectively to observe the effects of H19 or miR-675 expression on osteoblasts cell proliferation. The results showed that relative level of H19 or miR-675 was significantly decreased by siRNA or inhibitor transfection compared with the control (**Figure 3A** and **3C**). Consequently, miR-141 inhibitor was transfected into cells to assess the correlation between miR-141 and H19 or miR-675 during cell proliferation. Cell viability was significantly increased by miR-141 inhibitor transfection compared with the 24 h with time increasing ($P<0.05$), while cell viability was drastically suppressed by silencing H19 or miR-675 inhibitor with time increasing ($P<0.05$, **Figure 3B** and **3D**). However, cell viability was significantly decreased by co-transfection of siRNA-H19/miR-675 inhibitor and miR-141 inhibitor compared with the miR-141 inhibitor group with time increasing ($P<0.05$), suggesting that the promote role of miR-141 inhibitor on cell proliferation may be suppressed by silencing H19 or miR-675 inhibitor.

Expression of VEGF and IGF2 in hFOB1.19 cells

Since the former results revealed that miR-141 may play certain roles in regulating osteoblasts

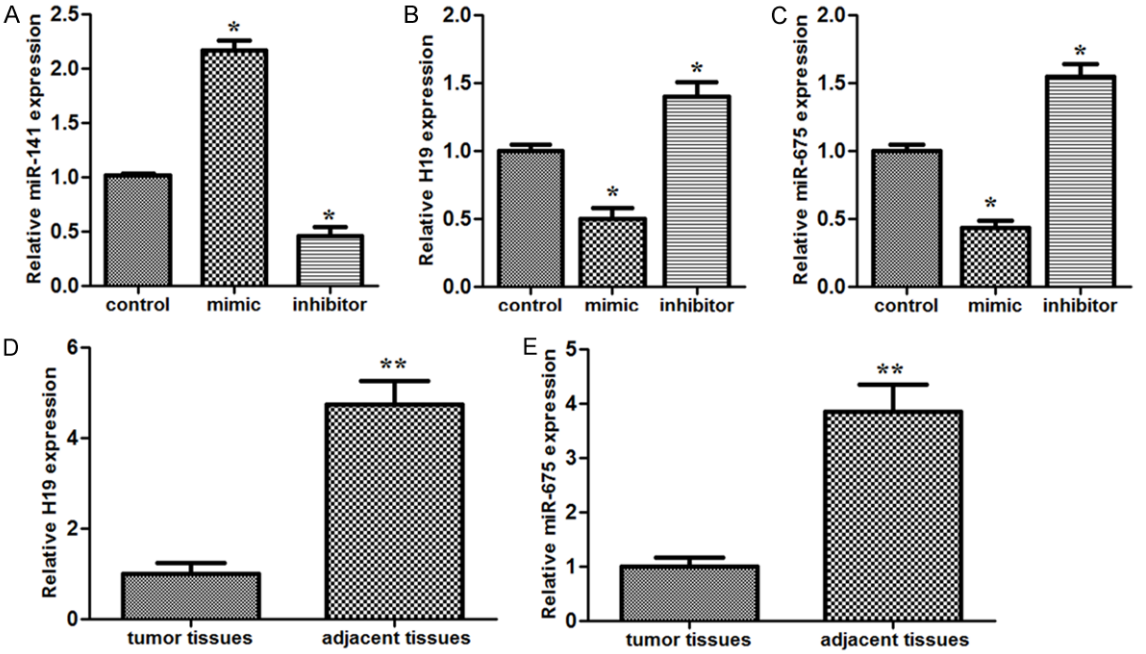

Figure 1. Expression of miR-141 and its target genes in osteoblasts and in tumor tissues. A: miR-141 mimic treatment significantly increased the miR-141 level in hFOB1.19 cells; B, C: Relative expression of H19 and miR-675 was significantly decreased by miR-141 overexpression; D, E: Relative level of H19 and miR-675 in adjacent tissues was significantly higher than that in tumor tissues. *: P<0.05 compared to the control; **: P<0.01 compared to the tumor tissues group.

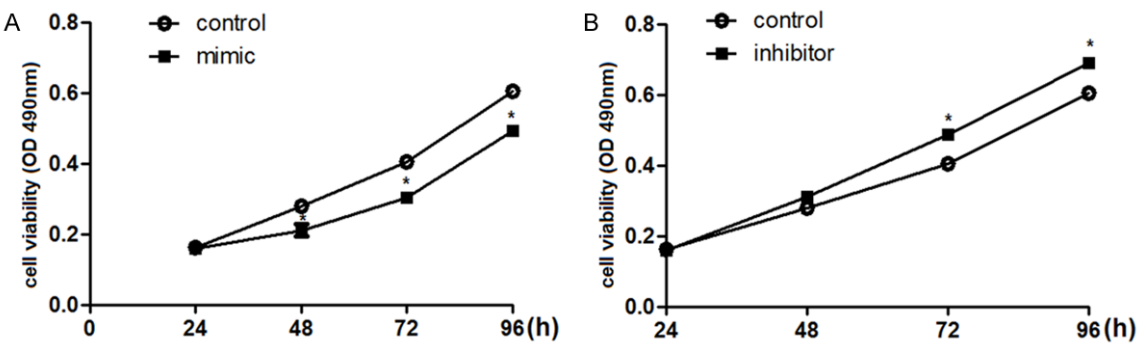

Figure 2. Influence of miR-141 expression on osteoblasts cell viability. A: miR-141 overexpression significantly decreased the osteoblasts cell viability from 48 h; B: Cell viability was significantly increased by miR-141 inhibitor compared to the 24 h with time increasing. *: P<0.05 compared to the control group (24 h).

proliferation via affecting the H19 and miR-675 expression, therefore, we further analyzed the expression of vascular endothelial growth factor (VEGF) and IGF2 to explore the potential regulatory mechanism (**Figure 4**). The results showed that VEGF and IGF2 levels were decreased by miR-141 overexpression (**Figure 4A**), also, protein levels of the two factors were also suppressed by silencing H19, which was reversed by co-transfection of silencing H19 and miR-141 mimic (**Figure 4B**). Besides, our results revealed that protein levels of VEGF and IGF2 were decreased by miR-675 inhibitor, indicating that miR-141 overexpression may suppress VEGF and IGF2 expression through down-regulating H19 or miR-675 in osteoblasts.

Effects of miR-141 expression on osteoblasts apoptosis

As is shown in **Figure 3** (part 3), the promote effect of miR-141 inhibitor on the cell prolifera-

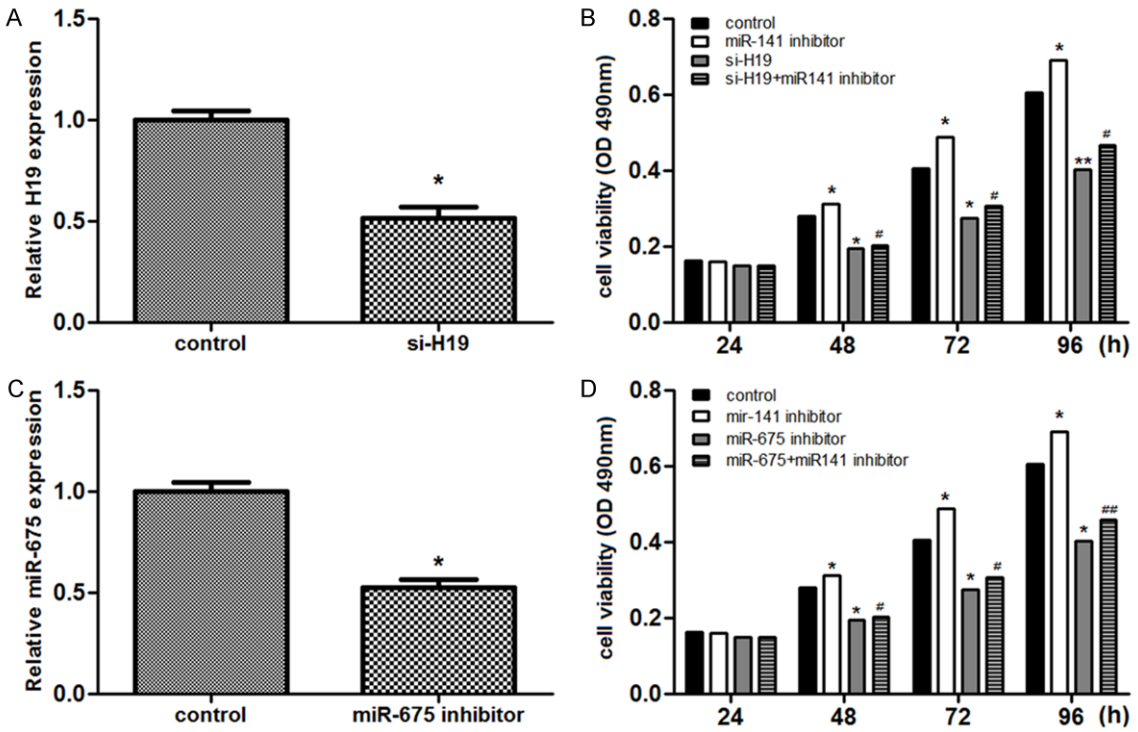

Figure 3. Effects of H19 or miR-675 expression on osteoblasts cell viability. A: Relative level of H19 was decreased by silencing H19; B: Compared with the control group, cell viability was significantly increased by miR-141 inhibitor treatment while was significantly decreased by silencing H19. Cell viability was significantly decreased by silencing H19 compared to the miR-141 inhibitor group; C: Relative level of miR-675 was decreased by miR-675 inhibitor treatment; D: Compared with the control group, cell viability was significantly increased by miR-141 inhibitor treatment while was significantly decreased by miR-675 inhibitor. Cell viability was significantly decreased by miR-675 inhibitor compared to the miR-141 inhibitor group. *: P<0.05 and **: P<0.01, compared to the control (24 h), #: P<0.05 and ##: P<0.01, compared to the miR-141 inhibitor group.

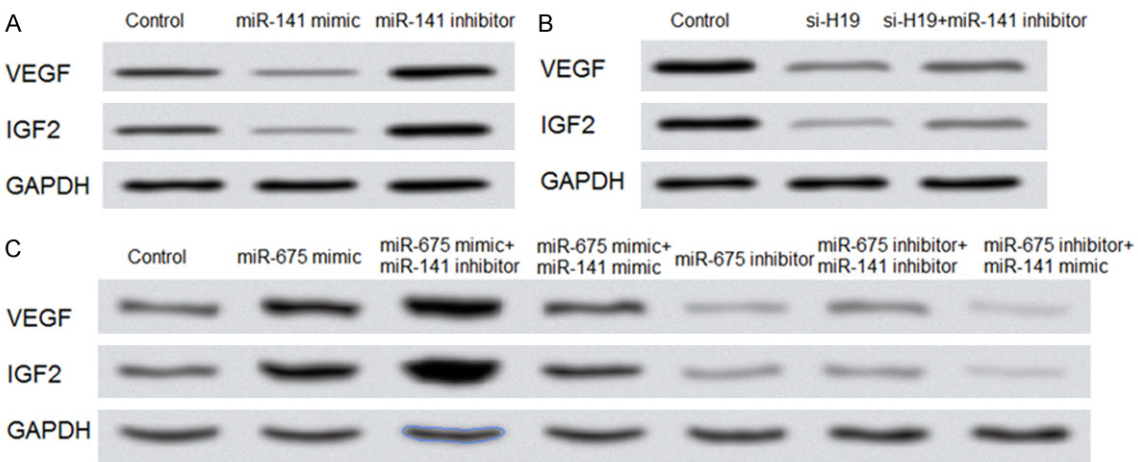

Figure 4. Expression of VEGF and IGF2 in osteoblasts. A: VEGF and IGF2 levels were decreased by miR-141 overexpression; B: Silencing H19 decreased the protein levels of VEGF and IGF2, and also suppressed the promote effects of miR-141 inhibitor on VEGF and IGF2 expressions; C: The VEGF and IGF2 expressions were suppressed by miR-675 inhibitor but were increased by miR-141 inhibitor.

tion was not inhibited by silencing H19 or miR-675 inhibitor, therefore, we further analyzed the potential influence of miR-141 on cell apoptosis (**Figure 5**). The results showed that per-

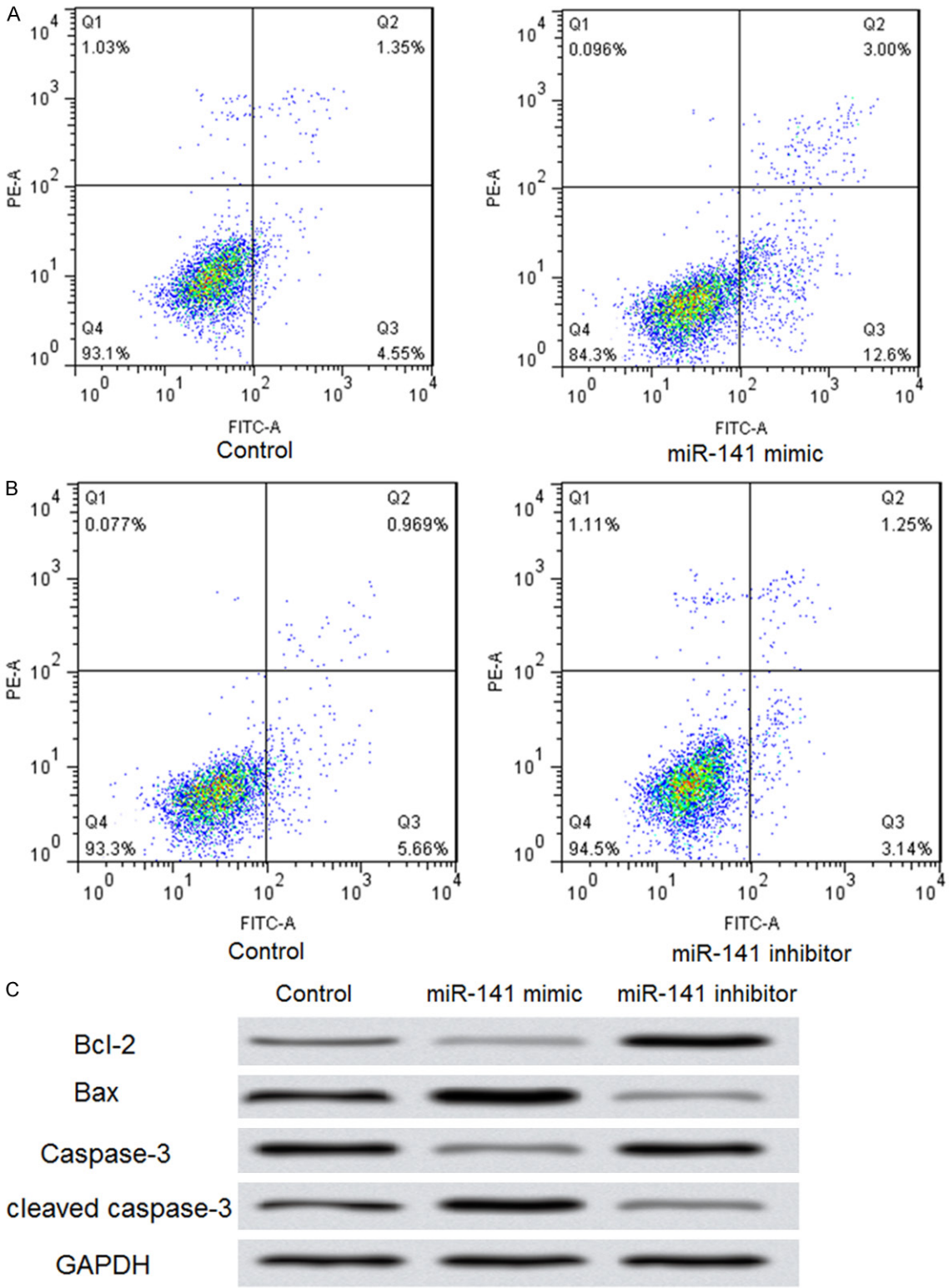

Figure 5. Effects of miR-141 on osteoblasts cell apoptosis. A: Compared to the control (4.55%), percentage of apoptotic cells was increased to 12.6% by miR-141 overexpression; B: Percentage of apoptotic cells was decreased to 3.14% by miR-141 inhibitor treatment compared with the control (5.66%); C: miR-141 overexpression increased the protein level of Bax and cleaved caspase-3, but decreased the protein levels of Bcl-2 and Caspase-3.

centage of apoptotic cells was increased to 12.6% by miR-141 mimic treatment compared with the control (4.55%; **Figure 5A**). However, the apoptotic percentage was decreased to 3.14% by miR-141 inhibitor treatment compared with the control group (5.66%; **Figure 5B**). Additionally, cell apoptosis-related proteins including Bcl-2/Bax, Caspase-3, and cleaved caspase-3 were also detected to explore the possible mechanism (**Figure 5C**). The results showed that the Bcl-2 and Caspase-3 levels were decreased while Bax and cleaved caspase-3 levels were increased by miR-141 mimic treatment, which was opposite to the results in miR-141 inhibitor group. These results indicated that miR-141 overexpression may contribute to osteoblasts cell apoptosis.

Discussion

Increasing evidence has demonstrated that miRNAs and lncRNAs play pivotal roles in disease biology, and miR-141 is involved in the osteoblasts differentiation [7, 8, 19]. LncRNA H19 and H19-derived miR-675 are associated with cell proliferation and migration in a variety of diseases [10, 13]. Until now, it has been reported that miR-141 could regulated H19 in gastric cancer [15]. In this study, we analyzed the role of miR-141 and H19 and H19-derived miR-675 on osteoblasts proliferation. In agreement with previous data, out data showed that miR-141 was down-regulated in osteosarcoma and the up-regulated miR-141 suppressed H19 and miR-675 expression. In addition, miR-141 down-regulation promoted osteoblasts proliferation but was blocked by the silencing H19 or miR-675 inhibitor, as well as the VEGF and IGF2 expression. Moreover, miR-141 overexpression promoted the osteoblasts apoptosis.

Xu et al. has proved that miR-141 functions as a tumor suppressor in human osteosarcoma [7]. Our results showed that miR-141 was down-regulated in tumor tissues compared to that in the adjacent tissues, but was overexpressed in hFOB1.19 cells by mimic transfection. Indicating that down-regulation of miR-141 may be correlated to osteosarcoma pathogen. Consequently, we further analyzed the effects of miR-141 expression on H19 and miR-675 levels in osteoblasts. Wu et al. have demonstrated that both H19 and miR-675 are highly expressed in osteoarthritis cartilage [20]. However, H19 was negatively regulated by miR-141 in gastric cancer cell proliferation and migration [15]. The results presented in this study showed that both H19 and miR-675 levels were significantly suppressed by miR-141 overexpression (**Figure 1**), suggesting the negative interaction between miR-141 and H19 and H19-derived miR-675 in osteoblasts.

Accordingly, cell viability was assessed to further investigate the deep correlation between miR-141 expression and H19 and miR-675. The down-regulated miR-141 was associated with gastric tumor cell growth, as well as osteoblast proliferation during bone formation [7, 21, 22]. In agreement with previous study, the results showed that osteoblasts proliferation was suppressed by miR-141 overexpression but was increased by down-regulated miR-141 (miR-141 inhibitor transfection; **Figure 2**), implying the suppress role of miR-141 in osteoblasts proliferation. Meanwhile, cell viability was suppressed by silencing H19 or miR-675 transfection (**Figure 3**). Zhou and his colleagues have proved that miR-141 could interacted with H19 and then play certain roles in gastric tumorigenesis [15], and our results showed that the promote role of miR-141 inhibitor on cell viability was significantly suppressed by silencing H19 or miR-675 inhibitor, implying that the promote effect of miR-141 down-regulation on osteoblasts proliferation may be suppressed by silencing H19 or miR-675 inhibitor.

VEGF is a cell proliferation-related factor that functions in various biological processes, including angiogenesis, vasculogenesis and endothelial cell growth, and high level of VEGF has been demonstrated to be associated with pathogenesis of osteoarthritis [23, 24]. IGF2 is also a cell proliferation-related factor, which is involved in the development of growth, and stimulated IGF2 is correlated to chondrocytes growth [25]. High level of VEGF or IGF2 was the contributor for osteoblasts proliferation [26, 27]. Association between miR-141 or H19 and VEGF or IGF2 in osteoblasts proliferation has not been fully discussed. However, Choi et al. proved that VEGF signal was negatively regulated by miR-200 family through targeting VEGF in angiogenesis [28]. The highly expressed IGF2 was correlated to miR-483-3p in renal childhood neoplasms [29], while miR-483 was associated to osteoblasts differentiation [30], indicating that IGF2 was involved in osteo-

blasts. Our results showed the levels of VEGF and IGF2 were down-regulated by miR-141 overexpression or silencing H19 or miR-675 inhibitor (miR-141 mimic; **Figure 4**); we speculated that miR-141 overexpression may play certain roles in regulating H19/miR-675 mediated osteoblasts proliferation by down-regulating VEGF and IGF2.

Interestingly, our data showed that the increased miR-141-induced osteoblast proliferation was partly suppressed by silencing H19 or miR-675 inhibitor (**Figure 3**), but it was not fully inhibited, implying that there may be else manners for miR-141 on influencing osteoblasts proliferation. Therefore, we further assessed whether miR-141 expression could affect osteoblasts apoptosis or not (**Figure 5**). miR-141 has been a biomarker for colon cancer prognosis through influencing apoptosis [31], and the down-regulated miR-141 induced cholangiocarcinoma cell apoptosis [32]. The apoptotic executioner of caspase-3 was activated in osteosarcoma [33], In this study, the osteoblasts apoptosis was induced by miR-141 overexpression (miR-141 mimic; **Figure 5**), also, the protein level of caspase-3 was down-regulated and the Bcl-2/Bax ratio were also decreased by miR-141 overexpression, implying that miR-141 overexpression contribute osteoblasts apoptosis. However, we speculated that the effect of miR-141 on osteoblasts apoptosis may be another factor for miR-141 on cell proliferation. However, deep mechanism are still investigated in our near future study.

To sum up, the data presented in our study reveals that miR-141 is interacted with lncRNA H19 and H19-derived miR-675 in osteoblasts proliferation. miR-141 overexpression suppressed osteoblasts proliferation but induced apoptosis via down-regulating H19 or miR-675 expression. Our study elucidates the interaction between lncRNA and miRNA in osteosarcoma, and may provide theoretical basis for the exploration of lncRNA in osteosarcoma pathogen and for the therapeutic target treatment for miR-141 in osteosarcoma. However, further experimental studies are still needed to explore the intensive mechanism.

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Address correspondence to: Yan Kang, Department of Joint Surgery, The First Affiliated Hospital of Sun Yat-sen University, No. 58, Zhongshan Second Road, Yuexiu District, Guangzhou 510080, China. E-mail: neokang@163.com

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