

lncRNA HOXA11-AS is involved in fracture healing through regulating mir-124-3p

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Abstract. – **OBJECTIVE:** Long noncoding RNAs (lncRNAs) have been reported to participate in many diseases. Fracture healing is one of these ordinary diseases. This study aims to identify how lncRNA HOXA11-AS affects the progression of fracture healing.

MATERIALS AND METHODS: RT-qPCR was performed to detect the level of HOXA11-AS. Moreover, function assays including cell growth assay and cell apoptosis assay were performed to explore how HOXA11-AS functions in fracture healing. Furthermore, the interaction between HOXA11-AS and mir-124-3p was studied by RT-qPCR, luciferase assay, and RNA immunoprecipitation assay. Rescue experiments were performed to verify the interaction between HOXA11-AS and mir-124-3p *in vitro*.

RESULTS: In the research, function assays revealed that HOXA11-AS overexpression inhibited cell proliferation, while HOXA11-AS knockdown promoted cell proliferation *in vitro*. Moreover, HOXA11-AS overexpression promoted cell apoptosis, while HOXA11-AS knockdown inhibited cell apoptosis *in vitro*. Furthermore, mechanism assays demonstrated that HOXA11-AS acts as a ceRNA via sponging mir-124-3p. Rescue assay demonstrated that HOXA11-AS suppressed cell proliferation and promoted cell apoptosis via targeting mir-124-3p.

CONCLUSIONS: These results indicate that HOXA11-AS could inhibit cell proliferation and promote cell apoptosis of osteoblast via sponging mir-124-3p, which may offer a new vision for interpreting the mechanism of fracture healing.

Key Words:

HOXA11-AS, mir-124-3p, Fracture healing.

Introduction

Along with the population aging, fractures become a big health problem as the most com-

mon injuries worldwide. Although the body has the ability of fracture healing, many risk factors can significantly delay fracture healing, such as advanced age, smoking, diabetes mellitus and anti-cancer drugs. Therefore, further exploring the mechanisms of fracture healing especially in patients with these factors is urgently required.

Evidence has proved that long noncoding RNAs participate in many diseases including fracture healing. lncRNA HOXA11-AS has been reported to function in many diseases. For example, HOXA11-AS enhances aggressiveness of gastric cancer by regulating PRC2¹. HOXA11-AS participates in epithelial-mesenchymal transition (EMT) process and promotes metastasis of breast cancer cells². HOXA11-AS functions as an oncogene in glioma and enhances cell invasion and migration *via* sponging miR-140-5p³. In addition, cell cycle progression and metastasis were enhanced by upregulating HOXA11-AS in gastric cancer⁴. HOXA11-AS induces EMT process of non-small cell lung cancer *via* suppressing miR-200b⁵. A recent study demonstrated that HOXA11-AS regulated the development and progression of osteosarcoma and its function was associated with miR-124-3p⁶. Moreover, BMP6 regulated by miR-124-3p participates in progress of fracture healing⁷. However, the role of HOXA11-AS in fracture healing remains unknown.

Our present study revealed that lncRNA HOXA11-AS inhibited proliferation and promoted apoptosis of osteoblasts *in vitro*. We further discovered the interaction between lncRNA HOXA11-AS and mir-124-3p as well as the possible mechanism.

Materials and Methods

Cell Lines

The American Type Culture Collection (ATCC, Manassas, VA, USA) provided us OS-732 cell line and 293T embryonic kidney cell line. Culture medium was as follows: 10% fetal bovine serum (FBS) Invitrogen Life Technologies (Carlsbad, CA, USA), penicillin, and Dulbecco's Modified Eagle Medium (DMEM) (Thermo Fisher Scientific, Waltham, MA, USA). Cells were cultured in humidified incubator with 5% CO₂ at 37°C.

Cell Transfection

Lentiviral small hairpin RNA and lentivirus targeting HOXA11-AS were synthesized. pLenti-EF1a-EGFP-F2A-puro vector (Biosettia Inc., San Diego, CA, USA) was used for cloning. Viruses were packaged in 293T cells, HOXA11-AS lentiviruses (plv-HOXA11-AS), lentiviral small hairpin RNA (sh-HOXA11-AS) and negative control (sh-ctrl). mir-124-3p mimics and inhibitor were provided by Genepharma Co., Ltd. (Shanghai, China).

RNA Extraction and qRT-PCR

Firstly, total RNA was separated with TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Then, RNA was reverse-transcribed to cDNAs via reverse Transcription Kit (TaKaRa Biotechnology Co., Dalian, China). qRT-PCR was performed on ABI 7500 RT-PCR detection system (Applied Biosystems, Foster City, CA, USA). The thermal cycle was as follows: 30 s at 95°C, 5 s at 95°C for 40 cycles, 35 s at 60°C.

Luciferase Assays

In our study, pGL3 vector (Promega, Madison, WI, USA) was used for colony of 3'-UTR of mir-124-3p or HOXA11-AS, wild-type (WT) 3'-UTR. mir-124-3p binding site in HOXA11-AS 3'-UTR, mutant (MUT) 3'-UTR was site-directed mutagenesis by quick-change site-directed mutagenesis kit (Stratagene, Cedar Creek, CA, USA). WT-3'-UTR or MUT-3'-UTR and miR-ctrl or mir-124-3p mimics were used for cell transfection. 48 h later, the dual luciferase reporter assay system (Promega, Madison, WI, USA) was used for the luciferase assays.

RNA Immunoprecipitation Assay (RIP)

RIP assay was performed utilizing Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore, Billerica, MA, USA). Co-precipitated RNAs were detected via RT-qPCR.

Cell Counting Kit-8 (CCK8) Assay

Cell viability of these treated cells was monitored every 24 h by CCK8 assay according to the protocol (Dojindo Molecular Technologies, Inc., Kumamoto, Japan). The absorbance was measured at 450 nm on the spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

Cell Apoptosis Analysis

Apoptosis rate of osteoblast was estimated with Annexin V-APC/7-AAD Apoptosis Detection Kit II (KeyGen Biotech Co., Ltd, Nanjing, Jiangsu, China) and comparison was performed on flow cytometry (FACScan, BD Biosciences, San Diego, CA, USA) programmed with CellQuest software (BD Biosciences, San Diego, CA, USA). The test was repeated thrice at least.

Statistical Analysis

SPSS 20.0 (SPSS IBM, Armonk, NY, USA) was used in our study. Student *t*-test was selected. We present data as mean $\pm$ SD. $p < 0.05$ was of statistically sense.

Results

HOXA11-AS Overexpression Inhibits Proliferation and Promotes Apoptosis

HOXA11-AS lentiviruses (plv-HOXA11-AS) and the empty vector (plv-ctrl) were synthesized and transduced into these cells. Then the HOXA11-AS expression was determined by qRT-PCR (Figure 1A). Furthermore, results of CCK8 assay showed that cell proliferation was inhibited after HOXA11-AS was overexpressed (Figure 1B). Meanwhile, cell apoptosis assay also showed that the apoptosis rate of these treated cells increased after HOXA11-AS was overexpressed (Figure 1C).

HOXA11-AS Knockdown Promotes Proliferation and Inhibits Apoptosis

Lentiviral small hairpin RNA (sh-HOXA11-AS) and the empty vector (sh-ctrl) were synthesized and transduced into these two cells. Then the HOXA11-AS expression was determined by qRT-PCR (Figure 2A). Furthermore, results of CCK8 assay showed that cell proliferation was promoted after HOXA11-AS was knocked down (Figure 2B). Meanwhile, cell apoptosis assay also

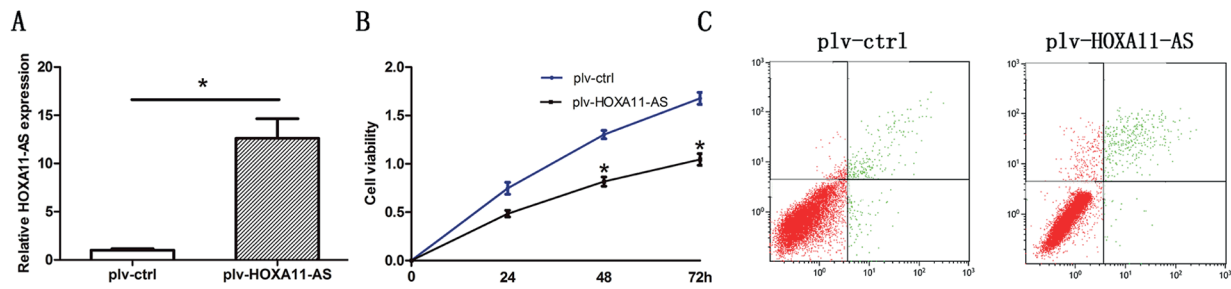


Figure 1. HOXA11-AS overexpression inhibits cell proliferation and promotes cell apoptosis *in vitro*. **(A)** Expression levels of HOXA11-AS relative to β -actin were determined in plv-HOXA11-AS group and plv-ctrl group by RT-qPCR. **(B)** Cell proliferation was detected in plv-HOXA11-AS group and plv-ctrl group by CCK8 assay. **(C)** Apoptosis rate was detected in plv-HOXA11-AS group and plv-ctrl group by cell apoptosis assay. Data are presented as the mean $\pm$ standard error of the mean. * $p < 0.05$.

showed that the apoptosis rate of these treated cells decreased after HOXA11-AS was knocked down (Figure 2C).

HOXA11-AS Interacts With mir-124-3p

DIANA LncBASE version 2.0 was used to predict the miRNAs that contained complementary base with HOXA11-AS. HOXA11-AS was predicted to harbor mir-124-3p binding sites (Figure 3A). Besides, mir-124-3p level in sh-HOXA11-AS group was higher compared with that in sh-ctrl group, while mir-124-3p level was lower in plv-HOXA11-AS group compared with that in plv-ctrl group (Figure 3B and 3C). Furthermore, the luciferase assay revealed that the luciferase activity of HOXA11-AS-WT cells with mir-124-3p mimics was reduced, while luciferase activity of HOXA11-AS-MUT cells with mir-124-3p was not obviously changed (Figure 3D). Meanwhile, RIP assay results demonstrated that mir-124-3p could be remarkably enriched in the HOXA11-AS group compared with control group, suggesting that HOXA11-AS might work as a mir-124-3p sponge (Figure 3E).

HOXA11-AS Suppresses Cell Proliferation and Promotes Cell Apoptosis Via Targeting mir-124-3p

CCK-8 assay and cell apoptosis assay were conducted in these treated cells. As the result, the inhibited cell proliferation caused by overexpressed HOXA11-AS could be rescued by mir-124-3p mimics (Figure 4A). Meanwhile, the promoted cell proliferation caused by HOXA11-AS knockdown could be rescued by mir-124-3p inhibitor (Figure 4B). Furthermore, the promoted cell apoptosis caused by HOXA11-AS overexpression could be rescued by mir-124-3p inhibitor (Figure 4C), while the inhibited cell apoptosis caused by HOXA11-AS knockdown could be rescued by mir-124-3p mimics (Figure 4D).

Discussion

Studies have demonstrated that numerous noncoding RNAs play important roles in many biological processes including tissue repairing. For instance, miR-92a were remarkably down-

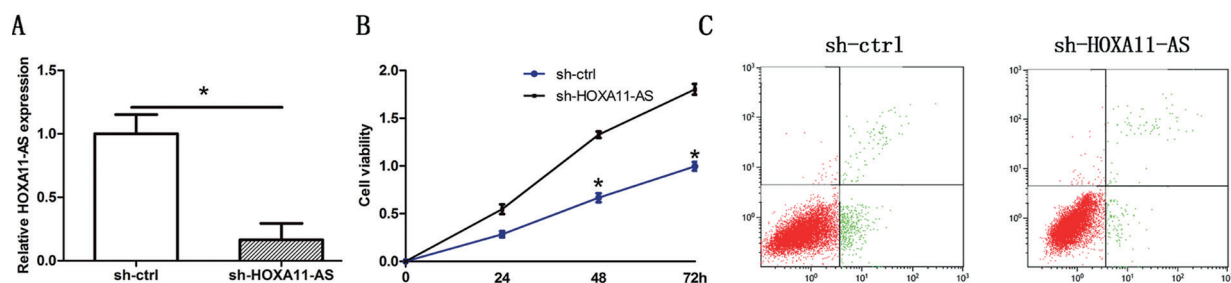


Figure 2. HOXA11-AS knockdown promotes cell proliferation and inhibits cell apoptosis *in vitro*. **(A)** Expression levels of HOXA11-AS relative to β -actin were determined in sh-HOXA11-AS group and sh-ctrl group by RT-qPCR. **(B)** Cell proliferation was detected in sh-HOXA11-AS group and sh-ctrl group by CCK8 assay. **(C)** Apoptosis rate was detected in sh-HOXA11-AS group and sh-ctrl group by cell apoptosis assay. Data are presented as the mean $\pm$ standard error of the mean. * $p < 0.05$.

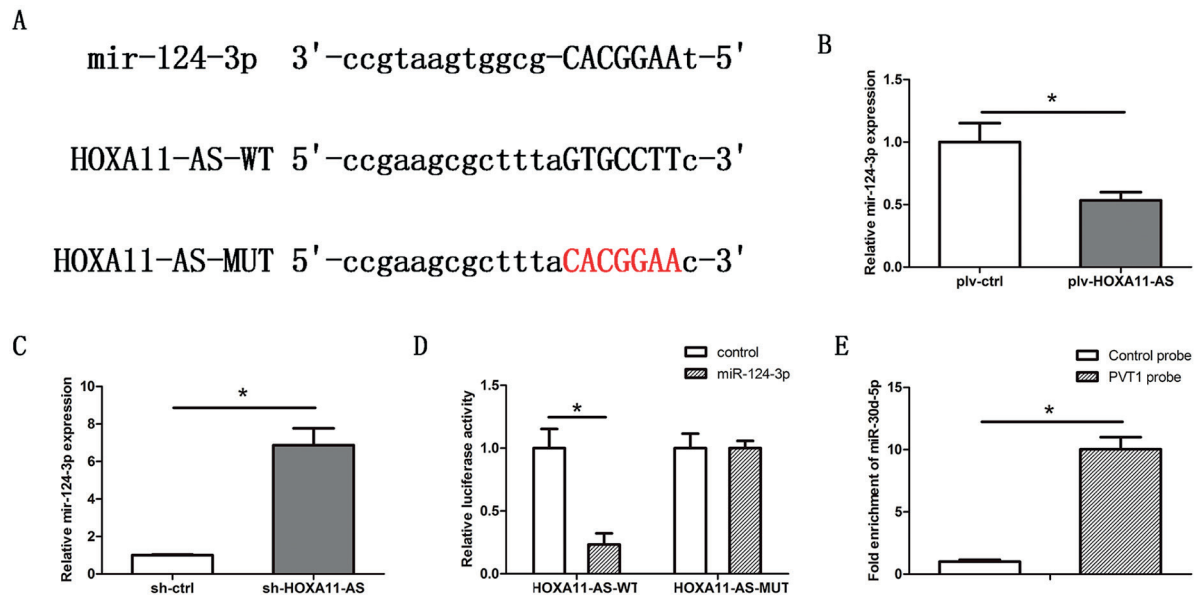


Figure 3. Interaction between HOXA11-AS and mir-124-3p. **(A)** The binding sites of mir-124-3p on HOXA11-AS. **(B)** Mir-124-3p expression was increased in sh-HOXA11-AS group compared with sh-ctrl group. **(C)** Mir-124-3p expression was decreased in plv-HOXA11-AS group compared with plv-ctrl group. **(D)** Co-transfection of mir-124-3p and HOXA11-AS-WT in OS-732 cells strongly decreased the luciferase activity, while co-transfection of mir-124-3p and HOXA11-AS-MUT did not change the luciferase activity either. **(E)** Mir-124-3p was significantly enriched by RNA immunoprecipitation (RIP) assay in the HOXA11-AS group compared with control. The results represent the average of three independent experiments. Data are presented as the mean $\pm$ standard error of the mean. * $p < 0.05$.

regulated in fracture patients⁸. Moreover, miR-140 is associated with short stature of skeletal phenotype⁹. On the other hand, miR-182 inhibits osteoblast differentiation and proliferation, and then functions in osteogenesis¹⁰. Furthermore, five miRNAs are newly discovered in serum of patients with osteoporotic fractures¹¹. Therefore, the mechanisms how noncoding RNAs regulate fracture healing needs to be studied. In the present study, HOXA11-AS was found to suppress cell growth and promoting cell apoptosis of osteoblasts. These data indicated that HOXA11-AS served as a suppressor and inhibited the progress of fracture healing.

Recent studies revealed that lncRNAs function in human diseases by binding to miRNAs. For example, lncRNA XIST enhances the invaded ability of stem cells through regulating miR-152 in human glioblastoma¹². Moreover, lncRNA CHRF contributes to regulating cardiac hypertrophy via sponging miR-489¹³. The interaction between lncRNA H19 and miR-675 is discovered in osteoblast differentiation and is associated with bone formation¹⁴. Furthermore, H19 functions in progression of osteoarthritis via regulating miR-675 *in vitro* conditions¹⁵. miR-124-3p was lower expressed in the serum of patients who

have low bone mass¹⁶. Our study showed that mir-124-3p bind directly to HOXA11-AS through a luciferase assay. mir-124-3p was significantly enriched by HOXA11-AS RIP assay. In addition, mir-124-3p expression could be upregulated with knockdown of HOXA11-AS, while mir-124-3p expression could be downregulated by overexpression of HOXA11-AS. All these results suggest that HOXA11-AS might suppress the progress of fracture healing via sponging mir-124-3p. We further used rescue experiments to verify the interaction between HOXA11-AS and mir-124-3p in osteoblast growth and apoptosis. We found that inhibition of growth by HOXA11-AS could be reversed through upregulating mir-124-3p. The promotion of apoptosis by HOXA11-AS could be reversed through upregulating mir-124-3p.

Conclusions

We showed that HOXA11-AS could inhibit cell proliferation and promote cell apoptosis of osteoblast via sponging mir-124-3p. These findings suggest that lncRNA HOXA11-AS may contribute to understanding the mechanism of fracture healing.

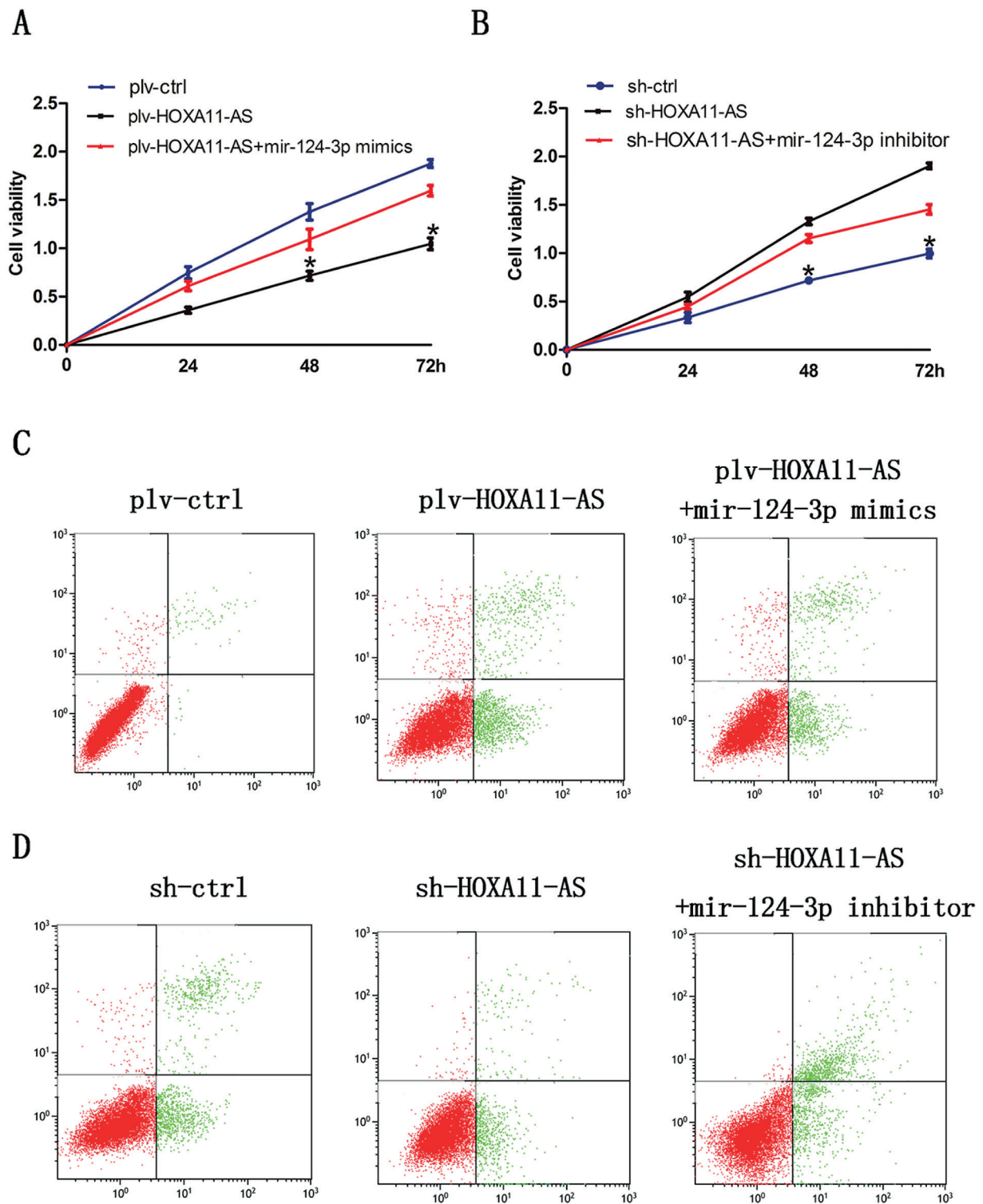


Figure 4. HOXA11-AS suppresses cell proliferation and promotes cell apoptosis via targeting mir-124-3p. **(A)** Cell proliferation was decreased by overexpressed HOXA11-AS, which could be rescued by mir-124-3p mimics. **(B)** Cell proliferation was increased by HOXA11-AS knockdown, which could be rescued by mir-124-3p inhibitor. **(C)** Cell apoptosis was increased by overexpressed HOXA11-AS, which could be rescued by mir-124-3p mimics. **(D)** Cell apoptosis was decreased by HOXA11-AS knockdown, which could be rescued by mir-124-3p inhibitor. The results represent the average of three independent experiments. Data are presented as the mean $\pm$ standard error of the mean. * $p < 0.05$.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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