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MicroRNA-7-5p regulates osteogenic differentiation of hMSCs via targeting CMKLR1

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Abstract. – OBJECTIVE: To investigate the function of microRNA-7-5p in human mesenchymal stem cells (hMSCs) and its underlying potential mechanism in osteogenic differentiation.

PATIENTS AND METHODS: Expression levels of osteogenic genes (ALP, RUNX2), microRNA-7-5p and CMKLR1 in hMSCs were detected by quantitative real time-polymerase chain reaction (qRT-PCR). After transfection of microRNA-7-5p mimics or inhibitor, the effect of microRNA-7-5p on osteogenic differentiation of hMSCs was detected by Alizarin red staining, ALP activity determination and Western blot, respectively. The potential target gene of microRNA-7-5p was predicted online and further verified by luciferase reporter gene assay. Rescue experiments were conducted to explore whether the effect of microRNA-7-5p on osteogenic differentiation of hMSCs could be reversed by CMKLR1.

RESULTS: Expression levels of ALP, RUNX2 and microRNA-7-5p were gradually elevated with the prolongation of osteogenic differentiation, whereas CMKLR1 was reduced. Overexpression of microRNA-7-5p increased levels of ALP and RUNX2. The amount of calcified nodules was increased after microRNA-7-5p overexpression. CMKLR1 was the target gene of microRNA-7-5p. The effect of microRNA-7-5p on osteogenic differentiation of hMSCs could be reversed by CMKLR1.

CONCLUSIONS: MicroRNA-7-5p promotes osteogenic differentiation of hMSCs via targeting CMKLR1.

Key Words:
MicroRNA-7-5p, CMKLR1, Human bone marrow mesenchymal stem cells, Osteogenic differentiation.

Introduction

Human mesenchymal stem cells (hMSCs) are bone marrow-derived stem cells with potentials of multipotent differentiation, self-renewal, tissue regeneration and anti-inflammation capacity[1]. Under specific induction conditions, hMSCs can differentiate into osteoblasts, cartilage, nerves, fat, muscle and liver. Besides, hMSCs present advantages of simple culture procedures, rapid proliferation and convenient preservation, which have been widely applied in gene therapy[2]. Researches[3,4] have pointed out that osteogenic differentiation of hMSCs is a well-ordered and precisely regulated programmed process. In recent years, microRNAs (miRNAs) have been well studied as novel regulatory factors[5]. Various studies have shown that miRNAs can regulate the differentiation of bone precursor cells and osteoblasts via osteogenic pathway[6,7]. MicroRNA-7-5p is involved in a variety of biological processes and is closely related to the occurrence of multiple tumors[8,9]. Functionally, microRNA-7-5p participates in cell proliferation, apoptosis, invasion and metastasis. However, the role of microRNA-7-5p in osteogenic differentiation is rarely reported. In the present study, the specific mechanism of microRNA-7-5p in regulating osteogenic differentiation of hMSCs is investigated.

Patients and Methods

Isolation and Culture of hMSCs

This study was approved by the Qingdao Municipal Hospital Ethic Committee and all subjects granted informed consent. 10 mL of bone marrow samples from patients with open fractures and tibial grafts were collected and placed in EDTA (ethylenediaminetetraacetic acid) anticoagulant tube. Patients with other systemic diseases, such as osteoporosis, tumors, and hematologic diseases, were excluded. Bone marrow samples were mixed with 10 mL of serum-free medium and centrifuged for removing lipid layer. Fircoll separation solution was slowly added along the tube

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wall, followed by 30-min gradient centrifugation. Monocyte layer was removed and cells were resuspended in medium containing 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA). Osteogenic differentiation was induced by Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS), 10 mmol/L sodium β-glycerophosphate, 50 μg/mL Vitamin C, 1% HEPES and 1% penicillin/streptomycin.

Cell Transfection

Third-passage hMSCs in good growth condition were selected for cell transfection according to the instructions of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Culture medium was replaced 6 h later.

RNA Extraction and Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

TRIzol kit (Invitrogen, Carlsbad, CA, USA) was used to extract the total RNA, which was then reversely transcribed into cDNA. After the complementary Deoxyribose Nucleic Acid (cDNA) was amplified, qRT-PCR was performed to detect the expressions of related genes. Primers used in this study were as follows: MicroRNA-7-5p, F: 5′-TGGAAGACTAGTGATTTT-3′, R: 5′-CTCAACTGGTGTCGTG-3′; CMKLR1, F: 5′-CGGGTGATAGGGGTGTTCCA-3', R: 5′-GCACACCCTGGCTTCTTTCT-3′; ALP, F: 5′-AAGGCTTCTTCTTGCTGGTG-3′, R: 5′-GCCTTACCCTCATGATGTCC-3′; RUNX2, F: 5′-ACTTCCTGTGCTCCGTGCTG-3′, R: 5′-TCGTTGAACCTGGCTACTTGG-3′; GAPDH, F: 5′-ACCCACTCCTCCACCTTTGA-3′, R: 5′-CTGTTGCTGTAGCCAAATTCGT-3′.

ALP Activity Detection

The hMSCs were fixed with 4% paraformaldehyde and 5% citric acid for 30 s. After phosphate-buffered saline (PBS) wash, cells were incubated with 0.2% naphthol and 0.2% diazonium salt for 15 min. ALP activity was finally detected.

Alizarin Red Staining

The hMSCs were induced for osteogenic differentiation. After 14-day induction, cells were washed with PBS twice, fixed with 4% paraformaldehyde for 15 min and stained with 1% alizarin red staining for 5 min. Calcified nodules were observed and captured using an inverted microscope.

Western Blot

Cells were lysed for protein extraction. The concentration of each protein sample was determined by a BCA (bicinchoninic acid) kit (Abcam, Cambridge, MA, USA). Protein sample was separated by gel electrophoresis and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After incubation with primary and secondary antibody, immunoreactive bands were exposed by enhanced chemiluminescence (ECL) method.

Bioinformatics Prediction

The binding condition of microRNA-7-5p and CMKLR1 was predicted by TargetScan, miRanda, and PicTar3.

Luciferase Reporter Gene Assay

The binding site of microRNA-7-5p and CMKLR1 was predicted to construct wild-type and mutant-type CMKLR1. Cells were co-transfected with 50 pmol/L microRNA-7-5p mimics or negative control and 80 ng wild-type or mutant-type CMKLR1 for 48 h, respectively. Luciferase activity was finally detected.

Statistical Analysis

We used Statistical Product and Service Solutions 16.0 software (SPSS Inc., Chicago, IL, USA) for statistical analysis. The quantitative data were represented as mean ± standard deviation ($\bar{x}\pm s$). The *t*-test was used for comparing differences between the two groups. $p<0.05$ was considered statistically significant (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

Results

Osteogenic Differentiation of hMSCs

After osteogenic differentiation of hMSCs, qRT-PCR assay was performed to detect the expressions of osteogenesis-related genes. It is found that the mRNA levels of ALP, RUNX2 and microRNA-7-5p were elevated with the prolongation of osteogenesis induction (Figure 1A-1C). On the contrary, CMKLR1 expression was gradually decreased in a time-dependent manner (Figure 1D).

MicroRNA-7-5p Promoted Osteogenic Differentiation of hMSCs

hMSCs were transfected with microRNA-7-5p mimics, inhibitor or negative control, respectively, followed by 7-day osteogenic differentia-

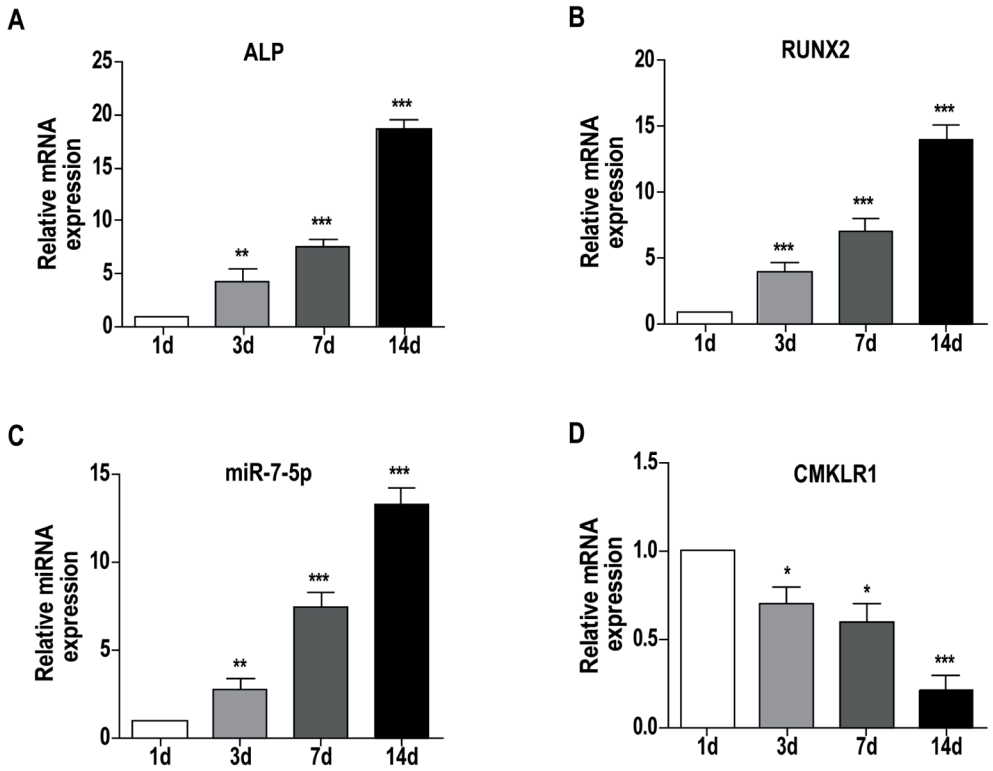

Figure 1. Osteogenic differentiation of hMSCs. ***A-C,*** The mRNA levels of ALP (***A***), RUNX2 (***B***) and microRNA-7-5p (***C***) were elevated with the prolongation of osteogenesis induction in hMSCs. ***D,*** CMKLR1 expression in hMSCs was gradually decreased in a time-dependent manner.

tion. Overexpression of microRNA-7-5p remarkably elevated expressions of ALP and RUNX2 in hMSCs. Knockdown of microRNA-7-5p obtained the opposite results (Figure 2A and 2B). Besides, overexpressed microRNA-7-5p elevated ALP activity and protein expression of RUNX2 (Figure 2C and 2D). After osteogenic differentiation for 14 days, the amount of calcified nodules was larger in hMSCs transfected with microRNA-7-5p mimics, indicating that microRNA-7-5p promotes bone calcification (Figure 2E).

MicroRNA-7-5p Directly Regulated CMKLR1

Bioinformatics predicted that CMKLR1 was the target gene of microRNA-7-5p. Wild-type and mutant-type CMKLR1 were first constructed (Figure 3A). Luciferase reporter gene assay demonstrated that microRNA-7-5p could bind to wild-type CMKLR1 (Figure 3B). QRT-PCR further verified that overexpressed microRNA-7-5p downregulated CMKLR1 expression, whereas knockdown of microRNA-7-5p upregulated CMKLR1 expression (Figure 3C).

The Promotive Effect of microRNA-7-5p on Osteogenic Differentiation of hMSCs was Reversed by CMKLR1 Overexpression

To further verify the regulatory effect of microRNA-7-5p on CMKLR1, rescue experiments were conducted. Elevated expressions of ALP and RUNX2 in hMSCs transfected with microRNA-7-5p mimics were reversed by CMKLR1 overexpression (Figure 4A and 4B). The increased ALP activity induced by microRNA-7-5p overexpression was also reversed after overexpression of CMKLR1 (Figure 4C). Western blot results indicated that upregulated protein expression of RUNX2 induced by microRNA-7-5p overexpression was remarkably reversed by CMKLR1 overexpression (Figure 4D).

Discussion

Cell differentiation of hMSCs is a key step in the process of bone construction. Under specific induction, hMSCs are sequentially differentiated into osteoprogenitor cells, preosteoblasts,

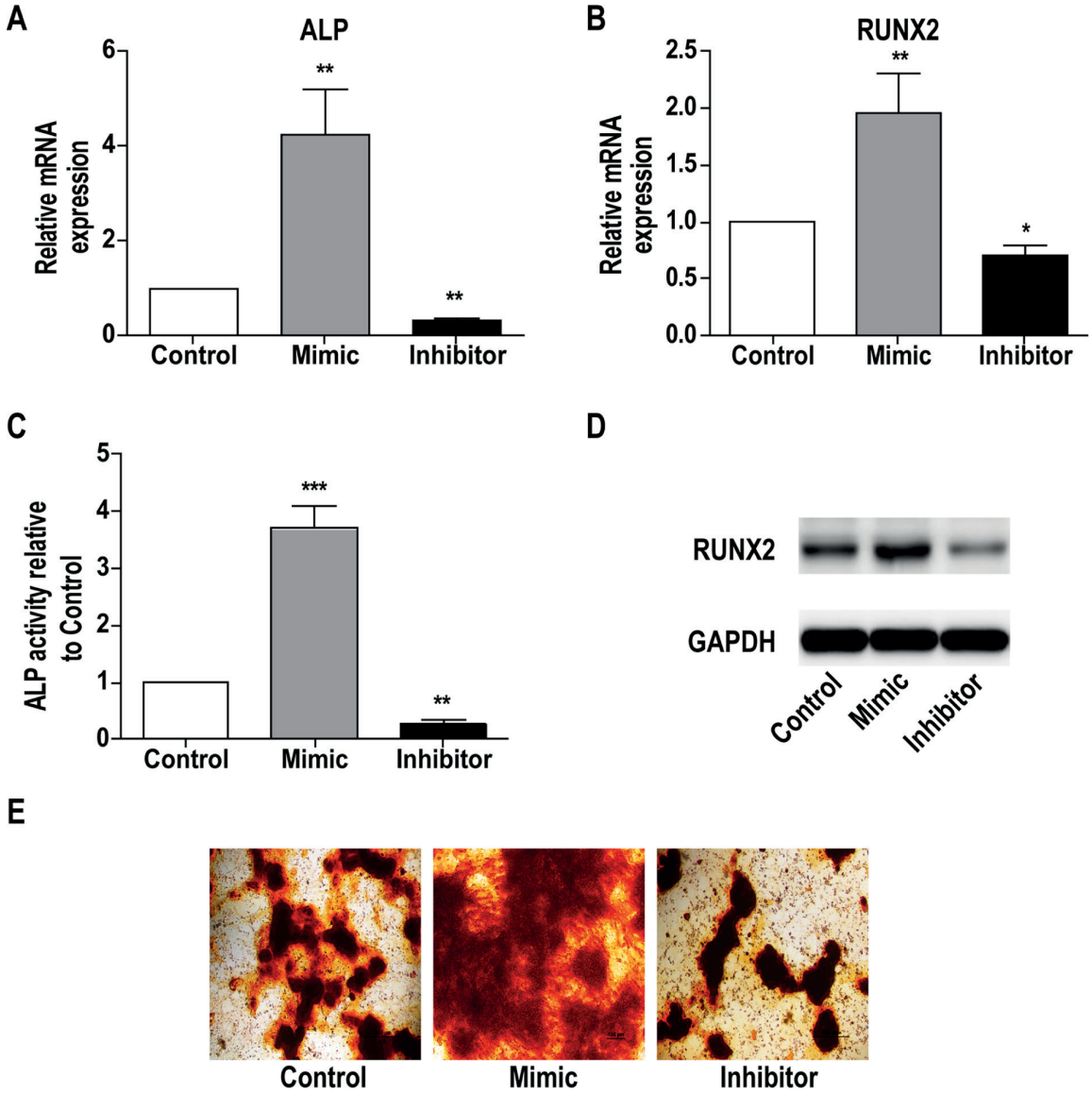

Figure 2. MicroRNA-7-5p promoted osteogenic differentiation of hMSCs. ***A-B,*** Overexpression of microRNA-7-5p remarkably elevated expressions of ALP and RUNX2 in hMSCs. Knockdown of microRNA-7-5p obtained the opposite results. ***C-D,*** Overexpressed microRNA-7-5p elevated ALP activity and protein expression of RUNX2. ***E,*** After osteogenic differentiation for 14 days, the amount of calcified nodules was larger in hMSCs transfected with microRNA-7-5p mimics.

transitional osteoblasts, secretory osteoblasts and osteoblasts[10,11]. Directional differentiation of hMSCs is regulated by various microenvironment factors. Recent studies pointed out that miRNAs could induce cell differentiation of hMSCs. However, the specific role of microRNA-7-5p in inducing osteogenesis is rarely reported. In the present study, expression levels of ALP and RUNX2 in hBMSCs were gradually increased with the prolongation of osteogenic induction time, suggesting that hMSCs could be differentiated into osteoblasts. Subsequently, we found that microRNA-7-5p expression was elevated in hMSCs during *in vitro* osteogenic differentiation, indicating that microRNA-7-5p may be involved in the regulation of osteogenic differentiation. MicroRNA-7-5p overexpression remarkably elevated RUNX2 expression, ALP activity and the amount of calcified nodules. We confirmed that microRNA-7-5p could promote the osteogenic differentiation of hMSCs. The 3'UTR sequence of binding site of microR-

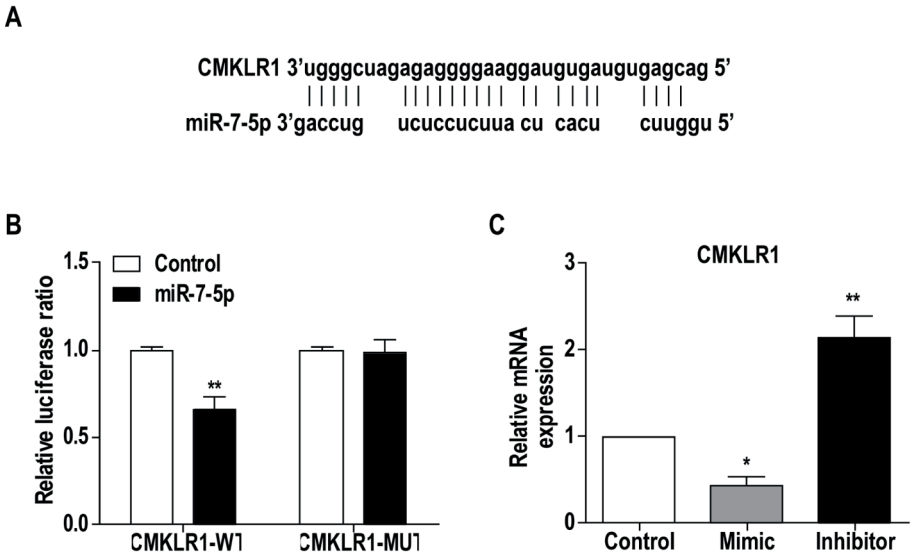

Figure 3. MicroRNA-7-5p directly regulated CMKLR1. ***A,*** Construction of wild-type and mutant-type CMKLR1. ***B,*** Luciferase reporter gene assay demonstrated that microRNA-7-5p could bind to wild-type CMKLR1. ***C,*** QRT-PCR verified that overexpressed microRNA-7-5p downregulated CMKLR1 expression, whereas knockdown of microRNA-7-5p upregulated CMKLR1 expression.

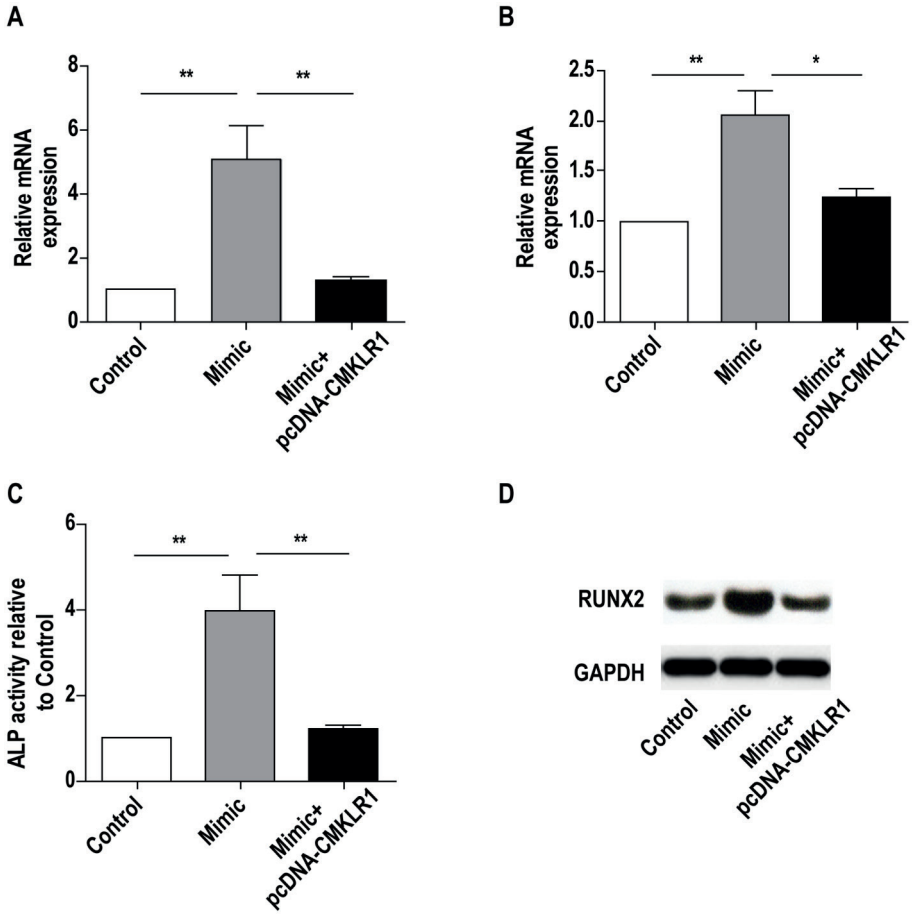

Figure 4. The promotive effect of microRNA-7-5p on osteogenic differentiation of hMSCs was reversed by CMKLR1 overexpression. ***A, B,*** Elevated expressions of ALP and RUNX2 in hMSCs transfected with microRNA-7-5p mimics were reversed by CMKLR1 overexpression. ***C,*** The increased ALP activity induced by microRNA-7-5p overexpression was reversed after overexpression of CMKLR1. ***D,*** Upregulated protein expression of RUNX2 induced by microRNA-7-5p overexpression was remarkably reversed by CMKLR1 overexpression.

NA-7-5p and CMKLR1 was predicted by TargetScan software. Wild-type and mutant-type CMKLR1 were constructed. Luciferase reporter gene assay and further qRT-PCR both indicated that CMKLR1 was the target gene of microRNA-7-5p. CMKLR1 is a receptor for chemerin and mainly expressed in the spleen, thymus, lymph nodes, and bone marrow cells[11-13]. Muruganandan et al[14] found that chemerin and its receptors CMKLR1 and CCRL2 participate in the differentiation of preosteoblasts 7F2 and bone marrow mesenchymal stem cells into osteoblasts and adipocytes, respectively. In this experiment, we found that the regulatory effect of microRNA-7-5p on osteogenic differentiation of hMSCs was reversed by CMKLR1 overexpression. In summary, this study found that microRNA-7-5p is highly expressed during osteogenic differentiation and promotes the differentiation of hMSCs into osteoblasts by inhibiting CMKLR1. Our results provide some theoretical foundations for relative diseases investigation.

Conclusions

MicroRNA-7-5p promotes osteogenic differentiation of hMSCs via targeting CMKLR1.

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Conflict of Interest

The Authors declare that they have no conflict of interest.

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