Park *et al. Journal of Orthopaedic Surgery and Research* (2024) 19:730
https://doi.org/10.1186/s13018-024-05163-3

Journal of Orthopaedic
Surgery and Research

RESEARCH ARTICLE

Open Access

CRISPR activation identifies a novel miR-2861 binding site that facilitates the osteogenesis of human mesenchymal stem cells

Seong-Ho Park[2], Jungwoo Kim[1], Hee-Jin Yang[1], Ju Yeon Lee[3], Chi Heon Kim[4,5], Junho K. Hur[3,6*] and Sung Bae Park[1,4*]

Abstract

We investigated the regulation of histone deacetylases (HDACs) by miR-2861 in the osteoblastic differentiation of human mesenchymal stem cells (MSCs) and miR-2861 binding site by CRISPR activation (CRISPRa). Transfection of miR-2861 into human MSCs was performed and the effect on osteoblast differentiation was analyzed. Using catalytically inactive Cas12a, the CRISPRa system induced targeted overexpression of endogenous miRNA and repressed the luciferase activities of reporters that contained functional miRNA target sites. The delivery of miR-2861 into MSCs enhanced osteoblast differentiation by decreased expressions of the *HDAC1*, *4* and *5* genes. The mechanism of *HDAC5* repression by miR-2861 in humans has not been fully elucidated. To this end, the *HDAC5* mRNA sequence was analyzed and a putative primate-specific miR-2861 binding site was identified in the 3′ untranslated region (3′-UTR). CRISPRa was applied to validate the putative binding site and an increase in endogenous miR-2861 was found to repress the expression of a reporter that contained the novel miR-2861 binding site. The delivery of miR-2861 to human MSCs enhanced osteoblast differentiation. In the 3′-UTR, the *HDAC5* repression was mediated by the miR-2861 binding site, and miR-2861 promoted osteoblast differentiation via the inhibition of *HDAC5* through a primate-specific miRNA binding site. Therefore, miRNAmiR-2861 with the CRISPRa methods might be a good biomaterial for osteogenesis augmentation.

Keywords CRISPR, MicroRNA, Osteogenesis, Histone deacetylase

Introduction

Population aging is an important issue in all countries, and chronic diseases, including osteoporosis, pose a burden to the aging population [1]. Older patients with or without osteoporosis, are likely to have low bone strength and incomplete bone repair owing to negative bone remodeling, and fragile fracture can result in significant morbidity and mortality [2, 3]. Accordingly, there are therapeutic needs related to strategies for osteoporosis treatment, bone healing, and positive bone remodeling.

In the aging process, bone loss is caused by several factors, including increased bone resorption, decreased self-renewal capacity, and functional impairment of mesenchymal stem cells (MSCs) that shifts from osteogenesis

*Correspondence:
Junho K. Hur
juhur@hanyang.ac.kr
Sung Bae Park
ddolbae01@naver.com
[1]Department of Neurosurgery, Seoul National University Boramae Medical Center, 20 Boramae-ro 5-gil, Dongjak-gu, Seoul 07061, Korea
[2]Department of Medicine, Major in Medical Genetics, Graduate School, Hanyang University, Seoul, Korea
[3]Hanyang Institute of Bioscience and Biotechnology, Hanyang University, Seoul, Korea
[4]Department of Neurosurgery, Seoul National University College of Medicine, Seoul, Korea
[5]Clinical Research Institute, Seoul National University Hospital, Seoul, Korea
[6]Department of Genetics, College of Medicine, Hanyang University, 222, Wangsimni-ro, Seongdong-gu, Seoul 04763, Korea

into adipogenesis [4, 5]. MSC differentiation to osteogenesis or adipogenesis could be affected by alterations of several cellular factors, including extracellular matrix, paracrine, nuclear lamina, epigenetic modifications, transcription factors, and autophagy [6]. To restore impaired osteogenesis in an aging population, the modulation of cell-intrinsic factors such as epigenetic modification and transcription factors might be more efficient than the alteration of cell-extrinsic factors for regulating the osteoblastic MSC differentiation without the co-stimulation of other intracellular pathways unconnected to osteogenesis. Osteoblastic differentiation is regulated by DNA methylation and histone modification, and histone deacetylase inhibitors (HDACIs), including HDAC1, 3, 4, 5, 7, and 9, suppress osteoclastogenesis and accelerate osteogenesis [7–10]. In addition, previous studies have shown that the gene expression of HDACs is regulated by micro RNAs (miRNAs), which are short non-coding RNAs that induce post-transcriptional regulation of the target gene at the mRNA level [6, 7].

miRNAs are short (18–25) nucleotides, single-strand, non-coding RNAs that regulate gene expression by inhibiting mRNA translation and inducing the degradation of target mRNAs [11–15]. Several miRNAs, such as miR-188, miR-22, miR-29b, miR-2861, and miR-449a, promote osteogenesis by inhibiting HDAC 1,4,5,6, and 9, respectively [11]. Because miRs are natural small RNAs that can selectively repress target transcription factors related to osteogenesis, they are potentially considered to be good therapeutic candidates, with fewer concerns related to unexpected side effects. Therefore, in this study, we aimed to investigate the potential therapeutic applications of miRNAs to regulate HDACs for promoting osteogenesis [9, 11]. To this end, miR-2861 was delivered to human MSCs to assess the modulation of HDAC1, 4, and 5. In humans, the miR-2861 binding site has not been identified. Therefore, we analyzed the *HDAC5* sequence and identified a putative miR-2861 binding site in the 3’ untranslated region (3’-UTR). Next, we validated the miR-2861 binding site by CRIPSR-mediated activation of endogenous miR-2861.

Materials and methods

Cultivation of human bone marrow MSCs

The human MSCs (BM027SS15-P2) used in the present study were obtained from the Catholic Institute of Cell Therapy, Catholic Medical Center, Seoul, Korea. The study protocol was approved by the Use Committee of the Clinical Research Institute, Seoul National University Hospital, Seoul, Korea (permit No.: 07-2020-6). These cells were maintained in a Dulbecco’s modified eagle medium (DMEM)-low-glucose (Biowest, MO, USA) with 20% fetal bovine serum (FBS) (Biowest) at 37 °C in an atmosphere of 5% CO_2. The medium was changed every 3–4 d. To expand the cells, the adhered monolayer was detached with trypsin/ethylenediaminetetraacetic acid (EDTA) (Gibco, Thermo Fisher, MA, USA) for 5 min at 37 °C. During in vitro passaging, the cells were seeded at a density of 8,000/cm^2 and stored in a freezer at −80 °C at 2–9 passages.

Transfection of miR-2861 and differentiation of MSCs

Three experiments were performed to assess the effects of miR-2861 (Sigma, Merck, Darmstadt, Germany) on osteogenic differentiation and determine the intracellular mechanisms: (1) cell proliferation, (2) intensity of alizarin red staining for differentiation of mature osteoblasts, and (3) gene expression related to osteoblast differentiation.

To determine the differentiation potential, MSCs from passage 5 were seeded overnight in a DMEM-low medium without FBS at a density of 100 cells/mm^2 in a six-well culture plate into three groups: MSCs alone (BMSC), MSCs + lipofectamine + miR-2861 (L-miR), and MSCs + miR-2861 alone (miR). After overnight incubation, the medium was changed with reduced serum medium (Opti-MEM, Gibco, Thermo Fisher), and miR-2861 was transfected with a concentration of 50 nM using the Lipofectamine 3000 reagent (Thermo Fisher) for 24 h in the L-miR-2861 group. The MSCs in the three groups were incubated continuously in osteogenic medium (OM) (StemPro Osteogenesis Differentiation Kit; Gibco, NY, USA), and cell proliferation and mineralization were measured at 7, 14, and 21 d.

Cell proliferation and osteoblast differentiation

The MSC osteoblastic differentiation was investigated by analyzing the proliferation and mineralization with alizarin red solution. In all experiments, the final miR-2861 concentration was 50 nM. Data were measured at 7, 14, and 21 d. For cell proliferation analysis, the cells were washed with phosphate-buffered saline (PBS) and incubated with 0.25 mL of 0.25% trypsin in 4 mM EDTA for 5 min at 37 °C. The cells were counted by hemocytometry [16]. Osteoblast differentiation was measured by the progression of mineralization, which was confirmed by alizarin red staining. Briefly, cells were washed with PBS and fixed with 70% ethanol for 1 h at 4 °C. After washing with deionized water, the cells were stained with fresh 2% alizarin red S solution (pH 4.2) for 30 min and washed five times with deionized water. Cell mineralization was examined by microscopy. For the quantification of alizarin red staining, the stained cells were incubated in 10% (w/v) cetylpyridinium chloride in 10 mM sodium phosphate for 1 h at room temperature to extract the alizarin red. An amount of 200 μL of media was moved into 96-well plates and analyzed by spectrophotometry at 562 nm. Osteoblast differentiation with the BMSC, L-miR-2861, and miR-2861 groups was measured using the same methods. All experimental groups were triplicated and their mean values were compared.

Gene expression

To assess the osteoblast-related gene expression, mRNA expression levels within the extracted specimens were measured using the reverse transcription–quantitative polymerase chain reaction (RT-qPCR). MSCs were seeded in 100 mm^2 six-well culture plates and treated with miR-2861 (50 nM), miR-2861 (50 nM) with lipofectamine, and miR-2861 alone in OM. The cells were harvested and the total RNA was extracted using Trizol (Thermo Fisher) at 7, 14, and 21 d, according to the manufacturer's instructions. One µg of RNA was reverse-transcribed into cDNA using AccuPower Rocket Script RT PreMix & MasterMix (Bioneer, Daejeon, Korea). RT–qPCR was performed in 7 µL water with 1 µL primer (10 pmol) and 10 µL of AccuPower 2X GreenStar qPCR MasterMix (Bioneer), and the products were analyzed on a LightCycler480 RT-PCR Instrument System (Roche, Basel, Switzerland). The initial pre-denaturation of cDNA was activated at 95 °C for 15 s followed by 40 denaturation cycles at 95 °C for 1 min, 60 °C for 30 s, and annealing and extension at 60 °C for 1 cycle/min. During PCR amplification, the amplified products were measured continuously by fluorescence emission.

The PCR primer sets for alkaline phosphatase (*ALP*), osteocalcin (*OC*), *HDAC1*, *HDAC4*, and *HDAC5*, Runt-related transcription factor2 (*RUNX2*), catenin beta-1 *(CTNNB1)*, *SMAD4*, and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) (all supplied by Bioneer) as the housekeeper genes were used for the RT–qPCR investigation. The expression level of each target gene was normalized to the internal *GAPDH* control and was represented as a relative expression. To confirm a constant expression level of the housekeeping gene during the total RNA extractions, *GAPDH* RT-PCR was performed. RT-PCR was quantified using the LightCycler480 (Roche) with the GAPD (GAPDH) endogenous control (SYBR probe).

Cell culture and transfection for CRISPRa

A human embryonic kidney 293T (HEK293T) cell line (purchased from ATCC, USA) cultured media consisting of high-glucose DMEM, 10% FBS, 100 U/mL penicillin, and streptomycin, at 90% confluency, was treated with 0.25% trypsin (all purchased from Welgene, Gyeongsan, Korea) and sub-cultured at 1/10 volume. The HEK293T cells were seeded in 24-well plates (NUNC, Thermo Fisher Scientific, Roskilde, Denmark) at a density of 1×10^5 cell/well and were transfected using Lipofectamine 2000 (Invitrogen, MA, USA), according to the manufacturer's instructions. The cells were transfected with 500 ng (each) of dAsCpf1-VPR [17] and crRNA expression vectors. The transfected cells were cultured for 48 h, and total RNA extraction was performed.

RNA extraction to qRT-PCR

The RNA extraction used Trizol (Thermo Fisher). On the day of RNA extraction, the HEK293T cells were harvested and treated with 1 mL lysis buffer. RNA extraction was performed according to the manufacturer's instructions; 1 µg of total RNA was used to synthesize cDNA using a High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Cheshire, UK), according to the manufacturer's instructions. miR-2861 cDNA was prepared from 1 µg total RNA, using has-miR142-3p stem loop primer (Cosmogenetech, Seoul, Korea) [18] and TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Cheshire, UK). For RT–qPCR, 1 µL cDNA was placed in each well of a 96-well reaction plate (Bio-Rad, CA, USA). Thunderbird™ Next, SYBR qPCR Mix (Toyobo, Osaka, Japan) provided the fluorescence signals used to detect target cDNA amounts. Relative mRNA expressions were determined by the ΔΔCt method, with U6 as endogenous control. Reactions were run on a CFX Connect Real-Time instrument (Bio-Rad).

Search and experimental validation of the miR-2861 binding site by CRISPRa using dual luciferase reporter assay

A computational search for putative miR-2861 target sites in the *HDAC5* gene was conducted using the TargetScanHuman 7.2 web tool (http://www.targetscan.org/vert_72)[19]. For experimental assessment, the putative miR-2861 target site was cloned into the pmirGLO Dual-Luciferase miR target expression vector (Promega, WI, USA) at the multicloning site using Mlu1 and Nhe1 (both from NEB, MA, USA) restriction enzymes.

CRISPRa was prepared by cloning the miR-2861 target CRISPR RNA (crRNA) into the U6 crRNA expression plasmid. The crRNA targeting miR-2861 was designed to target the CRISPR activator to the miR-2861 transcriptional start site based on the genome data at the National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov/). The miR-2861 reporter plasmids and CRIPR activator plasmids were transfected with HEK293T cells in a 24-well plate at 1×10^5 cell/well density using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Reporter plasmids (100 ng) were co-transfected into the HEK293T cells with 450 ng CRISPR activator protein dAsCpf1-VPR plasmids and 450 ng crRNA expression plasmids. Sample cells were cultured for 48 h, and the miR binding site was analyzed by conducting a dual-luciferase reporter assay using the Dual-Luciferase Reporter (DLR™) Assay System (Promega) according to the manufacturer's instructions. The luciferase signals were measured with GloMax Discover

Microplate Reader (Promega), and the firefly luciferase activity was internally normalized to *Renilla* luciferase activity.

Statistical analysis

For quantitative analysis, the experiments were conducted with biological replicates and statistical significance was determined by the two-tailed Student's *t* test and one-way analysis of variance after Bonferroni's correction. A *p*-value of less than 0.05 was considered significant.

Results

Delivery of mir-2861-enhanced osteogenesis in human MSCs

To assess whether increasing miR-2861 in the MSCs could facilitate osteogenesis, we transfected synthesized miR-2861 by lipofectamine into human MSCs (L-miRNA) and analyzed the effect using two control groups: untreated MSCs (BMSC) and MSC-treated miR-2861 without transfection reagent (miR). The MSC numbers in all three groups increased until 21 d after seeding. On day 21 after seeding, the MSC proliferation degree in the L-miR group was higher than that in both the nMSC group (L-miR [$4.26\times10^5 \pm 0.073\times10^5$] and the BMSC group [$4.15\times10^5 \pm 0.073\times10^5$]). The number of cells in the L-miR group on day 21 increased significantly compared with day 14 (Fig. 1A). Next, the MSC differentiation to mature osteoblasts was evaluated by determining the amounts of calcium deposits by alizarin red staining. The observed mineralization in the L-miRNA group appeared to be significantly higher than that of the control groups throughout the course of measurement on days 7, 14, and 21 (Fig. 1B, C). The mean alizarin red optical density values of the BMSC, L-miRNA, and miRNA groups on days 7, 14 and 21 were as follows: 7 d, 0.097 ± 0.005, 0.175 ± 0.007, and 0.159 ± 0.002; 14 d, 0.191 ± 0.008, 0.245 ± 0.005, and 0.192 ± 0.005; 21 d, 0.531 ± 0.008, 0.624 ± 0.007, and 0.520 ± 002. Notably, the miR-2861 group without transfection reagent (miR) showed increased alizarin red staining on day 7, but on days 14 and 21 no increment was detected. The alizarin staining results suggested that transfection of miR-2861 by lipofectamine into MSC enhanced the osteoblast differentiation.

Mir-2861-Mediated regulation of osteoblast-related genes

To understand how miR-2861 regulated the gene expression of genes related to osteogenesis, we transfected miR-2861 into MSCs and performed RT–qPCR on days 7, 14,

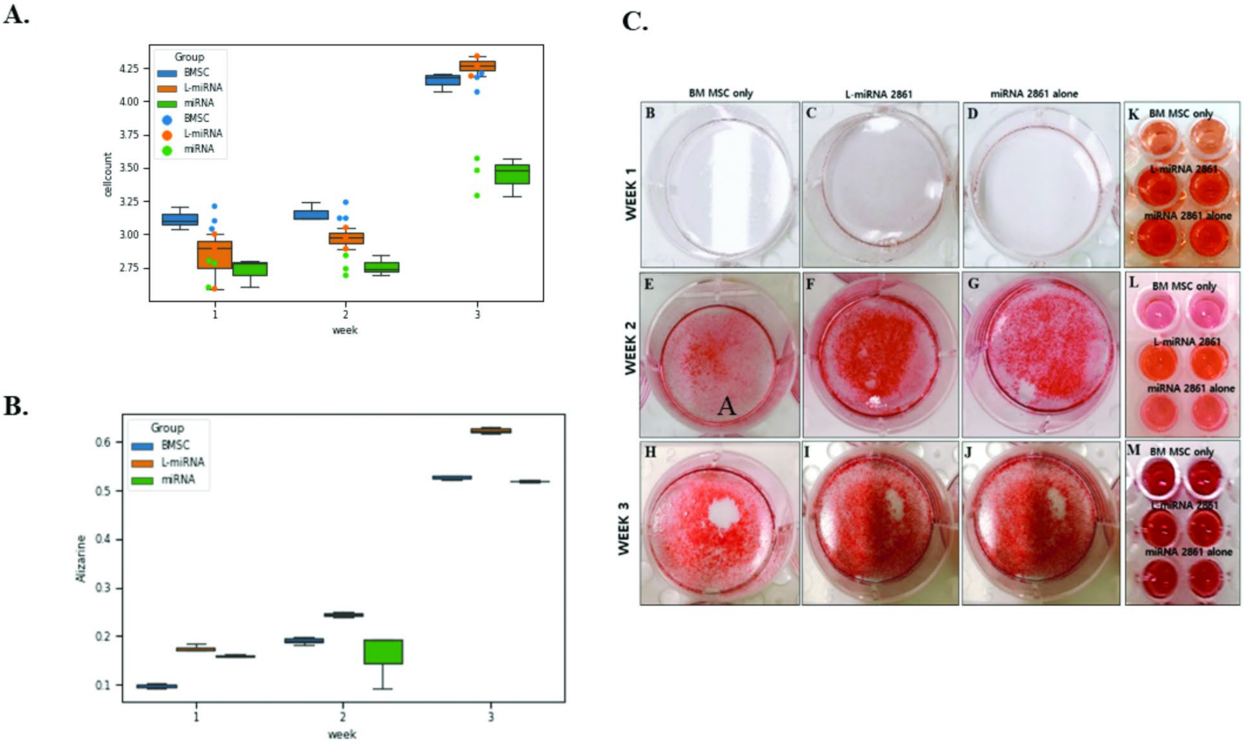


Fig. 1 In vitro MSC osteogenic differentiation properties. Cells were incubated in a medium containing PBS, Lipofectamine, and miR-2861 for 24 h. Then, cell proliferation (**A**) and the degree of alizarin red staining for mineral deposition (**B**) were investigated at 7, 14, and 21 days. In alizarin red staining at 21 days (**C**), the degree of staining in the L-miR group was higher than that in the BMSC and miR groups. PBS: phosphate buffer saline, BMSC: MSCs alone, L-miR: MSCs + Lipofectamine + miR-2861, miR: MSCs + miR-2861, MSC: mesenchymal stem cell

and 21 after seeding, to evaluate the expression levels of miR-2861 target genes related to osteogenic differentiation. We assessed *RUNX2* and *β-CATENIN*, which are known as the master transcription factors in osteoblast differentiation, *ALP* and *OC*, which are known to be related to osteoblast differentiation, and *HDAC1*,*4*, and *5*, which are known targets of miR-2861, miR-29b, and miR-449a, respectively [11]. The gene expression levels of *HDAC4* and *HDAC5* in the L-miR group were lower than those in the other groups (Fig. 2). After seeding, the gene expression level of *HDAC1* in the L-miRNA group was lower on day 14 and also lower than that in the nMSC group. The gene expression level of *RUNX2*, a master transcription factor in osteoblast differentiation, was higher in the L-miRNA group on days 14 and 21 than in the other groups. On day 21, the L-miRNA and miRNA groups showed increased expression levels of *β-CATENIN*, *OC*, and *ALP*. In the L-miRNA and miR-2861-only groups, the *HDAC1* and *SMAD4* gene expression levels were higher on day 7, and then decreased on day 21. The expression level of the *SMAD4* gene in the miR-2861-only group increased slightly on day 21. The change in gene expression level in *HDAC1* may be synchronized with that in *SMAD4*. The *SMAD* genes, including *SMAD4*, regulate the expression of transcriptional factors and cofactors important in osteoblasts (Dlx5 and RUNX2), and *SMAD 2/3* recruit *HDAC4/5* to counter RUNX2 activity [20, 21]. In dexamethasone-induced osteoblast differentiation, half LIM domains protein 2 binding to β-CATENIN augments the β-CATENIN transport into the nucleus, where it induces the transcription of RUNX2 [22]. The changes in gene expression levels suggest that miR-2861 regulates several aspects of the intracellular network of osteoblast differentiation (Fig. 3). miR-2861 activation inhibited the expressions of *HDAC1*, *4*, and *5* and concomitantly increased the expression of *RUNX2*. The gene expression of *RUNX2* was augmented by activation of the *β-CATENIN*, *ALP*, and *OC* genes, which are expressed in mature osteoblasts. The results suggest that miR-2861 regulates osteoblast differentiation by the inhibition of *HDAC1*, *4*, and *5*, which leads to increased gene expression of *RUNX2* and *β-CATENIN.*

Identification of a novel primate-specific miR-2861 binding site in the 3′ UTR of HDAC5 by CRISPRa

Previous studies using murine systems have shown that *HDAC5* was regulated by miR-2861 via a miR binding site in the gene body, but the reported miR-2861 binding site was not conserved in the human *HDAC5* gene. Therefore, the question of whether miR-2861 could directly repress *HDAC5* was investigated. To this end, sequence analyses of human *HDAC5* were conducted using TargetScan, and a putative primate-specific miR-2861 binding site was found in the 3’-UTR (Fig. 4A and Supplement Table 1). Next, we assessed whether the putative miR-2861 binding site was functional in repressing the target genes in human cells. To this end, dual-luciferase reporter systems were prepared that contained either the wild-type putative miR-2861 target (WT) or the mutant control sequence that contained mutations in the miRNA seed region (MT), to test whether the interaction of miR-2861 and the putative miR target site could induce a knockdown of the target gene expression. The miR-2861 target sequence was taken from the 3’-UTR region of HDAC5 mRNA, and the mutant control sequences contained base mismatches between the seed region of miR-2861 and the target sequence (Fig. 4B). It was anticipated that if the putative miR-2861 target was functional in cells, an overexpression of miR-2861 would result in repression of the reporter expression.

To this end, the CRISPR activator was used to induce overexpression of endogenous miR-2861 in HEK293T cells and assessed the expression of the miR-2861 reporter. For CRISPRa, we used a catalytically inactive form of AsCas12a fused to the VP64-p65-Rta tripartite transcription activator (dAsCas12a-VPR) (Fig. 4C) [17]. Previous studies have reported that increasing the number of crRNAs when using dCpf1-VPR enhances the efficiency of overexpression. Additionally, three crRNAs were designed to attract the dAsCas12a-VPR to the vicinity of the transcription start site of endogenous

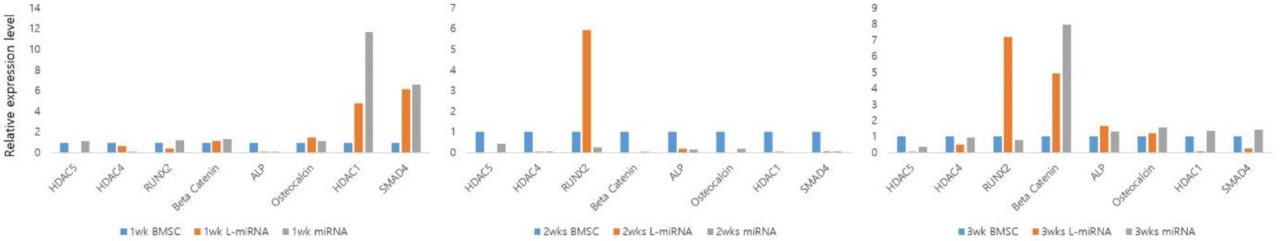


Fig. 2 Investigation of gene expression in relation to osteoblast differentiation. Cells were incubated in a medium containing PBS, Lipofectamine, and miRNAmiR-2861 for 24 h. Then, the expression levels of HDAC1, 4, and 5, SMAD4, Runx2, beta-catenin, ALP, and OC were examined at 1, 2, and 3 weeks using RT–qPCR. The data represent the fold change and compared with the PBS BMSC group

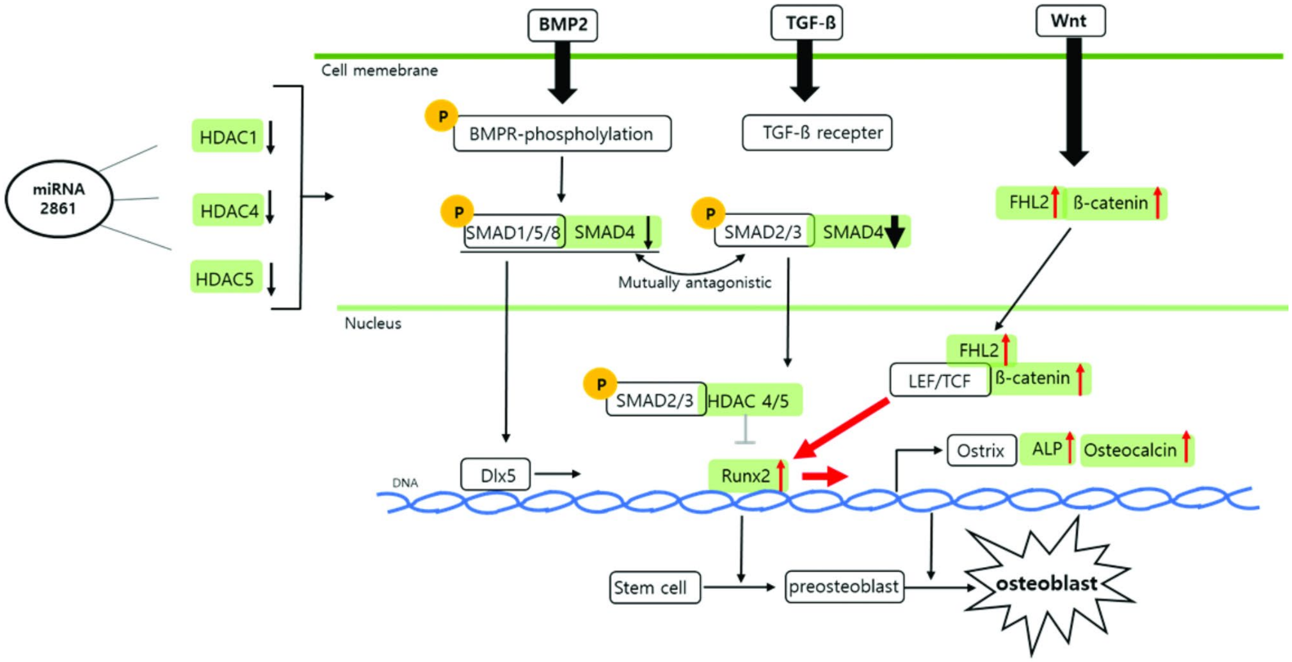


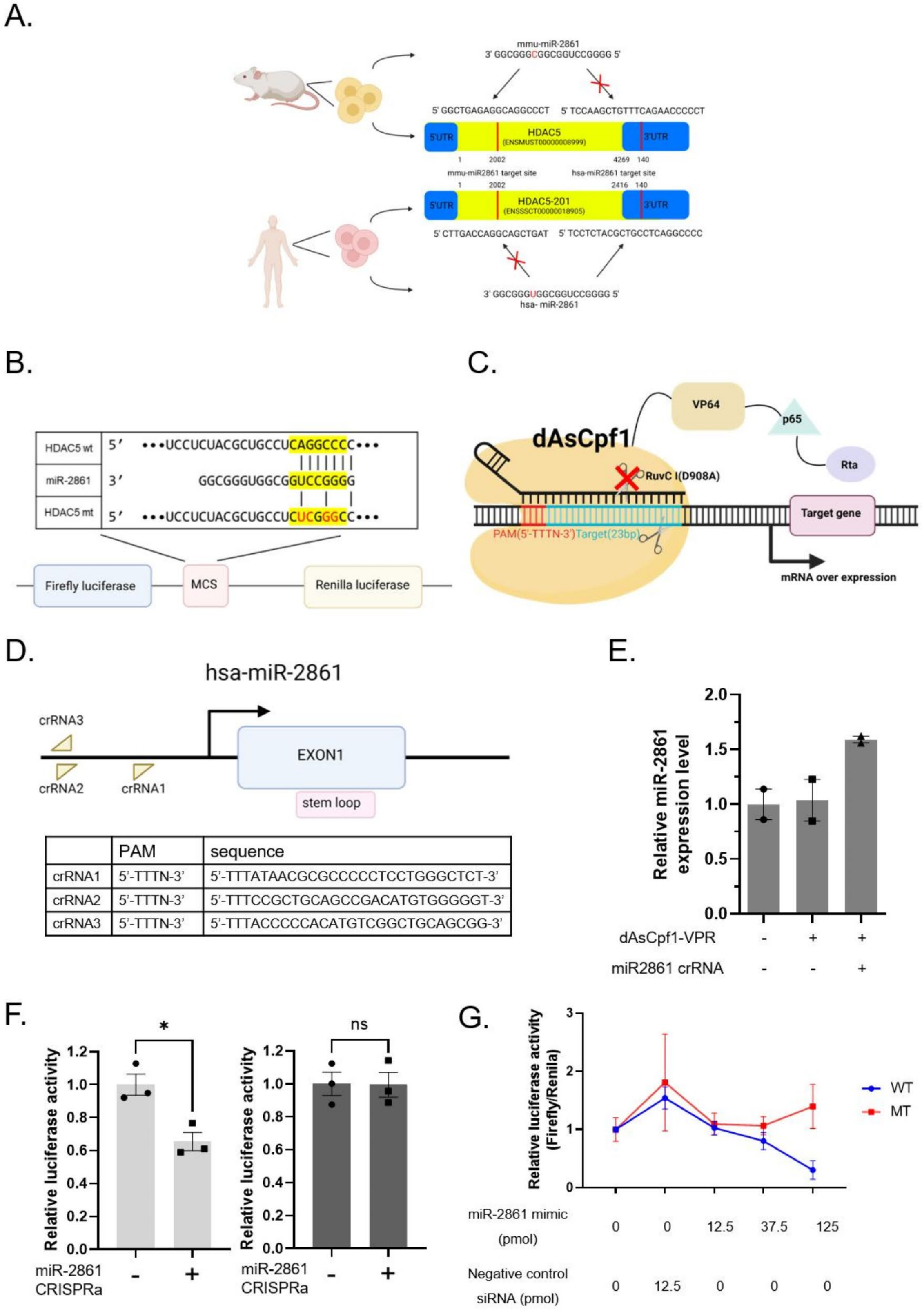
Fig. 3 Proposed intracellular mechanism related to osteoblast differentiation after miR-2861 activation. miR-2861 activation inhibited the expression of HDAC1, 4, and 5, followed by increased Runx2 expression. Runx2 gene expression was augmented by the activation of β-catenin and the ALP and OC genes expressed in mature osteoblasts

miR-2861(Fig. 4D). Next, we asked whether CRISPRa by miR-2861 by transfecting dAsCpf1-VPR and crRNA into HEK293T cells induced changes in the expression levels of the miR-2861 and luciferase reporter with the putative miR-2861 binding site. To assess the changes in miR-2861 expression level, we performed RT–qPCR at 2 d after transfection. We observed that the expression levels of miR-2861 increased by a factor of about 1.7 and upregulation of miR-2861 occurred when the experiment was replicated (Fig. 4E and Supplement Fig. 1A). Next, we sought to assess whether the luciferase reporter with the putative miR-2861 binding site from HDAC5 could be repressed by CRISPRa of endogenous miR-2861 via dAsCpf1-VPR. The dAsCpf1-VPR, crRNA, and the dual-luciferase reporter vector were co-transfected. The dual-luciferase reporter assay showed that CRISPRa of miR-2861 resulted in a reduction by a factor of about 0.4 of the luciferase reporter that contained the putative miR-2861 wild-type (WT) binding site from *HDAC5*. By contrast, the expression level of luciferase reporter with the HDAC5 mutant type (MT) remained unchanged by CRISPRa of miR-2861 (Fig. 4F).

Next, we asked if the reduction in the luciferase reporter level CRISPRa was mediated by miR-2861 and not by CRISPR off-target effects. To this end, exogenous miR-2861 was transfected into HEK293T cells and the levels of luciferase reporters were assessed. The reporter with the putative miR-2861 binding site from HDAC5 (WT) was repressed by exogenous miR-2861 in a concentration-dependent manner. By contrast, the expression level of a reporter with a mutant binding site was not significantly changed by transfection of exogenous miR-2861 (Fig. 4G); also, the luciferase expression was confirmed to be significantly different when 250 pmol of exogenous miR-2861 was transfected (Supplement Fig. 2A, B).

Discussion

In the present study, we investigated miR-2861 focusing on the modulation of other HDACs (HDAC1 and 4), including a known target, HDAC5, and related intracellular mechanisms in osteogenesis using human MSCs. Previous studies have shown that miR-2861 directly inhibits HDAC5 [11, 23]. However, other effects on HDAC1 and HDAC4 have not been clarified. In the RT–qPCR assessment in the present study, we found that after miR-2861 activation, these HDACs seem to be related. In the L-miR group, the gene expression of *HDAC4* had similar patterns to those of *HDAC5* for 21 d. During the first week of RT-qPCR, the *HDAC5* expression was severely inhibited owing to the direct inhibitory effect of miR-2861, whereas the expression level of HDAC1 was significantly high. However, the gene expressions of HDAC1, 4, and 5 were inhibited from 14 d after seeding. From week 2, the *RUNX2* gene expression increased as the master transcription factor of osteoblast differentiation



	PAM	sequence
crRNA1	5’-TTTN-3’	5’-TTTATAACGCGCCCCCTCCTGGGCTCT-3’
crRNA2	5’-TTTN-3’	5’-TTTCCGCTGCAGCCGACATGTGGGGGT-3’
crRNA3	5’-TTTN-3’	5’-TTTACCCCCACATGTCGGCTGCAGCGG-3’

Fig. 4 (See legend on next page.)

(See figure on previous page.)

Fig. 4 Dual luciferase reporter system of miR-2861. and endogenous miR-2861 overexpression by dAsCpf1-VPR. (**A**) Schematic diagram for the predicted interaction between miR-2861 and HDAC5 mRNA 3'UTR in human and mouse. (**B**) the luciferase reporter vector was constructed that inserted miR-2861 target site and mutation target site of HDAC5 3'UTR. Red indicated the mutant nucleotides. (**C**)Experimental scheme for gene overexpression by dAsCpf1-VPR. The ribonucleoprotein complex comprises catalytically inactive dAsCpf1 fused to the VPR and crRNA. (**D**) Design of crRNA targeting miR-2861. The triangle indicates the target position, and the table shows the sequence. (**E**) The dAsCpf1-VPR construct and crRNA targeting miR-2861 were co-transfected into HEK293T cells and the change in miR-2861 expression level was confirmed by qRT-PCR. Data shown are results from two biological replicates (error bars indicate the SEM) and normalized to U6 expression level. (**F**) Wild-type(gray), mutated-type(dark-gray) reporter system, dAsCpf1-VPR constructs and crRNA targeting miR-2861 were co-transfected into HEK293T cells. Data shown are results from three biological replicates (error bars indicate the SEM) and normalized to Renilla luciferase activity. The p values less than 0.05 were considered significant (*$p < 0.05$), and no significance between groups was indicated as ns. (**G**) Wild-type(blue line), mutated-type(red line) reporter system, miR-2861 mimic were transfected into HEK293T cells. Data shown are results from three biological replicates (error bars indicate the SEM)

in the L-miR group compared to the BMSC group [24]. β-CATENIN is a transcription factor in osteoblast differentiation, and ALP and OC are produced by osteoblast [24, 25]. The gene expression levels of *β-CATENIN*, *ALP*, and *OC* in the L-miR group were higher than those in the BMSC group at 21 d after seeding. The *SMAD4* and *HDAC1* gene expressions showed similar patterns for 21 d. Although the gene expressions of *HDAC1*, *4*, and *5* and *SMAD4* decreased in the L-miR group, those of *RUNX2*, *β-CATENIN*, *ALP*, and *OC* increased at 21 d after seeding. The counter effect of HDAC4/5 on RUNX2 activation seems to be hindered by miR-2861 activation, and the transcriptional effect of β-CATENIN on RUNX2 might not be either inhibited or activated. Therefore, the gene expression of *RUNX2* might increase and the osteoblastic differentiation might be augmented after miR-2861 activation.

Although our study demonstrates that miR-2861 enhances osteoblast differentiation as evidenced by increased mineralization through alizarin red staining, it is important to acknowledge that further validation is needed at the protein level. Specifically, the expression of key osteogenic markers such as ALP, Runx2, Osx, and collagen type 1 should be confirmed through Western blot analysis to ensure that the observed upregulation at the gene level translates to functional protein expression. This will provide a more comprehensive understanding of the molecular mechanisms underlying miR-2861's role in osteogenesis and address potential discrepancies between gene expression and protein synthesis.

In this study, CRISPRa was used to increase the expression levels of endogenous miR-2861. Also, a computational search was conducted to identify a putative miR-2861 binding site in 3'UTR of HDAC5. Next, we sought to verify whether the predicted miRNA binding site was functional. To this end, we performed luciferase reporter assays and observed that the putative HDAC5 binding of miR-2861 resulted in decreased gene expression in response to CRISPRa of endogenous miRNA-2861 dependent manner. Together, the results showed that CRISPRa could be applied to validate a putative miR-2861 target site. We expect that the CRISPRa methods could be more broadly applied for an miRNA mechanism study.

Conclusions

The putative HDAC5 binding of miR-2861 may have a positive effect on MSCs proliferation by decreased gene expression in response to the CRISPRa of endogenous miRNA-2861-dependent manner. Therefore, miRNAmiR-2861 with the CRISPRa methods might be a good biomaterial for osteogenesis augmentation.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13018-024-05163-3.

Supplementary Material 1

Supplementary Material 2

Acknowledgements
There is no additional acknowledge.

Author contributions

S.B. P. reports no disclosures. He is provided the study design and study supervision. And he wrote the manuscript.S. P reports no disclosures. He performed acquisition and analysis in the study. And he wrote the Methods and Results section in the present study.J.Y. L. report no disclosures. They performed acquisition and analysis in the study.C.H.K.and H.J.Y. reports no disclosures. He provided the study design and concept. J. K. H. reports no disclosures. He provided the study design.

Funding
This work was supported by grants from the National Research Foundation of Korea(NRF) funded by the Korean Ministry of Education, Science, and Technology (grant number RS-2023-NR076663 to J.K.H.) and from the Korea Health Technology R&D Project of the Korea Health Industry Development Institute (KHIDI) funded by the Korean Ministry of Health & Welfare (grant number RS-2022-KH125650 to J.K.H.).

Data availability

The data that support the plots within this paper and other findings including the code for the models are available from the corresponding author upon reasonable request.

Declarations

Ethic approval and conset to participate

Ethical review and approval were waived for this study due to no coflict of interest and ethical issue related to human subjects in the pressent study. The human MSCs (BM027SS15-P2) used in the present study were obtained from the Catholic Institute of Cell Therapy, Catholic Medical Center, Seoul, Korea. The study protocol was approved by Use Committee of the Clinical

Research Institute, Seoul National University Hospital, Seoul, Korea (permit No.: 07-2020-6). The informed consent was waived by Committee of the Clinical Research Institute, Seoul National University Hospital, Seoul, Korea (permit No.: 07-2020-6).

Competing interests

The authors declare no competing interests.

Received: 22 July 2024 / Accepted: 8 October 2024

References

1. Zhao C, Wong L, Zhu Q, Yang H. Prevalence and correlates of chronic diseases in an elderly population: a community-based survey in Haikou. PLoS ONE. 2018;13(6):e0199006. Epub 2018/06/15.
2. Park SB, Chung CK. Strategies of spinal fusion on osteoporotic spine. J Korean Neurosurg Soc Jun. 2011;49(6):317–22. Epub 2011/09/03.
3. Blume SW, Curtis JR. Medical costs of osteoporosis in the elderly Medicare population. Osteoporos Int Jun. 2011;22(6):1835–44. Epub 2010/12/18.
4. Coipeau P, Rosset P, Langonne A, Gaillard J, Delorme B, Rico A, et al. Impaired differentiation potential of human trabecular bone mesenchymal stromal cells from elderly patients. Cytotherapy. 2009;11(5):584–94. Epub 2009/07/25.
5. Stenderup K, Justesen J, Clausen C, Kassem M. Aging is associated with decreased maximal life span and accelerated senescence of bone marrow stromal cells. Bone Dec. 2003;33(6):919–26. Epub 2003/12/18.
6. Infante A, Rodriguez CI. Osteogenesis and aging: lessons from mesenchymal stem cells. Stem Cell Res Ther Sep. 2018;26(1):244. Epub 2018/09/28.
7. Husain A, Jeffries MA. Epigenetics and bone remodeling. Curr Osteoporos Rep Oct. 2017;15(5):450–8. Epub 2017/08/16.
8. Cantley MD, Fairlie DP, Bartold PM, Rainsford KD, Le GT, Lucke AJ, et al. Inhibitors of histone deacetylases in class I and class II suppress human osteoclasts in vitro. J Cell Physiol Dec. 2011;226(12):3233–41. Epub 2011/02/24.
9. Tan G, Cao X, Dai Q, Zhang B, Huang J, Xiong S, et al. A novel role for microRNA-129-5p in inhibiting ovarian cancer cell proliferation and survival via direct suppression of transcriptional co-activators YAP and TAZ. Oncotarget Apr. 2015;20(11):8676–86. Epub 2015/04/22.
10. Cantley MD, Zannettino ACW, Bartold PM, Fairlie DP, Haynes DR. Histone deacetylases (HDAC) in physiological and pathological bone remodelling. Bone Feb. 2017;95:162–74. Epub 2016/12/04.
11. Jing D, Hao J, Shen Y, Tang G, Li ML, Huang SH, et al. The role of microRNAs in bone remodeling. Int J Oral Sci Sep. 2015;14(3):131–43. Epub 2015/07/25.
12. Giordano L, Porta GD, Peretti GM, Maffulli N. Therapeutic potential of microRNA in tendon injuries. Br Med Bull May. 2020;15(1):79–94. Epub 2020/03/29.
13. Gargano G, Oliva F, Oliviero A, Maffulli N. Small interfering RNAs in the management of human rheumatoid arthritis. Br Med Bull Jul. 2022;9(1):34–43. Epub 2022/05/01.
14. Gargano G, Oliviero A, Oliva F, Maffulli N. Small interfering RNAs in tendon homeostasis. Br Med Bull Jun. 2021;10(1):58–67. Epub 2021/01/18.
15. Oliviero A, Della Porta G, Peretti GM, Maffulli N. MicroRNA in osteoarthritis: physiopathology, diagnosis and therapeutic challenge. Br Med Bull Jun. 2019;19(1):137–47. Epub 2019/05/09.
16. Nam SH, Lee Y, Ahn JH, Chung CK, Yang HJ, Park SB, et al. Augmented osteogenesis of mesenchymal stem cells using a fragmented Runx2 mixed with cell-penetrating, dimeric a-helical peptide. Eur J Pharm Sci Mar 1. 2020;144:105210. Epub 2020/01/04.
17. Choi J, Bae T, Byambasuren N, Park SH, Jo CH, Kim D, et al. CRISPR-Cpf1 activation of endogenous BMP4 gene for osteogenic differentiation of umbilical-cord-derived mesenchymal stem cells. Mol Ther Methods Clin Dev Jun. 2020;12:17:309–16. Epub 2020/02/06.
18. Chen C, Ridzon DA, Broomer AJ, Zhou Z, Lee DH, Nguyen JT, et al. Real-time quantification of microRNAs by stem-loop RT-PCR. Nucleic Acids Res Nov. 2005;27(20):e179. Epub 2005/11/30.
19. Agarwal V, Bell GW, Nam JW, Bartel DP. Predicting effective microRNA target sites in mammalian mRNAs. Elife Aug 12 2015;4. Epub 2015/08/13.
20. Chen G, Deng C, Li YP. TGF-beta and BMP signaling in osteoblast differentiation and bone formation. Int J Biol Sci. 2012;8(2):272–88. Epub 2012/02/03.
21. Wu M, Chen G, Li YP. TGF-beta and BMP signaling in osteoblast, skeletal development, and bone formation, homeostasis and disease. Bone Res. 2016;4:16009. Epub 2016/08/27.
22. Langenbach F, Handschel J. Effects of dexamethasone, ascorbic acid and beta-glycerophosphate on the osteogenic differentiation of stem cells in vitro. Stem Cell Res Ther. 2013;4(5):117. Epub 2013/10/01.
23. Hu R, Liu W, Li H, Yang L, Chen C, Xia ZY, et al. A Runx2/miR-3960/miR-2861 regulatory feedback loop during mouse osteoblast differentiation. J Biol Chem Apr. 2011;8(14):12328–39. Epub 2011/02/18.
24. Komori T. Regulation of osteoblast differentiation by transcription factors. J Cell Biochem Dec. 2006;1(5):1233–9. Epub 2006/06/24.
25. Bharadwaj S, Naidu AG, Betageri GV, Prasadarao NV, Naidu AS. Milk ribonuclease-enriched lactoferrin induces positive effects on bone turnover markers in postmenopausal women. Osteoporos Int Sep. 2009;20(9):1603–11. Epub 2009/01/28.

Publisher's note