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microRNA-21 Mediates Stretch-Induced Osteogenic Differentiation in Human Periodontal Ligament Stem Cells

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microRNAs (miRNAs) are short 20- to 22-nucleotide noncoding RNAs that negatively regulate the expression of target genes at the post-transcriptional level. The expression of specific miRNAs and their roles in the osteogenic differentiation of human periodontal ligament stem cells (PDLSCs) exposed to mechanical stretch remain unclear. Here, we found that stretch induced both osteogenic differentiation and the differential expression of miR-21 in PDLSCs. Furthermore, we identified activin receptor type IIB (*ACVR2B*) as a target gene of miR-21. Luciferase reporter assays showed that miR-21 interacts directly with the 3′-untranslated repeat sequence of *ACVR2B* mRNA. Mechanical stretch suppressed ACVR2B protein levels in PDLSCs, and this suppressive effect was modulated when endogenous miR-21 levels were either enhanced or inhibited. Both stretch and the expression of miR-21 altered endogenous ACVR2B protein levels and thus the osteogenic differentiation of PDLSCs. In addition, gain- and loss of function of ACVR2B mediated the osteogenic differentiation of PDLSCs. This study demonstrates that miR-21 is a mechanosensitive gene that plays an important role in the osteogenic differentiation of PDLSCs exposed to stretch.

Introduction

The periodontal ligament (PDL) is a complex, soft connective tissue with an abundant network of blood vessels that is embedded between the cementum and alveolar bone. Multipotent periodontal ligament stem cells (PDLSCs) have been isolated from the PDLs of extracted human teeth [1]. During orthodontic tooth movement, the mechanical stress applied to the teeth is transmitted to the alveolar bone by the PDL. Thus, PDLSCs are expected to play a pivotal role in alveolar bone remodeling during orthodontic tooth movement. A previous study showed that mechanical vibration promotes PDLSC osteogenic differentiation [2]. However, the mechanism underlying osteogenic differentiation of PDLSC subjected to mechanical force remains poorly understood.

microRNAs (miRNAs, miRs), which are classified as regulatory RNAs, are a recently identified family of small noncoding RNAs that regulate gene function post-transcriptionally [3]. MiR-21 is considered an onco-miRNA; increased MiR-21 expression was found in most human cancers with characteristics of promoting cell proliferation, migration, and anti-apoptosis [4,5]. Growing evidence suggests that miR-21 is also involved in mechanical-force–induced variation in cellular functions in vitro. A recent study demonstrates that shear stress modulates miR-21 expression. In turn, changes in miR-21 expression modulate endothelial cell (EC) apoptosis and EC nitric oxide synthase activity [6]. Oscillatory shear stress can induce activator protein-1 (AP-1)–dependent miR-21 expression, which directly targets peroxisome proliferator-activated receptor-α (PPARα) to inhibit its expression, thereby enabling activation of AP-1 and the promotion of monocyte adhesion [7]. Cyclic stretch also modulates miR-21 expression, suggesting that increased miR-21 expression is involved in the regulation of human aortic smooth muscle cell proliferation and apoptosis mediated by stretch [8].

Though miR-21 is believed to be important in many biological processes through regulation of gene expression under mechanical force, its role in the osteogenic differentiation of PDLSCs exposed to mechanical stretch remains to be elucidated. Accordingly, this study investigated the effect of stretch on miR-21 expression in PDLSCs and examined

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its functional roles in the regulation of stretch-induced osteogenic differentiation in PDLSCs.

Materials and Methods

Cell culture and isolation of PDLSCs

All protocols for handling human tissues were approved by the Research Ethics Committee of Shandong University, China. Informed consent was obtained from 12 donors aged 10–14 years and their parents. PDLSCs were isolated and cultured as previously reported [1], and the experiments used third-passage PDLSCs. The stem cell properties of PDLSCs were characterized using cell surface markers (STRO-1, CD146, CD34, and CD45) and by multi-differentiation. To detect osteogenic differentiation, cells were cultured in the presence of 100 μM L-ascorbate-2-phosphate, 10 mM β-glycerophosphate, and 10 nM dexamethasone for 3 weeks. To detect adipogenic differentiation, cells were cultured in the presence of 0.5 mM isobutylmethylxanthine, 0.5 mM hydrocortisone, 10 μg/mL insulin, and 60 mM indomethacin for 3 weeks. Calcification of the extracellular matrix was checked via Alizirin red staining. Oil Red O staining was used to identify lipid-laden fat cells.

Application of mechanical force

External mechanical stimulation was achieved using a Flexcell® FX-5000™ Tension System. Flexcell Amino silicone-bottomed plates were coated with 0.6 mg/mL collagen I solution (Sigma Aldrich). PDLSCs were seeded onto these plates at a density of $\sim 2.5 \times 10^5$ cells per cm^2. After the cultures reached ~80% confluence, the cells were serum deprived (2% serum) for 24 h. PDLSCs were then stretched in a Flexcell FX-5000™ Tension system (Flexcell International Corporation). We imposed 10% stretch at 1.0 Hz for 6, 12, 24, or 48 h. Control PDLSCs were cultured without stretching (static condition).

Real-time reverse transcription polymerase chain reaction

To measure miR-21 and mRNA levels of runt-related transcription factor 2 (*RUNX2*) and osteocalcin (*OCN*), total RNA was isolated from different PDLSCs groups using RNAisoTM Plus (TAKARA). After DNase treatment, 1 μg of the total RNA was reverse transcribed using has-miR-21 qPCR Primer Mix and PrimeScript® RT reagent Kit with gDNA Eraser (TAKARA). Relative transcript levels were measured by quantitative PCR in a 20 μL reaction volume, which included 10 μL SYBR®Premix Ex Taq™, 0.4 μL 10 μM forward primer (200 nM final), 0.4 μL 10 μM reverse primer (200 nM final), 100 ng Template DNA, and DEPC-treated water of approximately 20 μL, using Roche LightCycler® 480 sequence detection system (Roche Diagnostics GmbH) by SYBRPremix Ex Taq (TliRNaseH Plus; TAKARA). U6 snRNA or GAPDH was used as an internal control to quantify and normalize the results. Primers for U6 and miR-21 were TAKARA: D356-03 and TAKARA: DHM0189, respectively. Primer pairs used for *RUNX2*, *OCN*, *ACVR2B* and *GAPDH* are listed in Table 1. The PCR program was as follows: 95°C for 30 s, then 40 cycles of 95°C for 5 s, 60°C for 20 s, and 65°C for 15 s. The specificity of the reaction is given by the detection of the Tms of the amplification products immediately after the last reaction cycle. The value $2^{-\Delta\Delta CT}$ was used to comparative quatitation. All PCRs were performed in triplicate.

Table 1. Gene Primers

Gene name	*Primer (5′ to 3′)*	*Primer sequence*
RUNX2	Forward	CGAATGGCAGCACGCTATTAA
	Reverse	GTCGCCAAACAGATTCATCCA
OCN	Forward	TAGTGAAGAGACCCAGGCGCT
	Reverse	ATAGGCCTCCTGAAAGCCGA
ACVR2B	Forward	AGACACGGGAGTGCATCTACT
	Reverse	GCCTATCGTAGCAGTTGAAGTC
GAPDH	Forward	TCATGGGTGTGAACCATGAGAA
	Reverse	GGCATGGACTGTGGTCATGAG

Alkaline phosphatase activity assay

After being stretched, PDLSCs were stained to determine their alkaline phosphatase (ALP) activity. Following the manufacturer's protocol (Sigma-Aldrich), cells were fixed with 4% paraformaldehyde and stained with a solution of 0.25% naphthol AS-BI phosphate and 0.75% fast red violet (from the ALP activity kit). ALP activity was measured using the ALP activity kit and was normalized to the protein concentration in the sample.

Network analysis

The Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway (www.genome.jp/kegg) annotations of the miRNA targets was used for gene annotation. We chose pathways with $P < 0.05$ (based on Fisher's exact test).

Viral vector construction and transduction

Lentiviral pre-miR-21-GFP (pGC-LV-pre-miR-21-GFP) for hsa-miR-21-5p-overexpression, lentiviral anti-miR-21-GFP (pGC-LV-anti-miR-21-GFP) for hsa-miR-21-5p-inhibition, and empty lentiviral vector (pGC-LV-GFP) for miR-control were constructed by the Genechem Company. PDLSCs were seeded at 2.5×10^5 cells per cm^2. After the cultures reached ~80% confluence, cells were infected with lentiviruses in the presence of polybrene (5 μg/mL) for 6 h according to the manufacturer's instructions in the presence of virus at a multiplicity of infection of 20. The forward primers were synthesized by the Genechem Company: 5′-CGGCCGCGACTCTAGTTATC AAATCCTGCCTGACTG-3′ for hsa-miR-21-5p, and 5′-CCGGTTCAACATCAGTCTGATAAGCTATTTTTG-3′ for hsa-miR-21-5p-inhibition.

PcDNA3.1-ACVR2B for ACVR2B overexpression, pcDNA3.1-ACVR2Bsh for ACVR2B silencing, and pcDNA3.1 vector as a control were constructed by the Genechem Company. PDLSCs were seeded at 2.5×10^5 cells per cm^2. After the cultures reached ~80% confluence, cells were transfected with vectors using Lipofectamine™ 2000 (Invitrogen), following the manufacturer's protocol. Four hours later, the medium was changed and the cells were cultured in standard alpha modification of Eagle's medium with no additions, containing 10% fetal bovine serum, for 24 h.

After transfected, the cells underwent stretch (10% elongation, 1.0 Hz) for 24 h, as described in the previous section.

Luciferase assay

Luciferase assays were carried out in PDLSCs. 1×10^5 Cells were plated in 12-well plates at 24 h before transfection. After transfection with pGC-LV-pre-miR-21-GFP or pGC-LV-GFP, 30 ng of the pmirGLO-3′-UTR vector or the pmirGLO-3′-UTR mutation vector were co-transfected into cells using X-tremeGENE transfection reagent (Roche Applied Science). Renilla and firefly luciferase activities were measured with the Dual-Luciferase Reporter system (Promega) at 24 h after transfection. Firefly luciferase activity was normalized to Renilla luciferase expression for each sample. Each experiment was performed in triplicate.

Western blotting

After culture in the indicated conditions, confluent PDLSCs were lysed and the protein concentration of each lysate was determined using a protein assay kit (Bio-Rad Laboratories). Lysate proteins were resolved by electrophoresis, transferred to nitrocellulose membranes, and blotted with antibodies against actin and ACVR2B (1:500; Abcam).

Statistical analysis

All statistical calculations were performed with SAS9.2 statistical software. The student's *t*-test or one-way analysis of variance was performed for normally distributed data to determine statistical significance. A P value 0.05 was considered significant. If significance was found,

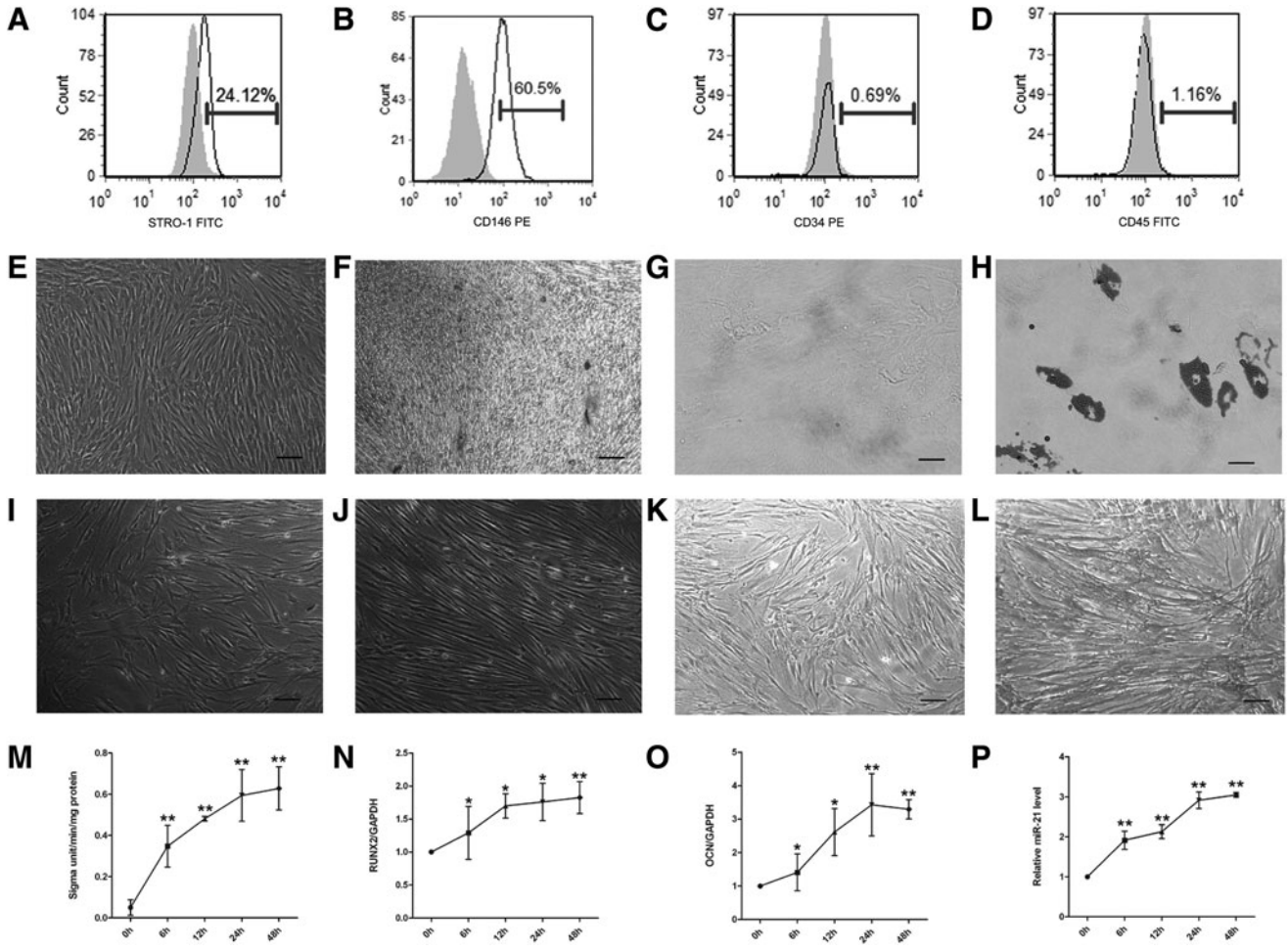

FIG. 1. Morphological changes and osteogenic differentiation of PDLSCs in response to stretch. The stemness properties of the PDLSCs were characterized. PDLSCs were positive for STRO-1 **(A)** and CD146 **(B)**, negative for CD34 **(C)** and CD45 **(D)**. **(E, F)** Alizirin red staining of PDLSCs showed odontogenic differentiation. **(G, H)** Oil red O-positive lipid clusters in PDLSCs indicated their adipogenic differentiation potential. **(I, J)** PDLSCs in the static group were oriented randomly, but PDLSCs in the stretch group were aligned in parallel to the direction of strain, resulting in an organized morphology. **(K, L, M)** Stretching PDLSCs increased ALP activity compared with static cells. **(N, O)** QPCR results showed that expression of the osteogenic genes RUNX2 and OCN increased in stretched PDLSCs. **(P)** QPCR results demonstrated that miR-21 expression increased when PDLSCs were stretched for increasing periods of time. The values in **(M, N, O,** and **P)** represent the mean ± SD for triplicate samples from a representative experiment (**$P < 0.01$, *$P < 0.05$ vs 0 h). GAPDH or U6 snRNA levels were used as internal controls for qPCR. Scale bar: **(E, F, I–L)** 40 μm; **(G, H)** 20 μm. ALP, alkaline phosphatase; PDLSCs, periodontal ligament stem cells.

the Dunnett-t (stretch for 6 h/12 h/24 h/48 h vs. 0 h) or Bonferroni (comparison between any two groups) was used for multiple comparisons. For non-normally distributed data, the Kruskal–Wallis test was performed, and then the Dunnett-t (stretch for 6 h/12 h/24 h/48 h vs. 0 h) or Bonferroni (comparison between any two groups) was used after the data were ranked.

Results

Morphological changes and osteogenic differentiation induced by stretching PDLSCs

First, we characterized the stem cell properties of PDLSCs. The cells were positive for the cell surface markers STRO-1 and CD146 (Fig. 1A, B) and negative for CD34 and CD45 (Fig. 1C, D). Standard culture medium did not induce any mineralized nodule and oil red O-positive lipid cluster formation (Fig. 1E, G). After 3 weeks of culture with osteogenic and adipogenic inductive cocktail, PDLSCs showed osteogenic and adipogenic differentiation according to Alizirin red and Oil red O staining (Fig. 1F, H). The stretched PDLSCs had a markedly altered morphology compared with static PDLSCs. Specifically, static PDLSCs spread out and were oriented randomly (Fig. 1I). However, stretched PDLSCs were elongated and were aligned in parallel to the direction of the applied strain (Fig. 1J). PDLSCs that were stretched for 6, 12, 24, or 48 h showed gradually increasing ALP activity compared with static cells; notably, ALP activity is an early marker of osteogenic differentiation (Fig. 1K–M). Stretching PDLSCs resulted in increased expression of the osteogenic genes RUNX2 and OCN (Fig. 1N, O). To examine the temporal dynamics of miR-21 expression, PDLSCs were exposed to mechanical stretch for various time periods. As detected, miR-21 was significantly increased at 6 h after stretching, and increased time dependently until 48 h (Fig. 1P).

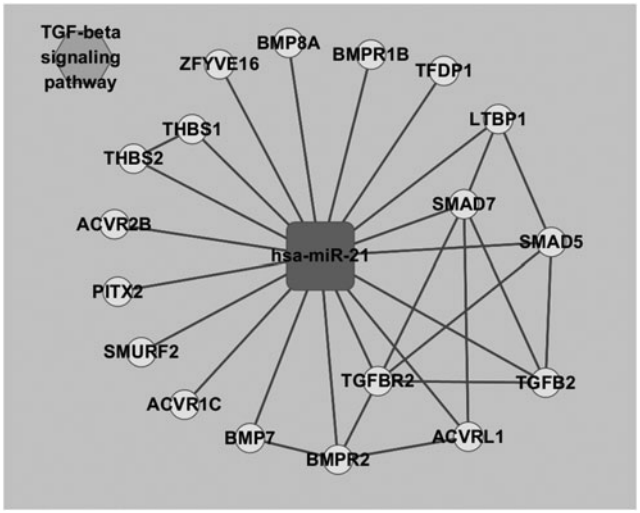

FIG. 2. The miR-21 gene network (TGF-β signaling pathway) was determined based on the KEGG pathway analyses. The constructed subnetwork of miR-21 and its potential target genes shows an enrichment of genes involved in the TGF-β signaling pathway. TGF-β, transforming growth factor-beta.

miR-21 target genes and microRNA-gene networks

The transforming growth factor-beta (TGF-β) signaling pathway contributes a great extent to osteogenic differentiation of MSCs in vitro [9,10]. Based on our KEGG data (Supplementary Tables S1 and S2; Supplementary Data are available online at www.liebertpub.com/scd), the putative target genes of miR21 that were underexpressed or overexpressed in stretched PDLSCs were involved in several signaling pathways associated with osteogenic differentiation. Among these, TGF-β signaling pathway is the most significant pathway. So,

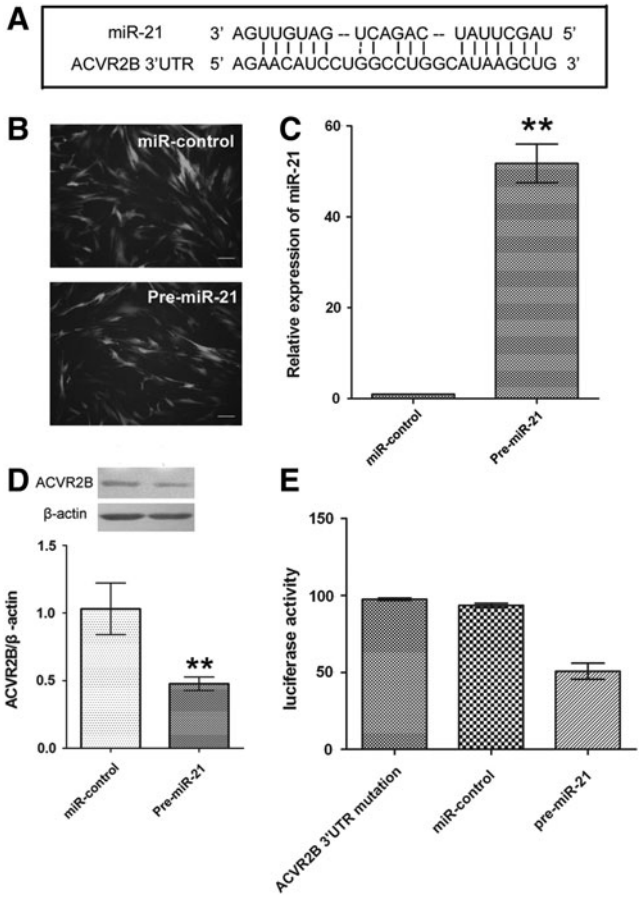

FIG. 3. miR-21 directly targets *ACVR2B* mRNA. **(A)** The 3′-UTR element of *ACVR2B* mRNA is partially complementary to the sequence of miR-21. **(B)** PDLSCs were transfected with pre-miR-21 or miR-control. Photomicrographs showed the ubiquitous expression of green fluorescence protein in cells transfected with either vector. **(C)** Overexpression of miR-21 was confirmed in pre-miR-21-transfected cells using qRT-PCR. U6 was used as the internal control. **(D)** Western blot analysis showed that the level of the ACVR2B protein decreased in the miR-21-transfected PDLSCs compared with miR-control cells. **(E)** Renilla and firefly luciferase activities were measured using the Dual-Luciferase Reporter system (Promega) at 24 h after transfection with pre-miR-21. The firefly luciferase activity was normalized to the Renilla luciferase expression for each sample. The values in **(C, E)** represent the mean ± SD for triplicate samples from a representative experiment (**$P < 0.01$). Scale bar = 40 μm.

we drew the diagram of an miR-21 gene regulatory network based on target genes in TGF-β signaling pathway (Fig. 2).

ACVR2B is a target of miR-21 in PDLSCs

To identify the molecular target of miR-21, we searched for predicted miR-21 targets with a focus on regulators of osteogenic differentiation. Bioinformatic analysis of miR-21 targets was performed based on the public database of human miRNAs miRGen (www.targetscan.org, www.microrna.org, http://pictar.bio.nyu.edu). Two closely related targets, the ACVR2B and the activin receptor type IC (ACVR1C) genes, were identified. We screened these two miR-21 targets and found that ACVR2B was a potential target of miR-21 (Fig. 3A). This was validated by western blot analysis and luciferase assays (Fig. 3B–E).

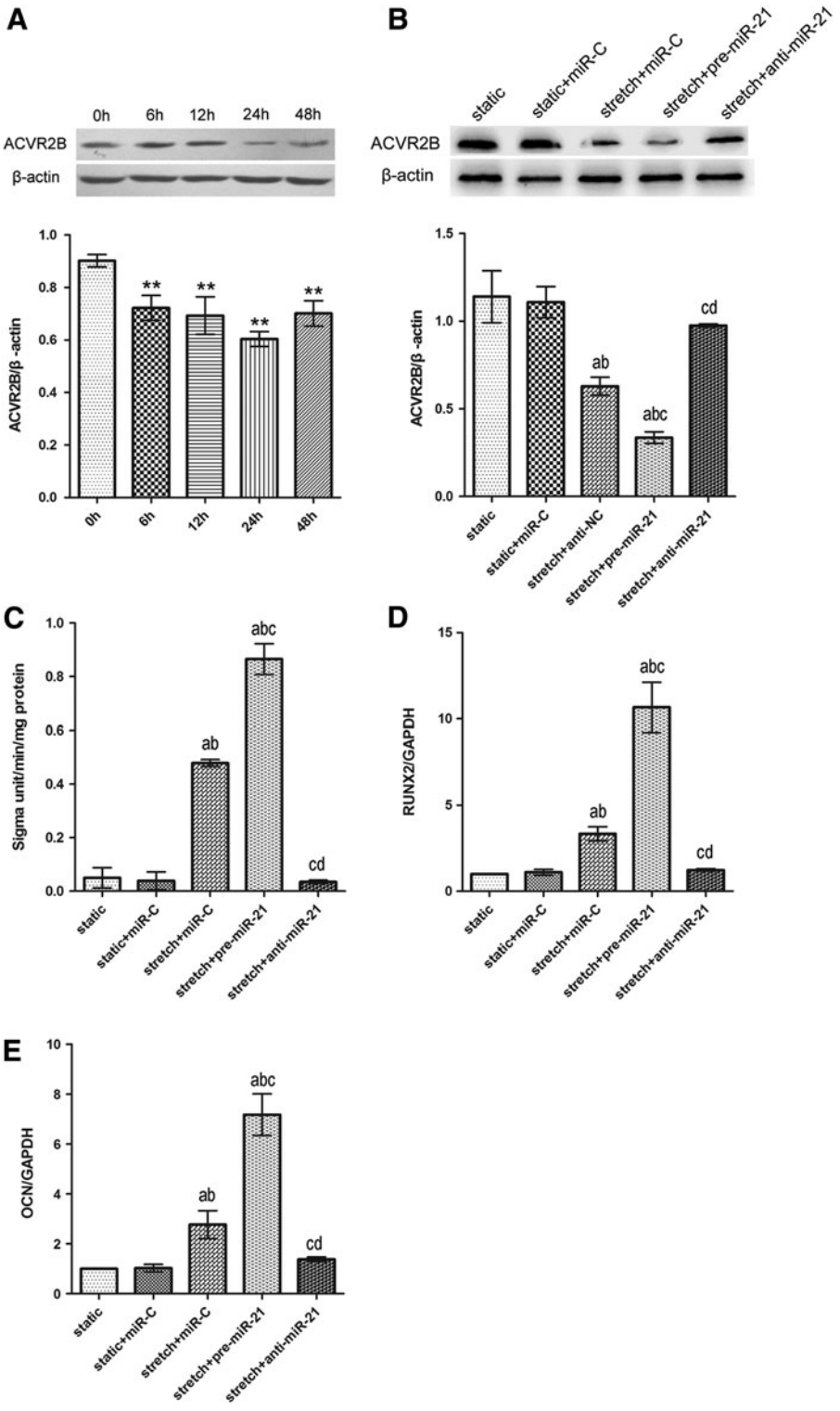

FIG. 4. miR-21 plays a role in the stretch-induced differentiation of PDLSCs by regulating the expression of the ACVR2B protein. **(A)** The effects of stretch on the expression of ACVR2B, a gene related to osteogenic differentiation. Confluent PDLSCs were exposed to 10% stretch for 0, 6, 12, 24, and 48 h. **(B)** Pretreatment with pre-miR-21 increased the inhibitory effect of stretch on ACVR2B expression, while pretreatment with anti-miR-21 blocked the inhibitory effect of stretch on ACVR2B expression. PDLSCs were pretreated with pre-miR-21, anti-miR-21, or miR-control for 72 h followed by stretching for 24 h, and ACVR2B expression was determined by western blotting. **(C)** Pre-miR-21 increased ALP activity, while anti-miR-21 partially attenuated the stretch-induced ALP activity compared with static and miR-control groups. **(D, E)** Pre-miR-21 increased the expression of the RUNX2 and OCN genes, while anti-miR-21 partially attenuated their expression. The values represent the mean ± SD for triplicate samples from a representative experiment (**$P < 0.01$ VS. 0 h. [a]Compared with static group, the difference was significant, $P < 0.05$; [b]Compared with static + miR-C group, the difference was significant, $P < 0.05$; [c]Compared with stretch + miR-C group, the difference was significant, $P < 0.05$; [d]Compared with stretch + pre-miR-21 group, the difference was significant, $P < 0.05$).

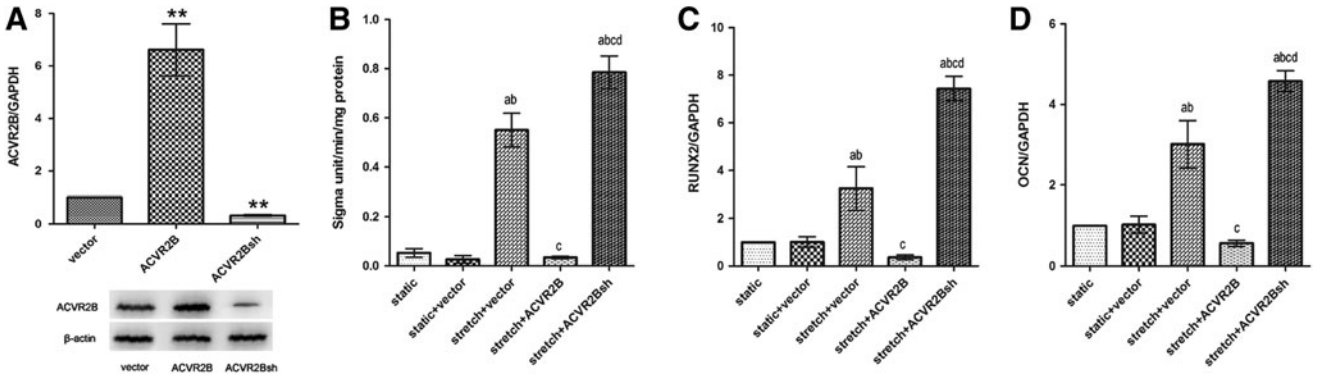

FIG. 5. Effect of ACVR2B on osteogenic differentiation of PDLSCs exposed to mechanical stretch. PDLSCs were transfected with PcDNA3.1-ACVR2B, pcDNA3.1-ACVR2Bsh, or pcDNA3.1 vector for 72 h followed by stretching for 24 h. **(A)** Real-time RT-PCR and western blot results showed that PDLSCs were overexpressed and silenced ACVR2B. **(B)** ALP activity. **(C, D)** Real-time RT-PCR shows expression of RUNX2 **(C)** and OCN **(D)** genes. The values represent the mean ± SD for triplicate samples from a representative experiment (**$P < 0.01$ vs. vector; [a]Compared with static group, the difference was significant, $P < 0.05$; [b]Compared with static + vector group, the difference was significant, $P < 0.05$; [c]Compared with stretch + vector group, the difference was significant, $P < 0.05$; [d]Compared with stretch + ACVR2B group, the difference was significant, $P < 0.05$).

miR-21 induces the stretch-initiated differentiation of PDLSCs by regulating ACVR2B protein expression

To investigate the effects of stretch on ACVR2B expression, we applied a 10% stretch to PDLSCs for 6, 12, 24, or 48 h. Compared with the static control cells (Fig. 4A), the expression level of the ACVR2B protein was decreased in the stretched cells. To examine the function of miR-21 in the stretch-induced osteogenic differentiation of PDLSCs, the PDLSCs were transfected with pre-miR-21 and anti-miR-21 before the stretch was applied. Pretreatment with pre-miR-21 increased the inhibitory effect of stretch on ACVR2B expression, while pretreatment with anti-miR-21 blocked the inhibitory effect of stretch on ACVR2B expression (Fig. 4B). Pre-miR-21 increased stretch-induced osteogenic differentiation as reflected by increased ALP activity and increased expression of the RUNX2 and OCN genes. In contrast, anti-miR-21 partially attenuated these effects (Fig. 4C–E).

Effect of ACVR2B on the osteogenic differentiation of PDLSCs exposed to mechanical stretch

To determine the role of ACVR2B in the differentiation of PDLSCs exposed to mechanical stretch, we overexpressed and suppressed ACVR2B before the stretch was applied. Ectopic ACVR2B expression and the knockdown efficiency were verified by real-time RT-PCR and western blot (Fig. 5A). Next, ALP activity and RUNX2 and OCN gene expression were assessed. We observed that ACVR2B overexpression partially inhibited the stretch-induced ALP activity and RUNX2 and OCN gene expression, while silencing ACVR2B enhanced ALP activity and RUNX2 and OCN gene expression (Fig. 5B, D).

Discussion

This study demonstrated that miR-21 was expressed at significantly higher levels in stretched PDLSCs than in static PDLSCs. It also confirmed the relationship between miR-21 and mechanical force-induced osteogenic differentiation of PDLSCs. We also showed that miR-21 regulated stretch-induced osteogenic differentiation of PDLSCs by targeting ACVR2B.

Many miRNAs play roles in mechanical force-induced variations in cellular function in vitro. For example, there are 34 upregulated miRNAs and 8 downregulated miRNAs that are differentially expressed in cyclically stretched versus nonstretched rat alveolar ECs [11]. Laminar shear stress modulates miRNA expression in ECs, and miR-19a plays an important role in the flow regulation of cyclin D1 expression and endothelial proliferation [12]. Mechanical stretch upregulates the expression of miR-26a, which attenuates the level of the endogenous glycogen synthase kinase-3β (GSK-3β) protein and induces hypertrophy in human airway smooth muscle cells [13]. Recent studies show that miRNAs play important roles in the proliferation and differentiation of osteoclasts, osteoblasts, and other types of cells [14–16]. Another recent study found that miRNA-218 decreased in differentiated PDLSCs and was identified as targeting RUNX2 to control osteogenic maturation [17]. However, the role of miR-21 in the osteogenic differentiation of PDLSCs exposed to mechanical stretch remains to be elucidated.

In the early part of this study, we found that stretch induced both osteogenic differentiation and a time-dependent increase of miR-21 in PDLSCs. However, it remains unclear how miR-21 functions in stretch-induced osteogenic differentiation in the cells. It has been demonstrated that miRNAs modulate gene expression by binding to target mRNAs, thereby inhibiting their translation or promoting their degradation [3]. As reported, many of the established targets of miR-21 are tumor suppressors, including PDCD4 [18], PTEN [19], and tropomyosin 1 [20]. Based on our KEGG data, the putative target genes of miR21 that were underexpressed or overexpressed in stretched PDLSCs were involved in TGF-β signaling pathway. ACVR2B, a

transmembrane serine/threonine receptor kinase, plays a crucial role in the activation of activin, which is a part of the TGF-β pathway and acts on cell growth and differentiation in different biological functions [21]. Next, luciferase reporter assay and western blotting analysis indicated that miR-21 mediated the osteogenic differentiation effect of stretch by directly targeting ACVR2B, which is a key regulator of osteogenic differentiation. In addition, we found that stretch-induced miR-21 expression increase was necessary for suppressing the ACVR2B protein level in stretched PDLSCs. More importantly, gain- and loss of function of ACVR2B further confirmed the logic link between miR-21 and ACVR2B in stretch-induced PDLSC osteogenic differentiation. This finding may explain the previous observation that knockdown of endogenous ACVR2B in bone marrow stromal cells reduces the suppressive effect of bone morphogenetic protein 3 on osteoblast differentiation [22]. These results show that the induction of miR-21 plays an important role in terms of mediating the effects of stretch.

In summary, this study found that stretch mediates miR-21 expression and that miR-21 plays a critical role in mechanical force-induced ACVR2B expression and osteogenic differentiation. However, the post-transcriptional control of osteogenic differentiation of PDLSCs subjected to mechanical force may require multiple miRNAs that act in a coordinated manner, and this merits further study.

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Author Disclosure Statement

The authors declare no competing financial interests.

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