MicroRNA-218, microRNA-191*, microRNA-3070a and microRNA-33 are responsive to mechanical strain exerted on osteoblastic cells

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Abstract. MicroRNA (miRNA) is an important regulator of cell differentiation and function. Mechanical strain is important in the growth and differentiation of osteoblasts. Therefore, mechanresponsive miRNA may be important in the response of osteoblasts to mechanical strain. The purpose of the present study was to select and identify the mechanoresponsive miRNAs of osteoblasts. Mouse osteoblastic MC3T3-E1 cells were cultured in cell culture dishes and stimulated with a mechanical tensile strain of 2,50 $\mu\varepsilon$ at 0.5 Hz, and the activity of alkaline phosphatase (ALP), mRNA levels of ALP, osteocalcin (OCN), and collagen type I (Col I), and protein levels of bone morphogenetic proteins (BMPs) inthe cell culture medium were assayed. Following miRNA microarray and reverse transcription-quantitative polymerase chain reaction analyses, differentially expressed miRNAs in the mechanically strained cells and unstrained cells were selected and identified. Using bioinformatics analysis, the target genes of the miRNAs were then predicted. The results revealed that the mechanical strain of 2,500 $\mu\varepsilon$ increased the activity of ALP, the mRNA levels of ALP, OCN and Col I, and the protein levels of bone morphogenetic protein(BMP)-2 and BMP-4 Continuous mechanical stimulation for 8 h had the most marked stimulant effects. miR-218, miR-191*, miR-3070a and miR-33 were identified as differentially expressed miRNAs in the mechanically strained MC3T3-E1 cells. Certain target genes of these four miRNAs were involved in osteoblastic differentiation. These findings indicated that a mechanical strain of 2,500 $\mu\varepsilon$, particularly for a period of 8 h, promoted osteoblastic differentiation, and the four mechanoresponsive miRNAs identified may be a potential regulator of osteoblastic differentiation and their response to mechanical strain.

Introduction

Mechanical strain is pivotal in bone remodeling as physiological dynamic loading promotes bone formation, whereas the absence of mechanical forces results in bone loss (1,2). In bone tissue, osteoblasts are important mechanical receptors, which can transform mechanical stimuli into biochemical signals for bone matrix formation and mineralization (3). Previous studies have demonstrated that mechanical forces are crucial regulators of osteoblastic proliferation, differentiation and apoptosis (4,5). However, the mechanism underlying the response of osteoblasts to mechanical strain remains to be fully elucidated, particularly the role of microRNAs (miRNAs; miRs) in the mecchano-response.

miRNAs are a class of small non-coding RNAs, typically 18-22 nucleotides in length, which repress gene expression at the post-transcriptional level by degrading their target mRNAs or through translational repression (6,7). miRNAs regulate cell proliferation, differentiation and apoptosis, and control physiological changes, including growth and development (6-8). Several miRNAs, which regulate bone formation or osteoblastic differentiation, have been found (9,10).

In previous years, certain mechanoresponsive or mechanosensitive miRNAs have been detected and identified in endothelial cells, chondrocytes and smooth muscle cells (11-13). For example, in mechanical strained chondrocytes, miR-365 is expressed at higher levels compared with unstrained cells, and regulates chondrocyte differentiation (12). Osteoblasts are a type of mechanoresponsive cell, therefore, the present study hypothesized that they contain mechanoresponsive miRNAs.

In the present study, mouse pre-osteoblastic MC3T3-E1 cells were stimulated with mechanical tensile strain, which was performed to stimulate osteoblastic differentiation. Subsequently, miRNA microarray and reverse

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transcription-quantitative polymerase chain reaction (RT-qPCR) analyses were performed to identify the presence of mechanoresponsive miRNAs.

Materials and methods

Application of mechanical strain to cultured cells. The MC3T3-E1 cells (provided by the Institute of Basic Medicine of Peking Union Medical College, Beijing, China), a mouse pre-osteoblastic cell line, at the third passage, were seeded into mechanical loading dishes, which were reformed from cell culture dishes (Nunc International, Roskilde, Denmark) in α-minimal essential medium (Invitrogen Life Technologies, Carslbad, CA, USA), supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Invitrogen Life Technologies).

At confluence, the medium was replaced with FBS-free medium, and the MC3T3-E1 cells were subjected to mechanical tensile strain of 2,500 $\mu\varepsilon$ at 0.5 Hz for different durations (0, 2, 4, 8, 12 and 24 h). The mechanical strain was generated by a specially designed four-point bending device (Institute of Medical Equipment, Academy of Military Medical Sciences, Tianjin, China), as previously described (14).

ALP activity assay. Following the induction of mechanical strain, the MC3T3-E1 cells were lysed by brief sonication on ice in radioimmunoprecipitation lysis buffer (Cw Biotech, Beijing, China), and the protein concentration of the cell lysates were measured using the Bichinchoninic Acid Protein Assay kit (Cw Biotech). The activity of ALP in the lysates was measured using a fluorometric detection kit (Nanjing Jiancheng Biotechnology Co., Ltd., Nanjing, China) using a p-nitrophenyl phosphate (Sigma-Aldrich, St. Louis, MO, USA) method, according to manufacturer's instructions. A single unit of ALP activity represented 1 μmol p-nitrophenyl phosphate hydrolyzed to p-nitrophenol/min, therefore the ALP activity in the proteins was expressed in U/g protein.

RT-qPCR. Total RNA was extracted from the cells using TRIzol reagent (Invitrogen Life Technologies), following which cDNA was synthesized using a Quant Script RT kit (Tiangen Biotechnology Co., Ltd., Beijing, China). qPCR was performed to detect the mRNA levels of ALP, OCN, Col I and glyceraldehyde3-phosphate dehydrogenase (GAPDH), as an internal reference, using SYBR Green I PCR mix (Cowin Biotech Co, Ltd., Beijing, China) on a Real-Time PCR system (7900; Applied Biosystems Life Technologies, Foster City, CA, USA), according to the manufacturer's instructions. The sequences of the primers are listed in Table I. The amplification reaction included a denaturation step at 94°C for 180 sec, followed by 40 cycles of 94°C for 15 sec, and annealing and extension at each annealing temperature for 30 sec at 60°C. The relative quantitative $2^{-\Delta\Delta Ct}$ method (15) was used to determine the mRNA levels of the PCR products relative to the control group.

Enzyme-linked immunosorbent assay (ELISA) of the protein levels of bone morphogenetic proteins (BMPs). Following mechanical strain, the cell culture medium was collected, and the protein levels of BMP-2 and BMP-4 in the culture
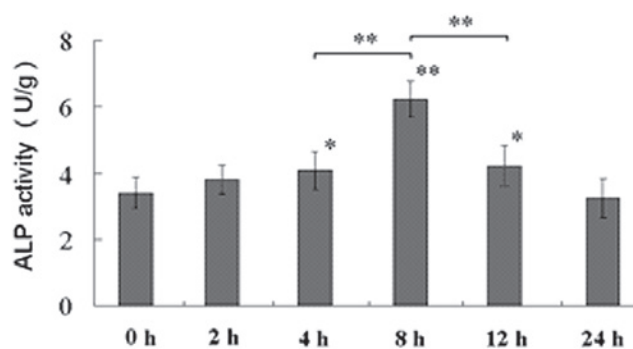

Figure 1. MC3T3-E1 cells were stimulated with a mechanical strain of 2,500 $\mu\varepsilon$ at 0.5 Hz for the indicated periods of time (0-24 h), following which the activity of ALP in the cells were assayed. The mechanical strain increased the activity of ALP in the cells, and mechanical strain for a duration of 8 h had the most marked effect on the activity of ALP. n=6, *P<0.05 and **P<0.01, compared with the control group (0 h), or between the indicated groups. ALP, alkaline phosphatase.

medium were detected using an ELISA kit (Wuhan Boster Bioengineering Co., Ltd., Wuhan China), according to the manufacturer's instructions. The absorbance was measured at 450 nm on a Multiskan FC ELISA reader (Thermo Fisher Scientific, Rockford, IL, USA), with the results presented as the percentage of activity change, compared with the unstrained control.

Microarray and RT-qPCR validation of miRNA. The Agilent Mouse miRNA microarray (Agilent Technologies, Santa Clara, CA, USA) was used to detect the miRNA expression levels in the MC3T3-E1 cells. The miRNA expression profiles of the mechanically strained cells were compared with the unstrained cells.

In brief, the total RNA extraction and miRNA enrichment procedures were performed using an mirVana™ miRNA Isolation kit (Ambion Life Technologies, Carlsbad, CA, USA), according to the manufacturer's instructions. Target labeling, hybridization, imaging and data processing were performed, according to the manufacturer's instructions, at CapitalBio Corporation (Beijing, China) using Agilent Mouse miRNA (Agilent Technologies), 8x60 K and Sanger miRBase V18.0 software (http://www.mirbase.org/). Data were acquired using Agilent Feature Extraction software version 10.7 (Agilent Technologies). Further data analyses were performed using GeneSpring GX 10.0 software (Agilent Technologies).

The expression levels of miRNA were confirmed using RT-qPCR at CapitalBio Corporation. The primers for RT-qPCR were synthesized by Invitrogen Life Technologies, and the sequences are shown in Table II. Following cDNA synthesis using Megaplex™ RNA RT mix, qPCR was performed using Power SYBR & Green PCR Master mix (ABI 4367659; Thermo Fisher Scientific). The reactions were incubated in a 96-well optical plate at 95°C for 10 min, followed by 40 cycles of 15 sec at 95°C, 1 min at 60°C (annealing and extension). Expression analysis was performed in triplicate for each sample. *Mus musculus* (mmu)-Actin was used as the normalization control. The miRNA expression levels were quantified using an ABI Prism 7300 Sequence Detection system (Applied Biosystems Life Technologies).

Table I. Primers used for qPCR analysis of mRNA.

Gene	Primer sequence (5'-3')	Product size (bp)	Annealing temperature (°C)
ALP	F: CGGGACTGGTACTCGGATAA	156	58
	R: ATTCCACGTCGGTTCTGTTC		
OCN	F: AGTCTGACAAAGCCTTCA	134	56
	R: AAGCAGGGGTTAAGCTCACA		
Col I	F: GGTATGCTTGATCTGTATCTG	130	58
	R: TCTTCTGAGTTTGGTGATACG		
GAPDH	F: ACCCATCACCATCTTCCAGGAG		
	R: GAAGGGGCGGAGATGATGAC	159	58

F, forward; R, reverse; ALP, alkaline phosphatase; OCN, osteonectin; Col I, collagen type I; GADPH, glyceraldehyde3-phosphatedehydrogenase.

Table II. Forward and reverse primers used for reverse transcription-quantitative polymerase chain reaction of microRNA.

MicroRNA	Oligonucleotide sequence (5'-3')
mmu-let-7e*	F: ATCCTATACGGCCTCCTAGCTT
	R: GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGGAAAG
mmu-miR-191	F: CAACGGAATCCCAAAAGCAG
	R: GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCAGCTG
mmu-miR-32	F: TGCCGTATTGCACATTACTAAGTT
	R: GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTGCAAC
mmu-miR-218	F: AGCCTTGTGCTTGATCTAACCA
	R: GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACACATGG
mmu-miR-210	F: CTGTGCGTGTGACAGCGG
	R: GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTCAGCC
mmu-miR-33	F: TGCGTGCATTGTAGTTGCATT
	R: GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTGCAAT
mmu-miR-3070a	F: TCGTAGTGCTACCGTCAGGGG
	R: GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTCTACC
UPL_U6	F: TTCCTCCGCAAGGATGACACGC
	R: GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGAGCCAACAAAAATAT
Universal reverse primer	GTGCAGGGTCCGAGGT

F, forward; R, reverse; miR, microRNA; mmu, *Mus musculus*.

Prediction of miRNA target genes. The TargetScan (http://wwwargetscan.org) and PicTar (http://www.pictar.org) software programs were used to predict the miRNA target genes. The target genes associated with osteoblastic differentiation or the response of the cell to mechanical strain, were selected.

Statistical analysis. To determine miRNAs, which were differentially expressed among the groups, Student's t-test was performed using SPSS version 12.0 (SPSS, Inc., Chicago, IL, USA). The experiments were repeated in triplicate. Statistical significance between the groups was measured using Student's t-test; $P<0.05$ was considered to indicate a statistically significant difference.

Results

Mechanical strain promotes osteoblastic differentiation. Following exposure of the MC3T3-E1 osteoblastic cells to a mechanical tensile strain of 2,500 $\mu\varepsilon$ at 0.5 Hz, the activity and mRNA level of ALP were elevated (Figs. 1 and 2), the mRNA expression levels of Col I and OCN were also increased (Fig. 2). In addition, the ELISA indicated that the mechanical strain increased the levels of BMP-2 and BMP-4 in the culture medium (Fig. 3). ALP, Col I, OCN, and BMP-2/4 are all markers of osteoblastic differentiation (16-18), therefore, the mechanical strain promoted osteoblastic differentiation of the MC3T3-E1 cells. These results demonstrated that mechanical strain for a duration
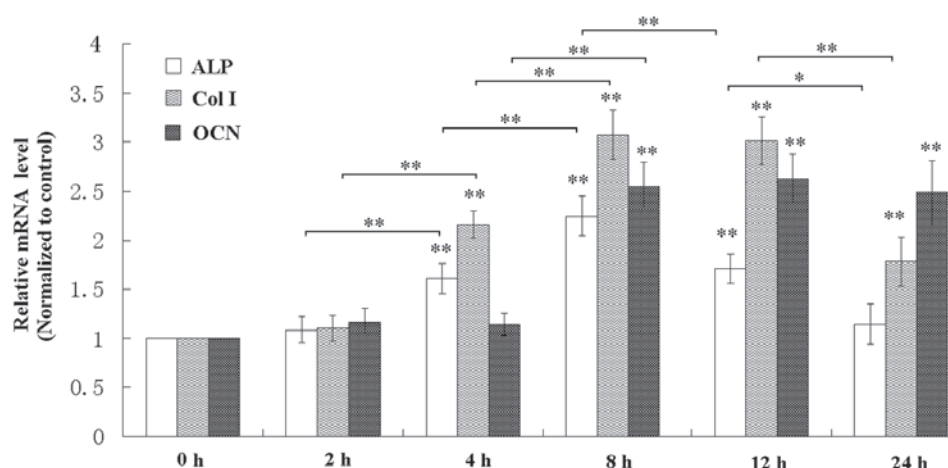
Figure 2. Following mechanical strain, the mRNA levels of ALP, OCN and Col I were detected using reverse transcription-polymerase chain reaction analysis. Mechanical strain for 8 h had the most marked effect on enhancement of the mRNA levels of ALP and OCN. n=6 *P<0.05 and **P<0.01, compared with the control group (0 h), or between the indicated groups. ALP, alkaline phosphatase; OCN, osteonectin; Col I, collagen type I.
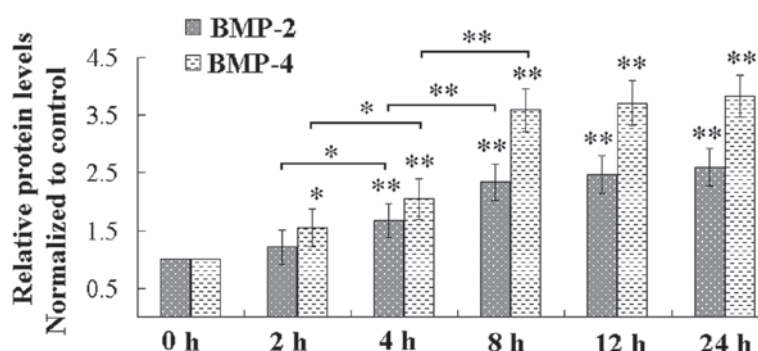

Figure 3. Following mechanical strain, the protein levels of BMP-2 and BMP-4 in the cell culture medium were detected using an enzyme-linked immunosorbent assay. The mechanical strain significantly increased the levels of BMP in the cell culture medium until 12 h, and mechanical strain for 8 h had the most marked effect on the increase of BMP.. n=7, *P<0.05 and **P<0.01, compared with the control (0 h) or between the indicated groups. BMP, bone morphogenetic protein.

of 8 h had the most marked effect on the enhancement of osteoblastic differentiation (Figs. 1-3).

Identification of four miRNAs responsive to mechanical strain applied to MC3T3-E1 cells. The microarray images are shown in Fig. 4. The results of the miRNA microarray indicated that the expression levels of five miRNAs (mmu-miR-191*, mmu-miR-3070a, mmu-miR-M1-2-3p, mmu-miR-let-7e*, mmu-miR-3470a and mmu-miR-) were higher in the mechanically strained group, compared with the unstrained control group, and the expression levels of four miRNAs (mmu-miR-32, mmu-miR-33, mmu-miR-5110 and mmu-miR-5121) were lower in the mechanically strained group, compared with the unstrained control group (Fig. 5A). The results of the RT-qPCR confirmed that the expression levels of miR-218, miR-191*, miR-3070a and miR-33 differed between the strained group and the unstrained group (Fig. 5B). Therefore, these four miRNAs were considered to be responsive to the mechanical strain, which was applied to the MC3T3-E1 cells.

Target genes of miR-218, miR-191, miR-3070a and miR-33 may be involved in osteoblast differentiation.* Using the

TargetScan and PicTar databases, the predicted target genes of the differentially expressed, mechanoresponsive, miRNAs, were determined. The target genes, which were identified aso being involved in osteoblast differentiation are shown in Fig. 6.

Discussion

Bones and the skeleton are responsive to dynamic mechanical loading, in which a suitable dynamic mechanical loading promotes bone formation and removal of mechanical loading reduces bone mass (19-21). The ability of bone tissues to respond to mechanical strain depends on the bone cells (22). Osteoblasts are located on the surface of bone and are bone-forming cells, which can be stimulated by dynamic mechanical strain *in vivo*.

Several studies have indicated that osteoblasts are responsive to mechanical strain (4,5,23,24). In the present study, the activity of ALP and the mRNA levels of ALP, Col I, OCN and BMP-2/4 in the cell culture were all increased, which confirmed that osteoblasts are also sensitive to mechanical strain *in vitro*. Additionally, the results of the present study indicated that mechanical strain for a duration of 8 h had the most marked effect on the enhancement of osteoblastic
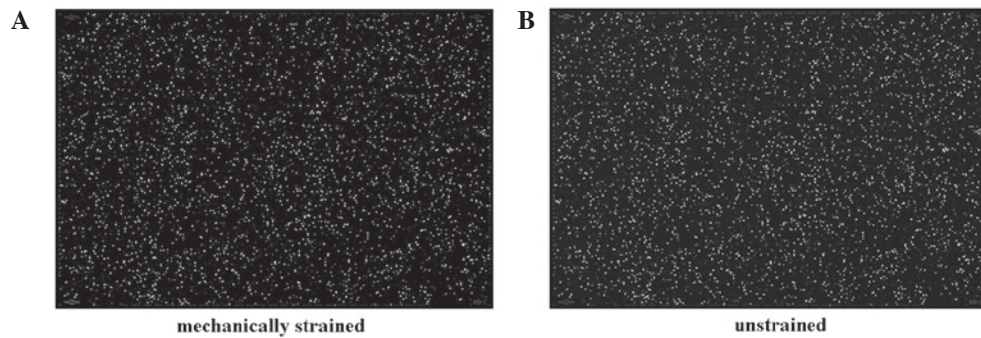
Figure 4. Different miRNAs in the MC3T3-E1 (A) mechanically strained cells (2,500 $\mu\varepsilon$ at 0.5 Hz for 8 h) and (B) unstrained cells, as detected by microarray. The microarray images exhibit the expression levels of every miRNA by luminescence. miRNA, microRNA.
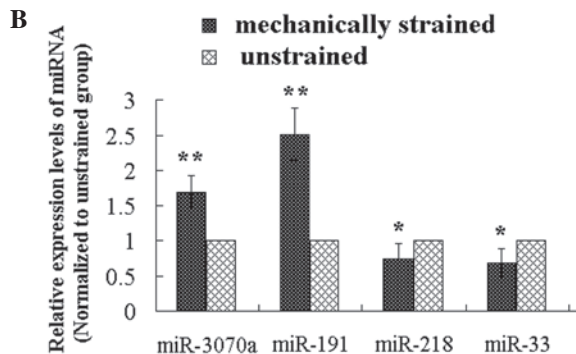

Figure 5. Differentially expressed miRNAs. (A) A total of nine differentially expressed miRNAs were selected using miRNA microarrays and (B) four differentially expressed miRNAs were identified using reverse transcription-quantitative polymerase chain reaction. n=6, $^*P<0.05$ and $^{**}P<0.01$, compared with the unstrained group. miRNA/miR, microRNA.

Figure 6. Putative target genes of differentially expressed miRNAs, involved in osteoblastic differentiation, with references. miRNA/miR, microRNA.

differentiation and was, therefore, selected for the following experiments.

Differentially expressed miRNAs in mechanical stimulated tissues or cells are regarded as mechanoresponsive, or mechanosensitive, miRNAs. In endothelial cells, chondrocytes and smooth muscle cells, the mechanoresponsive miRNAs have been identified, and are reported to be involved in cell differentiation (11-13). However, the mechanoresponsive miRNAs of osteoblasts remain to be fully elucidated.

In the present study, miRNA microarray and RT-qPCR analyses were performed, and the four mechanoresponsive miRNAs, miR-218, miR-191*, miR-3070a and miR-33 were identified. Using bioinformatics analysis, the target genes of the miRNAs were predicted and, of all the putative target genes, 19 genes were involved in osteoblast differentiation.

As mechanical strain was observed to promote osteoblastic differentiation, these mechanoresponsive miRNAs may be regulators of osteoblastic differentiation. These target genes require further verification, and the mechanism underlying the involvement of mechanoresponsive miRNAs in the mechanical response of osteoblasts requires further investigation.

In conclusion, the present study demonstrated that a mechanical strain of 2,500 $\mu\varepsilon$, particularly for a period of 8 h, promoted osteoblastic differentiation, and four miRNAs were identified as mechanoresponsive, which are potential regulators of osteoblastic differentiation and the response of osteoblasts to mechanical strain.

Acknowledgements

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