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MiR-494-3p induced by compressive force inhibits cell proliferation in MC3T3-E1 cells

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Mechanical stimuli regulate fundamental cell processes such as proliferation, differentiation, and morphogenesis. We attempted to identify microRNA (miRNA) whose expression is changed during compressive treatment in MC3T3-E1, a pre-osteoblastic cell line. Microarray analysis followed by reverse transcription-quantitative polymerase chain reaction revealed that compressive force at 294 Pa for 24 h in MC3T3-E1 cells increased levels of miR-494-3p, miR-146a-5p, miR-210-3p, and miR-1247-3p. Among these miRNAs, miR-494-3p was found to inhibit cell proliferation in MC3T3-E1 cells. Furthermore, cells subjected to compressive force showed slower cell growth compared with control cells. Levels of mRNA for fibroblast growth factor receptor 2 (FGFR2) and Rho-associated coiled-coil kinase 1 (ROCK1), which were predicted to be targets of miR-494-3p, were decreased by compressive force or overexpression of miR-494-3p mimics in MC3T3-E1 cells. Furthermore, binding sites of miR-494-3p within 3′-untranslated regions of *Fgfr2* and *Rock1* were determined using luciferase reporter assay. In conclusion, compressive force affected expressions of several miRNAs including miR-494-3p in MC3T3-E1 cells. Compressive force might inhibit cell proliferation in osteoblasts by up-regulating miR-494-3p followed by *FGFR2* and *ROCK1* gene repressions.

[**Key words:** Mechanical stimuli; Compressive force; MicroRNA; Osteoblasts; Cell proliferation]

Various tissues are exposed to mechanical stresses such as compressive, tensile, and shear stresses from their living environment and daily activities. Cells can perceive changes in their mechanical environments and promote alterations and adaptation in the tissue structure and function. Mechanical stimuli have been reported to regulate fundamental cell processes such as proliferation, differentiation, and morphogenesis (1).

Mechanical stimuli to bone play a critical role in maintaining bone mass and strength. For example, long-term bedridden and microgravity environment induce a disuse syndrome with decrease of bone mass (2,3) and training and vigorous activities enhance bone mass (4,5). To date, a large number of studies that investigate effects of mechanical stress on bone formation and function have been reported. In bone, mechanical stimuli are transmitted through extracellular matrix to osteoblasts, osteocytes, periosteal cells, and osteoclasts (6,7). Osteoblasts play an important role to transmit mechanical stimuli into biochemical signals and secrete bone matrix to promote the mineralization (7–11). However, whether mechanical stimuli affect other functions such as cell proliferation in osteoblasts and how cells convert the mechanical signal into biological signaling pathways, remain to be fully elucidated.

MicroRNAs (miRNAs) that are small non-coding RNA molecules with 18–25 nucleotides in length regulate gene expression by binding the 3′-untranslated regions (UTR) of their target mRNAs. MiRNAs are involved in many fundamental cell processes such as proliferation, differentiation, development, survival, and death (12–14). In osteoblasts, miRNAs have emerged as prominent factors to regulate the differentiation and bone formation (15). Therefore, it is conceivable that cell processes regulated mechanical stress in osteoblasts is mediated by miRNAs.

To clarify involvement of miRNAs in responses to mechanical stress on osteoblasts, we attempted to identify miRNAs regulated by continuous compressive force in mouse osteoblast cell line, MC3T3-E1. Among the candidate miRNAs up-regulated by compressive force, we focused miR-494-3p which have been reported to affect the cell proliferation in various cell-types (16,17), and examined the effects of miR-494-3p overexpression on cell proliferation and its target genes in MC3T3-E1.

MATERIALS AND METHODS

Application of compressive force MC3T3-E1 cells were seeded at a density of 4.0×10^5 cells per well in a 6-well plate with MEMα (Wako, Osaka, Japan) supplemented with 10% fetal bovine serum (Vita, Tokyo, Japan) and incubated to confluence. Cells were compressed for 24 h using a uniform compression method

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as previously described (18,19). Briefly, confluent cells were subjected to a static compressive force by placing a glass cylinder with lead weights onto the cells of a 6-well plate. The intensity of the force was controlled at 294 Pa by numbers of lead granules placed in the cylinder, because force of compression *in vivo* is assumed between 2.0 g/cm^2 (196 Pa) to 4.0 g/cm^2 (392 Pa) (10,11). A cell area in contact with the cylinder was marked beforehand on the bottom of the wells. Then, cells found exclusively in the area were used for the experiments as cells subjected to compressive force.

Microarray analysis Total RNA including miRNA from cells with or without compressive treatment was extracted by the ISOGEN II (Nippon Gene, Tokyo, Japan) by holding the hollow cylinder to the marked area as previously described (19). Each 100 μg of total RNA was subjected to miRNA microarray analysis using Agilent Technologies oligonucleotide microarray (Agilent Technologies, Santa Clara, CA, USA). Small RNA fractions were labeled with Cyanine3-pCp and loaded on miRNA array. After hybridization, signals were detected by Agilent Microarray Scanner (Agilent Technologies) and quantified by the software Feature Extraction (Agilent Technologies). The data were normalized to the average counts for all control spikes in each sample and analyzed by Gene Spring GX (Agilent Technologies).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) For measurement of miRNA levels, cDNA synthesis and RT-qPCR were performed by miScript PCR system (Qiagen, Hilden, Germany) according to manufacturer's protocol with specific forward primers (Table S1) and the universal reverse primer attached to the kit on a 7300 real-time PCR system (Applied Biosystems, Foster City, CA, USA). For measurement of mRNA levels, cDNA was synthesized using PrimeScript RT reagent Kit (Takara, Shiga, Japan) and RT-qPCR was performed using THUNDERBIRD SYBR qPCR Mix (Toyobo, Tokyo, Japan) with each specific primer set (Table S2). Each expression level of miRNA and mRNA was normalized to that of snoRNA420 and TATA-binding protein (*Tbp*) mRNA, respectively.

Cell proliferation assay At 24 h after MC3T3-E1 cells were seeded at 5.0×10^3 cells per well in a 24-well plate for 24 h, the cells were transfected with 50 nM miR-494 mimics (*mir*Vana; Life Technologies, Carlsbad, CA, USA), negative control oligonucleotide (Life Technologies), 30 nM *Fgfr2* and/or *Rock1* small interfering RNA (siRNA) (Sigma–Aldrich, St. Louis, MO, USA), or negative control siRNA (Sigma–Aldrich) by HiPerFect (Qiagen). At 0, 24, 48, 72, and 120 h after transfection, cell proliferation assay was carried out using a Cell Counting Kit-8 (Dojindo Labs, Kumamoto, Japan) according to the manufacturer's protocol. To examine the effect of compressive force on cell proliferation, MC3T3-E1 cells treated with or without compressive force for 24 h were collected by trypsinization and re-seeded at 5.0×10^3 cells per well in a 24-well plate. After 3, 24, 48, 72 and 96 h, the cell proliferation assay was performed in the same way.

Prediction of miRNA targets Target genes for miRNA were predicted using three web-based programs: Target Scan (www.targetscan.org), micro-RNA.org (www.microrna.org), and miRDB (www.mirdb.org).

Western blot analysis Cells subjected to compressive force or transfected with miR-494 mimics for 24 h were washed with phosphate-buffered saline twice and then lysed with lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 0.5% NP-40). The equal amounts of protein were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto Immobilon Transfer Membranes (Millipore, Bedford, MA, USA). Membranes were blocked with blocking reagent (Blocking One; Nacalai Tesque, Kyoto, Japan) and then incubated with Can Get Signal solution A (Toyobo) including antibody against fibroblast growth factor receptor 2 (FGFR2; Santa Cruz Biotechnology, Santa Cruz, CA, USA), Rho-associated coiled-coil kinase 1 (ROCK1; Santa Cruz Biotechnology), or β-actin (Sigma–Aldrich), followed by horseradish peroxidase-conjugated mouse or rabbit secondary antibody (GE Healthcare, Buckinghamshire, UK) in Can Get Signal solution B (Toyobo). Antigens were then visualized by enhanced chemiluminescence (Immobilon Western; Millipore). Optical density of protein band was calculated by Image J software (National Institute of Health, Bethesda, MD, USA).

Luciferase reporter assay To generate constructs of luciferase reporter plasmids, a 691 bp and a 1647 bp of 3′-UTR including putative miRNA binding sites in the *Fgfr2* and *Rock1* genes were amplified from cDNA prepared from MC3T3-E1 cells, respectively. Each PCR product was inserted at the 3'-end of the luciferase open reading frame in the pmirGLO Dual-Luciferase miRNA Target Expression Vector (Promega, Madison, WI, USA). Construction of luciferase reporter plasmids with lacking each putative miRNA binding site in the *Fgfr2* and *Rock1* genes was carried out by standard PCR-based site-directed mutagenesis. These reporter plasmids were co-transfected with 50 nM miR-494-3p mimics by Effectene Transfection Reagent (Qiagen) in HEK293 cells. After co-transfection for 24 h, the firefly and *Renilla* luciferase activities were measured using Dual Luciferase Assay Kit (Promega) on TriStar LB 941 Multi-label plate reader (Berthhold Technologies, Bad Wildbad, Germany). The firefly luciferase activity was normalized by *Renilla* luciferase activity.

Statistical analysis Data were expressed as the mean ± standard deviation (SD). Statistical analyses were performed using Student's *t*-test. Differences were considered to be significant when the *P*-value was less than 0.05.

RESULTS

MiRNA expression profile in MC3T3-E1 cells subjected to compressive force Effects of 294 Pa of compressive force for 24 h on miRNA expression in MC3T3-E1 cells were investigated. This condition was confirmed to not affect cell viability in the cells (data not shown). Microarray analysis showed 112 miRNAs (55 up-regulated and 57 down-regulated) with more than 1.5-fold expression changes by compressive treatment. Among them, 16 up-regulated miRNAs and 39 down-regulated miRNAs were conserved between human and mouse (Table 1).

Next, we performed RT-qPCR analysis to validate the microarray data. Among up-regulated miRNAs in microarray data, levels of miR-494-3p, miR146a-5p, miR-210-3p, and miR1247-3p were increased in the cells subjected to compressive force (Fig. 1). We focused on miR-494-3p with high basal expression levels and a high-fold increase (approximately 6.0-fold) by compressive force for further study.

Effects of miR-494-3p overexpression and compressive force on cell proliferation First, we attempted to investigate the effect of miR-494-3p overexpression on cell proliferation in MC3T3-E1 cells. MiR-494-3p mimics significantly inhibited cell proliferation (Fig. 2A). Next, we performed the cell proliferation assay in MC3T3-E1 cells that were trypsinized after 24 h compressive treatment and re-cultured for 24–120 h in non-

TABLE 1. Representative miRNAs shown to be up- and down-regulated by compressive force in microarray analysis.

Up-regulation		Down-regulation	
miRNA	Ratio	miRNA	Ratio
miR-1247-3p	88.2	miR-126-3p	54.0
miR-138-1-3p	52.3	miR-675-5p	29.0
miR-146a-5p	50.0	miR-192-5p	29.0
miR-210-5p	36.0	miR-378a-5p	25.0
miR-222-5p	20.0	miR-100-3p	23.0
miR-1199-5p	3.0	miR-24-1-5p	22.7
miR-210-3p	2.5	miR-466d-3p	16.6
miR-188-5p	2.5	miR-191-3p	15.1
miR-134-5p	2.1	miR-708-3p	14.0
miR-494-3p	1.8	let-7d-3p	12.9
miR-452-5p	1.8	miR-151-3p	11.7
miR-450a-2-3p	1.7	miR-30b-3p	11.0
mmiR-574-5p	1.7	miR-744-3p	11.0
miR-21a-3p	1.6	miR-335-3p	3.2
miR-432	1.6	miR-335-5p	3.0
miR-762	1.5	let-7c-1-3p	2.5
		miR-542-5p	2.1
		miR-101c	2.0
		miR-503-5p	1.9
		miR-186-5p	1.9
		miR-466c-5p	1.9
		miR-542-3p	1.8
		miR-99a-5p	1.7
		miR-7a-5p	1.7
		miR-100-5p	1.7
		miR-148b-3p	1.7
		miR-30c-2-3p	1.7
		miR-28a-5p	1.6
		miR-148a-3p	1.6
		miR-195a-5p	1.6
		miR-708-5p	1.6
		miR-450a-5p	1.6
		miR-28c	1.6
		miR-199b-5p	1.6
		miR-218-5p	1.6
		miR-125b-5p	1.6
		mmiR-497-5p	1.5
		miR-199a-3p	1.5

Up-regulated ratio represents the value of compressive force to control. Down-regulated ratio represents the value of control to compressive force.

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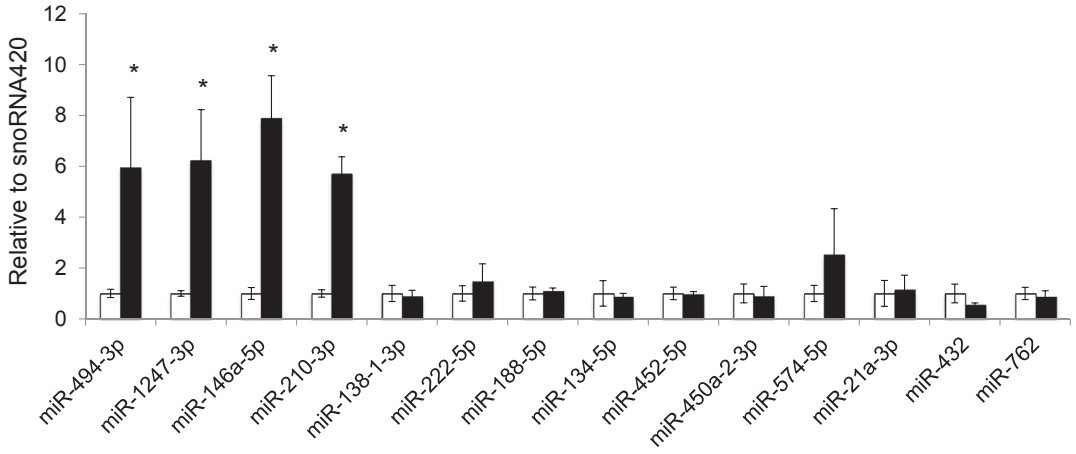

FIG. 1. Effect of compressive force on miRNA expression in MC3T3-E1 cells subjected to compressive force. Levels of miRNAs shown to be up-regulated by compressive force in microarray analysis were measured by RT-qPCR in MC3T3-E1 treated with (closed columns) or without (open columns) compressive force. The miRNA levels normalized by snoRNA420 are shown relative to those of the control cells. The graph is representative of 3 independent experiments. Data are the mean ± SD (n = 5). *$p < 0.05$.

compressive force-conditions. The cells treated beforehand with compressive force showed inhibition of cell proliferation and higher miR-494-3p levels compared with the cells without compressive force (Fig. 2B and C), suggesting that compressive force inhibits cell proliferation in osteoblasts and the effects are retained even after trypsinization and re-seeding of cells.

Identification of target genes of miR-494-3p in MC3T3-E1 cells From databases of Target Scan, miRDB, and microRNA.org, 568, 859, and 1067 human genes and 363, 506, and 672 mouse genes were obtained as targets of miR-494-3p, respectively. Among these candidate genes, we focused on *FGFR2* and *ROCK1* with higher scores in all algorithms, because these genes are known to be involved in cell growth (20,21). We examined *Fgfr2* and *Rock1* expression in MC3T3-E1 cells subjected to compressive force or transfected with miR-494-3p mimics. As expected, mRNA levels of *Fgfr2* and *Rock1* were decreased by both of compressive force and miR-494-3p overexpression (Fig. 3A and B). Western blots analysis revealed that protein levels of FGFR2 were decreased in MC3T3-E1 cells subjected to compressive force or transfected with miR-494-3p compared with the controls (Fig. 3C). On the other hand, ROCK1 protein levels did not change in both conditions (Fig. 3D). Next, we investigated the effect on cell proliferation in MC3T3-E1 cells transfected with siRNAs against *Fgfr2* and *Rock1*. Knockdown of either *Fgfr2* or *Rock1* inhibited cell proliferation and combined knockdown of these genes showed the synergistic effect (Fig. 3E).

Identification of miR-494-3p binding sites in the Fgfr2 and Rock1 genes Next, we performed luciferase reporter assay to identify miR-494-3p binding sites in these genes. Two putative binding sites for miR-494-3p were located in each 3′-UTR

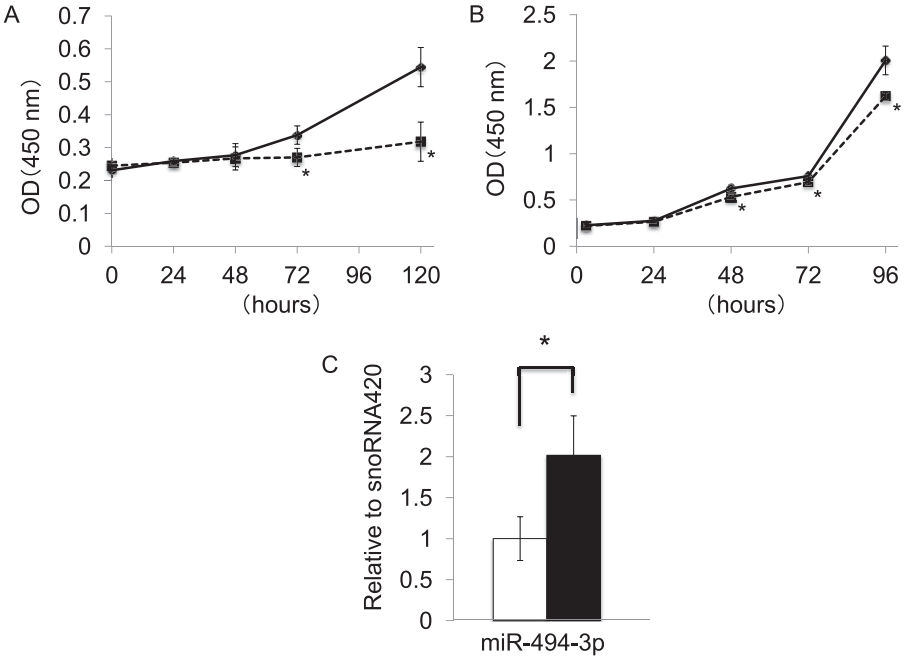

FIG. 2. Effect of miR-494-3p overexpression or compressive force on cell proliferation in MC3T3-E1 cells. (A) Cell proliferation were measured in MC3T3-E1 cells transfected with miR-494-3p mimics (dashed line) or negative control oligonucleotides (solid line). Data are the mean ± SD (n = 3). *$p < 0.05$. (B) Cell proliferation in MC3T3-E1 cells that were pre-treated with (dashed line) and without (solid line) compressive force for 24 h followed by trypsinization and re-seeding was evaluated. Data are the mean ± SD (n = 3). *$p < 0.05$. (C) At 12 h after re-seeding, miR-494-3p levels were measured by RT-qPCR in the cells pre-treated with (closed column) or without (open column) compressive force. Data are the mean ± SD (n = 7). *$p < 0.05$.

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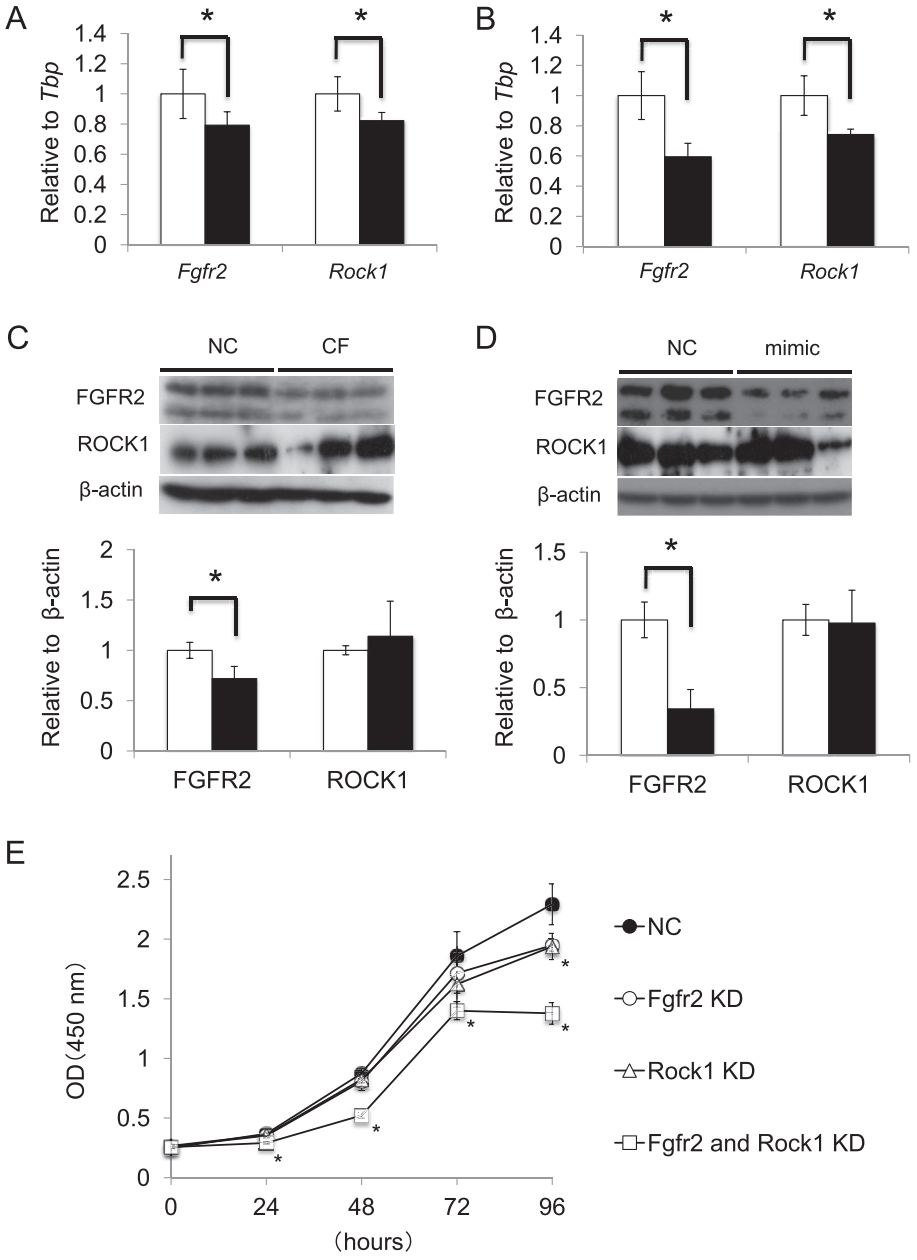

FIG. 3. Effect of compressive force and overexpression of miR-494-3p mimics on *Fgfr2* and *Rock1* expression in MC3T3-E1 cells. (A, B) Levels of *Fgfr2* and *Rock1* mRNA were measured by RT-qPCR in MC3T3-E1 cells treated with (closed columns) or without compressive force (open columns) (A), and transfected with miR-494-3p mimics (closed columns) or negative control oligonucleotides (open columns) (B). The mRNA levels normalized by *Tbp* mRNA levels are shown relative to those of the control cells. Data are the mean ± SD (n = 3). $^*p < 0.05$. (C, D) Representative images (upper) of Western blot for FGFR2 and ROCK1 in MC3T3-E1 cells treated with (CF) or without (NC) compressive force (C) and transfected with miR-494-3p mimics (mimic) or negative control oligonucleotides (NC) (D) are shown. The lower graphs represent band density ratio of the proteins to β-actin in the cells treated with (closed columns) or without (open columns) compressive force. Data are the mean ± SD (n = 5). $^*p < 0.05$. (E) Effect of knockdown of *Fgfr2* and *Rock1* on cell proliferation in MC3T3-E1 cells. Closed circles, control siRNA; open circles, *Fgfr2*; triangles, *Fgfr2*; squares, combined with *Fgfr2* and *Rock1*. Data are the mean ± SD (n = 4). $^*p < 0.05$.

sequence of the *Fgfr2* and *Rock1* genes (Fig. 4A). Luciferase reporter plasmids containing the wild-type 3′-UTR of *Fgfr2* and the mutated 3′-UTR that lacks either of binding site-1 or -2, or both of two binding sites were constructed (Fig. 4B). Each reporter plasmid was co-transfected with miR-494-3p mimics into HEK293 cells. Relative luciferase activities in the cells transfected with reporter plasmids except for those lacking both of the binding sites were significantly decreased in the presence of miR-494-3p mimics (Fig. 4C), indicating that either of binding site-1 or -2 in the *Fgfr2* gene is enough to be regulated by miR-494-3p. In the same way, binding sites of miR-494-3p in the *Rock1* gene were examined. Luciferase activity was decreased by miR-494-3p mimics in the cells transfected with the reporter vector containing wild-type of 3′-UTR in the *Rock1* gene and the 3′-UTR with deletion of binding site-2, but not that lacking the binding site-1 or both of the binding sites (Fig. 4D), suggesting that repression of *Rock1* mRNA by miR-494-3p depends on the binding site-1.

DISCUSSION

In this study, microarray and RT-qPCR analyses showed that miR-494-3p, miR146a-5p, miR-210-3p, and miR1247-3p were remarkably increased in MC3T3-E1 cells subjected to compressive force. In regard to miRNAs down-regulated by compressive force in microarray analysis, we did not attempt validation by RT-qPCR due to large

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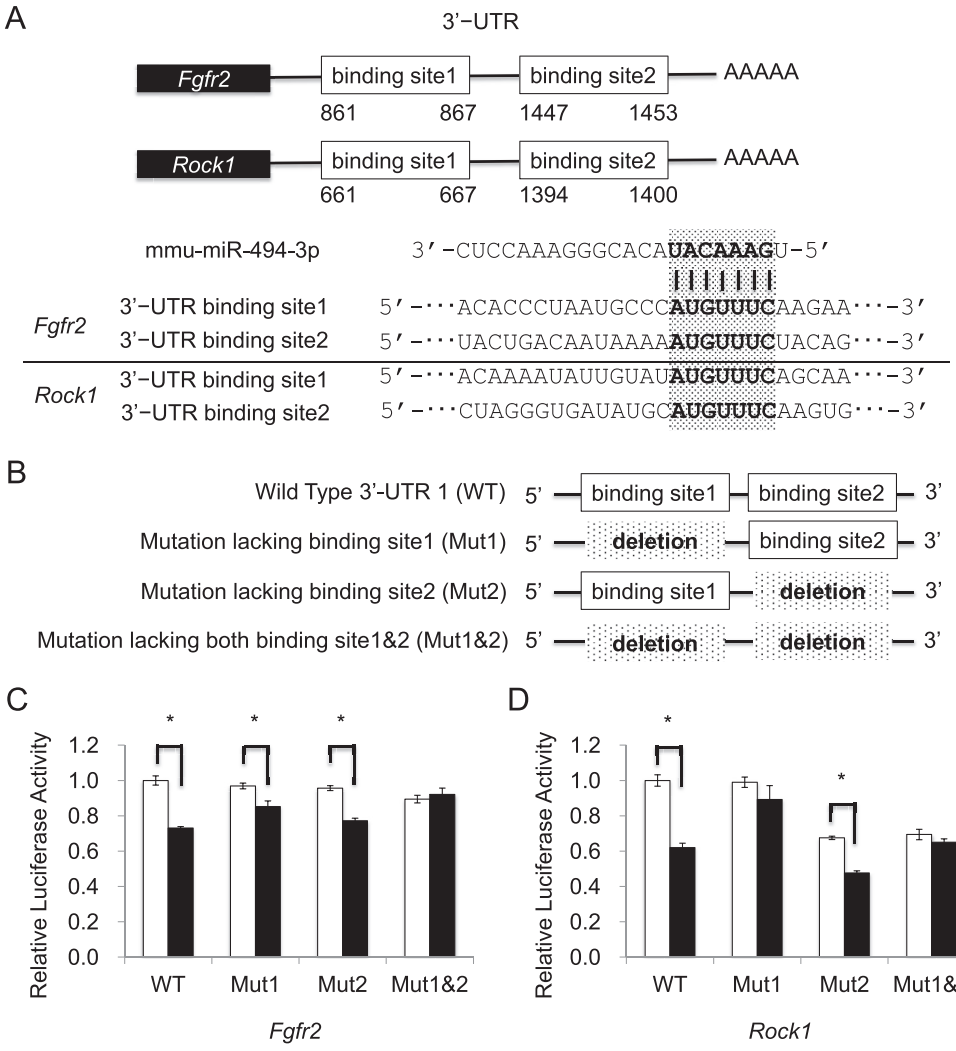

FIG. 4. Identification of binding sites for miR-494-3p in the *Fgfr2* and *Rock1* genes. (A) Two putative binding sites in each of the *Fgfr2* or *Rock1* gene were obtained from the miRbase sequence database. (B) Luciferase reporter plasmids including 3′-UTR sequences of wild-type (WT) and each mutant that lacks either of binding site 1 (Mut1) or 2 (Mut2), or both of binding sites (Mut1&2) in the *Fgfr2* and *Rock1* genes were constructed. (C, D) Luciferase assay using lysates in HEK293 cells transfected with each reporter construct with wild-type or mutated 3′-UTR of *Fgfr2* (C) and *Rock1* (D) in the presence of miR-494-3p mimics (closed columns) or negative control oligonucleotide (open columns) was performed. Luciferase activity is shown relative to that of cells transfected with negative control oligonucleotide. Data are the mean ± SD (n = 4). $^{*}p < 0.05$.

number of miRNA species. Among compressive force-induced miRNAs, miR-1247-3p was uninformative due to a minor form generated from the pre-miRNAs. MiR-146a-5p is induced in inflammatory condition to inhibit the proinflammatory cytokine signaling pathway (22,23) and suppress cell growth, migration, and invasion in some types of cancer (24,25). MiR-210 represents a major hypoxia-inducible miRNA and serves tumor suppressive functions in cell proliferation, mitochondrial respiration, arrest of DNA repair, and angiogenesis (26). MiR-494-3p has been reported to have contradictory functions in cell proliferation depending on difference of targets. It acts as an anti-apoptotic factor in human bronchial epithelial cells and myeloid-derived suppressor cells to target a gene encoding phosphatase and tensin homolog deleted from chromosome (PTEN) (27,28). On the other hand, overexpression of miR-494-3p decreases cell growth by targeting genes encoding KIT and cyclin-dependent kinase 6 in gastrointestinal stromal tumor cells (16) and cholangiocarcinoma (17), respectively. In this study, miR-494-3p overexpression in MC3T3-E1 cells suppressed cell growth, suggesting its inhibited property in cell proliferation. Thus, compressive force might inhibit cell proliferation in osteoblasts by up-regulating miR146a-5p, miR-210-3p, and miR-494-3p.

Effects of mechanical stimuli on cell proliferation in osteoblasts have not been definitive. For example, exposure to 0.5 Hz and 3500 με of mechanical tensile strain in MC3T3-E1 cells promotes cell proliferation through integrin-mediated ERK activation (29), and centrifugal forces increase cell proliferation in rat bone marrow osteoblast-like cells (8). In contrast, 98–588 Pa of compressive force induces apoptosis via induction of caspase-8 in human osteoblast-like cell line, MG63 (30), and 294 Pa of compressive force inhibits cell proliferation in human primary osteoblasts (10). This difference may be due to experimental conditions, types of mechanical stimuli, and cell types. In our condition, profile of up-regulated miRNA appeared to suggest an inhibitory effect of compressive force on cell proliferation. Because our experimental system that compressive force was applied to confluent cells, which do not have intercellular spaces to grow more, was difficult to examine the effects on further cell proliferation, we could not directly demonstrate an inhibitory effect. Instead, we showed inhibition of cell proliferation in the MC3T3-E1 cells that were trypsinized and re-seeded after treatment of compressive force.

We demonstrated that miR-494-3p targets the *Fgfr2* and *Rock1* genes in MC3T3-E1 cells. Overexpression of miR-494-3p mimics

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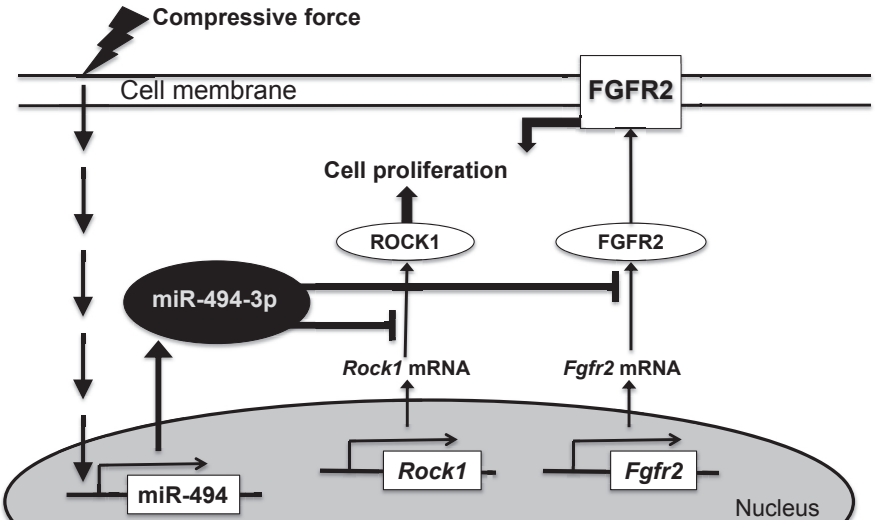

FIG. 5. Hypothesis of miR-494-3p-signaling in inhibition of osteoblasts proliferation by compressive force. Compressive force induces miR-494-3p expression in osteoblasts and miR-494-3p represses FGFR2 and ROCK1 expression to inhibit cell proliferation.

decreased expression of *Fgfr2* at both the protein and mRNA levels, whereas it decreased expression of *Rock1* at mRNA level, but not protein levels. As an explanation of this discrepancy, *Rock1* may have a slower protein turnover. MiRNA-494-3p has been reported to protect ischemia/reperfusion-induced cardiac injury by targeting genes encoding FGFR2, ROCK1, PTEN, leukemia inhibitory factor (LIF), and calcium/calmodulin-dependent protein kinase 2D (CAMK2D) (31). In our study, compressive force did not affect mRNA levels of *Pten*, *Lif*, and *Camk2d* in MC3T3-E1 cells (data not shown), suggesting that these genes may not be targets of miR-494-3p in osteoblasts subjected to compressive force.

FGFR2 belongs to a tyrosine kinase receptor family and the downstream-signal is involved in proliferation and differentiation of cells (32). In osteoblasts, FGFR2 promotes both osteogenic differentiation and cell proliferation via ERK signaling (20). ROCK1 also shows oncogenic characteristics in various tumors (33). Knockdown of ROCK1 decreases cell proliferation and viability, and induces apoptosis in osteosarcoma cell lines, KHOS and U−2OS (21). We demonstrated that knockdown of FGFR2 and ROCK1 inhibited cell proliferation in MC3T3-E1 cells, suggesting that down-regulation of these genes might be a cause of inhibited cell proliferation in MC3T3-E1 cells subjected to compressive force and transfection of miR-494-3 mimics (Fig. 5).

In conclusion, compressive force up-regulated expressions of miR-494-3p, miR146a-5p, and miR-210-3p, which have been reported as tumor suppressor miRNAs, in MC3T3-E1 cells. Among these RNAs, miR-494-3p was involved in the inhibition of osteoblast-proliferation, probably through down-regulation of FGFR2 and ROCK1 expressions. Our study contributes to the better understanding of molecular mechanisms in the response of bone to mechanical stress. Furthermore, miRNA as the signaling molecule may be a useful target for maintenance of bone health and treatment of bone diseases.

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jbiosc.2015.02.006

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