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Title: Compression and Tension Inversely Change Osteoprotegerin expression via miR-3198 in Periodontal Ligament Cells

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Abstract: Background: Osteoclastic bone resorption in the compression zone of periodontal ligament (PDL) plays a role in orthodontic tooth movement, and is regulated by the balance of RANKL and OPG. Compression downregulates OPG, conversely, tension upregulates OPG in PDL cells. However, the regulatory mechanism of OPG expression in PDL cells under different mechanical stresses remains unclear. Methods: To study microRNA (miRNA) expression profiles, compression (2g/cm^2) or tension (15%-elongation) was applied to immortalized human PDL (HPL) cells, and miRNA was extracted. The miRNA expression was analyzed using a human miRNA microarray, and the changes of the miRNA expression were confirmed by real-time RT-PCR. In addition, miR-3198-mimic and -inhibitor were transfected into HPL cells to understand the resulting OPG expression and production. Results: Certain miRNAs were expressed differentially under compression and tension. Some miRNAs including miR-3198 were upregulated only by compression. Real-time RT-PCR confirmed that compression induced miR-3198, but tension reduced it, in HPL cells. miR-3198-inhibitor upregulated and miR-3198-mimic reduced OPG in HPL cells. miR-3198-inhibitor rescued the compression-mediated downregulation of OPG. On the other hand, miR-3198-mimic reduced OPG expression under tension. Conclusion: We conclude that miR-3198 is upregulated by compression and is downregulated by tension, suggesting that miR-3198 downregulates OPG in response to mechanical stress.

Keywords: MicroRNA 1; osteoprotegerin (OPG) 2; orthodontic tooth movement (OTM) 3; miR-3198 4; mechanical stresses 5; periodontal ligament cells (PDL cells) 6; compression 7; tension 8

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1. Introduction

Orthodontic tooth movement (OTM) describes the orchestrated responses of periodontal tissues in response to physical force. During OTM, site-specific bone metabolisms take place simultaneously, i.e., osteoclastic bone resorption in the compression zone and osteoblastic bone formation in the tension zone of PDL [1-3]. Osteoclastogenesis is regulated mainly by receptor activator of nuclear-factor kappa-B ligand (RANKL; [4]. RANKL signaling is inhibited by osteoprotegerin (OPG), and a balance between RANKL and OPG contributes to the regulation of bone resorption [5]. The relationship between the ratio of RANKL/OPG and the progression of OTM has been extensively studies. Compression induces RANKL expression in periodontal ligament (PDL) cells [6-9] and reduces OPG expression [10,11], thereby increasing the RANKL/OPG ratio, and favoring RANKL-mediated osteoclastogenesis. On the other hand, tension increases OPG expression in PDL cells both in vivo [12,13] and in vitro [14-17]. However, the mechanism of OPG expression under different mechanical stresses in the PDL cells remains unclear.

Recently, the relationship between mechano-sensing and microRNA (miRNA) expression has become clearer. It is now understood that miRNAs in vascular endothelial cells play an essential role in shear stress-regulated endothelial responses [18]. In addition, miRNAs regulate osteo-chondral differentiation from stem cells [19]. It has been shown that miRNA-140 regulates cartilage development and homeostasis [20]. Furthermore, mechanical stress can induce expression of miRNAs that modulate the expression of osteogenic and bone resorption factors, leading to the impact of mechanical stress on bone remodeling [21]. These studies suggest that miRNAs play a role in the regulation of differential OPG expression in PDL cells under compression and tension.

We hypothesized that compression and tension induce different miRNA expression profiles, resulting in differential OPG expression in PDL cells. To test this hypothesis, we examined miRNA expression profiles under compression and tension, and explored which miRNA differentially altered the expression of OPG in response to mechanical stress. Then we focused on miR-3198, which was upregulated by compression and downregulated by tension. Augmentation and attenuation of miR-3198 by miRNA mimic and inhibitor, respectively, revealed that OPG expression was downregulated by miR-3198.

In the present study, we discovered that compression and tension differentially regulate miRNA expression. Importantly, we found that miR-3198, which was induced by compression and reduced by tension, downregulates OPG expression in PDL cells.

2. Results

2.1. miRNA expression is mediated by mechanical stress

We examined miRNA expression in HPL cells in three different groups using a microarray. The top 20 miRNAs that differed in their expression between the control and compression groups, the control and tension groups, and the tension and compression groups were identified (Table 1). Some miRNAs, such as miR-1268, -3648, -642b, and -135a, were upregulated in both the compression and tension groups compared with the control group. The data suggest that these miRNAs were upregulated by mechanical stress regardless of the type of stress. Furthermore, miRNAs such as miR-572, -663, -575, -3679-5p, UL70-3p, and -3198 were upregulated in the compression group both in comparison with the control group and the tension group, suggesting that these miRNAs are upregulated by compression. Upregulated miRNA in the tension group relative to the control group included miR-376a. These results suggest that, although some miRNAs are upregulated regardless of the type of mechanical stress, other miRNAs are upregulated only in response to a specific type of mechanical stress.

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97
98 Table 1 microarray analysis for miRNA expression

Cont. VS Comp.	Log2 Ratio	Cont. VS Tens.	Log2 Ratio	Tens. VS Comp.	Log2 Ratio
hsa-miR-1268	9.94	hsa-miR-3648	7.37	hsa-miR-4299	11.10
hsa-miR-572	7.73	hsa-miR-1268	6.57	hsa-miR-572	7.73
hsa-miR-663	7.60	hsa-miR-642b	6.31	hsa-miR-663	7.60
hsa-miR-3648	7.21	hsa-miR-135a*	5.35	hsa-miR-575	6.87
hsa-miR-575	6.87	hsa-miR-376a*	5.21	hsa-miR-3679-5p	6.68
hsa-miR-3679-5p	6.68	hsa-miR-4271	1.64	hcmv-miR-UL70-3p	6.57
hsa-miR-642b	6.64	hsa-miR-136	1.47	hsa-miR-3198	6.56
hcmv-miR-UL70-3p	6.57	hsa-miR-29b	1.36	hsa-miR-1305	6.47
hsa-miR-3198	6.56	hsa-miR-3663-3p	1.36	hsa-miR-1225-3p	6.31
hsa-miR-1305	6.47	hsv1-miR-H18	1.28	hsa-miR-125a-3p	6.16
hsa-miR-1225-3p	6.31	hsa-miR-3656	-1.04	hsa-miR-1246	6.14
hsa-miR-125a-3p	6.16	ebv-miR-BART13	-1.16	hsv1-miR-H17	5.88
hsa-miR-1246	6.14	hsa-miR-145	-1.28	hsa-miR-140-3p	5.79
hsv1-miR-H17	5.88	hsa-miR-181b	-1.35	hsa-miR-155	5.75
hsa-miR-654-5p	5.48	hsa-miR-181a-2*	-1.42	hsa-miR-654-5p	5.48
hsa-miR-135a*	5.43	hsa-miR-503	-4.99	hsa-miR-129-3p	5.37
hsa-miR-129-3p	5.37	hsa-miR-425*	-5.43	hsa-miR-874	5.36
hsa-miR-874	5.36	hsa-miR-425	-5.61	hsa-miR-425	5.26
hsa-miR-4299	5.32	hsa-miR-4299	-5.78	hsv1-miR-H7*	5.26
hsv1-miR-H7*	5.26	hsa-miR-155	-5.87	hsa-miR-485-3p	5.26

99
100 2.2. miRNAs targeting OPG are regulated by mechanical stress
101 Because OPG expression is regulated by mechanical stress in OTM, we investigated whether the
102 miRNAs that were upregulated by mechanical stress were also among the predicted miRNAs
103 targeting OPG. The miRNAs which target OPG were predicted by two databases (Table 2). miR-1207
104 was on neither list; therefore we used miR-1207 mimic and inhibitor as a negative control. We
105 identified miR-3198 on both of the lists, raising the possibility that compression-induced miR-3198
106 downregulates OPG expression.
107
108 Table-2 microRNAs which target OPG (TNFRSF11b)

microRNA.org				miRDB.org			
RANK and miRNA				RANK and miRNA			
1	hsa-miR-3163	26	hsa-miR-135a	1	hsa-let-7f-2-3p	26	hsa-miR-6870-3p
2	hsa-miR-586	27	hsa-miR-135b	2	hsa-miR-1185-1-3p	27	hsa-miR-936
3	hsa-miR-633	28	hsa-miR-200b	3	hsa-miR-1185-2-3p	28	hsa-miR-5692a
4	hsa-miR-656	29	hsa-miR-590-5p	4	hsa-miR-4262	29	hsa-miR-145-5p
5	hsa-miR-130b*	30	hsa-miR-21	5	hsa-miR-3163	30	hsa-miR-5195-3p
6	hsa-miR-548c-3p	31	hsa-miR-4255	6	hsa-miR-892c-5p	31	hsa-let-7c-3p
7	hsa-miR-590-3p	32	hsa-miR-4309	7	hsa-miR-5584-5p	32	hsa-miR-216a-5p
8	hsa-miR-577	**33**	**hsa-miR-3198**	8	hsa-miR-4729	33	hsa-miR-4753-3p
9	hsa-miR-579	34	hsa-miR-2054	9	hsa-miR-181a-5p	34	hsa-miR-590-5p
10	hsa-miR-576-5p	35	hsa-miR-936	10	hsa-miR-181c-5p	35	hsa-miR-3160-5p
11	hsa-miR-429	36	hsa-miR-380	11	hsa-miR-181d-5p	36	hsa-miR-429
12	hsa-miR-488*	37	hsa-miR-3172	12	hsa-miR-181b-5p	37	hsa-miR-200b-3p
13	hsa-miR-4262	38	hsa-miR-376a	13	hsa-miR-3942-3p	38	hsa-miR-200c-3p
14	hsa-miR-181a	39	hsa-miR-376b	14	hsa-miR-4766-3p	39	hsa-miR-765
15	hsa-miR-181b	40	hsa-miR-145	15	hsa-miR-4668-5p	40	hsa-miR-5582-5p

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16	hsa-miR-181c	41	hsa-miR-193b*	16	hsa-miR-3942-5p	41	hsa-miR-629-5p
17	hsa-miR-181d	42	hsa-miR-4307	17	hsa-miR-4294	42	hsa-miR-6892-5p
18	hsa-miR-1283	43	hsa-miR-765	18	hsa-miR-4703-5p	43	hsa-miR-577
19	hsa-let-7f-2*	44	hsa-miR-374a*	19	hsa-miR-6501-3p	44	hsa-miR-579-3p
20	hsa-miR-889	45	hsa-miR-29b-2*	20	hsa-miR-506-3p	45	hsa-miR-183-3p
21	hsa-miR-4294	46	hsa-miR-570	21	hsa-miR-124-3p	46	hsa-miR-6074
22	hsa-miR-187*	47	hsa-miR-188-3p	22	hsa-miR-3662	47	**hsa-miR-3198**
23	hsa-miR-200c	48	hsa-miR-222*	23	hsa-miR-130b-5p	48	hsa-miR-513b-3p
24	hsa-miR-506	49	hsa-miR-3170	24	hsa-miR-193b-5p	49	hsa-miR-7109-3p
25	hsa-miR-124	50	hsa-miR-1323	25	hsa-miR-5582-3p	50	hsa-miR-4309

2.3. Expression of miR-3198 and OPG was regulated differentially in compression and tension.

To confirm the change of miR-3198 by mechanical stress, real-time RT-PCR analysis was performed (Figure. 1a). Compression induced miR-3198 expression in HPL cells, whereas tension reduced miR-3198 expression. These results were consistent with the results of miRNA array analysis. OPG expression was reduced by compression but was induced by tension (Figure. 1b). Western blotting revealed that compression reduced protein level OPG in the culture supernatant (Figure. 1c). In addition, tension induced protein level OPG in the culture supernatant. These results suggest that miR-3198 downregulates OPG expression in response to mechanical stress.

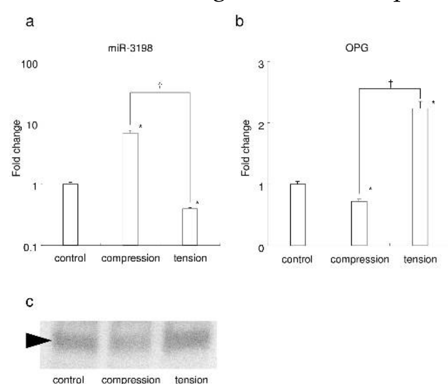

Figure 1. miR-3198 and OPG were regulated differentially by compression and tension.
The results of real-time RT-PCR analysis for miR-3198 (a) and OPG (b) expression in HPL cells are shown. Fold change from the control is displayed, with $P<0.05$ versus control indicated by * and $P<0.05$ between samples indicated by †.
(c) Representative image of the western blotting for OPG is shown.

2.4. miR-3198 gain-of-function and loss-of-function experiments.

We further examined the relationship between miR-3198 and OPG expressions through gain- and loss-of-function experiments. We found that transfection of miR-3198 inhibitor in HPL cells reduced miR-3198 expression (Figure. 2a), whereas transfection of miR-3198 mimic upregulated miR-3198 expression (Figure. 2b). Similarly, OPG mRNA expression was induced by miR-3198 inhibitor (Figure. 2c) and reduced by miR-3198 mimic (Figure. 2d). OPG protein levels were also increased by miR-3198 inhibitor (Figure. 2e) and decreased by miR-3198 mimic (Figure. 2f).

To clarify whether these phenomena were dependent on miR-3198 specifically, we examined the role of miR-1207, which was not predicted to target OPG. We found that the transfection of miR-1207 inhibitor reduced miR-1207 expression in HPL cells (Figure. 2g), and the transfection of miR-1207 mimic upregulated miR-1207 expression (Figure. 2h), consistent with the results of the miR-3198 inhibitor and mimic experiment. OPG expression was stable regardless of the transfection of miR-1207 inhibitor or mimic (Figure. 2i and 2j).

These results suggest that mechanical-stress-induced miR-3198 downregulates OPG expression in HPL cells.

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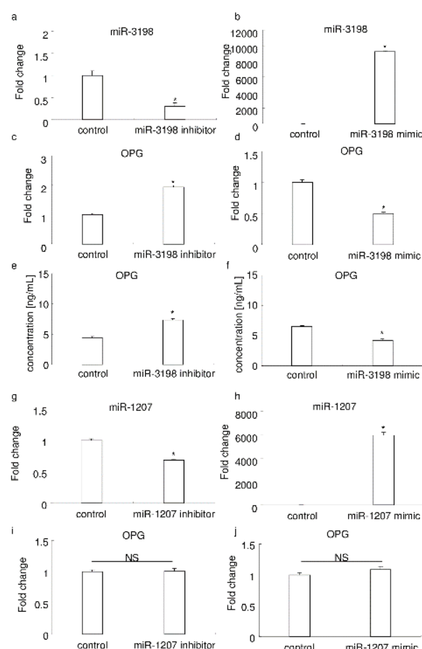

Figure 2. miR-3198 gain-of-function and loss-of-function experiments.
Results of real-time RT-PCR analysis for miR-3198 (a, b) and OPG (c, d) expression in HPL cells after transfection of miR-3198 inhibitor (a, c) and miR-3198 mimic (b, d). Fold change from the control is shown. The concentrations of OPG as measured by ELISA after transfection of miR-3198 inhibitor (e) and miR-3198 mimic (f) are shown. Results of real-time PCR analysis for miR-1207 (g, h) and OPG (i,j) expression in HPL cells under the transfection of miR-1207 inhibitor (g,i) and miR-1207 mimic (h, j). Fold changes from the control are shown. * indicates $P<0.05$ versus control and NS indicating there was no significant difference between samples.

2.5. miR-3198 regulates mechanical-stress-mediated OPG expression

Finally, we examined the role of miR-3198 in the regulation of mechanical-stress-mediated OPG expression. We found that OPG expression was reduced by compression, although transfection of miR-3198 inhibitor rescued the compression-mediated downregulation of OPG (Figure. 3a). In addition, there was no significant difference between the control and the compression + miR-3198 inhibitor groups, indicating that miR-3198 plays a role in the regulation of OPG expression under compression. Conversely, we found that under tension, augmentation of miR-3198 expression by miR-3198 mimic reduced OPG expression (Figure. 3b). There was a significant difference in the OPG expression levels between the control and the tension + miR-3198 mimic ($P = 0.03$).

Consistent with results of real-time PCR, OPG quantification by ELISA revealed that compression reduced OPG protein levels (Figure. 3c), whereas miR-3198 inhibitor prevented the compression-mediated reduction of OPG. In addition, tension upregulated OPG production (Figure. 3d). Similarly, we found that miR-3198 mimic significantly reduced the tension-mediated increase in OPG production.

Western blotting for OPG also revealed that the miR-3198 inhibitor prevented the compression-mediated reduction of OPG (Figure. 3e). On the other hand, miR-3198 mimic significantly reduced the tension-mediated increase in OPG production (Figure. 3f). These results indicate that miR-3198 downregulates OPG expression in HPL cells under mechanical stress.

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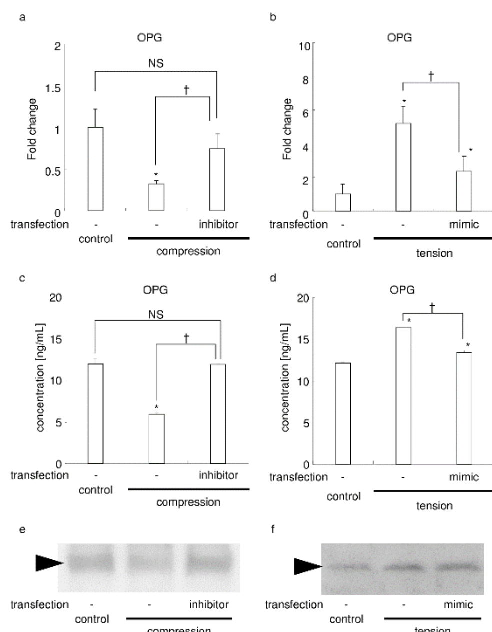

166
167 **Figure 3.** miR-3198 regulates the mechanical stress-mediated change of OPG expression.
168 Results of real-time RT-PCR analysis for OPG expression in HPL cells in the compression (a) and
169 tension (b) experiments. Fold change from the control are shown. Inhibitor: transfection of miR-3198
170 inhibitor. Mimic: transfection of miR-3198 mimic. Also shown are the OPG concentrations measured
171 by ELISA in the compression (c) and tension (d) experiments. * indicates P<0.05 versus control. †
172 indicates P<0.05 between samples. NS indicates there was no significant difference between samples.
173 (e and f) Representative image of the western blotting for OPG was shown.
174

175 **3. Discussion**

176 Osteoclastic bone resorption is tightly regulated by RANKL [4] in the periodontal ligament
177 during OTM [7,10]. Conversely, OPG, the decoy receptor to RANKL, inhibits osteoclastogenesis [22].
178 The RANKL/OPG ratio increases at the compression zone of the PDL during OTM [10]. In vitro
179 experiments have revealed that compression increases the RANKL/OPG ratio in PDL cells [9,23-25].
180 On the other hand, tension decreases RANKL/OPG ratio in PDL cells, mainly by the induction of
181 OPG expression [14-17]. Generally, OPG expression in the PDL cells usually increases under tension
182 and decreases under compression.
183 In this study, we examined whether miRNAs are involved in site-specific changes in OPG
184 expression during OTM. We found that miRNAs were differentially regulated by compression and
185 tension. In particular, miR-3198 was upregulated by compression and downregulated by tension.
186 Furthermore, we found that miR-3198 regulates OPG expression in response to mechanical stresses,
187 which is consistent with phenomena observed in the PDL during OTM; namely, osteoclastic bone
188 resorption in the compression zone and osteoblastic bone formation in the tension zone of the PDL
189 [3]. We previously reported that tension-induced TGF-beta participates in the upregulation of OPG
190 expression [15]. Our present results indicate that tension-induced OPG expression is reduced by the
191 overexpression of miR-3198 mimic, although we did find a significant difference in OPG expression
192 between control and tension + miR-3198 mimic groups. These results are consistent with our previous
193 report that tension-induced TGF-beta participates in the upregulation of OPG expression under
194 tension.
195 Regarding the relationship between OTM and miRNA, Chen et al. reported that miR-21
196 deficiency attenuated OTM via inhibition of alveolar bone resorption on both the compressive and
197 tensile sides [26]. In addition, Chang et al. reported the role of miRNA in tension force-induced bone
198 formation [27]. They concluded that miR-195-5p, miR-424-5p, miR-1297, miR-3607-5p, miR-145-5p,
199 miR-4328, and miR-224-5p were core miRNAs of tension force-induced bone formation. Liu et al.

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reported that miR-503-5p functions as a mechano-sensitive miRNA and inhibits bone marrow stromal cell osteogenic differentiation subjected to mechanical stretch and bone formation in OTM tension sides [28]. Chen et al. reported that cyclic stretch decreased, and compression increased, the expression of miR-29 in PDL cells, which directly interacts with Col1a1, Col3a1 and Col5a1 [29]. These studies reveal the relationship between mechanical stress-mediated miRNA expression and bone formation or tissue remodeling. However, the effects of miRNA on the RANKL/OPG ratio during OTM were unclear until now.

Regarding the regulation of OPG expression by miRNAs, miRs-21 [30,31], -145 [32], -146a [33], -150 [34], and -200 [35] have been reported to regulate OPG expression. Among them, miRs-21, -145, and -200 were thought to be direct regulators of OPG expression in the databases of miRDB.org and microRNA.org. Therefore, we presumed that the refinement of candidate miRNAs using the available databases was a sufficiently accurate method to choose candidates.

We found that miR-3198 plays a role in the regulation of the mechanical stress-mediated OPG expression, although the reciprocal regulatory mechanism of miR-3198 by compression and tension remains unclear. Some mechanical stresses induce differential intracellular signaling systems, such as G-proteins, calcium signaling, MAPK signaling, and nitric oxide signaling [36]. Further studies are needed to clarify the regulatory mechanism of miR-3198 by compression and tension. miR-3198 was identified in human tumor breast tissue [37], and is on 22q11.21 of the genome. It is important to confirm that miR-3198 downregulates OPG expression by mechanical stress in animal models. However, there is no orthologue of miR-3198 in mice or rats, which makes it difficult to conduct such experiments. Nevertheless, further confirmatory experiments are required.

In conclusion, we found that miRNAs were differentially regulated by compression and tension in PDL cells. Furthermore, miR-3198 downregulates OPG expression in PDL cells in response to mechanical stress.

4. Materials and Methods

4.1. Cells

Human immortalized periodontal ligament cell lines (HPL cells) were received from the University of Hiroshima, Hiroshima, Japan [38]. HPL cells were cultured in alpha modified Eagle's medium (Wako Pure Chemical, Osaka, Japan) containing 10% fetal bovine serum (Thermo Fisher Scientific, Waltham, MA) and supplemented with penicillin (100 U/mL) and streptomycin (100 µg/mL). All cells were cultured at 37°C in a 5% CO2 incubator.

4.2. Application of mechanical stress

Compressive force was applied to the HPL cells using a glass cylinder, as described elsewhere [9]. Briefly, a glass cylinder was placed over a confluent cell layer in the well of a 6-well plate. HPL cells were subjected to 2 g/cm2 of compressive force for 24 h. Cyclical tensile force was applied to HPL cells with a Flexercell Strain-Unit (Flexcell Corp., Hillsborough, NC, USA), as described elsewhere [15]. Briefly, PDL cells were pre-cultured in flexible-bottomed culture plates coated with type I collagen until confluent. The culture plates were then set on the rubber gasket of the Flexercell Strain Unit, and PDL cells were subjected to cyclical tensile force (15% elongation, 1 s stretch/1 s relaxation) for 24 h.

4.3. miRNA and RNA extraction

miRNA and RNA were extracted separately from HPL cells using the Nucleospin miRNA isolation kit (Macherey-Nagel, Düren, Germany), according to the manufacturer's instructions.

4.4. miRNA array analysis

The quality of RNA of the extracted miRNAs was examined by Agilent 2100 Bioanalyser (Agilent Technologies, Santa Clara, CA). RNA integrity numbers ranged from 8.7 to 9.5. miRNA expression in each sample was analyzed using a SurePrint G3 Human miRNA microarray 8×60K miRBase 16.0 (Agilent Technologies), according to the manufacturer's instructions.

4.5. Database analysis for miRNAs target prediction

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To identify candidate miRNA which targeted OPG, two target prediction databases, miRDB.org [39] and microRNA.org [40] were used. Candidate miRNAs were queried using "OPG" or "TNFRSF11B" as keywords.

4.6. Reverse transcription (RT) and real-time RT-PCR analysis

Isolated miRNA (2 μg each) were reverse-transcribed (RT) with the miScript II RT kit (Qiagen, Germantown, MD), according to the manufacturer's instructions. After reverse transcription, cDNA samples were diluted 5× with TE buffer. Real-time RT-PCR was performed using the miScript SYBR green PCR kit (Qiagen). The following PCR primers were used for the detection of miRNA: miR-1207 (MIMAT0005871), miR-3198 (MIMAT0015083), and RNU6B. Fold change of miR-3198 expression was calculated by using the Δ-Δ Ct method with RNU6B as a reference gene. Isolated RNA (500 ng) was reverse-transcribed using the iScript cDNA-Supermix (Bio-Rad, Hercules, CA, USA), according to the manufacturer's instruction. After reverse transcription, cDNA samples were diluted 5× with TE buffer. Real-time RT-PCR was performed using the SsoFast EvaGreen-Supermix (Bio-Rad). PCR primers used for the experiments were human OPG (forward, 5′-AAGGGCGCTACCTTGAGATAG-3′; reverse, 5′-GCAAACTGTATTTCGCTCTGGG-3′) and human ribosomal protein S18 (RPS18) (forward, 5′-GATGGGCGGCGGAAAATAG-3′; reverse, 5′-GCGTGGATTCTGCATAATGGT-3′). Fold changes of OPG expression were calculated by using the Δ-Δ Ct method with RPS18 as a reference gene.

4.7. miR-3198 gain-of-function and loss-of-function experiments

To observe the influence of miR-3198 on OPG expression, miR-3198 mimic (Qiagen) and miR-3198 inhibitor (Qiagen) were transfected into HPL cells using the TransIT-TKO® transfection reagent (Mirus Bio LLC, Madison, WI), according to the manufacturer's instructions. miR-1207 mimic (Qiagen) and miR-1207 inhibitor (Qiagen) were used as the negative control. miR mimic and miR inhibitor were used at a final concentration of 50 nM. The expression of miR-3198 was observed at 24 h after transfection. In some experiments, mechanical stress was applied to transfected HPL cells, beginning 12h after transfection.

4.8. OPG ELISA

The concentration of OPG in the culture supernatant was measured using an OPG ELISA kit (Boster Biological Technology, Pleasanton, CA), according to the manufacturer's instructions. Culture supernatants were diluted 5× prior to measurement.

4.9. Western blotting for OPG

The culture supernatant were subjected to electrophoresis on TGX Precast gels (BioRad), proteins were transferred to a PVDF membrane, which was blocked with PVDF Blocking Reagent (Toyobo Co. Ltd, Osaka, Japan), then incubated with the goat IgG anti OPG antibody (for figures 1c and 3e; Immundiagnostik AG, Bensheim, Germany) or rabbit IgG anti OPG antibody (for figure 3f; GeneTex, Irvine, CA, USA). After thorough washing with 0.5% Tween-20 in PBS (PBS-T), the membrane was incubated with a horseradish peroxidase-conjugated anti goat IgG antibody (R&D Systems, Inc., Minneapolis, MN, USA) or anti rabbit IgG antibody (Thermo Fisher Scientific, Waltham, MA, USA). Chemiluminescence was produced by using Luminata-Forte (EMD Millipore, Billerica, MA) and detected with LumiCube (Liponics, Tokyo, Japan).

4.10. Statistical analysis

All data are presented as mean ± SD. Multiple comparisons were performed by using Tukey's test. A P-value of $P < 0.05$ was considered statistically significant.

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308

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