MicroRNA-138 Inhibits Periodontal Progenitor Differentiation under Inflammatory Conditions

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Abstract

Inflammatory conditions as they occur during periodontal disease often result in decreased alveolar bone levels and a loss of connective tissue homeostasis. Here we have focused on the effect of microRNA-138 (miR-138) as a potential regulator of periodontal stem cells as they affect homeostasis during inflammatory conditions. Our data indicate that miR-138 was significantly upregulated in our periodontal disease animal model. Interaction of miR-138 with a predicted targeting site on the osteocalcin (OC) promoter resulted in a 3.7-fold reduction of luciferase activity in promoter assays compared with controls; and miR-138 overexpression in periodontal progenitors significantly inhibited OC (3.4-fold), Runx2 (2.8-fold), and collagen I (2.6-fold). Moreover, treatment with inflammatory modulators such as interleukin (IL)-6 and lipopolysaccharide (LPS) resulted in a significant 2.2-fold (IL-6) or 1.9-fold (LPS) increase in miR-138 expression, while OC and Runx2 expression was significantly decreased as a result of treatment with each inflammatory mediator. Further defining the role of miR-138 in the OC-mediated control of mineralization, we demonstrated that the LPS-induced downregulation of OC expression was partially reversed after miR-138 knockdown. LPS, miR-138 mimic, and OC small interfering RNA inhibited osteoblast differentiation marker alkaline phosphatase activity, while miR-138 inhibitor and OC protein addition enhanced alkaline phosphatase activity. Supporting the role of OC as an essential modulator of osteoblast differentiation, knockdown of miR-138 or addition of OC protein partially rescued alkaline phosphatase activity in periodontal ligament (PDL) cells subjected to LPS treatment. Our data establish miR-138 inhibitor as a potential therapeutic agent for the prevention of the bone loss associated with advanced periodontal disease.

Keywords: alveolar bone loss, periodontal ligament, stem cells, periodontal diseases, osteogenesis, osteocalcin

Introduction

The balance between anabolic and catabolic processes during the physiological turnover of periodontal connective tissues is maintained by stem cell populations that reside within the periodontal region, including periodontal ligament progenitors and alveolar bone osteoblasts (Liu et al. 2010). During periodontal disease, this balance between bone formation and resorption is disturbed, resulting in the inhibition of alveolar bone formation and regeneration (Liu et al. 2006; Graves et al. 2011; Wu et al. 2015). The inhibition of new bone formation as a result of periodontal disease suggests that the inflammatory process not only affects bone resorption and osteoclast activity but also reduces or inhibits the ability of periodontal progenitors to form new periodontal connective tissues, including alveolar bone.

The inflammatory response to acute and chronic stimuli is orchestrated by a multitude of regulatory factors (Paulsen et al. 2013), including transcription factors, epigenetic factors, RNA binding proteins, and microRNAs (miRs) (Tian et al. 2005; Hao and Baltimore 2009; Anderson 2010). MicroRNAs (miRNAs) are endogenous, small, noncoding RNAs that regulate gene expression through degradation of target mRNA or inhibition of translation (He and Hannon 2004). According to miRBase (v21), there are 1,881 annotated microRNA sequences in the human genome. These miRNAs are pivotal regulators of

diverse cellular processes such as proliferation, differentiation, apoptosis, survival, motility, and morphogenesis (Pillai 2005). Dysregulation of miRNAs is known to play major roles in many diseases, including inflammatory diseases such as periodontal disease (Xie et al. 2011; Perri et al. 2012; Plank et al.

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2013). These periodontal disease-related miRNAs have either been associated with the inhibition of proinflammatory cytokine secretion in human gingival fibroblasts (Xie et al. 2011) or with the modulation of osteogenic differentiation of periodontal ligament stem cells (Jin et al. 2011).

In the present study, we have focused on the role of miR-138 in periodontal inflammation. Previous studies have identified miR-138 as a potential inhibitor of osteogenesis (Eskildsen et al. 2011; Qu et al. 2014; Yan et al. 2014) and as a modulator of stem cell differentiation and tissue formation (Ye et al. 2012). Its known involvement in osteogenesis and stem cell differentiation establishes miR-138 as a candidate regulator of periodontal homeostasis and stem cell differentiation during periodontal inflammation. In the present study, we have therefore hypothesized that proinflammatory conditions alter periodontal ligament progenitor function through miR-138 by affecting their mineralization potential. Our study provides evidence for a direct relationship between proinflammatory factors and miR-138 expression in periodontal ligament progenitors and establishes a potential mechanism by which miR-138 modulates periodontal mineral homeostasis during inflammatory conditions.

Materials and Methods

Periodontitis Animal Model

Wistar rats (Charles River, Wilmington, MA, USA) were bred at the University of Illinois at Chicago (UIC) in strict accordance with the recommendations for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the UIC Committee on the Ethics of Animal Experiments. Animals received general anesthesia with a mixture of ketamine hydrochloride (100 mg/kg) and xylazine (5 mg/kg) administered via intraperitoneal injection, and a steel ligature was placed around the first maxillary molars. Periodontal pathogens *Porphyromonas gingivalis* ATCC 33277 (*Pg*) and *Fusobacterium nucleatum* ATCC 25586 (*Fn*) were used in this study (Xu et al. 2010). Equal volumes of *Pg* and *Fn* (10^9 colony-forming units [CFU]/mL) were mixed and 50 μ L of the cell suspension was applied to the gingival sulcus of both left and right maxillary first molars every other day for 14 d. Growth medium was used as a control. Inflammation levels in the periodontal ligament of periodontitis animals were determined by evaluating the ratio of polymorphonuclear leukocytes/mononuclear cells: 0, no inflammatory cells; 1, slight inflammation (a few inflammatory cells close to the junctional epithelium); 2, moderate inflammation (several inflammatory cells scattered through the gingival connective tissue); and 3, severe inflammation (more than one-third of the cells in the interproximal tissue were inflammatory cells) (Liu et al. 2006).

Tissue Collection and Processing

Rat maxillae were dissected from control and periodontitis animals for micro-computed tomography (CT) analysis, hematoxylin and eosin (H&E) staining, and RNA isolation (Lu et al.

2013). For H&E staining, the samples were fixed in formalin, dehydrated, and embedded in paraffin. For RNA isolation, periodontal ligament and alveolar bone tissues were collected and processed in liquid nitrogen. Subsequently, total RNAs were extracted for further analysis.

Micro-CT

Rat maxillae were analyzed using micro-CT. For this purpose, 3-dimensional X-ray CT images were acquired with an Xradia MicroXCT 400 (Xradia, Concord, CA, USA). Briefly, a 1024×1024 image matrix size over a 5.12-mm field of view was selected to create an isotropic voxel size of 5 microns. A total of 1,024 slices were acquired for each maxilla. No filtering processes were applied after the scan and reconstruction. During the scans, a 30-KeV, 6-Watt X-ray beam was generated to image the samples; a 5-s exposure time was used for each of the hundreds of projection images with a 0.25-degree step angle.

Real-Time Reverse Transcriptase Polymerase Chain Reaction Analysis

Total RNAs were extracted from collected tissues or cultured cells using the MicroRNA Easy kit (Qiagen, Germantown, MD, USA). MiR-138 levels were determined using TaqMan microRNA assay protocols (Life Technologies, Grand Island, NY, USA). The relative mRNA levels of OC, Runx2, Col1, Col3, SGK3, IL6, and DefA4 were examined using a quantitative 2-step reverse transcriptase polymerase chain reaction (RT-PCR) assay with gene-specific primer sets (OriGene, Rockville, MD, USA) as described previously (Dangaria et al. 2009; Dangaria et al. 2011). The relative expression level was computed using the $2^{-\Delta\Delta C_t}$ analysis method, where actin was used as an internal reference (Livak and Schmittgen 2001).

Western Blot Analysis

Total proteins were extracted as previously described (Atsawasuwan, Lu, Ito, Chen, et al. 2013). Protein samples were loaded at 10 μ g protein per well on 4% to 20% sodium dodecyl sulfate (SDS)–polyacrylamide gels. Osteocalcin antibody (Abbotect, Atlanta, GA, USA) was applied as primary antibody in 1:500 dilution. Immune complexes were detected with anti-rabbit secondary antibody (Abcam, Cambridge, MA, USA) and chemiluminescence reagents. The amount of protein expression was compared after normalization with β -actin (Abcam).

Cell Culture and Transfection

Human periodontal ligament (PDL) progenitor cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μ g/mL streptomycin at 37°C in a humidified incubator containing 5% CO₂. In a parallel study, PDL

progenitors were cultured in Mesenchymal Stem Cell Growth Medium—Chemically Defined (MSCGM-CD; Lonza, Valparaiso, IN, USA) to verify the effects of osteogenic differentiation and lipopolysaccharide (LPS) addition on miR-138 expression in a serum-free environment. For functional analyses, miRIDIAN Mimic Human hsa-miR-138-5p, control microRNA mimic (cel-miR-67 from *Caenorhabditis elegans*), miRIDIAN Hairpin inhibitor Human hsa-miR-138-5p, microRNA negative control (Dharmacon, Lafayette, CO, USA), and OC gene-specific small interfering RNAs (siRNAs) (On-TargetPlus SMARTpool; Dharmacon) were transfected into cells using DharmaFECT Transfection Reagent 1 as described previously (Chen et al. 2013; Jin, Chen, Cabay, et al. 2013). Osteogenic differentiation of PDL cells was induced using osteogenic induction medium containing 10^{-8} M dexamethasone, 10 mM β -glycerophosphate, and 50 μ g/mL ascorbic acid in culture medium. Inflammatory conditions were mimicked by adding interleukin (IL)-6 (10 ng/mL), LPS (10 μ g/mL), and tumor necrosis factor- α (TNF- α ; 10 ng/mL) to the culture medium.

MicroRNA Target Prediction

Base-pairing and minimum free energy (mfe) for the binding of miR-138 to the targeting sequences were predicted using the RNAhybrid program (<http://bibiserv.techfak.uni-bielefeld.de/rnahybrid>).

Dual Luciferase Reporter Assay

The luciferase reporter gene construct was created by cloning a 55-bp fragment from the 3'-untranslated region (3'-UTR; position 371–425 of the OC mRNA sequence NM_199173, containing the miR-138 targeting site) into the Xba I site of the pGL3-Control firefly luciferase reporter vector (Promega, Madison, WI, USA) as described previously (Jin, Chen, Liu, et al. 2013). The corresponding mutant construct was created by replacing the miR-138 targeting site with 5'-TATATATATA TATA-3'. All constructs were verified by sequencing. The reporter constructs and the pRL-TK vector (Promega) were cotransfected using Lipofectamine 2000 (Life Technologies). Luciferase activities were determined as described previously (Jiang et al. 2010; Chen et al. 2013) using a GloMax 20/20 luminometer (Promega). Experiments were performed in triplicates.

Alkaline Phosphatase Activity, Alizarin Red Staining, and Masson's Trichrome Staining

PDL cells were seeded into 24-well cell culture plates at a concentration of 5×10^4 cells/well and cultured under osteogenic induction conditions (10^{-8} M dexamethasone, 10 mM β -glycerophosphate, and 50 μ g/mL ascorbic acid). After 3, 7, or 14 d of culture, cells were stained with NBT/BCIP or alizarin red or subjected to Masson's trichrome staining (Atsawasuwan, Lu, Ito, Zhang, et al. 2013).

Biostatistics

Statistical analysis was performed using the SPSS 13.0 software (SPSS, Inc., an IBM Company, Chicago, IL, USA). Data were expressed as mean $\pm$ SEM and analyzed by analysis of variance with a significance level of $P < 0.05$. Experiments were repeated 3 times to ensure reproducibility.

Results

MicroRNA-138 Expression Is Upregulated in Periodontal Tissues of a Periodontitis Animal Model

To assess the role of miRs in diseased periodontal tissues, a periodontal inflammation and periodontitis animal model was established using ligature placement on the first maxillary molars and a bacterial challenge with *Pg* and *Fn* for 14 d. Three-dimensional reconstruction of micro-CT images from periodontitis rat maxillae demonstrated significant bone loss at the crest of the alveolar bone (Fig. 1A, B, E). The alveolar bone loss was accompanied by inflammatory cell infiltration in the periodontal ligament, as demonstrated by histochemical staining (Fig. 1B, scale 3 in periodontitis animals compared to scale 0 in control animals). On the molecular level (Fig. 1F), expression of the proinflammatory cytokine IL-6 (5.3-fold, $P < 0.001$) and Defensin A4 (4.1-fold, $P < 0.001$) was significantly increased. Mature microRNA-138 (miR-138) expression was also significantly upregulated (1.5-fold, $P < 0.05$). In contrast, expression of the matrix proteins Collagen I (Col 1A2, 2.5-fold, $P < 0.01$) and Collagen III (Col 3A1, 6.2, $P < 0.01$), as well as the bone marker protein osteocalcin (OC, 14.9-fold, $P < 0.01$), was significantly downregulated (Fig. 1F).

MicroRNA-138 Directly Targets OC mRNA

MicroRNA-138 has been reported to attenuate bone formation in vivo through the focal adhesion kinase signaling pathway (Eskildsen et al. 2011). To explore whether miR-138 is involved in inflammation-related alveolar bone loss and directly targets any bone-related proteins, the RNAhybrid program (Krüger and Rehmsmeier 2006) was used to predict the base pairing and the mfe for the binding of miR-138 to target sequences. Bioinformatics analysis revealed a predicted miR-138 targeting sequence located in the 3'-UTR between nucleotide 389 and 411 of the osteocalcin mRNA (Fig. 2A). This predicted miR-138 targeting site in the OC mRNA is a noncanonical targeting sequence (lacking a perfect complement seed sequence). However, a strong binding affinity between this target sequence and miR-138 is anticipated based on the calculated minimum free energy (mfe = -31.9 kcal/mol) (Fig. 2A). To test whether miR-138 directly interacts with this predicted targeting site in OC mRNA, dual luciferase reporter assays were performed using constructs containing this targeting site (Fig. 2B). When cells were transfected with the construct containing the

Figure 1. Upregulation of miR-138 in a periodontitis rat model. Three-dimensional reconstruction of micro-computed tomography (CT) images from control (A) and periodontitis (B) rat maxillae after challenge with *Porphyromonas gingivalis* and *Fusobacterium nucleatum* for 14 d. Note the alveolar bone loss at the bone crest of the first and second molars. (C, D) Hematoxylin and eosin staining of the periodontal tissues from control and periodontitis rats. The arrow points to the infiltration of inflammatory cells in the periodontal ligament of the periodontitis rat (D). Original magnification is $\times 200$. (E) Difference in the distance between the cervical margin of the alveolar crest and the cementum-enamel junction (CEJ) in our periodontitis rat model (Perio) compared to a healthy control (Con). Measurements were based on micro-CT images from animals subjected to periodontitis treatment, and the healthy controls were used as a reference. (F) The miR-138 and bone-related gene expression in the periodontal tissues of control and periodontitis rats was examined by quantitative reverse transcriptase polymerase chain reaction analysis, and U6 snRNA and actin were used, respectively, as an internal reference. The values are presented as mean $\pm$ SD from triplicates of 3 independent experiments. ab, alveolar bone; de, dentin; gg, gingiva; infl, inflammatory cells; pdl, periodontal ligament. Gene names: IL-6, interleukin 6; DefA4, defensin A4; Col 1A2 and Col 3A1, Collagen 1A2 and 3A; OC, osteocalcin; miR-138, microRNA 138. * $P < 0.05$. ** $P < 0.01$. *** $P < 0.001$.

miR-138 targeting site, luciferase activity was significantly reduced (3.6-fold, $P < 0.05$) compared with the cells transfected with a negative control. When the targeting site was mutated, the effect of miR-138 on the luciferase activity was abolished (Fig. 2B). To further confirm the effect of miR-138 on OC expression, the expression level of OC transcripts was determined using quantitative RT-PCR (qRT-PCR) analysis. Transient transfection of miR-138 mimic in PDL cells led to an increase of miR-138 levels from 5- to 20-fold as measured by

Figure 2. MiR-138 directly targets osteocalcin (OC) messenger RNA (mRNA). (A) A predicted miR-138 targeting sequence is located in the 3'-untranslated region (3'-UTR) between nucleotides 389 and 411 of the OC mRNA. The base pairing and the minimum free energy (mfe) for the binding of miR-138 to the targeting sequences were predicted using the RNAhybrid program. (B) Dual luciferase reporter assays were performed to test the interaction between miR-138 and its targeting sequence in the OC 3'-UTR using constructs containing the predicted targeting sequence and mutated sequences cloned into the 3'-UTR of the reporter gene. The constructs were transfected into periodontal ligament (PDL) cells, and the cells were cultured for 72 h in the presence of miR-138 mimic or negative control mimic. (C) In this study, PDL cells were treated with miR-138 mimic or negative control mimic. The relative level of OC mRNA was examined by quantitative reverse transcriptase polymerase chain reaction. Data represent at least 3 independent experiments with similar results. * $P < 0.05$. ** $P < 0.01$.

the TaqMan assay. The transfection of miR-138 mimic also resulted in a significant downregulation of OC expression (3.2-fold, $P < 0.01$), while knockdown of miR-138 enhanced OC expression (1.5-fold, $P < 0.05$) (Fig. 2C). These results confirmed that miR-138 directly interacts with its OC mRNA target site and regulates OC expression.

MicroRNA-138 Mediates Inflammatory Factor-Induced Inhibition of Bone-Related Gene Expression in Periodontal Ligament Progenitors

In previous studies, we have characterized our mouse periodontal ligament progenitors and demonstrated their multipotency and ability to differentiate into osteoblasts (Dangaria et al. 2009, 2011). Here, we provided additional evidence demonstrating that our human periodontal ligament progenitors have the ability to self-renew and form cell colonies (Appendix Fig. 1). Under induction conditions, these cells differentiated into

Figure 3. Inhibitory effects of miR-138 and inflammatory factors on periodontal ligament progenitor differentiation. **(A)** Changes in alkaline phosphatase activity in a cell culture system during periodontal ligament (PDL) progenitor differentiation and treatment with inflammatory factors. PDL cells were cultured for 7 d and subjected to osteogenic induction and treatment with interleukin (IL)-6, lipopolysaccharide (LPS), and tumor necrosis factor- α (TNF- α). Alkaline phosphatase activity was detected using NBT/BCIP as a substrate. **(B)** Time course of miR-138 expression in PDL progenitors. PDL cells were induced to differentiation using osteogenic induction medium with or without LPS challenge. The miR-138 levels were measured using quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) after 3, 7, and 14 d of osteogenic induction. **(C)** Effect of inflammatory factors on the expression of miR-138, osteocalcin (OC), and Runx2 genes. qRT-PCR amplification of mature miR-138, OC, and Runx2 transcripts from PDL cells after 7 d of culture with osteogenic induction and proinflammatory treatment. **(D)** Effect of miR-138 on OC, Runx2, and bone-related gene expression. Relative expression of OC, Runx2, Col 1A, and GST3 transcripts was measured from cells transfected with negative control and miR-138 mimics and normalized to β -actin transcripts levels. * $P < 0.05$. ** $P < 0.01$.

osteoblasts, neurons, oligodendrocytes, and astrocytes (Appendix Fig. 1). These human periodontal ligament progenitors were used to determine the effect of inflammatory conditions on periodontal progenitors subjected to osteogenic differentiation

conditions in the present study. Osteogenic differentiation of PDL cells was verified by an increase in alkaline phosphatase activity (Fig. 3A). Inflammatory factors IL-6, LPS, and TNF- α inhibited alkaline phosphatase activity (Fig. 3A). To confirm miR-138 as a negative regulator of osteoblast differentiation and to determine the role of miR-138 in inflammation, mature miR-138 expression was examined at 4 time points, D0, D3, D7, and D14, after osteogenic induction and LPS treatment (Fig. 3B). MiR-138 expression was downregulated at D3 (1.1-fold), reached the lowest level at D7 (1.6-fold), and remained downregulated at D14 (1.4-fold) (Fig. 3B) during osteogenic differentiation. In contrast, LPS treatment resulted in increased miR-138 expression at D3 (1.1-fold), peak expression levels at D7 (1.2-fold), and a return to initial levels at D14 (Fig. 3B). Statistically significant differences in miR-138 levels were observed between osteogenic induced cells with or without LPS treatment at both intervals D7 and D14 ($P < 0.05$). Changes in miR-138 expression patterns under osteogenic or inflammatory conditions were further confirmed when cells were cultured in MSCGM-Cd medium (Appendix Fig. 2). To investigate the effect of inflammation on the expression of miR-138 and its potential target genes, PDL cells were treated with inflammatory modulators IL-6, LPS, or TNF- α . qRT-PCR analysis revealed that in response to inflammatory conditions, miR-138 expression was increased (1.9-fold after LPS exposure, $P < 0.01$) while OC and Runx2 expression was decreased as a result of treatment with each inflammatory mediator (1.4-fold after LPS exposure, $P < 0.01$ for OC, and 1.5-fold after LPS exposure, $P < 0.01$ for Runx2) (Fig. 3C). To further study how miR-138 regulates OC and bone-related gene expression, a synthetic miR-138 mimic was used to force miR-138 expression. Overexpression of miR-138 inhibited OC, Runx2, and Col 1A2 expression (2.9-fold for OC, 2.9-fold for Runx2, and 2.5-fold for Col 1A2; $P < 0.01$), while

increasing GSK3 expression 2.2-fold ($P < 0.05$) (Fig. 3D). These data indicate that miR-138 plays a significant role in inhibiting osteogenic differentiation of PDL cells under inflammatory conditions.

MicroRNA-138 Inhibits Periodontal Ligament Progenitor Differentiation through OC Expression

To determine whether LPS inhibits OC expression in PDL progenitors through miR-138, PDL cells were transfected with microRNA negative control or miR-138 inhibitor. As evidenced by Western blot, LPS treatment decreased OC levels, while knockdown of miR-138 increased OC protein (Fig. 4A). Moreover, LPS-induced downregulation of OC expression was partially reversed when miR-138 was knocked down with our miR-138 hairpin inhibitor (Fig. 4A). To determine the effect of OC on osteogenic differentiation in PDL cells, OC expression was knocked down using OC siRNA. The OC siRNA reduced OC expression by 50%. Knockdown of OC expression also resulted in a downregulation of Runx2 (2-fold, $P < 0.01$) and Collagen I expression (3.6-fold, $P < 0.01$) (Fig. 4B). To further confirm that OC modulates osteogenic differentiation in PDL cells, OC protein was added to the cell culture medium at a concentration of 10 $\mu\text{g/mL}$. Addition of OC protein promoted collagen matrix formation as demonstrated by Masson staining (Fig. 4C) and significantly enhanced mineralization (Fig. 4D, F). OC protein also significantly reversed the LPS-induced decrease in collagen matrix formation and mineralization (Fig. 4C, D, F, $P < 0.05$). LPS, miR-138 mimic, and OC siRNA inhibited osteoblast differentiation marker alkaline phosphatase activity while miR-138 hairpin inhibitor and OC protein addition enhanced alkaline phosphatase activity (Fig. 4E). Densitometric evaluation revealed that differences were statistically significant (Fig. 4G, $P < 0.05$). Knockdown of miR-138 or addition of OC protein partially rescued alkaline phosphatase activity in PDL cells subjected to LPS treatment ($P < 0.05$), confirming the role of miR-138 as an inflammation-related modulator of periodontal mineralization (Fig. 4E, G).

Discussion

In the present study, we have used a periodontitis animal model and a PDL cell culture system to study the role of miR-138 in

Figure 4. Inflammatory factors inhibit periodontal ligament (PDL) progenitor differentiation through miR-138–modulated osteocalcin (OC) expression. PDL cells were transfected with microRNA negative control or miR-138 inhibitor and cultured in osteogenic induction medium for 3 d with or without lipopolysaccharide (LPS) treatment. Bone serum albumin or OC protein was added to the culture medium. (A) Western blot analysis of the blocking effect of miR-138– on LPS-induced decrease in OC expression. β-Actin served as a loading control. (B) OC expression was knocked down using OC small interfering RNA (siRNA). The knockdown efficiency and bone protein expression were evaluated using quantitative reverse transcriptase polymerase chain reaction. * $P < 0.05$. Masson staining of collagen matrix (C) and alizarin red staining of mineralization (D). PDL cells were treated with either OC protein or LPS alone or a combination and cultured in osteogenic induction medium for 14 d. Mineral nodules were stained in red and collagen matrix in purple. (E) Alkaline phosphatase activity of PDL progenitors. Cells were transfected with miR-138 mimic (miR-138m), miR-138 inhibitor (miR-138i), or OC siRNA (siOC); treated with LPS or OC protein; and stained with NBT/BCIP. (F) Densitometry comparison of alizarin red–stained mineralized matrix formed by PDL cells treated with either OC protein or LPS alone or a combination, as illustrated in D. (G) Densitometric analysis of alkaline phosphatase levels in PDL cells transfected with miR-138 mimic (miR-138m), miR-138 inhibitor (miR-138i), or OC siRNA (siOC) and treated with LPS or OC protein, as described in E.

PDL cell differentiation during inflammation. In our periodontitis animal model, alveolar bone loss was accompanied by inflammation and an increase in miR-138 expression. Our PDL culture model confirmed that inflammatory factors such as IL-6, LPS, and TNF- α upregulate miR138 expression. Our culture model also revealed that miR-138 directly targets osteocalcin mRNA and inhibits PDL cell differentiation toward an osteogenic lineage. Together, these findings support the concept that proinflammatory conditions in the periodontium inhibit mineralization through miR-138 upregulation and its inhibition of osteocalcin.

Our studies suggest that miR-138 is a negative regulator of stem cell and progenitor differentiation. The role of miR-138 as an inhibitor of progenitor differentiation is supported by previous studies, which demonstrated that miR-138 plays a significant role in the regulation of stem cell differentiation (Eskildsen et al. 2011; Ye et al. 2012). Moreover, enforced expression of miR-138 has been demonstrated to inhibit both osteogenic and adipogenic differentiation (Eskildsen et al. 2011; Yang et al. 2011). Explaining the negative regulatory effect of miR-138 on osteogenesis under inflammatory conditions, our studies indicate that inflammatory factors upregulate miR-138 expression, and miR-138 in turn suppresses bone-related protein expression and bone formation. In addition, it has been demonstrated that induction of miR-138 by proinflammatory cytokines causes endothelial cell dysfunction (Sen et al. 2014), which may further contribute to the destabilization of the periodontal connective tissue environment as a result of miR-138 upregulation under proinflammatory conditions.

Although miR-138-mediated suppression of osteoblast differentiation has been reported (Eskildsen et al. 2011), OC is the first identified bone marker gene directly regulated by miR-138. Our bioinformatics analysis revealed a miR-138 targeting site located directly within the 3'-UTR of the OC mRNA. While canonical microRNA target sites are characterized by a perfect 7-nt complement to positions 2 to 8 of the microRNA (Dai and Zhou 2010; Shin et al. 2010), this predicted miR-138 targeting site in the OC mRNA is a noncanonical targeting sequence that lacks a perfect complement seed sequence. However, the binding affinity ($mfe = -31.9$ kcal/mol) between this target sequence and miR-138 was predicted based on the calculated minimum free energy. The binding efficiency of this target site was much stronger compared with previously reported miR-138 target sites (mfe ranging from -22.6 to -26.3 for canonical target sites and -21.8 to -25.1 for the noncanonical target sites) (Jin et al. 2011). In the present study, we confirmed a direct interaction between miR-138 and this predicted targeting site in OC mRNAs using dual luciferase report assays and provided further verification by gene expression analysis. Together, these studies indicate that miR-138 directly affects OC bone protein expression through direct and noncanonical interactions.

Our study demonstrated that miR-138 inhibits periodontal ligament progenitor differentiation by regulating OC expression and modulating alkaline phosphatase activity, an early marker of osteoblast differentiation. Even though OC is the most abundant noncollagenous bone matrix protein (Wolf 1996) and is often used as a bone formation marker (Granéli et al. 2014), the function of OC related to bone development is not entirely understood (Ducy et al. 1996). However, recent studies demonstrated that OC facilitates mineralization and increases bone formation (Idelevich et al. 2011). In the present study, miR-138 upregulation during inflammatory conditions reduced both OC and Runx2, a master transcription factor that regulates bone differentiation during periodontal development and homeostasis (Luan et al. 2009), suggesting that miR-138 inhibits periodontal osteogenesis through multiple pathways. Together, these studies

illustrate the role of microRNAs in the regulation of periodontal stem cell fate during inflammatory disease.

Author Contributions

X. Zhou, X. Luan, contributed to conception, design, and data acquisition, drafted and critically revised manuscript; Z. Chen, C. Wu, contributed to conception and data acquisition, drafted and critically revised manuscript; M. Francis, X. Lu, contributed to design and data acquisition, drafted and critically revised manuscript; G. Gopinathan, contributed to design and data analysis, drafted and critically revised manuscript; W. Li, contributed to design, data acquisition, and analysis, drafted and critically revised manuscript; S. Li, contributed to conception, data analysis, and interpretation, drafted and critically revised manuscript; T.G.H. Diekwisch, contributed to conception, design, data acquisition, analysis, and interpretation, drafted and critically revised manuscript.

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