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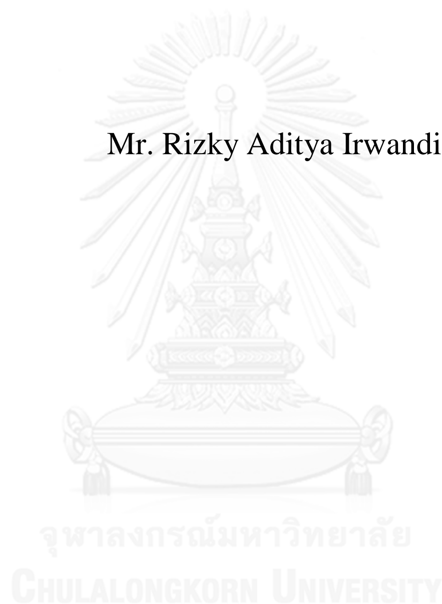
MICRORNA-302A-3P REGULATES PROSTAGLANDIN E_2-
INDUCED RANK LIGAND EXPRESSION IN HUMAN MANDIBULAR BONE-
DERIVED CELLS

Mr. Rizky Aditya Irwandi



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ริชกี้ อะดิตยา เออร์วานดิ : ไมโครอาร์เอนเอสามศูนย์สองเอ-สามพี ควบคุมการแสดงออกของแรงค์ไลแกนที่เหนี่ยวนำโดยพรอสตาแกลนดิน อีสอง ในเซลล์กระดูกจากขากรรไกรมนุษย์ (MICRORNA-302A-3P REGULATES PROSTAGLANDIN E_2-INDUCED RANK LIGAND EXPRESSION IN HUMAN MANDIBULAR BONE-DERIVED CELLS) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: อ. ทพญ. ดร. อัญชลี วัชรักษะ, 65 หน้า.

เนื่องจากแรงค์ไลแกนมีบทบาทที่สำคัญต่อกระบวนการสลายของกระดูก และการทำงานของไมโครอาร์เอนเอสามารถควบคุมการแสดงออกของยีน โดยเป็นการทำงานในระดับกระบวนการแปลรหัสจาก messenger RNA เป็นโปรตีน ไมโครอาร์เอนเอจึงเกี่ยวข้องกับกลไกที่สำคัญของกระบวนการทางชีววิทยารวมถึงกระบวนการสลายของกระดูกด้วย สำหรับการศึกษานี้ ต้องการศึกษาไมโครอาร์เอนเอที่ควบคุมการแสดงออกของแรงค์ไลแกน ในเซลล์กระดูกปฐมภูมิมนุษย์ โดยการใช้ inflammatory miRNAs PCR array (Qiagen) เมื่อใส่ PGE_2 ในเซลล์กระดูกปฐมภูมิมนุษย์ เพื่อเป็นการเลียนแบบภาวะอักเสบ จะทำให้มีการเพิ่มขึ้นของระดับแรงค์ไลแกน ในขณะที่การใส่ interferon-γ จะกดการแสดงออกของ RANKL จากการทดสอบโดยการใช้ PCR array ร่วมกับ qRT-PCR พบว่า ระดับของ miRNA-302a-3p จะลดลงเมื่อ RANKL เพิ่มขึ้น และระดับของ miRNA-302a-3p จะเพิ่มขึ้นเมื่อ RANKL ลดลง เมื่อใช้ TargetScanHuman 7.0 ทำนายเป้าหมายของ miRNA-302a-3p พบว่า miRNA-302a-3p น่าสามารถกดการแสดงออกของ PRKACB mRNA ซึ่งเป็นรหัสของ catalytic subunit ใน PKA signaling ตรงกับที่เคยมีรายงานก่อนหน้านี้ว่า กลไกการกระตุ้นการแสดงออกของ RANKL ใน HMBCs เกิดขึ้นผ่าน PKA signaling ดังนั้น miRNA-302a-3p จึงสามารถยับยั้งการแสดงออกของ RANKL โดยการยับยั้ง catalytic subunit เพื่อศึกษาบทบาทของ miRNA-302a-3p จึงทำการเพิ่มการแสดงออกของ miRNA-302a-3p ใน PGE_2-treated HMBCs ด้วยวิธี transfection ทำให้เพิ่มระดับของ miRNA-302a-3p ใน PGE_2-treated HMBCs ซึ่งปกติมี miRNA-302a-3p ในระดับต่ำ ส่งผลให้มีการลดระดับของ RANKL ใน HMBCs ในทางตรงกันข้าม IFNγ-treated HMBCs มีการแสดงออกของ RANKL ในระดับต่ำ พร้อมกับมี miRNA-302a-3p สูง จึงพบว่าการ transfection ด้วย miRNA-302a-3p inhibitor ทำให้เกิดการกระตุ้นการแสดงออกของ RANKL ใน HMBCs โดยสรุปแล้วพบว่าระดับของ miRNA-302-3p ส่งผลต่อการแสดงออกของ RANKL ใน HMBCs โดย miRNA-302a-3p สามารถกดการแสดงออกของ RANKL ใน HMBCs ผ่านทางการยับยั้ง cAMP/PKA signaling เมื่อการแสดงออกของ RANKL ถูกกดจึงอาจจะส่งผลต่อกระบวนการสลายของกระดูก.

สาขาวิชา ชีววิทยาช่องปาก ลายมือชื่อนิสิต ....

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RIZKY ADITYA IRWANDI: MICRORNA-302A-3P REGULATES PROSTAGLANDIN E_2-INDUCED RANK LIGAND EXPRESSION IN HUMAN MANDIBULAR BONE-DERIVED CELLS. ADVISOR: DR. ANJALEE VACHARAKSA, 65 pp.

Receptor activator of nuclear factor kappa-B (RANKL) plays an essential role in osteoclastogenesis. MicroRNAs (miRNAs) regulate gene expression at the post-transcriptional level in several biological processes including osteoclastogenesis. This study aimed to search for the candidate miRNA that regulates RANKL expression in human mandibular bone-derived cells (HMBCs) by using the inflammatory miRNAs PCR array (Qiagen). To mimic inflammation, PGE_2 treatment increases RANKL mRNA and protein in HMBCs whereas interferon-γ (IFNγ) suppresses RANKL expression. The miRNA profile of HMBCs in these conditions shows that miRNA-302a-3p, is down-regulated when RANKL increased, and up-regulated with RANKL suppression, and this result is confirmed by using qPCR. By using TargetScanHuman 7.0, the target of miRNA-302a-3p is predicted to be PRKACB mRNA that encodes catalytic subunit of PKA signaling. As RANKL expression in HMBCs is regulated through PKA signaling, miRNA-302a-3p therefore may play a role in this mechanism. To investigate its mechanism in RANKL expression, PGE_2-treated cells, that contain diminished level of miRNA-302a-3p, are transfected with miRNA-302a-3p mimic. When miRNA-302a-3p level is restored, HMBCs demonstrate decreased level of RANKL mRNA and protein in the presence of PGE_2. By contrast, IFNγ-treated cells show low level of RANKL with up-regulation of miRNA-302a-3p. Therefore, the transfection of miRNA-302a-3p inhibitor can suppress miRNA-302a-3p expression and increase RANKL mRNA and protein in HMBCs. Our results indicate that the level of miRNA-302-3p affects RANKL mRNA and protein expression in HMBCs. Since the target of miRNA-302a-3p may be PRKACB mRNA, when available, miRNA-302a-3p may decrease RANKL expression in HMBCs through suppression of cAMP/PKA signaling. The RANKL release by HMBCs may therefore influence the osteoclast differentiation and alveolar bone resorption during inflammation.

Field of Study:	Oral Biology	Student's Signature	....
Academic Year:	2015	Advisor' Signature	....

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Chapter 1: Introduction

In physiological condition, osteoclasts, osteocytes and osteoblasts involve in the bone resorption by interacting to each other to mediate osteoclastogenesis. Inflammatory cytokines, parathyroid hormone, growth factors or mechanical load-induced micro crack may stimulate osteocyte apoptosis, which attracts osteoclast precursors [1]. Osteoclast precursors receive a contact-dependent signal from receptor activator of nuclear κB ligand (RANKL), the membrane-bound protein expressed by osteoblasts, to differentiate into mature osteoclasts [2].

Periodontitis is a chronic inflammatory disease characterized by periodontal attachment loss and bone resorption. Inflammatory reaction against periodontal pathogen invasion results in tissue destruction. Inflammatory mediators, including tumor necrosis factor α (TNFα), interleukin 1β (IL-1β), interleukin 6 (IL-6) and prostaglandin E_2 (PGE_2), are detected in saliva and gingival crevicular fluid (GCF) of patients with periodontitis. Therefore, the inflammatory mediators can be used as salivary biomarkers of periodontal disease [3].

The mucosal cells can release inflammatory mediator including PGE_2 upon exposures to periodontal pathogens [4-6]. Released mediators attract circulating inflammatory cells including monocytes, mast cells and macrophage to accumulate at the site of infection. The increase of inflammatory mediators in periodontal tissue may regulate bone resorption through the release of receptor activator nuclear factor κB ligand (RANKL) by osteoblasts, gingival fibroblasts and periodontal ligament (PDL) cells [4, 6-8] and cells in monocyte lineage may differentiate into osteoclasts in a suitable condition with appropriate cell interaction [2, 9].

MicroRNAs (miRNAs) are single-stranded, small, non-coding RNAs, which inhibit gene expression at the post-transcriptional level. The mechanism of gene silencing is the complementary pairing of miRNA to targeted mRNA resulted in translation blockage and mRNA degradation [10]. Through this mechanism, miRNAs are involved in several biological processes including developmental, physiological, and pathological changes [11]. In bone metabolism, miRNA may act as promoter or inhibitor in osteogenesis or osteoclastogenesis [12-15]. In particular case resembling periodontitis such as arthritis [16], miRNA-146a was shown to inhibit the expression of TNFα and IL-1β [17], while in human primary monocytes, miRNA-187 inhibits IL-6 expression [18]. Interestingly, these recent reports demonstrate that microRNAs play a role in osteoclastogenesis through the inhibition of inflammatory mediator expression.

Level of PGE_2 in periodontal tissue is an important inflammatory biomarker relating to progression of periodontal disease. Accumulation of PGE_2 increases RANKL mRNA in many cell types including the primary bone cells [4, 6-8]. Since the epigenetic regulation was shown to essentially involve in RANKL expression in bone cells [19, 20], miRNAs might also have a role in RANKL up-regulation. In this study, we will investigate whether any miRNA(s) may be involved in RANKL expression in human mandibular bone-derived cells. This study may provide a basic knowledge for a novel approach of periodontal treatment by using the combination of miRNAs. The benefit of this approach is it may interrupt with the cellular signaling of inflammatory mediators and block disease progression.

1.1 Research question

Do microRNAs play a role in RANKL up-regulation in human mandibular bone-derived cells during PGE_2 stimulation?

1.2 Objectives and hypothesis

1.2.1 Objective 1

To demonstrate the up-regulation of RANKL in human mandibular bone-derived cells after PGE_2 stimulation

a. Hypothesis

PGE_2 treatment induces RANKL expression, both mRNA and protein, in HMBCs.

b. Experimental design

1. Human mandibular bone-derived cells were treated with 0.1µM PGE_2 for 24 h and total RNA was isolated afterwards. Reverse transcription followed by quantitative real time-polymerase chain reaction (qRT-PCR) were accomplished to estimate RANKL mRNA.
2. Human mandibular bone-derived cells were treated with 0.1µM PGE_2 for 24 h and the cell supernatant was collected afterwards. The soluble RANKL concentration was measured by ELISA.
3. Human mandibular bone-derived cells were treated with 0.1µM PGE_2 for 24 h and cell lysate was collected afterwards. The RANKL expression was detected by western blot.

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1.2.2 Objective 2

To search for the down-regulated miRNAs when RANKL is up-regulated during PGE_2 stimulation in human mandibular bone-derived cells

a. Hypothesis

Some miRNAs were down-regulated after PGE_2 stimulation in human mandibular bone-derived cells.

b. Experimental design

1. Human mandibular bone-derived cells were treated with 0.1μM PGE_2 for 24 h and total RNA was isolated afterwards. Reverse transcription followed by quantitative polymerase chain reaction (qRT-PCR) using Focused miScript miRNA PCR Array Human Inflammatory Responses & Autoimmunity (Qiagen, Hilden, Germany) were performed to identify the potential miRNAs. Using the online software of Qiagen Data Analysis Center (http://www.qiagen.com/th/shop/genes-and-pathways/data-analysis-center-overview-page/#), down-regulated miRNAs, showing more than 1.5 fold change in relative expression to untreated cells, were identified.
2. Human mandibular bone-derived cells were treated with 0.1μM PGE_2 for 24 h and total RNA was isolated afterwards. Reverse transcription followed by qRT-PCR were accomplished using PCR primer(s) specific to the sequences of the candidate microRNAs selected from microRNA PCR Array. Relative expression of the candidate miRNAs in treated cells was compared with untreated cells by using qRT-PCR.

1.2.3 Objective 3

To investigate the possible targets of the candidate miRNAs that is associated with PGE_2-induced RANKL up-regulation

a. Hypothesis

The candidate miRNAs may target genes that associate with the signaling pathway of PGE_2-induced RANKL up-regulation.

b. Experimental design

Using bioinformatics online software, TargetScanHuman 7.0 (http://www.targetscan.org), the target gene of the candidate miRNA was identified.

1.2.4 Objective 4

To overexpress or inhibit the candidate miRNA in human mandibular bone-derived cells and investigate RANKL mRNA and protein expression

a. Hypothesis

The candidate miRNA regulates RANKL expression in human mandibular bone-derived cells.

b. Experimental design

1. Human mandibular bone-derived cells were transfected by the candidate miRNA mimic or inhibitor and the viability of cells was assessed by MTT assay.
2. Human mandibular bone-derived cells were transfected by the candidate miRNA mimic or inhibitor before treatments and total RNA isolation followed by reverse transcription were accomplished

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afterwards. The level of the candidate miRNA was estimated by qRT-PCR to confirm the transfection.

3. Human mandibular bone-derived cells were transfected by the candidate miRNA mimic or inhibitor before treatments and total RNA isolation followed by reverse transcription were accomplished afterwards. The level of RANKL mRNA was estimated by qRT-PCR.
4. Human mandibular bone-derived cells were transfected by the candidate miRNA mimic or inhibitor before treatments and the cell supernatant was collected afterwards. The soluble-RANKL (sRANKL) concentration was measured by ELISA.
5. Human mandibular bone-derived cells were transfected by the candidate miRNA mimic or inhibitor before treatments and the cell lystes was collected afterwards. The membrane-bound RANKL expression was detected by western blot.

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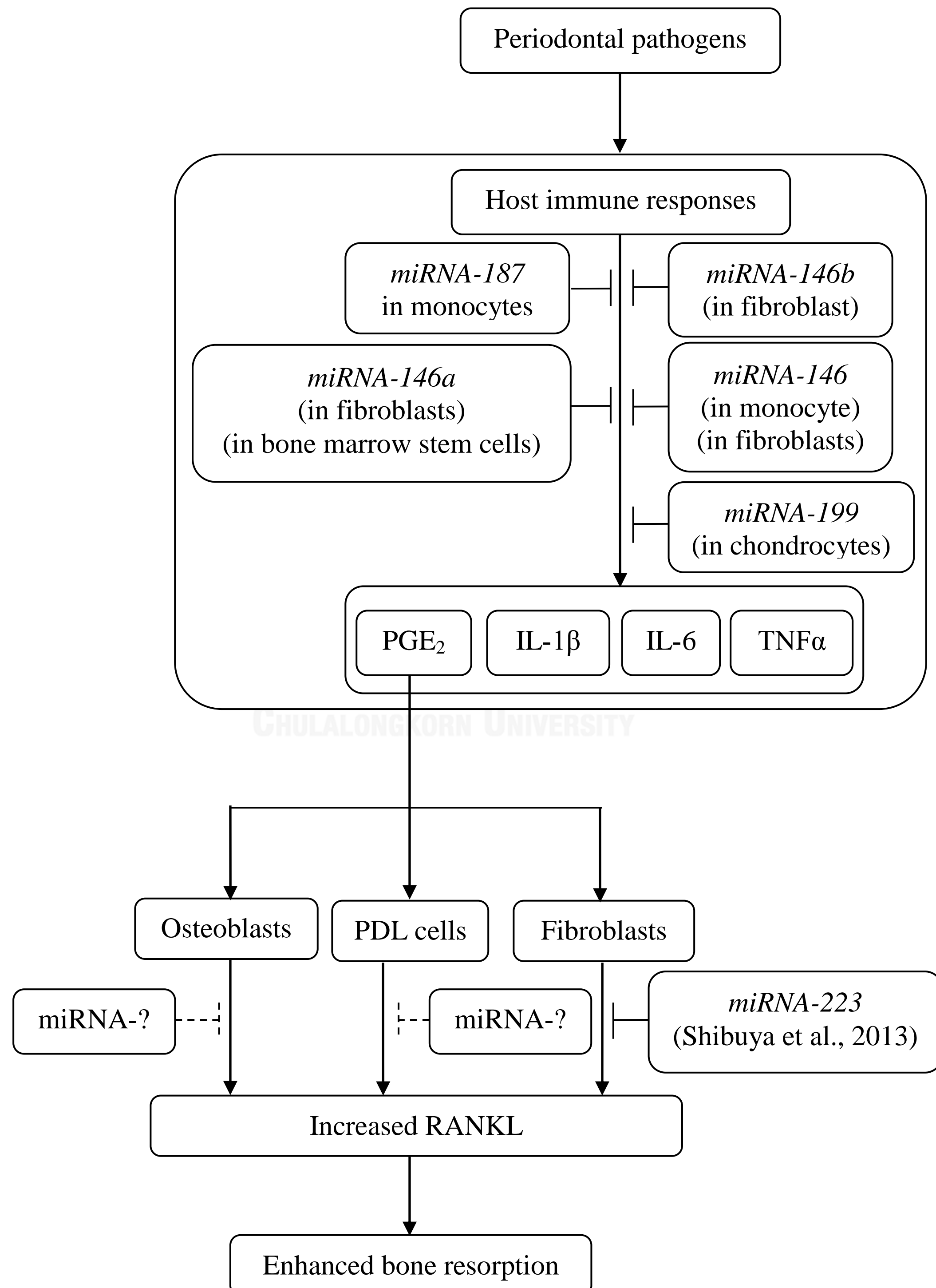
1.3 Expected benefit

The knowledge gained from this study will increase our understanding about the role of microRNAs in osteoblast as a response to PGE_2 induction and will give us an understanding of the biological response and the epigenetic mechanisms in human mandible-derived bone cells to PGE_2. Furthermore, it may suggest a possibility to use microRNAs to reduce osteoclastogenesis through targeting osteoblasts-derived RANKL expression.

1.4 Research design

Experimental research

1.5 Conceptual framework



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Chapter 2: Literature review

2.1 Physiologic bone resorption in bone remodeling

Bone resorption is a physiological event of bone metabolism. In physiologic bone remodeling, osteocytes, osteoclasts, and osteoblasts function to balance the process of bone resorption and bone formation. Bone remodeling process may be initiated due to several factors including hormones, mechanical load, growth factors, and cytokines [1]. Bone fatigue induces osteocyte apoptosis in the vicinity of micro-cracks followed by osteoclast recruitment [21]. Non-apoptotic osteocytes surrounding apoptotic osteocytes provide pro-osteoclastic signals which recruit osteoclasts to the microcrack region [22]. However, inadequate mechanical stimulation apparently induces osteocytes apoptosis in mice and also triggers osteoclast recruitment toward this apoptotic site followed by bone loss [23].

Role of osteoclasts in bone remodeling involves bone resorption process. Osteoclast derives from the monocytes/macrophage hematopoietic lineage [24] and its function in osteoclastogenesis is regulated by several transcription factors which are PU.1, c-Fos, MITF and NFATc1 [24]. Conditional knockout of PU.1 in mice resulted in osteopetrosis due to the absence of osteoclasts [25], similar to the osteopetrosis in c-Fos knockout mice [26]. MITF inhibits apoptosis therefore plays a role in osteoclast survival [27]. Embryonic stem cells lacking NFATc1 were defective of osteoclast formation relative to the wild type [28]. In summary, the transcription factors show an orchestral mechanism which is crucial in osteoclastogenesis instead of regulating osteoclastogenesis as a single component

[29]. Moreover, osteoclast precursors express colony-stimulating factor-1 receptor (c-Fms) and RANK whose ligands are M-CSF and RANKL, respectively. At the early stage, osteoclast precursors express only c-Fms while at the later stage,osteoclast precursors express both c-Fms and RANK [2].

Osteoblasts also have a role in osteoclastogenesis. Parathyroid hormone (PTH) [30] and mechanical strain [31] induce osteoblasts to release MMP-13, which is responsible for collagen degradation in bone remodeling [32]. Osteoblast mediates collagen degradation results in the exposure of an arginylglycylaspartic acid (RGD) for osteoclasts binding sites [33]. PTH also induces osteoblasts to release monocyte chemoattractant protein-1 (MCP-1) that recruits preosteoclasts into remodeling area [34]. In addition, osteoblasts express RANKL which binds to the receptor, RANK, on osteoclasts precursor cells and drives osteoclast differentiation [35]. Mice with conditional knockout of RANK [36] or RANKL [37] showed no osteoclast differentiation by the absence of cells positive for staining of tartrate-resistant acid phosphatase (TRAP).

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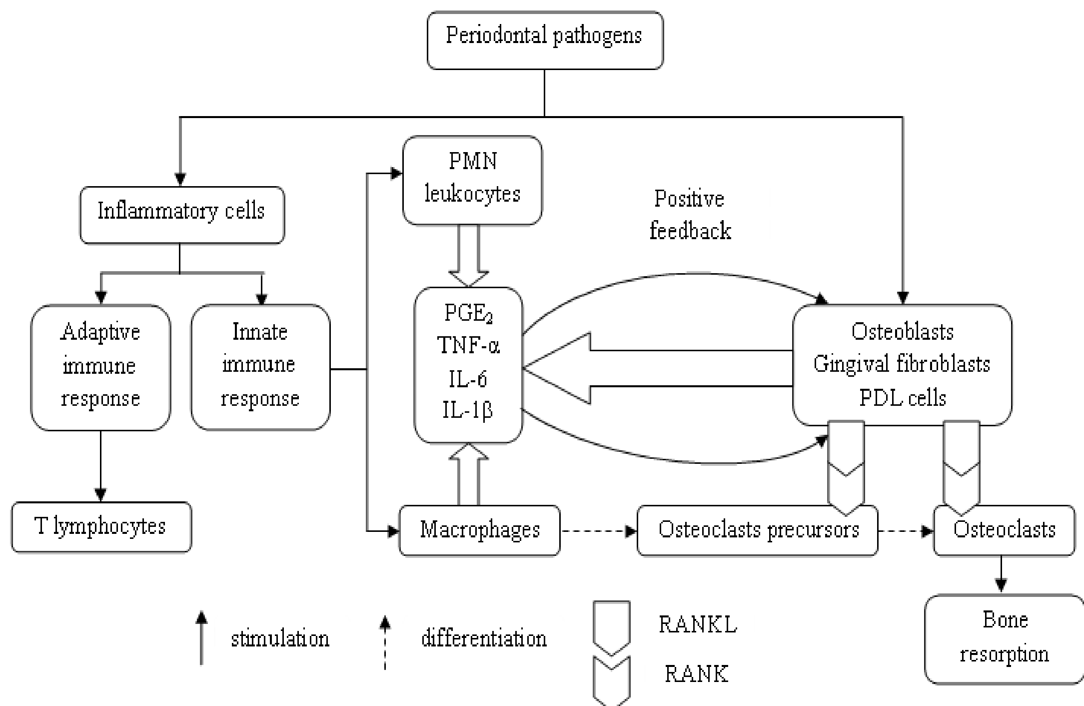
2.2 Inflammatory mediator-induced bone resorption in periodontitis

Periodontitis is a chronic inflammatory disease of periodontal tissue. The characteristics of this disease include attachment loss [38], and alveolar bone loss in periapical radiographs [39]. *Porphyromonas gingivalis* (*P. gingivalis*), *Aggregatibacter actinomycetemcomitans* (*A.actinomycetemcomitans*) and *Treponema denticola* (*T.denticola*) are periodontal pathogens associated with periodontal disease [40]. Periodontal pathogens and their virulent products interact with numerous cell types and activate host defense mechanisms. Tissue

destruction and alveolar bone resorption is a result of inflammatory responses against periodontal pathogens [41].

Studies of the inflammatory cascades in periodontitis have been reported. One of them concerns is involved several inflammatory mediators in the pathogenesis of periodontitis (Figure 2.1) [41]. This process is divided into five parts as follows: (1) the oral biofilm provides innate immunity, in which substances of pathogens such as lipopolysaccharide (LPS) are recognized, leading to mast cell stimulation by releasing vasoactive amines and TNFα, which induce resident cells of gingival tissue to release inflammatory mediators; (2) released inflammatory mediators summons inflammation in the tissue; (3) as the inflammatory mediators are released, PMN leukocytes become stimulated to release matrix metalloproteinase (MMP) followed by gingival tissue destruction; (4) antigen-presenting cells also recognize substances of pathogens and activate Th0 cells to evolve into several types of Th cells such as Th1, Th2, Treg and Th17. These Th cell groups produce several cytokines that can increase or inhibit inflammatory mediator production; (5) cytokines and PGE_2 are responsible for the ratio of RANKL, which promotes osteoclast differentiation and enhances bone resorption, and osteoprotegerin (OPG).



Figures 2.1: Summary of host response in periodontitis (modified from Yucel-Lindberg and Bage 2013) [41]

Inflammatory mediators such as TNFα, IL-1, IL-6, and PGE_2 are involved in the pathogenesis of periodontitis as shown in GCF and saliva of periodontitis patients at high level [42]. Pathogens, endotoxins and their related substances stimulate TNFα production in monocytes, macrophages or T cells. TNFα may lead to bone destruction by increasing osteoclast differentiation/proliferation in the presence of RANKL [43]. The TNF family cytokine RANKL induces the differentiation of osteoclasts in the presence of macrophage colony stimulating factor (M-CSF) and activates TNF receptor associated factor 6 (TRAF6), c-Fos and calcium signaling pathways, which are important for the induction and activation of nuclear factor of activated T cells (NFAT) c1, a key transcription factor in the process of osteoclastogenesis [2, 28]

Interleukin-1 beta (IL-1β) is cytokine that is also involved in periodontitis [44]. It enhances differentiation of osteoclast precursors and induces RANKL in several cell types, including osteoblasts, gingival fibroblasts and periodontal ligament fibroblasts [4, 6-8]. IL-1, which binds to two different receptors i.e. type 1 receptor (IL-1R1) and the receptor accessory protein (IL-1RAcp), recruits MyD88, IL-1 receptor-associated kinase (IRAK) and TRAF6 to mediate ERK and p38 MAPK signaling pathways [45]. RANKL expression is associated with both signaling pathways [46].

In the periodontal tissue, IL-6 is highly produced by leukocytes, macrophages, periodontal ligament cells and gingival fibroblasts [47]. IL-6 is up-regulated in response to IL-1, TNFα, virus, bacterial toxins, and LPS [48]. When expressed, IL-6 can induce the expression of RANKL mRNA and protein in mouse calvarialosteoblasts [49], and in fibroblast-like synoviocytes of rheumatoid arthritis patients [50] through the JAK/STAT signaling pathway.

PGE_2 is an inflammatory mediator that involves in vasodilatation and bone resorption [51]. PGE_2 is one the products of arachidonic acid conversion, which is released by cyclooxygenase-2 (COX-2). It has four activating receptors, E prostanoid (EP) 1, 2, 3 and 4 [52], but only EP2 and EP4 activating receptors have a role in PGE_2-induced bone resorption [53]. RANKL expression of mouse calvarial osteoblasts was increased after PGE_2 treatment. IL-6 not only induced RANKL expression in mouse calvarialosteoblasts but also induced PGE_2 expression because mouse calvarialosteoblasts with IL-6 treatment showed higher expression of COX-2, EP2, and EP4 protein expression than that of osteoblasts

without treatment [54]. Therefore, co-stimulation of IL-6 and PGE_2 may enhance bone resorption in mouse calvarial osteoblasts.

2.3 PGE_2-induced RANKL expression in osteoblasts and signaling pathway

Expression of RANKL is essential in osteoclastogenesis. RANKL is encoded by the TRANCE/TNFSF11 gene. When this gene was deleted, RANKL-knockout mice suffered from osteopetrosis due to the absence of TRAP-positive osteoclasts [55]. RANKL is expressed by several cell types including lymphocytes [56], fibroblasts [57], bone marrow stromal cells [35], chondrocytes [58], osteocytes [59] and osteoblasts [35]. Besides cytokines and PGE_2, PTHs and vitamin D3 were reported to stimulate RANKL expression. PTHs stimulation increased the number of osteoclasts in proximal tibial metaphyses of mouse due to increased RANKL expression *in vivo* [60]. Vitamin D3 stimulated RANKL expression by targeting a vitamin D responsive element (VDRE) in the RANKL gene promoter in a co-culture system between SaOS2 human osteosarcoma cells and human peripheral monocytic cells [61].

In periodontal tissue, *P. gingivalis* LPS stimulates PGE_2 formation in gingival fibroblasts [62] and upregulates RANKL/OPG expression ratio in primary human gingival fibroblasts [5]. In primary human PDL cells, PGE_2 and IL-1 increase RANKL expression *in vitro* [7] and PGE_2 formation due to mechanical stress increases RANKL [63]. Meanwhile, *P. gingivalis* and *T. denticola* stimulation increase PGE_2-induced RANKL expression in mouse calvarial osteoblasts [4]. Moreover, IL-1-induced PGE_2 expression increases RANKL expression in mouse calvarial osteoblasts and MG-63, osteoblastic

osteosarcoma cell line [8]. Table 2.1 shows the the role of PGE_2 in the up-regulation of RANKL expression in osteoblasts from several previous reports.

Table 2.1: Summary of PGE2-induced RANKL up-regulation in osteoblasts

Findings	Inducer	Cell type	References
PGE_2 stimulates RANKL expression in osteoblasts through EP4 involvement	-	Primary mouse calvarial osteoblasts	[64]
Pathogen-induced RANKL expression in osteoblasts is mediated by PGE_2 expression	*Porphyromonas gingivalis* *Treponema denticola* *Treponema soncranskii*	Primary mouse calvarial osteoblasts	[4]
IL-1-induced PGE_2 expression induce RANKL expression in osteoblasts	IL-1β	MG-63: human osteoblastic osteosarcoma cell line Primary mouse calvarial osteoblasts	[8]
PGE_2 stimulates more RANKL expression in osteoblasts than that in hPDL cells	-	Human osteoblastic cell line Human PDL cells	[65]

The signaling pathway of RANKL expression depends on the inducer and induced cell type. TNFα and IL-1β stimulated RANKL expression in bone marrow stromal cells through MKK3/6-p38 MAPK signaling pathways *in vitro* [66]. JAK/STAT mediated RANKL expression of IL-6-induced mouse calvarial osteoblasts [49] and fibroblast-like synoviocytes *in vitro* [50]. IL-33 stimulated RANKL expression through a mechanism dependent on the ERK and p38 MAPK pathways in mouse calvarial osteoblasts *in vitro* [46]. Combinatorial action between cAMP/PKA and calcineurin/NFAT pathways mediated PTH related protein (PTHrP)-induced RANKL expression in mouse calvarial osteoblasts [67].

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2.4 The role of microRNA in bone remodeling

MiRNAs are single-stranded small non-coding RNAs which targets coding RNAs, resulted in silencing the targeted RNAs. The miRNAs undergo base-pairing to the 3' untranslated region (3'UTR) of targeted mRNA then repress translation process by blocking mRNA translation and degrading the mRNA [10]. Through that mechanism, miRNAs may play a role in several biological cascades including developmental, physiological, and pathological events [11].

In bone metabolism, some miRNAs are involved as stimulatory or inhibitory factors of osteogenesis (Table 2.2). *miRNA-194* stimulates osteoblast differentiation by targeting chicken ovalbumin upstream promoter-transcription factor II (*COUP-TFII*) *in vitro* [15]. In mouse mesenchymal stem cells cultured in osteogenic medium, *miRNA-194* is up-regulated, while COUP-TFII is suppressed, by high levels of runt-related transcription factor 2 (RUNX2) [68]. This result is reversed by the addition of anti-*miRNA-194* [15]. *MiR-542-3p*, on the other hand, inhibits osteoblast differentiation and proliferation by targeting *BMP-7* and decreasing RUNX2-, type 1 collagen-, osterix-, and osteocalcin-specific mRNAs in mouse calvarial osteoblasts. *miRNA-542-3p* also inhibits bone formation and decreases the rate of mineral apposition in wild type and ovariectomized Balb/c mice [69].

MiRNAs also play a role in osteoclastogenesis. Protein inhibitor of activated STAT 3 (*PIAS3*) is a negative regulator for osteoclastogenesis by inhibiting transcriptional activity of microphthalmia-associated transcription factor (MITF) [70, 71] and *miRNA-9718* targets PIAS3 during osteoclast differentiation [14]. Transfection of *pre-miRNA-9718* into RAW 267.7 cells leads

to lower expression of PIAS3, but higher expression of osteoclastogenic markers such as nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 1 (NFATc1), c-Fos, MITF, and nuclear factor kappa B (NFκB). A higher number of TRAP-positive cells after *pre-miR-9718* transfection also indicate an increase in osteoclastogenesis. Conversely, intravenous injection of *miR-9718* inhibitor in both wild-type and ovariectomized mice leads to restored bone mineral density [14]. In contrast to *miRNA-9718*, *miRNA-34a* plays a role in the inhibition of osteoclastogenesis. In an *in vivo* model, *miRNA-34a* has been shown to target *TGIF2* [12], which leads to the repression of TGFβ-responsive genes [72] and the inhibition of osteoclast differentiation [73]. *miRNA-34a* knockout mice exhibit decreased *TRAP* mRNA and TRAP-positive cell number, but increased bone quantity, when compared to wild type mice [12]. *MiRNA-34a* is significantly down-regulated after an increase in RANKL or after treatment with rosiglitazone, a drug to induce bone loss. Further analysis shows that transfection of synthetic *pre-miRNA-34a* mimics into human peripheral mononuclear cells (PBMC) led to decreased TRAP-specific mRNA and TRAP-positive cell number. In contrast, transfection of an inhibitor of *pre-miRNA-34a* resulted in increased TRAP-specific mRNA and TRAP-positive cell number indicating *miRNA-34a* suppressed osteoclastogenesis *in vitro* [12]. Delivery of *miRNA-34a* using chitosan nanoparticles to the ovariectomized and two-cancer-cell-cardiac-injection mouse models results in higher bone quantity and lower bone metastatic rate compared with control ovariectomized mice [12].

Several miRNAs have been reported to be involved in the regulation of RANKL expression in various cell types, as summarized in Table 2.2. In cancer

research, *miRNA-335* has been shown to inhibit small cell lung cancer (SCLC) bone metastases by targeting insulin-like growth factor-1 receptor (*IGF1R*) and *RANKL* [74]. Xenografts of the SCLC SBC-5, but not SBC-3, cell line triggers the formation of osteolytic bone lesions in non-obese diabetic/severe-combined and immune-deficient (NOD/SCID) *IL2Rγ*^{null} mice that lack mature T cells, B cells, and functional NK cell [75]. A microarray study using the μParaflo®Microfluidic Biochip Microarray shows that 14 out of 833 miRNAs are down-regulated in SBC-5 mice when compared with SBC-3 mice. This phenotype is thought to arise from the lack of *miRNA-335* in SBC-5. Consistent with this, SBC-5 mice transfected with *miRNA-355* show lower levels of both IGF1R and RANKL protein expression and lower incidence of bone lesions [74].

The level of RANKL expression appears to be associated with osteoclastogenesis. In giant cell tumour of bone, *miRNA-106b* has been shown to inhibit osteoclastogenesis and osteolysis by directly targeting *RANKL*. Transfection of *miRNA-106b* into both giant cell tumour stromal cells and MG63 osteosarcoma cells directly targets *RANKL* mRNA, as shown by luciferase reporter assay, and reduces osteoclastogenesis *in vivo* [76]. In contrast, glucocorticoid treatment induces secondary osteoporosis with bone loss and fragility fracture [77]. In MC3T3-E1 cells and an *in vivo* model, glucocorticoid treatment increases the level of RANKL while decreasing the level of *miRNA-17/20a*. The bone loss associated with glucocorticoid treatment seems to be partly rescued by the addition of *miRNA-17/20a* [78]. These observations point to the role of miRNAs in RANKL regulation, which in turn affects bone metabolism.

Table 2.2: The miRNAs that are involved in bone metabolism and RANKL expression

miRNAs	Functions	Cell/tissue types	References
miR-194	Stimulation of osteogenesis through inhibition of COUP-TFII, RUNX2 suppressor protein.	primary mouse bone marrow stromal cells MC3T3-E1 cells *In vitro*	[15]
miR-542-3p	Inhibition of osteogenesis through suppression of bone morphogenetic protein (BMP-7)	Mouse calvarial osteoblasts *In vitro* Balb/c mice *In vivo*	[69]
miR-9718	Stimulation of osteoclastogenesis through inhibition of PIAS3	C57BL/6-derived bone marrow cells *In vitro* Wild type and ovariectomized C57BL/6 mice *In vivo*	[14]
miR-34a	Inhibition of osteoclastogenesis through suppression of transforming growth factor ß-induced factor II (TGIFII)	C57BL/6J-derived bone marrow cells *In vitro* *miR-34a* transgenic C57BL/6J mice *In vivo*	[12]
miR-335	Inhibition of small cell lung cancer bone metastases by suppression of IGF1R and RANKL expression	SBC-5: small cell lung cancer cell line *In vitro* C57BL/6J mice *In vivo*	[74]
miR-17 *miR-20a*	Inhibition of glucocorticoid-induced osteoclastogenesis by suppressing RANKL expression	Mouse calvarial osteoblasts: primary cells, MC3T3-E1 cell line, calvarial bone *In vitro* C57BL/6J mice *In vivo*	[78]
miR-106b	Inhibition of osteoclastogenesis and osteolysis through RANKL repression	Giant cell tumour *In vitro* Wild type and ovariectomized C57BL/6 mice *In vivo*	[76]

MiRNAs play a role in inflammatory mediator-induced osteoclastogenesis by targeting genes that encode inflammatory mediators or by ultimately affecting the release of these mediators and we summarized the relevant studies of it in Table 2.3. Rheumatoid arthritis shows chronic inflammatory characteristics similar to periodontitis [16]. The presence of TNFα and IL-1β in rheumatoid arthritis can induce *miRNA-146a* expression in synovial fibroblasts [79]. The NFκB-dependent induction of *miRNA-146a* targets *TRAF6* and *IRAK1* [80], which leads to the inhibition of TNFα, IL-1β, and IL-6 in human gingival fibroblasts [81]. IL10-induced *miRNA-187*, on the other hand, decreases IL-6 production in primary human monocytes *in vitro* [18]. The consequences of miRNA modulation that affects inflammatory mediator release can be inducer-dependent and cell type-dependent. For instance, bleomycin-induced senescent HCA2 cells express higher *miRNA-146a* compared with untreated cells, resulting in reduced IL-6 and IL-8 production through IRAK1 inhibition [82]. PgLPS-induced *miRNA-146* expression in human primary gingival fibroblasts, on the other hand, suppresses not only IL-6 but also IL-1β and TNFα via IRAK1 inhibition [81]. Moreover, miRNA can also be induced by cytokines affecting other cytokine release. For example, IL-10-induced *miRNA-187*, which suppressed TNFα and IL-6 through targeting TNFα-specific and NFκB inhibitor zeta-specific mRNA, respectively in human primary monocytes [18]. The effects of constitutive miRNA expression can vary between cell types. Fibroblast-like synoviocytes from rheumatoid arthritis patients express *miRNA-346*, which reduces LPS-induced TNFα production by destabilizing TNFα-specific mRNA [83]. In SJL mice-derived bone

marrow stem cells, on the other hand, *miRNA-146a* reduces PGE_2 production by targeting PGE_2 synthase 2-specific mRNA [84]. In human primary chondrocyte, *miRNA-199* also reduces PGE_2 production by targeting COX-2-specific mRNA [85]. However, constitutive miRNA expression can also modulate cytokine release constitutively and inductively, such as when *miRNA-125b* suppresses both constitutive and LPS-induced TNFα production in human primary umbilical monocytes [86].

Table 2.3: MiRNAs suppress inflammatory mediators released by different cell types

MiRNAs	Functions	Inducer	Cell/tissue type	References
miRNA-146	Suppression of IL-6 & IL-8 via targeting IRAK1	Senescent induction	Human foreskin fibroblasts cell line, HCA2 *In vitro*	[82]
	Suppression of IL-6, IL-1β and TNFα via targeting IRAK1	*P. gingivalis* LPS	Human primary gingival fibroblasts *In vitro*	[81]
miRNA-346	Suppression of LPS-activated TNFα production via tristetrapolin stabilization of TNFα-specific mRNA	-	Human primary rheumatoid arthritis fibroblast-like synoviocytes *In vitro*	[83]
miRNA-187	Suppression of TNFα via targeting TNFα-specific mRNA Suppression of IL-6 via targeting NFκB inhibitory zeta	IL-10	Human primary monocytes *In vitro*	[18]
miRNA-199	Suppression of PGE_2 synthesis via targeting COX-2 mRNA	-	Human primary chondroytes *In vitro*	[85]
miRNA-125b	Suppression of constitutive or LPS-stimulated TNFα production	-	Human primary umbilical monocyte *In vitro*	[86]
miRNA-146a	Inhibition of PGE_2 synthesis via targeting PGE_2 synthase 2 mRNA	-	SJL mice-derived bone marrow stem cells *In vitro*	[84]

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2.5 Clinical application of microRNA

The study of miRNA to use in therapeutic approach is still in progress, especially in miRNA-delivery systems. The miRNA stability inside the body, miRNA efficacy to arrive at targeted area as well as the off-target effect avoidance, miRNA administration for individuals and miRNA-delivery agent augmentation are the main concern for researcher to generate the most suitable delivery system in miRNA treatment [87]. One of delivery systems is using antibody-conjugated nanoparticles (NPs). As neuroblastoma expresses high level of GD_2 antigen, anti-GD_2 conjugated NPs delivers miRNA-34 precisely to the targeted area after intravenous administration of the neuroblastoma-induced mice. By this system, miRNA-34a reduces the tumor growth *in vivo* [88]. Only a few reports about the administration of microRNA are shown, however, the use of oligonucleotides has been licensed by the FDA to distribute commercially. Formivirsen can work through base-pairing to cytomegalovirus-specific RNA therefore intravitreal administration of phosphorothioate oligonucleotide fomivirsen can reduce CMV-mediated retinitis in patient [89]. In addition, Pegabtanib, an aptamer, can specifically bind to vascular endothelial factor (VEGF) and block age-related macular degeneration [90].

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Chapter 3: Materials and methods

3.1 Human mandibular bone-derived cell isolation and culture

Both human mandibular bone-derived cells and normal oral keratinocyte spontaneous immortalized (NOK-SI) were cultured and characterized as reported in our previous study [91]. All experimental protocol, were reviewed and approved by the local Research Ethics Committee (HREC/DCU 2015-042). Bone tissue was collected from patients with written consent in the Oral Surgery Departement, Chulalongkorn University Dental Hospital. Explanted cells were maintained in bone growth media: Dulbecco's Modified Eagle's Medium DMEM supplemented with 15% heat-inactivated fetal bovine serum (FBS), 1% L-glutamine and 1% Antibiotic-Antimycotic at 37°C in a humidified atmosphere of 5% CO_2 for 2-3 weeks. During incubation, the medium was changed at 24 hours and thereafter at 2 days intervals. Confluent cells were subcultured using 0.25% Trypsin-EDTA and plated at 1:3 ratio. Cells from passages 3–8 were used in the experiments.

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3.2 Normal oral keratinocyte spontaneous immortalized cell culture

Normal oral keratinocyte spontaneous immortalized (NOK-SI) were cultured and characterized as reported in our previous study [91]. NOK-SI cell line was derived from the retromolar area of a healthy volunteer and NOK-SI cells appear to be spontaneously immortalized through escaped cell senescence and became infinite number of passages [91, 92]. NOK-SI cells were maintained at 37°C in a humidified atmosphere of 5% CO_2 atmosphere in keratinocyte complete medium

(KCM) including defined keratinocyte serum-free medium (dk-SFM; Gibco) with growth supplement and 100 IU/ml Penicillin, 100 µg/ml Streptomycin, and 0.25 µg/ml Amphotericin B for 3 days. The 70% confluent cells were subcultured using 0.25% Trypsin-EDTA and plated at 1:4 ratio. Cells from passages 40–45 were used in the experiments.

3.3 Cell treatment

NOK-SI cell line were seeded at $2x10^5$ cells/well in 6-well plate overnight and challenged with 2.5 µg/mL *Porphyromonas gingivalis* lipopolysacharrides (PgLPS) (BioLegend, San Diego, CA, USA) for 24 h. The supernatant was collected and incubated with 1µg/ml anti-interferon γ (IFNγ) antibody (BioLegend, San Diego, CA, USA) for 30 minutes before being proceeded to PGE_2 treatment. The used concentration and time point was based on recommendation of manufacturer. Human mandibular bone-derived cells were seeded at $3x10^5$ cells/well in 6-well plate overnight and treated with 50:50 proportion of NOK-SI cell line supernatant or KCM and bone serum-free media (bone growth media without FBS but 1% Lactalbumin) containing 0.1 µM PGE_2 (Cayman Chemical Corp; Ann Arbor, MI, USA) and 1 ng/µl recombinant human IFNγ (rhIFN) (BioLegend, San Diego, CA, USA) for 24 hr. The used concentration of PgLPS, proportional combination of NOK-SI supernatant or growth media and bone serum-free media corresponded to our previous work [91] while PGE_2 concentration used in this study was according previous report [7].

3.4 Cell transfection

Shortly before transfection, human mandibular bone-derived cells were seeded at $3x10^5$ cells/well in 6-well plate. The cells were then transfected with 2.5 nM synthetic hsa-miR-302a-3p mimic, 25 nM hsa-miR-302a-3p inhibitor or 2.5 nM mimic control (Qiagen, Hilden, Germany) in bone growth media without FBS following the protocol of manufacturer. After 6 hr of transfection, the medium was replaced with bone growth medium. Human mandibular bone-derived cells were cultured for 24 hr and then subjected to cell treatment.

3.5 Cell viability assay

The viability of human mandibular bone-derived cells after transfection was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; USB Corporation, Cleveland, OH, USA) assay. The cells were seeded at $1x10^5$ cells/well in 24-well and subjected to cell transfection. After 6 hr of transfection, the medium was replaced with bone growth medium. After 24 hr, the medium was replaced with 0.5 ml MTT solution and incubated for 30 min at 37 ^{o}C. The formazan crystals were dissolved in a solubilization/stop solution containing 1:9 glycine buffer (0.1 M glycine/ 0.1 M sodium chloride pH 10) and dimethylsulfoxide (DMSO). The optical density was measured at 570 nm using a microplate reader (ELx800; BioTek, Winooski, VT, USA).

3.6 Quantitative real-time polymerase chain reaction (qRT-PCR)

All miRNA experiment was conducted using reagent from Qiagen, Hilden, Germany. The total RNA was isolated from human mandibular bone-derived cells using miRNeasy mini kit and reverse transcribed using miScript II RT kit (Qiagen, Hilden, Germany). Pooled cDNA (1µg) were collected from five patients at the same amount and miRNA expression profile of the treated cells was examined using human inflammatory response and autoimmunity miScript miRNA PCR array (MIHS-105Z) containing 84 inflammatory miRNAs primers according to the protocol of manufacturer. The data was analyzed by miScript miRNA PCR array data analysis online software at http://www.qiagen.com/th/shop/genes-and-pathways/data-analysis-center-overview-page/#. The miRNA expression of differentially expressed miRNAs from miRNA PCR array as well as after cell transfection and treatment with PGE_2 only or both PGE_2 and IFNγ were examined by qRT-PCR. The cDNA from extracted human mandibular bone-derived cells of each patient was used to assess miRNA expression using Quantitect SYBR green PCR master mix with miScript universal primer as reverse primer and hsa-miRNA-302a-3p: 5'-UAAGUGCUUCCAUGUUUUGGUGA-3' as forward primer. The results were normalized to the level of RNU6B (NR_002752) expression. To assess RANKL mRNA in human mandibular bone-derived cells after cell transfection and treatment with PGE_2 only or both PGE_2 and IFNγ, total RNA was extracted using Isol-RNA lysis reagent (S Prime Incorporation., Gaithersburg, MD, USA and cDNA was generated using Improm-II^{TM} reverse transcription reaction mix (Promega, Madison, WI, USA) followed by quantification using SYBR green

detection system (FastStart Essential DNA Green Master kit; Roche Diagnostic, IN, USA) with gene-specific primer (Sigma-Aldrich) as described previously [91] and GAPDH was used as an internal control. The relative expression of both miRNAs and mRNA were analyzed using $2^{-\Delta\Delta Ct}$ method [93].

3.7 MicroRNA target prediction

Base-pairing for the binding of candidate miRNA to the targeting sequence of genes was predicted using the TargetScanHuman v.7.0 (http://www.targetscan.org) [94]. Genes involved in PKA signaling was selected as previous study demonstrated that this intracellular signaling mediates PGE_2-RANKL up-regulation [7].

3.8 Enzyme-linked immunosorbent assay (ELISA)

Cell supernatants were collected from human mandibular bone-derived cells after transfection and treatment with PGE_2 only or both PGE_2 and IFNγ. Soluble-RANKL (sRANKL) protein was measured by ELISA according to the instruction of manufacturer. In brief, wells of ELISA microplate were coated overnight with 1μg/ml monoclonal mouse anti-human sRANKL. After blocking using 1% BSA in PBS, 100 μl samples were added into the wells and detected with 0.5 μg/ml biotinylated antigen-affinity purified goat anti-human sRANKL, avidin peroxidase as well as ABTS substrate (All from Peprotech, Rocky Hill, NJ, USA). The plates were read in microplate reader (BioTek, Winooski, VT, USA) at wavelength 450 nm.

3.9 Western blot

RANKL protein from the cell lysates of human mandibular bone-derived cells after transfection and treatment with PGE_2 only or both PGE_2 and IFNγ were assessed by western blot. Protein lysates were prepared with radioimmunoprecipitation assay (RIPA) buffer (150 mM NaCl, 1% NP40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris; pH 8.0) containing 1 mM PMSF (Merck Millipore, Darmstadt, Germany). Protein samples (40 μg) were fractionated by 12.5% SDS-PAGE. RANKL 2 μg/ml (Peprotech, Rocky Hill, NJ, USA) or an internal control β-actin (Cell Signaling, Danvers, MA, USA) (1:1000) was applied as primary antibody. Immune complex was detected with secondary antibody: horseradish peroxidase-conjugated anti-rabbit IgG (Enzo, Farmingdale, NY, USA) (1:2000) as well as the enhanced chemiluminescence (ECL) Western blot System (Thermoscientific, Rockford, IL, USA) then exposed to CL-Xposture film (Pierce, Thermal Scientific). Band density was quantified using ImageJ.

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3.10 Data analysis

The Saphiro-Wilk was used to confirm the normality of data. The data with normal distribution were analyzed by independent samples t-test for comparison between two groups and one-way ANOVA with Tukey HSD post hoc test for multiple comparisons while data with non-normal distribution were analyzed by Mann-Whitney for comparison between two groups and Kruskal-Wallis with Mann-Whitney post hoc test for multiple comparisons. We used SPSS software version 22.0 (SPSS. Inc, Chicago, IL, USA) with $p < 0.05$ was considered as

statistically significant. All experiments except miRNA PCR array were repeated at least three times to ensure reproducibility.

Chapter 4: Results

4.1 PGE_2 stimulates RANKL expression in human mandibular bone-derived cells

PGE_2 treatment (0.1 μM) stimulates RANKL up-regulation in human mandibular bone-derived cells. The level of RANKL mRNA in the cells after PGE_2 treatment was higher than that of untreated cells (Fig. 4.1A). This was also shown in protein which the soluble RANKL (Fig. 4.1B) and membrane-bound RANKL expression (Fig. 4.1C) were increased in PGE_2-treated cells compared with that of untreated cells

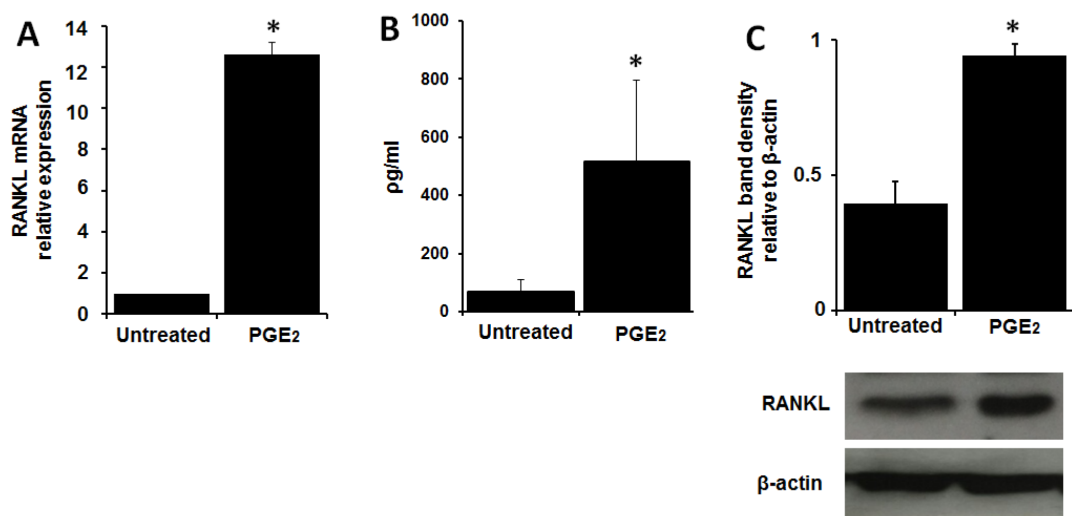


Figure 4.1: PGE_2 stimulates RANKL expression in human mandibular bone-derived cells. (A) RANKL mRNA was estimated by qRT-PCR. (B) Soluble RANKL concentration in the cell supernatant was measured by ELISA. (C) RANKL protein expression was detected by western blot and β-actin was used as internal control. The data were from 5 independent experiments using cell lines from 5 healthy donors Mean (±SD) are presented. *$P < 0.05$ indicates a significant difference using independent samples t-test.

4.2 The level of microRNA-302a-3p inversely relates to RANKL expression in human mandibular bone-derived cells

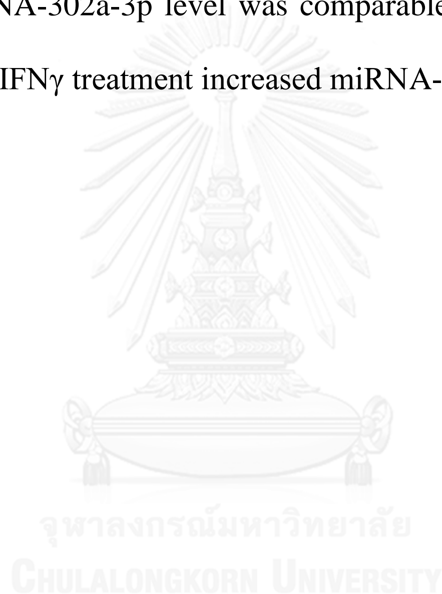
MiScript miRNA PCR Array Human Inflammatory Response & Autoimmunity (Qiagen) was used to assess 84 inflammatory miRNAs that might be involved in RANKL expression regulated by PGE_2 and epithelial-derived IFNγ. Seven miRNAs, which were down-regulated when RANKL was increased by PGE2 treatment, were demonstrated (Table 4.1).

Table 4.1: Regulation of miRNAs related to differential RANKL expression in human mandibular bone-derived cells

Treatment	PGE_2	PGE_2 NOK-SI Supernatant	PGE_2 NOK-SI Supernatant Anti-IFNγ antibody	PGE_2 rhIFNγ
RANKL Level	increased	decreased	increased	decreased
hsa-miR-106b-5p	0.55	0.71	0.69	1.27
hsa-miR-181d-5p	0.59	2.12	1.49	1.01
hsa-miR-23a-3p	0.54	1.60	2.49	0.79
hsa-miR-302a-3p	0.38	14.44	0.36	0.81
hsa-miR-302c-3p	0.46	0.87	0.83	0.69
hsa-miR-372-3p	0.28	1.06	1.02	0.69
hsa-miR-373-3p	0.42	1.33	1.27	0.69

From the expression pattern, miRNA-302a-3p demonstrated inverse relationship with RANKL expression. Human mandibular bone-derived cells expressed a low baseline level of RANKL expression which was increased after PGE_2 treatment. When IFNγ released by NOK-SI cells, or rhIFNγ, was added, the RANKL expression was decreased. To verify the result from MiScript miRNA PCR Array, miRNA-302a-3p level was demonstrated by using qRT-PCR (Fig. 4.2). Human mandibular bone-derived cells expressed miRNA-302a-3p while the baseline

RANKL was low. PGE_2 treatment decreased miRNA-302a-3p, when RANKL mRNA was increased. In contrast, miRNA-302a-3p level was restored close to untreated state by adding NOK-SI supernatant. Epithelial-derived IFNγ was shown to regulate RANKL expression in human mandibular bone-derived cells in the presence of PGE_2 (Khonsuphap et al, submitted manuscript), therefore the addition of anti-IFNγ antibody in NOK-SI supernatant, or rhIFNγ, were investigated. Using anti-IFNγ antibody to diminish the effect of IFNγ in epithelial supernatant, miRNA-302a-3p level was comparable with that of PGE_2 treatment group, whereas rhIFNγ treatment increased miRNA-302a-3p expression.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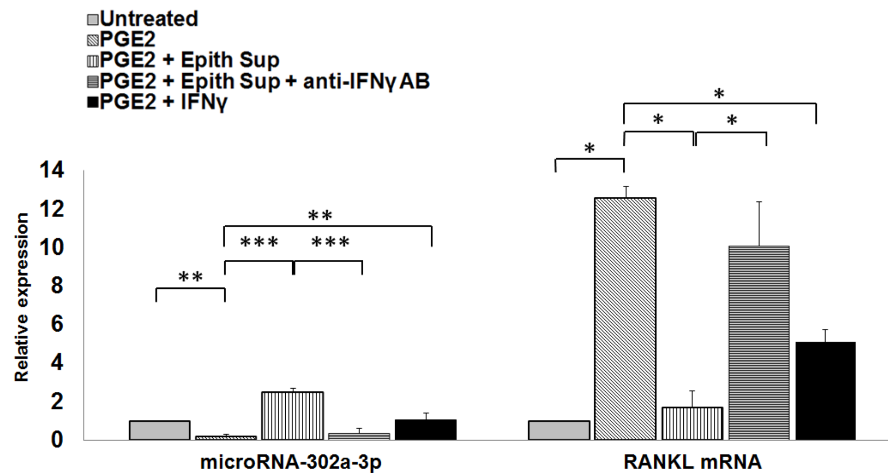

Figure 4.2: MiRNA-302a-3p levels are inversely related to RANKL mRNA expression. HMBCs were treated with PGE_2 or with PGE_2 and epithelial supernatant containing IFNγ (PGE_2 + Epith sup), for 24 h, and the expression of miRNA-302a-3p and RANKL mRNA was analyzed by qRT-PCR. The untreated cells expressed baseline levels of miRNA-302a-3p and RANKL mRNA, and RNU6B and GAPDH were used as internal controls, respectively. MiRNA-302a-3p was suppressed whereas RANKL was upregulated in the presence of PGE_2. MiRNA-302a-3p and RANKL mRNA were restored to baseline levels when the effect of PGE_2 was diminished by the addition of epithelial supernatant containing IFNγ (Epith sup). Using an anti-IFNγ antibody (anti- IFNγ AB) to neutralize the effect of IFNγ in epithelial supernatants, the miRNA-302a-3p level was comparable with that in the PGE_2 treatment group. Treatment with PGE_2 and recombinant human IFNγ (PGE_2 + rhIFNγ) confirmed a decrease in RANKL with miRNA-302a-3p upregulation relative to PGE_2 group. The data are from 3 independent experiments using cell lines from 3 healthy donors. Mean (± SD) values are presented. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ indicate a significant difference using one-way ANOVA followed by Tukey HSD analysis

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4.3 MicroRNA-302a-3p is predicted to target PRKACB mRNA in PKA signaling pathway

Bioinformatics analysis was performed by using Targetscanhuman 7.0 to predict the target gene of miRNA-302-3p. The seed region between nucleotides 2 to 8 of miRNA-302a-3p showed perfect and continuous base pairing to the sequence in the 3'UTR of the protein kinase A catalytic subunit B (PRKACB) mRNA at base position 1398 to 1404 (Fig. 4.3). Therefore, miRNA-302a-3p might target catalytic subunit β of protein kinase A in PKA signaling pathway. Since RANKL expression in Human mandibular bone-derived cells uses PKA signaling-dependent pathway, functional analysis of miRNA-302a-3p was further investigated in the next experiment.

PRKACB mRNA

5' UTR
Coding sequence
1398-1404
3' UTR
AAAAA
Methylation cap
miRNA-302a-3p

…CAGGUGGAAAUUCAAGCACUUU… **position 1398-1404 of PRKACB mRNA**

| | | | | | |

AGUGGUUUUGUACCUUCGUGAAU **hsa-miR-302a-3p**

Figure 4.3: Predicted base-pairing between miRNA-302a-3p and PRKACB mRNA by TargetScanHuman 7.0.

4.4 PGE_2 and IFNγ regulates RANKL expression in human mandibular bone-derived cells through microRNA-302a-3p

To investigate the role of miRNA-302a-3p on RANKL expression in the presence of PGE_2, we transfected the cells with miRNA-302a-3p mimic, or miRNA-302a-3p inhibitor. As evidenced by cell viability assay, transfection of miRNA-302a-3p mimic and inhibitor showed no effect on cell viability of human mandibular bone-derived cells (Figure 4.4).

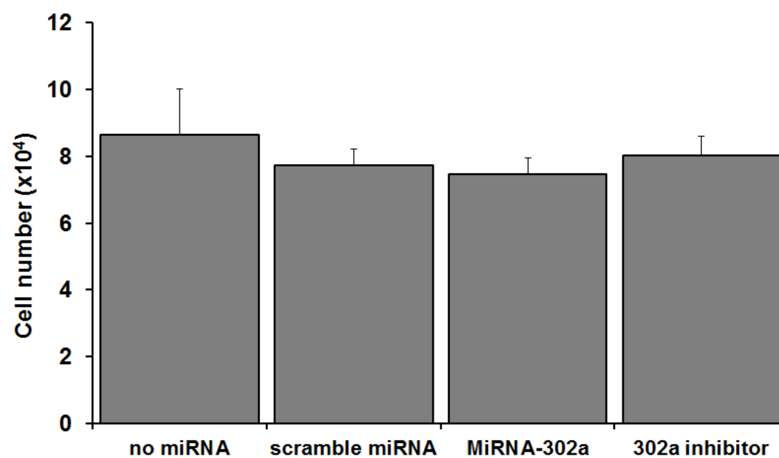

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Figure 4.4: Human mandibular bone-derived cells viability is unaffected by miRNA transfection. The cell viability was assessed by MTT assay.

Because PGE_2 treatment reduced miRNA-302a-3p with an increase of RANKL expression (Fig. 4.2B), we examined whether the transfection of miRNA-302a-3p mimic back to the cells in this condition could suppress RANKL expression. After transfection, increased level of miRNA-302a-3p was confirmed (Fig 4.5A). Although PGE_2 was present, RANKL mRNA (Fig 4.4B) and protein, both in soluble (Fig 4.5C) and membrane-bound forms (Fig 4.5D), were repressed in human mandibular bone-derived cells when miRNA-302a-3p was high.

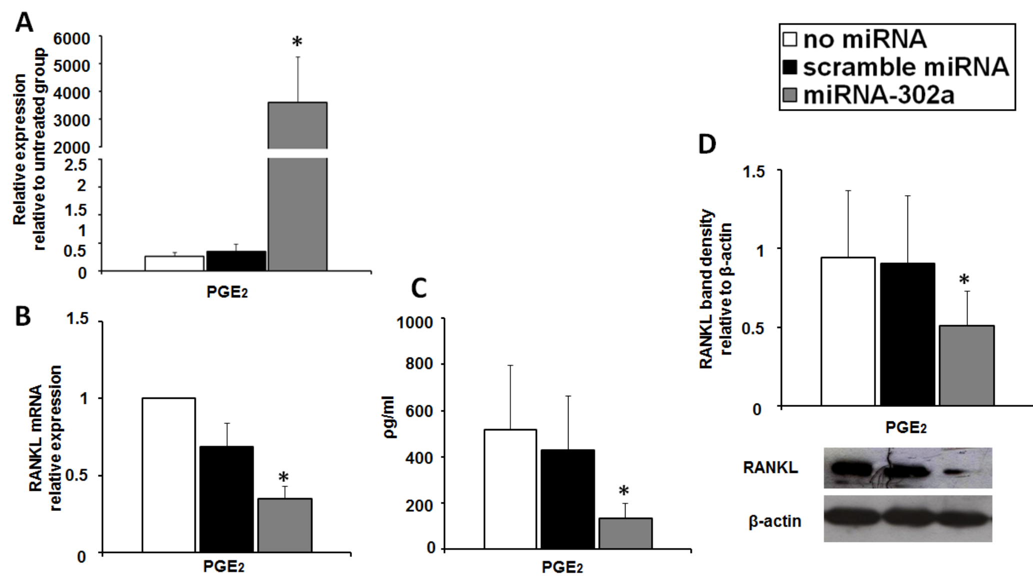

Figure 4.5: Transfection of microRNA-302a-3p mimic suppresses RANKL expression in human mandibular bone-derived cells. To investigate the role of miRNA-302a-3p, some HMBCs cells were transfected with miRNA-302a-3p mimic at 24h prior to PGE_2 treatment. When needed, some cells are treated with PGE_2, and the level of miRNA-302a-3p, or RANKL mRNA, or RANKL protein was analyzed at 24h post PGE_2 treatment. (A) Downregulation of miRNA-302a-3p in the PGE_2-treated HMBCs was demonstrated in non-transfected cells (no miRNA), or transfected with 2.5 nM scramble miRNA control (scramble miRNA). While, the increased level of miRNA-302a-3p was shown in cells transfected with 2.5 nM miRNA-302a-3p mimic (miRNA-302a) by qRT-PCR. RANKL mRNA (B), soluble-form RANKL protein (sRANKL) released in culture supernatant (C), and membrane-bound RANKL protein (D) were demonstrated by qRT-PCR, ELISA, and western blot, respectively. When level of miRNA-302a-3p was increased, RANKL expression was consistently suppressed in HMBCs. The expression of RNU6B, and GAPDH, were used as an internal control for miRNA-

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302a-3p, and RANKL expression, respectively. β-actin served as an internal control in western blot analysis. The data were from 5 independent experiments using cell lines from 5 healthy donors. Mean (±SD) are presented. $^*P < 0.05$ indicates a significant difference using Kruskal-Wallis with Mann-Whitney post hoc test (miRNA-302a-3p and membrane-bound RANKL) or one-way ANOVA followed by Tukey HSD analysis (RANKL mRNA, soluble RANKL).

Meanwhile, an addition of epithelial supernatant (Fig. 4.2C), or rhIFNγ (Fig. 4.2E) increased miRNA-302a-3p consistent with reduction of RANKL. When anti-IFNγ antibody was used to neutralize the effect of IFNγ in epithelial supernatant, miRNA-302a-3p level was diminished (Fig. 4.2D). By adding miRNA-302a-3p inhibitor, miRNA-302a-3p level was repressed (Fig. 4.6A). Consistently, reduction in miRNA-302a-3p was accompanied by up-regulation of RANKL mRNA (Fig. 4.6B), and both forms of RANKL protein (Fig. 4.6C and 4.6D) in cells treated with PGE_2 and rhIFNγ. These data indicate that microRNA-302a-3p regulates RANKL expression in Human mandibular bone-derived cells in the presence of PGE_2-IFNγ regulatory network.

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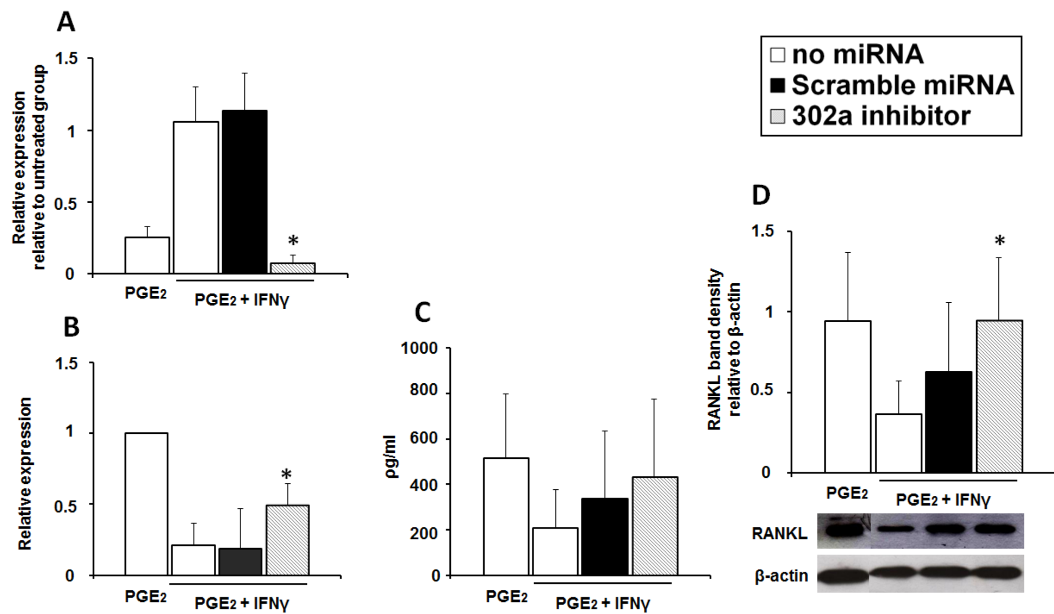

Figure 4.6: Transfection of microRNA-302a-3p inhibitor increases RANKL expression in human mandibular bone-derived cells. To confirm the role of miRNA-302a-3p, some HMBCs cells were transfected with miRNA-302a-3p inhibitor at 24h prior to PGE_2 and recombinant human IFNγ treatment (PGE_2+rhIFNγ). When needed, some cells were treated with PGE_2 and rhIFNγ, and the level of miRNA-302a-3p, or RANKL mRNA, or RANKL protein was analyzed at 24h post treatment. (A) Upregulation of miRNA-302a-3p in the PGE_2-rhIFNγ-treated HMBCs was demonstrated in non-transfected cells (no miRNA), or transfected with 2.5 nM scramble miRNA control (scramble miRNA). While, the decreased level of miRNA-302a-3p was shown in cells transfected with 25 nM miRNA-302a-3p inhibitor (302a inhibitor) by qRT-PCR. RANKL mRNA (B), soluble-form RANKL protein (sRANKL) released in culture supernatant (C), and membrane-bound RANKL protein (D) were demonstrated by qRT-PCR, ELISA, and western blot, respectively. When cells were transfected with 302a inhibitor, level of miRNA-302a-3p was decreased, and RANKL expression was consistently

upregulated in HMBCs. The expression of RNU6B, and GAPDH, were used for an internal control for RANKL, and miRNA-302a-3p expression, respectively. β-actin served as an internal control in western blot analysis. The data are from 5 independent experiments using cell lines from 5 healthy donors. *P < 0.05 indicates a significant difference using one-way ANOVA followed by Tukey HSD analysis (miRNA-302a-3p, RANKL mRNA, membrane-bound RANKL)

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Chapter 5: Discussion

Alveolar bone undergoes the dynamic process of bone remodeling that includes bone formation and resorption. This is a tightly regulated process that can be disturbed by many factors, such as hormone or inflammation. Loss of bone becomes apparent when the remodeling process is imbalanced. Such an interaction that cause bone resorption occurs in inflammatory diseases, including periodontitis. The functions of miRNAs studied in inflammatory diseases have become a focus of interest. For instance, a number of miRNAs differentially expressed in inflamed gingival tissue [95-98] and bone [99] have been identified using microRNA PCR array and microarray. In this study, PGE_2-treated primary bone cells derived from human mandible was used as an *in vitro* model to study the mechanism of RANKL signaling associated with bone resorption in inflammatory condition. The miRNAs associated with increase, or decrease, RANKL expression in human mandibular bone-derived cells were revealed based on the 84 miRNAs in the PCR array. We focused on miRNAs that were down-regulated after PGE_2 treatment in human mandibular bone-derived cells because this down-regulation suggests direct relationship of miRNAs to RANKL expression. The down-regulated miRNAs are miRNA-106b-5p, miRNA-181d-5p, miRNA-23a-3p, miRNA-302a-3p, miRNA-302c-3p, miRNA-372-3p and miRNA-373-3p, but only miRNA-302a-3p correlates with RANKL regulation in all treatments. The role of miRNA-302a-3p in RANKL regulation, or osteoclastogenesis, has never been reported. However, functions of miRNA-302a-3p was shown in many biological process by targeting the key molecules including chicken ovalbumin upstream promoter transcription factor II in mouse osteoblast [100], peroxisome

proliferator-activated receptor γ expression in pre-adipocyte cells [101], ATP-binding cassette A1 in mouse primary bone marrow-derived macrophages [102]. This suggests that the role of miRNA-302a-3p might be different between cell types. Moreover, miRNA-302a-3p also regulates cancer cell proliferation [103, 104]. However, those previous findings do not correspond with our study in which miRNA-302a-3p does not affect the cell proliferation of human mandibular bone-derived cells at all. This may occur because the cell type we used in this study was the normal cells and completely different with previous studies.

In this study, the suppression of miRNA-302a-3p in the presence of PGE_2-mimic inflammatory condition was consistent with the previous report that miRNA-302a-3p was down-regulated in inflamed gingiva [96]. The seed region of miRNA-302a-3p demonstrated complementary sequences to its predicted target, PRKACB mRNA, in PKA signaling pathway [105]. Because PGE_2 stimulates RANKL expression through cAMP/PKA signaling pathway [7], miRNA-302a-3p may suppress PRKACB, then RANKL was subsequently down-regulated. . Our results strongly support this hypothesis. The addition of miRNA-302a-3p mimic by transfection can suppress RANKL mRNA and both soluble and membrane bound forms of RANKL protein [106], although membrane-bound RANKL plays a major role to induce osteoclastogenesis [107]. On the other hand, the suppressive effect of IFNγ on RANKL expression in PGE_2-treated cells was partially reversed when the cells were previously transfected with miRNA-302a-3p inhibitor. Nonetheless, further study is required to elucidate the direct interaction between miRNA-302a-3p and PRKACB mRNA.

The profile of miRNA expression in tissue appears to be inconsistent among studies. This may be due to several factors including the differences in age, gender, genetic, and environmental factors of individuals, the different inclusion and exclusion criteria of the studies, and the miRNA array. In osteoarthritis, miRNA-9 and miRNA-98 are up-regulated in cartilage and bone tissue during inflammation [99], the level of miRNA-9, miRNA-98 was unchanged in our model. Although this is the first time that miRNA-302a-3p is shown to regulate RANKL expression, some miRNAs was shown to regulate RANKL expression [74, 76, 78]. By targeting RANKL mRNA, miRNA-17 and miRNA-20a inhibit glucocorticoid-induced osteoclastogenesis in mouse calvarial osteoblasts [78], and miRNA-106b inhibits osteoclastogenesis and osteolysis in giant cell tumor of bone [76]. In this study, miRNA-106b-5p, miRNA-17, and miRNA-20a level remains unchanged after PGE_2 treatment. It suggested that the expression level of these miRNAs is independent to PGE_2 and IFNγ stimulation in Human mandibular bone-derived cells, and the role of each miRNA could be different among cell types.

In tissue inflammation, accumulation of PGE_2 can stimulate RANKL expression in human PDL cells [7] and human mandibular bone-derived cells [91]. This may lead to osteoclast differentiation through RANK-RANKL intereaction [35]. On the other hand, oral epithelial cells may resolve osteoclastic-driven bone resorption by releasing IFNγ to attenuate PGE_2-induced RANKL [91]. IFNγ was previously reported to have dual role as a positive regulator to stimulates RANKL in T cell [108], or as negative regulator in RANKL-induced osteoclastogenesis [109]. Our study demonstrated that miRNA-302-3p is involved in RANKL PGE_2-induced RANKL expression in human mandibular bone-derived cells by regulating RANKL.

MiRNA-302a-3p inhibits RANKL up-regulation due to PGE_2 and the suppressive effect of IFNγ on PGE_2-induced RANKL expression is mediated by miRNA-302a-3p up-regulation. However, further study is required to examine the role of miRNA-302a-3p in the regulation of RANKL/RANK/OPG during osteoclastogenesis.

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2013 1st Winner of Densmart, National Dental Student Quiz, Faculty of Dentistry, Trisakti University, Jakarta, Indonesia

2012 1st Winner of Pepsodent Fokus Student Poster Award in National Dental Course Forum Faculty of Dentistry, Trisakti University, Jakarta, Indonesia

2012 3rd Place of Scientific Research Competition in the 39th Asia Pacific Dental Students Association Congress, Cairns, Queensland, Australia

2012 3rd Winner of Student Case Report, Faculty of Dentistry, University of Indonesia

2012 Dental Student Best Scientific Poster in the 1st Dental Research Exhibition and Meeting Muhammadiah University of Yogyakarta, Central Java, Indonesia

2011 1st Winner of Densmart, National Dental Student Quiz, Faculty of Dentistry, Trisakti University, Jakarta, Indonesia

2011 1st Runner Up of Sensodyne Student Poster Competition in the 5th Regional Dental Meeting and Exhibition, University of North Sumatra, Medan, Indonesia

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