Modulation of Interleukin-8 Protein Expression by Micro-RNA 181 in Human Pulpal Fibroblasts

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ABSTRACT

Johnah C. Galicia: Modulation of Interleukin-8 Protein Expression by Micro-RNA 181 in Human Pulpal Fibroblasts
(Under the direction of Asma A. Khan)

MicroRNAs (miRNA) regulate the synthesis of cytokines in response to Toll-like receptor (TLR) activation. miRNA-181 is differentially expressed in pulpitis, which represents an immune reaction to bacteria and is also the most common cause of emergency room visits due to dental pain. We employed an *in-vitro* model to determine the role of miRNA in pulpitis, which has been shown in several studies to overexpress Interleukin-8 (IL-8). Primary human dental pulp fibroblasts (HDPF) were stimulated with the TLR-2/4 agonist *P. gingivalis* lipopolysaccharide. An inversely proportional relationship between IL-8 and miRNA-181a was observed, which was validated by *in-silico* identification of a miR-181a binding site on the 3'UTR of IL-8 and by dual-luciferase assays. This is the very first report demonstrating miR-181a regulation of IL-8. Considering that both mir-181 and IL-8 have been implicated in various systemic conditions, the findings of this study extend well beyond the confines of Endodontics.

To my mother who did not only invest in her children's education, but also in kindness, love, gratefulness and joyful memories that held her family together. I miss you mama and see you in the resurrection morning.

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INTRODUCTION AND REVIEW OF LITERATURE

Discovery, Biogenesis and Function of MicroRNAs

MicroRNAs (miRNA) were discovered by a group of developmental biologists at Harvard University while studying the development of the nematode *C. elegans*. The report published by this group centered on a short sequence of 22 nucleotides, thus the term 'micro', which complemented with *lin-14* messenger RNA (mRNA) (Lee et al., 1993). As a consequence of this pairing, the translation of *lin-14* mRNA to LIN-14 protein that was essential to the nematode's development was impaired. This discovery led to hundreds of subsequent discoveries that catapulted miRNAs to the vertex of gene regulation research. At present, over 1000 miRNAs have been identified in humans, which potentially target over 30% of the human genome (Griffiths-Jones et al., 2008).

The biogenesis of miRNAs involves several steps that start in the cell nucleus and terminate in the cytoplasm by a coordinated process of cropping and dicing (Figure 1) (Kim, 2005). The step begins by transcription of intergenic or intragenic DNA sequences by RNA polymerase II that forms a one to three kilobase (kb) length sequence known as primary miRNA (Cai et al., 2004; Lee et al., 2004). Next, the enzyme Drosha and the RNA-binding protein Pasha (also known as DiGeorge syndrome critical region 8) cleave the primary miRNA into a shorter sequence of 70-100 nucleotide stem-loop structure called pre-miRNA (Denli et al., 2004; Gregory et al., 2004; Han et al., 2004; Landthaler et al., 2004).

Exportin-5 transports pre-mirRNA to the cytoplasm (Bohnsack et al., 2004; Lund et al., 2004; Yi et al., 2003) where it is further cleaved into its mature length of 14-24 double-stranded

oligonucleotide by an enzyme named Dicer (Bernstein et al., 2001; Hutvagner et al., 2001). The double strand then separates into two single strands. One of the strands transforms into a mature miRNA molecule incorporated into RNA induced silencing complex or RISC. This complex can complement with mRNA, inhibiting protein translation (Knight and Bass, 2001).

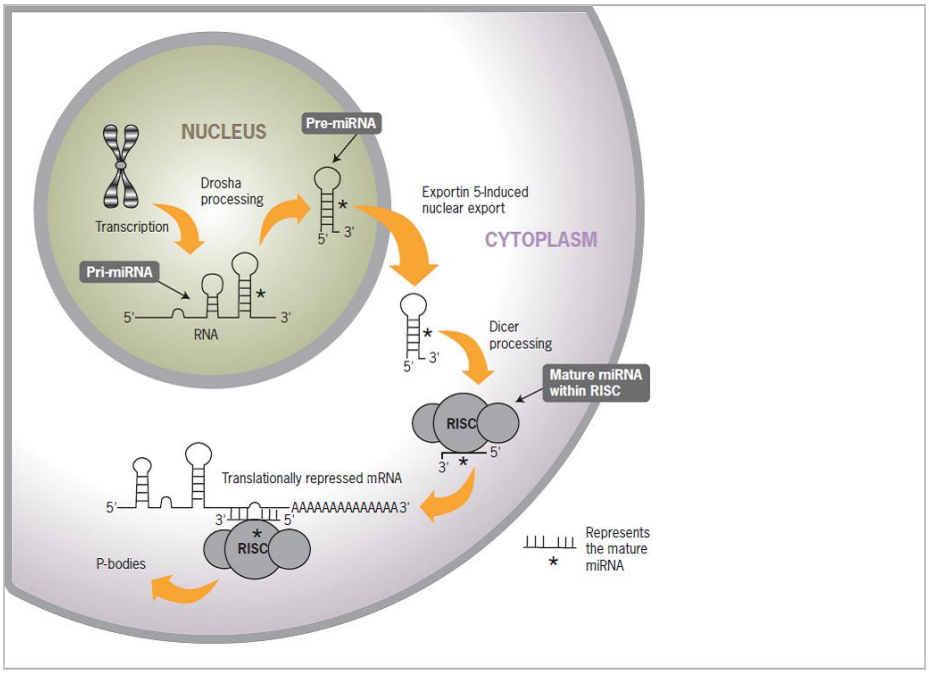

Figure 1. Biogenesis of micro-RNA (source: Life Technologies).

The miRNA-mRNA interaction depends on the degree of complementarity between their sequences. If there is a close to one hundred percent complementarity between miRNA and mRNA, mRNA can be cleaved and degraded; otherwise, mRNA translation is just repressed (Kim, 2005). Some miRNAs are produced at unusually high or low concentrations and consequently may bind more or less extensively to their mRNA targets. This dysregulation has been implicated in tumorigenesis where a disturbance in protein levels, identified as tumor markers, can be measured (Lu et al., 2005).

The degree of miRNA activity can also be altered if the concentration of the target mRNA changes during a physiological event like differentiation or development, or as the result of changes in the surrounding environment (Arvey et al., 2010). This fascinating area of miRNA-mRNA interaction has spurred studies on how disturbances in this interaction may be associated with pathological conditions like cancer (Genovesi et al., 2011; Kim et al., 2013; Li et al., 2011) and hepatitis (Janssen et al., 2013). The role of miRNAs in the regulation of gene expression makes them the key players in almost every biological function, both physiological and pathological (O'Connell et al., 2010).

micro-RNAs and the Immune Response

Toll-like receptors (TLR) are a class of proteins that recognize conserved pathogen structures, trigger innate immune responses and prime antigen-specific adaptive immunity (Eskan et al., 2008; Hajishengallis et al., 2006; Hirao et al., 2009; Kawai and Akira, 2010; Medzhitov et al., 1997). TLRs are a group of proteins expressed in the transmembrane region or in the cytoplasm of cells and recognize specific microbial patterns (Kopp and Medzhitov, 1999). TLR4 for example recognizes bacterial lipopolysaccharide (LPS) (Medzhitov et al., 1997). Upon microbial stimulation, TLR4 sends a cascade of downstream signals that trigger the cell to produce cytokines and /or chemokines that initiate the inflammatory process (Shimazu et al., 1999).

MiRNAs have recently emerged as an important post-transcriptional regulators of inflammation, metabolism and healing (van Rooij et al., 2012). As key regulators of inflammation, miRNAs have been linked to homeostatic responses to inflammatory stimuli by

TLR4 pathway activation (Xie et al., 2013b) and in various TLR-mediated immune responses to bacterial infection where miRNAs either suppressed the inflammatory response or reduced inflammatory triggers (Case et al., 2011; Liu et al., 2009).

Our recent microarray report showed differential expression of miRNAs in human dental pulp tissues that were clinically diagnosed to have pulpal inflammation or pulpitis (Zhong et al., 2012), a relatively common and painful dental disease that represents an immune response to bacterial infection (Love and Jenkinson, 2002). Pulpitis and its sequela, periapical periodontitis have been directly linked to the pathogenicity of the oral microflora (Adachi et al., 2007; Huang et al., 1999; Kakehashi et al., 1966; Love and Jenkinson, 2002; Siqueira et al., 2009). With the preceding information at hand, it elicits an interest to investigate the potential association between pulpal infection and changes in miRNA levels that may translate directly on how inflammatory triggers are stimulated or repressed.

mir-181 Family and their Role in Immune Response

The multiple validated gene targets of the mir-181 family (Table 1) underscore their role in regulating immune response and inflammation. A two to five fold increase in mir-181b and c expression was reported in inflamed periodontal tissues compared with healthy gingival tissues (Xie et al., 2011). In another similar study, mir-181b was found to be four times overexpressed in chronic periodontitis tissues compared with normal periodontal tissues (Lee et al., 2011). This increase in expression in diseased periodontal tissues seems to provide a link between infection and overexpression of mir-181 family.

Table 1. MiRNA-181 validated target genes

miRNA	Target gene	Gene product function	GO Term (Accession, Ontology)
mir-181a	DUSP5/6	Regulation of T Cell Receptor (TCR)-signalling and activation threshold; alteration of T helper (Th) subset differentiation	GO:0070373, Biological Process
	IL-6	Acute and chronic inflammation and the maturation of B cells; T helper (Th)17 differentiation	GO:0019981, Molecular Function
	STAT1	Cytokine-mediated signaling pathway; LPS-mediated signaling pathway	
	TGFB1	Adaptive immune response; positive regulation of collagen biosynthesis, chemotaxis, fibroblasts migration, and odontogenesis	GO:0034713, Molecular Function
	TLR-4	Toll-like receptor-4 (TLR) plays a fundamental role in pathogen recognition and activation of innate immunity	GO:0035662, Molecular Function
mir-181b	CCL8	Immune response, inflammatory response, chemokine activity, phospholipase activator	
	IL-6	Negative regulation of cytokine secretion; negative regulation of collagen biosynthetic process; positive regulation of acute inflammatory response; response to cold, heat and mechanical stimuli; T Helper (Th)17 differentiation	GO:0005138, Molecular Function
	MMP9	Cell response to IL-1, LPS; macrophage differentiation; response to heat and mechanical stimuli; positive regulation of apoptosis and angiogenesis	GO:0004229, Molecular Function
	TGFB1	Adaptive immune response; positive regulation of collagen biosynthesis, chemotaxis, fibroblasts migration, and ondontogenesis; T helper (Th)17 differentiation, T regulatory (Treg) differentiation	GO:0034713, Molecular Function
mir-181c	IL-2	Cytokine produced by T-cells in response to antigen or mitogen stimulation	GO:0005134, Molecular Function
	SOCS1	Cytokine mediated signaling pathway; negative regulator of JAK-STAT pathway; LPS response	
mir-181d	MMP9	Cell response to IL-1, LPS; macrophage differentiation; response to heat and mechanical stimuli; positive regulation of apoptosis and angiogenesis	GO:0004229, Molecular Function

Like pulpitis, periodontitis is a microbial disease that is directly correlated with the preponderance of a group of gram negative anaerobes called the red complex species (Ximenez-Fyvie et al., 2000). The root canal system, like the deep pockets in periodontitis, is an anaerobic environment. In fact, there is an overlap in the identified species between periodontitis that

progressed from gingivitis and apical periodontitis that resulted from pulpitis (Siqueira et al., 2009).

The difference in mir-181 family members identified in the two periodontitis studies above (Lee et al., 2011; Xie et al., 2011) can be attributed to subject variability in terms of disease stage or severity at the time the periodontal samples were collected

In terms of pain, which remains a diagnostic dilemma in endodontics and beyond, circulating levels of miR-181a and c are significantly lower in patients diagnosed with chronic regional pain syndrome (Orlova et al., 2011). miR-181a has been demonstrated to be a key regulator of AMPA-type glutamate receptors in neurons (Saba et al., 2012). miRNAs are known to modulate the expression and function of neuronal ions channels (Carrillo et al., 2011; Favereaux et al., 2011; Saba et al., 2012). These include the neuronal ion channel Cav1.2, a calcium channel implicated in chronic pain and NaV1.8, a sodium channel implicated in inflammatory pain and thermal hyperalgesia (Akopian et al., 1999; Favereaux et al., 2011; Laird et al., 2002; Zhao et al., 2011).

Extending outside the area of endodontics, circulating levels of mir-181b was found to be lower in patients with sepsis and in animal models of septic shock (Sun et al., 2012). In sepsis patients, each increase of one unit of miR-181b in blood is associated with an approximately 50% decrease in the odds of having sepsis. In an animal model of sepsis, the systemic administration of miR181-b reduces leukocyte influx in the vascular endothelium and decreases mortality. This response was attributed to the nuclear factor κβ (NF-κβ) targeting of mir-181b, hence the decrease in TNF-α induced vascular inflammation in mice (Sun et al., 2012).

Cytokines and mir-181 in Inflammation

Among the cytokines produced during inflammation, Interleukin-8 (IL-8) has gained significant attention due to its essential involvement in acute response to infection (Harada et al., 1994). IL-8 is a potent chemoattractant that is increased in pulpitis (Huang et al., 1999) and in gingival crevicular fluid collected from teeth with acute pulpitis (Karapanou et al., 2008). Its essential involvement and causative role in acute inflammation by recruiting and activating neutrophils have been firmly established (Gura, 1996; Harada et al., 1994).

In our study that compared normal and infected human dental pulps, we reported the differential expression of 36 miRNAs (Zhong et al., 2012). In another *in-vivo* study examining infected tissues surrounding the apices of teeth with infected root canal systems, 24 miRNAs were differently expressed (Chan et al., In press). The mir-181 family, which regulates a wide range of gene targets, was differentially expressed in both of these tissues. They have also been recently implicated in TLR-induced *in-vivo* increase in cytokine levels (Xie et al., 2013b). However, there have been no reports as yet on the role of these miRNAs in regulating the immune response in primary human dental pulpal fibroblasts (HDPF).

Both IL-8 and mir-181 family have been associated with other inflammatory conditions such as rheumatoid arthritis and neuroinflammation and inflammatory responses of various cell types like astrocytes and macrophages (Hutchison et al., 2013; Seitz et al., 1992; Sun et al., 2012; Xie et al., 2013a). Therefore, both IL-8 and mir-181 play important roles in inflammation.

Immunocompetence of the Dental Pulp

The role of bacteria in pulpal disease is well established (Kakehashi et al., 1966). These microorganisms invade the dentinal tubules of the susceptible tooth and eventually the dental pulp through a carious lesion, which is the most common etiology for pulpal inflammation and necrosis (Love and Jenkinson, 2002). As the carious lesion progresses deeper into the pulp-dentin interface, the microflora shifts from a Gram-positive to a more Gram-negative, anaerobic bacteria (Ozok et al., 2012).

Pulpal fibroblasts, the predominant resident cells of the dental pulp, express TLRs (Hirao et al., 2009) that, upon microbial infection, stimulates pulp cells to produce cytokines and chemokines (Adachi et al., 2007; Nagaoka et al., 1996; Park et al., 2004; Tokuda et al., 2001). Other cells types like endothelial cells in the dental pulp's blood vessels and the resident macrophages also express TLRs (Gallego et al., 2011; Hijiya et al., 2002). The dental pulp, therefore, is an immunocompetent tissue.

Inflammation of the pulp and periapical tissues is commonly associated with pain, and approximately 90% of dental emergency visits with pain as the chief complaint are attributable to activation of pulpal or periapical nociceptors (Hasselgren and Calev, 1994). The prevalence of periapical disease in the United States is estimated to be about 4.1% (Buckley and Spangberg, 1995). Despite the prevalence of endodontic disease and the great discomfort associated with it, the fundamental molecular aspects of its pathogenesis are still not fully understood. The current endodontic literature on pulpal immune response to microbial infection continues to expand; however, very little is known on the regulatory mechanism behind pulpal disease.

PURPOSE

The purpose of this study is to determine how pathogens modulate the expression of mir-181 family and to investigate this expression with the production of IL-8.

SPECIFIC AIMS

1) To characterize the expression of mir-181 family in primary HDPF;

2) To investigate if their expression level is influenced by TLR agonists;

3) To establish the regulatory mechanism of mir-181 with IL-8 in HDPF;

4) To determine if this regulatory mechanism is similar in macrophages, a resident immune cell in the dental pulp.

HYPOTHESIS

Mir-181 expression is influenced by bacterial stimulation that translates into modulation of IL-8 production in HDPF.

MATERIALS AND METHODS

The methods employed in this study are summarized below (Figure 2):

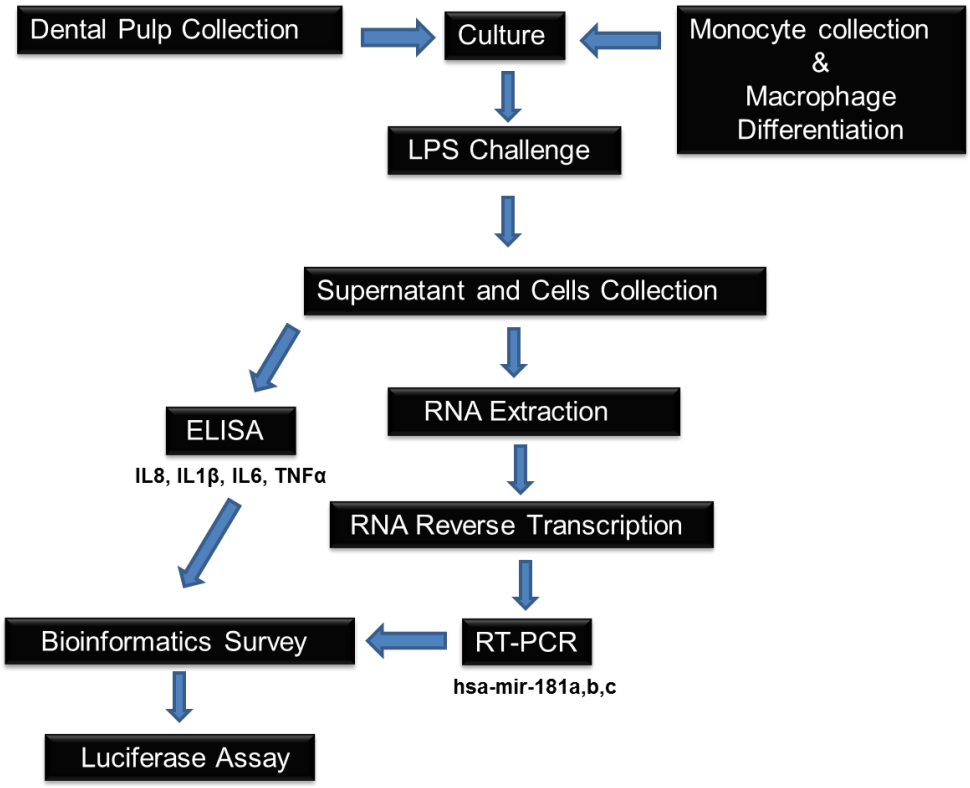

Figure 2. Flow diagram of the methods employed in this study.

Dental Pulp Tissue Collection and Culture

The study was performed with the approval of and compliance to the guidelines set by the Institutional Regulatory Board of the UNC Office of Human Research Ethics. Dental pulp tissue from a clinically normal tooth of three healthy donors was extirpated immediately after extraction for orthodontic reason (Figure 3a). The pulp tissue was obtained under informed consent.

HDPF cultures were established using a previously published protocol by Adachi et al (Adachi et al., 2007). In brief, minced pieces of pulp tissues were explanted into 35-mm culture dishes containing Dulbecco's modified Eagle's medium (DMEM) (Gibco, Grand Island, NY,

USA), supplemented with 10% fetal bovine serum (FBS) (Sigma, St. Louis, MO, USA), 1 mM sodium pyruvate (Gibco), and 50 IU/mL penicillin/50 3g/mL streptomycin (Gibco) at 37°C in a humidified atmosphere of 5% CO_2. Confluent primary cultures were harvested and subcultured. Morphologically fibroblastic cells obtained by this method were used as HDPF for experiments at passages 4 to 6 (Figure 3b).

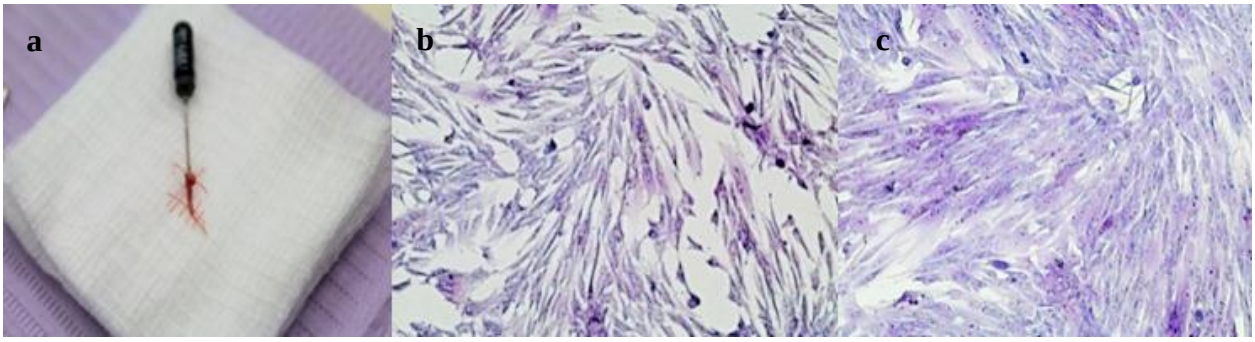

Figure 3. Human dental pulp tissue and fibroblasts in culture .**a**) Dental pulp extirpated from a donor patient; **b, c**) Cultured primary HDPF at different confluence (x10).

HDPF Stimulation

HDPFs were seeded in 6-well culture plates and incubated until a confluent monolayer of approximately 10^6 cells was established. The cells were then challenged with 1µg, 100ng or 10 ng of *Porphyromonas gingivalis* (*P. gingivalis)* W83 LPS (Pg-LPS) (Dr. David A. Scott, University of Louisville, KY, USA) at 1, 4 and 8 hours. Culture supernatants were collected and stored at -80 ^oC until use. Adherent HDPF were lysed for RNA extraction. Among the bacteria implicated in endodontic disease, *P. gingivalis* was utilized in this study because its presence in infected root canals has been repeatedly documented and its characteristics fit the nature of a root canal environment (Dougherty et al., 1998; Gomes et al., 1996; Kurihara et al., 1995)

Quantitative Real-time PCR

Total RNA was extracted from LPS-challenged HDPF and assessed for mir-181 a, b and c by real-time PCR using *Taq*-Man primers and probe sets (Applied Biosystems, Foster City, CA, USA). Normalization was performed using RNU-6B primer (Ucar et al., 2012). Relative quantification (RQ) values were analyzed using Excel spreadsheet from Microsoft (Redmond, WA). The experiments were carried out in three independent experimental set-ups.

ELISA

IL-1β, IL-6, IL-8 and TNF-α were measured by enzyme-linked immunosorbent assay (ELISA) using commercially available kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. The absorbance was read at 450 nm.

Luciferase Assay

IL8 3'UTR (~1.38 kb) was amplified by primers (Forward- GCACTCGAG TGTGTGGGTCTGT TGTAGGG; Reverse – ATGCGGCCGCTGACTGTGGAGTTTTGGCTG T) using pGL3-IL8 construct (kind gift of Dr. Richard Pestell Jefferson Medical College, Philadelphia, PA) as template. The amplified fragment (~950 bp) was digested by XhoI and NotI and cloned into psiCHEK^{TM}2 vector (Promega). The transfection was performed as described previously (Naqvi et al., 2013). Briefly, actively growing Human Embryonic Kidney 293 cells (HEK cells) were seeded in 96-well plate at a density of 2×10^4 cells per well in complete DMEM. The next day, cells were co-transfected using Lipofectamine 2000 (Invitrogen) with 80 ng of IL8 construct and different concentration (2pmol and 5pmol) of miR-181a-5p mimics (Qiagen). 36 h after transfection, luciferase activities were measured by Lumat (Turner

BioSystems, Sunnyvale, CA, USA). The fold change in luciferase activity was calculated as previously described (Naqvi et al., 2013).

MiRNA-181 Transfection in HDPF and Macrophages

Transfections with mir-181a were performed as described previously (Naqvi et al., 2013). HDPFs and differentiated macrophages were transfected with mir-181a mimic (5'AACAUUCA ACGCUGUCGGUGAGU) or inhibitor (5' AACAUUCAACGCUGUCGGUGAGU) (Qiagen) using HiPerfect reagent (Qiagen) according to manufacturer's instructions. Briefly, $1x10^6$ cells were seeded in six-well plates and transfected with miRNA mimics or inhibitor. 50nM was used for HDPFs while 50nM and 200nM were used for macrophages. Scrambled miRNAs were used as controls. After 36 hours of incubation with the mimics or antagomirs, LPS was added to HDPFs and was incubated for another one, four and eight hours before the supernatants were collected. Macrophages supernatants were collected at two and 20 hours post-incubation. Protein detection using ELISA was performed. Transfection efficiency of miRNAs was determined by SiGLO Green and SiGLO Red siRNA (Thermo Scientific) that served as surrogates for mir-181a. A transfection efficiency of over 80% was observed by dividing the number of fluorescent cells with total number of cells.

Statistical Analysis

One-way ANOVA with Tukey's post-hoc analysis was employed where statistical difference is noted in the study using Graph Pad (La Jolla, CA, USA). Relative quantification (RQ) values were analyzed using Excel spreadsheet from Microsoft (Redmond, WA).

RESULTS

Expression of Mir-181-a, -b and IL-8 in HDPF is Influenced by *P. gingivalis* LPS

To examine if the TLR response affect the expression of miR-181 family in HPDF, cells were challenged with *Pg* LPS. Figure 4a shows that mir-181a expression in HDPF is influenced by *P. gingivalis* in a time- and dose-dependent manner. Compared with the one-hour time point, the expression of mir-181a decreased at 4-hours. At the 8-hour time point, the expression level either remained relatively similar (10 ng ml^{-1} LPS) or increased slightly (100 ng ml^{-1} and 1 μg ml^{-1} LPS). This result is not surprising as a time- and dose-dependent stimulation by *P. gingivalis* LPS has been shown in other cell types (Herath et al., 2013; Tabeta et al., 2000). Mir-181b expression was only noted upon challenge with 1μg ml^{-1} *P. gingivalis* LPS (Figure 4b) while mir-181 c was not detected in this study. 1μg $ml^{-1}$of *P. gingivalis* LPS was used in the subsequent experiments as this dose had been widely used in other studies (Hajishengallis et al., 2006) and had shown the most consistent result in our experiments.

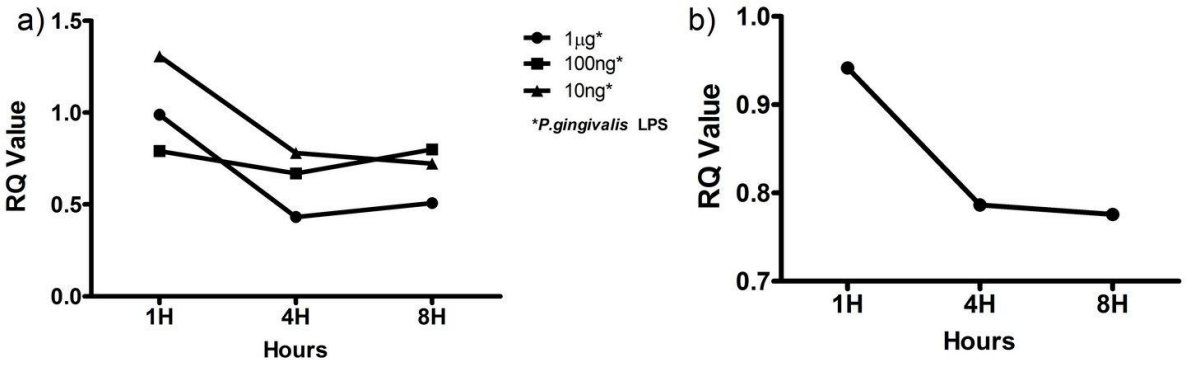

Figure 4. Mir-181a (**a**) and -181-b (**b**) expression upon LPS challenge. A time and dose response in mir-181a and b expression level is shown. Doses are in μg or ng/mL.

Induction of pro-inflammatory cytokines is a key feature of TLR signaling. We monitored the levels of IL-1β, IL-6, IL-8 and TNF-α in the supernatants of LPS stimulated HPDF. Among the cytokines assayed in this study, only IL-8 was detectable in the cell culture supernatant (Figure 5). IL-1β, IL-6 and TNF-α were all below minimum detectable dose (7.5 pg/mL); therefore, IL-8 was chosen to be further investigated in this study.

Interestingly, IL-8 was secreted in a time- and dose-dependent manner compared with LPS-free controls, which was below the minimum detectable dose of the assay.

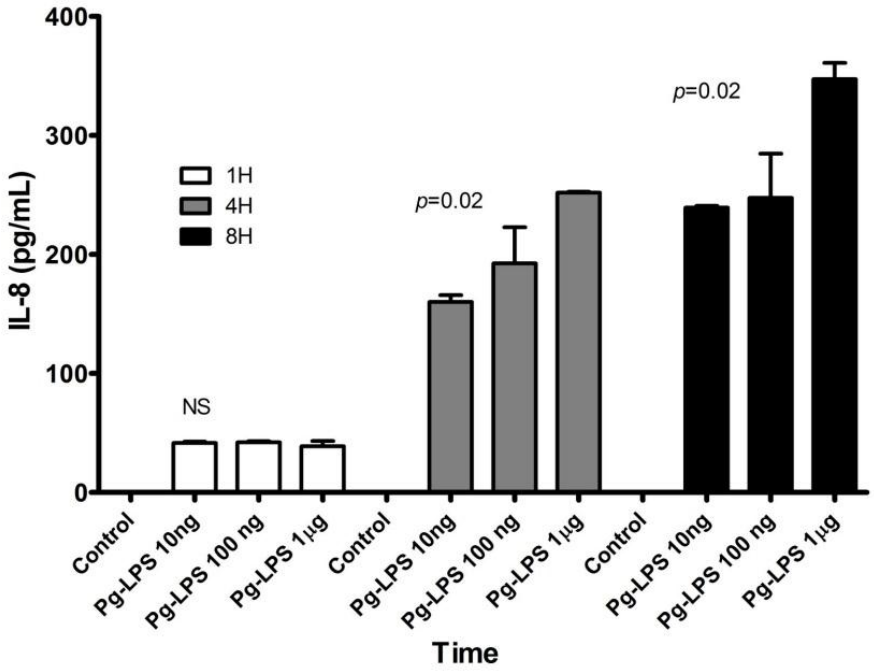

Figure 5. Interleukin-8 response to LPS stimulation at different doses and time points.IL-8 is released in a time- and dose-dependent manner. *P* values calculated using one-way ANOVA.

IL-8 and mir-181a and b Show an Inversely Proportional Relationship

From the dose and time dependent data we observed an inversely proportional relationship between mir-181a gene expression and IL-8 protein level (Figure 6a). The same relationship was observed between IL-8 and mir-181b (Figure 6b). This relationship suggests a possible modulatory role of mir-181a and miR-181b on IL-8 expression.

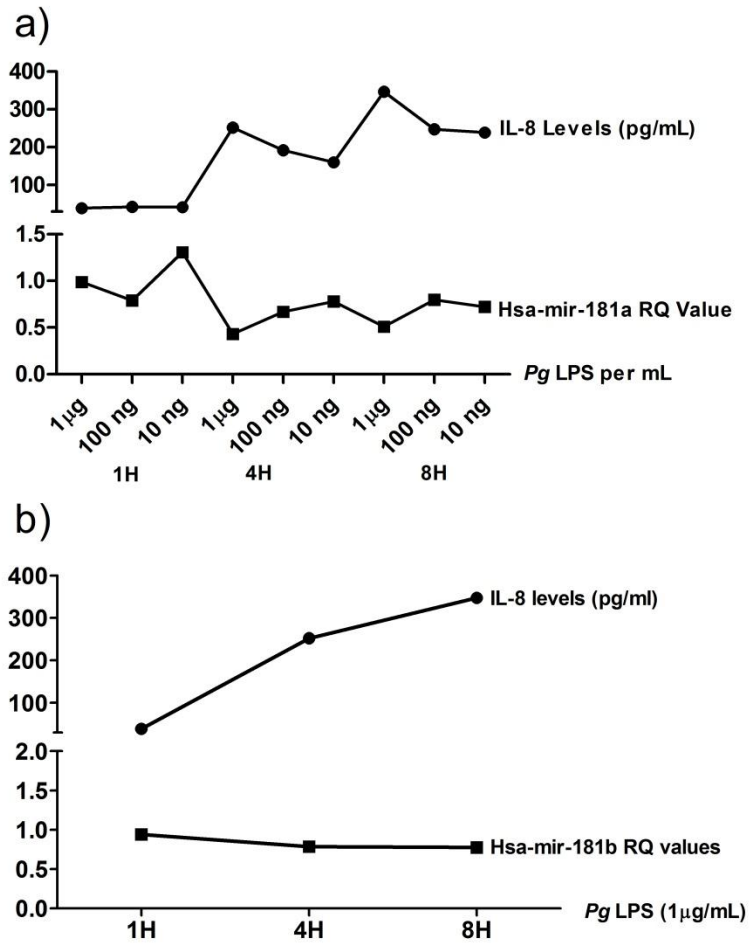

Figure 6. Relationship between level of IL-8 protein and hsa-mir181a (**a**) and -181b (**b**) .

IL-8 is Modulated by mir-181a

To investigate the possible mechanistic role of miR-181mediated regulation of IL8, we scanned the 3'UTR for miR-181 binding site. Bioinformatics analysis identified a novel miRNA binding region spanning 346-368 nts of IL8 3'UTR, as shown in Figure 7a. Importantly, the seed sequence is conserved in all the four miRNAs of miR-181 family. To validate functional miRNA-target interaction, dual luciferase assays were performed. HEK293 cells transfected with miR-181a show reduced luciferase activity compared with the control miRNA mimic (Figure 7b). Increasing the miR-181amimic concentration to 5 pmol had no further significant impact on the luciferase activity (Figure 7b). Thus, miR-181a directly regulates IL8 levels by interacting with the 3'UTR.

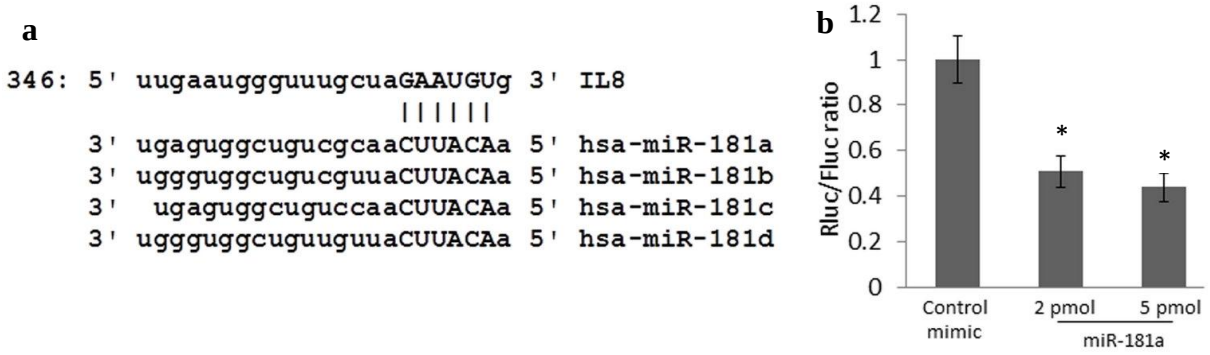

Figure 7. a) Complementarity between IL-8 and mir-181 family sequences. mirSVR score: -0.0308; PhastCons 0.5158. **b)** dual luciferase assay demonstrating the downregulation of IL-8 by mir-181a. **P*<0.05 compared with control mimic using one-way ANOVA.

Mir-181a Directly Binds to 3'UTR of IL-8

The luciferase assay results indicated that mir-181a directly binds to the 3' UTR of IL-8, thus capable of negatively regulating the activity of IL-8. In this experiment, we examined if overexpression of mir-181a would have a negative impact on IL-8 expression. Mir-181a mimics were transfected in both HDPFs and macrophages. Mir-181a antagonists were also transfected to determine if suppressing this miRNA's activity would increase the IL-8 protein levels upon LPS stimulation. As shown in the figure below (Figure 8), LPS challenge resulted in significantly increased IL-8 secretion in HDPFs. Interestingly, while the miRNA mimics significantly increased IL-8 upon LPS stimulation, the antagomirs significantly reduced IL-8 levels. This result is counterintuitive with the luciferase assay results. However, this is not surprising as a previous study has shown similar differences in luciferase and miRNA overexpression experiments (Tserel et al., 2011).

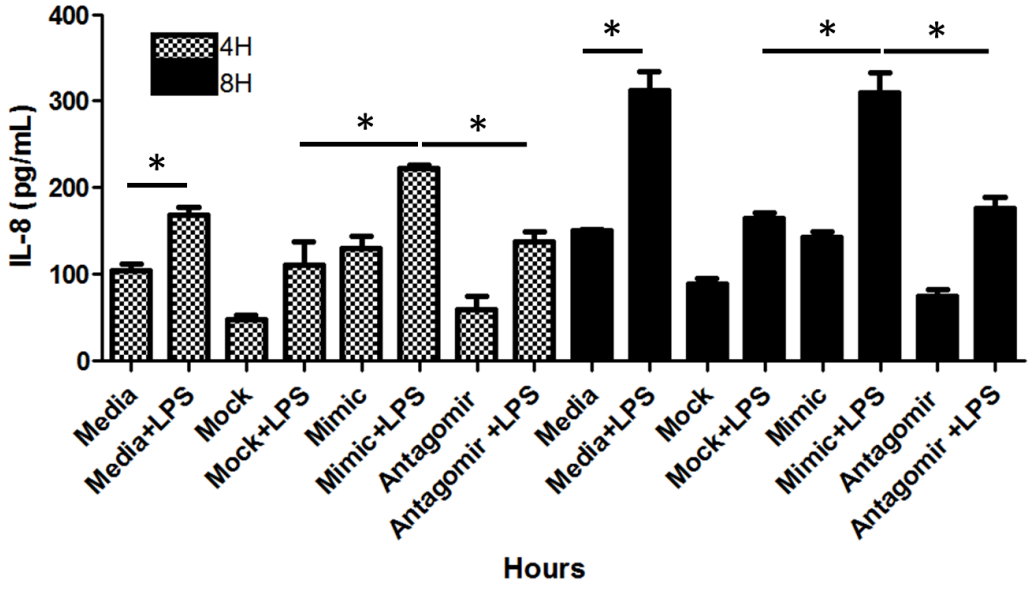

Figure 8. IL-8 response on LPS challenge and on mir-181a transfection with mimics or antagomirs with and without LPS stimulation. *P<0.05 using one-way ANOVA.

The results obtained from HDPFs above were also replicated in macrophages below (Figure 9), albeit without LPS challenge, indicating that overexpression of mir-181a in cells, independent of their type, function as a positive regulator of IL-8 while antagonizing this miRNA down regulates IL-8.

At two hours, no changes were detected in both doses of the mimics and the antagomirs; however, significant differences were observed at 20 hours, with the higher doses elucidating a higher IL-8 response. Control mimics – the mock controls – did not show any difference compared with the test samples. Modest IL-8 levels were obtained in this experiment as the macrophages were not challenged with LPS, thus no TLR stimulation ensued to trigger the release of cytokines.

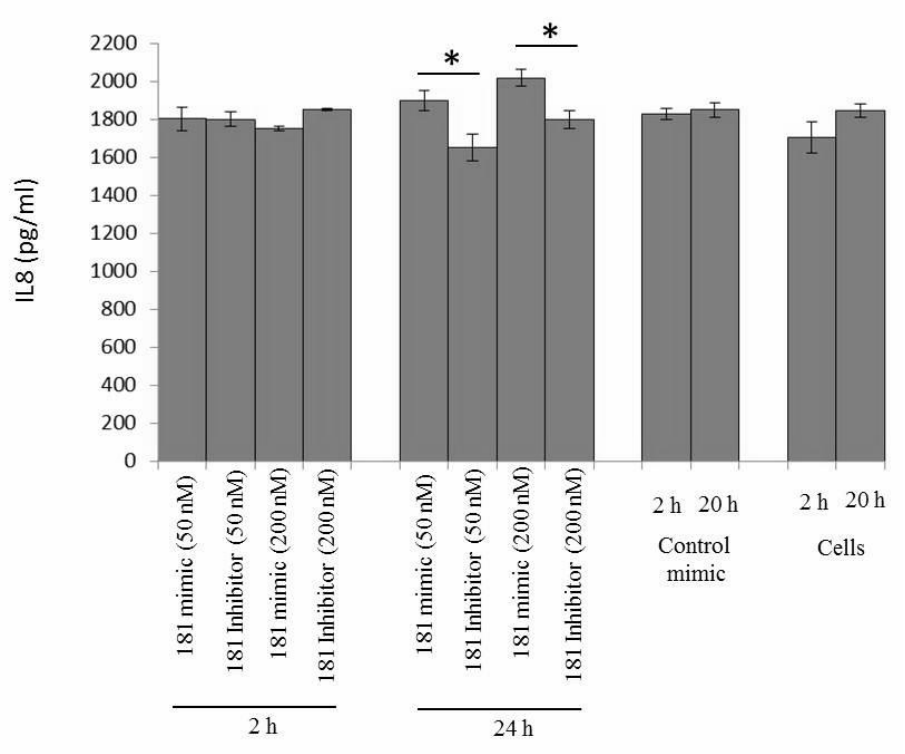

Figure 9. IL-8 levels in macrophages upon transfection with mir-181a mimics and antagomirs. *P<0.05 using One-way ANOVA.

DISCUSSION

Mir-181 is a critical micro-RNA that was largely thought to regulate lymphocyte development and homeostasis, among other important functions (Henao-Mejia et al., 2013). However, recent studies have also revealed its role in immunoregulation (Guo et al., 2013; Xie et al., 2013b). In this study, we have shown that mir-181 family is also expressed in non-leukocytic cells and that expression of both interleukin-8 and mir-181a and b are influenced by a TLR agonist in a time- and dose-dependent manner. Moreover, the inversely proportional relationship between mir-181a and IL-8 suggests that miR-181a directly binds to the 3'UTR of IL-8, an important inflammatory component of the pulpal immune response, and modulates its levels.

The findings above are important additions to our current understanding on the regulatory network governing the response of various cell types to microbial insults. Fibroblasts are primarily structural in function but their ability to respond to infection as first or second line of defense (after epithelial cells) is critical in limiting the spread of infection and in wound healing (McDougall et al., 2006).The presence of TLRs in HDPF confers immunocompetency on these cells (Hirao et al., 2009), giving merit to the importance of studying how their response is regulated. As part of the initial responders to microbial entry during the carious process, the capability of HDPF to recruit professional immune cells to the site of infection can be a double-edged sword that may halt or propagate the infectious process (Gura, 1996).

The polymicrobial etiology of pulpitis and the clinical implications associated with it make this disease an ideal model of immunoregulation. As a more gram negative, anaerobic bacterial species penetrate the pulp-dentin interface, pulpal cells, predominantly HDPF recognize

the conserved microbial patters through toll-like receptors (Hirao et al., 2009). Consequently, various inflammatory mediators are expressed that initiate and enhance the inflammatory process. We mimicked this response by utilizing an *in-vitro* model using HDPF stimulated with Pg-LPS. Our results agree with previous studies on the immunocompetence of pulpal fibroblasts (Adachi et al., 2007; Hirao et al., 2009; Nagaoka et al., 1996; Tokuda et al., 2001).

In this study, the detectable levels of IL-8 in Pg-LPS stimulated cells may recruit circulating immune cells (e.g. neutrophils) to the site of infection as shown *in-vivo* by Izumi et al (Izumi et al., 1995) and Olgart et al (Olgart et al., 1974). In their study, bacteria induced inflammatory changes in the dental pulp characterized by infiltration of immune cells, activation of the complement system due to the development of a local immune reaction, and the accumulation of arachidonic acid metabolism with the destruction of cellular components.

The conflicting data obtained in miRNA overexpression in both HDPFs (Figure 8) and macrophages (Figure 9) in comparison with the luciferase assay results (Figure 7b) create an area for further investigation. This difference is interesting because there are multiple predicted or validated mRNA targets for a single miRNA that could have a wide-ranging effect on the overall immune response.

Mir-181 family for example does not only complement to IL-8 as shown in this study, but it also targets other mRNAs whose protein products are essential for the downstream signaling of IL-8 such as TLR-4, STAT1 and SOCS1 (Table 1). Therefore, it is quite unexpected that overexpressing mir-181 to block IL-8 mRNA resulted in positive regulation of IL-8 and antagonizing it suppressed IL-8. One possible mechanism that could explain this phenomenon is

the switching of miRNA from repression to activation resulting in mRNA translational up-regulation instead of down-regulation in cells under cell cycle arrest (Vasudevan et al., 2007; 2008). With the *in-vitro* model that we have employed in this study, transfection with miRNA mimics and antagonists may have caused the HDPFs and the macrophages to undergo cell cycle arrest as shown in another study (Tserel et al., 2011). Therefore, depending on the cellular environment, mir-181 can either up- or down-regulate the target gene expression. At this point however, this extrapolation is purely speculative. Additional functional studies are definitely encouraged to further investigate the applicability of mir-181 mimics to down-regulate inflammatory response.

We confirmed for the very first time the regulation of IL-8 by mir-181a, a micro-RNA that has been shown to be differentially expressed in inflamed pulp (Zhong et al., 2012). Micro-RNAs posttranscriptionally regulate gene expression by targeting specific mRNAs for degradation or translational repression (Pauley and Chan, 2008). Their cytoplasmic levels directly influence the protein bioavailability of their targeted genes. We have shown in this study that the level of mir-181a is inversely proportional with secreted IL-8 level (Figure 5). We confirmed the repression of IL-8 by mir-181a using Luciferase assay (Figure 6b) and *in-silico* alignment analysis (Figure 6a). This pattern of negative regulation by micro-RNAs has been shown in other studies (Perry et al., 2008). Paradoxically, overexpression of mir-181a by transfection of mimics into HDPF and macrophages resulted in a significant increase of IL-8 and suppression of mir-181a using antagomirs resulted in decrease of IL-8 level.

CONCLUSION AND FUTURE DIRECTIONS

Developing a treatment modality that can be used to directly dampen inflammation and promote healing in various endodontic procedures like direct pulp capping, apical microsurgery, root resorption treatment, post-trauma management, apexification and apexogenesis sounds very encouraging. The results of this study can be, in theory, used to develop an miRNA based anti-inflammatory therapy not only in endodontics but in other inflammatory conditions as well where regulation of inflammatory markers by miRNA were reported (Case et al., 2011; Guo et al., 2013; Henao-Mejia et al., 2013; Hutchison et al., 2013; Sun et al., 2012; Ucar et al., 2012).

With the understanding that IL-8, a potent pro-inflammatory cytokine, can be directly regulated with a micro-RNA, a targeted therapy to control inflammation is imminent in the near future. This miRNA based therapeutics is already in large scale clinical trials in other fields (Janssen et al., 2013).

Another advancing field in miRNA research is miRNA diagnostics. At present, novel miRNA-based prognostic and diagnostic tools are being explored for cancers with poorer prognosis (Larne et al., 2013; Stenvang et al., 2008; Szafranska-Schwarzbach et al., 2011). As the field of miRNA research expands, it is predicted that the utility of these short sequenced nucleotide will have longer and broader applications in a myriad of conditions (van Rooij et al., 2012).

A critical barrier to progress in treatment of pulpitis is the incomplete understanding of the regulatory network governing this disease. This study provides evidence that the dental pulp possesses immunocompetent cells with an active regulatory network capable of responding to

microbial insults and modulating inflammatory response. Furthermore, the pathogenesis of pulpitis and its immunoregulation can serve as a model for other microbial diseases that provide less opportunity for *ex-vivo* or *in-vitro* studies due to their inaccessibility or critical location like the brain, lungs, or eyes.

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