microRNAs: Novel Players in the Regulation of Endodontic Disease

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ABSTRACT

Sheng Zhong: microRNAs: Novel Players in the Regulation of Endodontic Disease
(Under the direction of Dr. Asma Khan)

The purpose of this project was to examine the role of microRNA (miRNA) in endodontic disease. We tested the hypotheses that miRNAs are differentially expressed in infected pulps as compared to normal pulps and that exposure to lipopolysaccharide (LPS) induces changes in macrophage miRNA expression. miRNA expression in normal and infected human pulps (N=28) was profiled using microarray technology. Next CD14 positive monocytes isolated from fresh human buffy coats, and induced towards a macrophage phenotype were challenged with *E. coli* LPS for up to 8 hours and miRNAs expression was quantified using Nanostring technology. Of the 348 miRNAs expressed in the pulps, 36 were altered in infected pulps ($p<0.003$). The potential gene targets for these miRNAs included key mediators of the immune response. LPS challenge resulted in time-dependent changes in miRNA expression. Together our data provide support for the role of miRNAs in endodontic disease.

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CHAPTER 1

INTRODUCTION: microRNA in the pathogenesis of human endodontic disease

1.1 Endodontic disease and dental pain

According to the American Association of Endodontists, more than 14 million root canals are performed every year (http://www.aae.org/). A population-based longitudinal study - the Florida Dental Care Study (FDCS) reported that up to 13% of participants received endodontic treatment on at least one tooth during a four year period [1]. The prevalence of periapical disease is 4.1% in the US population [2]. In another epidemiological study, Makeeva et al. reported that the need for endodontic therapy was 31% [3]. The above mentioned studies highlight the prevalence of endodontic disease.

Pain is a common symptom of endodontic disease. Pau et al reviewed the prevalence and associated factors for dental pain and reported that the prevalence of oral and facial pain ranged between 40 to 44% [4]. According to the Executive Summary of the Surgeon General's report published in 2000, 22% of adults reported having experienced some form of orofacial pain in the past 6 months [5]. Up to 90% of the pain experienced by patients seeking emergency dental treatment is attributable to pulpal or/and periapical origin [6]. Every year, about 15 million working days are lost because of dental pain in the United States [5]. Despite the high prevalence and great discomfort suffered by patients, the fundamental molecular aspects of the pathogenesis of endodontic disease are still not fully understood, which hinders the effort to develop new ways of prevention and treatment.

1.2 The diagnosis and classification

The classification and diagnosis of endodontic diseases is based on American Board of Endodontics' Pulpal and Periapical Definitions (http://www.aae.org/certboard/currentnews/.)

Pulpal

Normal pulp: A clinical diagnostic category in which the pulp is symptom free and normally responsive to vitality testing.

Reversible pulpitis: A clinical diagnosis based on subjective and objective findings indicating that the inflammation should resolve and the pulp return to normal.

Irreversible pulpitis: A clinical diagnosis based on subjective and objective findings indicating that the vital inflamed pulp is incapable of healing.

Additional Descriptions

Symptomatic: Lingering thermal pain, spontaneous pain, referred pain.

Asymptomatic: No clinical symptoms, but inflammation produced by caries, caries excavation, trauma.

Pulp necrosis: A clinical diagnostic category indicating death of the dental pulp. The pulp is nonresponsive to vitality testing.

Previously treated: A clinical diagnostic category indicating that the tooth has been endodontically treated and the canals are obturated with various filling materials other than intracanal medicaments.

Previously initiated therapy: A clinical diagnostic category indicating that the tooth has been previously treated by partial endodontic therapy (eg, pulpotomy or pulpectomy).

Apical (Periapical)

Normal apical tissues: Teeth with normal periradicular tissues that are not abnormally sensitive to percussion or palpation testing. The lamina dura surrounding the root is intact and the periodontal ligament space in uniform.

Symptomatic apical periodontitis: Inflammation, usually of the apical periodontium, producing clinical symptoms, including painful response to biting and percussion. It may or may not be associated with an apical radiolucent area.

Asymptomatic apical periodontitis: Inflammation and destruction of apical periodontium that is of pulpal origin, appears as an apical radiolucent area, and does not produce clinical symptoms.

Acute apical abscess: An inflammatory reaction to pulpal infection and necrosis characterized by pain onset, spontaneous pain, tenderness of the tooth to pressure, pus formation and swelling of associated tissues.

Chronic apical abscess: An inflammatory reaction to pulpal infection and necrosis characterized by gradual onset, little or no discomfort, and the intermittent discharge of pus through an associated sinus tract.

1.3 Pathogenesis of endodontic disease

Endodontic disease is a result of polymicrobial infection of the root canal system and the host response. The role of microbes in pulpal and periradicular disease is well established [7] and over 460 bacterial taxa belonging to 100 genera and 9 phyla have been identified in endodontic infections [8]. Carious infection is by far the most common etiology for pulpal inflammation and necrosis. As the carious infection progresses from the tooth surface towards the pulp-dentin complex, both the innate and adaptive immunity are activated. Due to their peripheral positioning, odontoblasts are the first cells encountered by the pathogens and their byproducts followed by the more centrally located fibroblasts and immature dendritic cells [9]. Infiltration by macrophages and polymorphonuclear neutrophils constitutes the early response to frank bacterial infection of the pulp as well as to the diffusion of bacterial antigens through dentinal tubules. A diffuse infiltration with lymphocytes, plasma cells and macrophages occurs as the carious lesion approaches closer to the pulp chamber [10]. Microorganisms and their byproducts interact with immune cells via pattern recognition receptors such as Toll-Like Receptors (TLRs), Nod-like receptors (NLRs), Rig-I-like receptors (RLRs), C-type lectin receptors (CLRs) and the receptor for advanced glycation end products (RAGE). Receptor engagement induces transcriptional changes culminating in the release of numerous inflammatory and immune mediators such as cytokines (interleukin {IL}-1β, IL-6, tumor necrosis factor {TNF}-α), chemokines (IL-8), neuropeptides (substance P and calcitonin gene related peptide), and many others [11]. Increased blood flow, sprouting of neuronal terminals and

cytochemical changes occurs during pulpal inflammation [12] while complex and dynamic interactions take place among the neural elements, pulp cells, immunocompetent cells, pulpal tissue fluid, and vasculature, all of which contribute to the progression of endodontic disease. Although the pathogenesis of endodontic disease has been studied extensively, the genetic regulation of the inflammatory and immune response of the pulp is yet to be elucidated.

1.4 microRNA in inflammation and immune response

The discovery of microRNAs (miRNAs) is one of the major scientific breakthroughs in recent years and has revolutionized our understanding of gene regulation [13]. miRNAs are short single strand, non-coding RNA molecules (~22 nucleotides) that bind to complementary sequences of target messenger RNA transcripts (mRNAs) at the 3'untranslated region (3' UTR), resulting in translational repression or mRNA degradation [14]. miRNA was first discovered and named lin-4 in 1993, as a regulator of developmental timing in the nematode, *Caenorhabditis elegans* [15], [16]. In 2001, the term "microRNA" was introduced [17].

Given that miRNAs are one of the largest classes of gene regulatory molecules, understanding their physiological roles is of vital importance. Biochemical and genetic studies have revealed that miRNAs play an important role in a wide variety of biological processes, such as proliferation, differentiation, cell fate determination, apoptosis, signal transduction and organ development [13], [14]. Emerging evidence supports that abnormal miRNA expression is a common feature of various human diseases such as developmental abnormalities, cardiovascular disorders, cancer and inflammatory diseases [18],[19],[20],[21]. Thus, miRNAs represent an important target for potential therapeutic and diagnostic agents.

As our understanding of miRNA function continuous to develop, accumulating evidence supports the role of miRNA in inflammation and in the immune responses [22], [23]. The importance of miRNA in regulating the immune response is underlined by their unique expression in response to lipopolysaccharide (LPS). LPS is the major surface component of the cell envelope for virtually all gram-negative organisms and represents one of the conserved microbial structures responsible for

activation of the innate immune system. The first study on LPS-induced miRNA changes was performed on THP-1 human monocytes by Taganov et al. [23]. They reported up regulation of miRNA-146a, miRNA-155 and miR-132. Further characterization of miR-146a/b demonstrated that its induction by TNF-α and IL-1β treatments also in an NFκB-dependant manner. The direct targets of miR 146 include two key adapter molecules in the TLR/IL-1β pathway, tumor necrosis factor receptor-associated family TRAF6 and interleukin-1 receptor-associated kinase (IRAK1). The authors proposed that miR-146 functions as the effector arm of a negative feedback mechanism regulating the response to bacterial products [23]. Accordingly its expression may be critical in preventing excess inflammation. The induction of miR-155 by LPS was confirmed by another group of researchers who identified several genes involved in LPS signaling to be targeted by miR-155 [24]. This finding suggests that similar to miR-146, miR-155 is involved in the negative regulation of immune responses to LPS. Interestingly, they found that miR-155 not only represses but, by enhancing TNF-α translation, also favors the activation of the LPS/TNF-α pathway. In vitro and in vivo studies on miR-155 have implicated it in various immune functions involving innate as well as adaptive immune responses and the development of immune cells [25], [26]. miR-132 may also be responsible for limiting inflammation in response to bacterial and viral infection [27], [28].

Evidence of the role of miRNAs in fine tuning the immune response as well in acute and chronic inflammation continues to accumulate. Therefore, it is reasonable to hypothesize that miRNAs are involved in regulating gene expression associated with endodontic disease – a result of the immune and inflammatory response to microbial infection.

1.5 Previous studies and hypothesis

Although miRNAs are important regulators of cellular differentiation and function, very few studies have evaluated their role in oral diseases. A recent study reported differential expression of miRNA in inflamed and normal gingival tissues while another identified miRNAs which potentially link the molecular pathways between obesity and periodontal inflammation [29],[30]. However, the role of miRNA in endodontic disease is yet to be explored.

Primary endodontic infections are polymicrobial infections predominated by gram-negative anaerobic bacteria [31], [32], [33]. Infiltration by macrophages and polymorphonuclear neutrophils constitutes the early response to bacterial infection and their byproducts. The immune response is initiated upon recognition of conserved pathogen-associated molecular patterns (PAMPS) by transmembrane host pattern-recognition receptors (PRR) such as Toll-like receptors (TLR) . Lipopolysaccharide, a major component of the cell walls of gram-negative bacteria, is recognized by TLR4 [34]. The binding of LPS to TLR4 results in the activation of nuclear factor κ B, interferon response factor 3 and mitogen-activated protein kinase pathways resulting in the expression of several pro-inflammatory genes such as tumor necrosis factor-α (TNF-α), interleukin-18 (IL-18) and cyclooxygenase-2 (COX-2) [35]. Given the emerging role of miRNA in immune response, it is highly likely that they modulate the pulpal response to microbial infection.

In the residual cells of the dental pulp, TLR4 is expressed on odontoblasts and fibroblasts, both of which differ in their response to LPS [36], [37]. When the pulp is infected, the initial immune response includes infiltration by macrophages and polymorphonuclear neutrophils both of which express TLR4. A population of trigeminal neurons that express Transient Receptor Potential Vanilloid-1 (TRPV-1), an ionotropic channel that is expressed exclusively in nociceptors and is required for the development of inflammatory hyperalgesia, also co-express TLR4 [38]. Furthermore, LPS can directly activate trigeminal sensory neurons and sensitize TRPV1 via TLR4 pathways [39]. Thus suggesting that pain associated with bacterial infections of the dental pulp, may be due in part to the direct activation of nociceptors via TLR4.

The purpose of our first study was to characterize the miRNA expression profiles of normal and inflamed dental pulps and to identify their potential mRNA targets. We hypothesized that inflamed dental pulps differentially express miRNAs as compared to normal dental pulps. The purpose of our second study was to gain further insight on the role of miRNAs in regulating the immune response to endodontic infections. We hypothesized that miRNA is responsive to LPS challenge, a common

endodontic pro-inflammatory mediator, and induces expression changes of miRNAs predicted to target key genes of the innate immune response.

The information obtained from this study will help further our understanding of epigenetics in the pathogenesis of endodontic diseases and may elucidate potential therapeutic targets to more effectively control pulpal inflammation and reduce odontogenic pain.

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CHAPTER 2

Differential expression of microRNAs in normal and inflamed human pulps

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Abstract

Introduction: MicroRNAs (miRNAs) are small non-coding RNAs which regulate gene expression via post-transcriptional repression. They are critical to normal cellular function and bioinformatic predictions indicate that at least one third of all messenger RNAs may be regulated by miRNAs. While both the innate and adaptive immune response are known to be regulated by miRNAs, their role in regulating endodontic disease is yet to be explored. The purpose of this study is to examine the differential expression of miRNAs in normal and inflamed human dental pulps and to explore their functional gene targets. **Methods:** After obtaining informed consent we collected normal and inflamed human pulps (N=30). Microarray and molecular biology techniques were then used for gene profiling and identifying functional gene targets. **Results:** Of the 335 human miRNAs identified in the pulp tissues, 3 miRNA- miR-150*, miR-584 and miR-766 were significantly upregulated in inflamed pulps as compared to normal pulps ($p<0.003$). Thirty three miRNAs were down regulated in the inflamed pulps ($p<0.003$). The false discovery rate for these findings is estimated to be approximately 5%. The potential gene targets for these miRNAs include pro-inflammatory cytokines as well as other key mediators of the immune and inflammatory response to infection. **Conclusions:** Our data identifies differential expression of miRNAs in healthy and diseased human dental pulps. These findings highlight the intricate and specific roles of miRNA in inflammation and immunity, both of which are key aspects of pulpal pathology.

Introduction:

Endodontic disease characterized by inflammation of the pulp and periapical tissues is fairly prevalent with more than 14 million root canal procedures being performed every year (http://www.aae.org/). The Florida Dental Care Study (FDCS), a population-based longitudinal study reported that up to 13% of its participants received endodontic treatment on at least one tooth during a four year period [1]. The prevalence of periapical disease in the United States is estimated to be about 4.1% [2]. 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 [3]. 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.

Endodontic disease is a result of microbial infection of the root canal system and the host response. The role of microbes in pulpal and periradicular disease is well established [4] and over 460 bacterial taxa belonging to 100 genera and 9 phyla have been identified in endodontic infections [5]. Carious infection is by far the most common etiology for pulpal inflammation and necrosis. As the carious infection progresses from the tooth surface towards the pulp-dentin complex, both the innate and adaptive immunity are activated. Due to their peripheral positioning, odontoblasts are the first cells encountered by the pathogens and their byproducts followed by the more centrally located fibroblasts and immature dendritic cells [6]. The inflammatory and immune mediators released in the inflamed pulp include interleukin (IL)-6, IL-8, monocyte-chemo attractant protein-1, prostaglandin E2 and inducible nitric oxide synthase [7].

While information regarding the inflammatory and immune responses to microbial infection of the dental pulp continues to accumulate, very little is known about the genetic regulation of pulpal disease. microRNAs (miRNA) have recently emerged as important regulators of gene expression in diverse biological processes including inflammation, immune response, and osteoclastic bone resorption [8]. [9]. They are small (~20-22 nucleotide), single stranded, non-coding RNA molecules that bind to complementary sequences within the 3' untranslated region (UTR) of target mRNA species, evoking messenger RNA (mRNA) degradation or translational repression [10]. Bioinformatic predictions indicate that in humans at least one third of all mRNAs may be regulated by miRNAs [11].

miRNAs are known to regulate both the innate and adaptive immune responses. For example, miR-223 plays a crucial role in granulocyte differentiation [12]. miR-223 mutant mice spontaneously develop inflammatory lung pathology and exhibit exaggerated tissue destruction after endotoxin challenge [13]. In macrophages, interferon-β and toll-like receptor (TLR) ligands can induce the

upregulation of miR-155 via both the Nuclear Factor-kappa B and Jun N-terminal Kinase pathways [14]. miR-155 deficient dendritic cells are unable to induce efficient T-cell activation in response to antigens [15]. In contrast, miR-125b is downregulated upon lipopolysaccharide (LPS) stimulation in macrophages. This suggests miR-125b is required to ensure a proper inflammatory response by macrophages in response to microbial stimuli [15]. These studies highlight the central role of miRNA in regulating immunity and the inflammatory response.

Although miRNAs are important regulators of cellular differentiation and function, very few studies have evaluated their role in oral diseases. A recent study reported differential expression of miRNA in inflamed and normal gingival tissues [16] while another identified miRNAs which potentially link the molecular pathways between obesity and periodontal inflammation [17]. However, the role of miRNA in endodontic disease is yet to be explored. The purpose of this study is to characterize miRNA expressed in normal and inflamed dental pulps and to identify their potential mRNA targets. We hypothesize that inflamed dental pulps differentially express miRNAs as compared to normal dental pulps.

Materials and Methods:

Study participants and sample collection: This study was approved by the Institutional Review Board, at the University of North Carolina at Chapel Hill. Written informed consent was obtained from 28 participants who were recruited from the School of Dentistry, University of North Carolina at Chapel Hill. The inclusion criteria were ≥12 years old and American Association of Anesthesiologists status I or II. Patients who had a compromised immune system or those who were taking medications known to influence the immune response were excluded from the study. Participants were enrolled into 2 groups based on the pulpal status of the teeth being treated. Normal pulps were extirpated from healthy third molars or teeth extracted for orthodontic purpose. These did not include teeth with carious lesions or deep (≥ 5mm) probing defects. Inflamed pulps were extirpated from carious teeth diagnosed with irreversible pulpitis defined as either carious pulpal exposure or the presence of spontaneous pain and an exaggerated and lingering response to cold (1,1,1,2-tetrafluoroethane) [18].

After obtaining informed consent, local anesthesia was administered. For teeth diagnosed with normal pulps, the teeth were extracted and the root canal system immediately accessed. The pulp was then extirpated using a sterilized barbed broach or Hedstrom hand file. For teeth diagnosed with inflamed pulps, rubber dam isolation was obtained and the tooth and rubber dam were disinfected with 0.2% chlorhexidine gluconate. The carious tooth structure was removed and then the root canal system was accessed. Pulp tissue was collected using a sterilized barbed broach or

Hedstrom hand file. Pulp tissue was gently separated from the instrument and placed in a sterile eppendorf tube with 0.5ml RNAsafer Stabilizer Reagent (VWR, Bridgeport, NJ). All samples were stored at -80^{o} C until processing.

RNA isolation and miRNA microarray: Samples were thawed on ice and centrifuged at 4^{o}C for 2 minutes at 12,000 rpm to remove the stabilizer reagent. Total RNA was extracted using the miRNeasy Mini kit (Qiagen, Valencia, CA) according to manufacturer's instructions. The RNA was quantitated using the NanoDrop (Thermo Scientific, Wilmington, DE) and RNA integrity assessed using the 2100 Bioanalyzer (Agilent, Foster City, CA). The miRNA expression profiles of normal and inflamed pulps were interrogated using Human miRNA Microarrays (V3) and the miRNA Complete Labeling and Hyb Kit (both from Agilent Technologies, Santa Clara, CA). The microarrays consist of glass slides containing 8 identical 15K oligonucleotide microarrays incorporating probes for 866 human and 89 human viral miRNAs represented from the Sanger miRBase 12.0. The procedure was performed as described previously [19]. Briefly, 300 ng of total RNA samples were dephosphorylated, denatured by dimethyl sulfoxide, and then immediately transferred to ice-water bath for ligation. The samples were incubated with T4 RNA ligase at 16^{o}C in a circulating water bath for 2 hours. Labeled miRNAs were desalted through Micro Bio-spin 6 columns (Bio-Rad, Hercules, CA) for purification. Samples were hybridized at 55^{o} C for 20 hours at 20 rpm in a rotating hybridization oven. The microarray slides were then washed using fresh wash buffer. Finally, the slides were scanned using the Agilent Microarray Scanner and the Agilent Feature Extraction Software version 10.5.1.1 (both from Agilent, Foster City, CA).

Bioinformatics miRNA analysis and target selection: Potential mRNA target genes for differentially expressed miRNAs in normal and inflamed dental pulps were identified using PUBMED and miRWalk (http://www.rna.uni-heidelberg.de/apps/zmf/mirwalk/index_html). miRWalk is a comprehensive database that provides information on miRNA from human as well as mouse and rat on their predicted as well as validated binding sites on their target genes. It is based on a comparison of computed mRNA 3' UTRs miRNA binding sites with 8 miRNA-target prediction programs: Diana-microT (http://diana.cslab.ece.ntua.gr/microT/), miRanda (http://www.microrna.org/microrna/home.do), miRDB (http://mirdb.org/miRDB/), Pictar (http://pictar.mdc-berlin.de/), Pita (http://genie.weizmann.ac.il/pubs/mir07/mir07_prediction.html), RNA22 (http://cbcsrv.watson.ibm.com/rna22.html), RNAhybrid (http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/submission.html), and Targetscan (http://www.targetscan.org/). Candidate mRNAs were selected if they were identified as miRNA targets in at least 5 out of 8 databases and were linked to immunity, inflammation and pain by GO Biological Process

(www.geneontology.org). Results from both miRWalk and PUBMED search were integrated to reach our final results.

Statistical analysis: For microarray data analysis, any expression value that was lower than the reported error for that particular gene (which includes negative expression values) was set to be equal to the estimated error rate. Quantile normalization was applied to the expression data. To identify genes that were differentially expressed in each group, we applied a permutation test to test the null hypothesis that the mean expression of each gene was the same in both groups. An exact hypothesis test was used since the sample size was small. We used the resulting p-values to estimate the false discovery rate q-value when the differential expression of each miRNA is called "significant". For each resulting p-value, we computed the q-value, which is defined to be the false discovery rate when all tests with a p-value less than or equal to the given p-value are called "significant." Since we performed multiple hypothesis tests, the usual $p<0.05$ threshold for significance was too liberal for this analysis. However, using the Bonferroni-adjusted significance threshold ($p<0.05/335$) would be too conservative and result in a less of power given our small sample size. Thus, for each p-value, we computed the q-value, which is defined to be the false discovery rate when all tests with a p-value less than or equal to the given p-value are called "significant" [20]. This allows us to control for type I error while avoiding the power loss that would result from using a Bonferroni correction.

Results:

Twenty-eight patients were recruited into the study. The total number of teeth from which pulps were collected is 30. The demographic data of the study subjects is summarized in Table 1. No differences were noted in gender distribution between the two groups although subjects from whom inflamed pulps were collected were significantly older than those from whom normal pulps were collected ($p<0.001$). The latter group consisted primarily of younger individuals undergoing tooth extraction for orthodontic reasons or for removal of 3rd molars. None of the teeth in the normal group were extracted due to carious or periodontal pathology. In the inflamed group 12 teeth had carious pulpal exposure with no recent history of spontaneous pain or thermal sensitivity while the remaining 6 teeth had a carious lesion and were associated with spontaneous pain and/or lingering response to cold.

Three hundred and thirty-five human miRNAs were identified that were expressed in both normal and inflamed human pulp tissues (Supplemental Data Table). Of these, 36 miRNAs were differentially expressed in inflamed pulps as compared to normal pulps (Fig 1). Three miRNAs, namely miR-150*, miR-584 and miR-766 were upregulated in inflamed pulps as compared to normal pulp. The fold changes for miR-150*, miR-584, and miR-766 were 2.71, 4.54 and 2.17

respectively. Thirty three miRNAs were downregulated in inflamed pulps as compared to normal pulps. The most significantly repressed expression was noted for miR-664*, miR-214*, and miR-152 which had a fold change of 7.11, 4.23 and 4.05 respectively. The q-values for these miRNAs varied between 0.032 and 0.047, suggesting that no more than 5% of these miRNAs are spurious findings whose apparent group differences are due to chance.

To identify validated and predicted targets for the miRNAs differentially expressed in inflamed pulps we first searched the miRWALK database and identified 1045 miRNAs known to regulate inflammation. These included 10 of the 36 miRNAs that were differentially expressed in inflamed pulps. The role of these miRNAs in regulating inflammation was confirmed using PUBMED. The target genes comprise key mediators of the inflammatory response to infection including those involved in the detection of microbial ligands (toll-like receptor 4{TLR4}), chemotaxis (chemokine ligands 8 and 3 {CCL8, CCL3}), proteolysis (MMP9, matrix metalloprotease 9), pro-inflammatory and anti-inflammatory cytokines (IL1, IL6, IL-10, TNF α) and signal transduction molecules (mitogen-activated protein kinase 3 and 8). Importantly, the target genes identified also included those known to suppress inflammation such as suppressor of cytokine signaling 1 (SOCS1) and macrophage migration inhibitory factor (MIF) (Table 2). The results of *in silico* studies predict that some of the miRNAs that were differentially regulated in inflamed pulps target genes involved in sensing thermal, mechanical and electrical stimuli (Table 3).

Discussion:

miRNAs regulate gene expression by binding to target mRNAs and initiating the degradation or translational repression of mRNAs [10]. They were first discovered as a regulator of developmental timing in *Caenorhabditis elegans* in 1993 [21], [22]. To date, 1921 unique mature human miRNA sequences have been identified in the human genome (miRbase 18, Nov 2011, http://microrna.sanger.ac.uk/) and most protein-coding genes are likely to be regulated by miRNAs [11]. The high sequence conservation of many miRNAs among distantly related organisms highlights the significance of their function. Indeed, biochemical and genetic studies have revealed that miRNAs act as key regulators in cell development, differentiation, proliferation, and apoptosis [10]. Expression and mechanistic studies now link miRNAs to major human diseases ranging from cancer to inflammation.

The role of miRNA in regulating inflammation and the immune response is very complex since a single miRNA can regulate dozens or maybe even hundreds of different mRNAs. Conversely, a single mRNA can be targeted by multiple unique miRNAs. miRNA can also have different functional characteristics based on the cell types where it is expressed and can serve as negative

regulators of gene expression. Indeed, several miRNAs such as miRNA-155, miRNA-9 and miRNA-146 are part of a negative feedback mechanism in the innate immune response and may limit excessive inflammation [14], [23], [24]. In this and other ways miRNAs are thought to “fine-tune” the inflammatory response.

In our study, 3 miRNAs, namely miR-150*, miR-584 and miR-766 were significantly upregulated in inflamed pulps as compared to normal pulps. Our finding that only 3 miRNA's were upregulated is probably conservative. Our choice of 3 miRNA's ensured that the false discovery rate (estimated by the q-value) is no more than 0.05. Data in the supplementary Table indicates that there is strong evidence that additional miRNA's are upregulated. However, classifying any additional miRNA's as "signficantly upregulated" would have resulted in a significant increase in the false discovery rate.

Thirty-three miRNAs were downregulated in inflamed pulps as compared to normal pulps. miR-152, a negative regulators of the innate response and antigen presenting capacity of dendritc cells, inhibits the production of cytokines including IL-12, IL-6, TNF-α, and IFN-β [25]. miR-148, a negative regulator of the innate response was downregulated by two fold. Similar to miR-152, it also functions to regulate the antigen presenting capacity of dendritic cells [25]. Several of the miRNA 181 family members were downregulated. These included miR-181a, known to regulate IL-6 [26], miR-181b known to regulates CCL8 [27], miR-181c which regulates IL-2 [28] and miR-181d which regulates MMP9 [29] expression. These findings highlight the intricate and specific roles of miRNA in inflammation and immunity, key aspects of pulpal pathology.

Animal models of acute and inflammatory pain report altered expression of several miRNAs [30]. Clinical studies also report altered miRNA expression in painful diseases such as rheumatoid arthritis, osteoarthritis, complex regional pain syndrome, systemic lupus erythematosus and others [31], [32]. For example, the expression of miRNA let-7c is correlated with hypesthesia (decreased sensation to mechanical stimulation) in patients with complex regional pain syndrome [32]. Our data also show altered expression of let-7c in inflamed pulps as compared to normal pulps. The potential targets of the miRNAs which were altered in the present study target kinases involved in intracellular signaling pathways known to be involved in the sensitization of primary afferent nociceptors.

Another class of short RNA molecules, small interfering RNA (siRNA), has also been identified as sequence-specific posttranscriptional regulators of gene expression. Similar to miRNA, they are small non-coding RNA oligonucleotide molecules produced by Dicer, a double-stranded RNA-specific enzyme of the RNAse III family [33]. siRNA and miRNA are incorporated into related RNA-induced silencing complexes (RISCs), termed siRISC and miRISC, respectively. This process may indicate the functional similarity of miRNA and siRNA. However, much evidence suggests that siRISCs and miRISCs are distinct types of complex due to different biogenesis, maturation and

subsequent assembly of siRNAs and miRNAs into silencing complexes. Many endogenous miRNAs and their RISCs are genetically programmed to regulate gene expression and thus are important for the growth and development of an organism. By contrast, siRNAs are produced from dsRNAs that are often synthesized *in-vitro* or *in-vivo* from viruses or repetitive sequences introduced by genetic engineering. Thus, siRNAs have been proposed to function in antiviral defense, silencing mRNAs that are overproduced or translationally aborted, and guarding the genome from disruption by transposons [34], [35].

The limitations of this study include the small sample size and low RNA yields. The small sample size required us to use a permutation test rather than a more conventional t-test, which potentially reduces the power to detect group differences. Due to the limited amounts of tissue that could be obtained from some samples, pooling of some samples was required to reach the minimum required miRNA concentration for probe labeling prior to microarray hybridization. Additional studies using larger cohort populations as well as PCR confirmation of the array data will be required. Functional studies will be required to confirm and validate our *in-silico* findings. Despite these limitations, our data has identified differential expression of miRNAs in healthy and diseased pulps and has identified mRNA targets linked to the host inflammatory and immunological responses.

Primary endodontic infection is dominated by gram negative anaerobes. As such future studies should address the impact of these microorganisms and their byproducts on miRNA expression profiles. It will also be necessary to compare/contrast the expression of miRNAs between normal and inflamed periapical tissues as well as to compare periapical and pulpal miRNA profiles. *In-situ* hybridization will aid in identifying the cellular sources of the miRNAs. Importantly, deregulated miRNA expression has been reported in autoimmune and inflammatory conditions such as rheumatoid arthritis and osteoarthritis and recent studies have identified differential miRNA expression in periodontal tissues suggesting that miRNA may also play a contributory role in endodontic pathogenesis. In conclusion, our study provides novel insights into the pathogenesis of endodontic lesions and may identify potential novel therapeutic targets for future investigation.

Statement regarding conflict of interest

We affirm that we have no financial affiliations (e.g., employment, direct payment, stock holdings, retainers, consultantships, patent licensing arrangements or honoraria) or involvement with any commercial organization with direct financial interest in the subject or materials discussed in this manuscript not have any such arrangements existed in the past three years.

Protection of human subjects

The informed consent of all human subjects who participated in the experimental investigation reported or described in this manuscript was obtained after the nature of the procedure and possible discomforts and risks had been fully explained

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Legends

Fig 1. Differential expression of miRNAs in normal (n=12) and inflamed (n=18) human pulps. miR-150*, miR-584 and miR-766 were significantly upregulated in inflamed pulps as compared to normal pulps ($p<0.003$). Thirty three miRNAs were down regulated in the inflamed pulps ($p<0.003$). Data was analyzed using an exact hypothesis test and are expressed as log values.

Table 1. Study participant demographics (N=28). Mann-Whitney U test was used to compare age and McNemar's Chi-square test was used to compare gender distribution between the two groups. Age is presented as mean ± standard deviation. Pain history is defined as patients' report of spontaneous or thermally evoked pain in the past 14 days.

Table 2. miRNAs differentially expressed in normal and human dental pulp. Potential miRNA target genes were identified using PUBMED and miRWalk (http://www.rna.uni-heidelberg.de/apps/zmf/mirwalk/index_html).

Table 3. miRNAs which potentially regulate the response to thermal, mechanical and electrical stimuli. Potential effects were analyzed using www.AMIGO.com

Supplemental data table. List of human miRNAs identified in normal and inflamed pulps.

TABLE 1

Variables	Normal pulp (n=12)	Inflamed pulp (n=18)
Mean Age (StdDev)	18 (3.00)	37 (14.42)
Gender		
Female	5 (42%)	10 (56%)
Male	7 (58%)	8 (44%)
Pain History	0%	6 (33%)

Table 2

miRNA	Target gene	Gene product function	GO Term (Accession, Ontology)
hsa-let-7c	IL-13	Cellular response to mechanical stimuli; negative regulation of TGF beta production; positive regulation of macrophage activation; response to LPS	GO:0005144, Molecular Function
hsa-miR-128	TNF	Chronic inflammatory response to antigenic stimuli; LPS mediated signaling; regulation of cytokine signaling involved in immune response; positive regulator of chemokin ligand 2 production; apoptosis	GO:0032640, Biological Process
hsa-miR-140-3p	IL-1B	Cellular response to mechanical stimuli, response to heat; LPS mediated signaling; positive regulation of IL-2, 6 and 8; positive regulator of JNK cascade, nitric oxide production, VEGF production, and prostaglandin synthesis	GO:0050702, Biological Process
hsa-miR-148a	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	GO:0005138, Molecular Function
hsa-miR-150*	IL6	Negative regulation of cytokine secretion; negative regulation of collagen biosynthetic process; positive regulation of acute inflammatory response; response to cold, heat and mechanical stimuli	GO:0005138, Molecular Function
	NFKB1	Anti-apoptosis; negative regulaiton of cytokine production, reponse to bacterium; reponse to oxidative stress	GO:0034977, Cellular Component

	JAK2	Activation of MAKK activity, GPCR signaling; positive regulation of IL-1 beta production, positive regulation of nitric oxide synthesis pathway; response to LPS, TNF, IL-12	GO:0042977, Biological Process
	IRAK2	Activation of innate immune response, inflammatory response, IL-1 signaling; activation of MAPK pathway, JNK cascade, LPS mediated signaling, MyD88 dependent toll like receptor signaling pathway, Regulation of NFKB, TLR1-4 signaling, protein phosphorylation	
hsa-miR-152	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	GO:0005138, Molecular Function
	TLR-4	A pattern recognition receptor that binds bacterial lipopolysaccharide (LPS) to initiate an innate immune response	GO:0035662, Molecular Function
	MAPK8	MAPK activity; cellular reponse to mechanical stimuli; induction of apoptosis by extracellular, intracellular and chemical stimuli; innate immune response	GO:0007254, Biological Process
hsa-miR-181a*	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	GO:0005138, Molecular Function
	TGFB1	Adaptive immune response; positive regulation of collagen biosynthesis, chemotaxis, fibroblasts migration, and ondontogenesis	GO:0034713, Molecular Function

	STAT1	Cytokine mediated signaling pathway; LPS mediated signaling pathway	GO:0042508, Biological Process
hsa-miR-181a-2*	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	GO:0005138, Molecular Function
	STAT1	Cytokine mediated signaling pathway; LPS mediated signaling pathway	GO:0042508, Biological Process
hsa-miR-181b	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	GO:0005138, Molecular Function
	CCL8	Immune response, inflammatory response, chemokine activity, phospholipase activator	
	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	GO:0034713, Molecular Function
hsa-miR-181c	SOCS1	Cytokine mediated signaling pathway; negative regulator of JAK-STAT pathway; LPS response	
	IL-2	Cytokine produced by T-cells in response to antigen or mitogen stimulation	GO:0005134, Molecular Function
has-miR-181d	MMP9	Cell response to IL-1, LPS; macrophage differentiation; response to heat and mechanical stimuli;	GO:0004229, Molecular Function

		positive regulation of apoptosis and angiogenesis	
hsa-miR-192	IL-1B	Cellular response to mechanical stimuli, response to heat; LPS mediated signaling; positive regulation of IL-2, 6 and 8; positive regulator of JNK cascade, nitric oxide production, VEGF production, and prostaglandin synthesis	GO:0050702, Biological Process
	TNF	Chronic inflammatory response to antigenic stimuli; LPS mediated signaling; regulation of cytokine signaling involved in immune response; positive regulator of chemokin ligand 2 production; apoptosis	GO:0032640, Biological Process
hsa-miR-199a-5p	MAPK1	MAPK activity, T call and B cell activity, macrophage activation, chemotaxis, induction of apoptosis, LPS signaling pathway, MAPKK pathway, sensory perception of pain, synaptic transmission	
	MAPK8	Cellular response to mechanical stimuli, activation of pro-apoptotic genes, JNK cascade, activation of MyD 88 dependent and independent TLR signaling pathways, NGF signaling pathway, TLR1-4 signaling pathway	GO:0007254, Biological Process
	MAPK9	Cell response to IL-1, LPS; macrophage differentiation; response to heat and mechanical stimuli; positive regulation of apoptosis and angiogenesis	GO:0004229, Molecular Function
	MAPK14	Angiogenesis, LPS mediated signaling pathway	
hsa-miR-	PTEN	Angiogensis, apoptosis, cell migration, cell proliferation, NGF receptor	GO:0051800, Molecular Function

214*		signaling	
	TGFBR2	Apoptosis, B cell tolerance, T cell tolerance	
hsa-miR-30a*	TGFBR1	Activation of MAPKK activity; collagen fibril organization; negative regulation of the apoptotic process; positive regulation of cell proliferation; response to electrical stimuli; response to PGE stimulation; wound healing	
	CCL13	Immune response; inflammatory response; eosinophil chemotaxis	
	TNFSF9	Apoptotic process; immune response; cell proliferation; cytokine activity	
hsa-miR-451	MIF	Lymphokine involved in cell-mediated immunity, immunoregulation, and inflammation; suppression of anti-inflammatory effects of glucocorticoids	GO:0035718, Molecular Function
hsa-miR-455-3p	IL-10	Anti-inflammatory cytokine	GO:0019969, Molecular Function
hsa-miR-455-5p	IL-10	Anti-inflammatory cytokine	GO:0019969, Molecular Function
hsa-miR-584	MAPK8	Cellular response to mechanical stimuli, activation of pro-apoptotic genes, JNK cascade, activation of MyD 88 dependent and independent TLR signaling pathways, NGF signaling pathway, TLR1-4 signaling pathway	GO:0007254, Biological Process
hsa-miR-766	HSF1	The product of this gene is a heat-shock transcription factor. Transcription of heat-shock genes is rapidly induced after temperature stress.	

hsa-miR-98	IL-1B	Cellular response to mechanical stimuli, response to heat; LPS mediated signaling; positive regulation of IL-2, 6 and 8; positive regulator of JNK cascade, nitric oxide production, VEGF production, and prostaglandin synthesis	GO:0050702, Biological Process
	TNF	Chronic inflammatory response to antigenic stimuli; LPS mediated signaling; regulation of cytokine signaling involved in immune response; positive regulator of chemokin ligand 2 production; apoptosis	GO:0032640, Biological Process

TABLE 3.

Response to	miRNA
Heat	hsa-miR-181b, hsa-miR-199b-5p, hsa-miR-199b-5p, hsa-miR-150*
Cold	hsa-miR-15b, hsa-miR-150*
Electrical	hsa-miR-30a*
Mechanical	hsa-let-7c, hsa-miR-142-3p, hsa-miR-150*, hsa-miR-181b, hsa-miR-199a-5p, hsa-miR-199b-5p

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CHAPTER 3

Lipopolysaccharide Induced Changes in microRNA Expression

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Abstract

Introduction: microRNAs (miRNAs) have emerged as important regulators of the innate and adaptive immune response. The role of miRNA in modulating the immune response to endodontic infection is yet to be fully explored. We previously identified altered expression of 36 miRNAs in inflamed human pulps extirpated from carious teeth as compared to normal pulps The purpose of the present study is to investigate the effect of lipopolysaccharide (LPS) on the expression of these 36 miRNAs. **Methods**: CD14 positive monocytes were isolated from human buffy coats, cultured and induced to macrophages. The macrophages were then challenged with *Escherichia coli* 0111:B4 LPS (5ng/ul) and incubated for 1, 4 and 8 hours. Controls were macrophages not exposed to LPS. At the end of the incubation period, total RNA was extracted, and miRNAs quantified by nanostring technology. Data were analyzed using Kruska-Wallis test. **Results:** The expression of 4 miRNAs, miR-199a-5p, miR-214, miR-455-5p and miR-629, was significantly decreased at the 4 hour time point as compared to controls ($p < 0.05$). **Conclusion:** These results provide further support for the role of these miRNAs in modulating the pulpal and periapical response to infection.

Introduction:

Primary endodontic infections are polymicrobial infections predominated by gram-negative anaerobic bacteria [1], [2], [3]. The initial immune response to bacterial infection involves the recognition of conserved pathogen-associated molecular patterns such as lipopolysaccharide (LPS), lipoteichoic acid and peptidoglycans by members of the toll-like receptor (TLR) family. Lipopolysaccharide, a major component of the cell walls of gram-negative bacteria, induces a robust immune response in both pulpal and periapical tissues [4], [5], [6]. It is recognized by TLR4 which is expressed on the surfaces of diverse cell types including epithelial cells, polymorphonuclear leukocytes, mast cells, dendritic cells and monocytes/macrophages [7].

Infiltration by macrophages and polymorphonuclear neutrophils constitutes the early response to frank bacterial infection of the pulp as well as to the diffusion of bacterial antigens through dentinal tubules. The binding of LPS to TLR4 expressed on the cell surfaces of immune cells results in the activation of nuclear factor κ B, interferon response factor 3 and mitogen-activated protein kinase pathways inducing the expression of several pro-inflammatory genes such as tumor necrosis factor-α (TNF-α), interleukin 18 (IL-18) and cyclooxygenase-2 (COX-2) [8]. TLR4 signaling is terminated by endocytosis, ubiquitylation and lysosomal degradation which results in pathogen clearance as well as in priming of the adaptive immune response [9].

The discovery of microRNAs (miRNAs) has greatly expanded our understanding of the mechanisms that regulate gene expression. miRNAs are small (18-22 nucleotides), single stranded, noncoding RNA oligonucleotides. They regulate gene expression by binding to the 3'-UTR of target messenger RNAs (mRNAs) to down-regulate gene expression at the post-transcriptional level either by translational repression or mRNA degradation [10]. To date, 1921 miRNAs are encoded in the human genome and they regulate a wide variety of biological processes including proliferation, differentiation, cell fate determination, apoptosis, signal transduction and others. A growing body of evidence supports the key role of miRNAs in the activation of both the innate and adaptive immune response [11], [12], [13].

We recently reported the expression of miRNAs in human dental pulps [14]. Using microarray analysis, 335 miRNAs were identified, of which 36 miRNAs were differentially expressed in inflamed pulps as compared to normal pulps. The potential gene targets for these differentially expressed miRNAs included pro-inflammatory cytokines as well as other key mediators of the immune response to infection. The purpose of the present study is to gain further insight on the role of miRNAs in regulating the immune response to endodontic infections. For this purpose we challenged CD14+ human macrophages with *E. coli* LPS, a widely accepted

model to examine the innate response to microbial infection [15]. We hypothesize that challenge of these cells with LPS induces changes in expression of miRNAs linked to host defense.

Materials and Methods:

Cell culture and reagents: Freshly drawn human buffy coat (Oklahoma Blood Institute) from three different healthy donors was used to isolate monocytes by following Magnetic Cell Sorting (MACS) protocol, modified from the Miltenyi MACS general protocol [16]. Positive selection protocol was followed to select CD14 positive monocytes. Cells were grown in High-glucose Dulbecco's Modified Eagle Medium (DMEM, Gibco BRL, Grand Island, NY, USE), supplemented with 2mM L-glutamine (Gibco, Carlsbad, CA) and 50ul/ml gentamicin (Gibco, Carlsbad, CA) in a humidified incubator with 5% CO2 at 37^{o}C for macrophage differentiation. After four days of cell culture, the media was changed to DMEM supplemented with 10% fetal bovine serum, 2mM L-glutamine (Gibco, Carlsbad, CA) and 50ul/ml gentamicin (Gibco, Carlsbad, CA). On day 6, primary macrophages were stimulated by *Escherichia coli* 055:B5 LPS (Sigma, St. Louis, MO) at 5ng/ul and incubated for 1, 4, and 8 hours. Controls were macrophages not treated with LPS. At the end of the incubation period, cells were lysed and total RNA was extracted by using miRNeasy Mini kit (Qiagen, Germantown, MD). All samples were sent to Genomics and Bioinformatics Core, Lineberger Comprehensive Cancer Center at University of North Carolina at Chapel Hill for miRNA quality check.

nCounter miRNA Expression Assay: The nCounter® miRNA Sample Preparation Kit (Nanostring tech, Seattle, WA) was used for ligating unique oligonuceotide tags onto miRNAs, allowing these short RNAs to be detected. The miRNA tag ligation reaction was performed in a background of total RNA. Briefly, sample preparation involves a multiplexed annealing of the specific tags to their target miRNA, a ligation reaction, and an enzymatic purification to remove the unligated tags. Control RNA included in the nCounter miRNA Sample Preparation Kit allows the user to monitor the ligation efficiency and specificity through each step of the reaction. After ligation, all samples were sent to Genomics and Bioinformatics Core, Lineberger Comprehensive Cancer Center at University of North Carolina at Chapel Hill for hybridization and miRNA quantification. Data Collection was carried out in the nCounter Digital Analyzer. Digital images were processed and the barcode counts were tabulated in a comma separated value (CSV) format.

Statistical analysis: For nCounter expression data analysis, each miRNA Panel Assay contains 6 positive ligation controls, 8 negative controls and 5 housekeeping genes to assess technical performance in gene expression. The data produced by the nCounter Digital Analyzer were exported as a Reporter Code Count (RCC) file. Geometric mean of the top 100 targets with the highest counts was calculated to normalize the data in order to eliminate variability unrelated to

the sample. Due to small sample size, Kruskal-Wallis test was performed to detect any difference of miRNA gene expression levels at different time points. Pairwise comparisons were adjusted by Bonferroni correction. A p-value ≤ 0.05 was considered significant. miRNAs which showed significantly changed gene expressions were selected and plotted to show the trend. All the statistical analysis was conducted in SAS 9.2 (SAS, Cary, NC).

Results:

Significant changes were noted in the expression levels of four miRNAS- 199a-5p, miR-214, miR-455-5p and miR-629 when the data was analyzed using the non-parametric Kruskall-Wallis test (Table 1). We then used pairwise comparison, a parametric test to compare expression levels at each time point. Pairwise comparisons reveal that levels of miR-199a-5p in macrophages lysed 1 hour after the LPS challenge did not differ from that of the controls. However expression of miR-199a-5p levels at 4 and 8 hours after LPS challenge were significantly lower than that of the controls ($p < 0.05$). Expression of miR-214 did not show any significant difference from the controls when pairwise comparisons were performed. Levels of miR-455-5p and miR-629 were significantly lowered at the 4 hour time point as compared to the one-hour time point ($p < 0.05$).Validated and predicted targets for these 4 miRNAs were obtained by searching the miRWALK database (Table 2). Other miRNAs which showed similar trends were miR-95, miR-152 and miR-584.

Discussion:

Innate immunity constitutes the first line of defense by which the host recognizes and responds to invading pathogens or their conserved molecular patterns. Given the emerging role of miRNA in the immune response, it is highly likely that they modulate the pulpal response to microbial infection. We recently reported that several miRNAs are differentially expressed in inflamed pulps collected from carious teeth as compared to normal pulps, suggestive of the role of these miRNA in the immune response of the pulp [14]. The results of the present study provide further support for this as the expression of some of the same miRNAs were altered following LPS challenge.

As in other tissues in the body, the inflammatory response of the pulp represents a balance between the pro- and anti-inflammatory mediators designed to effectively neutralize or eliminate the pathogens and at the same time, minimize deleterious effects on the host tissues. The role of miRNAs as fine tuners of this inflammatory response is exemplified by their regulation of cytokine expression. miR-455-5p which is known to regulate the anti-inflammatory cytokine IL-10, was downregulated at the 4 hour time point following the LPS challenge under the experimental conditions of this study. The downregulation of this miRNA increases the expression

of IL-10 and thus result in an anti-inflammatory effect. At 8 hours after the LPS challenge the levels of miR-455-5p were higher than that at the 4 hour time point which likely signals a return to baseline levels. Similar patterns of expression were noted with miR-199a-5p and miR-214 known to regulate NFκB a central mediator of the innate immune response leading to inflammation. miR-199a downregulates Iκκ2 a key activator of the NFκB pathway [17]. Lack of miR-214 expression contributes to constitutive NF-κB activation [18], The role of miR-214 is further supported by the finding that in our in vivo study that expression of miR-214 in infected pulps was repressed by a fold change of 4.23 as compared to normal pulps [14].

In addition to its regulation of the immune response miR-199a-5p is also reported to regulate the response to heat and mechanical stimulation. This is of additional interest to our specialty as the response to mechanical and thermal stimuli are routinely used in the clinic to diagnose pulpal and periapical status. Future studies should explore the relationship between the expression of this miRNA, activation or sensitization of sensory neurons and the pulpal response to thermal and mechanical stimuli.

TLRs belong to a family of receptors that recognize conserved parts of microbial components known as pathogen-associated molecular patterns. To date, 13 different TLRs (TLR1-13) have been identified which recognize various microbial products, DNA and RNA viruses, fungi and protozoa [19]. These receptors play a key role in linking pathogen recognition to the induction of innate immunity, inflammation and eventually adaptive immunity. TLR4 is an important cell surface receptor which recognizes LPS as well as endogenous ligands such as heat shock proteins (HSP60 and HSP70), the extra domain A of fibronectins, oligosaccharides of hyaluronic acid, heparan sulfate and fibrinogen. In the dental pulp TLR4 is also expressed on odontoblasts and it is likely that odontoblasts are among the first cells to respond to microbial infection of the pulp due to their peripheral location [20]. While it was initially reported that fibroblasts do not express TLRs, it was later reported that pulpal fibroblasts express TLRs and that pulpal fibroblasts and odontoblasts differ in their response to LPS [20], [21]. A population of trigeminal neurons that express Transient Receptor Potential Vanilloid-1 (TRPV-1), an ionotropic channel that is expressed exclusively in nociceptor and is required for the development of inflammatory hyperalgesia, also co-express TLR4 [22]. Furthermore, LPS can directly activate trigeminal sensory neurons and sensitize TRPV1 via TLR4 pathways [23]. Thus suggesting that pain associated with bacterial infections, may be due in part to the direct activation of nociceptors via TLR4.

The model used in this study was LPS evoked changes in macrophage miRNA expression. LPS is the major surface component of the cell envelope for virtually all gram-negative organisms

and represents one of the conserved microbial structures responsible for activation of the innate immune system. It is composed of three regions- lipid A, a short core oligosaccharide, and the O-antigen polysaccharide [24]. The lipid A domain is also known as endotoxin, and is the bioactive component recognized by TLR4. While the synthesis of lipid A is a highly conserved process there is some diversity among the lipid A structures of various organisms. These differences are likely due to the action of latent enzymes that modify the canonical lipid A molecule. As such it is likely that the innate immune responses evoked by different microorganisms or even by different strains of the same microorganism are not completely alike. Future studies should evaluate changes in miRNA expression evoked by different gram-negative microorganisms.

LPS induced miRNA changes have been evaluated using different sources of LPS, cell types as well as different models. Exposure to aerosilised LPS induced rapid and transient increase in the expression of 46 miRNA in the mouse lung [25]. This increase was correlated with a reduction in the expression of TNF-alpha, keratinocyte-derived chemokine (KC) and MIP-2, suggesting a potential role for miRNAs in the regulation of inflammatory cytokine production. Contrary to the results of our study which show a decrease in miRNA 214, it was up regulated at 1 and 3 hours after stimulation but not at the 6 hour time point. Other studies on LPS induced miRNA changes report that miR-147, miR-155 and miR-125b were induced in LPS-stimulated mouse macrophages in vitro as well as under in vivo conditions in the lungs of LPS-treated mice [26], [27]. These miRNAs were not downregulated in our study. An in vivo study of human leukocyte microRNA response to endotoxemia reported an upregulation of miR-143 and downregulation of miR-146b, miR-150, miR-342, and let-7g 4 hours after E.coli LPS infusion [28]. In our study evaluating differences in macrophage miRNA expression, none of these miRNA was differentially regulated. The differences between the results of these aforementioned studies and our data may be attributed to differences in source of LPS, time points examined, cells types examined as well several other experimental conditions.

In addition to the source of LPS, the effect of LPS on miRNA expression is also dependent upon the cell type examined and the stimulation time. We chose to focus on macrophages as they, along with polymorphonuclear neutrophils, constitute the primary cells types which infiltrate the pulp in its initial response to infection. We examined miRNA expression at 3 different time points- 1, 4 and 8 hours after the LPS challenge. While the expression of miRNAs decreased in a linear fashion at 1 and 4 hours, the expression at the 8 hour time point was higher than that at the 4 hour time point. There are two potential explanations for this. The first is that this increase in expression is due to a negative feedback mechanism which facilitates the return of miRNA expression to basal levels as the infection is resolved. The second explanation is

that this is reflective of endotoxin tolerance, also referred to as LPS hyporesponsiveness or refractoriness. This is a phenomenon where cells show reduced responsiveness towards repeated endotoxin stimulation and has been reported in both *in vivo* and *in vitro* studies [29].

In this study we used a relatively new approach NanoString nCounter assay to evaluate changes in miRNA expression. NanoString nCounter has been shown to be a novel, effective alternative to SYBR green real-time PCR [30], [31]. This technology enables the digital quantification of multiplexed target RNA molecules using color-coded barcodes and single-molecule imaging. This direct detection of targets with molecular barcodes eliminates the use of reverse transcription or amplification. It gives discrete counts of RNA transcripts and is capable of providing a high level of precision and sensitivity at less than one transcript copy per cell. By providing discrete counts of RNA transcripts, the nCounter overcomes the saturation limitations of microarrays while avoiding the complex sequence analysis necessitated by RNA-Seq. The platform can quantify up to 800 different RNA targets simultaneously in up to 12 samples per run [32].

In conclusion, the results of this study provide evidence of the role of miRNA in modulating the pulpal response to infection. Future studies on these fine tuners of the immune response may lead to development of novel biomarkers for the resolution of endodontic infection.

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Legends

Fig 1. Gene expression levels of miRNAs in human macrophages treated with 5ng/ul E. *coli* LPS at 1 hour, 4 hours and 8 hours. Controls were macrophages not challenged with LPS. Each time point has 5 duplicates from three human donors. Data was analyzed using the Kruskal-Wallis test to look for global difference in expression. Horizontal axis is the incubation time (hours) of macrophages with LPS. Vertical axis is the Wilcoxon Score calculated from Kruskal-Wallis test. Interquartile range (IQR) with maximum observation below 75% quartile (not shown) and minimum observation above 25% quartile (not shown) was shown with outliers plotted as open circle. Median (line) and mean (open diamond) were also shown for each boxplot.

Table 1. Wilcoxon Scores of miRNA gene expression in human macrophages treated with 5ng/ul E. *coli* LPS. Kruskal-Wallis test was used to detect the global difference across different time points.

Table 2 Potential miRNA target genes identified using PUBMED and miRWalk (http://www.rna.uni-heidelberg.de/apps/zmf/mirwalk/index_html).

IκBK Inhibitor of κ B kinase activity; IL-10 Interleukin -10; MAPK Mitogen Activated Protein Kinase; MAP2K3 Mitogen Activated Protein Kinase Kinase 3; NOS3 Nitrous Oxide Synthase 3; PI3 Phosphatidylinositol-3-kinase; PTGS2 prostaglandin G/H synthase; TCF3 Transcription factor 3; TLR4 Toll-Like Receptor 4 ; VEGF A Vascular Endothelial Growth Factor A

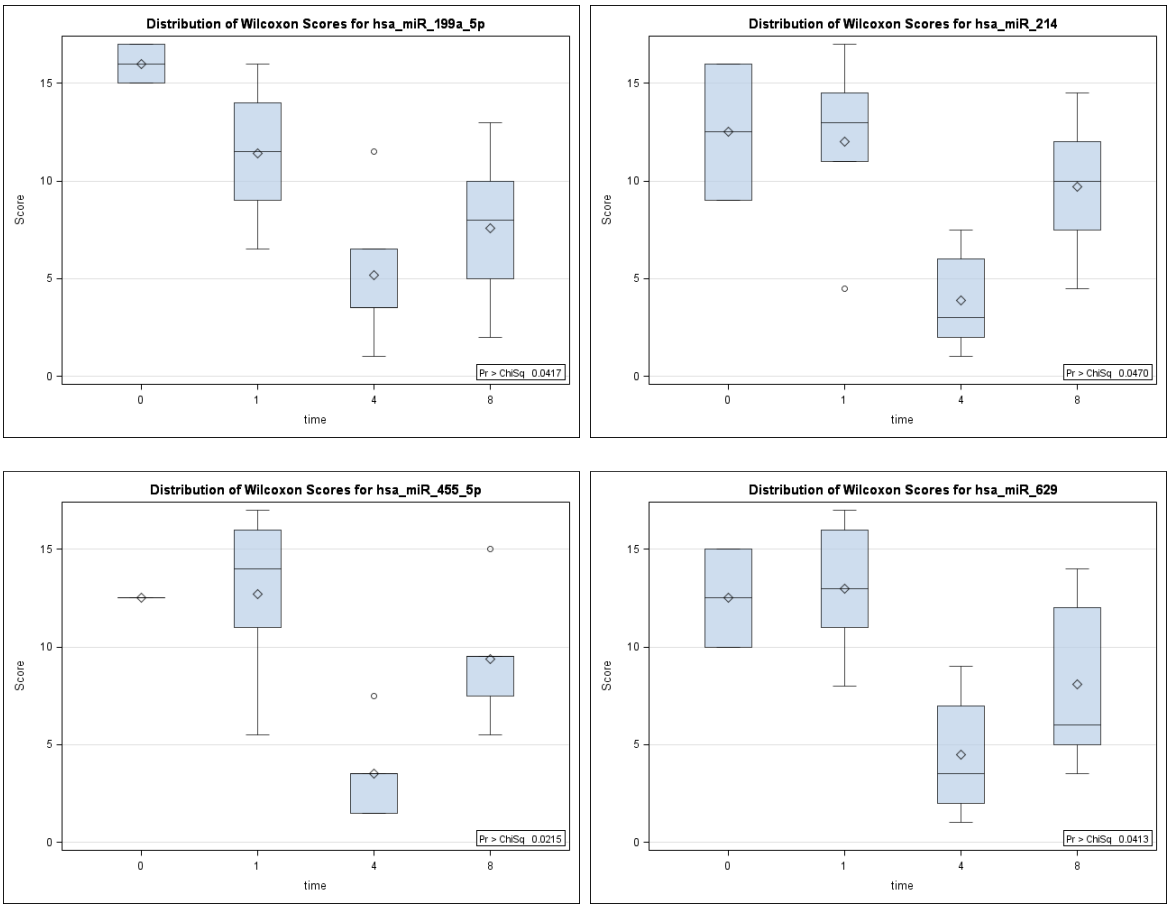
Table 1.

	Control	1 hr	4 hr	8 hr	p-value
miR-199-5p	16.0	11.4	5.2	7.6	0.041
miR-214	12.5	12.0	3.9	9.7	0.047
miR-455-5p	12.5	12.7	3.5	9.4	0.021
miR-629	12.5	13.0	4.5	8.1	0.041

Table 2.

miRNA	Target gene	Gene product function (Gene ontology)
hsa-mir-199a-5p	IκBKB	Activation of NFκB by causing dissociation of the inhibitor (GO:008384)
	PI3	1-Phosphatidylinositol-3-kinase activity (GO:0016303) 1-Phosphatidylinositol-3-kinase binding (GO:0005942)
	MAPK8	c-Jun N-terminal kinase cascade (GO:007254)
hsa-mir-214	MAP2K3	Innate immune response (GO:004587) Regulation of cytokine biosynthetic process (GO:0042035) Stress activated MAP kinase (GO:0051403)
	TCF3	Immunoglobulin V(D)J recombination (GO:0033152)
	VEGF3	Activation of protein kinase activity (GO: 0032147) Angiogenesis (GO:0001525) Anti-apoptosis: (GO:0006916) Basophil chemotaxis (GO:0002575) Platelet activation (GO:0030168) Platelet degranulation (GO:0002576) Positive regulation of cell adhesion (GO:0008284) Positive regulation of cell migration) (GO:0030335)
	NOS3	Postive regulaiton of angiogenesis (GO:0045766) Postive regulaiton of apoptotic process (GO:0043065) Response to mechanical stimulus (GO:0009612)
hsa-455-5p	IL-10	Anti-inflammatory cytokine (GO:0019969)
	MAPK3	Innate immune response (GO:0045087) Cellular response to mechanical stimulus (GO:0071260) LPS mediated signaling pathway (GO:0031663)
	TLR4	TLR4 binding (GO:00035662) TLR4 signalling pathway (GO:0034142)
	PTGS2	Cyclooxygenase pathway (GO:0019371)
Hsa-mir-629	DICER1	RNA induced silencing complex (GO:0070578)

Fig 1.


Distribution of Wilcoxon Scores for hsa_miR_199a_5p
Score
time
Pr > ChiSq 0.0417
Distribution of Wilcoxon Scores for hsa_miR_214
Score
time
Pr > ChiSq 0.0470
Distribution of Wilcoxon Scores for hsa_miR_455_5p
Score
time
Pr > ChiSq 0.0215
Distribution of Wilcoxon Scores for hsa_miR_629
Score
time
Pr > ChiSq 0.0413

Appendix:

miRNAs indentified from human dental pulp

miRNA	p-value	q-value	Control	Infected	Fold change
ebv-miR-BART10	1.0000	0.9393	4.5105	4.5520	1.0092
ebv-miR-BART12	0.8801	0.9393	37.5032	42.2115	1.1255
ebv-miR-BART13	0.9306	0.9393	127.5078	139.9364	1.0975
ebv-miR-BART16	1.0000	0.9393	18.3056	18.1418	0.9911
ebv-miR-BART19-3p	0.1667	0.4236	104.0139	59.4092	0.5712
ebv-miR-BART3*	0.9457	0.9393	8.0235	8.4790	1.0568
hcmv-miR-UL70-3p	0.0189	0.1168	98.2564	526.2155	5.3555
hcmv-miR-US4	0.0051	0.0518	19.6653	91.2402	4.6397
hiv1-miR-H1	0.0088	0.0757	38.6154	17.9092	0.4638
hsa-let-7a	0.0088	0.0757	28646.6910	15599.2800	0.5445
hsa-let-7a*	0.0808	0.2921	7.2542	2.1100	0.2909
hsa-let-7b	0.0202	0.1196	20045.5179	10227.0913	0.5102
hsa-let-7c	0.0038	0.0514	7850.2395	3397.7731	0.4328
hsa-let-7d	0.4722	0.7059	1629.9985	1403.9502	0.8613
hsa-let-7d*	0.9369	0.9393	38.1074	35.4442	0.9301
hsa-let-7e	0.0152	0.0968	2790.0500	1685.1946	0.6040
hsa-let-7f	0.0076	0.0696	18116.5711	9737.5921	0.5375
hsa-let-7g	0.0467	0.2071	4124.7448	3317.9801	0.8044
hsa-let-7i	0.6780	0.8592	2882.7065	3054.7174	1.0597
hsa-miR-1	0.7260	0.8648	11.5347	14.6721	1.2720
hsa-miR-100	0.0366	0.1825	2363.4006	1281.7890	0.5423
hsa-miR-101	0.2449	0.5334	1358.1739	1218.1971	0.8969
hsa-miR-103	0.7172	0.8648	1047.3447	1287.4903	1.2293
hsa-miR-106b	0.1023	0.3316	561.3318	1053.4491	1.8767
hsa-miR-107	0.1755	0.4410	1174.0291	1831.2033	1.5598
hsa-miR-10a	0.0644	0.2558	48.1833	22.7166	0.4715
hsa-miR-10b	0.0404	0.1916	747.7225	425.0666	0.5685
hsa-miR-10b*	0.1818	0.4543	8.2248	5.6797	0.6906
hsa-miR-1180	0.4318	0.6717	9.1646	12.1785	1.3289
hsa-miR-1181	0.5013	0.7295	29.3836	42.8212	1.4573
hsa-miR-1182	0.0114	0.0857	24.8683	61.7162	2.4817
hsa-miR-1183	0.0417	0.1955	21.7586	56.7090	2.6063
hsa-miR-1202	0.0265	0.1426	670.8306	3281.3922	4.8915
hsa-miR-1207-5p	0.0227	0.1302	705.6625	2409.5722	3.4146
hsa-miR-1208	0.3396	0.6113	22.6383	18.3131	0.8089

hsa-miR-1224-5p	0.0051	0.0518	47.4526	146.8995	3.0957
hsa-miR-1225-3p	0.0227	0.1302	48.2332	81.7192	1.6943
hsa-miR-1225-5p	0.0379	0.1836	397.5281	1666.6704	4.1926
hsa-miR-1226*	0.0101	0.0795	19.8843	58.7958	2.9569
hsa-miR-1228	0.0669	0.2612	69.3185	108.4586	1.5646
hsa-miR-1229	0.0631	0.2554	18.2877	24.9271	1.3631
hsa-miR-1234	0.1503	0.4001	81.2372	121.4765	1.4953
hsa-miR-1237	0.0859	0.2993	33.6534	51.6670	1.5353
hsa-miR-1238	0.3270	0.6019	54.8295	69.2305	1.2626
hsa-miR-124	0.0215	0.1262	6.2570	25.2032	4.0280
hsa-miR-1246	0.8939	0.9393	977.9141	2990.0414	3.0576
hsa-miR-1247	1.0000	0.9393	108.7658	106.0561	0.9751
hsa-miR-1249	0.0202	0.1196	12.4693	26.6332	2.1359
hsa-miR-125a-3p	0.2033	0.4820	39.3370	125.7030	3.1955
hsa-miR-125a-5p	0.1301	0.3811	733.9798	537.0483	0.7317
hsa-miR-125b	0.0354	0.1782	15874.6413	8868.4202	0.5587
hsa-miR-125b-2*	0.0139	0.0942	68.9511	35.2653	0.5115
hsa-miR-126	0.5152	0.7376	1995.7185	2519.9889	1.2627
hsa-miR-126*	0.7538	0.8725	165.1176	147.4389	0.8929
hsa-miR-1260	0.4419	0.6790	1872.3708	1422.8516	0.7599
hsa-miR-1268	0.5758	0.7753	210.4317	1363.2090	6.4782
hsa-miR-1271	0.0088	0.0757	74.4464	29.0017	0.3896
hsa-miR-127-3p	0.0051	0.0518	104.0996	38.2738	0.3677
hsa-miR-1274a	0.2917	0.5781	250.9281	188.9223	0.7529
hsa-miR-1274b	0.9419	0.9393	2117.4903	2005.2448	0.9470
hsa-miR-1275	0.7929	0.9001	535.7787	605.7335	1.1306
hsa-miR-128	0.0013	0.0318	91.5650	32.8906	0.3592
hsa-miR-1280	0.0884	0.3033	354.1818	220.4608	0.6225
hsa-miR-1281	0.1174	0.3584	32.0168	55.8417	1.7441
hsa-miR-1287	0.0126	0.0905	14.2737	127.6899	8.9458
hsa-miR-1288	0.8232	0.9193	14.1655	15.3530	1.0838
hsa-miR-1290	1.0000	0.9393	87.5215	2208.5465	25.2343
hsa-miR-1299	0.0240	0.1348	19.2152	141.9657	7.3882
hsa-miR-1300	0.0795	0.2921	24.8682	54.3709	2.1864
hsa-miR-1305	0.5467	0.7642	85.8215	72.2057	0.8413
hsa-miR-1306	0.6717	0.8561	10.1347	16.4740	1.6255
hsa-miR-1308	0.0013	0.0318	652.1375	339.2507	0.5202
hsa-miR-130a	0.2992	0.5829	1440.7472	1165.0170	0.8086
hsa-miR-130b	0.6465	0.8409	77.1995	88.6157	1.1479
hsa-miR-132	0.6717	0.8561	81.1316	59.7462	0.7364
hsa-miR-132*	0.1856	0.4560	21.7700	12.7820	0.5871

hsa-miR-1321	0.2576	0.5474	8.5147	12.7222	1.4942
hsa-miR-133b	0.2601	0.5501	10.8784	20.3504	1.8707
hsa-miR-134	0.5758	0.7753	61.3375	229.0911	3.7349
hsa-miR-135a*	1.0000	0.9393	50.0319	57.7190	1.1536
hsa-miR-135b	0.3750	0.6410	6.1894	10.5258	1.7006
hsa-miR-136	0.0114	0.0857	238.7032	77.4286	0.3244
hsa-miR-137	0.1919	0.4637	64.6901	20.6956	0.3199
hsa-miR-139-3p	0.5303	0.7532	104.9398	147.8199	1.4086
hsa-miR-139-5p	0.7096	0.8648	76.8688	91.2016	1.1865
hsa-miR-140-3p	0.0025	0.0474	1521.8173	652.3199	0.4286
hsa-miR-140-5p	0.0038	0.0514	1053.2866	567.5443	0.5388
hsa-miR-142-3p	0.0240	0.1348	583.5928	6825.3298	11.6954
hsa-miR-142-5p	0.0417	0.1955	39.3820	562.2198	14.2761
hsa-miR-143	0.1174	0.3584	289.0058	214.4241	0.7419
hsa-miR-144	0.0354	0.1782	168.5734	7858.0077	46.6147
hsa-miR-144*	0.0290	0.1516	31.4526	907.8164	28.8630
hsa-miR-145	0.0139	0.0942	1668.8910	970.3419	0.5814
hsa-miR-145*	0.0114	0.0857	87.5738	46.0550	0.5259
hsa-miR-146a	0.2727	0.5659	359.9826	711.4285	1.9763
hsa-miR-146b-5p	0.1288	0.3786	391.8908	300.0214	0.7656
hsa-miR-1471	0.3283	0.6019	31.6584	66.6496	2.1053
hsa-miR-148a	0.0013	0.0318	1119.6881	519.7603	0.4642
hsa-miR-148b	0.0152	0.0968	185.2397	112.7180	0.6085
hsa-miR-149	0.0139	0.0942	214.3567	66.0932	0.3083
hsa-miR-149*	0.0038	0.0514	11.1623	52.7091	4.7221
hsa-miR-150	0.1843	0.4560	333.9235	855.8605	2.5630
hsa-miR-150*	0.0013	0.0318	82.6264	223.6804	2.7071
hsa-miR-151-3p	0.9470	0.9393	212.4303	215.9055	1.0164
hsa-miR-151-5p	0.0278	0.1467	1146.7101	805.1739	0.7022
hsa-miR-152	0.0013	0.0318	288.8991	71.2592	0.2467
hsa-miR-155	0.3990	0.6586	61.7784	212.5610	3.4407
hsa-miR-15a	0.0379	0.1836	2164.9182	3413.4131	1.5767
hsa-miR-15b	0.0341	0.1748	1478.2727	3508.6887	2.3735
hsa-miR-15b*	0.7790	0.8912	1.5939	1.8746	1.1761
hsa-miR-16	0.0139	0.0942	3482.4776	11799.9250	3.3884
hsa-miR-16-2*	0.0581	0.2428	9.3209	40.1815	4.3109
hsa-miR-17	0.3586	0.6263	389.1794	522.6848	1.3430
hsa-miR-17*	0.8106	0.9142	79.4376	75.2103	0.9468
hsa-miR-181a	0.0051	0.0518	4898.9443	2884.3670	0.5888
hsa-miR-181a*	0.0013	0.0318	229.3456	79.9602	0.3486
hsa-miR-181a-2*	0.0013	0.0318	75.6226	22.3590	0.2957

hsa-miR-181b	0.0013	0.0318	789.5319	287.8950	0.3646
hsa-miR-181c	0.0013	0.0318	384.4149	152.0355	0.3955
hsa-miR-181c*	0.0126	0.0905	21.5815	10.8633	0.5034
hsa-miR-181d	0.0013	0.0318	147.6726	52.3475	0.3545
hsa-miR-182	0.8939	0.9393	1.6298	1.7345	1.0643
hsa-miR-1825	0.2109	0.4933	32.2281	49.4506	1.5344
hsa-miR-1826	0.9331	0.9393	82.1720	85.3930	1.0392
hsa-miR-183	0.2311	0.5172	9.2914	17.1459	1.8454
hsa-miR-185	0.0997	0.3295	152.9673	358.7073	2.3450
hsa-miR-186	0.0013	0.0318	432.4502	262.1019	0.6061
hsa-miR-188-5p	0.0770	0.2866	62.1128	346.0385	5.5711
hsa-miR-18a	0.0480	0.2105	11.1915	58.1817	5.1987
hsa-miR-18b	0.1111	0.3500	5.0967	29.8392	5.8547
hsa-miR-190	0.6679	0.8550	22.5982	19.8081	0.8765
hsa-miR-191*	0.1288	0.3786	42.7488	59.6084	1.3944
hsa-miR-1914*	0.4697	0.7045	59.0378	132.5253	2.2448
hsa-miR-1915	0.0189	0.1168	432.5165	2047.4160	4.7337
hsa-miR-192	0.0013	0.0318	218.2222	120.7019	0.5531
hsa-miR-193a-3p	0.0063	0.0612	372.8698	234.3399	0.6285
hsa-miR-193a-5p	0.5429	0.7625	148.5886	211.4506	1.4231
hsa-miR-193b	0.4356	0.6740	291.9273	382.7243	1.3110
hsa-miR-193b*	0.0278	0.1467	21.1650	60.4719	2.8572
hsa-miR-194	0.3043	0.5829	91.3281	72.3982	0.7927
hsa-miR-195	0.0316	0.1638	3298.8834	1607.6921	0.4873
hsa-miR-197	0.4369	0.6748	603.7597	470.2987	0.7790
hsa-miR-198	0.2008	0.4773	11.1785	19.1736	1.7152
hsa-miR-199a-3p	0.0404	0.1916	12973.1563	6867.5768	0.5294
hsa-miR-199a-5p	0.0025	0.0474	3683.8899	1542.7673	0.4188
hsa-miR-199b-5p	0.0038	0.0514	1170.8235	428.7759	0.3662
hsa-miR-19a	0.0114	0.0857	415.2070	852.5489	2.0533
hsa-miR-19b	0.0126	0.0905	1661.1323	2817.9674	1.6964
hsa-miR-19b-1*	0.0631	0.2554	6.9977	2.4323	0.3476
hsa-miR-200b	0.0038	0.0514	40.0300	11.2034	0.2799
hsa-miR-200c	0.4419	0.6790	7.0047	5.4586	0.7793
hsa-miR-202	0.0732	0.2760	27.0387	42.6488	1.5773
hsa-miR-203	0.1503	0.4001	10.5269	4.9085	0.4663
hsa-miR-204	0.6982	0.8648	1477.2428	1259.8633	0.8528
hsa-miR-205	0.0202	0.1196	35.3234	6.5342	0.1850
hsa-miR-20a	0.0114	0.0857	834.1078	1445.6139	1.7331
hsa-miR-20a*	0.0051	0.0518	67.3933	41.2388	0.6119
hsa-miR-20b	0.0783	0.2889	294.0199	436.5729	1.4848

hsa-miR-21	0.0682	0.2637	9364.6806	21954.7563	2.3444
hsa-miR-21*	0.1048	0.3361	10.1487	30.6410	3.0192
hsa-miR-210	0.8283	0.9224	244.3486	231.9133	0.9491
hsa-miR-214	0.0290	0.1516	1867.7155	1117.1573	0.5981
hsa-miR-214*	0.0013	0.0318	92.3948	21.8417	0.2364
hsa-miR-215	0.0038	0.0514	125.0024	62.6732	0.5014
hsa-miR-218	0.0492	0.2150	488.2283	250.5187	0.5131
hsa-miR-22	0.5152	0.7376	3919.1596	4602.6300	1.1744
hsa-miR-22*	0.7146	0.8648	38.3275	42.0314	1.0966
hsa-miR-221	0.5227	0.7460	169.8557	144.8234	0.8526
hsa-miR-221*	0.6591	0.8511	9.8192	7.5088	0.7647
hsa-miR-222	0.8182	0.9181	52.7456	59.9631	1.1368
hsa-miR-223	0.1654	0.4216	518.6274	7254.1765	13.9873
hsa-miR-223*	0.5758	0.7753	1.8630	13.0873	7.0250
hsa-miR-224	0.9331	0.9393	28.3999	24.9164	0.8773
hsa-miR-23a	0.0253	0.1383	11426.7172	8159.4524	0.7141
hsa-miR-23a*	1.0000	0.9393	14.3530	14.9419	1.0410
hsa-miR-23b	0.0051	0.0518	6851.8934	3762.8775	0.5492
hsa-miR-24	0.4116	0.6586	4291.1098	3591.0546	0.8369
hsa-miR-24-1*	0.0013	0.0318	64.4343	18.1635	0.2819
hsa-miR-25	0.2285	0.5142	606.6401	888.4644	1.4646
hsa-miR-26a	0.0265	0.1426	7531.3256	4721.6824	0.6269
hsa-miR-26b	0.1540	0.4055	5110.3714	4181.0180	0.8181
hsa-miR-27a	0.2841	0.5746	2198.3544	1891.2429	0.8603
hsa-miR-27b	0.0051	0.0518	2874.7109	1633.3507	0.5682
hsa-miR-28-5p	0.0202	0.1196	297.1007	206.8257	0.6961
hsa-miR-296-5p	0.1629	0.4176	25.8691	17.7883	0.6876
hsa-miR-29a	0.1338	0.3858	3722.6054	3224.8897	0.8663
hsa-miR-29a*	0.0013	0.0318	35.5984	16.4770	0.4629
hsa-miR-29b	0.5568	0.7734	2161.2207	2289.0477	1.0591
hsa-miR-29b-1*	0.8245	0.9193	15.5867	13.5085	0.8667
hsa-miR-29c	0.4053	0.6586	2527.1958	2738.6983	1.0837
hsa-miR-29c*	0.0013	0.0318	109.5854	65.9356	0.6017
hsa-miR-301a	0.2121	0.4949	114.9874	171.1726	1.4886
hsa-miR-30a	0.0013	0.0318	870.2322	377.4380	0.4337
hsa-miR-30a*	0.0025	0.0474	159.7698	71.4553	0.4472
hsa-miR-30b	0.0038	0.0514	4332.8267	2043.6311	0.4717
hsa-miR-30b*	0.0025	0.0474	55.2462	27.1528	0.4915
hsa-miR-30c	0.0013	0.0318	1407.3432	740.6189	0.5263
hsa-miR-30c-1*	0.7475	0.8720	7.1919	6.4047	0.8905
hsa-miR-30c-2*	0.0922	0.3139	15.1358	11.0005	0.7268

hsa-miR-30d	0.0025	0.0474	2879.9717	1203.2359	0.4178
hsa-miR-30e	0.0013	0.0318	664.5918	410.9393	0.6183
hsa-miR-30e*	0.0051	0.0518	215.3577	128.3605	0.5960
hsa-miR-31	0.5619	0.7753	209.4239	128.1489	0.6119
hsa-miR-31*	0.2487	0.5377	82.9032	37.5199	0.4526
hsa-miR-32	0.2753	0.5659	73.5058	58.9773	0.8023
hsa-miR-32*	0.8939	0.9393	23.8888	22.5281	0.9430
hsa-miR-320a	0.8699	0.9393	890.4515	861.4080	0.9674
hsa-miR-320b	0.8674	0.9393	1942.0737	1782.1034	0.9176
hsa-miR-320c	1.0000	0.9393	2206.3788	2210.7287	1.0020
hsa-miR-320d	0.6881	0.8645	3534.9918	3221.6202	0.9114
hsa-miR-324-3p	0.3384	0.6113	412.7404	355.2257	0.8607
hsa-miR-324-5p	0.0038	0.0514	190.2616	110.1162	0.5788
hsa-miR-326	0.9331	0.9393	6.7695	6.6270	0.9789
hsa-miR-328	0.7109	0.8648	74.1162	61.4702	0.8294
hsa-miR-330-3p	0.8131	0.9147	7.6610	8.9431	1.1674
hsa-miR-331-3p	0.1250	0.3725	952.1451	682.2852	0.7166
hsa-miR-335	0.0076	0.0696	242.7163	71.2304	0.2935
hsa-miR-338-3p	0.6679	0.8550	2897.8332	3605.8405	1.2443
hsa-miR-339-3p	1.0000	0.9393	1.9859	3.6670	1.8465
hsa-miR-33a	0.0480	0.2105	50.1077	25.4785	0.5085
hsa-miR-33b*	0.0240	0.1348	11.1997	20.0032	1.7860
hsa-miR-340	0.3333	0.6085	58.6063	45.2638	0.7723
hsa-miR-340*	0.1414	0.3972	35.8799	22.9842	0.6406
hsa-miR-342-3p	0.2955	0.5804	729.3682	594.3485	0.8149
hsa-miR-342-5p	0.6856	0.8626	43.5423	57.5894	1.3226
hsa-miR-345	0.7020	0.8648	17.0098	20.6600	1.2146
hsa-miR-34a	0.0152	0.0968	1279.9032	743.1676	0.5806
hsa-miR-34b*	0.0013	0.0318	176.3477	71.6194	0.4061
hsa-miR-361-3p	0.7551	0.8728	113.7774	102.5295	0.9011
hsa-miR-361-5p	0.0152	0.0968	448.9015	260.1396	0.5795
hsa-miR-362-3p	0.7753	0.8903	19.0158	16.4391	0.8645
hsa-miR-362-5p	0.9369	0.9393	31.7264	33.4611	1.0547
hsa-miR-363	0.0960	0.3218	417.6578	315.5593	0.7555
hsa-miR-365	0.0152	0.0968	1079.4873	690.5566	0.6397
hsa-miR-370	0.5341	0.7537	22.6346	25.8596	1.1425
hsa-miR-371-5p	0.0101	0.0795	29.4939	92.1165	3.1232
hsa-miR-374a	0.0025	0.0474	343.6555	251.7001	0.7324
hsa-miR-374b	0.0051	0.0518	250.6887	164.7677	0.6573
hsa-miR-376a	0.0038	0.0514	138.3893	39.1435	0.2829
hsa-miR-376c	0.0025	0.0474	175.2335	45.9690	0.2623

hsa-miR-377	0.0038	0.0514	88.3959	27.8990	0.3156
hsa-miR-378	0.8119	0.9145	20.2511	21.7814	1.0756
hsa-miR-381	0.0189	0.1168	29.2276	11.1050	0.3799
hsa-miR-382	0.0644	0.2558	16.4093	6.9943	0.4262
hsa-miR-423-3p	0.0985	0.3265	23.7756	11.8336	0.4977
hsa-miR-423-5p	0.5341	0.7537	173.2040	152.8789	0.8827
hsa-miR-424	0.8460	0.9282	338.7993	370.1684	1.0926
hsa-miR-424*	0.0101	0.0795	209.3531	17.6368	0.0842
hsa-miR-425	0.1073	0.3405	87.1185	187.0093	2.1466
hsa-miR-425*	0.1843	0.4560	29.1470	41.0596	1.4087
hsa-miR-432	0.6490	0.8430	14.2186	11.4937	0.8084
hsa-miR-450a	0.9508	0.9393	7.6458	6.7704	0.8855
hsa-miR-451	0.0177	0.1114	5474.2829	72800.6684	13.2987
hsa-miR-452	0.0833	0.2976	23.5726	7.7073	0.3270
hsa-miR-454	0.0846	0.2993	74.0738	141.1872	1.9060
hsa-miR-455-3p	0.0025	0.0474	5418.6196	1868.1993	0.3448
hsa-miR-455-5p	0.0013	0.0318	320.7794	94.7597	0.2954
hsa-miR-483-3p	0.4116	0.6586	66.7066	100.6692	1.5091
hsa-miR-483-5p	0.0202	0.1196	161.7515	350.2826	2.1656
hsa-miR-484	0.2134	0.4953	180.0970	131.8517	0.7321
hsa-miR-485-3p	0.6768	0.8592	23.6773	20.7862	0.8779
hsa-miR-486-5p	0.0265	0.1426	113.7982	1969.8190	17.3098
hsa-miR-489	0.0644	0.2558	7.2785	3.7383	0.5136
hsa-miR-490-5p	0.9432	0.9393	3.8196	3.8608	1.0108
hsa-miR-494	0.5063	0.7297	1175.7980	956.0122	0.8131
hsa-miR-497	0.0051	0.0518	1739.5055	690.2066	0.3968
hsa-miR-500	0.0758	0.2843	8.0961	15.0508	1.8590
hsa-miR-500*	0.1124	0.3527	16.9391	28.6132	1.6892
hsa-miR-501-3p	0.6654	0.8542	3.8944	5.2836	1.3567
hsa-miR-501-5p	0.1578	0.4118	5.4182	11.0359	2.0368
hsa-miR-502-3p	0.2904	0.5781	15.1890	21.5311	1.4175
hsa-miR-505	0.0682	0.2637	58.8956	44.0548	0.7480
hsa-miR-505*	0.4899	0.7250	10.7614	14.2235	1.3217
hsa-miR-513a-5p	0.4886	0.7243	266.9359	227.1030	0.8508
hsa-miR-513b	0.9419	0.9393	24.6155	22.7570	0.9245
hsa-miR-513c	0.2083	0.4900	14.6786	7.7158	0.5257
hsa-miR-526b	0.0088	0.0757	5.0259	14.6628	2.9175
hsa-miR-532-3p	0.6982	0.8648	38.4455	44.6108	1.1604
hsa-miR-532-5p	0.0859	0.2993	43.9807	29.1955	0.6638
hsa-miR-539	0.0088	0.0757	11.4548	4.5343	0.3958
hsa-miR-542-3p	0.7348	0.8648	10.4682	5.5547	0.5306

hsa-miR-542-5p	0.8447	0.9282	10.3749	8.2498	0.7952
hsa-miR-545	0.0859	0.2993	19.7982	12.0558	0.6089
hsa-miR-548c-5p	0.0101	0.0795	23.5231	13.3752	0.5686
hsa-miR-550	0.0051	0.0518	5.7190	15.6216	2.7315
hsa-miR-557	0.0859	0.2993	17.2702	43.0384	2.4921
hsa-miR-564	0.2891	0.5781	25.4395	20.7634	0.8162
hsa-miR-572	0.0391	0.1886	88.5138	296.8865	3.3541
hsa-miR-574-3p	0.3699	0.6348	496.1446	364.1758	0.7340
hsa-miR-574-5p	0.2361	0.5245	446.6473	325.1913	0.7281
hsa-miR-575	0.0846	0.2993	176.6420	661.8849	3.7470
hsa-miR-582-5p	0.3157	0.5884	11.4194	37.0018	3.2403
hsa-miR-584	0.0013	0.0318	28.8978	131.3303	4.5447
hsa-miR-590-5p	0.1768	0.4429	143.5408	121.8756	0.8491
hsa-miR-595	0.8068	0.9123	17.4458	19.4055	1.1123
hsa-miR-601	0.0038	0.0514	14.6651	60.7574	4.1430
hsa-miR-610	0.0227	0.1302	10.6682	23.6155	2.2136
hsa-miR-622	0.0051	0.0518	9.9572	41.3232	4.1501
hsa-miR-623	0.3220	0.5953	62.1587	49.8349	0.8017
hsa-miR-625	0.9811	0.9393	72.2370	70.6996	0.9787
hsa-miR-629	0.0025	0.0474	17.0413	7.3293	0.4301
hsa-miR-629*	0.6818	0.8615	4.7573	3.5380	0.7437
hsa-miR-630	0.2790	0.5684	413.3049	2021.5284	4.8911
hsa-miR-631	0.0556	0.2344	10.2500	52.7415	5.1455
hsa-miR-636	0.0644	0.2558	16.5876	26.7151	1.6105
hsa-miR-638	0.0354	0.1782	708.6618	2014.5045	2.8427
hsa-miR-652	0.0076	0.0696	38.7776	106.2421	2.7398
hsa-miR-654-3p	0.0051	0.0518	41.1544	10.9868	0.2670
hsa-miR-654-5p	0.2955	0.5804	4.2069	20.8182	4.9486
hsa-miR-660	0.4924	0.7257	65.2540	85.2999	1.3072
hsa-miR-663	0.0051	0.0518	54.3424	134.9091	2.4826
hsa-miR-664	0.0051	0.0518	85.6747	48.0338	0.5607
hsa-miR-664*	0.0013	0.0318	89.9709	12.6459	0.1406
hsa-miR-671-5p	0.0076	0.0696	119.4726	775.3642	6.4899
hsa-miR-7	0.0783	0.2889	10.9530	42.5278	3.8828
hsa-miR-7-1*	0.6793	0.8596	5.6800	6.8323	1.2029
hsa-miR-720	0.8939	0.9393	10892.7170	9978.3919	0.9161
hsa-miR-744	0.1364	0.3880	60.9696	40.9959	0.6724
hsa-miR-760	0.0139	0.0942	14.2662	89.9243	6.3033
hsa-miR-765	0.0126	0.0905	82.9355	147.9731	1.7842
hsa-miR-766	0.0013	0.0318	38.5325	83.6600	2.1712
hsa-miR-769-5p	0.0013	0.0318	49.2510	22.0029	0.4467

hsa-miR-873	0.7525	0.8725	20.4371	8.8695	0.4340
hsa-miR-874	0.7601	0.8763	210.5200	221.7386	1.0533
hsa-miR-877	0.0051	0.0518	2.8616	19.8211	6.9266
hsa-miR-877*	0.0657	0.2574	16.4466	25.8944	1.5745
hsa-miR-885-3p	0.0253	0.1383	1.9997	11.9630	5.9825
hsa-miR-886-3p	0.1856	0.4560	437.2845	299.7835	0.6856
hsa-miR-887	0.9912	0.9393	3.7734	9.2924	2.4626
hsa-miR-9	0.3169	0.5884	83.2583	54.6865	0.6568
hsa-miR-9*	1.0000	0.9393	45.6192	44.2111	0.9691
hsa-miR-923	0.0808	0.2921	112352.5542	65294.4075	0.5812
hsa-miR-92a	0.2247	0.5109	1292.1626	1661.9903	1.2862
hsa-miR-93	0.1414	0.3972	205.6646	344.3354	1.6743
hsa-miR-933	0.0581	0.2428	8.5009	20.9069	2.4594
hsa-miR-934	0.1616	0.4168	3.2144	15.0196	4.6726
hsa-miR-936	0.0051	0.0518	9.0560	22.5650	2.4917
hsa-miR-939	0.0051	0.0518	160.0824	479.0070	2.9923
hsa-miR-940	0.0038	0.0514	229.9230	436.3230	1.8977
hsa-miR-95	0.0013	0.0318	595.3604	159.7282	0.2683
hsa-miR-96	0.0379	0.1836	19.4747	51.0820	2.6230
hsa-miR-98	0.0013	0.0318	280.3399	147.4352	0.5259
hsa-miR-99a	0.0177	0.1114	2657.6460	1234.1716	0.4644
hsa-miR-99b	0.0505	0.2194	333.0540	248.9550	0.7475
hsv1-miR-H1	0.0341	0.1748	34.7196	133.3991	3.8422
hsv1-miR-H6	0.2424	0.5305	12.9311	26.5704	2.0548
kshv-miR-K12-10b	0.0985	0.3265	1.6834	6.2161	3.6925
kshv-miR-K12-3	0.0152	0.0968	291.4937	1110.3206	3.8091

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