C. Stoecklin-Wasmer¹, P. Guarnieri²,
R. Celenti¹, R.T. Demmer³,
M. Kebschull^{1,4}, and
P.N. Papapanou^{1*}

¹Division of Periodontics, Section of Oral and Diagnostic Sciences, Columbia University College of Dental Medicine, 630 West 168th St., PH-7 E 110, New York, NY 10032, USA;

²Division of Bioinformatics, Biomedical Informatics Shared Resources, Columbia University, New York, NY, USA;

³Department of Epidemiology, Mailman School of Public Health, Columbia University, New York, NY, USA; and

⁴Department of Periodontology, Operative and Preventive Dentistry, University of Bonn, Bonn, Germany; *corresponding author, pp192@columbia.edu

J Dent Res 91(10):934-940, 2012

ABSTRACT

To gain insights into the *in vivo* function of miRNAs in the context of periodontitis, we examined the occurrence of miRNAs in healthy and diseased gingival tissues and validated their *in silico*-predicted targets through mRNA profiling using whole-genome microarrays in the same specimens. Eighty-six individuals with periodontitis contributed 198 gingival papillae: 158 ‘diseased’ (bleeding-on-probing, PD > 4 mm, and AL ≥ 3 mm) and 40 ‘healthy’ (no bleeding, PD ≤ 4 mm, and AL ≤ 2 mm). Expression of 1,205 miRNAs was assessed by microarrays, followed by selected confirmation by quantitative RT-PCR. Predicted miRNA targets were identified and tested for enrichment by Gene Set Enrichment Analysis (GSEA). Enriched gene sets were grouped in functional categories by DAVID and Ingenuity Pathway Analysis. One hundred fifty-nine miRNAs were significantly differentially expressed between healthy and diseased gingiva. Four miRNAs (hsa-miR-451, hsa-miR-223, hsa-miR-486-5p, hsa-miR-3917) were significantly overexpressed, and 7 (hsa-miR-1246, hsa-miR-1260, hsa-miR-141, hsa-miR-1260b, hsa-miR-203, hsa-miR-210, hsa-miR-205*) were underexpressed by > 2-fold in diseased vs. healthy gingiva. GSEA and additional filtering identified 60 enriched miRNA gene sets with target genes involved in immune/inflammatory responses and tissue homeostasis. This is the first study that concurrently examined miRNA and mRNA expression in gingival tissues and will inform mechanistic experimentation to dissect the role of miRNAs in periodontal tissue homeostasis and pathology.

KEY WORDS: gene expression, transcriptome, periodontitis, pathogenesis, inflammation, human.

DOI: 10.1177/0022034512456551

Received May 15, 2012; Last revision July 5, 2012; Accepted July 6, 2012

A supplemental appendix to this article is published electronically only at <http://jdr.sagepub.com/supplemental>.

© International & American Associations for Dental Research

MicroRNAs and Their Target Genes in Gingival Tissues

INTRODUCTION

MicroRNAs (miRNAs) are small, non-coding, single-stranded, ~22-base RNA sequences that play critical roles in post-transcriptional regulation, either by repressing translation initiation and protein synthesis, or by inducing degradation of messenger RNA (mRNA; Selbach *et al.*, 2008; Dai and Ahmed, 2011). Over 1,000 human miRNAs have been identified to date (Griffiths-Jones *et al.*, 2006; Dai and Ahmed, 2011). Complete sequence complementarity between a miRNA and its potential target mRNA is not required to suppress gene expression, and matching of as few as 6 base-pairs may be sufficient (Thomson *et al.*, 2011). Consequently, a single miRNA has the potential to control the translation of hundreds of genes simultaneously and may thus have a significant impact on protein expression (Baek *et al.*, 2008). Differential miRNA expression in cells involved in the innate and adaptive immunity has been shown to regulate cell development and function (O’Connell *et al.*, 2010). In humans, miRNA expression has been shown to affect the pathobiology of several diseases such as cancer (Sumazin *et al.*, 2011), but also inflammatory diseases including lupus (Dai *et al.*, 2007) and rheumatoid arthritis (Nakasa *et al.*, 2008). The expression of miRNAs in periodontal tissues and their potential function in the context of the pathobiology of periodontal diseases are largely unexplored. To date, only two small-scale studies have reported on the occurrence of selected miRNAs in gingival tissues (Xie *et al.*, 2011; Perri *et al.*, 2012).

In this study, we first describe the occurrence of miRNAs in healthy and inflamed gingival tissues from 86 well-phenotyped patients with periodontitis, and subsequently validate their *in silico*-predicted targets by examining mRNA profiles in the same specimens. We hypothesized that the concomitant study of miRNA and mRNA expression in gingival tissues would provide new insights into the *in vivo* gene-regulatory function of miRNAs in human periodontitis.

MATERIALS & METHODS

Study Participants and Tissue Samples

This report is based on 198 gingival tissue samples from 86 patients with periodontitis (age range 13-76 yrs). They comprised all available archived, homogenized tissue specimens from an original collection of 310 tissue samples from 120 individuals, earlier analyzed with respect to whole-genome

transcriptomes and subgingival bacterial profiles (Papapanou *et al.*, 2009; GEO accession number GSE16134). Details on patient selection, tissue harvesting, and assessment of whole-genome mRNA profiles by microarrays (U-133Plus2.0; Affymetrix, Santa Clara, CA, USA) were reported earlier (Demmer *et al.*, 2008). Enrollees did not include current smokers or patients with diabetes mellitus or other systemic conditions. The tissue samples analyzed here originated from 158 periodontally ‘diseased’ sites [with bleeding on probing (BoP), probing depth (PD) > 4 mm, and attachment level (AL) ≥ 3 mm] and 40 ‘healthy’ sites (no BoP, PD ≤ 4 mm, and AL ≤ 2 mm). The study was approved by the Institutional Review Board, Columbia University Medical Center.

Tissue Processing, RNA Labeling, miRNA Microarray Hybridization, and Real-time RT-PCR Validation

These procedures are described in detail in the Appendix. In brief, miRNA profiling was performed on the Agilent Platform with microarrays carrying probes for 1,205 human and 144 human viral miRNAs. In a subset of 16 samples from eight patients, each contributing one healthy and one diseased tissue sample with RIN ≥ 7, we used quantitative RT-PCR to validate expression of the 6 top differentially modulated miRNAs (3 overexpressed and 3 underexpressed in diseased tissues).

miRNA Differential Expression

miRNA expression profiles were analyzed with R (The R Development Core Team, 2005) and Bioconductor statistical frameworks (Gentleman *et al.*, 2004). Raw data were pre-processed with AFE (Agilent), and quality control was performed using the limma (Smyth, 2005) and AgiMicroRNA (Romero, 2011) packages. Signals were background-corrected by fitting a normal plus exponential convolution model, and quintile-normalized between arrays using the RMA algorithm (Irizarry *et al.*, 2003). Probes were filtered to eliminate controls and undetected miRNAs using default parameters. We validated miRNA microarray data by calculating the Pearson correlation coefficient between the log₂ ratio of miRNA expression of each pair (healthy/diseased sample from the same patient) and the corresponding RT-PCR value.

miRNA Target Identification

The mRNA expression profiles for each sample were retrieved from GEO series GSE16134. Signals were pre-processed and normalized as earlier described (Papapanou *et al.*, 2009). We used limma to fit the linear model and to compute the moderated *t* statistic. Differential expression was assessed, accounting for the correlation between biological replicates (multiple samples from the same patient for a given condition, when available) and corrected for multiple comparisons (Benjamini and Hochberg, 1995). miRNA targets were identified by the cupid algorithm (Sumazin *et al.*, 2011), which integrates sequence-based predictions from the MIRANDA, Pita, and Targetscan databases, cross-species conservation of predicted sites, and miRNA and gene expression data, with emphasis on site multiplicity in 3'UTRs to predict miRNA targets.

miRNA Target Enrichment Analysis

We summarized the mRNA data on the gene-level after excluding probes with lower interquartile range. Subsequently, we computed Spearman correlation coefficients between the expression of each miRNA and its predicted targets in each specimen and restricted the sets to anti-correlated genes at a False Discovery Rate adjusted *p* value (FDR) of < 0.1. We then constructed a library including the miRNA-target sets for the miRNAs showing a significant (FDR < 0.05) modulation in gingival health and disease. We considered only gene sets (sets of miRNA targets) with ≥ 10 and ≤ 2,000 genes/set and tested them for possible enrichment using GSEA (Mootha *et al.*, 2003; Subramanian *et al.*, 2005). Next, we identified the enriched gene sets of the miRNAs that showed a significant (FDR < 0.05) modulation in healthy and diseased tissues. Identified enriched gene sets were grouped into functional categories by the use of DAVID (Huang *et al.*, 2009a,b) and IPA (Ingenuity® Systems, Redwood City, CA, USA).

RESULTS

We identified 159 miRNAs with significant (FDR < 0.05) differential expression between healthy and diseased gingival tissues (Appendix Table 1). Of these, 91 were overexpressed and 68 were underexpressed in disease vs. health. Table 1 reports the top 50 differentially expressed miRNAs in diseased gingiva, along with the corresponding fold-change (FC) values describing their expression relative to that in healthy tissues. The Fig. depicts a heat map illustrating expression levels for the top 25 differentially expressed miRNAs in either direction.

The 6 miRNAs selected for qRT-PCR validation were hsa-miR-451, hsa-miR-223, hsa-miR-486-5p, hsa-miR-1246, hsa-miR-1260, and hsa-miR-141. The Spearman correlation coefficient between microarray and qRT-PCR-based log₂ ratio of expression was 0.80 (*p* = 7.93 × 10⁻¹²), indicating close agreement.

Table 2 presents the number of predicted gene targets of the top miRNAs, as well as the subset that was actually modulated in the gingival tissues, *i.e.*, the number of genes with statistically significant (FDR < 0.05) inverse expression. Overall, approximately 25% of the predicted gene targets were statistically down-regulated, and 7% were differentially modulated with an absolute FC of ≥ 1.5.

miRNA target enrichment analysis identified 242 gene sets. Appendix Table 2 lists the significantly enriched gene sets (FDR < 0.05) of the differentially expressed miRNAs (FDR < 0.05) in diseased or healthy tissues. Twenty-nine miRNAs overexpressed and 31 miRNAs underexpressed in diseased tissues had significantly enriched targets. The Appendix Fig. depicts the enrichment plots of the top two enriched gene sets associated with either overexpressed or underexpressed miRNAs in diseased gingival tissues.

Next, we sought to identify processes seemingly modulated by miRNA activity. Analysis with DAVID revealed statistically significantly regulated GO terms for 12 miRNAs (Appendix Table 3). Among these, hsa-miR-210 was associated with the highest number of significant terms, with “Phosphorus metabolic process”, “Phosphate metabolic process”, and “Response to wounding” emerging as the highest-ranked GO groups. Table 3 depicts elements of the functional analysis (IPA) for the hsa-miR-210 gene set.

Table 1. List of the Top Differentially Expressed miRNAs between Diseased and Healthy Gingival Tissues at FDR < 0.05

(A) Top 25 miRNAs with Increased Expression in Diseased Compared with Healthy Gingiva

miRNAs Overexpressed in Disease	FC
hsa-mir-451	2.63
hsa-mir-223	2.53
hsa-mir-486-5p	2.46
hsa-mir-3917	2.08
hsa-mir-671-5p	1.94
hsa-mir-140-3p	1.87
hsa-mir-4306	1.85
hsa-mir-483-5p	1.81
hsa-mir-575	1.80
hsa-mir-145	1.76
hsa-mir-4299	1.72
hsa-mir-155	1.70
hsa-mir-363	1.69
hsa-mir-185	1.68
hsa-mir-650	1.68
hsa-mir-148a	1.66
hsa-mir-3679-5p	1.63
hsa-mir-16	1.57
hsa-mir-3652	1.57
hsa-let-7i	1.54
hsa-mir-497	1.51
hsa-mir-126	1.51
hsa-mir-29a	1.49
hsa-mir-125a-3p	1.49
hsa-mir-1202	1.48

(B) Top 25 miRNAs with Decreased Expression in Diseased Compared with Healthy Gingiva

miRNAs Underexpressed in Disease	FC
hsa-mir-1246	0.33
hsa-mir-1260	0.44
hsa-mir-141	0.46
hsa-mir-1260b	0.46
hsa-mir-203	0.46
hsa-mir-210	0.47
hsa-mir-205*	0.49
hsa-mir-3156-5p	0.50
hsa-mir-720	0.56
hsa-mir-1274a_v16.0	0.56
hsa-mir-4286	0.58
hsa-mir-1224-5p	0.60
hsa-mir-31	0.62
hsa-mir-33a	0.62
hsa-mir-99a	0.62
hsa-mir-31*	0.63
hsa-mir-1914*	0.64
hsa-mir-3692*	0.65
hsa-mir-205	0.65
hsa-mir-30e	0.66
hsa-mir-136	0.67
hsa-mir-200a	0.67
hsa-mir-200c	0.68
hsa-mir-1274b_v16.0	0.69
hsa-mir-3911	0.70

Table 2. Total Number of Predicted Gene Targets of the Top 50 Differentially Expressed miRNAs using the Cupid Algorithm, and Number of Statistically Significantly (FDR < 0.05) Negatively Correlated Genes ('Inversely expressed targets')

(A) Top 25 miRNAs Overexpressed in Diseased When Compared with Healthy Gingiva

miRNAs Overexpressed in Disease	Number of Predicted Targets	Inversely Expressed Targets
hsa-mir-451	257	77
hsa-mir-223	798	235
hsa-mir-486-5p	138	41
hsa-mir-3917	0	0
hsa-mir-671-5p	112	30
hsa-mir-140-3p	381	104
hsa-mir-4306	393	109
hsa-mir-483-5p	223	64
hsa-mir-575	371	115
hsa-mir-145	272	82
hsa-mir-4299	220	64
hsa-mir-155	1028	314
hsa-mir-363	313	87
hsa-mir-185	392	97
hsa-mir-650	240	73
hsa-mir-148a	663	179
hsa-mir-3679-5p	0	0
hsa-mir-16	642	183
hsa-mir-3652	0	0
hsa-let-7i	398	98
hsa-mir-497	290	71
hsa-mir-126	79	19
hsa-mir-29a	520	147
hsa-mir-125a-3p	206	65
hsa-mir-1202	149	46

(B) Top 25 miRNAs Underexpressed in Diseased when Compared with Healthy Gingiva

miRNAs Underexpressed in Disease	Number of Predicted Targets	Inversely Expressed Targets
hsa-mir-1246	339	51
hsa-mir-1260	173	40
hsa-mir-141	261	57
hsa-mir-1260b	180	47
hsa-mir-203	667	154
hsa-mir-210	952	238
hsa-mir-205*	615	128
hsa-mir-3156-5p	0	0
hsa-mir-720	148	36
hsa-mir-1274a_v16.0	0	0
hsa-mir-4286	164	44
hsa-mir-1224-5p	239	42
hsa-mir-31	334	71
hsa-mir-33a	259	48
hsa-mir-99a	114	23
hsa-mir-31*	1051	226
hsa-mir-1914*	126	26
hsa-mir-3692*	0	0
hsa-mir-205	313	69
hsa-mir-30e	259	59
hsa-mir-136	621	161
hsa-mir-200a	252	62
hsa-mir-200c	339	69
hsa-mir-1274b_v16.0	0	0
hsa-mir-3911	0	0

Figure. Heat map illustrating miRNA expression in diseased and healthy gingival tissue samples. Expression values have been transformed using z-scores [(expression value - mean)/StDev]. Color shading reflects expression level, with red indicating high expression and blue low expression. Note that the differential expression of miRNAs between healthy and diseased gingival tissues was not driven by a subset of samples, but was observed rather uniformly.

Within the primary category “Cellular and Molecular Functions” (Table 3A), the top 20 functional annotations were strongly associated with “Cell Death” and “Cellular Movement”. With respect to “Physiological System Development and Function” (Table 3B), the 20 most significantly enriched functional annotations mapped to “Hematological System Development and Function” and “Immune Cell Trafficking”.

DISCUSSION

This is the first study to examine concurrently the expression of miRNAs and mRNAs in healthy and diseased gingival tissues, and provides the foundation for further mechanistic experimentation to dissect the role of miRNAs in gingival tissue homeostasis and pathology.

We have earlier presented gingival tissue transcriptomic data from the same patients (Demmer *et al.*, 2008; Papapanou *et al.*, 2009), and demonstrated substantial differential gene expression between states of periodontal health and disease. Here, we extend this work and analyze differential expression of miRNAs in the same samples. So far, two small-sized studies have examined differential expression of miRNAs in gingival tissues but have adopted different approaches. Xie *et al.* (2011) pooled total RNA samples from 10 periodontally healthy and 10 diseased gingival tissue samples and examined differential expression of miRNAs after hybridization of a single pair of aggregate samples. Therefore, no meaningful comparisons with the current data are feasible. Perri *et al.* (2012) collected healthy and diseased gingival tissues from 10 obese and 10 non-obese periodontitis patients and analyzed expression of 88 candidate miRNA species using targeted PCR arrays. Our dataset corroborates the reported overexpression of hsa-miR-185 in diseased

tissues. However, in contrast to Perri *et al.*, we observed a higher expression of hsa-miR-30e and hsa-miR-210 in healthy gingiva.

Among the miRNAs found to be significantly overexpressed in disease in our study, hsa-miR-155 was reported to regulate immune responses, primarily by activating Toll-like and interleukin-1 receptors (O’Connell *et al.*, 2007; Tsitsiou and Lindsay, 2009; Quinn and O’Neill, 2011). Recent work demonstrated induction of hsa-miR-203 in gingival epithelial cells upon *in vitro* infection by *Porphyromonas gingivalis* (Moffatt and Lamont, 2011). In our dataset, expression of hsa-miR-203 was higher in healthy gingiva. This particular miRNA is one of the most abundantly expressed in keratinocytes under physiological conditions, plays an important role in the induction of differentiation and the repression of proliferation in skin stem cells (Lena *et al.*, 2008) and is down-regulated in instances of pronounced proliferation, as seen in carcinomas (Rentoft *et al.*, 2011). Therefore, the observed lower expression of hsa-miR-203 in diseased tissues is compatible with the reported significantly higher net proliferative rate of both the junctional and the oral epithelium in states of disease (Nagarakanti *et al.*, 2007). Among the up-regulated miRNAs in diseased gingiva, hsa-miR-223 has been shown to be involved in granulopoiesis, and hsa-miR-146a was identified as a regulator of immune responses (Dai and Ahmed, 2011; Quinn and O’Neill, 2011). In a mouse model, expression of miR-146a was increased in the gingival tissues upon infection by several periodontal pathogens and regulated cytokine production in response to microbial ligands (Nahid *et al.*, 2009, 2011). Clearly, the cellular origin of the identified miRNAs in our dataset is impossible to define, since the analyzed tissue lysates included epithelium, fibroblasts, and infiltrating inflammatory cells. In addition, subject-based factors, such as gender, and/or environmental exposures may also

Table 3. Functional Analysis (IPA) Results for the hsa-miR-210 Gene Set

(A) Modulation of "Cellular and Molecular Functions"

Functional Annotation	p-value	Categories					
		Cell Death	Carbohydrate Metabolism	Cellular Movement	Cellular Development	Cellular Growth and Proliferation	Cellular Function and Maintenance
Apoptosis	3.76×10^{-16}	x					
Cell death	1.30×10^{-14}	x					
Metabolism of carbohydrate	6.42×10^{-14}		x				
Migration of cells	2.55×10^{-13}			x			
Cell movement	2.99×10^{-13}			x			
Cell movement of phagocytes	1.69×10^{-12}			x			
Differentiation	1.74×10^{-12}				x		
Differentiation of cells	2.15×10^{-12}				x		
Cell movement of leukocytes	1.04×10^{-11}			x			
Proliferation of cells	1.45×10^{-11}					x	
Leukocyte migration	1.84×10^{-11}			x			
Growth of cells	3.18×10^{-11}					x	
Function of leukocytes	1.07×10^{-10}						x
Cell movement of neutrophils	1.38×10^{-10}			x			
Differentiation of blood cells	2.46×10^{-10}				x		
Apoptosis of tumor cell lines	6.30×10^{-10}	x					
Homing of cells	1.20×10^{-9}			x			
Chemotaxis of cells	1.37×10^{-9}			x			
Cell death of tumor cell lines	2.42×10^{-9}	x					
Chemotaxis of leukocytes	2.54×10^{-9}			x			

(B) Modulation of "Physiological System Development and Function"

Functional Annotation	p-value	Categories							
		Organismal Survival	Tissue Morphology	Hematological System Development and Function	Immune Cell Trafficking	Tissue Development	Hematopoiesis	Connective Tissue Development and Function	Skeletal and Muscular System Development and Function
Organismal death	1.38×10^{-16}	x							
Quantity of cells	1.95×10^{-14}		x						
Quantity of leukocytes	7.95×10^{-14}		x	x					
Quantity of blood cells	9.21×10^{-13}		x	x					
Cell movement of phagocytes	1.69×10^{-12}			x	x				
Cell movement of leukocytes	1.04×10^{-11}			x	x				
Formation of tissue	1.31×10^{-11}					x			
Leukocyte migration	1.84×10^{-11}				x				
Development of connective tissue	2.19×10^{-11}					x			
Cell movement of neutrophils	1.38×10^{-10}			x	x				
Tissue development	1.48×10^{-10}					x			
Quantity of lymphocytes	1.52×10^{-10}		x	x					
Differentiation of blood cells	2.46×10^{-10}			x					
Survival of organism	1.13×10^{-9}	x							
Hematopoiesis	2.12×10^{-9}			x			x		
Chemotaxis of leukocytes	2.54×10^{-9}			x	x				
Development of bone	2.55×10^{-9}					x		x	
Cell movement of myeloid cells	3.88×10^{-9}			x	x				
Migration of phagocytes	3.92×10^{-9}			x	x				
Angiogenesis	4.54×10^{-9}								x

The Table lists the top 20 functional annotations in each primary category, ranked by ascending p-value.

have affected the differential miRNA expression, beyond the inflammatory status of the tissues.

We sought to validate the predicted targets of the identified differentially modulated miRNAs by examining the actual mRNA expression of these particular genes in our transcriptomic database. We observed that, overall, approximately 25% of the predicted gene targets of the highly expressed miRNAs in gingival health or disease were indeed down-regulated. However, it must be realized that the computational identification of miRNA targets based on sequence complementarity is not flawless, but represents the only source of genome-wide rapid identification of putative miRNA targets in the absence of experimental target validation (Alexiou *et al.*, 2009). The main prediction feature used in most miRNA target identification software is the sequence alignment of the miRNA 'seed' region (*i.e.*, 7-8 bp located at the 5' end of the miRNA) and the corresponding motifs in the 3' untranslated region of a protein-coding gene (Doench and Sharp, 2004; Lewis *et al.*, 2005). However, miRNA-binding sites may also reside, albeit infrequently, within the coding regions (Baek *et al.*, 2008). Therefore, experimental identification of miRNA targets is essential for the optimization of existing algorithms and will result in target prediction computations that reflect more accurately the underlying biological processes (Alexiou *et al.*, 2009).

GSEA revealed that, among the top overexpressed 25 miRNAs in disease, 14 showed enrichment of their gene targets in health. In other words, occurrence of these miRNAs decreased the level of their target mRNAs in a statistically significant manner. Among the top 25 miRNAs underexpressed in disease, 17 showed enrichment. These observations are in accordance with the canonical concept that miRNAs predominantly induce degradation or destabilization of their target gene mRNA by reducing the levels of available mRNAs rather than by mediating translational repression with persistence of repressed mRNA (Baek *et al.*, 2008; Selbach *et al.*, 2008; Guo *et al.*, 2010).

Twelve miRNAs were associated with statistically significant regulation of several functional groups in GO analysis. Of these, hsa-miR-210 is of particular interest, since (i) both the miRNA and its gene targets were differentially modulated in both healthy and diseased gingiva, and (ii) the involved genes are significantly affiliated with a variety of biological processes linked to periodontal inflammation. Specifically, hsa-miR-210 is the most consistently and predominantly up-regulated miRNA in response to hypoxia (Huang *et al.*, 2010). This observation is corroborated by our transcriptomic dataset, in which GO analysis identified 'response to hypoxia' as a biological process significantly attributed to the hsa-miR-210 gene set. Hypoxia-inducible factors (HIFs) control cellular responses to hypoxia, and hsa-miR-210 was shown to be induced by HIFs, but also to form a feed-forward loop, contributing to the maintenance of high HIF (Devlin *et al.*, 2011). A member of the HIF family, HIF-1 α , plays an important role in defenses against bacterial infection in keratinocytes; HIF-1 α regulates production of cathelicidin, an antimicrobial peptide shown to confer protection against group A *Streptococcus* infection (Peyssonnaud *et al.*, 2008). Interestingly, normal skin is hypoxic, and HIF-1 α is extensively expressed in normal epidermis (Boutin *et al.*,

2008). This concept may also be important in the homeostasis of the gingival tissues, given the observed significant overexpression of hsa-miR-210 in health. Interestingly, HIF-1 α protein expression was markedly higher in pathological gingival tissue samples from periodontitis patients than in healthy samples from periodontitis-free control individuals, although HIF-1 α mRNA levels were not significantly different between the two groups (Ng *et al.*, 2011). Thus, regulation of HIFs by hsa-miR-210 seems to be important in host tissues exposed to a chronic microbial challenge such as skin and oral mucosal surfaces.

IPA analysis of the modulated targets of hsa-miR-210 showed their involvement in "cellular movement" and "immune cell trafficking", suggesting that it may influence the migration of immune and inflammatory cells in the periodontal tissues. Recent work demonstrated that expression of hsa-miR-210 in cultured endothelial cells stimulated by growth factors resulted in chemotaxis and increased angiogenic potential (Fasanaro *et al.*, 2008).

In summary, we have identified specific miRNAs that are overexpressed in healthy or periodontitis-affected gingiva, and have validated for the first time particular genes among their predicted targets that are indeed concomitantly modulated, several of which are involved in tissue homeostasis and inflammatory/immune responses. The precise cellular origins of these particular miRNAs within the gingival tissues, and the experimental validation of their potential functions, warrant further investigation.

ACKNOWLEDGMENTS

This work was supported by the NIH/NIDCR (DE015649 and DE021820), the NCATS-NCRR/NIH (CTSA UL1RR024156), and Colgate-Palmolive, NJ, USA. Dr. Kebschull was supported by the German Research Foundation (DFG KFO208 TP6 & TP9), the German Society for Periodontology (DGP), and the DGZMK. The authors declare no potential conflicts of interest with respect to the authorship and/or publication of this article.

REFERENCES

- Alexiou P, Maragkakis M, Papadopoulos GL, Reczko M, Hatzigeorgiou AG (2009). Lost in translation: an assessment and perspective for computational microRNA target identification. *Bioinformatics* 25:3049-3055.
- Baek D, Villen J, Shin C, Camargo FD, Gygi SP, Bartel DP (2008). The impact of microRNAs on protein output. *Nature* 455:64-71.
- Benjamini Y, Hochberg Y (1995). Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J R Statist Soc B* 57:289-300.
- Boutin AT, Weidemann A, Fu Z, Mesropian L, Gradin K, Jamora C, *et al.* (2008). Epidermal sensing of oxygen is essential for systemic hypoxic response. *Cell* 133:223-234.
- Dai R, Ahmed SA (2011). MicroRNA, a new paradigm for understanding immunoregulation, inflammation, and autoimmune diseases. *Transl Res* 157:163-179.
- Dai Y, Huang YS, Tang M, Lv TY, Hu CX, Tan YH, *et al.* (2007). Microarray analysis of microRNA expression in peripheral blood cells of systemic lupus erythematosus patients. *Lupus* 16:939-946.
- Demmer RT, Behle JH, Wolf DL, Handfield M, Kebschull M, Celenti R, *et al.* (2008). Transcriptomes in healthy and diseased gingival tissues. *J Periodontol* 79:2112-2124.
- Devlin C, Greco S, Martelli F, Ivan M (2011). miR-210: more than a silent player in hypoxia. *IUBMB Life* 63:94-100.
- Doench JG, Sharp PA (2004). Specificity of microRNA target selection in translational repression. *Genes Dev* 18:504-511.

- Fasanaro P, D'Alessandra Y, Di Stefano V, Melchionna R, Romani S, Pompilio G, *et al.* (2008). MicroRNA-210 modulates endothelial cell response to hypoxia and inhibits the receptor tyrosine kinase ligand Ephrin-A3. *J Biol Chem* 283:15878-15883.
- Gentleman RC, Carey VJ, Bates DM, Bolstad B, Dettling M, Dudoit S, *et al.* (2004). Bioconductor: open software development for computational biology and bioinformatics. *Genome Biol* 5:R80.
- Griffiths-Jones S, Grocock RJ, van Dongen S, Bateman A, Enright AJ (2006). miRBase: microRNA sequences, targets and gene nomenclature. *Nucleic Acids Res* 34(Database issue):D140-D144.
- Guo H, Ingolia NT, Weissman JS, Bartel DP (2010). Mammalian microRNAs predominantly act to decrease target mRNA levels. *Nature* 466:835-840.
- Huang da W, Sherman BT, Lempicki RA (2009a). Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat Protoc* 4:44-57.
- Huang da W, Sherman BT, Lempicki RA (2009b). Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. *Nucleic Acids Res* 37:1-13.
- Huang X, Le QT, Giaccia AJ (2010). MiR-210—micromanager of the hypoxia pathway. *Trends Mol Med* 16:230-237.
- Irizarry RA, Hobbs B, Collin F, Beazer-Barclay YD, Antonellis KJ, Scherf U, *et al.* (2003). Exploration, normalization, and summaries of high density oligonucleotide array probe level data. *Biostatistics* 4:249-264.
- Lena AM, Shalom-Feuerstein R, Rivetti di Val Cervo P, Aberdam D, Knight RA, Melino G, *et al.* (2008). miR-203 represses 'stemness' by repressing DeltaNp63. *Cell Death Differ* 15:1187-1195.
- Lewis BP, Burge CB, Bartel DP (2005). Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell* 120:15-20.
- Moffatt CE, Lamont RJ (2011). *Porphyromonas gingivalis* induction of microRNA-203 expression controls suppressor of cytokine signaling 3 in gingival epithelial cells. *Infect Immun* 79:2632-2637.
- Mootha VK, Lindgren CM, Eriksson KF, Subramanian A, Sihag S, Lehar J, *et al.* (2003). PGC-1 α -responsive genes involved in oxidative phosphorylation are coordinately downregulated in human diabetes. *Nat Genet* 34:267-273.
- Nagarakanti S, Ramya S, Babu P, Arun KV, Sudarsan S (2007). Differential expression of E-cadherin and cytokeratin 19 and net proliferative rate of gingival keratinocytes in oral epithelium in periodontal health and disease. *J Periodontol* 78:2197-2202.
- Nahid MA, Pauley KM, Satoh M, Chan EK (2009). miR-146a is critical for endotoxin-induced tolerance: implication in innate immunity. *J Biol Chem* 284:34590-34599.
- Nahid MA, Rivera M, Lucas A, Chan EK, Kesavalu L (2011). Polymicrobial infection with periodontal pathogens specifically enhances microRNA miR-146a in ApoE(-/-) mice during experimental periodontal disease. *Infect Immun* 79:1597-1605.
- Nakasa T, Miyaki S, Okubo A, Hashimoto M, Nishida K, Ochi M, *et al.* (2008). Expression of microRNA-146 in rheumatoid arthritis synovial tissue. *Arthritis Rheum* 58:1284-1292.
- Ng KT, Li JP, Ng KM, Tipoe GL, Leung WK, Fung ML (2011). Expression of hypoxia-inducible factor-1 α in human periodontal tissue. *J Periodontol* 82:136-141.
- O'Connell RM, Taganov KD, Boldin MP, Cheng G, Baltimore D (2007). MicroRNA-155 is induced during the macrophage inflammatory response. *Proc Natl Acad Sci USA* 104:1604-1609.
- O'Connell RM, Rao DS, Chaudhuri AA, Baltimore D (2010). Physiological and pathological roles for microRNAs in the immune system. *Nat Rev Immunol* 10:111-122.
- Papapanou PN, Behle JH, Kebschull M, Celenti R, Wolf DL, Handfield M, *et al.* (2009). Subgingival bacterial colonization profiles correlate with gingival tissue gene expression. *BMC Microbiol* 9:221.
- Perri R, Nares S, Zhang S, Barros SP, Offenbacher S (2012). MicroRNA modulation in obesity and periodontitis. *J Dent Res* 91:33-38.
- Peyssonnaud C, Boutin AT, Zinkernagel AS, Datta V, Nizet V, Johnson RS (2008). Critical role of HIF-1 α in keratinocyte defense against bacterial infection. *J Invest Dermatol* 128:1964-1968.
- Quinn SR, O'Neill LA (2011). A trio of microRNAs that control Toll-like receptor signalling. *Int Immunol* 23:421-425.
- Rentoft M, Fahlen J, Coates PJ, Laurell G, Sjöström B, Ryden P, *et al.* (2011). miRNA analysis of formalin-fixed squamous cell carcinomas of the tongue is affected by age of the samples. *Int J Oncol* 38:61-69.
- Romero P (2011). Pre-processing and differential expression analysis of Agilent microRNA arrays using the AgiMicroRna Bioconductor library. *BMC Genomics* 12:64.
- Selbach M, Schwanhauser B, Thierfelder N, Fang Z, Khanin R, Rajewsky N (2008). Widespread changes in protein synthesis induced by microRNAs. *Nature* 455:58-63.
- Smyth G (2005). limma: linear models for microarray data. In: *Bioinformatics and computational biology solutions using R and Bioconductor*. Gentleman R, Carey V, Huber W, Irizarry R, Dudoit S, editors. Berlin: Springer-Verlag, pp. 397-420.
- Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, *et al.* (2005). Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc Natl Acad Sci USA* 102:15545-15550.
- Sumazin P, Yang X, Chiu HS, Chung WJ, Iyer A, Llobet-Navas D, *et al.* (2011). An extensive microRNA-mediated network of RNA-RNA interactions regulates established oncogenic pathways in glioblastoma. *Cell* 147:370-381.
- The R Development Core Team (2005). R: a language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing.
- Thomson DW, Bracken CP, Goodall GJ (2011). Experimental strategies for microRNA target identification. *Nucleic Acids Res* 39:6845-6853.
- Tsitsiou E, Lindsay MA (2009). microRNAs and the immune response. *Curr Opin Pharmacol* 9:514-520.
- Xie YF, Shu R, Jiang SY, Liu DL, Zhang XL (2011). Comparison of microRNA profiles of human periodontal diseased and healthy gingival tissues. *Int J Oral Sci* 3:125-134.