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ORIGINAL ARTICLE

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hsa-miR-374b-5p regulates expression of the gene U2AF homology motif *(UHM) kinase 1*

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Abstract

Objective: We aimed to identify a microRNA (miRNA) that is significantly upregulated in blood and in cells of the oral mucosa upon exposure to the periodontitis main risk factors oral inflammation and tobacco smoke, to subsequently identify its target gene and to describe the molecular mechanism of gene regulation.

Background: miRNAs are associated with many disorders. Array-based miRNA expression studies indicated a number of differentially expressed miRNAs in the pathology of oral diseases. However, these miRNAs mostly lacked replication, and their target genes have remained unknown.

Methods: 863 miRNAs were analyzed in blood from 18 PD cases and 70 controls (Geniom Biochip). Selected miRNAs were analyzed for upregulation in the inflamed oral mucosa of PD patients using published miRNA expression profiling studies from gingival cells. hsa-miR-374b-5p mimic was overexpressed in primary gingival fibroblasts (pGFs) from 3 donors, and genome-wide mRNA expression was quantified (Clarion Array). Gene-specific regulation was validated by qRT-PCR and Luciferase activity in HeLa cells.

Results: hsa-miR-374b-5p showed >twofold change (FC) in 3 independent studies performed in blood, gingival tissues, and cells. After hsa-miR-374b-5p overexpression, genome-wide expression analysis showed *UHMK1* as top 1 downregulated gene in pGFs ($p = 2.5 \times 10^{-04}$, fold change = −1.8). Reporter genes demonstrated that hsa-miR-374b-5p downregulates mRNA levels ($p = .02$; FC = −1.5), leading to reduction in protein activity ($p = .013$, FC = −1.3).

Conclusions: hsa-miR-374b-5p is upregulated in blood and ginvial cells exposed to oral inflammation and tobacco smoke and regulates *UHMK1*, which has a role in osteoclast differentiation.

KEYWORDS
blood, inflammation, microRNA, periodontitis, tobacco

1 | INTRODUCTION

Periodontitis is a very common inflammatory disease of the oral cavity, and it is characterized by inflammation of the oral mucosa that causes gingival bleeding, loss of connective tissue, alveolar bone loss, and subsequent tooth loss. In the etiology of periodontitis, persistent oral inflammation is the most important risk factor,[1] followed by smoking[2,3] This implies that oral inflammation and smoking can be considered "environmental" variables with a causal role for onset and progression.

Inflammation and smoking[4] affect microRNA (miRNA) expression in gingival tissues and blood and may modulate pathogenesis. MicroRNAs (miRNAs) are small noncoding RNAs, with an average of 22 nucleotides in length. miRNAs interact with the 3' untranslated regions (3' UTRs) of target messenger RNA transcripts (mRNAs) to suppress gene expression through mRNA degradation or, more rarely, by translational repression (reviewed in[5,6]). Giving evidence of their importance, miRNAs comprise up to 5% of animal genes,[7] making them one of the most abundant classes of regulators. Individual miRNAs can modulate multiple mRNA targets, and a single mRNA can be regulated by different miRNAs.[8] As a consequence, miRNAs are involved in a variety of biological processes and act in complex networks that control a variety of target genes.[9] miRNA expression that is atypical to a healthy state is associated with many human diseases.[10] The identification of differentially expressed miRNAs and of the target genes of their regulation directly has potential to elucidate the molecular mechanisms that are involved in pathogenesis. Accordingly, various studies generated array-based profiles of miRNA expression in healthy and inflamed oral tissues that have a role in the pathogenesis of oral inflammatory diseases and reported differential miRNA expression in the oral mucosa,[11–15] saliva,[16,17] gingival crevicular fluid,[18] and peripheral blood.[19] However, miRNA profiles vary due to differences in the analyzed tissues, tissue collection sites, or collection methods, which influence the distribution of cell types that contribute differently to miRNAs profiles. miRNA levels are also affected by isolation and quantification methods, and importantly, by the environmental and genetic background of the donor. Accordingly, the lists of differentially expressed miRNAs from different studies showed little overlap, making their interpretation difficult. Some miRNAs are secreted into extracellular fluids like venous blood[20] and to identify miRNAs that are differentially expressed between health and disease in blood; we recently quantified miRNAs with a miRNA expression array to identify characteristic blood-borne miRNA patterns of various complex diseases including PD.[19] In the current study, we aimed to find a miRNA that, upon exposure to the periodontitis main risk factors oral inflammation and tobacco smoke, is significantly upregulated in blood and in cells of the oral mucosa. To compensate for random variation of miRNA levels and thus to reduce discovery of false positives, we searched for miRNAs that had been discovered independently by at least two different genome-wide expression studies of oral tissues, in addition to our own study that investigated differential miRNA expression in blood. Subsequently, we aimed to identify a target gene of its regulation to better understand the molecular processes that lead to periodontitis.

2 | MATERIALS AND METHODS

2.1 | Study populations

The study population was described in detail in a previous paper.[19] In brief, 18 untreated PD cases of German or Dutch genetic background were collected at the University Dental Clinics of Bonn, Dresden, Kiel (all Germany), and Amsterdam (the Netherlands). Inclusion criteria were ≥7 teeth with bone loss >1/3 of the root length or attachment loss of ≥5 mm in conjunction with ≥50% bleeding on probing (BoP). Exclusion criteria were any acute or chronic condition and allergies. The clinical characteristics of the patients are summarized in Table 1. Patients did not smoke on the day of blood donation and did not receive antibiotics 6 months before blood donation. All patients were <55 years of age, with 39% smokers and 33% females. The mean age of first diagnosis was 41 years with an age range from 20 to 55. The number of affected teeth was on average 13, with a range between 7 and 28. The control sample consisted of 70 German generally healthy age-matched blood donors from Heidelberg, Homburg, Saarbrücken, and Würzburg. The mean age at the time of enrollment was 44 years, and the female: male ratio in the sample was 2:1. The controls reported in a questionnaire to be free of periodontitis and other systemic diseases. All participants gave written informed consent. Approval for the project was received from the local ethics committees.

2.2 | miRNA extraction of blood samples

5 ml peripheral blood of PD patients and healthy donors was extracted using PAXgene Blood RNA tubes (BD, Franklin Lakes, New Jersey USA), and the total RNA including the miRNAs were isolated using the miRNeasy kit according to the manufacturer's instructions (Qiagen GmbH, Hilden). The detailed protocol of miRNA extraction was described in.[19]

2.3 | Microarray screening for differentially expressed blood secreted microRNAs

Total miRNAs were quantified on the Geniom Biochip array "miRNA Homo sapiens" and analyzed using the microarray-based screening approach described in previous studies.[21,22]

To identify significantly differentially regulated miRNAs, we performed parametric t tests with a Benjamini-Hochberg adjusted significance level of 0.001.

TABLE 1 Description of the study sample

ID	Age at blood donation	Age of diagnosis	sex	Affected teeth[a]	Current smoker	Center
CP 107714	40	20	Female	9	Non-smoker	Bonn
CP 107711	43	43	Female	25	Non-smoker	Kiel
CP 107712	53	53	Female	10	unknown	Bonn
CP 107715	54	54	Female	>10	Non-smoker	Dresden
CP 109080	35	Unknown	Female	8	smoker	Amsterdam, NL
CP 105277	48	25	Male	9	Non-smoker	Bonn
DPC 108591	50	27	Male	11	smoker	Bonn
CP 108592	39	39	Male	>10	smoker	Dresden
CP 115282	40	40	Male	7	Non-smoker	Dresden
CP 115286	49	40	Male	14	smoker	Kiel
CP 109081	50	50	Male	>10	Non-smoker	Dresden
CP 107713	50	51	Male	8	Non-smoker	Bonn
CP 109078	54	54	Male	>10	Non-smoker	Dresden
CP 107719	58	55	Male	21	smoker	Kiel
CP 1089082	36	Unknown	Male	7	Non-smoker	Amsterdam, NL
CP 108590	30	Unknown	Male	28	Non-smoker	Kiel
CP 108593	50	Unknown	Male	10	Non-smoker	Bonn
CP 108594	53	Unknown	Male	>10	Non-smoker	Dresden

[a]> 30% bone loss, measured from the enamel junction to the root tip.

2.4 | Selection criteria of miRNAs for follow-up experiments

miRNAs that showed significantly higher expression in the blood of PD patients compared to healthy controls were selected for the identification of their target genes if they corresponded to the following criteria: (I) $p < 5 \times 10^{-06}$, (II) a twofold change threshold, that is, only miRNAs that are in venous blood of PD cases compared to healthy controls up- or downregulated at least by a factor of 2 were further considered, and (III) differential upregulation (>twofold) in at least two independent array-based miRNA expression profiling studies of human gingival tissues and cells that were exposed to the PD risk factors inflammation or tobacco smoke metabolites. For the identification of microarray studies that analyzed differential miRNA expression in the gingiva, we used the PubMed literature search terms miRNA, microarray, periodontitis, periodontal cells, oral inflammation, smoke, gingiva, and oral mucosa.

2.5 | Cell culture

Primary cell cultures were generated and cultivated as previously described by our group.[23] In brief, primary gingival fibroblast cells (pGFs) were cultured in cell growth medium (DMEM, 10% FCS, 1% Amphotericin B, 1% Pen/Strep, 1% non-essential amino acids). One day prior to transfection, the pGFs (passage 3–4) were seeded in 6-well tissue culture plates (TPP Techno Plastic Products) (1.8×10^5 cells per well). HeLa cells were cultivated in cell growth medium (Earle's MEM, 10% FCS, 2 mM L-glutamine, 1% non-essential amino acids, 1% Pen./Strep.). One day before transfection, HeLa cells were seeded in 6-well tissue culture plates (TPP Techno Plastic Products) (7×10^4 cells per well).

2.6 | miRNA transfection

Each of the 3 independent pGFs cultures was transfected in three technical replicates (N = 9) with three mirVana mimics (ThermoFisher Scientific): mirVana miRNA mimic miR374b-5p was used to mimic miRNA hsa-miR-374b-5p. mirVana miRNA mimic Negative Control #1 was used for normalizing between samples. mirVana miRNA mimic miR-1, that specifically downregulates the expression of the gene Protein Tyrosine Kinase 9 (*PTK9*), was used as a positive control. The miRNAs were transfected using Lipofectamine RNAiMAX reagent according to the manufacturer's instructions. 24 hours after transfection, the cells were washed twice with PBS. Cell disruption and total RNA extraction were carried out using the RNeasy Mini Kit (Qiagen).

2.7 | Genome-wide gene expression analysis

Total RNA was extracted from pGFs using the RNeasy Mini Kit (Qiagen GmbH, Hilden). 3 biological independent cell cultures from 3 different donors were transfected in triplicates with mirVana mimic miR374b-5p, miRNA mimic negative control #1 and mirVana mimic miR-1. RNA isolated from mimic miR374b-5p and negative control #1 transfected cells was hybridized in biological triplicates with the Human Clariom D Expression Array (Affymetrix) as previously

described.[24] The array design covered the transcribed human genome with >540 000 transcripts of all known coding and noncoding splice variants. Expression data were analyzed using Partek Genomics Suite software (Partek). Each RNA transcript was represented by multiple independent 25mer oligonucleotides (probes) that were combined as a probe set to generate a reliable signal with high sensitivity and specificity. The signals were normalized logarithmic (basis 2), and mean signals for all perfect match (PM) probes on the array were calculated. Signal differences between the miRNA and control transfected cells were analyzed using parametric t tests.

RNA was isolated from mirVana mimic miR-1 (positive control) transfected cells to validate downregulation of *PTK9* by qRT-PCR (see Figure S1).

2.8 | Prediction of microRNA targets

To predict biological targets of miRNAs, we searched the 3'UTR of mRNA sequences for the presence of conserved 8mer and 7mer sites that matched the seed region of miRNA hsa-miR-374b-5p, using the online tool TargetScanHuman (version 7.1).[25]

2.9 | Cloning of the *UHMK1* 3'UTR into the reporter plasmid pGL4.24

Genomic DNA (gDNA) was extracted from pGFs using the AllPrep DNA/RNA/miRNA Universal Kit (Qiagen). The purified gDNA was used as a PCR-template. 2055 bp of the *UHMK1* 3'UTR, that included the predicted binding sites of miRNA hsa-miR-374b-5p, was cloned into the plasmid pGL4.24 (Promega). The PCR product was amplified using Phusion High-Fidelity PCR Polymerase (NEB) with forward and reverse primers containing the XbaI restriction site. The primer sequences were as follows: fwd-primer 5'-AATTGGCCTCTAGATGC CTGGAAATAGCCTTGGT-3' and rev-primer 5'-AATTGGCCTCTAG ATCTGCTGGAAAAGATAGGG C-3' (XbaI site underlined). The PCR product was purified using QIAquick gel extraction kit (QIAGEN) and ligated to the XbaI digested plasmid pGL4.24 (Promega). The modified plasmid was purified using the QIAprep Spin Miniprep Kit.

2.10 | Transfection of miRNA mimics and reporter plasmids into HeLa cells

HeLa cells were transfected in three biological replicates with the reporter gene plasmids pGL4.24 including the *UHMK1* 3' UTR and pRL-SV40 as Renilla Luciferase control reporter vector (Promega) in a ratio 1:10 using jetPEI transfection reagent (Polyplus transfection). 24 hours after transfection of the reporter gene plasmids pGL4.24 and pRL-SV40, the cells were transfected with the mirVana miRNA mimic miR374b-5p and the mirVana miRNA mimic Negative Control #1. The transfection was performed using Lipofectamine RNAiMAX Reagent. After additional 24 hours, the HeLa cells were washed twice with PBS. Subsequent cell disruption with Passive Lysis Buffer and luciferase measurement was carried out using Dual-Luciferase Reporter Assay system (Promega). The activities of the reporter gene assays were quantified as relative light units that were normalized as the ratio of firefly luciferase activity to *renilla* luciferase activity. Relative luciferase activity was calculated as the ratio of the average (Firefly/*Renilla*) from the sample (mirVana miRNA mimic miR374b-5p) to the average (Firefly/*Renilla*) from the control (mirVana miRNA mimic Negative Control #1). Data were analyzed using a t test.

HeLa cells were also transfected in three biological replicates with the reporter gene plasmid pGL4.24 using jetPEI transfection reagent (Polyplus transfection) followed by transfection with mirVana miRNA mimics after 24 hours. The transfection was performed using Lipofectamine RNAiMAX Reagent. After additional 16 hours, the HeLa cells were washed twice with PBS and total RNA extraction was carried out using the RNeasy Mini Kit (Qiagen). Subsequently, transcript levels were quantified by qRT-PCR. Transcript levels of the Luciferase gene (LUC) were normalized to GAPDH expression. The relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method. Data were analyzed using t test.

2.11 | Quantitative RT-PCR

The effects of the mirVana miRNA mimics on pGFs gene expression and HELA cells were quantified by qRT-PCR using the CFX Connect System (Bio-Rad) in combination with SYBR Select Master Mix (Thermo Fisher Scientific) as recently described by us.[23,26] In brief, 500 ng of the total RNA of the miRNA mimic-transfected pGFs was transcribed into cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). qRT-PCR was performed using SYBR Select Master Mix (Applied Biosystems). The expression of the gene *GAPDH* (fwd-primer 5'-GCATCTTCTTTTGCGTCG; rev-primer 5'-TGTAAACCATGTAGTTGAGGT) and *PTK9* (fwd-primer 5'-AGCTCAACTATGTGCAGTTGGAAA-3', rev-primer 5'-ACGAGCTGAATCCTTGGGAA) was quantified for up- and down-regulated genes using the $2^{-\Delta\Delta CT}$ method as described previously.[27]

From the transfected HELA cells, 500 ng of the total RNA was transcribed into cDNA as described above. Total RNA was treated with DNase I (Roche) followed by RNA precipitation until no traceable band could be detected by PCR using primers that annealed to the pGL4.24 plasmid backbone (fwd-primer: 5'-TTCAACCCAGTCAGCTCCTT-3', rev-primer 5'-CAAGAACTCTGTAGCACCGC-3'). Differential expression of the Luciferase gene (*LUC*), that was encoded by the plasmid pGL4.24, was quantified by qRT-PCR with primers that annealed to *LUC* (fwd-primer 5'-ACGTGCAAAAGAAGCTACCG-3', rev-primer 5'-GGCAAATGGGAAGTCACGAA-3').

2.12 | Luciferase-based reporter assay

Hela cells were transfected with the Dual-Luciferase reporter gene plasmids pGL4.24 and pRL-SV40 (Promega) in a 1:10 ratio. 24 hours

later, the miRNA mimics were transfected, and another 24 hours later, luciferase protein activity was measured with an Orion Microplate Luminometer (Berthold) according to the manufacturer's instructions.

3 | RESULTS

3.1 | Identification of miRNAs that are upregulated in blood and gingiva of PD patients

Out of 863 miRNAs that were quantified with the Geniom Biochip array,[19] 29 miRNAs were significantly higher expressed ($p < 5 \times 10^{-6}$) in blood from PD cases compared to healthy controls, 23 of which showed >twofold upregulation (Table 2, Table S1). hsa-miR-374b-5p was >twofold upregulated in blood of PD cases ($p = 2.1 \times 10^{-6}$) and was additionally reported by miRNA array studies to be >twofold upregulated in biopsies of the oral masticatory mucosa from PD cases compared to healthy controls[13] and in nicotine-treated human periodontal ligament-derived stem cells (PDLSC).[28] No other of the 29 blood-borne miRNAs was reported by ≥2 miRNA array studies with a FC >2. Following our pre-assigned selection criteria, we chose hsa-miR-374b-5p to identify target genes of its regulatory function (Figure 1).

3.2 | Genome-wide expression profiling of the effects of hsa-miR-374b-5p on primary human gingival fibroblasts

We transfected 3 cultures of pGFs from unrelated healthy donors with hsa-miR-374b-5p mimic and quantified the expression with the Clariom D Expression Array. A total of 21 448 individual transcripts were detected in pGFs, and the expression was compared to pGFs that were transfected with miRNA mimic negative control #1.

We hypothesized that effects of a single miRNA on a gene are generally small and because of that biological variation between the transfected cell cultures and technical variation between individual miRNA mimic transfections could result in false-positive findings. This is why we performed a pooled analysis of the expression array data. On the transcript level, miRNAs generally suppress gene expression through mRNA degradation. This is why we focused on downregulated genes. Genes that were downregulated in the pooled analysis are shown in Table 3 and Table S2. Genes that showed upregulation after transfection of miRNA hsa-miR-374b-5p mimic are listed in Table S3. The gene *UHMK1* (U2AF homology motif [UHM] kinase 1) showed the smallest p-value in the pooled analysis with $p = 2.5 \times 10^{-4}$. It showed the third largest fold change downregulation, with FC = −1.77.

TABLE 2 List of upregulated genes with $p < 5 \times 10^{-6}$ in whole blood from PD cases compared to healthy controls.

miRNA (miRBAv20)	*p*-value	Fold change
hsa-miR-744-5p	1.30E−12	2.76
hsa-miR-1228-3p	2.53E−11	5.72
hsa-miR-758-3p	1.56E−10	28.70
hsa-let-7d-5p	2.30E−10	4.02
hsa-miR-567	2.54E−09	20.22
hsa-miR-1281	2.54E−09	7.02
hsa-miR-361-5p	4.12E−09	2.75
hsa-miR-1182	2.45E−08	24.77
hsa-miR-1181	2.45E−08	1.87
hsa-miR-765	3.73E−08	17.85
hsa-miR-548h-5p	1.28E−07	11.03
hsa-miR-146b-5p	1.28E−07	1.86
hsa-miR-199b-5p	1.69E−07	19.90
hsa-miR-384	1.69E−07	3.13
hsa-miR-9-3p	2.26E−07	3.97
hsa-miR-421	2.52E−07	3.92
hsa-miR-653-5p	3.52E−07	34.38
hsa-miR-637	3.92E−07	15.49
hsa-miR-187-5p	3.92E−07	2.46
hsa-miR-125a-3p	4.40E−07	16.25
hsa-let-7b-3p	6.62E−07	20.38
hsa-miR-522-3p	9.56E−07	7.21
hsa-miR-490-3p	1.33E−06	0.66
hsa-miR-1256	1.33E−06	0.56
hsa-miR-551b-5p	1.67E−06	3.10
hsa-miR-634	1.67E−06	1.66
hsa-miR-374b-5p	**2.06E−06**	**2.16**
hsa-let-7f-2-3p	2.31E−06	11.01
hsa-miR-191-5p	3.87E−06	1.45

From the total of 863 miRNAs, hsa-miR-374b (marked in bold) solely showed increased expression with a FC >2 in blood of PD patients, in addition to ≥ 1 miRNA expression study of gingival cells and tissues.

3.3 | Validation of the effect of hsa-miR-374b-5p on *UHMK1* expression

Extensive multiple independent testing in a genome-wide expression analysis increases the chances of false-positive findings. Thus, statistical associations require validation on the molecular biological level. Two conserved 8mer sites in the 3'UTR of *UHMK1*, located 961 and 2859 nucleotides upstream of the end of the 3'UTR, matched the seed region of hsa-miR-374b-5p. We cloned a 2055 nucleotide sequence of the 3'UTR that included the two 8mer sites to the poly-adenylation site of the luciferase gene in the reporter vector pGL4.24. miRNAs usually interact with the 3'UTRs of the target mRNAs to suppress gene expression through mRNA degradation. To

give evidence that hsa-miR-374b-5p suppressed the target mRNA on the transcript level, as was suggested by the expression array data, we quantified the expression of the reporter gene after miRNA transfection using qRT-PCR. 16 hours after miRNA transfection, luciferase mRNA expression showed −1.5 FC downregulation in HeLa cells, with $p = .02$ (Figure 2A).

To demonstrate that this effect on the protein level, we quantified luciferase activity of the reporter gene 24 hours after miRNA transfection in HeLa cells. In this experiment, luciferase activity showed −1.3-fold change, with $p = .013$ (Figure 2B).

4 | DISCUSSION

In this study, we identified miRNA hsa-miR-374b-5p as a transcriptional repressor of *UHMK1*. We showed that hsa-miR-374b-5p expression results in decreased *UHMK1* transcript levels in gingival fibroblasts and gave mechanistic evidence that overexpression of hsa-miR-374b-5p results in decreased transcript levels and protein activity of a reporter gene that expressed the *UHMK1* 3'UTR. These experiments indicated that hsa-miR-374b-5p interacts with the 3'UTR of *UHMK1* to suppress gene expression through mRNA degradation. By using gingival fibroblasts and HeLa cells, we showed that the molecular mechanism of this effect is independent of a specific cell type.

UHMK1 encodes a serine/threonine protein kinase that promotes cell cycle progression through G1 by phosphorylation of the cyclin-dependent kinase inhibitor 1B (provided by RefSeq,[29]). It was shown that *UHMK1* knockdown decreased osteoblast and increased osteoclast differentiation, but *UHMK1* expression increased osteoblast and decreased osteoclast differentiation.[30] Additionally, *UHMK1* is genome-wide associated with bone mineral density.[30] Taken together, this suggests a role of *UHMK1* in the regulation of bone homeostasis. Additionally, *UHMK1* does not only promote osteoblast differentiation[30] but also cell cycle progression.[29] Successful resolution of inflammation requires transition from the inflammatory to the proliferative phase, a key step in the healing process of oral inflammation.

It is possible that individual cases of our sample developed periodontitis for different reasons. This is because in a few cases periodontitis was diagnosed at young adults (<30 years of age) whereas other cases developed periodontitis >50 years of age. This implies a general role of miRNA-374b-5p in the etiology of periodontitis.

A limitation of our study was that the small effect sizes on the individual gene level and the resulting *p*-values did not allow statistical correction for the number of individual comparisons in the expression profiling, for example, by applying the false discovery rate or Bonferroni correction thresholds. This impeded discrimination of a true positive finding from type I errors in this experiment. In the analysis that combined all replicates, *UHMK1* showed the most significant downregulation of all mRNAs expressed in pGFs. Here, the effects of the single experiments were collectively adding, indicating robustness of our finding. We did not perform a biological pathway analysis, because we were interested in the identification of a specific target gene of miR-374b-5p regulation. Furthermore, we questioned that the small *in vitro* effect of the overexpressed miRNA would result in significant changes of an entire biological pathway, impeding the extraction of biological meaningful hypotheses. However, in an natural *in vivo* situation, that is subject to multiple physiological factors that often act over time, the effects of miRNA regulation may also be observed on the level of biological pathways.

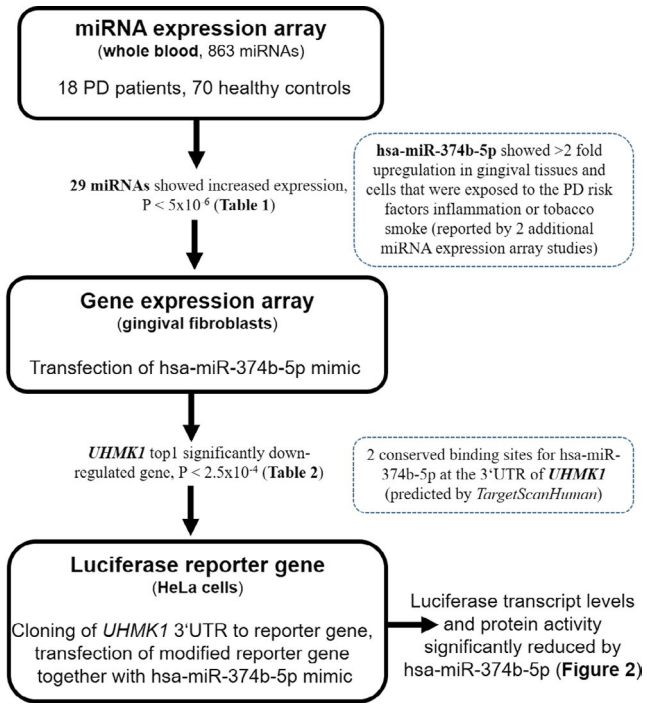

FIGURE 1 Workflow of the study. 29 miRNAs showed significant upregulation in whole blood of 18 PD patients compared to 70 healthy controls (Geniom Biochip). Of these, only hsa-miR-374-5p showed increased expression in more than one study that used various miRNA arrays to quantify miRNA expression in gingival tissues and cells that were exposed to inflammation or nicotine as risk factors of PD. To identify the target gene of hsa-miR-374-5p regulation, a hsa-miR-374-5p mimic was transfected into primary gingival fibroblasts (3 biological replicates) and changes in expression of protein coding genes were monitored (Clariom D expression array). 24 hours after transfection, *UHMK1* was identified as the top 1 most significantly downregulated gene. 2 conserved hsa-miR-374-5p binding sites were predicted at the 3'UTR of *UHMK1* (TargetScanHuman). To proof the molecular mechanism of miR-374-5p regulation of *UHMK1*, we cloned the *UHMK1*-3'UTR sequence that included the predicted miR-374-5p binding sites to the reporter gene Luciferase. After co-transfection of a hsa-miR-374-5p mimic and the modified reporter gene into HeLa cells, Luciferase transcript levels and protein activity were significantly reduced. These results give evidence that hsa-miR-374-5p binds to the 3'UTR of *UHMK1* to suppress *UHMK1* gene expression at the post-transcriptional level indicating that the miRNA-mediated regulation is exerted through mRNA degradation but not through translational repression [Colour figure can be viewed at wileyonlinelibrary.com]

TABLE 3 Genes with <−1.5-fold change downregulation at $p < .005$ after miR-374b-5p mimic transfection in pGF cells for 24 hours

Chr.	Gene	*p*-value	Fold change	GWAS catalog entries
1	*UHMK1*	.00025	−1.767	Bone mineral density, Takayasu arteritis, emotional problems
16	*HSBP1*	.00031	−1.530	chronic obstructive pulmonary disease, scoliosis
10	*CACUL1*	.00040	−1.505	Height, obesity
14	*STYX*	.00122	−2.017	—
19	*ZNF30*	.00196	−1.563	—
12	*TAS2R19*	.00245	−1.609	Bitter taste perception
11	*MS4A7*	.00265	−1.798	myeloid leukemia, taurine metabolite levels
1	*TMED5*	.00319	−1.894	HDL levels, educational attainment, myeloid leukemia
6	*SLC25A27*	.00397	−1.629	FEV/FEC ratio, QRS complex, thyroiditis, hyperthyroidism
17	*METTL16*	.00424	−1.567	HDL levels, mean corpuscular hemoglobin, insomnia
1	*CSRP1*	.00424	−1.624	—
15	*RAB8B*	.00462	−1.670	mean corpuscular hemoglobin, schizophrenia, social communication impairment

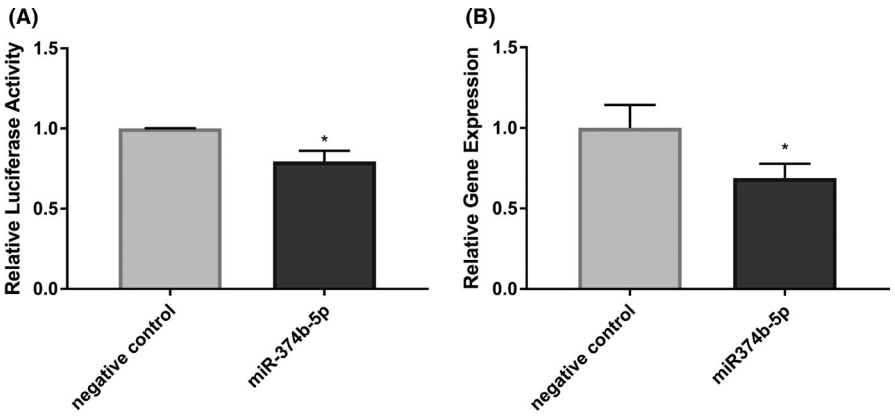

FIGURE 2 miR 374b-5p downregulates Luciferase-*UHMK1*-3'UTR reporter gene expression. Significantly reduced activity of the Luciferase-*UHMK1*-3'UTR reporter gene was observed (A) on the protein level ($p = .013$; FC = −1.3) and (B) on the transcript level ($p = .02$; FC = −1.5) following 24 h after miR 374b-5p transfection

We observed downregulation of *UHMK1* in the mRNA gene expression profiling experiment. However, this observation could be a false-positive finding and did not provide direct biological evidence for an inhibitory effect of miR-374b-5p. Thus, it was necessary to prove on the molecular level that the downregulated transcript sequence represented a real physiological target of miR-374b-5p. To this end, we cloned the 3'UTR sequence stretches of *UHMK1* that included the bioinformatically predicted conserved binding sites for miR-374-5p to a reporter gene. Because mRNA degradation by miRNA binding at the 3'UTR is a general mechanism and as such should be independent of the cell type and the genomic context, we used HeLa cells, which is an accepted cell model that allows efficient transfection. This experiment proved that miR-374-5p downregulates the transcript levels of any gene that includes the two conserved 3'UTR binding sites of miR-374-5p in the 3'UTR of *UHMK1*, indicating generality of this function. By measuring both Luciferase transcript levels and protein activity, we showed that hsa-miR-374-5p binds to the 3'UTR of *UHMK1* to suppress *UHMK1* gene expression at the post-transcriptional level. The observed effect size was consistent with other studies that quantified luciferase activity to validate the regulatory effect of miRNAs.[31,32] This indicated that the miRNA-mediated suppression is exerted at the mRNA level, probably by mRNA degradation, and not on the protein level through translational repression. However, it is currently unclear how the diverse mechanisms of miRNA-mediated regulation are interrelated.[6]

We note, that the 3'UTR of *UHMK1* is comprised of 7.200 kb, which exceeds the average length of human 3'UTR of 800 nucleotides ~10-fold.[33] 3'UTRs were likely shaped under selection to acquire or eliminate miRNA target sites.[34] Accordingly, the average 3'UTR length differs considerably in correlation with tissue specificity of gene expression. For instance, genes that are expressed ubiquitously and involved in basic cellular processes such as ribosomal protein genes have sixfold shorter 3'UTRs than tissue-specific

genes. The long 3'UTR sequence of *UHMK1* indicates stringent regulation, which might reflect potential damage from misregulation. 95% of genes with conserved target sites have just one site for a single miRNA, but most identified genetic targets contain multiple sites for the specific miRNA.[35,36] In the current study, we identified two conserved 8mer binding sites for hsa-miR-374b-5p in the 3'UTR of *UHMK1*. This supports the specific regulatory role of hsa-miR-374b-5p for *UHMK1* expression adding to the results of our *in vitro* experiments.

In conclusion, the current study identified miRNA hsa-miR-374b-5p to be upregulated in blood and gingiva of periodontitis patients and identified *UHMK1* as target gene. This gene has a role in the regulation of osteoblasts and osteoclasts homoeostasis. Our study demonstrated that the integration of microarray data and biological experiments is an efficient approach to identify the biological targets of pathophysiological relevant miRNAs and supports the idea that blood contains disease-specific miRNAs.

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DATA AVAILABILITY STATEMENT

The Geniom Biochip array "miRNA Homo sapiens" of this study are openly available in Gene Expression Omnibus (GEO; accession ID GSE31568). The Human Clariom D Expression Array data of this study are available on request from the corresponding author.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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