ARTICLE
Journal of Cellular Biochemistry 116:640–647 (2015)

Journal of Cellular Biochemistry

lncRNA Expression Signatures in Periodontitis Revealed by Microarray: The Potential Role of lncRNAs in Periodontitis Pathogenesis

Yaguang Zou,[1] Chun Li,[2] Fangpeng Shu,[3] Zhihui Tian,[1] Wenan Xu,[1] Huiyong Xu,[1] Hua Tian,[1] Rong Shi,[4*] and Xiangming Mao[3*]

[1]*Department of Stomatology, NanFang Hospital, Southern Medical University, Guangzhou 510515, China*
[2]*Nursing Department, NanFang Hospital, Southern Medical University, Guangzhou 510515, China*
[3]*Department of Urology, NanFang Hospital, Southern Medical University, Guangzhou 510515, China*
[4]*Institute of Genetic Engineering, Southern Medical University, Guangzhou 510515, China*

ABSTRACT

Periodontitis, a common chronic inflammatory disease of the periodontium, is caused by dental plaque formation induced by microorganisms. Recent studies have demonstrated that lncRNAs play a critical role in the regulation of gene expression and in the pathogenesis of diseases. To demonstrate that periodontitis is associated with lncRNAs, microarray analysis was used to detect differently expressed lncRNAs in chronic periodontitis and adjacent normal tissues. The results of some differently expressed lncRNAs were further confirmed using real-time PCR. A total of 8925 differentially expressed lncRNAs were detected, including 4313 upregulated lncRNAs and 4612 downregulated lncRNAs. Further lncRNA subgroup analysis showed there were 589 enhancer-like lncRNAs, 238 homeobox (HOX) cluster lncRNAs, and 1218 Rinn′s lincRNAs, of which 656 lincRNAs were upregulated and 562 lincRNAs were downregulated. Therefore, we confirmed that lncRNAs were differently expressed in chronic periodontitis tissues compared with adjacent normal tissues, indicating that lncRNAs may exert partial or key roles in periodontitis pathogenesis and development. Taken together, this study may provide potential targets for future treatment of periodontitis and novel diagnostic biomarkers for periodontitis. J. Cell. Biochem. 116: 640–647, 2015.

KEY WORDS: CHRONIC PERIODONTITIS; lncRNA; MICROARRAY; ENHANCER-LIKE lncRNA

Chronic periodontitis, the most common form of periodontitis, is a complex infectious disease of the periodontium caused by a group of bacterial pathogens in dental plaque. It has been demonstrated that periodontal tissue inflammation can induce loss of connective tissue and bone support for the teeth, thereby leading to loosening of teeth and formation of lesions elsewhere. The prevalence rate of chronic periodontitis in Chinese adults is about 50%, and is the leading cause of adult tooth loss. Therefore, early diagnosis and investigation on the pathogenesis of periodontitis is important for prevention and treatment of periodontal disease [Arigbede et al., 2012].

In recent years, with the discovery and study of ncRNAs, the complex relationship between ncRNAs and diseases has raised concern. In the human genome, approximately 93% of the sequence can be transcribed, in which only 2% have protein-coding function, whereas 98% of the transcriptome are non-protein-coding functional non-coding RNA (noncoding RNA, ncRNA) [Consortium, 2004]. Based on the molecular size, ncRNAs can be classified into small ncRNA and long ncRNA (lncRNA). MicroRNAs (miRNAs) are the best studied short ncRNAs and have been confirmed to be involved in paradentitis development [Kapranov et al., 2007; Ulveling et al., 2011].

LncRNAs are defined as ncRNAs that are longer than 200 nucleotides, which have been shown to be alternatively spliced, polyadenylated, and developmentally regulated in eukaryotes, including antisense, intergenic transcripts, and epigenetic regulators [Okazaki et al., 2002; Guttman et al., 2009]. Many studies demonstrate that deregulated expression of lncRNAs is closely correlated with the diversity of multigenetic diseases. The comprehensive function of lncRNAs in biological processes occurs through

Y. Zou, C. Li and F. Shu contributed equally to this work.

*Correspondence to: Rong Shi and Xiangming Mao, No. 1838, North Guangzhou Avenue, Guangzhou 510515, Guangdong Province, China. E-mail: shirongphd@126.com; 13802503635@126.com

Manuscript Received: 20 November 2013; Manuscript Accepted: 6 November 2014
Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 16 November 2014
DOI 10.1002/jcb.25015 • © 2014 Wiley Periodicals, Inc.

various mechanisms, such as gene expression regulation, dosage compensation, genomic imprinting, nuclear organization and compartmentalization, and nuclear-cytoplasmic trafficking [Clark and Mattick, 2011]. Recent studies have illustrated that abnormal expression of lncRNAs is closely associated with the development of disease [Gibb et al., 2011]. Through whole genome sequence analysis, lncRNA ultra-conservative elements were found to be related to proto-oncogenes in normal cells, which can inhibit apoptosis. Abnormal expression of these ultra-conservative elements can lead to cell malignant transformation, thereby inducing tumorigenesis. Meanwhile, neurodegenerative disease researchers found that various anomalies in lncRNA expression are related to subcellular localization changes [Mikkelsen et al., 2007; Xu et al., 2011]. However, the expression of lncRNAs and their biological functions in paradentitis remain unknown.

In this study, lncRNA expression profiles were obtained using microarray assay in two pairs of chronic periodontitis tissues samples compared with adjacent normal samples, several of which were evaluated by qPCR. The results demonstrate that lncRNA expression profiles may serve as new molecular biomarkers or a new basis for the diagnosis of periodontitis.

MATERIALS AND METHODS

TISSUE SAMPLES

For microarray analysis, two cases of chronic periodontitis tissues samples (271212, 1231) and two cases of normal tissue samples (1229, 14) were obtained from the Department of Stomatology of the NanFang Hospital, Southern Medical University. Meanwhile, 15 cases of normal tissue samples and 30 cases of chronic periodontitis tissue samples from 2013. 01 to 2013. 12 were selected to confirm the microarray analysis results. The diagnosis of chronic periodontitis was established on the basis of clinical and radiographic criteria. The patients with chronic periodontitis had more than one periodontal pocket with a depth of $\geq$5 mm, with at least one pocket having $\geq$5 mm loss of attachment and all gingival samples were obtained from the teeth with a probing depth of $\geq$5 mm, swelling of the marginal gingiva, and bleeding corresponding to gingival sulcus bleeding indexes above 3 according to Mühlemann and Son [1971]. Following surgery, excised tissue specimens were immediately placed in liquid nitrogen and subsequently frozen at -70°C. Written informed consent was obtained from all patients, and the study was approved by the Ethical Committee of the NanFang Hospital, Southern Medical University.

RNA EXTRACTION

TRIzol Reagent (Invitrogen Life Technologies) was used to extract RNA from the tissue samples. The total RNA from each sample was quantified using NanoDrop ND-1000, and RNA integrity was assessed by standard denaturing agarose gel electrophoresis.

RNA LABELING AND ARRAY HYBRIDIZATION

Sample labeling and array hybridization were performed according to the Agilent One-Color Microarray-Based Gene Expression Analysis protocol (Agilent Technology) with minor modifications. Briefly, mRNA was purified from total RNA after removal of rRNA (mRNA-ONLY^{TM} Eukaryotic mRNA Isolation Kit, Epicentre). Each sample was then amplified and transcribed into fluorescent cRNA along the entire length of the transcripts without 3′ bias through a random priming method. The labeled cRNAs were purified using the RNeasy Mini Kit (Qiagen). The concentration and specific activity of the labeled cRNAs (pmol Cy3/μg cRNA) were measured using NanoDrop ND-1000. A total of 1 μg of each labeled cRNA was fragmented by adding 11 μL of 10× blocking agent and 2.2 μL of 25× fragmentation buffer. The resulting mixture was heated at 60°C for 30 min, after which 55 μL of 2× GE hybridization buffer was added to dilute the labeled cRNA. The hybridization solution (100 μL) was dispensed into the gasket slide and assembled to the lncRNA expression microarray slide. The slides were incubated for 17 h at 65°C in an Agilent hybridization oven. The hybridized arrays were washed, fixed, and scanned using the Agilent DNA microarray scanner (part number G2505C).

The Arraystar human lncRNA array v2.0 (8 × 60K, Arraystar) was designed for profiling both lncRNAs and protein-coding RNAs in the human genome. A total of 33,045 lncRNAs and 30,215 coding transcripts were collected from authoritative data sources, including RefSeq, UCSC Knowngenes, Ensembl, and other related sources in the literature.

DATA ANALYSIS

Agilent Feature Extraction software (version 11.0.1.1) was used to analyze the obtained array images. Quantile normalization and subsequent data processing were performed using the GeneSpring GX v11.5.1 software package (Agilent Technologies). After quantile normalization of the raw data, lncRNAs and mRNAs that had at least two out of four samples with flags in Present or Marginal ("All Targets Value") were chosen for further data analysis. Differentially expressed lncRNAs and mRNAs with statistical significance between the two groups were identified through volcano plot filtering. Hierarchical clustering was performed using the Agilent GeneSpring GX software (version 11.5.1). GO analysis and Pathway analysis were performed in the standard enrichment computation method.

lncRNA CLASSIFICATION AND SUBGROUP ANALYSIS

Subgroup analysis was performed using homemade scripts. Results are provided in the lncRNA Classification and Subgroup Analysis Folder. Enhancer-like lncRNAs were identified using the GENCODE annotation of human genes. Rinn's lncRNAs were identified based on papers by John Rinn. Human homeobox transcription factors (HOX) cluster lncRNAs were also identified based on the study of Rinn. Long intergenic lncRNAs (lincRNAs), enhancer-like lncRNAs, and their nearby coding genes (distance $<$300 kb) were listed to analyze the association of lncRNA genes with nearby mRNA-coding genes.

CONFIRMATION OF DIFFERENTIALLY EXPRESSED lncRNAs BY REAL-TIME QUANTITATIVE RT-PCR

Real-time PCR was performed to validate some of the differently expressed lncRNAs confirmed by microarray analysis. Briefly, 2 μg of total RNA was converted to cDNA using SuperScript^{TM} III Reverse Transcriptase according to the manufacturer's protocol (Invitrogen).

qPCR was performed using an ABI PRISM7900 system (Applied Biosystems). The 10 μL PCR solution included 1 μL of cDNA product and 5 μL of 2× PCR Master Mix (Superarray). The reactions were incubated at 95°C for 10 min, followed by 40 cycles of 95°C for 10 s and 60°C for 60 s. The primers used in this study were shown in (Table S10). All reactions were run in triplicate. After reaction, the threshold cycle value (CT) data were determined using default threshold settings, and the mean CT was determined from the duplicate PCRs. Human GAPDH was used for normalization. The expression levels of lncRNAs were measured in terms of CT and then normalized to GAPDH using $2^{-\Delta\Delta Ct}$ [Schmittgen and Livak, 2008].

WESTERN BLOT

Chronic periodontitis tissue samples and normal tissue samples were homogenized in ice-cold CHAPS buffer (20 mMHepes, pH 7.4, 140 mM NaCl, 10 mM CHAPS, 2 mM EDTA, 1 mM EGTA, and Complete Protease Inhibitor Cocktail), and incubated on the thermoshaker for 30 min at 4°C. The lysates were then subjected to 15% SDS-PAGE, and proteins were transferred to a nitrocellulose membrane (Roche Biosciences, Germany). The membrane was blocked with 5% nonfat milk powder in Tris-buffered saline (TBS) (10 mM Tris-HCl, pH 7.2, 150 mM NaCl) for 4 h. After being washed three times with TBS with 0.1% Tween 20 (TBS-T), the membrane was then incubated with IFN-gamma (R&D Systems China, 1:800), IL16 (R&D Systems China, 1:800), IL6 (Cell Signaling Technology, 1:800), IL8 (R&D Systems China, 1:800) and GAPDH (Novus Biologicals, 1:1000) antibodies for 2 h. Antibodies binding were visualized by a colorimetric reaction catalyzed by peroxidase-conjugated goat anti-rabbit antibody (1:10,000 dilution in TBS; Promega).

STATISTICAL ANALYSIS

The lncRNAs expression differences between normal tissue samples and chronic periodontitis tissue samples were analyzed using Student′s *t* test within SPSS (Version 16.0 SPSS Inc.). A *P* value <0.01 was considered statistically significant.

RESULTS

lncRNA MICROARRAY DATA PROFILE

The lncRNAs were carefully collected from the most authoritative databases such as RefSeq, UCSC Knowngenes, Ensembl, and other related sources in the literature. Each transcript is represented by a specific exon or splice junction probe that can accurately identify individual transcripts. Total RNA was extracted from periodontitis tissues and normal tissue samples. Then the RNA was purifed and used for cRNA synthesis. Subsequently, array hybridization was performed with Arraystar Human LncRNA Microarray v2.0. Using the reliable data consisting of more than 33,045 lncRNAs and 30,215 mRNAs, differently expressed lncRNAs were identified from chronic periodontitis tissues samples and adjacent normal tissues. To identify differentially expressed lncRNAs with statistical significance, volcano plot filtering between the two groups (fold change ≥ 2.0, *P*-value ≤ 0.05) was employed. The results are shown in Table S1 and Table S2, which confirmed 8925 differently expressed lncRNAs (fold change ≥ 2.0, accounting for 40.84% of the total lncRNAs), among which 4313 lncRNAs (accounting for 19.73%) were upregulated, whereas 4612 lncRNAs (accounting for 21.10%) were downregulated. A collection of top 18 deregulated lncRNAs (9 upregulated and 9 downregulated lncRNAs) is listed in Table I.

lncRNA CLASSIFICATION AND SUBGROUP ANALYSIS

Recently, some classes or clusters of lncRNAs such as enhancer lncRNAs, Rinn lincRNAs, HOX lncRNAs have been identified to perform specific function in human cells, which are more likely involved in the occurrence and development of many diseases to exert different functions. Harrow and his colleagues defined a set of lncRNAs in human cell lines as lncRNAs with enhancer-like function [Harrow et al., 2006; Ørom et al., 2010]. Depletion of such lncRNAs led to decreased expression of neighboring protein-coding genes. Here, we found a list of lncRNAs with enhancer-like function in chronic periodontitis tissues compared with normal tissue samples (Table S3), in which 997 enhancer lncRNAs were found, and 589 were obviously differently expressed. Rinn et al. [Guttman et al., 2009; Khalil et al., 2009] found a set of lincRNAs, which may significantly influence cancer, immune signaling, and stem cell biology. Based on the study method by Rinn [2009], we profiled the data of all probes for lincRNAs in chronic periodontitis tissues compared with normal tissue samples. As shown in Table S4, 1218 differently expressed lincRNAs were detected, among which 656 were upregulated and 562 were downregulated.

Rinn et al. [2007] characterized the transcriptional landscape of the four human Hox loci and identified a total of 407 discrete transcribed regions in the four Hox loci. Using current genome annotations, the transcribed regions were partitioned into known Hox gene exons, introns, and intergenic transcripts. Most of these intergenic transcripts did not show any coding potential in all six transcriptional frames. These lncRNAs expressed in a temporal and site-specific fashion probably used the same enhancers as the HOX genes and may have global regulating functions as the HOX. Table S5 presents the profiling data of all probes in the four HOX loci, targeting 407 discrete transcribed regions, lncRNAs, and coding transcripts. As shown in Table S5, 134 coding transcripts were detected, with 82 differently expressed lncRNAs, whereas 264 transcribed ncRNAs (including introns and intergenic transcripts) were identified, 156 of which were significant differently expressed.

mRNA MICROARRAY DATA PROFILE

In order to gain insight into the function of possible targets of lncRNAs, GO and pathway annotation were applied to analyze the deregulated mRNAs. Up to 18,696 coding transcripts were detected in two pairs of samples (Table S6). Among these transcripts, an average of 4956 mRNAs (accounting for 26.51%) were upregulated in chronic periodontitis tissues compared with the matched normal tissues, whereas an average of 2565 mRNAs were downregulated (accounting for 13.72%) (Table S7). GO and Pathway analysis showed that differentially expressed mRNAs may be involved in cytokine-cytokine receptor interaction, peroxisome, lysosome, endocytosis, ECM-receptor interaction, metabolism, MAPK signaling pathway, etc. (Figs. 1 and 2). These results support the viewpoint that periodontitis is a chronic inflammatory disease [Arigbede et al., 2012].

TABLE I. A Collection of Deregulated lncRNAs Detected Using Microarray in Periodontitis Patients

Seqname	GeneSymbol	Type	Source	Chrom	Strand	Relationship	Regulation ([T] vs [C])	Absolute fold change ([T] vs [C])	*P*-value
AW293169	lincRNA-ZNF507	noncoding	lincRNA	chr19	+	intergenic	up	103.9061113	9.38056E-05
EC495588	lincRNA-RCN2	noncoding	lincRNA	chr15	–	intergenic	up	96.51054978	0.000647423
CB112975	lincRNA-UBL3–1	noncoding	lincRNA	chr13	+	intergenic	up	71.48499298	0.01690132
uc002zxb.3	ZDHHC8P	noncoding	UCSC_knowngene	chr22	–	intergenic	up	44.47452679	0.011743262
NR_001275	CELP	noncoding	RefSeq_NR	chr9	+	intergenic	up	43.3265723	8.42079E-05
ENST00000458343	KRT42P	noncoding	Ensembl	chr17	–	intergenic	up	41.64351667	5.63212E-05
ENST00000439490	AC007241.3	noncoding	Ensembl	chrY	–	intergenic	up	39.69735005	0.004463084
HIT000395572		noncoding	H-invDB	chr1	–	intergenic	up	38.76777549	0.006511677
ENST00000394467	RP13–147D17.1	noncoding	Ensembl	chrX	–	intergenic	up	34.65091274	0.000421092
BE244504	lincRNA-FAM110A	noncoding	lincRNA	chr20	–	intergenic	down	1185.608516	0.00982956
CR617239		noncoding	RNAdb	chr7	+	intronic antisense	down	134.7195907	0.002657778
CR617239		noncoding	RNAdb	chr7	+	natural antisense	down	134.7195907	0.002657778
NR_027466	LOC647288	noncoding	RefSeq_NR	chr13	–	intergenic	down	126.5766693	0.000535325
ENST00000400646	AC007275.3	noncoding	Ensembl	chrY	–	intergenic	down	122.6568716	0.001432316
NR_015407	LOC339535	noncoding	RefSeq_NR	chr1	–	intergenic	down	122.3560646	0.000342342
ENST00000424182	RP11–402P6.6	noncoding	Ensembl	chrX	+	intergenic	down	85.12342236	0.036373736
ENST00000509019	AC007790.1	noncoding	Ensembl	chr7	–	intronic antisense	down	84.69653242	0.000881699
uc010bcp.1	AK057625	noncoding	UCSC_knowngene	chr15	+	natural antisense	down	83.65949881	0.000210935

NEARBY CODING GENE FUNCTION ANALYSIS

Previous studies indicated that lncRNAs have intrinsic cis-regulatory capacity by tethering to its own locus, which may function as epigenetic factors [Ørom and Shiekhattar, 2011]. Previously, Harrow et al. [2006] found that depletion of enhancer-like lncRNAs can lead to decreased expression of their neighboring protein-coding genes. Therefore, elucidating the differently expressed lncRNA and their nearby protein-coding genes is an important approach to infer the functions of lncRNA [Caley et al., 2010]. In this study, 452 differentially expressed enhancer-like lncRNAs were identified, as well as their nearby coding genes (distance <300 kb) (Table S8). Furthermore, Table S9 shows 904 differentially expressed lincRNAs and nearby coding gene pairs (distance <300 kb). As shown in Table II, different lncRNAs can regulate a common gene, whereas a single lncRNA can be regulated by different genes.

REAL-TIME QUANTITATIVE PCR AND WESTERN BLOT VALIDATION

For confirmation of the microarray analysis results, two susceptibility genes (IL6, IL8) were detected and found upregulated, which was consistent with previous results [Davanian et al., 2012]. Five differently expressed mRNAs (PI3, IFNG, IL16, CDKN2B, and MYD88) and their related lncRNAs (RP3–461P17.10, lincRNA-IFNG-2, lincRNA-KIAA1199, CDKN2BAS, and lincRNA-CDON-1) were then tested. The results were consistent with the expression patterns obtained from microarray analysis (Fig. 3). To further confirm the results, more samples (15 cases of normal tissue samples and 30 cases of chronic periodontitis tissue samples, The demographic data and clinicopathological features of the patients were summarized in Table III) were collected and we confirmed that the expression of RP3–461P17.10, lincRNA-IFNG-2, lincRNA-KIAA1199, CDKN2BAS, and lincRNA-CDON-1 were consistent with the results of microarray analysis (Fig. 4). Then,

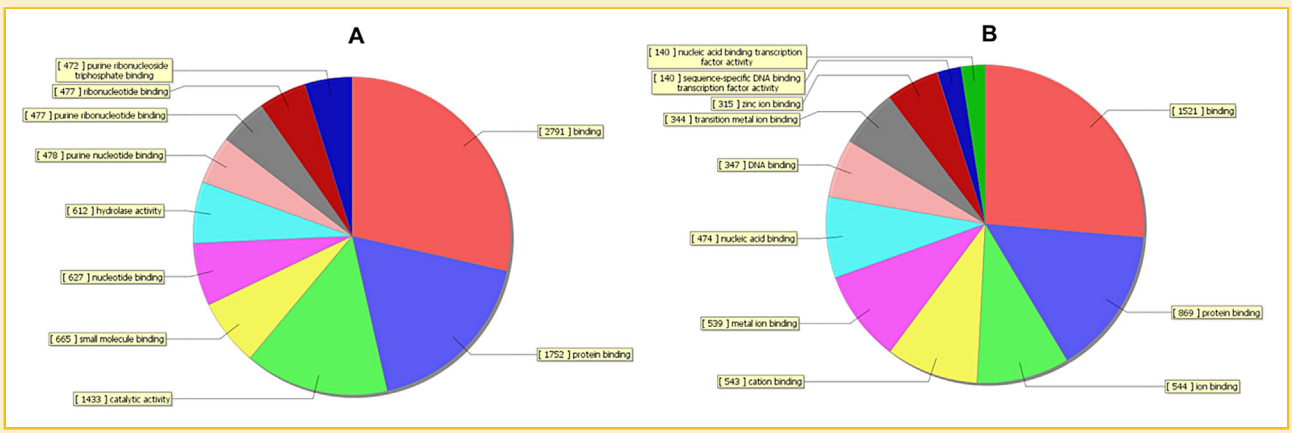

Fig. 1. GO Molecular Function Classification of mRNAs. (A) upregulated genes; (B) downregulated genes.

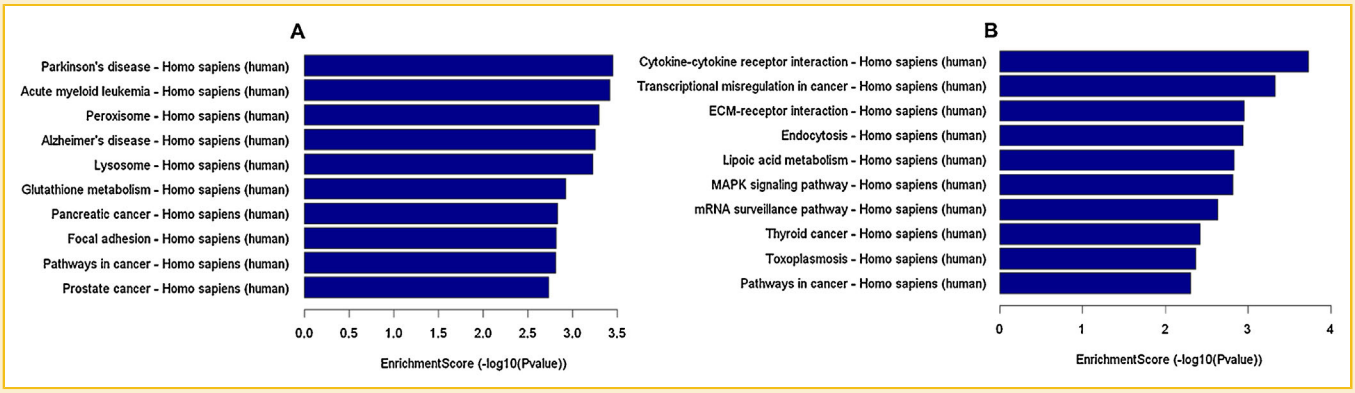

Fig. 2. Pathway analysis of differently expressed genes (mRNAs). (A) upregulated genes; (B) downregulated genes.

western blot was used to test some chronic periodontitis tissue samples and normal tissue samples, and the results showed similar expression patterns obtained from microarray analysis (Fig. 5).

DISCUSSION

Periodontitis is a bacteria-induced chronic inflammatory disease that involves multiple disruptions to the periodontium. The pathogenesis of periodontitis is mainly caused by toxins secreted by pathogenic microorganisms which can induce host immune disorders, release inflammatory mediators, and cause damage to local periodontal tissue [Darveau, 2010].

Differential expression of lncRNAs is becoming recognized as one of the important factors controlling gene expression and epigenetic regulation in the evolution of species, embryonic development, metabolism, and disease development [Mercer et al., 2009]. However, the functional role for the vast majority of these unique genes is still in question. Gupta et al. [2010] found that HOX-transcribed antisense RNA (HOTAIR) was significantly upregulated in metastatic breast tumors with 2000-fold increase. HULC is highly expressed in primary liver tumors and in colorectal carcinomas that metastasized to the liver. Further study demonstrated that miR-372 can repress the expression of PRKACB to inhibit the function of CREB, which can promote HULC transcription [Wang et al., 2010]. Considering the important role of lncRNA, the lncRNA expression profiles in the tissue of periodontitis patients were evaluated to reveal the potential role of lncRNAs in the pathogenesis of periodontitis. Microarray techniques revealed 8925 differentially expressed lncRNAs (fold change >2.0), of which 4313 were upregulated and 4612 were downregulated in periodontitis tissue when compared with normal adjacent tissue.

Rinn et al. [Guttman et al., 2009; Khalil et al., 2009; Ørom et al., 2010] found a set of lincRNAs and human (HOX) cluster lncRNAs. Moreover, Harrow and his colleagues defined numbers of lncRNAs in human cell lines as lncRNAs with enhancer-like function [Schmittgen and Livak, 2008], which can play different roles in gene regulation. For example, the lincRNA BDNFOS (NR_033314) is the natural antisense transcript of BDNF. Modarresi et al. [2012] demonstrated that inhibition of BDNFOS upregulates BDNF mRNA by two-fold to seven-fold. Microarray analysis shows

TABLE II. Some Differentially Expressed LncRNAs and Their Nearby Coding Genes (Distance <300 kb)

GeneSymbol	*P*-value-LncRNAs	Fold change-LncRNAs	Regulation-LncRNAs	Genome relationship	NearbyGene	Nearby GeneSymbol	*P*-value-mRNAs	Fold change-mRNAs	Regulation-mRNAs
RP11-296O14.3	0.006216528	26.593534	down	upstream	NM_004905	PRDX6	0.000719298	4.872687	up
AP000282.3	0.016932003	25.51497	down	downstream	NM_207584	IFNAR2	0.00269859	3.480801	down
AP000282.3	0.016932003	25.51497	down	downstream	NM_000874	IFNAR2	0.000388462	2.6185656	down
AP000282.3	0.016932003	25.51497	down	downstream	NM_207585	IFNAR2	0.030071905	2.0512574	down
RP3-461P17.10	0.0086662	3.903414	up	upstream	NM_002638	PI3	0.000206	61.082283	up
RP1-300I2.2	0.011022008	3.0897377	up	upstream	NM_002638	PI3	0.000206049	61.082283	up
lincRNA-IFNG-2	0.0478133	7.2941446	up	downstream	NM_000619	IFNG	0.0005166	20.446806	down
	0.007617182	3.9246998	down	downstream	NM_000619	IFNG	0.000516624	20.446806	down
lincRNA-DYRK2-2	0.028020509	3.2389712	up	upstream	NM_000619	IFNG	0.000516624	20.446806	down
AC005294.1	0.03190556	2.3591645	down	downstream	NM_000619	IFNG	0.000516624	20.446806	down
lincRNA-CDON-1	0.0004792	5.157475	down	upstream	NM_001039661	TIRAP	0.0011296	3.479414	up
lincRNA-KIAA1199	0.0081883	4.272878	down	upstream	NM_172217	IL16	0.0063795	2.0575085	down
lincRNA-IL1RL1	0.005369412	3.7445376	up	upstream	NM_003854	IL1RL2	0.010453484	7.6985235	up
lincRNA-MAP4K4	0.015719736	3.2388942	down	upstream	NM_003854	IL1RL2	0.010453484	7.6985235	up
lincRNA-IL1RL1	0.005369412	3.7445376	up	upstream	NM_004633	IL1R2	0.037348356	3.178611	up
lincRNA-MAP4K4	0.015719736	3.2388942	down	upstream	NM_004633	IL1R2	0.037348356	3.178611	up
lincRNA-IL1RL1	0.005369412	3.7445376	up	upstream	NM_000877	IL1R1	0.035737593	2.2034247	down
lincRNA-MAP4K4	0.015719736	3.2388942	down	upstream	NM_000877	IL1R1	0.035737593	2.2034247	down

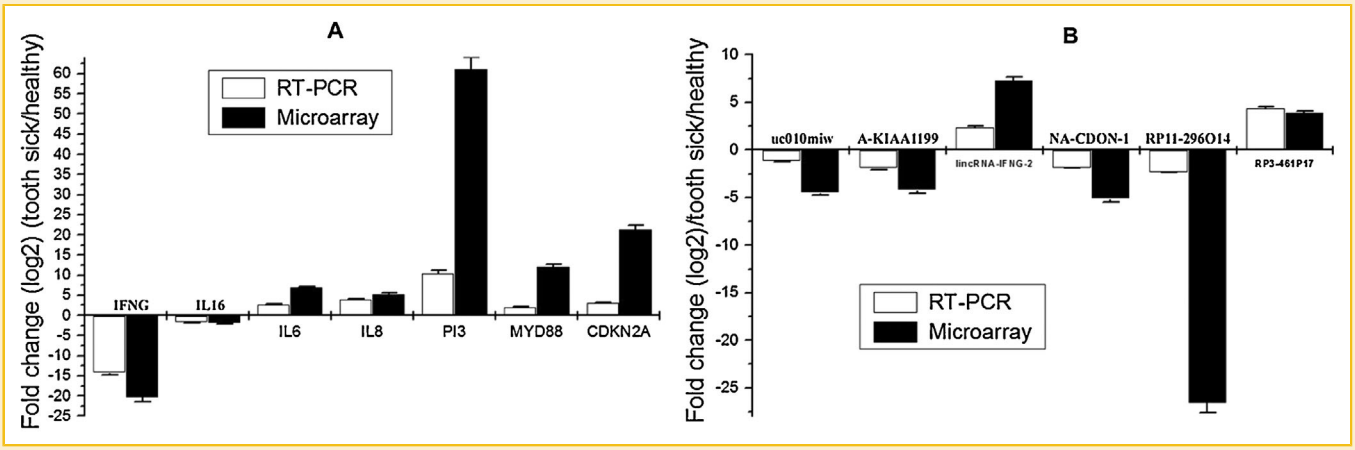

Fig. 3. qPCR results compared with the microarray analysis. (A) Differentially expressed genes (IL6, IL8, MYD88, PI3, CDKN2A, IL16, and IFNG) detected using microarray were validated by qPCR. (B) qPCR analysis results of CDKN2BAS (uc010miw.1), lincRNA-KIAA1199, lincRNA-IFNG-2, lincRNA-CDON-1, RP11–296O14.3, and RP3–461P17.10, which were consistent with microarray analysis. Experiments were carried out in triplicate and the results were expressed as mean values.

downregulation of BDNFOS in periodontitis tissue (fold change = 2.79, *P*-value = 0.03), whereas the two transcripts of BDNF (NM_001143811, NM_001143805) were upregulated (fold change = 7.84, *P*-value = 0.01; fold change = 3.85, *P*-value = 0.03). However, the other two BDNF transcripts were downregulated (NM_001143815: fold change = 4.05, *P*-value = 0.01; NM_001143807: fold change = 2.46, *P*-value = 0.03). Therefore, we speculate that lncRNA can regulate transcripts through different mechanisms and deserves our further study.

HOTAIR was the first discovered lncRNA which can function through trans-regulation of gene expression [Kaneko et al., 2010; Kim et al., 2013]. Clinical studies showed that HOTAIR expression levels are correlated with tumor metastasis, recurrence, and prognosis in breast, colon, and liver cancers and other tumor tissues. In this study, the transcript HOTAIR (ENST00000455246) was found to be upregulated in periodontitis (fold change = 3.06, *P*-value = 0.01), whereas another transcript NR_003716 was downregulated (fold change = 18.54, *P*-value = 0.002). Therefore, alternative splicing also exists in lncRNAs, and different lncRNA transcripts may play different regulatory functions.

TABLE III. Demographic Characteristics of the Study Population and Clinical Parameters (mean ± SD)

Characteristics	Non-chronic periodontitis (n = 15)	chronic periodontitis (n = 30)
Age (years)	38.2 ± 6.3	44.6 ± 5.5
M/F (n)	6/9	14/16
PI (%)	8.5 ± 6.8*	60.5 ± 19.4
BoP (%)	4.7 ± 3.6*	66.2 ± 11.7
PD (mm)	2.1 ± 0.6*	4.2 ± 0.9
CAL (mm)	1.2 ± 0.1*	4.6 ± 1.3

M, male; F, female; PI, plaque index; BoP, bleeding on probing; SUP, suppuration; PD, probing depth; CAL, clinical attachment level.

A recent study has shown that lncRNA are related with its neighboring protein-coding genes [Ponjavic et al., 2009]. Thus, correlation analysis between lncRNAs and its neighboring mRNA would elucidate the functions of lncRNA and contribute to the understanding of the relationship between lncRNA and periodontitis. For example, the lncRNAs RP3–461P17.10 and RP1–300I2.2 are located upstream of PI3 gene and are transcribed from the sense strand of PI3 gene, which encodes the protein Elafin, an endogenous serine protease inhibitor of trappin family [Verrier et al., 2012]. In this study, we confirmed that RP3–461P17.10 and RP1–300I2.2 were upregulated in periodontitis. PI3 was also upregulated obviously (Table II), indicating that the lncRNAs RP3–461P17.10 and RP1–300I2.2 may exert a synergistic effect on PI3 expression. RP11–296O14.3 is reproduced and located upstream of Peroxiredoxin 6 (PRDX6) antisense strand. Evidence suggests that PRDX6 is a novel thiol-dependent antioxidant that functions to scavenge particular hydroperoxides in the cell and mediate specific signals [Ye et al., 2005]. The microarray results showed that RP11–296O14.3 was downregulated in periodontitis, whereas PRDX6 was upregulated. We speculate that RP11–296O14.3 may exert its function by repressing PRDX6 expression.

In a study consisting of 15 patients with periodontitis and atherosclerosis, researchers demonstrated higher expression of IFNGR1 and IFNGR2, as well as lower expression of IFNG [Niedzielska and Cierpka, 2010]. In the present study, IFNG was downregulated in periodontitis. Four IFNG neighboring lncRNAs were detected, among which lincRNA-CDON-1 was downregulated. lincRNA-CDON-1 was transcribed in the antisense strand of TIRAP. TIRAP is an adapter protein, which associates with Toll-like receptors (TLRs) to mediate signal transduction [Couture et al., 2012]. TIRAP was also found to be upregulated in periodontitis. Therefore, lincRNA-CDON-1 may be involved in signaling pathways associated with TLRs in periodontitis pathogenesis.

To verify the results of the microarray analysis, real-time PCR and western blot were performed, which identified the periodontitis

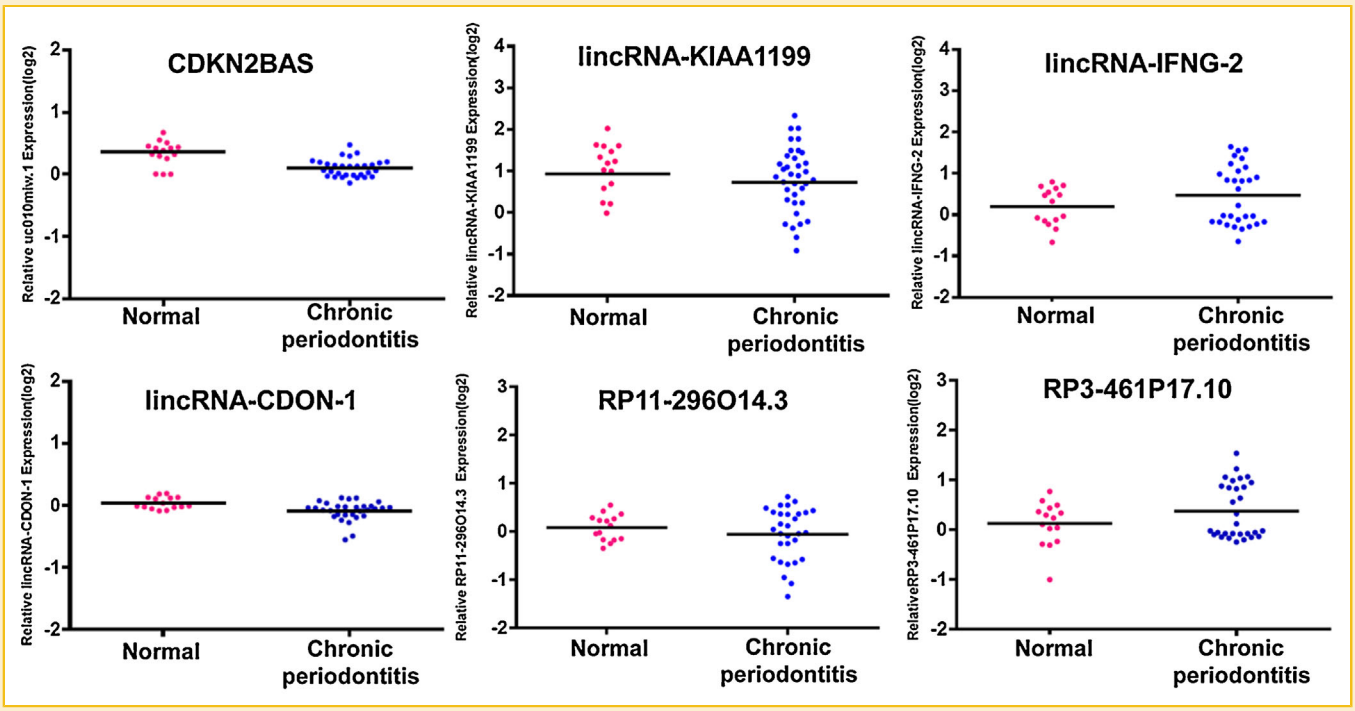

Fig. 4. Real-time qPCR analysis showed expression of CDKN2BAS (uc010miw.1), lincRNA-KIAA1199, lincRNA-IFNG-2, lincRNA-CDON-1, RP11–296O14.3 and RP3–461P17.10 in normal tissue samples and chronic periodontitis tissue samples, and they were significantly different ($P < 0.01$).

susceptibility genes IL6, IL8 were upregulated. These results are consistent with a previous study [Davanian et al., 2012]. Some differently expressed lncRNAs and its neighboring genes are consistent with the microarray results. We further collected periodontitis samples and confirmed that the expression of RP3–461P17.10, lincRNA-IFNG-2, lincRNA-KIAA1199, CDKN2BAS, and lincRNA-CDON-1 were consistent with microarray analysis results. Schaefer et al. [2011] detected the expression of CDKN2BAS, CDKN2A, CDKN2B, and CDK4 in healthy and inflamed gingival epithelium (GE) and connective tissue (CT), and a significantly higher expression of CDKN2BAS was found in healthy CT compared with GE ($P = 0.004$). Microarray and real-time PCR results confirm that the lncRNA CDKN2BAS was downregulated in periodontitis (fold change = 4.50, P-value = 4.91809E-05), whereas both CDKN2A and CDKN2B were upregulated.

In conclusion, our study reveals for the first time that lncRNAs are differentially expressed in chronic periodontitis tissuesamples compared with the adjacent normal tissues, indicating critical functions of lncRNA in the pathogenesis of periodontitis. Further research is required to determine whether these lncRNAs can serve as new therapeutic targets and diagnostic biomarkers in periodontitis.

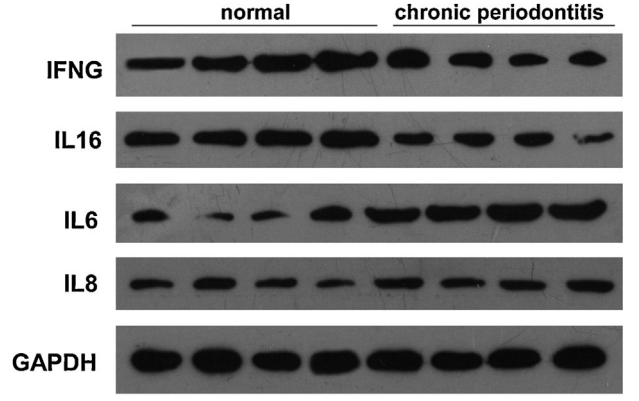

Fig. 5. Western blot analysis showed expression of IFNG, LI16, IL6, and IL8 in normal tissue samples and chronic periodontitis tissue samples.

ACKNOWLEDGEMENT

Microarray experiments were performed by KangChen Bio-tech, Shanghai, China.

REFERENCES

Arigbede AO,Babatope BO, Bamidele MK. 2012. Periodontitis and systemic diseases: A literature review. J Indian Soc Periodontol 16:487–491.

Caley DP, Pink RC, Trujillano D, Carter DR. 2010. Long noncoding RNAs, chromatin, and development. Sci World J 10:90–102.

Clark MB, Mattick JS. 2011. Long noncoding RNAs in cell biology. Semin Cell Dev Biol 22:366–376.

Consortium IHGS. 2004. Finishing the euchromatic sequence of the human genome. Nature 431:931–945.

Couture LA, Piao W, Ru LW, Vogel SN, Toshchakov VY. 2012. Targeting Toll-like receptor (TLR) signaling by Toll/interleukin-1 receptor (TIR) domain-containing adapter protein/MyD88 adapter-like (TIRAP/Mal)-derived decoy peptides. J Biol Chem 287:24641–24648.

Darveau RP. 2010. Periodontitis: A polymicrobial disruption of host homeostasis. Nat Rev Microbiol 8:481–490.

Davanian H, Stranneheim H, Båge T, Lagervall M, Jansson L, Lundeberg J, Yucel-Lindberg T. 2012. Gene expression profiles in paired gingival biopsies from periodontitis-affected and healthy tissues revealed by massively parallel sequencing. PLoS One 7:e46440.

Gibb EA, Brown CJ, Lam WL. 2011. The functional role of long non-coding RNA in human carcinomas. Mol Cancer 10:38.

Gupta RA, Shah N, Wang KC, Kim J, Horlings HM, Wong DJ, Tsai MC, Hung T, Argani P, Rinn JL, Wang Y, Brzoska P, Kong B, Li R, West RB, van de Vijver MJ, Sukumar S, Chang HY. 2010. Long non-coding RNA HOTAIR reprograms chromatin state to promote cancer metastasis. Nature 464:1071–1076.

Guttman M, Amit I, Garber M, French C, Lin MF, Feldser D, Huarte M, Zuk O, Carey BW, Cassady JP, Cabili MN, Jaenisch R, Mikkelsen TS, Jacks T, Hacohen N, Bernstein BE, Kellis M, Regev A, Rinn JL, Lander ES. 2009. Chromatin signature reveals over a thousand highly conserved large non-coding RNAs in mammals. Nature 458:223–227.

Harrow J, Denoeud F, Frankish A, Reymond A, Chen CK, Chrast J, Lagarde J, Gilbert JG, Storey R, Swarbreck D, Rossier C, Ucla C, Hubbard T, Antonarakis SE, Guigo R. 2006. GENCODE: Producing a reference annotation for ENCODE. Genome Biol 7(Suppl1:S4):1–9.

Kaneko S, Li G, Son J, Xu CF, Margueron R, Neubert TA, Reinberg D. 2010. Phosphorylation of the PRC2 component Ezh2 is cell cycle-regulated and up-regulates its binding to ncRNA. Genes Dev 24:2615–2620.

Kapranov P, Cheng J, Dike S, Nix DA, Duttagupta R, Willingham AT, Stadler PF, Hertel J, Hackermüller J, Hofacker IL, Bell I, Cheung E, Drenkow J, Dumais E, Patel S, Helt G, Ganesh M, Ghosh S, Piccolboni A, Sementchenko V, Tammana H, Gingeras TR. 2007. RNA maps reveal new RNA classes and a possible function for pervasive transcription. Science 316:1484–1488.

Khalil AM, Guttman M, Huarte M, Garber M, Raj A, Rivea Morales D, Thomas K, Presser A, Bernstein BE, van Oudenaarden A, Regev A, Lander ES, Rinn JL. 2009. Many human large intergenic noncoding RNAs associate with chromatin-modifying complexes and affect gene expression. Proc Natl Acad Sci USA 106:11667–11672.

Kim K, Jutooru I, Chadalapaka G, Johnson G, Frank J, Burghardt R, Kim S, Safe S. 2013. HOTAIR is a negative prognostic factor and exhibits pro-oncogenic activity in pancreatic cancer. Oncogene 32:1616–1625.

Mercer TR, Dinger ME, Mattick JS. 2009. Long non-coding RNAs: Insights into functions. Nat Rev Genet 10:155–159.

Mikkelsen TS, Ku M, Jaffe DB, Issac B, Lieberman E, Giannoukos G, Alvarez P, Brockman W, Kim TK, Koche RP, Lee W, Mendenhall E, O'Donovan A, Presser A, Russ C, Xie X, Meissner A, Wernig M, Jaenisch R, Nusbaum C, Lander ES, Bernstein BE. 2007. Genome-wide maps of chromatin state in pluripotent and lineage-committed cells. Nature 448:553–560.

Modarresi F, Faghihi MA, Lopez-Toledano MA, Fatemi RP, Magistri M, Brothers SP, van der Brug MP, Wahlestedt C. 2012. Inhibition of natural antisense transcripts in vivo results in gene-specific transcriptional upregulation. Nat Biotechnol 30:453–459.

Mühlemann HR, Son S. 1971. Gingival sulcus bleeding–a leading symptom in initial gingivitis. Helv Odontol Acta 15:107–113.

Niedzielska I, Cierpka S. 2010. Interferon gamma in the etiology of atherosclerosis and periodontitis. Thromb Res 126:324–327.

Okazaki Y, Furuno M, Kasukawa T, Adachi J, Bono H, Kondo S, Nikaido I, Osato N, Saito R, Suzuki H, Yamanaka I, Kiyosawa H, Yagi K, Tomaru Y, Hasegawa Y, Nogami A, Schönbach C, Gojobori T, Baldarelli R, Hill DP, Bult C, Hume DA, Quackenbush J, Schriml LM, Kanapin A, Matsuda H, Batalov S, Beisel KW, Blake JA, Bradt D, Brusic V, Chothia C, Corbani LE, Cousins S, Dalla E, Dragani TA, Fletcher CF, Forrest A, Frazer KS, Gaasterland T, Gariboldi M, Gissi C, Godzik A, Gough J, Grimmond S, Gustincich S, Hirokawa N, Jackson IJ, Jarvis ED, Kanai A, Kawaji H, Kawasawa Y, Kedzierski RM, King BL, Konagaya A, Kurochkin IV, Lee Y, Lenhard B, Lyons PA, Maglott DR, Maltais L, Marchionni L, McKenzie L, Miki H, Nagashima T, Numata K, Okido T, Pavan WJ, Pertea G, Pesole G, Petrovsky N, Pillai R, Pontius JU, Qi D, Ramachandran S, Ravasi T, Reed JC, Reed DJ, Reid J, Ring BZ, Ringwald M, Sandelin A, Schneider C, Semple CA, Setou M, Shimada K, Sultana R, Takenaka Y, Taylor MS, Teasdale RD, Tomita M, Verardo R, Wagner L, Wahlestedt C, Wang Y, Watanabe Y, Wells C, Wilming LG, Wynshaw-Boris A, Yanagisawa M, Yang I, Yang L, Yuan Z, Zavolan M, Zhu Y, Zimmer A, Carninci P, Hayatsu N, Hirozane-Kishikawa T, Konno H, Nakamura M, Sakazume N, Sato K, Shiraki T, Waki K, Kawai J, Aizawa K, Arakawa T, Fukuda S, Hara A, Hashizume W, Imotani K, Ishii Y, Itoh M, Kagawa I, Miyazaki A, Sakai K, Sasaki D, Shibata K, Shinagawa A, Yasunishi A, Yoshino M, Waterston R, Lander ES, Rogers J, Birney E, Hayashizaki Y; FANTOM Consortium; RIKEN Genome Exploration Research Group Phase I & II Team. 2002. Analysis of the mouse transcriptome based on functional annotation of 60,770 full-length cDNAs. Nature 420:563–573.

Ponjavic J, Oliver PL, Lunter G, Ponting CP. 2009. Genomic and transcriptional co-localization of protein-coding and long non-coding RNA pairs in the developing brain. PLoS Genet 5:e1000617.

Rinn JL, Kertesz M, Wang JK, Squazzo SL, Xu X, Brugmann SA, Goodnough LH, Helms JA, Farnham PJ, Segal E, Chang HY. 2007. Functional demarcation of active and silent chromatin domains in human HOX loci by noncoding RNAs. Cell 129:1311–1323.

Schaefer AS, Richter GM, Dommisch H, Reinartz M, Nothnagel M, Noack B, Laine ML, Folwaczny M, Groessner-Schreiber B, Loos BG, Jepsen S, Schreiber S. 2011. CDKN2BAS is associated with periodontitis in different European populations and is activated by bacterial infection. J Med Genet 48:38–47.

Schmittgen TD, Livak KJ. 2008. Analyzing real-time PCR data by the comparative C(T) method. Nat Protoc 3:1101–1108.

Ulveling D, Francastel C, Hubé F. 2011. When one is better than two: RNA with dual functions. Biochimie 93:633–644.

Verrier T, Solhonne B, Sallenave JM, Garcia-Verdugo I. 2012. The WAP protein Trappin-2/Elafin: A handyman in the regulation of inflammatory and immune responses. Int J Biochem Cell Biol 44:1377–1380.

Wang J, Liu X, Wu H, Ni P, Gu Z, Qiao Y, Chen N, Sun F, Fan Q. 2010. CREB up-regulates long non-coding RNA, HULC expression through interaction with microRNA-372 in liver cancer. Nucleic Acids Res 38:5366–5383.

Xu K, Wang X, Shi Q, Chen C, Tian C, Li XL, Zhou RM, Chu YL, Dong XP. 2011. Human prion protein mutants with deleted and inserted octarepeats undergo different pathways to trigger cell apoptosis. J Mol Neurosci 43:225–234.

Ye P, Simonian M, Nadkarni MA, Decarlo AA, Chapple CC, Hunter N. 2005. Identification of epithelial auto-antigens associated with periodontal disease. Clin Exp Immunol 139:328–337.

Ørom UA, Derrien T, Beringer M, Gumireddy K, Gardini A, Bussotti G, Lai F, Zytnicki M, Notredame C, Huang Q, Guigo R, Shiekhattar R. 2010. Long noncoding RNAs with enhancer-like function in human cells. Cell 143:46–58.

Ørom UA, Shiekhattar R. 2011. Long non-coding RNAs and enhancers. Curr Opin Genet Dev 21:194–198.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher's web-site.