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MicroRNA-146a and microRNA-155 show tissue-dependent expression in dental pulp, gingival and periodontal ligament fibroblasts *in vitro*

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Abstract: MicroRNAs (miRNAs) are small non-coding RNAs showing a tissue-specific expression pattern, and whose function is to suppress protein synthesis. In this study, we hypothesized that expression of miRNAs would differ among fibroblasts from dental pulp (DPF), gingiva (GF) and periodontal ligament (PLF) *in vitro*. Once established by an explant technique, DPF, GF and PLF were collected for RNA isolation and subjected to a miRNA microarray. Next, cells were stimulated with *E. coli* lipopolysaccharide (LPS) for 24 h and then collected for RNA isolation. Expression of miR-146a and miR-155 was investigated by qPCR. Microarray screening revealed several miRNAs that showed specifically high expression in at least one of the fibroblast subtypes. These molecules are potentially involved in the regulation of extracellular matrix turnover and production of inflammatory mediators. Microarray analysis showed that both miR-146a and miR-155 were among the miRNAs expressed exclusively in GF. qPCR demonstrated significant upregulation of miR-146a only in GF after LPS stimulation, whereas basal expression of miR-155 was higher in GF than in the other cell subtypes. LPS downregulated the expression of miR-155 only in GF. Our results suggest that the expression and regulation of miR-146a and miR-155 are more pronounced in GF than in DPF and PLF.
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Keywords: microRNAs; fibroblasts; inflammation; lipopolysaccharide.

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Introduction

Fibroblasts are resident connective tissue cells present throughout the entire human body, playing a major role in production and remodeling of the extracellular matrix. They also have important functions related to the innate immune response such as identification of bacterial products and recognition and production of inflammatory mediators (1). However, it has been clearly demonstrated that the biological behavior of fibroblasts is related to the type of connective tissue from which they originate (2-4). Such properties include membrane receptor expression (3) and cytokine release kinetics (2,3,5-7).

Bacterial sensing by resident cells is essential for host defense against pathogens. Recognition of byproducts and cell wall compounds such as lipopolysaccharide (LPS) is triggered by a complex system of molecules known as pattern recognition receptors (PRRs). Among

them, toll-like receptors (TLRs) are known as one of the major families, and are considered key components for recognition of a wide spectrum of pathogen-associated molecular patterns (PAMPs). The myeloid differentiation (MD) complex 2-TLR4 is responsible for recognition of LPS, a cell wall component of Gram-negative bacteria. Once activated, TLR4 initiates an intracellular signaling cascade that results in transcription of genes for inflammatory mediators such as cytokines. The TLR signaling pathway is tightly regulated, and negative signaling of these molecular cascades is essential for homeostasis (8,9). Recently, a novel group of molecules, the microRNAs (miRNAs), have been shown to regulate TLR signaling by fine tuning (10-12).

miRNAs are a class of small non-coding RNAs known to act as regulators of protein synthesis at the post-transcriptional level (13). miRNA pairing within the 3'-untranslated region (UTR) of the target mRNA results in translation inhibition or mRNA cleavage depending on the degree of miRNA complementarity with the corresponding target mRNA. Regardless of the mechanism involved, protein synthesis is impaired even in the presence of mRNA expression (13). miRNAs are currently known to be involved in a broad range of biological events such as cell differentiation, carcinogenesis and inflammation (13,14). Also importantly, miRNA expression shows a tissue-specific pattern (15,16).

Oral inflammatory diseases such as gingivitis and pulpitis are known to modulate the expression of miRNAs (17-19). Also in the context of infectious diseases, miR-146a and miR-155 have been shown to have a role in innate immune responses, especially the negative feedback of cell signaling in response to bacterial sensing (10-12,18). Furthermore, miRNAs have recently emerged as molecules that are intimately involved in regulation of the immune response through silencing of molecules in the TLR pathway. Interleukin-1 receptor-associated kinase (IRAK)-1 and tumor necrosis factor (TNF) receptor-associated factor (TRAF)-6 are known to be targets of miR-146a (10,11), leading to downregulation of proinflammatory cytokines such as TNF-α, IL-6 (11,12) and IL-1β (12). In turn, miR-155 is known to inhibit SH2 domain-containing inositol-5'-phosphatase (SHIP)-1 (20) and supressor of cytokine signaling (SOCS)-1 (21), both of which are involved in the regulation of proinflammatory signals (20,21). Both miR-146a and miR-155 are known to target TLR4 signaling molecules, whereas miR-155 is also able to abrogate the initiation of the TNFR signaling cascade (22). Taken together, these data suggest important roles of miR-146a and miR-155 in the fine tuning of inflammation signaling events. In inflamed gingival tissue, the expression of miR-146a and miR-155 is up- and down-regulated, respectively, in comparison with healthy tissue (18), suggesting the modulation of miR-146a and miR-155 by inflammation in oral connective tissues. However, the extent to which fibroblasts are involved in this biological process is unclear.

The available data clearly indicate that miRNAs have an important role in the regulation of cell signaling triggered by bacterial sensing, and thus maintenance of tissue homeostasis. Nevertheless, the extent to which the tissue-specific expression of miRNAs, especially miR-146a and miR-155, reflects the response of fibroblasts during modulation of the local inflammation process is still unclear. In the present study, we hypothesized that the expression and immune regulation of miRNAs would differ among dental pulp, gingiva and periodontal ligament fibroblasts.

Materials and Methods

Fibroblast culture

The experiments were performed with the approval of the Ethics Committee for Human Research of the Bauru School of Dentistry, University of São Paulo (Proccess #15/2007), in accordance with Declaration of Helsinki, 1975 (revised in 2000). With written informed consent, teeth and gingival tissues were donated by patients undergoing lower third molar removal. Cultures of human fibroblasts from dental pulp, gingiva and periodontal ligament from the same donors (n = 3) (2 females and 1 male, 16-26 years old) were established by means of the explant technique as described elsewhere (5,7,23). Immediately after removal, the tissues were kept separately under aseptic conditions. After being minced, fragments were incubated in Dulbecco's modified Eagle medium (DMEM) (Gibco, Invitrogen Corp., Carlsbad, CA, USA) supplemented with 10% fetal bovine serum and antibiotics (100 µg/mL penicillin, 100 µg/mL streptomycin, 0.5 mg/mL amphotericin B) (Gibco). Explants were maintained at 37ºC in an atmosphere of 5% CO_2 and 95% air at 100% humidity. Cells were used between the fourth and eighth passages. Phenotypic characterization of fibroblasts in culture was performed as described previously by our group (6,7).

Fibroblast stimulation and RNA isolation

Fibroblasts were seeded into 24-well plates at 5×10^4 cells/well. After 24 h to allow cell attachment, medium alone or containing lipopolysaccharide (LPS) from *Escherichia coli* (Sigma-Aldrich, St. Louis, MO, USA) at a concentration of 10 µg/mL was added to the wells

Table 1 miRNAs expressed exclusively in dental pulp (DPF), gingival (GF) and periodontal ligament fibroblasts (PLF) *in vitro*

Cell type	miRNA (fold increase)	Candidate targets	Validated functions
DPF	miR-24 (0.7384)	IL1A, IL1R1, MMP14	Collagen synthesis and cellular migration (25)
	miR-605 (0.5907)	LPS-induced TNF factor	Activation of apoptosis (45)
	miR-488 (0.5907)	FGFR3	Upregulation of MMP-13 (26)
	miR-181b (0.5907)	integrin β8	Reduction of proinflammatory cytokines release (29,31)
	miR-589 (0.5907)	TRAF6, MMP16,	Activation of *COX-2* gene transcription (30)
	miR-32 (0.5907)	fibrillin 1, fibrillin 2, integrin α6	Targets TRAF3 (27)
	miR-212 (0.7384)	MMP16	Downregulation of IRAK4 leading to immunological tolerance (28)
	let-7e (0.7384)	IL10	Downregulation of TLR4 (21)
GF	miR-34a (0.7384)	FGF23, IL6R	Targets TGF-β signalling (33)
	miR-20b (0.7384)	CCL1, CXCL14	Downregulation of VEGF (32)
	miR-190 (0.5907)	fibrillin 1	Unrelated functions
	miR-591 (0.5907)	thrombospondin 2	Unrelated functions
	miR-153 (0.7384)	TGFBR2, FGFR2	Regulation of epithelial to mesenchymal transition in oral cancer (39)
	miR-612 (0.7384)	integrin α5	Unrelated functions
	miR-365 (0.5907)	FGF1, integrin α10	Negative regulation of IL-6 (34)
PLF	miR-9 (0.7384)	fibrillin 1, fibrillin 2, NADPH oxidase 4, CXCL11, TGFBR2, IL6R, CXCR4, ALP, MMP15	Neuroconvertion of human dermal fibroblasts (35)
	miR-484 (0.7384)	MMP14	Unrelated functions
	miR-520e (0.5907)	TGFBR2	Unrelated functions
	miR-202 (0.7384)	MMP1, IL6R, IL10, periostin, fibromodulin, DMP1, IL6, TGFBR3, thrombospondin 1, CCL7, integrin α11	Unrelated functions

Levels of miRNA expression are presented in parenthesis based on microarray data analysis. miRNA expression is shown as log2 differences (a value of 1 being equivalent to a 2-fold change).
IL: interleukin, -R: -receptor, MMP: matrix metalloproteinase, COX: cyclooxygenase, LPS: lipopolysaccharide, TNF: tumor necrosis factor, FGF: fibroblast growth factor, TRAF: TNF receptor-associated factor, IRAK: interleukin-1 receptor-associated kinase, TGF: transforming growth factor, CCL: chemokine C-C motif ligand, CXCL: chemokine C-X-C motif ligand, TGFBR: TGF-β receptor, VEGF: vascular endothelial growth factor, NADPH: nicotinamide adenine dinucleotide phosphate, ALP: alkaline phosphatase, DMP: dentin matrix protein.

in duplicate. After 24 h, cells were collected with 1 mL of TRIzol (Invitrogen) for isolation of total RNA by guanidinium isothiocyanate phenol-chloroform extraction, as described previously (5,7). Briefly, RNA was isolated through cells homogenization, chloroform extraction, isopropanol precipitation, and washing with 75% (volume/volume) ethanol. After reconstitution with nuclease-free water (12 µL), RNA was quantified using a spectrophotometer (Nanodrop 1000, Thermo Scientific, Wilmington, DE, USA) at wavelengths of 280 and 260 nm.

miRNA expression by microarrays

To determine the overall expression of miRNAs in fibroblasts in relation to their tissue of origin, dental pulp (DPF), gingiva (GF) and periodontal ligament fibroblasts (PLF) from one donor were chosen (male, 25 years old, free of systemic or periodontal disease). After RNA isolation, 500 ng of total RNA from each sample was hybridized using a Cy3 Alexa Fluor using NCode miRNA rapid labeling system (Cat. # MIRLSRPD-20) (Invitrogen) in accordance with the manufacturer's instructions (24). Labeled samples were separately incubated on microarray slides (NCode Multi-Species miRNA microarray kit V2, Invitrogen) for 18 h while protected from light. The slides were washed in decreasing concentrations of saline-sodium citrate (SSC) buffer and dried after centrifugation. The arrays were scanned (GenePix 4000B Scanner, Molecular Devices, Sunnyvale, CA, USA), and after alignment the data obtained were analyzed using the JMP Genomics Software package (SAS Institute Inc., Cary, NC, USA) using Ward's method for hierarchical clustering. miRNA expression was represented as log2 differences (one point higher meaning two-fold higher

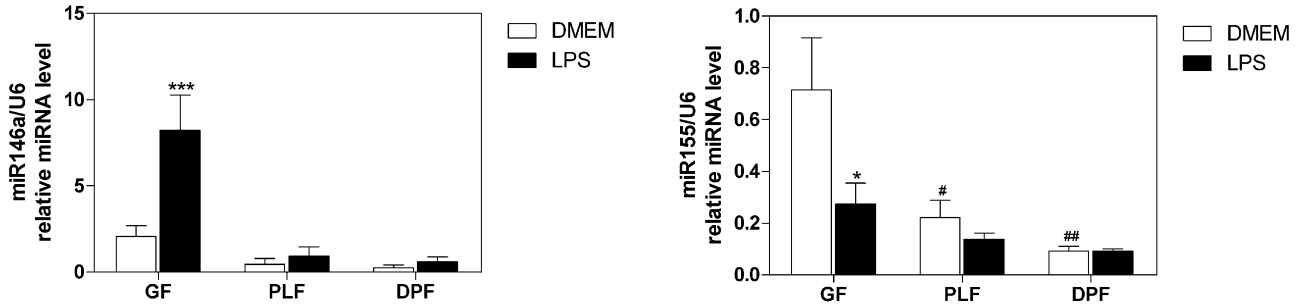

Fig. 1 Expression of miR-146a and miR-155 in fibroblasts from dental pulp, gingiva and periodontal ligament *in vitro*. Dental pulp (DPF), gingival (GF) and periodontal ligament fibroblasts (PLF) were stimulated with 10 µg/mL LPS for 24 h (solid bars) prior to RNA isolation. Control cells were maintained in DMEM only (clear bars). Data are presented as means and standard errors ($n = 3$). Detection and quantitation of miR-146a (left panel) and miR-155 (right panel) were performed by means of qPCR using the U6 gene as a reference for data normalization.

* Significant differences between untreated cells from the same tissue. # Significant differences between cells from distinct tissues under the same experimental conditions. * or # $P < 0.05$, ## $P < 0.01$, and *** $P < 0.001$.

expression).

Determination of miR-146a and miR-155 expression using qPCR

The differential expression of miR-146a and miR-155 demonstrated by microarray analysis and its eventual modulation by LPS were investigated using quantitative polymerase chain reaction (qPCR).

Reverse transcription (RT) of 10 ng of total RNA was performed using specific miRNA assays (miR-146a assay #468; miR-155 assay #479; RNU6B assay #1093 - Taqman miRNA assays, Applied Biosystems, Life Technologies, Darmstadt, Germany) employing the corresponding RT primers together with a miRNA Reverse Transcription Kit (Applied Biosystems) in accordance with the manufacturer's instructions. Once synthesized, complementary DNA obtained for each sample and target was amplified by qPCR using the PCR primers for the above-mentioned miRNA assays and Universal Mastermix II (Applied Biosystems) in a qPCR instrument (ViiA 7 Real-Time PCR System, Applied Biosystems). Based on the manufacturer's instructions, the cycling parameters were 50°C for 2 min, 95°C for 10 min and 40 cycles of 95°C for 15 s and 60°C for 60 s. Data normalization was performed on the basis of the endogenous control (U6B). Efficiency of the primers sets was confirmed prior to the experiments. Comparisons among samples were made by comparative Ct analysis based on a reference sample from the untreated group.

Statistical analysis

Statistical analysis was performed by two-way ANOVA followed by Bonferroni post-hoc test using GraphPad Prism 5.0 software (La Jolla, CA, USA). The level of significance was set at $P < 0.05$.

Results

Determination of miRNA expression by microarray analysis

An exploratory analysis of miRNAs of DPF, GF and PLF from the same donor was performed in order to investigate molecules that were differentially expressed by these cells under basal conditions. The miRNAs that exhibited the highest levels of expression detected exclusively in each cell type are shown in Table 1. Some predicted targets based on a search of www.targetscan.org are also displayed.

The immuno-miRs miR-146a and miR-155 were not among the miRNAs showing the highest expression, but both were detected exclusively in GF. Considering the role of these miRNAs in regulation of the TLR4 signaling cascade (10,11,20,21), they were chosen for further validation.

Detection of miR-146a and miR-155 by qPCR

The expression of miR-146a and miR-155 by DPF, GF and PLF detected by qPCR is shown in Fig. 1. MiR-146a expression under basal conditions was slightly, but not significantly, higher for GF. Interestingly, stimulation with LPS resulted in a four-fold increase of miR-146a expression in GF, a three-fold increase in DPF, and a two-fold increase in PLF (Fig. 1, left panel). However, only the increased expression in GF was significant ($P = 0.0003$). In contrast to miR-146a, basal expression of miR-155 in GF was three- and six-fold higher in comparison with PLF and DPF, respectively ($P = 0.0393$)

(Fig. 1, right panel). Conversely, LPS challenge resulted in a 2.5-fold decrease in the expression of miR-155 in GF ($P = 0.0025$), while non-significant alterations were observed for the other cell subtypes.

Discussion

miRNAs are small non-coding nucleotides involved in a broad range of biological events, acting as molecules for fine tuning of protein synthesis. The role of miRNAs in inflammatory and immune responses has been demonstrated previously (10-12,18). The TLR-related miRNAs, miR-146a, miR-146b and miR-155, have been shown to be involved in inflammation-related signaling, while miR-155 also has some role in fibroblast differentiation (18). miRNAs have also emerged as molecules exhibiting a high degree of tissue specificity (15,16). Considering previous reports that have demonstrated distinct differences among fibroblasts in terms of bacterial sensing and immune responses in relation to their tissue of origin (2,3,5-7), we sought to investigate corresponding differences in the expression of their miRNAs.

Microarray analysis was performed to identify possible miRNA signatures in fibroblasts from distinct oral sites: dental pulp, gingiva and periodontal ligament. Hierarchical clustering analysis of the resulting data showed that some miRNAs were expressed at higher levels exclusively in a specific cell subtype relative to the others, as shown in Table 1. Based on target prediction using the algorithm www.targetscan.org, these miRNAs appeared to be involved in extracellular matrix production and remodeling, as observed by combinatorial matching with distinct extracellular matrix components and matrix metalloproteinases genes. Additionally, these miRNAs appeared to be involved in the synthesis of distinct growth factors, cytokines, chemokines and also signaling molecules, such as TRAF6, involved in innate immune responses. Interestingly, in GF, none of the predicted targets of the miRNAs were related to MMPs, whereas in DPF, unlike GF and PLF, chemokines were not found among these targets. Based on previous studies, miRNAs found exclusively in DPF were strongly related to matrix extracellular synthesis (25) and remodeling (26), and also regulation of the innate immune (21,27,28) and inflammatory (29-31) responses. On the other hand, GF showed high expression of miRNAs involved in the regulation of growth factors playing important roles in tissue repair (32,33) and also IL-6 reduction (34). Finally, among the miRNAs exhibiting higher expression in PLF, one was found to be involved in cytodifferentiation (35). Taken together, these findings suggest a possible role of miRNAs in the modulation of matrix turnover and inflammatory responses by oral tissue-derived fibroblasts.

Previous studies have demonstrated modulation of miR-146a and miR-155 expression resulting from bacterial sensing and innate immunity events (10-12,18,36). One study (18) supported these alterations in gingival inflamed tissues. For this reason, we chose miR-146a and miR-155 for validation by means of qPCR, despite the fact that these miRNAs were not found among those showing the highest expression in microarrays. In the present study, the basal expression of miR-146a was slightly higher in GF than in DPF and PLF (Fig. 1, left panel). Under LPS stimulation, the expression of miR-146a tended to be upregulated in the three cell subtypes. However, this increased expression was statistically significant only in GF. Our findings are in accordance with previous reports (10-12,22,36,37). Increased expression of miR-146a under pro-inflammatory conditions has been demonstrated previously in inflamed tissues (18) and after stimulation of TLR2, TLR4, TLR5, tumor necrosis factor receptor (TNFR) and interleukin-1 receptor (IL1R) (10). In this context, miRNAs may act as a novel class of TLR and cytokine receptor signaling regulators (10). In particular, miR-146a has been found to target the TLR signaling molecules IRAK1 and TRAF6 (10,11). Consequently, miR-146a is involved in release of IL-1β, IL-6, IL-10 and TNF-α by gingival fibroblasts stimulated *in vitro* by *Porphyromonas gingivalis* LPS (12). These findings strongly suggest that miR-146a is involved in down-regulation of TLR signaling, and consequently in negative regulation of the innate immune response (10,12).

A previous study has detected a 5- to 25-fold increase in the expression of miR-146a in dental pulp cells stimulated with 1 μg/mL LPS (36). Our data revealed a non-significant increase of miR-146a expression in DPF in the presence of LPS, which was smaller in extent than that observed by Wang and colleagues. These differences might have been due to the experimental conditions employed, such as the culture medium, the type of LPS and its concentration, as well as the number of passages of the dental pulp cells.

In contrast to miR-146a, basal expression of miR-155 was significantly higher in GF than in DPF and PLF (Fig. 1, right panel). The presence of endotoxin downregulated its expression to a similar degree in the three cell subtypes. Previous studies have investigated the regulation of miR-155 during inflammation. In RAW264.7 macrophages, miR-155 expression was shown to increase in a concentration-dependent manner upon stimulation with *Salmonella typhimurium* LPS, unlike miR-146a, which showed an all-or-nothing type of response (22).

These data demonstrate distinct expression patterns for miR-155 and miR-146a in murine macrophages (22). Honda et al. (11) demonstrated upregulation of miR-155 expression in THP-1 cells following stimulation with *P. gingivalis* LPS and *E. coli* LPS. On the other hand, in inflamed gingival tissues, this molecule was shown to be downregulated (18), thus corroborating our findings for gingival fibroblasts. In a mouse model of atherosclerosis, expression of miR-155 was found to be unaltered in maxillary gingival tissue with periodontal disease, while miR-146a was upregulated (37). The expression of miR-155 was shown to be highly variable among different tissues (15), and therefore we may speculate that not just the basal expression of miR-155, but also its modulation during innate immune events, might show a tissue-specific pattern.

During infectious oral diseases, bacterial sensing by resident and immune cells is essential for host immune defense. Cellular signaling resulting from activation of innate immunity receptors, such as TLR, leads to transcription of genes for cytokines and inflammatory mediators, among other biological events. Along these cell signaling cascades, miRNAs may act to silence some of the involved molecules. The role of miR-146a and miR-155 in this context has already been demonstrated (10,22,37). The expression of both miR-155 and miR-146a is regulated by LPS (10). miR-146a has been shown to target IRAK1 and TRAF6, which are TLR4 signaling molecules (10). On the other hand, miR-155 is known to target SHIP-1 (20) and SOCS-1 (21), molecules that are involved in the downregulation of proinflammatory responses induced by innate immunity (20,21). Additionally, miR-155 is inhibited by IL-10, an anti-inflammatory cytokine, resulting in upregulation of SHIP-1 (38). In GF, miRNA-146 was shown to inhibit the production of IRAK-1, a TLR4 signaling molecule, by binding directly to the 3'-UTR of the corresponding mRNA (39). Translational repression of the human receptor of angiotensin II (AT1) in lung fibroblasts is also one of the known functions of miR-155 (40). The role of AT1 in nuclear factor -κB (NF-κB) activation and cytokine release has been shown previously (41), thus suggesting another important molecular mechanism of inflammation control accomplished by miR-155 expression. Additional targets of miR-146a and miR-155 together with the mechanisms that regulate their expression are issues that require further and deeper investigation.

It is well known that fibroblasts from distinct anatomic sites display peculiar phenotypes (2,3) that reflect differences in tissue function (1). Moreover, once activated, fibroblasts may take on new patterns of extracellular matrix remodeling (42,43) or even assume new functions, as in the case of cytokine production (3,44). Smith et al. (1) have highlighted differences among fibroblasts from distinct tissues as a possible determinant of local susceptibility to disease. Together with a previous report (18), our data support the hypothesis that fibroblasts contribute to the regulation of miR-146a and miR-155 observed in inflamed gingival tissues. The molecular mechanisms involved in the tissue identity of fibroblasts remain unknown. On the other hand, since their discovery, miRNAs have emerged as tissue-specific molecules (13) and it is reasonable to assume the existence of miRNA signatures for fibroblasts according to their tissue of origin.

To our knowledge, no comparative study of miR-146a and miR-155 expression among LPS-challenged fibroblasts from different oral tissues has been reported previously. Our data clearly demonstrate a distinct pattern in the modulation of these molecules in GF in comparison with DPF and PLF. Therefore, we may speculate that differences observed previously in fibroblast phenotypes with regard to cytokine secretion and TLR modulation might be due to the distinct expression profile of miRNAs in these cells. Additionally, the role of resident cells in miRNA tissue specificity as well as the role of the tissue microenvironment in the distinct cellular regulation of miRNAs is an intriguing issue that requires further investigations.

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