

# Micro RNAs In Periodontal Disease – A Review

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## KEYWORDS

microRNA; biogenesis; profiling; innate immunity; acquired immunity; bone remodelling

## ABSTRACT

microRNAs (miRNAs) constitute a family of small, non-coding RNA molecules that regulate gene expression and protein expression. microRNAs have influence on a broad range of physiologic and pathologic conditions. They are also considered as promising biomarkers especially when they are secreted extracellularly. In the inflammatory pathways, they dysregulate the molecular processes and contribute to the development of chronic inflammatory diseases including periodontitis. In this review, we provide an overview of miRNA characteristics, biogenesis, mechanisms of action and profiling methods. In addition, the role of miRNAs in the pathobiology of periodontitis, especially those pertaining to the cellular and molecular pathways of inflammation has been considered to enhance our understanding of the pathobiology of periodontitis.

## INTRODUCTION

The central dogma of molecular biology, introduced by Crick in 1958, states that biological information is transferred from DNA to messenger RNA (mRNA) and to protein in a linear and sequential manner [1]. The microRNAs (miRNAs) take a prominent role in this context. They are the recently discovered family of small non-coding RNA molecules that is 22 nucleotides long. Their function is either to inhibit the initiation of mRNA translation into proteins or to induce degradation of mRNA molecules. Thus they have massive effects on protein translation. About 60% of all protein-coding genes are targets of miRNAs [2]. They have a major role in the normal development and are critical in many biological processes. They can be secreted into the extracellular fluids like plasma, serum, tears, colostrum, saliva etc. From there, they will be delivered to target tissues to act in an autocrine, paracrine or endocrine fashion thereby modulating the cellular activities. Thus miRNAs have a hormone

like behaviour too. This property enables its use as a biomarker for diagnostic applications in diseases like cancer, cardiovascular and autoimmune diseases.

The first described miRNA was lin-4 by Victor Ambros and Gary Ruvkun [3,4] based on work carried out in the *Caenorhabditis elegans* nematode. Since then, additional miRNAs have been discovered to a total of 2,588 mature miRNAs in humans and 1,915 miRNAs in mice [5]. Regulation of protein expression by miRNAs has an important role in homeostasis as well as pathology. Aberrant miRNA can dysregulate both the innate and adaptive immune responses. This dysregulation develops into chronic inflammatory diseases. This review aims to provide an overview of basic miRNA biology and its relation to inflammatory periodontal disease.

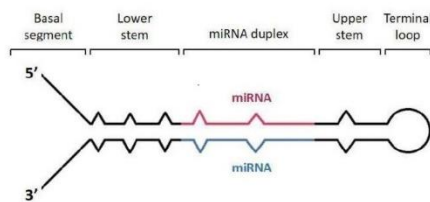
## miRNA CHARACTERISTICS

Mature miRNAs are typically 22 nucleotides long but their origin is from much longer primary transcripts. The biogenesis of mature miRNA starts

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from DNA sequences. RNA polymerase II enzyme transcribes the DNA sequences to primary transcript, known as the pri-miRNA. The primary transcripts are usually 100 to 1000 nucleotides long and has a characteristic stem loop structure. The cleavage of primary transcripts give rise to pre-miRNA/ precursor miRNA that are 70 to 100 nucleotide long and has a hairpin loop structure. Further cleavage of pre-miRNA by the RNA polymerase III enzyme results in a miRNA duplex strand with a 3' and 5' end. The duplex strand is composed of a guide strand and passenger strand. Guide strand is thermodynamically more stable and gets incorporated into the RNA-induced silencing complex (RISC) where they are bound by a member of the Argonaute (AGO) family of RNA-binding proteins. The passenger strand is thermodynamically less stable and are often degraded (Figure: 1).



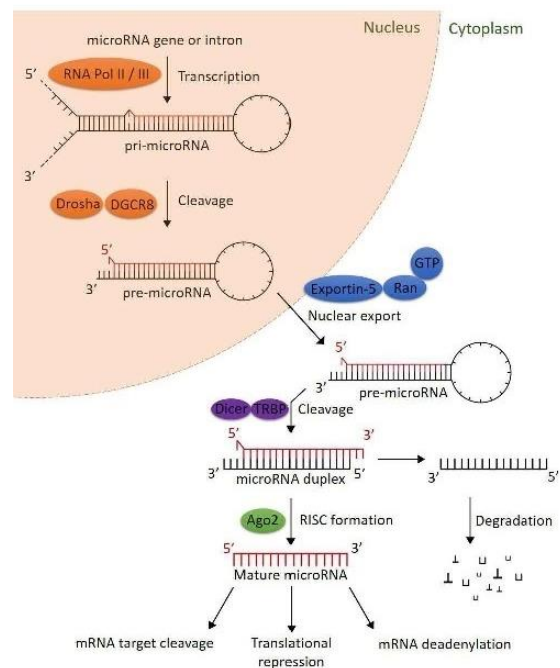
**Figure 1:** The typical stem-loop structure of miRNA. The typical stem loop structure of miRNA. It consists of a terminal loop about 10 nucleotide long, a miRNA duplex approximately 22 nucleotide long, a lower stem 11 nucleotide long and two flanking segments, 9 nucleotide long with a 3' and 5' end. The miRNA duplex consists of a guide strand and passenger strand. The guide strand is the functional strand and the passenger strand is often degraded.

MicroRNA represents about 0.01% of total RNA by weight but the average copy number of an individual miRNA species has been estimated as 500/cell. They represent only a tiny fraction of total RNA quantity yet they are numerous because of the much lower molecular weight compared to mRNA.

### BIOGENESIS OF miRNA

The generation of genome-encoded miRNA precursors occurs in the nucleus and their transportation and further processing takes place in the cytoplasm [6]. Genes encoding for miRNAs can be found between the protein-coding genes, as polycistronic transcripts under their own

promoters, or in the intronic regions of protein-coding genes that often encode multiple end-product miRNAs [7]. Transcription of miRNA genes is mainly by RNA polymerase II and RNA polymerase III [8]. The RNA polymerase II enzyme cleaves the DNA sequences. They produce long pri-miRNAs. The maturation process involves two consecutive cleavage steps by RNase III enzymes DROSHA and DICER. A heterotrimeric complex formed by DROSHA and its partner DiGeorge syndrome critical region gene 8 (DGCR8) [9] crops the pri-miRNA into pre-miRNAs [10]. Pre-miRNA is then exported to cytoplasm with the help of Exportin-5-Ran-GTP complex [11] where DICER cleaves it and forms a mature miRNA duplex. Dicer acts together with Argonaute-2(AGO2). Mature miRNA is approximately 22-nucleotide long and is composed of guide-strand and passenger strand duplexes and the process is referred to as 'dicing'. This is the classical (canonical) pathway of biogenesis (Figure: 2).



**Figure 2:** The canonical pathway of miRNA biogenesis. The canonical pathway of miRNA biogenesis. It involves the production of pri-miRNA by RNA polymerase II or III followed by cleavage of pri-miRNA by the microprocessor complex (DROSHA-DGCR8) in the nucleus. The resultant, pre-miRNA is exported from nucleus to cytoplasm by Exportin 5- Ran-GTP complex. In the cytoplasm it is again cleaved by DICER and TRBP, the double stranded RNA binding protein, to form mature miRNA duplex. The guide strand gets incorporated into the RISC along with Ago2 protein and takes part in mRNA cleavage,

translational repression or deadenylation where as the passenger strand undergoes degradation.

Then there is the alternative processing pathways of pre-miRNA. It is independent of either Drosha or Dicer, i.e. formed either by Drosha independent pathway or by Dicer independent pathway. The imprecisions in Drosha and Dicer cleavage of miRNA precursors lead to formation of length variant or shifted sequenced [12] miRNAs called 'isomiRs' [13,14]. Most isomiRs vary by one or more bases at the 3' and/or 5' end, in size, from the canonical miRNA. The variations at the 5' end are functionally relevant because they mediate most of the miRNA-mRNA interactions [15]. These isomiRs can regulate the same targets as the canonical miRNAs [16].

### **MECHANISM OF ACTION**

The miRNAs binds to specific regions on the target mRNA. Depending upon the region, the effect they produce also varies. Studies have shown that the miRNAs, when binding to specific sequence at the 3'UTR of target mRNA induce translational repression, mRNA de adenylation and decapping [17,18]. When they bind to 5'UTR and coding region, they have silencing effect on gene expression. When the interactions are with promoter region, they can induce transcription [19, 20, 21].

The guide strand along with AGO2 protein forms a ribonucleoprotein complex termed the miRNA-induced silencing complex (miRISC). Of the four paralogs of AGO family, AGO2 most frequently takes part in construction of miRISC [22] along with the trinucleotide repeat containing protein 6 (TNRC6). These are also known as glycine-tryptophan 182-kDa protein, GW182). GW182 provides the scaffolding needed to recruit other effector proteins like poly(A)-deadenylase complexes PAN2-PAN3 and CCR4-NOT following miRNA: target mRNA interaction (50) [23].

PAN2/3 helps in initiating deadenylation of mRNA which is then completed by CCR4-NOT. The bound guide strand directs the interactions of miRISC with partially complementary sequences in target mRNAs. These complementary sequences are known as miRNA response elements (MREs) and they determine the target specificity of miRISC.

They are most commonly located in the 3'-untranslated region.

The degree of complementarity is the key factor that determines the fate of target mRNA [24]. When the miRNA: MRE interactions are fully complementary, target mRNA cleavage occurs through AGO2 endonuclease activity [24]. Majority of miRNA: MRE interactions in animal cells are not fully complementary [23]. When there are mismatches, AGO2 endonuclease activity is prevented. AGO2 acts similar to the other non endonucleolytic AGO family members like AGO1, 3 and 4 leading to target mRNA decay.

### **miRNA PROFILING**

miRNA plays a major role in regulation of processes including tissue differentiation, development and maintenance of an organism. Hence they can be used to identify the tissue differentiation stages of diseases like cancer. The miRNA released into extracellular fluids show high stability and resist degradation both at room temperature and under deleterious conditions. Their measurability is also greater when compared to proteins because they can be amplified as required. Thus they find application as biomarkers for diagnosis of diseases and also for forensic purposes.

Sample processing and RNA extraction are the vital steps needed for miRNA profiling. A wide range of cell and tissue sources including the body fluids serve as samples for miRNA extraction. The principles of isolation of miRNAs in a cell or tissue sample is same like that for total RNA, but may need special precautions to preserve the small RNA fraction [29]. The major approaches used currently for profiling are quantitative reverse transcription-PCR (qRT-PCR), hybridisation-based methods and high throughput sequencing. In qRT-PCR, reverse transcription of miRNA to cDNA takes place which is then subjected to quantitative polymerase chain reaction followed by real time monitoring of the reaction products. This method can be used for absolute quantification of miRNA. A limitation is that only known miRNAs can be detected. Moreover it is labour intensive if large experiments are to be carried out.

Hybridisation based methods include micro arrays, which is one of the first methods that can identify large number of miRNAs by parallel analysis. They have lower specificity when compared to other methods, hence the results obtained should be validated by qRT-PCR or northern blot. This is a least expensive method. The microarray hybridization assays can also only detect miRNAs that have already been described and cannot be used for absolute quantification.

RNA-sequencing is another major approach for miRNA profiling. They are helpful to detect both already described and new miRNAs and their sequence variants [30]. But data analysis require computational support and this method cannot quantify miRNA. A faster and less biased technique currently in use is the single molecule real-time sequencing (SMRT). Their high error rates and cost makes them less acceptable for wide use [31]. The applications of miRNA profiling includes novel miRNA detection, understanding various gene regulatory network processes, developmental biology etc. Those in body fluids have applications in forensic setting also.

#### **miRNAs IN PERIODONTAL DISEASE**

Lee et al. (2011), Xie et al. (2011), Perri et al. (2012), Stoecklin-Wasmer et al. (2012) were the few investigators who did pioneer studies for knowing the expression of miRNAs in healthy and diseased gingival tissues [32, 33, 34, 35]. Of these, the largest study available so far was by Stoecklin-Wasmer et al, in 2012 [34] where they evaluated 86 systemically healthy, non-smoking subjects with moderate to severe periodontitis. In their study, 40 gingival tissue samples harvested from periodontally healthy sites and 158 from diseased sites were subjected to genome-wide miRNA profiling. Microbial colonization patterns in the proximal periodontal pockets [36] were also available from these tissue biopsies. The results showed that there's about 159 miRNAs that are differentially expressed between healthy and diseased gingival tissues. Of these, 91 were over expressed and 68 were under expressed in disease versus health. This study have validated for the first time particular genes involved in tissue

homeostasis and inflammatory/immune responses [34].

In all these studies, the miRNA expression assessed, was done in whole tissue gingival biopsies mixed with other cellular components. These cellular components may show variations in their proportions significantly between health and disease. Hence, it is uncertain whether the increased expression of a specific miRNA, seen in any condition, is a true increased transcription of the particular sequence, or is because of the proliferation of any cellular component that harbours the transcript, or a combination of both. This is a major limitation in most of the miRNA studies. But there is enough evidence that suggests particular miRNAs have specific role in molecular and cellular pathways involved in periodontal tissue inflammation.

#### **miRNAs IN INNATE IMMUNE RESPONSE**

The innate immune response is the host's first line defence against pathogens. This is characterised by the interaction of pattern-recognition receptors like toll-like receptors, with the pathogens. These receptors, through the intracellular signalling cascades, trigger direct cellular responses, like cytokine production [37]. In periodontal diseases, toll-like receptor-2 and toll-like receptor-4 interacts with specific pathogens and their biofilm components like lipopolysaccharide from *Porphyromonas gingivalis*, *Aggregatibacter actinomycetemcomitans* and other gram-negative periodontal species [38].

The toll-like receptor binding interactions can be down regulated by pathways that involve the NF-KB (nuclear factor kappa light chain enhancer of activated B cells) family. The NF-KB family is targeted by several miRNAs, important one being the miR-146a. When the bacterial stimuli acts via Toll-like receptors-2/4/5/9, NF-KB activation occurs which can regulate miR-146a. They trigger a negative feedback loop that down regulates the two key NF-KB/tumour necrosis factor signalling adapter molecules, namely interleukin-1 receptor-associated kinase-1 and tumour necrosis factor receptor associated factor-6 [39]. Hence, this is an immunosuppressive miRNA that regulates the

immune responses in periodontitis. It is significantly up-regulated in inflamed gingival tissues when compared to healthy gingiva. Moreover, the Toll-like receptor-2-mediated inflammatory responses were suppressed by miR-146a in the keratinocytes [40] and macrophages [41] preventing the development of excessive inflammation [42].

Another miRNA, miR-155, also have a role in innate immunity acting via the NF-KB pathway [43,44]. Toll-like receptor ligation by both bacterial and viral stimuli or the stimulation by cytokines, signals NF-KB pathways causing indirect induction of miR-155. [45,46]. In periodontal ligament cells, the induction of miR-155 down-regulates NF-KB signalling and promotion of cell differentiation [47]. They mediate type I interferon response to infection, [48] a feature often associated with aggressive periodontitis [49]. They are also capable of suppressing the expression of inositol phosphatase SHIP critical for the survival of primary gingival epithelial cells infected with *P.gingivalis* [50]. They induce activation of natural killer cells [51] and increases the production of interferon- $\gamma$  [52].

Two additional miRNAs that have natural killer cell regulatory roles are miR-30e and miR-200a. miR-30e inhibits natural killer cell cytotoxicity [53], whereas miR-200e negatively regulates interleukin-12 signalling in natural killer cells [54]. These miRNAs have reduced expression in periodontitis and hence explains the increased activation of natural killer cells and higher interferon- $\gamma$  production associated with periodontal tissue destruction [55, 56]. Both miR-146b and miR-155 can mediate the increase in Toll-like receptor responsiveness in macrophages, which is triggered by leukotriene B4. Leukotriene B4 levels positively correlates with severity of periodontitis [57]. Dysregulation of expression of these micro RNAs in *P.gingivalis* infected primary oral epithelial cells can induce Toll-like receptor signalling [58, 59]. Studies have confirmed that the anti-inflammatory agent triclosan can modulate the expression of these miRNA, thereby normalizing the Toll like receptor signalling [60].

Lethal-7i is a member of the highly conserved lethal-7 miRNA family [61] that directly targets the Toll-like receptor-4. They are found to be overexpressed in periodontitis affected gingival tissues. miR-31 is another miRNA down regulated in periodontitis. It is a negative regulator of NF-KB [62] thus containing the excessive NF-KB-mediated inflammation. Opposing this action, miR-486 acts by disrupting the multiple NF-KB negative feedback loops, thereby sustaining NF-KB signalling [63]. Thus they are also strongly overexpressed in periodontitis lesions.

miR-141, is a major miRNA associated with gingival health. They target mitogen-activated protein kinase MAP4K4. In periodontal disease, miR-141 is down regulated, inducing MAP kinase signalling and its pro-inflammatory down-stream events [64]. Leukocyte migration is mediated by the chemokine stromal-cell derived factor-1 (SDF-1/CXCL12). This factor along with its receptor CXCR4 [68,69] is strongly up regulated in periodontitis. This is also a target of miR-141. The neutrophil migration from the gingival micro-vasculature into the periodontal pocket is also mediated by chemotactic factors like interleukin-8 and granulocyte chemotactic protein-2 (GCP2/CXCL6) [66]. miR-451 [67] is a dicer independent miRNA that can suppress neutrophil chemotaxis via the p38 MAPK pathway [68] and is strongly induced in periodontitis.

The innate and adaptive immunity bridges by means of dendritic cells. These are antigen presenting cells that mediate cellular response. Thus dendritic cell signalling is a crucial step that drives the immune responses in periodontal infections and these signalling pathways are also regulated by miRNAs [70]. miR-155 plays role in dendritic cell maturation and function whereas miR-146a impair Toll- signalling in dendritic cells to reduce the amount of cytokines produced by these cells [71].

miR-45 also reduce cytokine secretion by infected dendritic cells using a negative feedback loop [72]. Their levels are often high in the inflamed gingiva. miR-148a was also known to impair innate response and antigen presentation of dendritic cells by interfering with intracellular calcium homeostasis [73]. miR-29a is another miRNA with elevated

expression in diseased gingival tissues that down-regulates interleukin-23. This particular micro RNA can be induced by NOD2 signalling, a pathway recently implicated in the pathogenesis of periodontal disease [74]. Thus the innate immune responses are often regulated by several miRNAs that can either inhibit or trigger inflammatory pathways, mostly via the NF-KB signalling [75].

#### **miRNAs IN ADAPTIVE IMMUNE RESPONSE**

The adaptive immune system provides immunological memory to the host. It involves both T-and B-cell mediated responses. Adaptive immunity plays role in established periodontal lesions and this accounts for the high proportions of antibody-producing plasma cells in these lesions [76]. Several miRNAs are potent regulators of adaptive immunity with suspected roles in periodontal disease. miR-146a plays a significant role in B cell development and induce a brake on autoimmunity [77]. This miRNA exerts an immune restriction that is critical for adequate B cell response [78]. miR-650 is another micro RNA that influences the proliferation capacity of B cells [79]. Both these are found to be up-regulated in periodontitis lesions. miR-155 plays a role in innate as well as adaptive immune response [80]. They regulate type I interferon signalling and control CD8 T-cell responses [81,82]. They activate cytokine gene expression in Th17 and T regulatory cells [83]. Through the dendritic cell signalling, they indirectly activate Th17 responses [84] and by inhibition of interferon-gamma signalling, they induce Th1 differentiation of CD4+ cells [85]. miR-210 acts contrary to miR-155 and are under-expressed in periodontitis lesions. They impair the immune suppressive functions of regulatory T-cells [86]. When down-regulated, they promote regulatory T-cell signalling [87]. miR-155 bring about biological effects by direct action on target genes. Moreover, there is a hierarchy of miRNAs that are directly or indirectly regulated by miR-155, bringing about certain biological effects. For example, miR-155 deficiency results in lower levels of miR-455 in murine dendritic cells [88]. Thus miRNA signalling has a crucial role in the regulation of adaptive immunity in periodontal disease.

#### **microRNAs IN BONE REMODELLING**

The miRNAs implicated in healthy or periodontitis-affected gingival tissues have specific role in bone homeostasis also. miR-148a, induced by receptor activator of NF-KB ligand (RANKL), [89] is a micro RNA mediating osteoclastogenesis in CD14+ peripheral blood mononuclear cells. On the contrary, miR-29a modulates signalling in human osteoblasts leading to osteoblast differentiation [90]. miR-31 is found to be down regulated in periodontitis affected gingival tissues. They mediate osteoclastogenesis and repress osteogenesis leading to impaired osteoclast formation and reduced bone resorption [91, 92, 93, 94] Similarly, miR-200c is also down regulated in periodontitis. They are known to induce odontogenesis and osteoclastogenesis [27], thereby regulating the excessive bone loss in periodontal inflammation.

#### **CONCLUSIONS**

Since the discovery of miRNAs in the earlier 1990s, numerous studies have been made on their production, effects on gene expression and involvement in various physiological and pathological events. Most of these studies were conducted in vitro but to know the endogenous miRNA functions, in vivo studies are also required. miRNAs can modulate the expression of target mRNAs in different pathways. They may also regulate other non-coding RNAs at post transcriptional levels so the miRNA regulatory cascades are not limited to protein coding transcripts. It is now a known fact that circulating miRNAs can serve as biomarkers for diseases. They also play important roles in intercellular communication. Some miRNAs can even interact with cell surface receptors, such as Toll like receptors thus having hormone like activities too. miRNAs have key role in regulating gene expression in mammalian cells affecting several molecular and cellular pathways. Their specific role in maintaining homeostasis of periodontal tissues is well documented. Future studies are needed to expand these early findings, thus aiming to understand the differential expression of several miRNAs between 'healthy' and 'diseased' gingival tissues. This can ultimately enhance our understanding of the pathobiology of periodontal diseases.

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The author reports no conflicts of interest. The author alone is responsible with the content of this article.

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