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Mini Review

The role of microRNAs in the pathogenesis of rheumatoid arthritis

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Recently, microRNAs (miRNAs or miRs) have been found to play a role in the pathogenesis of rheumatoid arthritis (RA). The expression levels of miRNAs vary in different cell types and thus, the function of miRNAs should be distinct depending on the type and status of the cell. Inflammatory signals induce the expression of some specific miRNAs, such as miR-146a and miR-155, which are known to be up-regulated in the synovium in RA. These miRNAs presumably play a role in RA pathogenesis by regulating the inflammatory status. However, the precise functions of each miRNA in the pathogenesis of RA remain to be discovered. Generation of conventional knockout mice for each miRNA has been a rate-limiting step in gaining further insights into the role of miRNAs in arthritis and autoimmune diseases. However, recent developments in gene editing technologies, such as TALEN (transcription activator-like effector nuclease) and CRISPR/Cas9 systems, allowed the generation of precisely targeted miRNA knockout mice within a short time and at a cheaper cost, thereby accelerating *in vivo* studies of miRNA functions. These new technologies have allowed a more systematic and comprehensive analysis of the role of miRNAs in RA. This will lead to a better understanding of RA pathogenesis and, hopefully, to a discovery of new molecular targets for RA therapy.

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Introduction

MicroRNAa are small noncoding RNAs that regulate gene expression by binding to the 3' untranslated region (3' UTR) of target mRNAs. Several miRNAs are up- or down-regulated in synoviocytes and immune cells in inflamed arthritic joints and therefore they are thought to be involved in the pathogenesis of arthritis (Refs. 1, 2, Fig. 1). Among various types of immune cells infiltrating into the arthritic joints, $CD4^+$ T cells are particularly important for the pathogenesis of RA. Recent findings suggest that $CD4^+$ T cells can be regulated by several miRNAs in RA. However, the precise role of miRNAs in the development of arthritis is still largely unknown, partly because it is difficult to generate knockout mice for each individual miRNA, but also due to problems in the delivery of therapeutic mimetics and inhibitors of miRNAs. Recently, novel genome editing methods, such as TALEN and CRISPR/Cas9 system, have been developed and successfully utilized in the generation of mouse knockouts[3, 4]. These new methods can significantly shorten the time required to make a novel miRNA knockout mouse line. In this review, we summarize the recent findings concerning the roles of miRNAs in RA and introduce a new strategy for miRNA research using the gene editing technique.

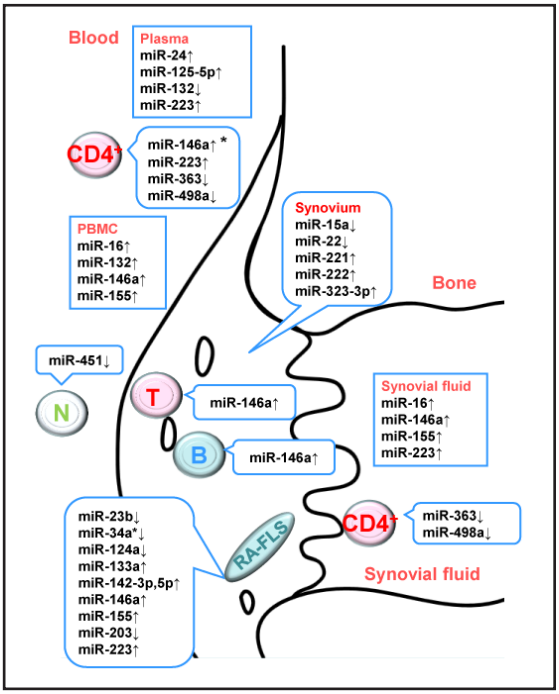

Fig. 1 Expression of miRNAs in the human arthritic joint

Expression of miRNAs in human joints affected by the rheumatoid arthritis is compared to that in the osteoarthritis condition or in healthy controls. T: $CD3^+$ T cell, B: $CD79a^+$ B cell, N: Neutrophil. ↑ : Up-regulated, ↓ : down-regulated miRNA compared to levels in osteoarthritis or healthy controls., *: The expression of miR-146a in $CD4^+$ T cells from the PBMC pool is debatable.

Essential role of miRNAs in T cells

Functions of miRNAs have been extensively examined during the past decade[5]. Almost all precursor miRNAs are recognized and processed into double-stranded miRNAs by the endonuclease Dicer protein. Dicer is essential for the mouse embryogenesis. A genetic deletion of Dicer in mice leads to embryonic lethality at the early stage of embryogenesis[6]. To explore the function of miRNAs in $CD4^+$ T cells that are known to play a critical role in RA and other autoimmune diseases, conditional Dicer-deficient $CD4^+$ T cells were generated and examined for the phenotype. The $CD4^+$ T cells in Dicer deficient mice showed heightened sensitivity to T cell receptor (TCR) signaling, IL-2 production, and proliferation in the absence of co-stimulation[7, 8].

Foxp3 positive cells (regulatory T cells, Tregs) act to repress the excess immune reaction, thus a dysregulation of Tregs may be involved in RA pathogenesis. A $CD4^+$-specific Dicer depletion reduced Treg numbers and resulted in the immune pathology[9]. Foxp3-specific Dicer-deficient cells showed a lack of their immunosuppressive activity *in vivo* and the Foxp3-specific Dicer-deficient mice rapidly developed a fatal systemic autoimmune disease. These findings suggest that T cells may utilize miRNAs during immune reactions, whereas a dysregulation of the miRNA expression could be a cause of autoimmune disorders.

Function of miR-146a and miR-155 in rheumatoid arthritis

A deficit of Dicer halts the biogenesis of almost all miRNAs. To identify specific miRNAs that play a critical role in $CD4^+$ T cells, several miRNAs have been examined for their potential functions in the immune activity and RA pathogenesis. Among them, the roles of miR-146a and miR-155 in RA are well established. The expression of miR-146a was first reported in the synovium derived from patients with RA in 2008[10, 11]. MiR-146a is an anti-inflammatory mediator because its major target genes are *TRAF6* and *IRAK1* that mediate inflammatory signals[12]. Three prime-UTR of *TRAF6* has four binding sequences

for miR-146a and the number of binding sites of mRNA 3' UTR against miRNAs is important for its inhibitory effect on the target expression. T cells were activated and general inflammation was induced in miR-146a knockout mice. T cells lacking miR-146a were hyperactive in both acute antigenic responses and chronic inflammatory autoimmune responses. T cells without the Treg from miR-146a knockout (KO) mice induced the mononuclear cell infiltration into tissues with subsequent severe tissue destruction.

MiR-155 knockout mice developed neither synovial inflammation nor cartilage or bone destruction after the collagen immunization unlike miR-146a deficiencies[13, 14]. Th17 cytokines IL-17 and IL-22 were found to be significantly reduced in miR-155 knockout mice compared to wild type after CD3 stimulation *in vitro*. An overexpression of miR-155 induced the production of TNFα, IL-6, and IL-8 in monocytes. MiR-155 was up-regulated in T cells, B cells, and synovial fibroblasts in the arthritic joint and this modulation could enhance the inflammation of the synovium.

MiR-146a and -155 expressed in Tregs have been reported to regulate immune responses to several diseases but not during RA. Recently, Zhou Q and colleagues reported that a decreased expression of miR-146a and miR-155 contributed to an abnormal Treg phenotype in patients with RA. MiR-146a facilitates a pro-inflammatory phenotype of Tregs via the increase in the STAT1 activation[15]. The latter report revealed a role of miR-146a in Tregs during RA. MiR-146a and miR-155 were induced by inflammatory signals and expression of these miRNAs in peripheral blood mononuclear cells (PBMCs) was higher in patients with RA, compared to osteoarthritis patients or healthy controls (HC) in Fig.1. However, Zhou Q et al. did not observe differences in the miR-146a expression levels in $CD4^+$ T cells derived from RA patients and from HC. TCR signaling reduced miR-146a expression in both Tregs and CD25-$CD4^+$ cells. It was proposed that the difference could be caused by the differences in the backgrounds of the patients. MiR-146a expression may be influenced by disease progression, medical treatment, and disease duration (stage of disease). MiRNA profiles are different in naïve Tregs and memory Tregs derived from patients with RA, so the miRNA signature can predict the Treg phenotype[16].

Other miRNAs in RA pathogenesis

In addition to miR-146a and miR-155, many other miRNAs have been reported to be up- or down-regulated in RA synovium (Refs. 1, 2, Fig.1). Almost all RA related miRNAs were expressed in PBMC or fibroblast-like synoviocytes (FLS). MiR-451 is expressed in neutrophils and is unique as it does not require Dicer for its maturation process[17, 18]. MiR-451 expression in neutrophils isolated from patients with RA was lower than that in healthy controls[19]. A potential function of miR-451 may be the suppression of the neutrophil chemotaxis via p38 MAPK (mitogen-activated protein kinase) repression.

The expression levels of miR-23b and miR-223 have been shown to be related to disease activity in an arthritis animal model. MiR-23b was expressed in human FLS, mouse primary kidney cells and astrocytes, which may be subjected to autoimmune responses. MiR-23b acts as a suppressor of IL-17-associated autoimmune inflammation by targeting *TAB2, TAB3*, and *IKK-a*[20]. On the other hand, IL-17-dependent reduction of miR-23b in FLS causes an up-regulation of the target inflammatory genes resulting in a prolonged inflammation.

MiR-223 regulates $CD3^+$ T cell functions and osteoclasts differentiation. Overexpression of miR-223 suppressed the IL-10 production in $CD3^+$ T cells[21]. Introduction of lentiviral vectors expressing the miR-223 target sequence, that could inhibit native miR-223 functions as a decoy, lowered the arthritis scores and decreased osteoclastogenesis during the collagen-induced arthritis (CIA), suggesting that miR-223 could be a therapeutic target for RA[22].

In addition to miRNAs within intact cells, circulating miRNAs in RA patients are reported to be potential markers of disease progression. The expression of both miR-16 and miR-223 in sera were not significantly different between established RA and HC. However, both miR-16 and miR-223 could be predictors for the disease outcome in only early RA[23]. At this moment, it is unclear how these circulating miRNAs are involved into RA pathogenesis.

TALEN and CRISPR/Cas9 system for miRNA knockout

Human miRNAs are coded in intragenic (1072 miRNAs) or intergenic (799 miRNAs) regions of protein-coding genes[24]. Intragenic miRNA contains intronic and exonic miRNAs. Intronic miRNAs usually share common transcriptional mechanisms and thus expression patterns with its host gene, although some exceptional intronic miRNAs are transcribed by the independent transcription units of host genes[25]. The expression correlation between intragenic miRNAs and their host genes across five tissues are

summarized in the miRAD web-database. While most RA-related miRNAs described above are intergenic miRNAs that can be independently transcribed as protein-coding genes, miRNAs such as miR-23b, miR-24, and miR-133a are intragenic miRNAs. MiR-23b is an intronic miRNA and there is a strong positive correlation between the expression of this miRNA and the host gene (rho=1, Spearman's rank correlation). MiR-23b and its host gene may share their transcriptional regulation. Analysis of microarray data could not clearly distinguish between miR-24-1 and miR24-2. These two mature miRNAs have the same sequence. MiR-24-1 is intronic (rho=0.9), whereas miR-24-2 is an intergenic miRNA. MiR-133a also comprises two miRNAs derived from distinct genomic loci: miR-133a-1 is intronic (rho=-0.7) and miR-133a-2 is exonic (rho=1).

To analyze the relationship between a host gene and an intragenic miRNA, it is necessary to compare the phenotypes of mouse mutants bearing loss-of-function mutations for this miRNA and its host gene. However, it used to be difficult to generate double mutants or even a single mutant without affecting the expression of the host gene. TALEN and CRISPR/Cas9 systems are powerful technologies by which knockout mice can be generated without using ES cell lines, i.e., within a shorter period compared to the typical time required for creating a knockout by conventional gene targeting methods. In case of the miRNA knockout using TALEN, both the guide and the opposite, “passenger” miRNA strands can be depleted by a single double-strand break in the genomic sequence of the precursor miRNA[4]. We recently generated miR-146a KO mice using TALEN system. MiR-146a mutants had 2 to 32 base pairs deletion of nucleotides containing the cutting site FokI on the mature guide strand miRNA. Simultaneous disruption of the guide and passenger miRNA strands by a single TALEN-mediated deletion is possible because the precursor miRNA cannot be maintained as a hairpin structure. TALEN and CRISPR/Cas9 systems also permit a specific deletion of the miRNAs in the genome without affecting its host gene, Therefore, TALEN and CRISPR/Cas9 systems may become an extremely useful tool to reveal the relationships between miRNAs and their host genes. TALEN system enables the deletion of clusters containing several miRNAs using two pairs of TALENs[26]. CRISPR/cas9 system also delete large-scale genome[27]. TALEN and CRISPR/cas9 systems enable short/long editing of genome for the generation of genome-modified animals to clarify function of miRNAs. We expect that the number of studies using miRNA knockout mice created by means of the TALEN and CRISPR/cas9 system will undoubtedly increase in the near future.

Summary

Expression of specific miRNAs in RA has been demonstrated by many studies. Among them, the function of some miRNAs gradually have become clear in inflammatory conditions. However, the role of these miRNAs in the pathogenesis of RA is still largely unknown. To clarify the role of miRNAs in RA pathogenesis, it is important to identify miRNA-target genes interactions and the function of miRNAs in various cell types. TALEN or CRISPR/cas9 systems could be powerful technology to clarify the function of miRNAs.

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Conflict of interests

No potential conflicts of interest are disclosed.

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