Recent findings regarding the effects of microRNAs on fibroblast-like synovial cells in rheumatoid arthritis

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ABSTRACT
Rheumatoid arthritis (RA) is a systemic autoimmune disease with severe joint inflammation and destruction characterized by marked hyperplasia of the lining layer of the synovium. Fibroblast-like synovial cells (FLS) is a key cellular component within the synovia; it plays pivotal roles in RA pathogenesis by unfavorable behaviors such as producing inflammatory cytokines and chemokines, and hyperproliferation. MicroRNAs are evolutionarily conserved small non-coding RNAs (length is 18–25 nucleotides) that regulate gene expression at the post-transcriptional level. There is increasing interest in the involvement of microRNAs in autoimmune diseases including RA. Recent studies revealed the regulation of the function of FLS by microRNAs. Here, we review the known functional microRNAs in RA and summarize the potential uses of these small molecules in the treatment of RA.

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1. Introduction
Rheumatoid arthritis (RA) is a chronic and severe inflammatory disease, characterized by progressive bone and cartilage destruction, resulting in joint destruction and function disability. RA affects approx. 1% of the world’s population [1]. Recent progress in the treatment of RA (such as the use of biologics) has provided better outcomes including the achievement of remission, but not all patients with RA will obtain such benefits due to economic constraints, inadequate responses, and other problems. The etiology and pathogenesis of RA remain largely unknown, and there is a great need to elucidate the pathogenesis of RA and to develop satisfactory therapeutic options.

One of the well-known characteristics of RA pathology is marked hyperplasia of the lining layer of the synovium, leading to the destruction of articular cartilage and bone. Fibroblast-like synovial cells (FLS) is thus suspected to play a pivotal role in the pathogenesis of RA. In fact, RA-FLS contribute to the production of pro-inflammatory cytokines, small molecule mediators of inflammation, and collagenolytic enzymes that degrade the extracellular matrix [2]. In addition, RA-FLS shows prolonged cell death which come from the imbalance of apoptosis and over-proliferation.

MicroRNAs (miRNA) are small non-coding RNA molecules that are implicated in the regulation of post-transcriptional gene expression. miRNAs suppress protein synthesis by destabilizing target mRNAs or inhibiting mRNA translation by base-pairing with the 3’ untranslated region (3’-UTR) of target mRNAs [3]. There is growing evidence that the deregulation of miRNAs is implicated in the pathogenesis of human diseases [4–6]. In addition to the potential involvement of miRNAs in pathogenesis, recent studies revealed the potential of several miRNAs as therapeutic target [7,8].

Many research groups have investigated the roles of miRNAs in the pathogenesis of RA. For example, the serum miR-24, miR-26a and miR-125a were increased in RA patients [9], and miR-451 was expressed by T cells from RA patients and associated with disease activity [10]. As mentioned above, FLS play a crucial role in the pathogenesis of RA. The elucidation of unfavorable behaviors of RA-FLS via miRNA deregulation will contribute to the clarification of RA’s pathogenesis and to miRNA-targeted therapy. The use of miRNAs to improve the function of RA-FLS might become a one of treatment option in RA treatment. This review therefore focuses on the potential roles played by miRNAs in RA-FLS. We summarize the current understanding of the roles of miRNAs in RA-FLS, and we highlight the functions of RA-FLS in the production of cytokines, chemokines and collagenolytic enzymes, hyper-proliferation, and the therapeutic potential of miRNAs based on the osteogenic ability of RA-FLS.
2. The biogenesis and functions of microRNAs

The biogenesis and maturation of miRNAs occurs in multiple steps. First, within the cell nucleus, RNA polymerase II transcribes the genes to the primary microRNA(pri-miRNA), which is characterized by a hairpin. Next, pri-miRNAs are processed by the multimeric protein complex called Drosha and the DiGeorge syndrome critical region protein 8 (DGCR8) into the precursor miRNA(pre-miRNA), which is shorter than pri-miRNA (70–80-nt) [11]. The pre-miRNA is then recognized by exportin 5 and transported to the cytoplasm. Subsequently, in the cytoplasm, Dicer, an endonuclease RNase III, cleaves pre-miRNA to release the miRNA/miRNA* duplex. A mature duplex miRNA is loaded onto argonaute (AGO) proteins, after which the guide strand generates the RNA-induced silencing complex (RISC), which suppresses the expression of target genes by complementary binding to the target mRNAs. In the RISC/mRNA complex, mature miRNA recognizes its target mRNA by the small (6–8-nt) miRNA fragment named the "seed" region. Complementary binding between the seed region and the 3’-UTR of target mRNA leads to translational repression or degradation [12,13]. Through these steps, microRNAs mediate the repression of that target. Each miRNA regulates hundreds to thousands of target genes [14].

3. Cytokine, chemokine and collagenolytic enzyme targeting by microRNAs in RA-FLS

Persistent and excessive cytokine and chemokine production is a hallmark of RA, and it may play a critical role in disease pathogenesis and amplification. RA-FLS is known as one of the cell types that produce excessive amounts of cytokines and chemokines in RA. The production of collagenolytic enzymes such as matrix metalloproteinases (MMPs) from RA-FLS also contributes to RA pathogenesis. The regulations of these productions in RA-FLS by miRNAs has been reported; the regulation can be either "direct" on the target, or "indirect" via other target genes that influence the production of cytokines, chemokines and collagenolytic enzymes.

miR-203 was highly expressed in RA-FLS and, an enforced expression of miR-203 increased the production of MMP-1 and interleukin (IL)-6. Moreover, the basal expression of IL-6 was correlated with the basal expression of mir-203 on RA-FLS [15]. miRNA expression is known to be affected by inflammation, and to simulate an inflammatory condition such as the active phase of RA, lipopolysaccharide (LPS) has been used in experiments. Phillipe et al. reported that LPS-mediated RA-FLS showed a lower expression of miR-20a compared to control RA-FLS. miR-20a directly targeted apoptosis signal-regulating kinase (ASK) 1, a key component of the toll-like receptors 4 pathway, and the overexpression of miR-20a decreased the production of C-X-C motif chemokine ligand(CXCL)-3 and IL-6 from RA-FLS. These findings suggested that miR-20a controls the release of proinflammatory cytokines and chemokines by controlling the ASK1 expression in RA-FLS [16].

miR-346 has been described as a modulator of both the expression of IL-18 and tumor necrosis factor (TNF)-α in RA-FLS. LPS-mediated RA-FLS showed an increased expression of miR-346, and the knockdown of miR-346 in LPS-mediated RA-FLS increased the expression of IL-18 by indirectly inhibiting Bruton’s tyrosine kinase expression [17]. Not only IL-18, TNF-α expression was also upregulated by knockdown of miR-346 via tristetraprolin (TTP), an RNA-binding protein that inhibits TNF-α synthesis [18]. miR-17 is known to be related to TNF-α signaling. The expression of miR-17 was significantly low in the serum and synovial tissues of patients with RA, and TNF-α signaling-related genes such as TNF receptor associated factor (TRAF)2, cellular inhibitor of apoptosis protein (cIAP)1, and cIAP2 were reduced by miR-17 in TNF-α stimulated RA-FLS. Moreover, the overexpression of miR-17 reduced the expression of TRAF2, resulting in a reduction of the production of IL-18, IL-8, MMP-1 and MMP-13 in RA-FLS. These results provided evidence of the role of miR-17 as a negative regulator of TNF-α signaling [19].

The relationship between miR-19 and RA-FLS was reported in two studies. miR-19 was downregulated in LPS-mediated RA-FLS. The overexpression of miR-19 decreased the expression of Toll-like receptor (TLR)2 protein in RA-FLS. In parallel, the secretion of IL-6 and MMP-3 were also decreased. Thus, miR-19 seemed to regulate the release of IL-6 and MMP-3 by controlling TLR2 expression [20]. Another study reported that miR-19 suppressed negative regulators of NF-κB (such as tumor necrosis factor alpha-induced protein(TNFAIP)3 and Rnf11), and the enforced expression of miR-19 exacerbated the inflammatory activation of RA-FLS by controlling NF-κB signaling [21]. The NF-κB signaling pathway is a key pathway that contributes to the abnormal stromal environment created by RA-FLS via the secretions of proinflammatory cytokines and growth factors [22].

In addition to the above findings, it has been indicated that several miRNAs modulate NF-κB signaling in RA-FLS. The expression of miR-18a was upregulated by TNF-α in an NF-κB-dependent manner in RA-FLS. MMP-1, IL-6, IL-8, and monocyte
chemoattractant protein (MCP)-1 were upregulated following the enforced expression of miR-18a in RA-FLS. In parallel, the enforced expression of miR-18a strongly activated NF-κB signaling by targeting TNFAIP3, an NF-κB pathway inhibitor. In summary, miR-18a might contribute to chronic inflammation in the joints through a positive feedback loop in NF-κB signaling [23]. Also, miR-10a controls NF-κB signaling. The knockdown of miR-10a, which was downregulated in RA-FLS as compared to osteoarthritis (OA)-FLS, accelerated the degradation of inhibitor of NF-κB (IkB) and NF-κB activation by targeting interleukin-1 receptor-associated kinase 4 (IRA4K4), TGF-β-activated kinase 1 (TAK1), and β-transducin repeat-containing protein 1 (BTRC). This activation of NF-κB signaling significantly promoted the production of various inflammatory cytokines, including IL-1β, TNF-α and IL-6 [24].

4. Modulation of apoptosis and cell invasion by microRNAs in RA-FLS

The prolonged cellular lifespan is a fundamental characteristic of activated FLS in RA. The acquired anti-apoptosis property of RA-FLS, results in hyperplastic synovial tissue (which is also called pannus) and the destruction of cartilage and bone. Several miRNAs regulate the apoptosis property of RA-FLS. miR-34a* was decreased in RA-FLS compared to OA-FLS, and the enforced expression of miR-34a* in RA-FLS increased apoptosis. X-linked inhibitor of apoptosis (XIAP) was considered as a target of miR-34a, namely miR-34a* decreased XIAP and increased apoptosis [25]. miR-155, whose altered expression in RA-FLS was considered as a target of miR-34a, namely miR-34a* decreased XIAP and increased apoptosis [25]. miR-155, whose altered expression in RA-FLS was reported in three studies, decreased the proliferation and invasion of RA-FLS, but the targeted genes have not been explored [26].

miR-15a was found to be downregulated and to contribute to apoptosis resistance, since miR-15a is a negative regulator of the B cell lymphoma 2 (Bcl-2), a suppressor of cell apoptosis [27]. Another study showed that the overexpression of miR-15a in synovium increased cell apoptosis due to Bcl-2 dysregulation [28]. miR-221 was upregulated in LPS-mediated RA-FLS, and the inhibition of miR-221 led to increased apoptosis and decreased cell migration as well as invasion. Furthermore, the inhibition of miR-221 decreased the expressions of survivin and XIAP [29].

As a not-targeted apoptosis-related protein, miR-663 has shown a relationship with the proliferation of RA-FLS. miR-663 was increased in synovium from RA patients, and the overexpression of miR-663 suppressed the expression of adenosomatous polyposis coli (APC) in RA-FLS. This APC suppression was followed by the activation of canonical Wnt signaling, resulting in FLS proliferation [30]. In another study, miR-124a was especially low in RA-FLS compared to OA-FLS, and its overexpression on RA-FLS suppressed their proliferation and arrested the cell cycle at the G1 phase. One of the targets of miR-124a was cyclin-dependent kinase (CDK)2, the cell cycle regulator, and thus miR-124a inhibited cell proliferation [31]. Detailed analyses of miR-124a were performed using a rat model of RA, and the overexpression of miR-124a improved the disease severity and suppressed the FLS proliferation in the model [32].

With respect to miRNA, little knowledge is available regarding whether miRNA contribute to RA pathogenesis as part of the inflammatory response during the early stage or late stage. The function of miR-188-5p, which was down-regulated in RA synovial tissue, was analyzed using both aggressive RA-FLS (early-passage RA-FLS) and less-aggressive RA-FLS (long-time cultivated RA-FLS). miR-188-5p was downregulated in both type of cells, but less downregulation was observed in the less-aggressive RA-FLS. After exposure to IL-1β, RA-FLS showed levels of miR-188-5p expression that were decreased by approx. 20%. The overexpression of miR-188-5p diminished the migration ability in both the aggressive and less-aggressive RA-FLS, but this effect in the less-aggressive RA-FLS was less pronounced than the effect of migration in the aggressive RA-FLS. These results suggested that the contribution of miR-188-5p to RA pathogenesis was more important in the early phase of RA [33].

Related to phosphoinositide-3-kinase (PI3K) and miRNA, the miR-126 expression was negatively correlated with the expression of phosphatidylinositol 3-kinase regulatory subunit beta (PIK3R2) in RA-synovial tissues. The overexpression of miR-126 decreased PIK3R2 protein, promoted proliferation, and reduced the apoptosis of RA-FLS, whereas the inhibition of miR-126 increased the apoptosis [34].

It is known that a hypoxic environment in a joint may be one of the causes of hyperplasia in RA [35]. miR-191 was upregulated in RA-FLS cultured in hypoxia (3% O2) compared to those cultured in normoxia (21% O2). The overexpression of miR-191 increased cell proliferation by promoting G1/S transition of the cell cycle and suppressing apoptosis induced by cell starvation. CCAAT/enhancer binding protein β (C/EBPβ) was confirmed as a target gene of miR-191 in RA-FLS and, the enforced expression of C/EBPβ rescued miR-191-induced cell proliferation. Thus, the miR-191-C/EBPβ signaling pathway seemed to mediate the hypoxia-induced cell proliferation in RA [36].
thylation of miR-124a gene was confirmed by a RA-FLS. After treatment with 5-AzadC, the deme-
cantly increased the miR-124a expression in (5-AzadC), a DNA methylation inhibitor, signifi-
miR-17 TRAF2, cIAP1 Downregulated in synovial tissue NF-

5. DNA methylation-related microRNAs in RA-FLS

In addition to miRNAs, other epigenetic alterations have been known to contribute to the etiology and progression of several diseases including RA. DNA methylation is one of the most prevalent epigenetic alterations in RA [37–39]. Several miRNAs have been shown to be associated with DNA methylation. miR-29 is one of the DNA methylation-related miRNAs. Re-methylation might be a therapeutic option in RA because the genomic DNA in RA-FLS is generally hypomethylated. Betaine affects DNA methylation, and miR-29 was increased in RA-FLS treated with betaine. The DNA methyltransferase (DNMT)3A was defined as a target of miR-29 in RA-FLS. Thus, miR-29 might repress the re-methylation effect of betaine [40]. Miao et al. showed that miR-152 was downregulated in an arthritis rat model, and the overexpression of miR-152 in FLS decreased the expression of DNMT1. The reduction of DNMT1 caused an upregulation of secreted frizzled-related protein (SFRP)4, the negative regulator of the WNT signaling pathway. An overexpression miR-152 inhibited the activation of the canonical WNT pathway and resulted in a reduction of FLS proliferation. Thus, miR-152-mediated DNA methylation could inhibit the WNT signaling pathway through SFRP4 expression [41].

Other studies revealed that DNA methylation regulated the expression of miRNAs. Zhou et al. showed that treatment with 5-Aza-2'-deoxycytidine (5-AzadC), a DNA methylation inhibitor, significantly increased the miR-124a expression in RA-FLS. After treatment with 5-AzadC, the deme-
thylation of miR-124a gene was confirmed by a methylation-specific polymerase chain reaction (PCR). The inhibition of miR-124a cancelled the IL-1β-mediated proliferation of RA-FLS caused by 5-AzadC treatment. This suggested that the methylation of miR-124a genes contributed to the IL-1β-mediated proliferation of RA-FLS [42]. As mentioned in the section entitled “Cytokine, chemo-
kine and collagenolytic enzyme targeting by microRNA in RA-FLS,” miR-203 is related to cyto-
kine production. The role of DNA methylation about miR-203 was further analyzed in that study, pro-inflammatory cytokines such as TNF-α and IL-1β did not increase the miR-203 expression; however, DNA demethylation with 5-azacytidine (5-azaC) increased miR-203 expression [15].

6. microRNA promote the osteogenic
differentiation of RA-FLS

RA-FLS conserve mesenchymal properties, and in vitro studies have shown that appropriate stimu-
lation in culture induces the differentiation of FLS into osteoblasts [43]. If proliferating FLS in RA joints could be differentiated into osteoblasts, this might become a treatment option for RA. miR-218 was upregulated during an osteogenic induction medium-mediated osteogenic differentiation of RA-
FLS in the early phase (until 12 h) and was then downregulated in the late phase (until 21 days). Most interestingly, RA-FLS could be differentiated into osteoblast by miR-218. Roundabout1 (ROBO1), known as an osteogenic-related gene [44], was downregulated by the overexpression of miR-218 in RA-FLS, whereas ROBO1 was upregulated by the knockdown of miR-218. Both the overexpression of miR-218 and the knockdown of ROBO1 decreased

| microRNA | Targets | Expression in RA-FLS | Related pathway | Reference |
|----------|---------|----------------------|----------------|-----------|
| miR-203  | Not identified | Upregulated | NF-κB signaling | [15] |
| miR-20a  | ASK1     | Downregulated in response to LPS | TLR signaling | [16] |
| miR-346  | BTB      | Upregulated in response to LPS | TLR signaling | [17,18] |
| miR-17   | TRAF2, cIAP1 | Downregulated in synovial tissue | NF-κB signaling | [19] |
| miR-19   | TNFAIP3, Rnf1 | Downregulated in response to LPS | TLR signaling | [20,21] |
| miR-18a  | TNFAIP3  | Upregulated in response to TNF-α | NF-κB signaling | [23] |
| miR-10a  | IRAK4, TAK1, BTRC | Downregulated | NF-κB signaling | [24] |
| miR-34a  | XIAP     | Downregulated | [25] |
| miR-155  | IKBKE    | Upregulated | [26] |
| miR-15a  | Bcl-2    | Downregulated | [27,28] |
| miR-221  | XIAP     | Upregulated in response to LPS | Wnt signaling | [29] |
| miR-663  | APC      | Upregulated | [30] |
| miR-124a | CD2K     | Downregulated | [31] |
| miR-188  | Not identified | Downregulated | [33] |
| miR-126  | PIK3R2   | Upregulated in synovial tissue | PI3K-AKT signaling | [34] |
| miR-191  | C/EBPβ  | Upregulated in hypoxia environment | [36] |
| miR-29   | DNMT3    | DNA-methylation related | DNA-methylation related | [40] |
| miR-152  | DNMT1    | DNA-methylation related | Wnt signaling | [41] |
| miR-218  | ROBO1    | Upregulated during osteogenic differentiation | Wnt signaling | [45] |

5-AZa-deoxycytidine; LPS: lipopolysaccharide; TLR: Toll-like receptor; TDF: TNF receptor associated factor; cIAP: cellular inhibitor of apoptosis protein; TNFAIP: tumor necrosis factor alpha-induced protein; IRAK: interleukin-1 receptor-associated kinase; TAK: TGF-β-activated kinase; BTRC: β-transducing repeat-containing protein; XIAP: X-linked inhibitor of apoptosis; IKBKE: Inhibitor of Nuclear Factor Kappa B Kinase Subunit Epsilon; Bcl: B cell lymphoma; APC: adenomatous polyposis coli; CDK2: cyclin-dependent kinase; PI3K: phosphatidylinositol 3-kinase regulatory subunit beta; C/EBPβ: CCAAT/enhancer binding protein β; DNMT: DNA methyltransferase; ROBO1: Roundabout1.
the expression of Dickkiof (DKK)-1 (which has been shown to be a potent inhibitor of Wnt/β-catenin signaling) in RA-FLS. These results suggested that miR-218 modulated the osteogenic differentiation of RA-FLS through ROBO1/DKK-1 axis, and induction of miR-218 has potential to be therapeutic option of RA [45].

7. Concluding remark

Recent studies revealed that miRNAs play critical roles in RA-FLS, which may be involved in the pathogenesis of RA and potential therapeutic options for RA (Table 1). Of course, many immune-competent cells such as T cells and macrophages contribute to RA pathogenesis, but fibroblast-like synovial cells are also one of the key effector cells in RA and have the potential to become a therapeutic target. miRNAs (small non-coding RNAs implicated in several diseases including autoimmune diseases) have been revealed to modulate unfavorable behaviors of RA-FLS such as "tumor-like" invasion and aberrant cytokine production. Although much work remains to be performed to gain an integrated overview of the miRNAs related to RA-FLS, such research may lead to the clarification of the critical roles of miRNA in RA-FLS. These miRNAs are interesting candidates for next-generation drugs for RA.

Disclosure statement

No potential conflict of interest was reported by the authors.

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