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MicroRNAs in mucosal inflammation

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Abstract

Of the total human body's surface, the majority is internal surface, belonging to the lungs (100 m²) and intestinal tract (400 m²). In comparison, the external surface area, belonging to the skin, comprises less than 1% (2 m²). Continuous exposure of the mucosal surface to external factors (e.g., pathogens, food particles) requires tight regulation to maintain homeostasis. MicroRNAs (miRNAs) have gained noticeable attention as playing important roles in maintaining the steady-state of tissues by modulating immune functions and inflammatory responses. Accordingly, associations have been found between miRNA expression levels and human health conditions and diseases. These findings have important implications in inflammatory diseases involving pulmonary and intestinal mucosa, such as acute lung injury or inflammatory bowel disease. In this review, we highlight the known biology of miRNAs and discuss the role of miRNAs in modulating mucosal defense and homeostasis. Additionally, we discuss miRNAs serving as potential therapeutic targets to treat immunological conditions, particularly mucosal inflammation.

Keywords

miRNA; Mucosal inflammation; Mucosal immunity; Epithelial inflammation; Epithelial immune response; Barrier function; Pulmonary inflammation; Acute and chronic lung disease; Inflammatory bowel disease

Introduction

MicroRNAs (miRNAs), single-stranded, non-coding RNA molecules consisting of 20–24 nucleotides that bind to messenger RNAs (mRNAs) and act as posttranscriptional regulators. They are involved in the control of almost all cellular functions and influence the expression of genes in various regulatory networks. Accordingly, miRNAs are considered to be critical players in the development and pathophysiology of human diseases [1–5]. The first study reporting the discovery of miRNAs was in 1993 in *C. elegans* [6]. Almost a decade later, miRNAs were discovered in mammals. These studies showed miRNAs to regulate critical processes, such as developmental timing, cell differentiation and proliferation, and cell death [7, 8]. Subsequent screening studies turned out to identify miRNAs with tissue- and cell-specific functions [7, 9] and that miRNA expression levels correlated with various human

diseases and health conditions such as mucosal inflammation [10, 11]. The steady-state maintenance of the mucosal surface involves various regulatory networks that work to thwart homeostatic threats, such as microbes, toxins, and allergens. A significant body of work has shown miRNAs are a crucial part of controlling these regulatory networks, helping to maintain mucosal barrier integrity or counterbalancing inflammatory responses [12–14]. For example, the process of mucosal barrier migration of granulocytes during mucosal inflammatory disease is regulated by miRNA-involved networks to prevent inflammatory responses from becoming excessively aggressive, thus limiting collateral tissue damage and ensuring appropriate resolution (Neudecker et al., Neutrophil-epithelial miR-223 shuttling dampens acute lung injury. *Science Translational Medicine*, in press). This review aims to provide readers with an understanding of the roles of miRNAs during physiologic and inflammatory processes impacting mucosal surfaces, particularly in the lung and the digestive tract. Moreover, we highlight the most recent miRNAs involved in mucosal inflammation and their potential use in the clinic in the near future.

Biological functions

MiRNA production

The first steps in the biogenesis of miRNAs is the same as for the majority of protein-coding genes, while the later steps in achieving the mature miRNA differ from other small RNAs (Fig. 1). During biogenesis, RNA polymerase II (Pol II) generates long primary transcripts (pri-miRNAs) [15] in the nucleus, which are processed within the nucleus to precursor miRNA (pre-miRNA) by Drosha, an miRNA exclusive RNase III enzyme. During the Drosha-dependent enzymatic step, RNase III associates with DiGeorge syndrome critical region 8 (DGCR8), a nuclear protein, and produces a hairpin-structured shorter precursor — the pre-miRNA. This pre-miRNA gets exported into the cytoplasm where another shortening process is conducted by a second RNase III enzyme, Dicer. The Dicer-dependent enzymatic step creates an unstable, short, double-stranded miRNA duplex of which one strand becomes the mature miRNA. Here, the single-stranded mature miRNA is incorporated into the RNA-induced silencing complex (RISC). RISC uses the single-stranded miRNA as a template for recognizing target RNA transcripts to cleave [16–20]. At present, a total of 2588 human mature miRNA sequences have been identified [21].

Regulation of miRNA expression

Similar to their production and maturation, the process regulating miRNA expression is complex and tightly regulated. Thus, any interruptions or deviations in the steps of biogenesis can alter miRNA expression and, as a consequence, modify gene expression. Indeed, various studies have shown that dysregulated miRNA expression leads to deleterious downstream effects, such as cell-cycle arrest, cellular senescence, and tumorigenesis [1]. Drosha and Dicer are crucial to miRNA biogenesis. Their critical importance is emphasized by the fact that the loss of either one of the enzymes is embryonically lethal [22, 23]. Studies have also revealed diverse regulatory mechanisms that control tissue-specific miRNA expression. This includes the selective export of specific pre-miRNAs into the cytoplasm and variations in miRNA stability and decay rates [24–26]. In addition, classical transcription factors (TFs) are also involved in the regulation of miRNAs, regulating their

expression positively or negatively in a tissue- or developmental-specific manner. Finally, negative and positive feedback loops between miRNAs and TFs appear to be a common phenomenon [27, 28], which further highlights the complexity of these regulatory networks. Due to the relevance of miRNAs in regulation of physiological and pathological processes, such as inflammation, understanding the modulation of miRNAs expression is crucial.

Mechanisms of target regulation

For the most part, miRNAs bind to target sequences in the 3' untranslated region (3'UTR) of mRNAs. In rare cases, miRNAs have been shown to bind to sequences outside the 3'UTR [29–32]. The interaction between the miRNA and mRNA (miRNA-mRNA) induces the nuclease activity of RISC and prevents translation of the target mRNA (Fig. 1). In general, about two thirds of all human genes are thought to be regulated by posttranscriptional mechanisms of miRNAs [33].

miRNA-mediated gene expression modulation involves regulating both mRNA and protein [34–36]. Indeed, the interaction of miRNA with target mRNA not only inhibits protein translation, but can also result in direct degradation of the protein-coding transcript [37]. As mentioned, miRNAs can function in regulatory loops—providing negative or positive feedback, which are important for critical cellular processes, such as cell differentiation and immune responses. The fact that miRNAs are frequently found to be involved in these regulatory loops points out the essential importance of miRNAs. Feedback loops often allow for more appropriate responses towards external factors and stressors, such as occurs during inflammation, hypoxia, or ischemia [38, 39], by modulating the strength of the signal. For example, miR-146 functions as a key mediator of the innate immune response. Pro-inflammatory factors, such as interleukin-1 (IL-1) and tumor necrosis factor alpha (TNF α) induce the expression of miR-146 [40]. During inflammation miR-146 fine tunes the immune response acting in a negative feedback loop [41].

MiRNAs in mucosal immune functions

Immune challenges of mucosal organs

The mucosal surface can be seen as being “under fire”, as it is constantly exposed to diverse external factors, including environmental antigens as well as commensal and pathogenic microbes (Fig. 2). Most antigens enter the body across the epithelial barriers of the mucosa lining the respiratory and the gastrointestinal tract. Compared to the skin, the pulmonary and intestinal interfaces are thin boundaries but equally serve as a robust barrier and an exchange interface. Mucosal surfaces are masters in carrying of multiple tasks. For example, the intestinal mucosa has to assure adequate nutritional intake, cope with unwanted digestive tract content, and protect from loss of water and tissue salts all at the same time. The higher the integrity of mucosal barriers, the better their ability to perform these tasks to maintain homeostasis.

Mucosal irritations can impair mucosal barrier function. Increased barrier permeability allows for uncontrolled, bidirectional passage of substances, which can have severe consequences including inducing acute inflammatory responses and the development of

allergies, or disease with chronic immune dysfunctions. Therefore, regulating physiological responses to maintain mucosal homeostasis is essential.

Epithelial cells constitute the mucosal surfaces of the gut and the lung and are important to conducting mucosal immunity via two main functions. First, they provide a mechanical barrier adhering tightly with neighboring cells. Via diverse types of intercellular adhesions (e.g., tight junctions), the epithelial cells separate the parenchyma from the external environment. Here, there is a flexible boundary controlling para- and transcellular cell trafficking and transport of molecules through specialized junctions. This facilitates cellular functions and also allows for intercellular communication. This cellular crosstalk is essential for mucosal homeostasis and represents an important element in mucosal defense.

Regulating the initiation and resolution of innate and adaptive immune response during mucosal inflammation, the mucosa is also critically involved in indirect immune defense. Epithelial cells attract inflammatory cells by releasing a variety of chemokines, such as interleukin-8 (IL-8), which recruits neutrophils (polymorphonuclear neutrophils, PMNs) to sites of injury [42–44]. Moreover, mucosal expression of adhesion molecules facilitates the transmigration of inflammatory cells, such as PMNs and mononuclear cells, into inflamed tissues [45, 46]. In orchestrating various innate and the adaptive immune responses, the epithelium finds itself in permanent contact with resident (e.g., tissue macrophages, dendritic cells) and non-resident immune cells (e.g., PMNs or monocytes). Intercellular communication between epithelial and resident or non-resident immune cells, such as PMNs, presents a crucial part in mucosa immunity and serves as a major element in mucosal defense. A rising number of studies in mucosal inflammation implicate that tissue infiltrating PMNs, classically understood to be pro-inflammatory, also have protective roles, as they help to minimize collateral damage. For example, PMNs have been reported to release anti-inflammatory microvesicles [47, 48] modulating immune response in other immune cells or inflammatory gene expression in resident cells (Neudecker et al., Neutrophil-epithelial miR-223 shuttling dampens acute lung injury. *Science Translational Medicine*, in press). Other studies attributed an essential role to PMNs during epithelial repair [49]. Also, mounting evidence shows that alveolar and intestinal epithelial cells have the ability to upregulate major histocompatibility complex class I and II to allow the epithelial cells to function as antigen presenting cells [50–53]. In this process, the epithelial immune-modulatory factors serve not only for directing an appropriate immune response but also to ensure to limit the steady-state inflammation and to convey tolerance against commensal bacteria.

The mucosal microbiome plays an essential role in the mucosal immune defense; the intestinal commensal microbiome is composed of estimated 10^{13} to 10^{14} bacterial cells [54]. In the face of this number of potentially harmful organisms, the intestinal mucosal barrier function is critical to avoiding the onset of uncontrolled mucosal inflammation. Similar to the intestine, the pulmonary mucosa closely interacts with the lung microbiome, (reviewed in detail here [55]). Changes in microbial community composition can affect mucosal immunity and promote the development of pulmonary disorders, such as chronic obstructive pulmonary disease (COPD) [56] or asthma [57].

Taken together, irregularities in the processes during direct or indirect mucosal immune response, an imbalanced microbial environment, or pathogen entry can affect the integrity of the mucosal barrier resulting in uncontrolled mucosal inflammation. Studies focusing on the immune responses induced and controlled by epithelial cells are of great interest.

Inflammatory diseases of mucosal tissues include well-known diseases, such as acute lung injury, lung fibrosis, asthma, and COPD, as well as inflammatory bowel disease, including ulcerative colitis (UC) or Crohn's disease (CD).

MicroRNAs in mucosal functions

A number of studies have demonstrate miRNAs are essential for mucosal integrity and barrier function. For example, conditional knockout of Dicer in the skin induces significant defects in barrier function of the epithelium [58]. In the same mice, conditional Dicer knockout in lung epithelial cells, during early pulmonary development, revealed failure in the development of epithelial branches. The lungs showed defects in the connection of the epithelium with subepithelial cells and increased epithelial cell death [59]. Certain miRNAs have been found to play essential roles in proper mucosal surface development and proliferation. *MIR-194* was shown to play a role in hepatocyte nuclear factor 1 alpha (HNF1-alpha)-mediated gene regulation during intestinal epithelial cell differentiation. HNF1-alpha, a well-known transcription factor, induces miR-194 expression, thereby expanding its own direct gene regulating transcriptional activity by additional modulation through miR-194 [60]. In pulmonary epithelial development, expression of the *miR-17-92*, a miRNA cluster known to promote tumorigenesis, is highly expressed at early developmental stages, but its levels decrease in later stages. The lethality of experimental embryonic overexpression of mir-17-92 was traced to the ability of miR-17-92 to promote high proliferation rates of undifferentiated lung epithelial progenitor cells [61]. Besides the proper epithelial development, miRNAs are also crucially involved in the maintenance of the barrier function during inflammation. For example, *miR-21* was shown to impair intestinal barrier integrity [62] through downregulating gene RhoB (Ras homolog gene family, member B). Accordingly, miR-21 caused the disruption of tight junctions in intestinal epithelial cells resulting in reduced transepithelial electrical resistance and increased mucosal permeability. *miR-122a* represents another miRNA implicated in the regulation of intestinal epithelial tight junctions, thereby contributing to changes in barrier function [63]. Studies revealed that during inflammation, TNF- α induces the epithelial expression of miR-122a thereby causing the degradation of its target gene occludin, resulting in an increased mucosal permeability. By mediating the impairment of the mucosal barrier, these miRNAs contribute to the development of mucosal inflammation.

miRNA-dependent regulation of immune cell development and function is also critically involved in mucosal immune defense and include regulatory functions in immune cells, such as PMNs. A prominent miRNA involved in regulating innate immune responses, including the control of PMN development and function, is *miR-223* [64]. The differentiation of human PMNs was shown to be controlled by a regulatory circuitry involving miR-223, pointing towards an essential role for miR-223 during granulopoiesis [27, 28, 64]. MiRNA expression is regulated dynamically during development, but is also very potently regulated in response to immune stimuli (Fig. 3). External factors like pathogens can induce changes

in miRNA expression, as do endogenous signal molecules, such as cytokines or chemokines [72, 73]. As shown long before, stimulation of an immune response affects the expression of inflammatory mediators in epithelial cells of the intestine and the lung [42, 43, 74, 75]. Studies in pulmonary epithelial cells focusing on the posttranscriptional machinery during inflammation have revealed changes in miRNAs expression along with altered mRNA content [76]. This up- or downregulation of miRNAs during inflammation will affect target mRNAs, the magnitude of the protein-mediated effects, and subsequently immune responses. Depending on their target, miRNAs can suppress inflammatory pathways or promote immune responses. In the case of increased pro-inflammatory miRNAs, this can cause an uncontrolled inflammatory response with increased collateral damage and the induction of auto-immune disease. Or, in the event of increased anti-inflammatory miRNAs, this can result in an insufficient immune response leading to chronic tissue dysfunction.

MiRNAs during mucosal inflammation

MiRNAs in acute and chronic lung disease—The pulmonary mucosa is an essential player in the pathogenesis of acute lung injury. Pulmonary inflammation is characterized by disrupted alveolar-capillary membrane integrity and lung mucosal barrier along with massive infiltration of immune cells caused by epithelial cell released inflammatory mediators. Screening studies, using various murine and rat models, have revealed specific miRNA expression patterns characterizing the acute inflamed pulmonary tissue. Additionally, it was found that pulmonary miRNA expression varies depending on the specific mechanism of lung injury. MiRNA expression patterns have been analyzed in lung injury caused by mechanical ventilation [77–79], by administration of oleic acid [80], induced by pathogens including endotoxin exposure [81–84] or induced by radiation or hyperoxia [85, 86]. In vitro studies using cyclic mechanical stretch modeling mucosal injury showed changes in epithelial miRNAs expression [87]. Mucosal miRNAs represent potential players in the regulation of pathways involved in injury-associated alterations of the alveolar epithelial permeability and in the induction of the inflammatory response. Studies with direct cytokine stimulation of human lung alveolar epithelial cells using interleukin-1 β (IL-1 β) also induced changes in miRNA expression, and moreover, showed dynamic expression alterations of specific miRNAs over time [72]. This finding of miRNA expression alterations in pulmonary tissue following the induction of lung injury and dynamic epithelial expression changes suggest the critical involvement of miRNAs in the regulation of mucosal immune response during acute lung injury.

Looking more specifically at the impact of lung injury on the pulmonary mucosa, several studies identified functional miRNAs in the pulmonary epithelium. Analysis of pulmonary miRNA expressions in lipopolysaccharide (LPS)-induced lung injury, revealed that inflammation is accompanied by downregulation of *miR-16*. In functional studies in pulmonary epithelial cells, miR-16 was found to act as an anti-inflammatory miRNA, inhibiting the expression of the two pro-inflammatory genes, TNF- α and interleukin-6 (IL-6) [66]. Similar results have been demonstrated in a different model of lung injury, induced by pulmonary exposure to hyperoxia. Again, the induction of lung injury resulted in decreased levels of pulmonary miR-16. Subsequent functional studies using epithelial overexpression of miR-16, during the exposure of excess supply of oxygen, revealed a

critical target for miR-16. MiR-16 was found to regulate a sodium channel that has the potential to promote edema resolution in lung inflammation and injury [88]. Another miRNA, *miR-302b* was found to be induced in murine alveolar epithelial cells stimulated with *Pseudomonas aeruginosa* [89] via activation of toll-like receptors (TLRs). Pulmonary epithelial overexpression of miR-302b was shown to repress the activation of pro-inflammatory NF- κ B (nuclear factor kappa-light-chain-enhancer of activated B cells) signaling, thereby repressing the acceleration of inflammation promoting cytokine production and increasing the survival of *P. aeruginosa*-infected mice. Acting in a negative feedback to TLR-mediated immune response, IRAK4 (interleukin-1 receptor-associated kinase 4), an activator of NF- κ B, was identified to be the direct target of miR-302b during respiratory bacterial infections.

In contrast to the miRNAs reported in the previous paragraph, *miR-155* was found to act pro-inflammatory in acute lung injury [90], which is in accordance with its role reported in other inflammatory disease. Inducing acute fetal lung injury in a nonhuman primate model of choriodecidual infection, analysis of changes in fetal lung gene expression revealed significant changes in miR-155 expression. Similarly, miR-155 expression level changes have been observed in cytokine stimulated fetal airway epithelial (FeAE) cells [90]. Functional studies using mucosal overexpression of miR-155 led to increased epithelial production of pro-inflammatory cytokines, such as Interleukin-6 (IL-6) and interferon (IFN)- γ inducible protein (CXCL10/IP-10). Interestingly, this pro-inflammatory activity was discussed to serve a protective role during lung development. In infected lungs, fibroblast growth factor 9 (FGF9), associated with abnormal lung development and identified to be a direct target of miR-155, was increased. The induction of miR-155 in the infected lungs may therefore represent a feedback mechanism to limit the increase in FGF9 that could result in aberrant lung development.

Various studies found miRNAs intervening in the control of epithelial cell apoptosis. During endotoxin (LPS)-induced pulmonary epithelial cell inflammation *miR-135a* and *miR-203* were found to promote cell apoptosis via regulating BCL2 [91] and by inactivating the signaling through the PI3K/Akt (Phosphatidylinositol-4,5-bisphosphate 3-kinase/AKT also called protein kinase B, PKB) pathway [82]. LPS-induced mucosal inflammation in lung epithelial cells revealed a significant increase in the expression of miR-135a. Anti-apoptotic Bcl-2 was identified to represent a direct miR-135a target and to be markedly reduced during mucosal inflammation, resulting in increased cell apoptosis. Vice versa, the inhibition of miR-135a led to restored levels of Bcl-2 and attenuated epithelial cell death. Similarly, miR-203 was significantly upregulated in pulmonary tissue of LPS-challenged mice and its epithelial overexpression led to increased epithelial apoptosis rates. Experimental overexpression of miR-203 uncovered its direct regulatory activity on phosphoinositide 3-kinase catalytic subunit alpha (PIK3CA), whose decrease affected the PI3K/Akt pathway. Inversely, the suppression of miR-203 ameliorated the LPS-induced pulmonary inflammation and interstitial pneumonia.

During inflammation, many resident cells find themselves in close contact with different cell types, particularly immune cells. Intercellular communication between resident cells and infiltrating immune cells is crucial to ensure proper response during inflammation and

resolution. Here, miRNAs are emerging as critical mediators of cell-to-cell communication. In particular, *miR-223* has gained attention as being important for intercellular communication during pulmonary inflammation, mediating signals between the alveolar epithelium and lung-infiltrating PMNs ((Neudecker et al., Neutrophil-epithelial miR-223 shuttling dampens acute lung injury. Science Translational Medicine, in press); Fig. 4). Experimentally, the transfer of miRNA-223 from PMNs to epithelial cells was found to be protective. Once transferred into the alveolar epithelium, miR-223 downregulates the pro-inflammatory target gene PARP-1, resulting in diminished epithelial inflammation and cell injury. Moreover, the pulmonary overexpression of miR-223 was protective in sterile and bacterial infection models of murine acute lung injury. While many studies have revealed an anti-inflammatory and protective role for miR-223 in acute inflammatory settings, a very recent study by *Leuenberger* et al. found miR-223 to have a disease-promoting role in chronic obstructive pulmonary disease [92]. Their in vitro experiments in pulmonary endothelial cells revealed a regulatory effect of miR-223 on the expression of histone deacetylase 2 (HDAC2), a critical actor in COPD, suppressing inflammatory gene expression. Reduced levels of HDAC2 have been described in patients with COPD. Additionally, in in vivo murine COPD models as well as in COPD patient cohorts, miR-223 consistently revealed elevated pulmonary expression levels, and was inversely correlated with the expression of HDAC2. Moreover the elevated levels of miR-223 were associated with an altered expression profile of chemokines amplifying pro-inflammatory signals. Another study that found miR-223 to be critical in a chronic inflammation focused on its role in the control of tuberculosis (TB) [93]. While miR-223 was found to be significantly upregulated in TB patients and during experimental murine TB, its deletion generated a highly susceptible phenotype. Further analyses indicated a causal link between the lack of miR-223 and downregulated chemoattractants CXCL2 and CCL3, as well as pro-inflammatory IL-6 in myeloid cells. This altered chemo- and cytokine milieu results in inadequate pulmonary immune cell recruitment ultimately leading to neutrophil-driven lethal inflammation. Therefore this study revealed, that miR-223 is not only critical in directly controlling myeloid cell development and activity, or preventing collateral damage by mediating communication between resident and myeloid cells, but is also highly involved in regulating the dimension of leukocyte infiltration.

miRNA expression analysis has also been performed in post lung injury stages, such as resolution and recovery, and during the development of fibrosis. Fibrosis is another type of inflammatory lung disease, involving the pulmonary mucosa, which results from repetitive alveolar epithelial injury and subsequent aberrant dysregulated repair processes. In particular, dysfunctional intercellular communication of pulmonary epithelial and mesenchymal cells is thought to present a critical event in the pathology of lung fibrosis [94, 95]. In bleomycin-induced lung injury, miR-101 was found to be induced, while the protein levels of transcription factor Sox9 were downregulated. Sox9 turned out to be a direct *miR-101* target, and its suppression adequate recovery after lung injury. Therefore, appropriate epithelial Sox9 expression appears to be essential for proper lung recovery after lung injury [68]. One of the first miRNAs with a role in preventing fibrosis was identified by Pandit et al. [69]. *Let-7d* was identified among several dysregulated miRNAs comparing idiopathic pulmonary fibrosis (IPF) tissue with healthy control subjects. The suppression of

let-7d was found to be mediated by SMAD3, an intracellular mediator for transforming growth factor beta (TGF- β) signals. The downregulation of let-7d by SMAD3 transcriptional activity resulted in a pro-fibrotic effect. Restoration of epithelial let-7d expression levels therefore represents an essential role in preventing pulmonary fibrosis.

Another prominent miRNA in pulmonary fibrosis is *miR-21*. Murine lungs from experimental fibrosis and tissue samples from patients with IPF displayed increased miR-21 levels. While *Liu et al.* localized miR-21 primarily in myofibroblasts [96], later studies from *Yamada et al.* found epithelial induction of miR-21 [97]. They found the expression of miR-21 increased with experimental epithelial-mesenchymal transition (EMT), while its in vitro inhibition prevented EMT, indicating that miR-21 acts in a pro-fibrotic manner [97]. The impact of miR-21 on fibrosis is even more emphasized by the fact that days post-initiation of experimental lung fibrosis, a strong anti-fibrotic effect and milder course of disease was observed with miR-21 suppression. In pulmonary fibroblasts, miR-21 was found to directly target SMAD7. SMAD7 is an inhibitory component of the TGF- β pathway, which is a major player in fibrotic diseases [98]. Thus the inhibition of SMAD7 represents a potential mechanism for miR-21 to mediate its pro-fibrotic effect. During the process of EMT, epithelial cells transit into cells with more mesenchymal features. miRNAs involved in this event therefore hold potential regulatory relevance in the development of fibrosis. miR-26a is one of these miRNAs, as it negatively regulates HMGA2, a crucial regulatory factor of EMT in vitro and in vivo [70]. miR-26a was found to be downregulated in experimental murine fibrosis as well as in patients with IPF [70, 71]. Overexpression of miR-26a prevented fibrogenesis in vitro and in vivo by suppressing connective tissue growth factor (CTGF) gene expression and collagen production. In addition, miR-26a was found to be part of a regulatory feedback loop, reflecting its strong regulatory involvement in the fibrotic process. Here, while TGF- β 1-mediated phosphorylation of SMAD3 suppresses miR-26a, miR-26a inhibits the nuclear translocation of p-SMAD3 through directly targeting the key regulator of this translocation, SMAD4 [71]. Therefore, though regulating several critical fibrosis-related genes, miR-26a can be seen as one of the key anti-fibrotic miRNAs. As discussed above, critical microRNAs involved in acute or chronic mucosal inflammation of the lung are summarized in Table 1.

MiRNAs in inflammatory bowel disease—In the intestinal mucosa the epithelial cells also prove crucial to regulating tissue homeostasis. They constitute a mechanical and biophysical barrier, function to maintain an immunological environment that allows co-inhabitation with commensal bacteria, and provide to appropriate immune response against invading pathogens. Dysregulated epithelial function with loss of the mucosal barrier and immune regulatory activity results in mucosal inflammation and disease. Together with increased bacterial translocation, epithelial barrier failure is thought to play a central role in intestinal inflammatory disease, such as inflammatory bowel disease (IBD), which includes CD and UC.

One of the first screening studies in IBD compared miRNA expression patterns in colon biopsies from patients at different stages of the disease to healthy control subjects. In total, this study revealed 11 differentially expressed miRNAs in active UC compared to controls. Among these *miR-192* was found to be predominantly expressed in the colonic epithelial

cells and showed reduced levels in active UC [99]. Additional studies identifying differentially regulated miRNAs in active UC have supported the presumption that miRNAs are critically involved in the development and progression of inflammatory bowel diseases [100]. Indeed, in CD, mucosal biopsies demonstrate distinct miRNA expression patterns that distinguish active CD from healthy controls [10]. While most studies in CD focused on colonic biopsies, one study also investigated differences in the miRNAs expression between ileal and diverse colonic biopsies. Distinct differences in specific miRNA expression levels were observed and suggest that miRNAs are differentially involved in IBD [101]. Recent efforts have also been made to investigate potential miRNA expression pattern differences in UC versus CD. These studies revealed specific aberrant miRNA expression patterns for UC that differ from those in CD [102]. A very recent study in duodenal biopsies from pediatric patients found two miRNAs, *miR-146* and *miR-155*, with increased expression in inflamed mucosa biopsies [103]. Interestingly, expression levels of both miRNAs in small intestinal epithelial cells or primary duodenal fibroblasts could be reduced by treatment with anti-inflammatory TGF- β , indicating a close association of these miRNAs with pro-inflammatory pathways.

As mentioned earlier, one of the first reports of miRNA in IBD was based on the finding of decreased epithelial *miR-192* expression in active UC. Subsequent studies revealed a critical role for miR-192 in regulating colonic mucosal chemokine expression. MiR-192 and its newly identified target gene, macrophage inflammatory peptide (MIP)-2 alpha, a chemokine expressed by epithelial cells, were found to act in a feedback loop. Epithelial miR-192 directly regulates the expression of MIP-2 alpha, while the induction of MIP-2 alpha simultaneously decreases miR-192 expression [99]. Similarly, *miR-193a-3p* was found to dampen mucosal inflammation [13] by directly regulating PepT1, a di/tripeptide transporter that uptakes bacterial products. Downregulation of miR-193a-3p was accompanied with upregulation of PepT1 in inflamed colon tissues of patients with UC. Overexpression of miR-193a-3p decreased PepT1 expression and, most likely via suppression of the NF- κ B pathway, resulted in significantly milder dextran sodium sulfate-(DSS) induced colitis. Interestingly, antibiotic treatment similarly ameliorated experimental colitis, indicating that the anti-inflammatory effect of miR-193a-3p is mediated by negative regulation of PepT1 and its response to the microbiota.

Several studies also found elevated colonic expression of *miR-146a* in human IBD patients [67, 102, 103]. MiR-146, representing a key player in controlling the adaptive immune response [104], is also a critical player in the regulation of the T cell function in the intestine. MiR-146a is known to hold a fundamental role in the regulation of the suppressive regulatory T cells (Tregs), thereby controlling T helper cell (Th1) responses [105]. In the intestine, miR-146a was found to be critical in regulating the expansion of certain intestinal T cell subtypes, including T helper 17 cells (Th17), Tregs, and T follicular helper cells (Tfh) [67]. Interestingly, in mucosal inflammation of the intestine, miR-146a not only regulated immune responses, but was also shown to influence the barrier function. Deficiency in miR-146a was accompanied with a more resistant phenotype during experimental murine colitis showing a more enhanced intestinal barrier function [67]. *MiR-223* that was also discussed within the scope of pulmonary mucosal inflammation, only recently, was found to also orchestrate the intestinal immune responses [106]. Initial studies of intestinal biopsies

revealed elevated miR-223 expression levels in patients with active IBD, as well as in preclinical models of intestinal inflammation. Subsequent studies in miR-223 knockout mice revealed an exacerbated course of myeloid-driven experimental colitis in the absence of miR-223, and mechanistical analyses revealed that this phenotype was characterized by enhanced NLRP3 inflammasome expression with elevated IL-1 β (Interleukin-1 β). Strikingly, targeted mutation of the miR-223 binding site in the NLRP3 3'UTR phenocopied the features of the miR-223 knockout mice during colitis. Consequently, therapeutic overexpression of miR-223 dampened intestinal inflammation and attenuated experimental colitis, implicating miR-223 as a promising therapeutic target during intestinal inflammation.

During mucosal inflammation, DSS also induces epithelial cell apoptosis. *MiR-150* found to be upregulated in DSS-colitis, showed to be directly involved in regulating the increased cell death. High expression levels of miR-150 in experimental colitis and in human UC biopsies were associated with reduced expression levels of c-Myb, a transcription factor known to regulate the anti-apoptotic Bcl-2. Bcl-2 showed equally repressed expression levels. Direct targeting of c-Myb by miR-150 in mucosal inflammation suggested a crucial role for this miRNA in maintaining epithelial barrier integrity [107]. Similarly, increased levels of *miR-21* present in UC correlates with increased disruption of the intestinal barrier function. Experimental epithelial overexpression of miR-21 led to increased mucosal barrier permeability. Studies show that the mechanism underlying this observation involves impaired tight junctions of the intestinal mucosa resulting from miR-21 targeting Ras homolog gene family member B (RhoB) [62].

Currently, one of the miRNAs relevant in IBD that seems promising to be translated into clinical therapy is *miR-214* [65]. MiR-214 was found to be upregulated in colon biopsies of patients with active colitis or colitis-associated cancer (CAC) as well as in preclinical models of colitis. Further studies revealed that these increased levels of miR-214 are induced by IL-6 induced signal transducer and activator of transcription 3 (STAT3) signaling and correlated with elevated activity of NF- κ B. This increased activity of pro-inflammatory pathways was triggered by a feedback loop mediated by the downstream action of miR-214. Here, miR-214 was found to reduce levels of PDLIM2 (PDZ and LIM domain-containing protein) and PTEN (phosphatase and tensin homolog), thereby increasing phosphorylation of AKT (also Protein kinase B, PKB), and finally activating NF- κ B. Blocking miR-214 was sufficient to break this feedback loop and resulted in a lesser degree of severity of experimental colitis and decreased incidence and quantity of tumor development in experimental CAC. Therapeutic benefits have been seen in colonic biopsies from UC patients with active disease following ex vivo miR-214 inhibition [65]. Therefore, miR-214 is a promising therapeutic target to control the progression of IBD (Fig. 5).

Besides the disrupted epithelial barrier function, another hallmark of inflammatory bowel disease is increased attraction of infiltrating immune cells. In two different models of experimental colitis, murine 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced and interleukin-10 (IL-10) knockout chronic colitis, epithelial *miR-141* and CXCL12 β /total-CXCL12 (C-X-C motif chemokine 12 isoform β /total C-X-C motif chemokine 12) showed opposed expression levels. Similar observations were seen in CD patients. Direct miR-141-

mediated regulation of CXCL12 β was shown to be crucial in the modulation of leukocyte infiltration and trafficking. Depending on the level of miR-141 experimental colitis is milder or takes a more severe course. Therefore, miR-141 appears to be essential in regulating the colonic immune cell recruitment [14]. Several studies found the well-known, inflammation-related *miR-155* to also be upregulated in patients with active UC [100, 108]. Functional studies showed epithelial miR-155 directly regulates Forkhead box protein O3a (FOXO3a). Thereby, miR-155 partially mediates pro-inflammatory activity since the decrease of FOXO3a results in activation of the NF- κ B signaling pathway [108]. In a very recent study, experimental TNBS induced colitis and inflamed mucosa from patients with active CD showed raised levels of *miR-124*. The study identified that miR-124 directly targets aryl hydrocarbon receptor (AHR) and that AHR is inversely correlated within inflamed intestinal epithelial cells. AHR is involved in the immune response and is critical in the regulation of the mucosal immune homeostasis. Its downregulation via experimental over-expression of miR-124 revealed exacerbated intestinal inflammation during TNBS colitis, while miR-124 inhibition revealed ameliorated experimental colitis [109]. MicroRNAs critically involved in processes during inflammation of the intestinal mucosa are summarized in Table 2.

Extracellular microRNAs—The existence of extracellular miRNAs was not discovered until lately [111, 112]. Since then it has been obvious that miRNAs can be secreted by one cell and taken up by another one. In this way, miRNAs can be involved in intercellular communication between cells, even cells in different tissues. The process of miRNA transfer from one cell to another, named miRNA shuttling, can modulate the gene expression in the recipient cell. This miRNA shuttling is gaining more and more attention and rising evidence attributes a significant role of miRNA exchange to cell-cell communication. It is accepted that the miRNA based crosstalk among different cell types is critically involved in various biological processes, including immune responses. Here, miRNAs can not only be transferred between immune cells to orchestrate acute immune responses [110, 113, 114], but also mediate signals from immune to resident tissue cells (Neudecker et al., Neutrophil-epithelial miR-223 shuttling dampens acute lung injury. Science Translational Medicine, in press). One example of miRNA shuttling between immune cells was recently shown by Alexander et al. [114]. Their study elegantly elucidated the role of intercellular shuttling of extracellular miR-155 and miR-146a among dendritic cells. Both miRNAs candidates, known to be highly involved in the regulation of inflammation, critically affected the inflammatory responses in endotoxin-challenged mice. Here, cell-to-cell transfer of miR-155 induced inflammation, whereas intercellular shuttling of miR-146a dampened inflammatory gene expression in recipient cells. Similarly, but taking place between different cell types during inflammation, intercellular transfer of miR-223 from neutrophils to resident alveolar epithelial cells attenuated pulmonary mucosal inflammation (Neudecker et al., Neutrophil-epithelial miR-223 shuttling dampens acute lung injury. Science Translational Medicine, in press). Here, further analyses revealed that miR-223 repressed the pro-inflammatory gene PARP-1. Interestingly, other studies have also shown that miRNA transfer between resident cells and immune cells, specifically macrophages, plays an important role in regulating intestinal mucosal inflammation [115]. Indeed, studies in DSS-induced colitis revealed that intestinal epithelial PepT1 induced secretion of miRNA-23b, a miRNA known to be involved in inflammatory bowel disease [101]. Subsequently, miR-23b was taken up in

recipient intestinal macrophages and modulated their gene expression by silencing a specific target gene, Marcksl-1. Thereby, miR-23b shuttling regulated macrophage activity and as a result influenced colitis severity.

Challenges and conclusions

This review has highlighted the roles of mucosal miRNAs in the regulation of processes maintaining mucosal homeostasis and immune defense. MiRNAs are involved in the regulation of mucosal barrier functions and orchestrate innate and adaptive immune responses. Increased or decreased miRNA expression can significantly contribute to the protection or pathophysiology of acute or chronic mucosal inflammation, such as that seen with lung injury or inflammatory bowel disease.

Having gained insights about the role of mucosal miRNAs in inflammatory disease from diverse in vitro and in vivo studies as well as analysis of human tissue, the future challenge lies in translating this knowledge into successful clinical therapies. Using miRNAs as targets for therapy has obvious future potential (see Table 3) and partially is already an existing approach [116]. In terms of ease of access of the therapeutic agent the fact that mucosal surfaces are in direct contact to the external environment is beneficial. There are several technical approaches existing to restore downregulated miRNAs, including administering miRNA-mimics [117] or double-stranded miRNAs with chemical modifications improving their stability and increasing the probability of their cellular uptake. Theoretically, suppressing unwanted miRNA expression is even easier to achieve by using sugar-modified RNA molecules, so-called locked nucleic acids (LNA) [116]. However, miRNA based therapies also imply challenges to overcome. For example, many miRNAs regulate more than one gene target. This can have unwanted effects depending on target cell type and organ. For example, therapeutic overexpression of miR-26a in pulmonary fibrosis could be a promising approach, but one has to take into account that miR-26a was also shown to facilitate gliomagenesis in vivo [118]. Similarly, aiming to restore miR-192 levels in the therapy of UC requires consideration of the pro-fibrotic component that might accompany a miR-192 overexpression in other organs, such as the kidney [119]. Accordingly tissue-specific delivery of miRNAs is of rising interest.

Taken together, even though miRNA research in mucosal disease appears to be still in its infancy, we believe that the relevance of miRNAs in mucosal inflammation is emerging. Recent bench to bedside translation of the first miRNA successfully passing a clinical phase IIa study [116] is supporting this assumption. Future investigations uncovering more details about miRNA-mediated regulation in inflammatory diseases, such as acute lung injury or colitis, hold potential of miRNAs to be utilized in diagnostics and as potential therapeutic targets.

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Fig. 1. MicroRNA biogenesis and function. Initially, in the nucleus miRNAs are generated as large transcripts called primary miRNAs (pri-miRNA) by RNA polymerase II (pol II). In the following step, a shorter hairpin structured, precursor miRNAs (pre-miRNA) is synthesized by RNase nuclease, Drosha associated with DiGeorge syndrome critical region 8 protein (DGCR 8). After exportation from the nucleus, cytosolic RNase nuclease Dicer further processes the hairpin-structured miRNA leading the unstable double-stranded short duplex miRNA. Here, only the mature strand gets incorporated into the RISC (RNA-silencing complex) for targeted gene repression. Functional repression of the target gene depends on miRNA-messenger RNA (mRNA) interaction in the 3' untranslated region (3' UTR) of the target mRNA. That way, one single miRNA can bind to many different mRNA targets and can therefore hold a wide regulatory impact. And, nonetheless, one target mRNA can be repressed by a variety of miRNAs, all together contributing to a robust miRNA-mediated regulation

Fig. 2.

Immune challenges of mucosal organs. Together the lung and the intestine represent the main mucosal organs of the body. The large surface area is the main contact surface of the organism to the outside world. Being permanently exposed to diverse external factors including environmental antigens and commensal and pathogenic microbes, the mucosal surface can be seen as constantly “under fire”. A homeostatic mucosal environment is essential for keeping the balance between serving as a robust barrier and functioning as an exchange interface. Inflammatory metabolites and microbial products as well as impaired immune responses and bacterial translocation may affect this equilibrium, while an intact mucosal barrier together with the innate and adaptive immune system function to counter any disturbance

Fig. 3.

Immune functions of microRNAs. MicroRNAs (miRNAs) contribute to the mucosal immunity on diverse levels. They orchestrate and modulate immune responses, such as regulating NF- κ B pathways by miR-214 in the intestinal epithelium [65] or miR-16 which directly regulates the expression of TNF α and interleukin-6 in the pulmonary epithelium during acute lung injury [66]. MiRNAs take also control of the adaptive immune response, e.g., miR-146 represents an important regulator of the suppressive regulatory T cells (Tregs) in the intestine [67]. Intercellular communication between the immune cells and resident mucosa is also involving miRNAs. Neutrophil-epithelial transfer of miR-223 mediates critical anti-inflammatory regulatory signals during pulmonary inflammation (Neudecker et al., Neutrophil-epithelial miR-223 shuttling dampens acute lung injury. *Science Translational Medicine*, in press). Barrier integrity is another major component ensuring proper defense against any invading pathogens. Here miRNAs are involved in regulations affecting the mucosal barrier function. For example miR-21 and miR-122a directly influence mucosal permeability by regulating the expression of epithelial tight junctions [62, 63]. In later stages of mucosal inflammation including the resolution, remodeling and repair processes miRNAs are also critically involved and e.g., promote adequate recovery such as miR-101 which is important for proper lung recovery after lung injury [68]. Epithelial let-7d is essential in preventing remodeling and development of pulmonary fibrosis after inflammation and lung injury [69]. Similarly, miR-26a is acting in an anti-fibrotic manner [70, 71]

Fig. 4.

Protective role of miR-223 in acute lung injury. During lung injury and pulmonary inflammation neutrophils (polymorphonuclear neutrophils, PMNs) are recruited to the sides of injury infiltrating the inflamed lung. Neutrophil derived miR-223 mediates anti-inflammatory signals by being secreted and transferred to alveolar epithelial cells. In the epithelium, miR-223 downregulates the pro-inflammatory target gene PARP-1, thereby dampening mucosal inflammation and cell injury (Neudecker et al., Neutrophil-epithelial miR-223 shuttling dampens acute lung injury. Science Translational Medicine, in press)

Fig. 5.

Therapeutic inhibition of miR-214 in ulcerative colitis. During intestinal injury miR-214 is induced by IL-6 induced signal transducer and activator of transcription 3 (STAT3) (*left side of the dotted line*). Elevated miR-214 levels inhibit PDLIM2 (PDZ and LIM domain-containing protein) and PTEN (phosphatase and tensin homolog) representing direct miR-214 target genes. Inhibition of PDLIM2 and PTEN allow for phosphorylation of AKT (also protein kinase B, PKB), and finally activation of NF- κ B. This pro-inflammatory pathway is triggered by a positive feedback loop aggravating and driving inflammation. Blocking miR-214 (*right side of the dotted line*) can break this feedback loop. Elevated PDLIM2 and PTEN level prevent phosphorylation of AKT, and consequently impede activation of NF- κ B. In experimental colitis this therapeutic inhibition of miR-214 resulted in a lesser degree of severity of disease and reduced tumor growth in experimental colitis-associated cancer (CAC) [65]

Table 1**MicroRNAs critical in pulmonary mucosal inflammation**

microRNA	Mode of action	In vitro/in vivo model	Target genes	Ref.
Pulmonary mucosa				
miR-223 (↑ in murine TB, murine model of COPD and in sterile or bacterial acute lung injury)	Reducing myeloid pulm. recruitment; pro-inflammatory in pulm. endothelia; anti-inflammatory in pulm. epithelia	Murine TB model Murine COPD model Ventilator- and <i>S.aureus</i> -induced murine lung injury	CXCL2, CCL3 HDAC2 PARP-1	[92] [(Neudecker et al., Neutrophil-epithelial miR-223 shuttling dampens acute lung injury. Science Translational Medicine, in press), 91]
miR-302b (↑ in murine infected lungs)	Anti-inflammatory and pro-survival	Murine alveolar epithelial cells, <i>P. aeruginosa</i> -infected mice	IRAK4	[88]
miR-155 (↑ in inflamed human FeAE, and in infected fetal monkey lungs.)	Pro-inflammatory	Human fetal airway epithelial (FeAE) cells, group B- streptococcal-induced lung injury in fetal monkeys	FGF9	[89]
miR-135a (↑ in LPS-challenged pulmonary epithelial cells)	Pro-apoptotic, pro-inflammatory	LPS stimulation in pulmonary epithelial cells (A549)	BCL2	[90]
miR-16 (↑ LPS-induced acute lung injury)	Anti-inflammatory	Pulmonary epithelial cells (A549), murine LPS-induced acute lung injury	TNFα, IL-6	[87]
miR-101 (↑ in acute lung injury)	Pro-repair/pro-recovery	Bleomycin-induced murine lung injury	Sox9	[95]
Let-7d (↓ in fibrosis)	Anti-fibrotic	Tissue samples from IPF (Idiopathic pulmonary fibrosis) patients	HMGA2	[68]
miR-21 (↑ in fibrosis)	Pro-fibrotic	Bleomycin-induced murine lung fibrosis	5MAD7	[69]
miR-26a (↓ in fibrosis)	Anti-fibrotic	Pulm. epithelia and fibroblasts (A549, MRC-5), murine bleomycin-induced fibrosis	HMGA2 p-SMAD4	[70, 98]

Table 2**MicroRNAs critical in intestinal mucosal inflammation**

microRNA	Mode of action	In vitro/in vivo model	Target genes	Ref.
Intestinal mucosa				
miR-21 (↑ in ulcerative colitis biopsies)	Reduces intestinal barrier function	Intestinal epithelial cell line (Caco-2)	RhoB	[61]
miR-122a (↑ in TNFα-induced affected permeability)	Increasing intestinal permeability	Intestinal epithelial cell line (Caco-2), mouse small intestine ex vivo	Occludin	[62]
miR-150 (↑ in murine colitis, in ulcerative colitis biopsies)	Pro-apoptotic and reduces intestinal barrier function	Murine DSS-colitis	C-Myb	[105]
miR-214 (↑ in active colitis biopsies and in murine colitis and colitis- associated cancer)	Pro-inflammatory	Murine DSS-colitis	PDLIM2	[106]
miR-141 (↑ in murine colitis, in Crohn's disease biopsies)	Modulating leukocyte infiltration and trafficking	TNBS-induced and IL-10-knockout experimental murine colitis	CXCL12β	[14]
miR-146 (↑ in pediatric Crohn's disease, in human ulcerative colitis and Crohn's disease biopsies)	Impaired barrier function and anti-immunoprotective inflammation	DSS-colitis in miR-1464 ^{-/-} mice	Various genes in barrier function and immunoprotection	[101–103]
miR-155 (↑ in pediatric Crohn's disease, in ulcerative colitis biopsies)	Pro-inflammatory	TNFα-treated intestinal epithelial cell line (HT29)	FOXO3a	[97, 100, 106]
miR-124 (↑ in murine colitis, in patients with Crohn's disease)	Pro-inflammatory	TNBS-induced murine colitis	AHR	[65]
miR-23b (↑ in patients with Crohn's disease, ↓ in murine colitis, but secretion ↑)	Mediating intercellular communication	Murine DSS-colitis in PepT1 overexpressing mice	Marcksl	[100, 110]

Table 3Examples of ongoing clinical trials involving miRNAs (<https://www.clinicaltrials.gov>)

Disease (intervention)	Patient population	Purpose of study	ClinicalTrials.gov identifier (status)
Inflammatory bowel disease	Ulcerative colitis, Crohn's disease, Healthy non-IBD colitis	Evaluation of the diagnostic value of microRNAs in inflammatory bowel disease	NCT02020382 (recruiting)
Asthma (budesonid)	Asthmatics, healthy non-asthmatic controls	Evaluate the role of miRNAs in Th2-driven inflammation in asthma	NCT01484691 (recruiting)
Acute lung inflammation (bronchial segmental endotoxin instillation, 4 ng/kg)	Healthy individuals (18 to 45 years) with no significant medical problem, no medication, non-smoker	Identification of cell-associated and secreted miRNAs with specific types of resident and inflammatory cells in the lung	NCT02392442 (recruiting)
Recurrent malignant pleural mesothelioma (MPM) and non-small cell lung (NSCLC) (miR-16 mimic, or TargomiRs; TargomiRs are targeted minicells containing a microRNA mimic)	Patients with recurrent MPM and NSCLC	Dose-finding studies in patients with recurrent MPM or NSCLC (phase I study, TargomiRs as 2nd or 3rd line treatment)	NCT02369198 (recruiting)