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MicroRNAs and Other Non-Coding RNAs in Inflammation

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Catherine M. Greene
Editor

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Editor
Catherine M. Greene
Respiratory Research, Department of Medicine
Royal College of Surgeon in Ireland
Education and Research Centre, Beaumont Hospital
Dublin, Ireland

Series Editors
Michael J. Parnham
J.W. Goethe University
Frankfurt am Main
Germany

Achim Schmidtko
Institute of Pharmacology and Toxicology
University Witten/Herdecke
Witten
Germany

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Preface

Inflammation is central to the development, progression, and outcome of infectious and noninfectious diseases, whether they be chronic or acute. There have been significant advances in inflammation research over the past 20–30 years. These have led to our current understanding of how blood and tissue cells interact and detailed knowledge regarding the intracellular mechanisms that control inflammatory cell activation. With the relatively recent discovery of noncoding RNA (ncRNA), a further level of complexity in the control of inflammatory processes at a molecular level is now known to exist. ncRNAs are likely to represent multiple new targets for potential anti-inflammatory and immunomodulatory therapy. As we learn more about the biology of ncRNA, it will reveal new information on the underlying inflammatory pathology of many diseases.

ncRNAs are a recently identified class of regulatory molecules. As a superclass, ncRNA can be subdivided into two major subgroups: (1) long noncoding RNAs (lncRNAs) greater than 200 nucleotides in length, with diverse biological functions that largely impact on gene expression and protein function, and (2) microRNAs (miRNAs), 20–25 nucleotide RNAs involved in the translational regulation of gene expression. Other short ncRNAs (<200 nucleotides) with house-keeping roles such as transfer RNA, ribosomal RNA, and small nucleolar RNAs also exist. It is estimated that humans can express approximately 2,000 miRNAs and over 30,000 lncRNAs. Each ncRNA can regulate the expression of specific genes, and collectively ncRNAs have a fundamental role in controlling almost all biological processes and are amongst the newest therapeutic targets known.

Studies into understanding the role of miRNAs and other ncRNAs in innate immunity and inflammation are burgeoning. Although less is known regarding the role of lncRNA compared to miRNA in the inflammatory process, overall our knowledge has advanced greatly since the discovery of the first miRNA in the mid-1990s. In this book a team of international scientists offer their insights into this popular and rapidly expanding area. Each chapter presents succinct overviews intended to explain basic concepts and to highlight recent advances in a specific topic related to miRNA and ncRNA inflammation research. Chapters “The Biology

of MicroRNA" and "The Biology of Long Non-coding RNA" describe the fundamental biology of miRNA and lncRNA. In chapters "MicroRNA Regulation of Neutrophil Function," "How Noncoding RNAs Contribute to Macrophage Polarization," and "Endogenous Control of Dendritic Cell Activation by miRNA," the roles of miRNA and ncRNA in the maturation and function of neutrophils, macrophages, and dendritic cells—major cells involved in the inflammatory process—are reviewed. The remaining chapters describe what is known regarding the roles of ncRNA in a selection of common, medically important human inflammatory conditions ranging from acute infectious diseases such as viral infection to chronic inflammation-related disorders including rheumatoid arthritis, obesity, Crohn's disease, and diabetes. Finally, the biomedical diagnostic and prognostic use of ncRNA is explained, using hepatocellular carcinoma as an example.

The overall aim of this book is to provide an up-to-date single resource that collates current knowledge on the latest developments in microRNA and other noncoding RNA in inflammation research. In the future by focussing closely on what we learn about the contribution of ncRNA to inflammatory disease pathology and by carefully analyzing the molecular mechanisms that both control and are regulated by inflammatory events, it is likely that we will identify new therapeutic strategies based on ncRNA that can be developed for a range of inflammatory diseases.

Dublin, Ireland Catherine M. Greene

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Contributors

Stephan Blüml Division of Rheumatology, Medical University of Vienna, Vienna, Austria

Oliver Brain Translational Gastroenterology Unit, John Radcliffe Hospital, Oxford, UK

Scott Chaffee Heart and Lung Research Institute, Ohio State University Medical Center, Columbus, OH, USA

Jack B. Cowland Granulocyte Research Laboratory, Department of Hematology, Rigshospitalet, Copenhagen, Denmark

Huachun Cui Division of Pulmonary, Allergy, and Critical Care Medicine, Department of Medicine, University of Alabama at Birmingham, Birmingham, AL, USA

Amitava Das Heart and Lung Research Institute, Ohio State University Medical Center, Columbus, OH, USA

Chiara de Santi Respiratory Research, Department of Medicine, Royal College of Surgeons in Ireland, Dublin, Ireland

Betty Diamond The Center for Autoimmune and Musculoskeletal Diseases, The Feinstein Institute for Medical Research, Manhasset, NY, USA

Johanna K. DiStefano Diabetes, Cardiovascular and Metabolic Diseases Division, Translational Genomics Research Institute, Phoenix, AZ, USA

Eliana Goncalves-Alves Division of Rheumatology, Medical University of Vienna, Vienna, Austria

Catherine M. Greene Respiratory Research, Department of Medicine, Royal College of Surgeons in Ireland, Education and Research Centre, Beaumont Hospital, Dublin, Ireland

Laurence Josset Department of Microbiology, Laboratoire de Virologie et Centre National de Référence virus influenzae Laboratoire VirPatH EA4610, Faculté de Médecine Lyon Est, Université Claude Bernard Lyon 1, Lyon, France

Michael Karbiener RNA Biology Group, Institute of Molecular Biotechnology, Graz University of Technology, Graz, Austria

Michael G. Katze Department of Microbiology, School of Medicine, University of Washington, Seattle, WA, USA

Sun Jung Kim The Center for Autoimmune and Musculoskeletal Diseases, The Feinstein Institute for Medical Research, Manhasset, NY, USA

Matthew W. Lawless Experimental Medicine, School of Medicine and Medical Science, University College Dublin, Catherine McCauley Centre, Mater Misericordiae University Hospital, Dublin 7, Ireland

Fatjon Leti Diabetes, Cardiovascular and Metabolic Diseases Division, Translational Genomics Research Institute, Phoenix, AZ, USA

Gang Liu Division of Pulmonary, Allergy, and Critical Care Medicine, Department of Medicine, University of Alabama at Birmingham, Birmingham, AL, USA

Paul J. McKiernan Respiratory Research, Department of Medicine, Royal College of Surgeons in Ireland, Dublin, Ireland

Sashwati Roy Heart and Lung Research Institute, Ohio State University Medical Center, Columbus, OH, USA

Victoria Saferding Division of Rheumatology, Medical University of Vienna, Vienna, Austria

Marcel Scheideler RNA Biology Group, Institute of Molecular Biotechnology, Graz University of Technology, Graz, Austria

Chandan K. Sen Heart and Lung Research Institute, Ohio State University Medical Center, Columbus, OH, USA

Alison Simmons Translational Gastroenterology Unit, John Radcliffe Hospital, Oxford, UK

MRC Human Immunology Unit, Weatherall Institute of Molecular Medicine, John Radcliffe Hospital, Oxford, UK

Matthew Taila Diabetes, Cardiovascular and Metabolic Diseases Division, Translational Genomics Research Institute, Phoenix, AZ, USA

Jennifer Tisoncik-Go Department of Microbiology, School of Medicine, University of Washington, Seattle, WA, USA

Jun Zhao Experimental Medicine, School of Medicine and Medical Science, University College Dublin, Catherine McCauley Centre, Mater Misericordiae University Hospital, Dublin 7, Ireland

Part I
Biology of ncRNA in Inflammation

The Biology of MicroRNA

Chiara de Santi and Catherine M. Greene

Abstract MicroRNAs (miRNAs) are small non-coding RNA molecules involved in mRNA regulation at a post-transcriptional level. The first miRNA was discovered in 1993, and since then many branches of research have been explored to fully understand the miRNA world. Studies regarding the biogenesis process have highlighted different pathways according to miRNA gene localization, although the biochemical mechanism is not completely clear yet. In animals, miRNAs act mainly as negative regulators through translation inhibition, but recent evidence has shown their ability to stimulate mRNA degradation through recruiting decapping enzymes and nucleases. The "canonical" binding site of miRNAs is located within the 3′UTR of the mRNA target, but the coding sequence and the 5′UTR can also be bound by miRNAs. Although they mainly play a negative role at a post-transcriptional level, a few miRNAs have been reported to actually enhance mRNA expression. Altered patterns of miRNAs, due to genetic alterations, defects in the biogenesis process, epigenetic modification or aberrant expression of miRNA genes, are associated with many pathological contexts, including cancer and inflammatory diseases. Although the wider world of small RNAs has to be further explored, these regulators have already been shown to play a crucial role in all biological processes.

1 Introduction

MicroRNAs are small non-coding RNA molecules, 20–25 nucleotides long, highly conserved in the plant and animal world. They play a major role in post-transcriptional regulation processes, mainly silencing target mRNAs and thus decreasing their corresponding protein levels. Physiological and pathological mechanisms are affected by these small molecules, and the scientific community is showing more and more interest in non-coding RNA in general and microRNAs

C. de Santi (✉) • C.M. Greene
Respiratory Research, Department of Medicine, Royal College of Surgeons in Ireland Education and Research Centre, Beaumont Hospital, Dublin, Ireland
e-mail: cdesanti@biologia.unipi.it

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in particular. In this chapter, we aim to explain the basic biology of microRNAs, from the first miRNA discovered in the early 1990s to the last update in terms of biological mechanisms underlying miRNA biogenesis, function and altered expression.

2 The Discovery of MicroRNAs

Before 1993, small RNA molecules were known to be involved in essential cellular processes such as messenger RNA (mRNA) splicing [1] and RNA editing [2]. In 1993, the first microRNA (miRNA) was characterised as an important player in post-transcriptional regulation of mRNA in nematodes by Lee and collaborators [3]. The authors, focusing on *C. elegans* post-embryonic development, found that *lin-4* produced two non-coding RNAs of approximately 61 (*lin-4*L) and 22 (*lin-4*S) nucleotides with complementary sequences to the 3′UTR (untranslated region) of *lin-14* mRNA. These small transcripts were able to bind the 3′UTR of *lin-14* mRNA and to decrease protein levels without affecting the mRNA stability. The authors suggested that the inhibition of protein synthesis was operated by *lin-4* small RNAs either indirectly (via modification of *lin-14* mRNA or its localization in a sub-cellular compartment not accessible to ribosomes) or directly (via steric hindrance towards the translation machinery). This phenomenon was considered strictly correlated to the larval development of the nematode and only in 2000 was the second miRNA characterised, again in *C. elegans* [4]. Reinhart and collaborators showed that *let-7* encoded a temporally regulated small RNA (21 nucleotides) complementary to sequences in the 3′UTRs of numerous genes (i.e. *lin-14*, *lin-28*, *lin-41*, *lin-42* and *daf-12*) involved in the larval development of *C. elegans*. *Let-7* could directly regulate the protein level of *lin-41*, whereas *lin-4* was involved in *lin-14* and *lin-28* post-transcriptional regulation. These two small RNAs were suggested to be part of a pathway that regulates a temporal sequence of events in *C. elegans* larval development. The official name "microRNA" was used for the first time in 2001, when three papers, all published in the same issue of *Science*, showed the existence of small RNAs involved in post-transcriptional regulation of target mRNAs in both vertebrates and invertebrates [5–7]. Since then, miRNAs have been studied in many organisms and the latest version of the miRNA database (*miRBase*, http://www.mirbase.org/) contains 24,521 microRNA loci from 206 species, corresponding at about 30,000 mature miRNAs [8]. This list is ever expanding and contains at the moment ~2,000 human mature microRNAs, representing 1–3 % of all genes in *Homo sapiens* [9].

3 Localization, Biogenesis and Expression of miRNAs

MicroRNAs can be classified into five groups according to their genomic locations relative to introns and exons (Fig. 1) [10]:

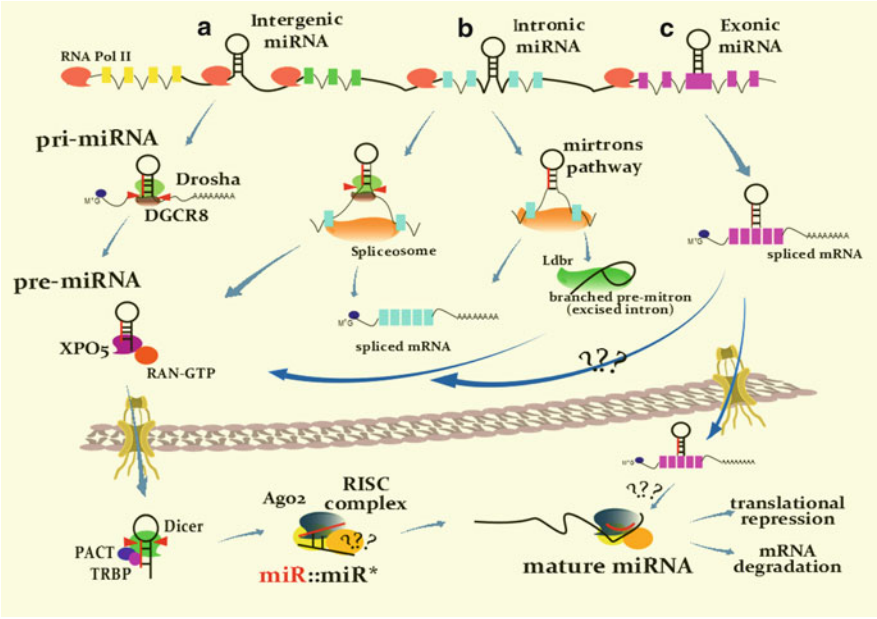

Fig. 1 Biogenesis of microRNAs. MicroRNA biogenesis depends on the genomic localisation of miRNA genes. (A) Intergenic miRNA genes are transcribed by RNA Pol II in pri-miRNAs and then cropped by Drosha-DGCR8 complex in pre-miRNAs and moved by XPO5 in the cytoplasm. They are then cleaved by Dicer and the double strand miR::miR* is loaded in the RISC complex. The mature miRNA is selected and can guide the catalytic machine to a target mRNA and act as a negative regulator. (B) Intronic miRNAs are processed before (or during) splicing. Drosha cleaves the miRNA hairpin that enters into the miRNA pathway. Mirtrons are non-canonical intronic small RNAs produced from spliced introns and debranching. They bypass the Drosha-processing step because Lariat debranching enzyme (Ldbr) resolves the secondary structure of the excised intron into a hairpin structure resembling pre-miRNAs. (C) Exonic miRNA biogenesis is poorly understood. The unspliced nuclear transcript is mainly processed to pre-miRNA. The spliced transcript (still containing the miRNA hairpin) can be exported to the cytoplasm, but it is not clear if miRNA hairpin is processed to a mature form or not

1. "Intergenic miRNAs"—term used when miRNA genes are located between two consecutive protein-coding genes.

2a. "Intronic miRNAs in non-coding transcripts"—e.g. the miR-15a~16-1 cluster found in one intron of a non-coding RNA gene, *Leu-2* [11].

2b. "Intronic miRNAs in protein-coding transcripts"—e.g. miR-126 located within the fifth intron of the *EGFL-7* gene on chromosome 9 and suggested to regulate the innate immune response in cystic fibrosis [12].

3a. "Exonic miRNAs in non-coding transcripts"—e.g. miR-155, one of the most important miRNAs in the immune response [13], located in the third exon of a non-coding gene, BIC.

3b. "Exonic miRNAs in protein-coding transcripts"—e.g. miR-198 located in the 11th exon of the *FLST1* gene, their co-regulation was studied in wound healing [14].

Some miRNAs are defined as "mixed" when, depending on alternative splicing, they can be located either in exons or introns [10]. Intergenic miRNAs are transcribed from their own promoters, whereas intragenic (i.e. exonic and intronic) miRNAs are preferentially located in the same orientation as the host genes [15] and frequently co-expressed with them [16]. About 50 % of total miRNAs are located in close proximity to others [10], and they usually produce long polycistronic transcripts (clusters) that are then processed into mature miRNAs [15].

Biogenesis of canonical miRNAs Canonical miRNA biogenesis in animals undergoes a stepwise process: (1) miRNA genes are transcribed in the nucleus by RNA polymerase II into primary nascent miRNA transcripts called "pri-miRNAs". These are characterised by stem-loop structures (containing the future mature miRNAs), a 3′ poly-A-tail and a 5′ methylated cap and they can be up to several hundred nucleotides long, particularly when a miRNA cluster is transcribed [17]. Some miRNA genes are transcribed by RNA polymerase III, in particular those that are close to *Alu* repeats (transposable elements in the human genome reviewed by Deininger [18]) [19]. (2) Pri-miRNAs are then recognised by a multiprotein complex called the "microprocessor complex" that cleaves them in 60–100 nt products named "pre-miRNAs" [20]. The two main core components of the microprocessor are Drosha and Di George syndrome Critical Region 8 (DGCR8). The double-stranded RNA-binding protein DGCR8 binds to the base of the stem-loop structure and guides the positioning of the RNase type-III enzyme Drosha, which constitutes the catalytic centre of the complex. Drosha cleaves the double-stranded stem about 11 bp from the base and generates a 2-base 3′ overhang [21]. (3) This particular structure is recognised by Exportin 5 (XPO5) that, together with its "energetic" partner Ran-GTP, moves pre-miRNAs from the nucleus into the cytoplasm through nuclear pore complexes [22]. In addition to its transport role, XPO5 also stabilises pre-miRNAs by preventing degradation by exonucleases [23]. (4) Once in the cytoplasm, the hairpin precursors are recognised and cleaved near to the terminal loop by another RNAse type-III enzyme, Dicer, generating miRNA duplexes ~22 nt long with 3′ and 5′ overhangs [15]. These dsRNA duplexes are referred to as miRNA:miRNA* and contain both the mature miRNA strand and its complementary passenger strand (*). Dicer was first characterised as a key protein of the RNA interference (RNAi) machinery [24] and then investigated as a miRNA processing factor [25]; it is highly conserved and is reported to be associated with dsRNA binding proteins such as trans-activation response RNA-binding protein (TRBP) [26] and protein activator of PKR (PACT) [27]. (5) When miRNA duplexes are produced, they associate with Argonaute (Ago) proteins (in particular, Ago2), are recruited by TRBP and form the RNA-induced silencing complex (RISC) [26]. In detail, one of the strands, the one that will become the mature miRNA, is loaded into RISC, whereas the miRNA* strand is degraded or expressed at a lower level. Generally, the strand

having the less stable base pairing at the 5′-end is chosen as the guide strand; the ability to assess the thermodynamic properties of the duplex is mediated by RISC [28]. Ago2 represents the engine of the RISC complex and its structure has been studied in depth in order to understand the RNAi biochemical mechanism. Ago2 has three major domains [29]: *PAZ* (N-terminal, ~20 kDa) which specifically recognises the two base 3′ overhangs of miRNA duplexes generated after Dicer cleavage; *MID* which contains a highly specific binding pocket for the 5′ end of the miRNA and *PIWI* (C-terminal, ~40 kDa) which shows nuclease activity and is involved in the mechanism of action of miRNAs. As part of the RISC complex, the mature miRNA can guide the catalytic machine to a target mRNA and act as a negative regulator at a post-transcriptional level.

Biogenesis of intronic miRNAs The majority of miRNA loci are located in intronic regions [30]. Intronic miRNAs share the same promoter with their host genes, so unspliced pre-mRNAs contain miRNA sequences folded as hairpin structures in intron(s) [31]. It was generally assumed that this class of miRNA was processed from excised introns, in parallel to what happens to intronic small nucleolar RNAs [32], but Kim and Kim showed that Drosha cleavage of miRNA hairpins might take place after the assembly of the early spliceosome complex but before or at the same time as proper intron excision [30]. Thus, the pre-miRNA enters the miRNA pathway and the rest of the transcript undergoes the splicing process to produce a mature mRNA for protein synthesis. “Mirtrons”, a class of non-canonical intronic miRNAs, were first identified in *D. melanogaster* and *C. elegans* in 2007 and their processing bypasses Drosha cleavage [33]. In their case the spliced intron product is not linear but adopts a lariat between the 5′ end and the 3′ branchpoint. Following the resolution of this structure by the lariat debranching enzyme Ldbr, the resulting hairpin structure, resembling a pre-miRNA, can be transferred to the cytoplasm by XPO5, cleaved by Dicer, loaded into RISC and act as a canonical microRNA. Some mirtrons have short extensions at either the 3′ or 5′ end of the hairpin ([33] and [34], respectively) that require a further exonucleolytic trimming in order to be recognised by XPO5 and enter the miRNA pathway. Mitrons have also been described in mammals [35] and are reported to be involved in acute lymphoblastic leukaemia [36] and inflammatory lymphangiogenesis [37].

Biogenesis of exonic miRNAs The biological mechanism underlying exonic miRNA biogenesis is poorly understood. Recently Slezak-Prochazka and collaborators used miR-155 and its host gene *BIC* as a model of exonic miRNA biogenesis and evaluated whether unspliced and/or spliced transcripts were used as template for miRNA processing [38]. The authors suggested that the unspliced nuclear transcript is mainly processed to pre-miRNA, and is then transported in the cytoplasm where it follows classical miRNA biogenesis. Moreover, they showed that alternatively, the spliced transcript still containing the miRNA hairpin can be exported to the cytoplasm; however in this case the miRNA hairpin is not fully available for processing to its mature form. Thus the biochemical details of this process are not clear yet.

4 miRNA Function on Target mRNAs

In mammals microRNAs are predicted to regulate the expression of about 60 % of protein-coding genes [39] and are involved in almost all the physiological and pathological processes investigated to date [40]. Generally, miRNAs regulate gene expression at a post-transcriptional level by base pairing to miRNA recognition elements (MRE) located in the 3′UTR of target mRNAs. The miRNA::mRNA base pairing can be perfect or limited to the so-called "seed region", i.e. the 5′ miRNA sequence that provides most of the pairing specificity [41].

Perfect complementarity The consequence of perfect miRNA::mRNA pairing, mainly present in plants [42], is target degradation (or "slicing"), which leads to a decrease in both mRNA and protein levels. Specifically, Ago2 cleaves the target mRNA between nucleotides complementary to the 10th and 11th bases of the miRNA guide strand [43] then 3′ deadenylation and 5′ decapping occur before complete mRNA degradation by exonucleases, e.g. XRN4, in *Arabidopsis* [44].

Partial complementarity In this case, only the seed region is required for miRNA::mRNA binding. The mRNA region complementary to the miRNA seed sequence is called the "seed match". The most common seed types are the following [45]:

1. 6mer site: this is considered the minimal region required (6 nt) for miRNA::mRNA interaction; there is perfect pairing of the region from the second to the seventh nucleotide of miRNA.
2. 7mer-A1 site: perfect pairing between bases 2–7 and the first nucleotide is an adenine.
3. 7mer-m8 site: perfect pairing from the second to the eighth nucleotide of the miRNA.
4. 8mer site: perfect pairing for bases 2–8 and the first nucleotide is an adenine.

Knowledge of the details about miRNA::mRNA interaction sites is crucial for bioinformatic tools for target prediction that are based on both the binding free energy and conservation between different species of miRNA::mRNA pairing.

In the case of partial complementarity, miRNAs act as negative regulators inhibiting the translation of an mRNA target through two proposed mechanisms [46]: (1) miRNAs can stop the initiation of translation by repressing m^7G cap recognition by eIF4E, thus preventing the recruitment of the 40S ribosomal subunit or by interfering with the 60S joining; (2) miRNAs can interfere with translation after the very initial steps by repressing ribosome elongation or inducing ribosome drop-off and nascent protein chain degradation. These mechanisms lead to a decrease of protein but not mRNA levels. Recent papers also showed that miRNAs are involved in target mRNA degradation in animals but differently than in plants. Indeed, while in plants an endonucleolytic cleavage by Ago2 occurs within the region complementary to the miRNA, in animals miRNAs might direct mRNA targets towards the degradation machinery [46]. GW182 is a protein component of the RISC complex that interacts with the Ago2 *MID*/*PIWI* domain, recruits CCR4-NOT1 (carbon catabolite repression 4—negative on Tata-less) deadenylase and

binds poly-A binding proteins (PABP), promoting the deadenylation process by putting in contact the poly-A tail of an mRNA with the deadenylation machine. Then the mRNA is either degraded by 3′–5′ exonucleases or, first, the decapping enzyme DCP1-DPC2 complex removes the m^7G cap, and then degradation is completed by the 5′–3′ exonuclease Xrn1 [46]. Recent evidence supports the idea that miRNA machinery components and proteins involved in the repression process might not be located in the cytoplasm but organised in structures that promote the efficiency of this mechanism. P-bodies ("processing bodies") were first discovered in 1997 by Bashkirov and collaborators and described as specialised loci for mRNA degradation, since the exonuclease Xrn1 was found to be localised there [47]. Nowadays, most of the components of both miRNA and repression machinery are found to be localised in these cytoplasmatic structures (for instance, Ago2, GW182, DPC1-2, Xrn1, CCR4, NOT1) [48]. Thus, P-bodies are considered as the sites of the final step of mRNA degradation triggered by miRNAs, although their precise role in miRNA-mediated repression needs to be further investigated since it is not clear whether they are essential or not for the efficacy of this mechanism [46].

As mentioned, the main miRNA binding sites are localised in the 3′ UTR of target mRNAs. Despite this, binding sites in the coding sequence (CDS) or in the 5′ UTR have been predicted and experimentally validated [49–51], although their efficacy in negatively regulating gene expression is lower than 3′UTR binding sites [52].

Collectively, miRNAs are principally negative regulators of gene expression. Nonetheless, some have been shown to activate translation of their target mRNAs in particular cellular conditions including quiescence [53] or stress [54] by binding to the 3′ or 5′ UTR of target genes, respectively. Tsai and collaborators reported miR-346 binding to the 5′UTR of *RIP140* mRNA and enhancing its translation by promoting the association between mRNA and polysome fractions [55]. Surprisingly, miR-373 was shown to promote *E-Cadherin* and *CSDC2* transcription by binding to complementary sequences located in the promoters of these genes [56]. This finding raises the question: how can a miRNA act in a nuclear context? In vitro studies suggested that the "AGUGUU" sequence at the 5′ end of miR-29b might direct its nuclear import [57]; a very similar sequence (AGUGCUU) is also present in miR-373 [58]. Little information exists regarding miRNA nuclear import (only Ago2 was shown to be able to transport small RNA from the cytoplasm to the nucleus [59]), their ability to contact with DNA and their role in the regulation of gene transcription.

5 Altered miRNA Expression

MicroRNAs are expressed in a spatio-temporal manner and are sensitive to metabolic or physiological conditions. Since they are involved in the regulation of almost all aspects of cellular biology, it is not surprising that their expression is finely controlled and that alterations in miRNA expression patterns can lead to

various diseases. Here we describe the main mechanisms that can affect miRNA expression, and that are thus deregulated in pathological contexts, with a main focus on inflammatory diseases and alterations of the immune system.

Genetic alterations Genomic amplifications or deletions and chromosomal rearrangements or mutations can affect miRNA genes to the same extent they alter protein-coding genes. Actually, the first paper proving an association between miRNA and cancer showed a deletion of the 13q14 region in chronic lymphocytic leukaemia (CLL), where miR-15 and miR-16 are located, and thus defined them as tumour suppressors [11]. Interestingly, the miR-15/-16 locus is also affected by genetic duplication in the 3q25.33 region (to prevent the loss of expression of these two microRNAs) and by germline mutations in about 15% of CLL patients [60]. No genetic or chromosomal alterations have yet been linked with inflammation or the immune system; however single nucleotide polymorphisms (SNPs) in miRNA genes or target sites are involved in these processes (discussed later).

MiRNA machinery defects Altered expression of or the presence of mutations in genes involved in the miRNA biogenesis machinery leads to a general deregulation of the miRNA pathway. In cancer, many papers have reported down-regulation of the miRNA machinery genes, such as Drosha [61] or Dicer [62], and global repression of miRNA maturation is known to enhance transformation and tumorigenesis in animal models [63]. Nonetheless, other reports show up-regulation of these genes [64, 65]; thus the effect of altered miRNA machinery might be tissue specific. In the immune system, the role of some miRNA machinery proteins was studied through disruptions of their genes in animal models. For instance, deletion of Ago2 in hematopoietic cells affects early development of lymphoid and erythroid cells; Dicer-deficient B lymphocytes show decreased cell survival and antibody production; and conditional knock out of Dicer or Drosha in T_{reg} cells leads to the development of autoimmune inflammatory disease [66]. Moreover, a mouse model of lupus erythematosus is characterised by decreased levels of Dicer mRNA in T_{reg} cells [67]. PACT, a protein associated with Dicer, was found to be up-regulated in chronic rhinosinusitis [68], but this represents nearly the only report about human inflammatory diseases and deregulation of miRNA machinery; thus more studies are needed to assess their role in this pathological context.

RNA editing RNA editing is a cellular mechanism where specific nucleotide sequences within a transcribed RNA undergo different modifications. In animals, A-to-I editing is the most common and involves hydrolytic deamination of adenosine to inosine mediated by adenosine deaminases acting on RNA (ADARs) [69]. A-to-I editing was reported to affect different points of miRNA biogenesis and function: (1) the processing of pri-miRNAs by the Drosha/DGCR8 complex is altered, and edited pri-miRNA can be degraded by Tudor-SN, a nuclease specific to I-dsRNA; (2) the Dicer cleavage step of edited pre-miRNA is suppressed; (3) when an edited mature miRNA is expressed and its seed sequence is modified, it can target a different set of mRNAs compared to its unmodified partner; thus gene expression might be strongly affected in tissues where a substantial proportion of

pri-miRNAs is edited [69]. The effects of edited miRNAs on disease outcome or progression need further investigations. In cancer, unedited miR-376a* stimulates glioma cell migration and invasion, whereas the edited miR-376a* can suppress these features, mainly due to the different sets of target genes bound by both miRNAs [70]. ADAR1 was found to be up-regulated in a mouse model of inflammation [71], but to date no edited miRNAs have been reported to be involved in any human inflammatory disease.

Epigenetic modifications Epigenetics is a term that refers to inheritable modifications in gene activity that are not caused by a change in DNA sequence but by several other alterations such as DNA methylation or histone modification. About half of miRNA genes are located in CpG islands and thus are likely to be affected by the DNA methylation machinery [72]. Epigenetic mechanisms controlling miRNA expression have been described in inflammatory diseases, for instance, the miR-199a promoter was found to be hyper-methylated in chronic obstructive pulmonary disease and this affects the unfolded protein response (UPR) in monocytes [73]. Another example involves increased methylation of miR-124a in colitis-associated cancer patients compared to healthy volunteers and was suggested as a promising marker for individual risk of developing colitis-associated cancer [74]. No data are available on epigenetic histone modification in inflammatory diseases, but it is suggested to be another important regulatory pathway in cancer growth [75], chemoresistance [76] and stem-like properties [77].

Alteration of transcription factors binding miRNA gene promoters Extracellular and intracellular stimuli can lead to alterations in transcription factor binding to the promoters of miRNA genes. Thus, pathological contexts often show deregulated miRNA expression compared to normal physiological conditions, and this affects the expression of target genes that are regulated at a post-transcriptional level. This phenomenon is so important that the term "oncomir" was coined in 2006 to define a primary transcript (OncomiR-1) highly expressed in many cancer cell lines [78] and used since then to describe miRNAs playing an important role in cancer. Some oncomirs act as oncogenes targeting tumour suppressor genes and leading to malignant transformation when over expressed. In contrast, some of them target oncogenes and, to the same extent as protein-coding tumour suppressor genes, they are reported to be down-regulated in cancer.

Various inflammatory diseases are reported to be associated with altered miRNA expression and have been investigated through high-throughput analyses [79–81]. Many other papers focus on only one or a few altered miRNAs in an attempt to explain the biological mechanism underlying the alteration and its effects on disease pathology. miR-155 is considered a master regulator of the immune response as a pro-inflammatory agent, and is reported to be deregulated in numerous inflammatory diseases including multiple sclerosis, rheumatoid arthritis and endotoxemia, via its ability to target various negative regulators of inflammation including *SHIP1* and *SOCS1* [82]. miR-146a represents its negative counterpart. Its expression is altered in lupus erythematosus, rheumatoid arthritis and Sjogren's

syndrome [82], and it can mute the immune response by targeting *IRAK1* and *TRAF6* involved in the NF-κB pathway [13]. Recently, less well-characterised miRNAs were reported to be altered in other diseases and to play an active role in the pathological condition. For instance, miR-145, miR-223 and miR-494, up-regulated in the bronchial epithelium of cystic fibrosis patients [12], were reported to target *CFTR* through luciferase assays and western blots and to contribute, together with inflammatory processes, to the regulation of ΔF508 *CFTR* [83]. MiR-149 was shown to be down-regulated in osteoarthritis and to target many cytokine genes involved in the pathology such as *IL1β*, *TNFα* and *IL6* [84].

6 miRSNP

Single nucleotide polymorphisms (SNPs) are the most common genetic variation in the human genome and can affect introns, intergenic regions or coding regions of genes (leading to altered protein sequence). A single nucleotide polymorphism falling in a miRNA target site commonly called "miRSNP" [85] can affect the strength of miRNA::mRNA binding and thus the post-transcriptional regulation of that mRNA. Several elements have to converge in order for a miRSNP to be considered functional: (1) the SNP must have an association with a physiological or pathological process, (2) both the miRNA and its predicted target must be expressed in the tissue, and (3) the allelic changes must result in differential binding of the miRNA and affect expression of the target gene. Nowadays databases recording polymorphisms in putative miRNA binding sites, e.g. PolymiRTs [86], are available online, although experimental validation is required to test the three previous characteristics. Increasingly, papers suggest that miRSNPs can have important phenotypic consequences. In the past studies reporting their association with the risk of various types of cancer, such as breast [87], ovarian [88], colorectal [89] and lung [90], have been published. Moreover, miRSNPs were found to be involved in chemoresistance [85], risk of myocardial infarction [91] and the performance of diagnostic biomarkers [92]. Concerning inflammatory diseases, Tan and collaborators showed altered binding of miR-148a, miR-148b and miR-152 to the *HLA-G* mRNA in asthma due to the presence of a polymorphism in its 3′UTR [93]. In inflammatory bowel diseases, miRNA-mediated regulation was reported to occur for *IL-23R* since the rs10889677 variant in its 3′UTR could enhance *IL-23R* mRNA and protein levels due to loss of binding capability for Let-7e and Let-7f [94]. A miRSNP located in the 3′UTR of the *BSG* gene and affecting miR-492 binding site was found to be associated with risk of psoriasis [95].

SNPs can also occur within miRNA gene sequences and are again reported to be associated with risk of tumours, such as in childhood leukaemia [96], breast cancer [97] and cervical carcinoma [98]. In inflammatory or autoimmune disorders, miR-146a rs2910164 has been associated with asthma [99] whilst miR-196a-2

rs11614913 plays a functional role in vitiligo through the differential allele modulation of TYRP1 target gene [100].

7 Conclusions/Perspective

Despite the acquired knowledge about miRNA regulation, further investigations are needed to fully understand their role in the physiology and pathology of inflammation and the immune system. Although they have been proposed as diagnostic biomarkers and possible therapeutic targets in cancer [101–103], their clinical use is less well known in immune disorders. A recent review by Zeng and co-authors summarised the available knowledge about circulating miRNAs that could be used as biomarkers in autoimmune diseases, but suggested that more studies with larger number of recruited subjects should be carried out [104]. In conclusion, findings about the fundamental biological role played by miRNAs suggest the potential use of these small molecules in the clinic; collaborative and interdisciplinary efforts together with novel approaches are required to succeed in this goal.

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The Biology of Long Non-Coding RNA

Paul J. McKiernan and Catherine M. Greene

Abstract The majority of human transcribed loci do not encode protein. Long non-coding RNAs (lncRNAs) are an emerging class of non-coding RNA that are implicated in the regulation of gene expression at almost every level. Although their mechanisms of action are relatively unknown, general mechanistic themes are emerging. They have the ability to affect gene expression through a spectrum of interactions with RNA, proteins and possibly DNA. With their capacity to act both in *cis* and in *trans*, they can guide epigenetic-modifier complexes or transcription factors to particular genomic sites. These lncRNAs have been implicated in a variety of cellular processes, including chromatin modification, genomic imprinting and cell cycle regulation. This chapter examines their biology, conservation, function and roles in disease as well as various approaches to their characterisation.

1 Introduction

Given the fact that humans are complex organisms, it was expected that we would have a large number of protein-coding 'genes', estimated at approximately 100,000. However, since the draft human sequence became available in 2001 [1, 2] this number is now estimated to be in the region of 20,000–25,000. GENCODE (v20) currently lists this number at 19,942, Ensembl (v76) at 20,389 and EntrezGene at 20,658 (Aug. 2014). The collaborative consensus coding sequence (CCDS) project is attempting to unify the discrepancies between annotations of protein-coding genes, and agree on a core set. As of August 2014, its conservative strategy lists the number of protein-coding genes universally agreed upon at 18,800. Thus, it is becoming increasingly apparent that much of the human genome encodes non-coding RNA, as only ~1.2 % of the genome is protein-coding [3]. Many studies over the last few decades have led to the speculation as to why so many parts of the

P.J. McKiernan (✉)
Respiratory Research, Department of Medicine, Royal College of Surgeons in Ireland, Dublin, Ireland
e-mail: pauljmckiernan@rcsi.ie

C.M. Greene
Respiratory Research, Department of Medicine, Royal College of Surgeons in Ireland, Education and Research Centre, Beaumont Hospital, Dublin, Ireland

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human genome are transcribed but do not code for protein. The Encyclopaedia of DNA Elements (ENCODE) consortium was established in 2003 by the US National Human Genome Research Institute to identify all the functional elements in the human genome and other transcriptomes. Recently the ENCODE project has reported that transcription of three-quarters of the genome is possible in human cells [4]. Until recently, however, non-coding RNA had been viewed as 'junk' or non-functional. The traditional view of many in the molecular biology field was that the only functional transcripts were those that are involved in coding for protein (messenger RNA, mRNA) or 'housekeeping' such as translation (ribosomal or transfer RNA, rRNA and tRNA) and splicing (small nuclear, snRNA). The ENCODE consortium has identified the involvement of 80.4 % of the human genome in at least one biochemical RNA—and/or chromatin-associated event in at least one cell type [5]. Delving into the functionality of regions of the genome, this consortium identified that within ENCODE-annotated functional regions, the number of non-coding variants is at least as large as those that lie in protein-coding genes. Small regulatory non-coding RNAs and microRNA (miRNA) in particular have been studied in detail, and the methods used to identify and annotate these are becoming relatively mature. Beyond these, very little is known about larger, long non-coding RNAs (lncRNA).

lncRNAs are loosely defined as RNAs that are over 200 nucleotides (nt) in length that have no protein-coding capacity. The 200 nt limit is somewhat arbitrary, but is generally agreed upon, to distinguish these from other small ncRNAs such as miRNA, small nucleolar RNAs (snoRNA) and piwi-interacting RNAs (piRNA). The first of these lncRNAs, then viewed as exotic RNA molecules, were identified in the late 1980s as having involvement in genomic imprinting, whereby one allele is expressed from either the paternal or maternal chromosome in diploid mammalian cells. One of these lncRNAs, and one of the most studied to date is *X* (*inactive*) *specific transcript* (*XIST*). *XIST* plays a central role in the process of X chromosome inactivation (XCI), whereby one of the two X chromosomes in females is inactivated, to equal male dosage of X chromosome gene products. *XIST* RNA (19 kb in humans, 17 kb in mice) is transcribed from the inactive X chromosome, spreads over and coats this chromosome *in cis*, leading to its inactivation [6, 7]. *XIST* physically associates with and recruits Polycomb repressive complex 2 (PRC2), which is involved in the trimethylation of histone H3 lysine 27 (H3K27me3) of the X chromosome, and ultimately leads to its inactivation [8]. As will be discussed later in this chapter, lncRNAs have been implicated in regulating numerous other cellular processes from meiotic entry to retrotransposon silencing and hormone signalling to pathogen susceptibility.

Microarray technology, and its ability to simultaneously determine the expression of thousands of loci throughout a chromosome or genome, drove the early discovery of lncRNA. With the advent of these microarrays, combined with the draft genome sequences, evidence for the pervasive transcription of non-coding RNAs has begun to arise. Two initial studies estimated that the number of lncRNA genes may be on a par with that of protein-coding genes [9, 10]. However, early studies suggesting that lncRNAs were functionally important were accepted with

scepticism. Some have suggested that these transcripts are by-products of transcription or 'transcriptional noise' [11]. More recently, there has been a shift away from this view, with an explosion of studies assigning important functions to lncRNA.

2 lncRNA Classification: Genomic Context

As such diversity of species of lncRNA exists, efforts are under way to classify these based on genomic context. It must be noted that this classification does not provide any information on the specific functions of each lncRNA as this approach groups similar lncRNAs, based on their anatomical position with respect to protein-coding genes. The first of these groups is described more by what they are not, rather than what they are. These are referred to as long intervening (or intergenic) ncRNA (lincRNA). These transcripts are those that are found between protein-coding genes, with no overlap with either protein-coding or other lncRNA genes. Most of these have been identified from chromatin signatures, when examining trimethylation of histone H3 lysine 4 (H3K4me3) and histone H3 lysine 36 (H3K36me3), markers of the promoters and the transcribed units of RNA polymerase II (Pol II)-actively transcribed genes, respectively. The majority of lincRNAs, like other stable Pol II products, are capped and polyadenylated and many are spliced, generally with alternative isoforms and fewer exons than their coding counterparts [12]. Some of the best known lncRNAs fall into this category, including *XIST* [13, 14], *HOTAIR* [15] and *H19* [16].

The second class of lncRNA diversity are the natural antisense transcripts (NAT). These are transcribed from the opposite DNA strands of other annotated transcript units, and thus share sequence complementarity. As many as 70 % of sense strands have been reported to have antisense counterparts [17, 18]. The overlap between sense–antisense (S/AS) pairs may be full or partial, with the latter being more common. NATs appear to be more highly enriched around the 5′ promoter or 3′ terminator ends of the sense RNA [19]. Some well-characterised S/AS paired lncRNAs fall within the NAT category, such as the *XIST/TSIX* pair [20].[1] *TSIX* initiates silencing of *XIST* expression from the active X chromosome [21], but more intriguingly, *Tsix* has an additional antisense companion in mice, *Xite*, the *cis*-acting activator of *Tsix* [22]. Some of these NATs lie within imprinted regions, such as the lncRNA pair *Kcnq1/Kcnq1ot1* [23], and the *Insulin-like growth-factor type-2 receptor/Antisense Igf2r RNA* (*IGF2R/AIR*) coding/non-coding RNA pair [24].

A third class of lncRNA are housed within intronic regions of other transcribed units. These have received less attention and remain somewhat controversial as to whether they should be classified as lncRNA. Some are known to harbour small

[1] This highlights the inadequacies of classification based on genomic context, as *XIST* is also characterised as a lincRNA.

ncRNA such as miRNA and snoRNAs. Many are of the opinion that the majority of these lncRNAs may in fact be fragments of pre-mRNA or resulting from immature mRNAs; however this view is changing. A study by Louro et al. [25] has revealed that about 81 % of all spliced human protein-coding genes have transcriptionally active introns. There are over 30,000 conserved structured RNA elements predicted in the human genome; about one-third of these reside within introns of annotated protein-coding genes [26]. Whether this class of lncRNA arises from mRNA splicing or is actually independently transcribed remains largely unknown; however the studies referred to above indicate that these ncRNAs are stable and possibly functional. The best-known example of a functional lncRNA from this class controls seasonal timing of flowering in plants (known as vernalisation) through the 1.1 kb *COLDAIR*, a cold-inducible intronic lncRNA. This lncRNA recruits PRC2 in *cis* leading to trimethylation of histone H3 lysine 27 (H3K27me3) to silence the flowering regulator gene *FLC* [27].

lncRNAs may also arise from transcription of pseudogenes. These are often the relics of other coding genes that have lost their coding ability as a result of a variety of mutations. These may arise from tandem gene duplication or retrotransposons. Data from the ENCODE project provide experimental evidence that up to 10 % of pseudogenes are transcribed in at least one of 12 human tissues examined and a survey of additional ENCODE-generated transcription data increases the estimate of this proportion to nearly 20 % [28]. Having been likened to 'genomic fossils', emerging evidence suggests that a small proportion of these are biologically functional. For example, the expression of the tumour suppressor PTEN is regulated by *PTENpg1*, a *PTEN* pseudogene, through its microRNA sponging activity. In fact, there are two known *PTENpg1* antisense (AS) RNA isoforms, α and β, which have opposite functions in the regulation of PTEN [29]. The β AS-RNA isoform interacts with *PTENpg1* in the cytoplasm, stabilises it and up-regulates PTEN protein levels by enhancing the sequestration of microRNAs. On the other hand, the α AS-RNA remains in the nucleus and recruits chromatin regulators to the *PTEN* promoter, silencing its transcription [29]. Recently, *Lethe*, a pseudogenic lncRNA, has been shown in mice to function in negative feedback signalling to NF-κB, by interacting with the NF-κB subunit RelA to inhibit RelA-DNA binding and block target gene activation [30].

lncRNAs may arise from transcripts that are produced from ultraconserved regions (UCRs). There are 481 described UCRs, areas of the genome that are longer than 200 bp, that are completely conserved (100 % identity, without insertions or deletions) in human, mouse and rat genomes and most of which are also conserved in chicken and dog genomes [31]. 68 % of these are transcribed (T-UCRs) and processed into 200–799 nt sized RNAs [32]. Although many are dysregulated in diseases such as cancers, their overall functions remain a mystery; some have been shown to be involved in microRNA processing [33], while others are believed to possess long-range enhancer activity [34].

A final broad category of lncRNAs include enhancer-associated RNAs, divergent transcripts and promoter-associated transcripts. Enhancers are sequences that regulate transcriptional activities from a distance, independent of their orientation

or position in relation to the affected transcriptional unit [35]. How enhancer regions function is as of yet not fully understood. The classical view of their mechanism of action is that a physical interaction takes place between enhancer regions and promoter regions and an intervening chromatin 'loop', probably mediated by DNA-binding proteins. However, a novel class of lncRNA, termed enhancer RNA (eRNA), has been observed to be transcribed bi-directionally from enhancer sequences [36–38]. Recently, an enhancer-like lncRNA, *HOTTIP*, was found to interact directly with the adaptor protein WRD5 driving H3K4 trimethylation and gene transcription [39].

3 Evolutionary Conservation of lncRNA

The proportion of lncRNA that is functional is as of yet unknown. A large amount of these transcripts may well be non-functional. The transcriptional machinery, like all biochemical processes, is not impeccable, and can lead to spurious RNAs with no function [11]. These transcripts would possess minimal fitness cost or benefit and it may be easier to tolerate these than to evolve more rigorous regulatory mechanisms to avoid their production [12]. On a different level, perhaps the product of transcriptional activity does not matter, but its activity does. These would include RNAs involved in the regulation of gene expression via transcriptional interference [40]. There is general agreement that some lncRNAs are functional while others are not, but opinions vary as to these respective proportions. If, say, only 10 % of lncRNAs are functional out of 14,470 lncRNAs listed by GENCODE (v20), for example, this would imply that over 1,400 lncRNA loci generate transcripts with biological function. lncRNAs, however, are poorly conserved in general. Mammalian lncRNAs tend to lack known orthologs in non-vertebrates, which is in contrast to mRNAs and many other classes of ncRNA. Long non-coding RNA sequences seem to evolve very rapidly; for instance, only approximately 12 % of mouse and human lincRNAs appear to be conserved in the other species [41, 42]. Recent reports suggest that while the transcripts are less conserved than protein-coding RNAs, the promoter regions of lncRNAs are often as conserved as the promoters of coding genes [43, 44]. Furthermore, exonic sequences of many lncRNA show clear evidence of evolutionary constraint. Ponjavic et al. [45] have described a conservative set of 659 lincRNAs out of over 3,000 full-length lncRNA, whose individual genomic sequences exhibit evolutionary constraint, a hallmark of functionality. These had reduced nucleotide substitutions, insertions and deletions both within their promoters and sequences. Although this study in itself suggests that most lncRNAs are not under strong evolutionary constraint, this does not directly infer a lack of function. Evolutionary constraint can be generally estimated from the nucleotide substitution rate in a given functional sequence. This rate is about 10 % for protein-coding sequences, but is much higher in ncRNA sequences, on average 90–95 % [46]. This is understandable, as nucleotide substitutions tend to be less deleterious in ncRNA sequences, as compared to coding sequences. This may

explain the rapid evolution of lncRNAs. Interestingly, a GENCODE study has estimated that about a third of lncRNAs have arisen in the primate lineage only [43]. Perhaps more pertinent to lncRNA function, purifying (negative) selection has recently been validated for secondary structures of human ncRNA transcripts [47, 48]. For example, specific helices in the secondary structure of the lncRNA *steroid receptor RNA activator* (*SRA*) are highly conserved, while one junction containing branching helices has 57 % of its bases fully conserved across all mammals [49]. Recent studies examining conservation of the lncRNA *HOTAIR* have postulated an over-importance of nucleotide conservation when it comes to probing lncRNA functions. Its murine homologue *mHotair* was found to contain only two exons, whereas the human homologue contains six and contained poor overall sequence homology [50]. Diederichs [51] has suggested that there are four dimensions of lncRNA conservation: (1) the nucleotide sequence; (2) structure (despite a lack of sequence homology lncRNAs from different species may fold into similar secondary or tertiary structures); (3) the functions of lncRNA from different species may be similar despite apparent lack of similar sequence or structures; and they may be transcribed from (4) syntenic loci (regions of chromosomes with preserved co-localised genes across different species) whereby the locus of transcription is conserved.

4 Methods for Identification/Annotation

Tiling microarrays were the first to identify lncRNAs on a genome-wide scale. Unlike traditional DNA microarrays which contain probes for sequences of known or predicted transcripts, tiling microarrays contain oligonucleotide probes covering the entire length of a defined section of DNA, allowing for the unbiased interrogation of expression of the whole or part of the genome. This means that these microarrays are capable of identifying novel lncRNA without prior knowledge about their exact genomic location [52]. As traditional microarrays can only interrogate the expression of known lncRNAs in a given RNA sample, these are not used for the identification of novel lncRNAs, but with the increasing pool of known lncRNAs, these microarrays are still very useful in determining differential expression, with ease of use and a relatively low cost.

Recent advances in DNA sequencing technologies have led to the rise of RNA sequencing (RNA-seq), and the ability to sequence cDNA derived from cellular RNA using next generation sequencing (NGS) technologies. RNA-seq offers many advantages over microarray approaches. RNA-seq works on a genome-wide scale with single nucleotide resolution, allowing for the exact identification of exon and intron boundaries. Compared with probe-based microarrays, RNA-seq has relatively low background noise levels and a much larger dynamic range and offers the ability to detect transcripts with low expression [53]. However, this approach does suffer from some limitations. It is relatively costly to perform and the downstream data analysis can be quite time consuming. Nevertheless, RNA-seq allows for

unbiased ncRNA detection and expression quantification across the entire transcriptome. Prior to beginning RNA-seq, decisions must be made whether to use total or polyadenylated RNA, as the presence of rRNA and tRNA can severely reduce the diversity of a cDNA library during amplification [53]. Given the high percentage of RNA transcripts that are not polyadenylated (estimated at 40 % [54]), some studies have suggested that depletion of rRNA may be superior in producing reliable expression data, as opposed to using polyadenylated RNA [55, 56]. A combination of the advances in DNA tiling microarray and, especially, high-throughput RNA-seq technologies, coupled with the completion of the human genome project, has resulted in an unprecedented ability to identify lncRNA and other ncRNAs.

DNA is densely packed into a highly ordered chromatin structure, a DNA–protein complex in the nucleus. Chromatin signatures or signatures of histone modification can be very useful in identification of lncRNAs. Large-scale chromatin immunoprecipitation (ChIP) in combination with hybridisation to tiling arrays (ChIP-chip) provided evidence for the use of histone marks to indicate specific regulatory elements [57, 58]. For example, the relative abundances of H3K4me3 and H3K4me1 are accurate predictors of promoters and enhancers, respectively [58]. Observations that the transcriptional start sites and transcribed units of genes actively transcribed by Pol II are marked by H3K4me3 and H3K36me3 motifs, respectively, led to the search for these 'K4-K36 domains' in lncRNA loci. Guttman and colleagues have identified approximately 1,250 candidate lncRNA loci in mice cell lines based on this approach [59].

Once potential lncRNAs have been identified, by whatever method, they must be evaluated for protein-coding capacity. Transcripts representing known protein-coding genes must be eliminated in the first instance. This can be achieved by examining the overlap with protein-coding annotated genomic coordinates, available from several sources such as RefSeq, Ensembl or UCSC. Next the protein-coding capacity of a transcript of interest should be assessed. This may perhaps be the most challenging aspect in lncRNA identification, as non-coding RNAs are loosely defined. Typically, most lncRNAs with known non-coding functions have multiple potential open reading frames (ORFs) that might be translated inefficiently or be translated into non-functional proteins (e.g. due to rapid degradation), or might not be translated at all [12]. Some may have a dual nature, acting both as ncRNA and protein-coding RNA. Many lncRNAs, due to their considerable length, should by chance contain an ORF of at least 100 amino acids [60]. Thus, it is argued that it is impossible to clearly define a binary separation between coding and ncRNA and is probably better to use imperfect grading criteria to preferentially identify transcripts that are less likely to code for functional proteins [12]. Some features of coding transcripts help in the differentiation between these and their non-coding counterparts: (1) coding regions tend to be much lengthier than would be expected by chance [60]; (2) selective pressures during evolution tend to bias nucleotide substitutions in coding sequences (giving rise to higher rates of substitution in silent positions of codons); (3) non-random codon usage dictates the nucleotide frequencies of functional ORFs; (4) known protein domains are typically

present in protein-coding transcripts; and (5) coding regions are likely to bear similarities to sequences in protein databases [12]. Experimental approaches to examine coding potential include testing whether a particular transcript is located in the cytoplasm (e.g. through RNA fluorescence in situ hybridisation (FISH) or cell fractionation followed by RNA-seq), if it associates with ribosomes (e.g. ribosome profiling) [14], and whether it can produce peptides when translated in vitro [61, 62]. Subcellular localisation or more specifically access to translational machinery, however, is of limited aid in evaluating coding potential, as many lncRNAs, contrary to popular perception, are not localised in the nucleus alone, but in the cytoplasm. Advances in bioinfomatic and experimental tools to distinguish non-coding from coding transcripts will be paramount to enhance the speed at which true lncRNA are identified.

5 Ascribing Functionality to lncRNA

With the acknowledgement that many lncRNAs or their secondary structure are conserved, the next question is what are their functions? To determine functionality, different approaches have been employed. One of the first steps along this route is to examine expression patterns, to identify the type of cells, biological processes, proteins and/or disease(s) associated with a candidate lncRNA. Direct association with proteins or cellular expression and localisation can be determined by RNA FISH [63]. FISH is a useful tool in starting to probe function; however there is a need for a larger scale association analysis. A commonly used approach is *guilt by association* analysis [41, 59, 63, 64]. Tight correlation with the expression of certain known genes, for example, can help in the process of ascribing functionality. *Guilt by association* examines the co-expression of candidate lncRNA with protein-coding mRNAs or gene groups. Therefore, on this basis, hypotheses are generated as to potential functions and regulators of the candidate lncRNA. This approach has proven useful and has predicted diverse roles from stem cell pluripotency to cancer [8]. This and other methods that rely on co-expression networks provide good starting points in the search for function, yet the full scope of lncRNA function is far from understood, and comprehensive loss- or gain- of function experiments are required.

Although there are many strategies available for loss-of-function studies of protein-coding genes, many of these are not suitable for lncRNA analysis, due to the absence of translation, and the presence of less well-characterised RNA functional domains. Therefore, the best approach is one that stops the lncRNA from being transcribed. There are currently some available methods to achieve this. The entire lncRNA gene may be deleted or replaced by a reporter gene cDNA (e.g. lacZ) with a leading KOZAK sequence (a translation initiation signal). This is normally carried out using homologous recombination. This strategy leaves the endogenous promoter region intact, but may affect neighbouring gene expression by removing *cis* regulatory DNA elements that may be located in the lncRNA locus [41]. Other

methods include the deletion of promoters or the integration of a premature polyadenylation cassette to create a strong stop signal at the beginning of the transcriptional unit [65].

Two recent studies have performed large-scale loss-of-function studies in mice [66, 67]. The first of these demonstrated that knockdown of intergenic lncRNA in embryonic stem cells (ESCs) has important consequences on gene expression patterns, of comparable magnitude to that of well-studied ESC regulators [66]. Interestingly, this study showed that lincRNAs primarily affect expression of genes in *trans*, and that some of these are essential for maintenance of pluripotency. The second of these studies demonstrated that hundreds of lncRNAs are regulated during adipogenesis, and most of these were specific to adipose tissue, a common theme in lncRNA expression; tissue-specific expression. Knockdown of several of these lncRNAs each globally perturbed the adipogenic gene expression signature [67]. These studies provide a glimpse of the global effects of lncRNA function.

To determine direct interactions between lncRNA and either protein, RNA or DNA different approaches may be used. When probing RNA–protein interactions, good success has been achieved in vivo on a genome-wide level based on microarray or high-throughput sequencing. This approach is generally based on the use of an antibody against a protein of interest, followed by enrichment of RNA bound to this protein (RNA immunoprecipitation, RIP), and subsequent analysis of bound RNAs via microarray (RIP-chip [68]) or sequencing (RIP-seq [69]). However, these approaches cannot distinguish direct from indirect interactions, and the resulting read length is too large for determining the actual binding site by sequencing. These disadvantages can be overcome by the addition of a cross-linking step prior to enrichment (cross-linking and immunoprecipitation, CLIP). There are currently some variations in methods that combine CLIP with high-throughput sequencing (CLIP-seq), including HITS-CLIP, PAR-CLIP, CRAC, and iCLIP. For a detailed review see [70]. The underlying principle is to cross-link RNA to protein in vivo prior to cell lysis, to avoid additional interactions at or after this step. Fewer high throughput experimental methods exist to determine RNA–RNA and RNA–DNA interactions. RNA–DNA interactions may be examined by CHIRP-seq which is again based on cross-linking between the ribonucleoprotein complex, RNA of interest and its target DNA [71]. Modifications to a recent experimental high-throughput protocol, CLASH-seq [72], might be useful in the identification of RNA targets of lncRNA. This CLASH-seq method is a variant of CLIP-seq that has been used to determine AGO-bound miRNA targets with more precision.

6 Functions of lncRNA

Unlike other small ncRNAs such as microRNA, lncRNAs have a much broader functional repertoire (Fig. 1). As only relatively few lncRNAs have been studied in detail to date, many more functions are likely to be assigned in the future. An

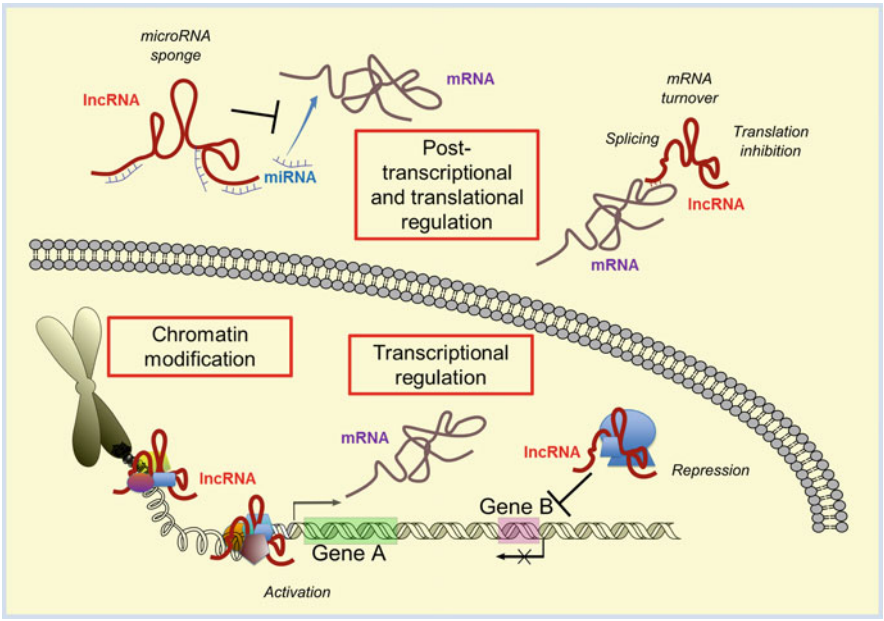

Fig. 1 Functions of long non-coding RNA. LncRNAs have a diverse repertoire of functions. lncRNAs are involved in chromatin remodelling through recruiting or guiding chromatin-modifying complexes to specific DNA loci. lncRNAs can directly or indirectly activate or repress the transcription of protein-coding genes by modulating the localisation or activity of general transcriptional machinery and/or specific regulatory components. lncRNA can also modulate protein expression and activity by post-transcriptional mechanisms such as splicing, capping, translation initiation and mRNA degradation and through the production of endogenous siRNA and targeting of microRNA

emerging theme is the ability of lncRNA to regulate gene expression, in *cis* for nearby genes or in *trans* regardless of genomic location.

Chromatin modification In the 1970s, in an attempt to isolate the principal components associated with euchromatin and heterochromatin, Paul and Duerksen [73] found that biochemically purified chromatin was associated with twice as much RNA as DNA, raising the idea that RNA may be involved in directing chromatin structure and gene expression. Many years later another study provided strong evidence for a structural role for RNA in sustaining the spatial organisation of pericentromeric heterochromatin in conjunction with histone modifications [74]. Perhaps central to this is the ability of lncRNAs to bring DNA sequences and regulatory DNA-binding proteins into close proximity, and therefore to create a docking platform for the recruitment of other epigenetic modifiers. Early insights into lncRNA function revealed that recruitment of the activating Trithorax/MLL and the inhibitory PRC2 chromatin modifiers to specific genomic regions occurs by *HOXA transcript at the distal tip* (*HOTTIP*) [39] and *HOX transcript antisense RNA* (*HOTAIR*) [15], respectively. *HOTAIR*, for example, is a lincRNA transcribed from

the *HOXC* locus on chromosome 12 and targets PCR2 to silence *HOXD* and hundreds of genes on other chromosomes [15], and was one of the first *trans*-acting transcripts to be identified. In a similar fashion, the lncRNA *ANRIL* (*antisense non-coding RNA in the INK4 locus*) recruits the polycomb chromatin modifier CBX7 to silence the *INK4a* tumour suppressor allele by H3K27 trimethylation [75]. Indeed recent studies have revealed an abundant interaction between Polycomb repressor proteins with up to 20 % of expressed lncRNAs, thus indicating the prominence with which lncRNAs direct the inscription and erasure of epigenetic chromatin marks [76]. This most likely extends to other chromatin-modifying proteins, with lncRNA having been identified to associate with at least 12 of these to date [66].

Transcriptional regulation lncRNAs are also known to bypass chromatin-modifying complexes and interact directly with promoters, basal transcriptional machinery and transcription factors [77]. The lncRNA *Airn* (*antisense to Igf2r RNA non-coding*) is transcribed from the paternal chromosome and overlaps the promoter of the maternally expressed Igf2r (insulin-like growth factor 2 receptor) gene to silence it [78]. The precise mechanism by which this occurs remained a mystery until recently. It was experimentally determined that *Airn*-mediated silencing of *Igfr2* occurs via the formation of a transcriptional overlap, i.e. transcription alone and not through its RNA product [79]. This may perhaps be the simplest mechanism of lncRNA function, when the act of transcription, rather than the transcribed product itself, regulates gene expression. lncRNAs can significantly affect the transcriptional output by interfering with Pol II activity in other ways. For example, the *7SK* lncRNA prevents the transcription factor PTEFβ from phosphorylating the Pol II carboxy-terminal domain, and represses transcriptional elongation [80]. The lncRNA *B2* generally represses Pol II activity upon heat shock by binding to and inhibiting phosphorylation of its carboxy-terminal domain by transcription factor II human (TFIIH). Several lncRNAs can act directly on specific transcription factors. For example, the growth arrest specific 5 (*GAS5*) lncRNA represses glucocorticoid receptor (GR)-mediated transcription by folding into a structure that mimics the binding site of the GR to act as a decoy glucocorticoid response element (GRE), thus competing with DNA GREs for binding to the GR [81]. In contrast, depending on whether it recruits the transcriptional repressor MeCP2 or the activator DLX2 to gene regulatory elements, *Evf2* can act as either a co-repressor or co-activator [82, 83]. The diversity in the mechanisms by which lncRNA is involved in transcriptional regulation alone is exemplified by non-coding repressor of NFAT (*NRON*). This lncRNA represses the transcription of NFAT by inhibiting nuclear trafficking of the transcription factor NFAT (nuclear factor of activated T cells) [84]. It is thought that this occurs by competing with NFAT for binding to the transport receptor importin-β. As these examples show, there are many levels on which lncRNAs can regulate the transcription of other genes, and more will likely be found in the future.

Post-transcriptional and translational regulation lncRNAs can also be involved in controlling post-transcriptional processes including splicing, capping, polyadenylation, turnover and transport of mRNAs and subcellular localisation of proteins. The patterns of alternative splicing of mRNA can lead to protein

diversification in higher eukaryotes and is where lncRNA can have a profound effect. The nuclear lncRNA *MALAT1* (metastasis-associated lung adenocarcinoma transcript 1) has recently been implicated in alternative splicing. *MALAT1* interacts with and sequesters several serine/arginine (SR) splicing factors to nuclear speckle domains. Although depletion of this lncRNA increased the phosphorylation and abundance of SR proteins, it inhibited their accumulation in nuclear speckles [85, 86]. lncRNA has also been implicated in both protecting against and accelerating decay of mRNA. An example of the former is the conserved NAT for β-secretase-1 (BACE1), a central enzyme associated with Alzheimer's disease. Augmentation of this lncRNA (*BACE1-AS*) protects *BACE1* mRNA from degradation by RNase in vitro, whereas depletion of this NAT reduces *BACE1* mRNA and protein levels in the mouse brain and in neuronal cell culture [87]. Recently, Faghihi et al. [88] have provided evidence that *BACE1-AS* competes with miR-485-5p for binding to a site in exon 6 of *BACE1* mRNA. On the other hand, a group of transcripts are involved in activating the Staufen 1 (STAU1)-mediated messenger RNA decay (SMD) of specific mRNAs. SMD regulates diverse classes of translationally active mRNAs that have a binding site for the dsRNA binding protein STAU1 in their 3′UTRs. Interestingly, this group of cytoplasmic *trans*-acting lncRNAs, termed half-STAU1-binding site RNAs, were found to facilitate the formation of STAU1 binding sites through imperfect base pairing, via *Alu* repeat elements, leading to the degradation of the target mRNA [89]. lncRNAs can also influence the translation of mRNAs. The mouse Ubiquitin carboxy-terminal hydrolase L1 (*Uchl1*) gene has been found to be regulated by its antisense transcript (*Uchl1-AS*). This NAT increases Uchl1 protein synthesis through an unknown mechanism that depends upon a 73 nt motif that partially overlaps the 5′ end of its sense *Uchl1* mRNA partner transcript, and an embedded inverted short interspersed nuclear elements (SINE) B2 repeat element [90]. Another example is expression of *Zeb2NAT*, the NAT lncRNA of the transcription factor Zeb2 which increases the translation of Zeb2 protein [91].

7 lncRNAs and Disease

Aberrant lncRNA expression has been associated with many human diseases. Notwithstanding that lncRNAs are poorly understood, their roles in cancer have been more extensively studied. Many lncRNAs exhibit altered expression in cancerous cells and are controlled by tumour suppressor and oncogenic pathways such as MYC, NF-κB and p53 [8, 59, 92, 93].

Depending on the cancer type, the heritable component of cancer susceptibility can be quite significant. Interestingly, the majority of genome-wide association studies (GWAS) identify cancer risk loci outside of protein-coding regions [94]. Recent analysis of the comprehensive National Human Genome Research Institute GWAS catalogue, filtered for cancer-related SNPs identified that only 3.3 % change the amino acid sequence, with the majority located in intergenic

(44 %) or intronic (40 %) regions of protein-coding genes, further implicating the involvement of non-coding loci in cancer [94]. Although initially discovered as a predictive biomarker for lung cancer metastasis and survival [95], recent studies have established that *MALAT1* acts by inducing the expression of metastasis-associated genes [96]. In fact, antisense oligonucleotides directed to *MALAT1* prevent metastasis formation after tumour implantation in mice [96]. In approximately one-quarter of human breast cancers *HOTAIR* is highly induced and its expression in primary tumours is a powerful predictor of eventual metastasis and death [97]. Overexpression of *HOTAIR* in vivo drives breast cancer metastasis, in part by re-targeting PRC2 occupancy patterns genome-wide, altering the positional identity of cancer cells to more resemble embryotic fibroblasts [97]. Elevated levels of *HOTAIR* are also predictive of metastasis and disease progression in colon, pancreatic, liver and other cancers [98–100]. Three intimately linked tumour suppressor genes, *p16INK4a*, *p14ARF* and *p15INK4b*, encoded in the 35 kb *INK4B–ARF–INK4A* locus on chromosome 9, are regulated by the lncRNA *ANRIL*. *ANRIL* is upregulated in leukocytes from patients with leukaemia and in prostate cancer tissue [101, 102].

Interestingly, *ANRIL* is located in the chromosome 9p21 locus which is the most highly implicated marker of coronary artery disease, myocardial infarction and large-vessel ischemic stroke in GWAS data [103, 104]. In fact some of these SNPs identified by GWAS that are most strongly associated with susceptibility to atherosclerotic vascular disease lie within *ANRIL* which may regulate the splicing of lncRNA and the production of circular *ANRIL* RNA transcripts [105]. Although *ANRIL* is known to regulate genes by recruiting PCR complexes to direct H3K27me3 repressive methylation, the molecular mechanisms at play in cardiovascular disease are as of yet unknown [75]. Natural antisense transcripts have been characterised for seven candidate genes potentially involved in hypertension [106]. One of these genes, natriuretic peptide precursor A (*NPPA*), is usually only expressed in foetal atrial and ventricular myocardium, being downregulated in the ventricles after birth. It has, however, been shown to be reactivated in the ventricles during hypertrophy and heart failure, and therefore is considered a highly conserved marker for heart disease [107]. Alternative splicing of this gene has been shown to be modulated by its NAT *NPPA-AS* suggesting a role for this lncRNA in cardiovascular disease [106].

lncRNAs have been implicated in the pathophysiology of neurodevelopmental disorders, especially those associated with genomic printing, such as Angelman syndrome and Prader–Willi syndrome [108]. lncRNAs have been proposed to influence the pathogenesis of fragile X syndrome (FXS). This syndrome is characterised by a trinucleotide repeat expansion in the 5′ UTR of the *FMR1* gene that encodes the neuronal development protein, FMRP. Two lncRNAs are produced from the FMR1 gene locus: *ASFMR1* and *FMR4*. *ASFMR1* is silenced in FXS patients, and alternative splicing of this lncRNA appears to exhibit pre-mutation-specific profiles [109, 110]. *FMR4* is also silenced in FXS patients, but in contrast to *ASFMR1*, it is upregulated in pre-mutation carriers [111].

Recent studies have implicated a central role of lncRNA in inflammatory processes. Genome-wide expression analysis has indicated that inflammatory stimuli induce the expression of hundreds of lncRNAs [30, 112]. In fact, thousands are expressed across many stages of T cell development and differentiation [113]. Many animal studies have been performed to examine the expression and function of lncRNAs in response to immune activation. In chicks, rapid lysozyme induction by LPS stimulation in monocytes requires initial transient synthesis of *LINoCR*, an upstream antisense lncRNA. *LINoCR* transcription appears to displace the enhancer-blocking protein CTCF and induce chromatin remodelling, inducing expression of the lysozyme gene [114]. *NeST* (also known as *Tmevpg1*) is a lincRNA residing near the IFN-γ gene (*IFNG*). By controlling the expression of IFNG in $CD8^+$ T cells this lincRNA also controls the susceptibility to *Salmonella* pathogenesis and the persistence of Theiler's virus in mice [115, 116]. An important role for lncRNA in controlling gene expression has been revealed in macrophages. Numerous lncRNAs were increased in mouse bone marrow-derived macrophages exposed to the TLR2 ligand Pam3CSK4 [112]. One of these, *lincRNA-Cox2*, appears to act as a master regulator of inflammatory gene expression in these cells. For example, this lincRNA inhibits the basal expression of IFN-stimulated genes by physically interacting with multiple heterogeneous nuclear ribonucleoproteins (hnRNPs). This lincRNA is also essential for the TLR-induced upregulation of IL-6 and many additional genes associated with immune pathways. Another lincRNA *THRIL* also interacts with hnRNPs, in this case hnRNPL, to regulate TNFα expression in the THP-1 human macrophage cell line [117]. A recent study has shown the involvement of a pseudogenic lncRNA *Lethe* in negative feedback signalling to NF-κB [30]. This lncRNA is selectively induced by pro-inflammatory cytokines via NF-κB or glucocorticoid receptor agonists and binds RELA, the p65 subunit of NF-κB, inhibiting DNA binding and target gene expression.

At a pathological level, more lncRNAs are being examined in cells from patients with various inflammatory or immune disorders. Cystic fibrosis is a lethal autosomal recessive disorder, characterised by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene. Characterised primarily by the development of chronic inflammatory lung disease, inflammatory processes play critical roles in disease progression. Recently, an aberrant lncRNA expression profile has been identified in bronchial samples from people with cystic fibrosis as compared to non-CF controls [118]. Perry et al. [119] recently identified a number of lncRNAs that are differentially expressed in primary human airway smooth muscle cells treated with the corticosteroid dexamethasone and propose that some of these act as microRNA sponges, with possible implications in asthma and chronic obstructive pulmonary disease (COPD). The lncRNA *DQ786243* is significantly overexpressed in peripheral blood mononuclear cells from clinically active Crohn's disease (CD) patients compared with clinically inactive CD patients or healthy controls [120]. Finally, lnc-IL7R was one of many lncRNAs differentially expressed in response to LPS stimulation in human peripheral blood mononuclear cells [121]. Interestingly, this lncRNA which overlaps with 3′UTR of the human

interleukin-7 receptor α-subunit gene (IL7R) was significantly upregulated in LPS-treated cells and is functionally capable of diminishing the LPS-induced inflammatory response, as knockdown of this lncRNA resulted in elevated expression of LPS-induced IL-6, IL-8, E-selectin and VCAM.

8 Conclusion/Perspective

The non-coding RNA revolution of the past two decades has given insight into the pervasiveness of ncRNA transcription. Although still in the early days of assigning function to these molecules, it is clear that this emerging class of molecules provide a new layer to our current understanding of the regulation of gene expression. Much more work, however, is required to carefully evaluate their functional contributions. Improvements in bioinformatic tools and annotation will help to speed up the process of selecting functional lncRNAs. While their roles in cancer are better known, they are increasingly being regarded as an important class of molecules in other human diseases. Ultimately, a more comprehensive understanding of the expression and biological roles of these lncRNA may reveal new targets for therapeutic intervention in a myriad of diseases, including those associated with dysregulated inflammation.

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MicroRNA Regulation of Neutrophil Function

Jack B. Cowland

Abstract Neutrophil maturation in the bone marrow, granulopoiesis, is a tightly regulated process governed by a temporal and successive expression of transcription factors and appropriate stimulation by cytokines from the surrounding stromal tissue of the bone marrow. Observations published over the last decade have, however, made it increasingly clear that posttranscriptional fine-tuning of protein expression by microRNAs (miRNAs) also plays a very important role in this process. In this chapter the influence of miRNAs on normal neutrophil development and biology is reviewed. The majority of miRNAs alter their expression profile at the point in granulopoiesis where proliferation ceases and terminal differentiation commences indicating a major role of miRNAs in fine-tuning of cell cycle regulatory proteins and transcription factors governing this transition. Some miRNAs are induced by external stimuli of the mature neutrophil and act to fine-tune the innate immune action of the cell.

1 Introduction

Neutrophil granulocytes are crucial for the combat of intruding microorganisms and are major players in the innate immune system. Granulopoiesis, the development of neutrophil granulocytes in the bone marrow, is a stringently regulated stepwise process where the myeloblast (MB) differentiates through the intermediate cell stages promyelocyte (PM), myelocyte (MC), metamyelocyte (MM), band cell (BC), and segmented cell (SC) to be released as a mature polymorphonuclear neutrophil (PMN) into peripheral blood [1] (Fig. 1). Cell proliferation ceases at the MC stage whereupon the cells enter the terminal phases of differentiation [2, 3]. If the PMNs do not sense an inflammatory challenge in the tissue, they will circulate in peripheral blood with a half-life of about 8 h after which they undergo apoptosis and are removed by phagocytosis by macrophages and dendritic cells. In

J.B. Cowland (✉)
Granulocyte Research Laboratory, Department of Hematology, Rigshospitalet, Copenhagen, Denmark
e-mail: jack.cowland@regionh.dk

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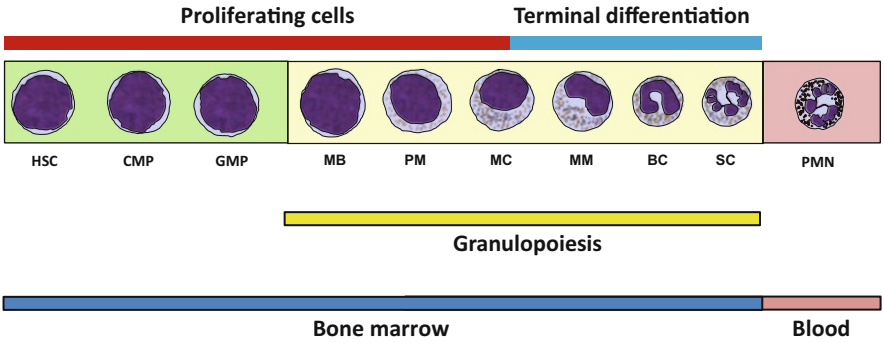

Fig. 1 Schematic representation of neutrophil development in bone marrow. The hematopoietic stem cell (HSC) can differentiate to all blood cell lineages. When entering the myeloid lineage the cells proceed through the intermediate multipotent "common myeloid progenitors" (CMP) and "granulocyte-macrophage progenitors" (GMP) before reaching the first recognizable cell of the committed neutrophil lineage, the myeloblast (MB). Granulopoiesis starts with the MB that differentiates to a promyelocyte (PM) and following to a myelocyte (MC) at which stage proliferation ceases and terminal differentiation commences with metamyelocytes (MM) and next band cells (BC) and segmented neutrophilic cells (SC), which are released to peripheral blood as mature PMNs

the case of an infection, neutrophils are recruited to the infectious site by diapedesis through the endothelial lining of the blood vessel and migration along a chemotactic gradient in the tissue. Once at the infectious site neutrophils kill the microorganisms by phagocytotic engulfment, release of antimicrobial proteins and enzymes by degranulation, or trapping and killing of the microorganisms by NETosis [4, 5]. For a more comprehensive review of the everyday life of neutrophils see [1, 4, 5].

2 miRNAs and Granulopoiesis

Granulopoiesis is orchestrated by the temporal expression of a small handful of transcription factors with Runx1, PU.1, C/EBP-α, C/EBP-ε, and Gfi-1 as important players [6]. Correct timing of transcription factor expression and cell proliferation is essential for proper neutrophil development. Fine-tuning by microRNAs has been shown to play an important role in keeping a correct balance of these proteins in the cell. The importance of miRNAs for proper granulopoiesis is demonstrated by the lack of definitive maturation of neutrophils from myeloid precursors with a *Cebpa*-Cre driven deletion of *Dicer1* [7]. Both in vitro and in vivo this resulted in blast accumulation and myeloid dysplasia.

MicroRNAs (miRNAs) are small (20–23 nts) single-stranded RNA molecules that bind to complementary miRNA recognition elements (MREs) predominantly located in the 3′-untranslated region (3′-UTR) of target mRNAs [8]. The effect of miRNAs is downregulation of protein expression by translational inhibition or

mRNA destabilization [8]. miRNAs are produced as pri-miRNAs encoded by a unique miRNA gene or contained within an intron of a protein-encoding precursor-mRNA. The pri-miRNA is processed in the nucleus by the DGCR8:Drosha RNase III complex to the pre-miRNA, that is exported to the cytoplasm. Following, the pre-miRNA is processed by a second RNase III enzyme, Dicer, to form a partially homologous double-stranded miRNA:miRNA* (guide strand:passenger strand) RNA complex from which one of the strands is loaded into the RNA-induced silencing complex (RISC) that mediates the binding between the miRNA and its target mRNA, while the other strand usually is degraded [8]. A single mRNA can be regulated by several miRNAs, and each miRNA targets different mRNAs [8, 9]. A more comprehensive review of miRNA biogenesis is found elsewhere in this book.

Initially, data regarding miRNA expression during granulopoiesis was primarily obtained by examining peripheral blood neutrophils or neutrophil precursor cell lines differentiated towards a mature PMN by addition of cytokines or chemical inducers such as all-trans retinoic acid (ATRA). The drawback of this approach is that the two most commonly used human cell lines (HL-60 and NB4) are of leukemic origin and immortalized and thus must have a disrupted cell cycle regulation. Furthermore these cells are unable to form secondary or tertiary granules indicating a major deficiency in their regulation of terminal differentiation [10]. The most extensively examined murine cell model is the myeloblastic-derived 32Dcl3 cell line that can be induced to neutrophil differentiation by exchanging IL-3 with G-CSF in the growth medium [11]. This cell line is able to recapitulate most of the features of the normal granulocyte during in vitro granulopoiesis, although synchronous differentiation is not easy to achieve making subtle changes in expression difficult to detect. Recently, two papers were published describing miRNA profiles in cells purified from human bone marrow by flow-cytometric sorting [12] and a combined density centrifugation and immunomagnetic-depletion protocol [13], respectively. The latter is the most comprehensive report on the in vivo expression profiles of miRNAs in neutrophil precursors from bone marrow and mature neutrophils in peripheral blood and tissue, and consequently will be referred to throughout the text as the reference profile for miRNA expression during normal granulopoiesis. In that paper the miRNA profiles in three bone marrow-derived cell populations containing MB/PMs, MC/MMs, and BC/SCs and peripheral blood PMNs were analyzed by hybridization to an Affymetrix array. Of the 1,105 miRNAs analyzed, 135 were found to be differentially expressed and to fit into one of six different expression profiles [13] (Fig. 2). The vast majority of changes in miRNA expression occur at the transition from MB/PMs to MC/MMs indicating a role for many miRNAs in regulation of cell cycle factors or proteins governing terminal neutrophil differentiation. Only a few miRNAs alter their expression pattern beyond the BC stage including peripheral blood PMNs and extravasated neutrophils [13].

Dysregulation of miRNAs can have a major impact on neutrophil development as clearly exemplified in human acute myeloid leukemias (AMLs) where perturbations of the miRNA pool often are observed [9, 14]. The biology of miRNA in AMLs, however, is not the scope of this chapter which focuses instead on the role of

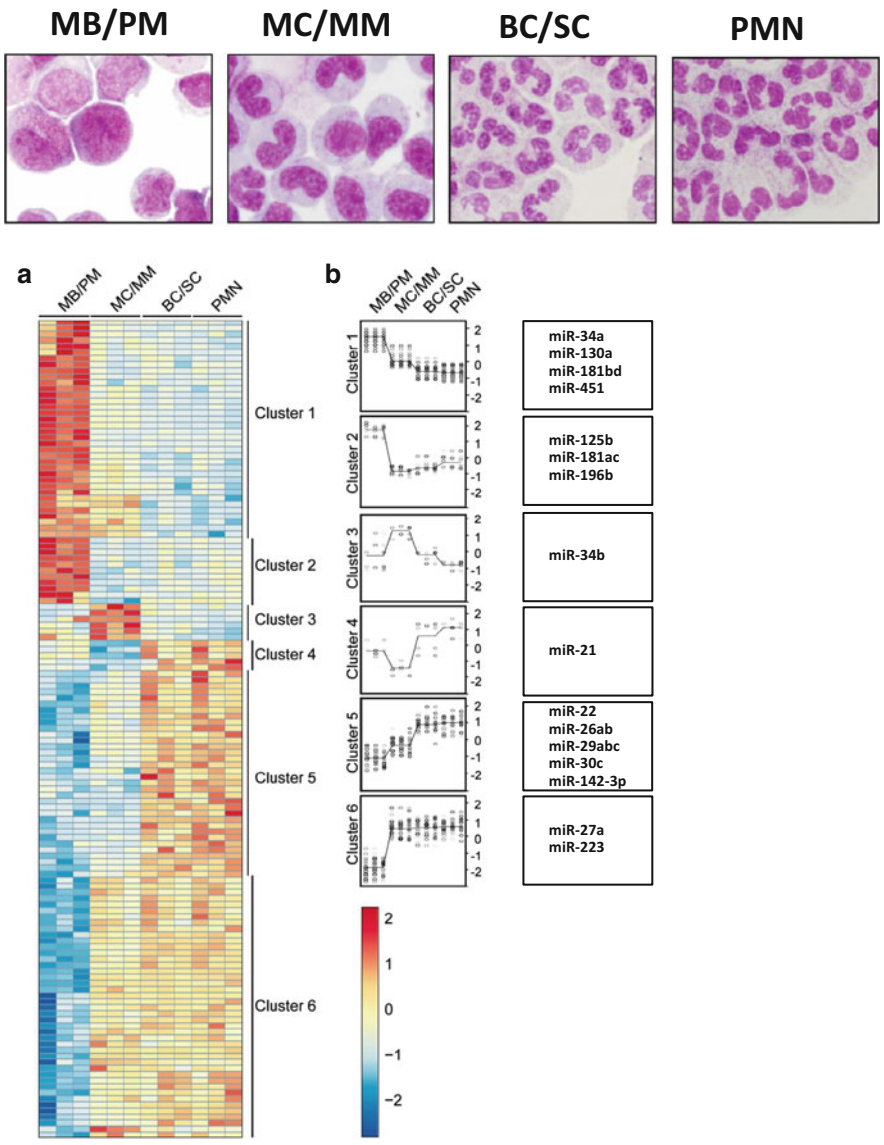

Fig. 2 miRNA expression analysis of neutrophil precursors from human bone marrow. *Top*: Cytospins of the MB/PM, MC/MM, BC/SC, and PMNs populations from one representative donor after immunomagnetic depletion of non-neutrophil cells. *Bottom*: (**a**) Heatmap illustrating the different miRNA expression profiles for the 135 differentially regulated miRNAs in bone marrow and peripheral blood (*red* indicates high expression and *blue* low expression). Each *brick row* downwards represents one donor. The four different cell populations are marked on the top of the heatmap and the cluster classification is shown on the right side. (**b**) Using hierarchical cluster analysis (hclust) the cluster affiliation of the different miRNAs was determined showing the different expression profiles on a Log2 scale. Each *bullet* indicates the expression level of a single miRNA in one donor and the *line* represents the average expression. The differentially expressed miRNAs mentioned in the text are listed in the *boxes* to the right. The figure is based, in part, on figures previously published in [13]

miRNAs in normal development and function of the PMN with special emphasis on transcription factors, cell cycle regulation, and intracellular signaling in response to external stimuli.

3 miRNAs and Transcription Factors

Runx1 expression is required for definitive hematopoietic stem cell (HSC) formation and maturation of both the lymphoid and myeloid lineages [15]. In granulopoiesis Runx1 expression peaks in the most immature precursors and is downregulated at the MC stage where proliferation ceases [3, 16]. This fits the role of Runx1 as a regulator of cell cycle as demonstrated in e.g., murine myeloblastic 32Dcl3 cells where overexpression of Runx1 was shown to shorten the G_1 phase [17]. Runx1 promotes cell proliferation in hematopoietic cells by inhibiting p21Cip1 and inducing cyclin D2 gene expression [17]. The Runx1 transcript carries two closely located miR-27a MREs in the 3′-UTR and translation of the Runx1 mRNA is repressed by overexpression of miR-27a/b [18]. The expression profile of miR-27a in normal granulopoiesis with 2–3-fold higher expression in MCs and more mature cells than in MB/PMs supports a model where miR-27a acts as an inhibitor of Runx1 production in MC/MMs [13]. miR-27a also represses Runx1 synthesis during megakaryopoiesis demonstrating the importance of this microRNA as a regulator of different hematological pathways [19]. Transcription of miR-27a (as part of the miR-23a microRNA cluster) is controlled by the transcription factor PU.1 and promotes myeloid differentiation over B-cell development [20]. Expression of PU.1 is low in MB/PMs and high in more mature precursors [16], a pattern that mimics that of miR-27a in normal granulopoiesis [13].

Runx1 is also involved in transcriptional regulation of the *MIR223* gene [21]. Correct expression of miR-223 is of major importance for proper granulopoiesis as demonstrated by the accumulation of myeloid blasts as a result of heterochromatic silencing of the miR-223 gene by the RUNX1-RUNX1T1 (AML/ETO) oncoprotein [21], a process that can be reversed by reintroducing miR-223 into the cells. Expression of miR-223 can be detected as early as in the HSC and increases as granulocytic progenitors proceed through the “common myeloid progenitor” (CMP) and “granulocyte-macrophage progenitor” (GMP) stages of differentiation [22]. In the normal course of granulopoiesis, starting with the first identifiable neutrophil precursor, the myeloblast, the level of miR-223 increases a further 5–6-fold when reaching the MC stage and remains high throughout the terminal steps of granulopoiesis [13]. This strongly suggests that another transcription factor besides Runx1 regulates transcription of this miRNA as the expression profile of miR-223 does not coincide with that of Runx1 in the terminal stages of maturation. A plausible candidate is C/EBP-α, which along with transcription factor NFI-A is part of a regulatory circuitry involving miR-223 [23]. In neutrophil precursors expression of miR-223 is

repressed by binding of NFI-A to the *MIR223* gene promoter. Emergence of C/EBP-α with neutrophil differentiation, however, results in displacement of NFI-A by C/EBP-α on the *MIR223* gene promoter with increased miR-223 expression as result. This initiates a negative feedback loop where miR-223 inhibits translation of the NFI-A mRNA thus further relieving the repressive effect of NFI-A on the *MIR223* gene [23].

No miRNA affecting C/EBP-α expression in neutrophils has yet been identified, whereas C/EBP-ε, that like C/EBP-α is obligate for neutrophil development [24], has been shown to be repressed by miR-130a [25]. C/EBP-α is a key factor for commitment of CMPs and GMPs towards the granulocytic lineage as demonstrated by the selective and complete block in neutrophil differentiation in *Cebpa* knockout mice [26]. Expression of C/EBP-α is decisive for whether GMPs mature along the monocytic or granulocytic path. Lack of C/EBP-ε expression results in the formation of atypical granulocytes and a differentiation arrest at the MC stage [24]. C/EBP-α and C/EBP-ε are bi-functional proteins that act both as transcription factors and as regulators of cell proliferation through direct interaction with proteins of the cell cycle machinery (see below). Mice with a targeted deletion of the *Cebpe* gene are characterized by attenuated and dysplastic granulopoiesis with accumulation of MCs and MMs in the bone marrow and the presence of abnormal hyposegmented neutrophils in peripheral blood [24]. These mice also lack expression of secondary and tertiary granule proteins such as lactoferrin, cathelin B9, and MMP9 [27], a trait mimicked in humans with specific granule deficiency (SGD) due to an inactivating point mutation of the *CEBPE* gene [28]. In the course of normal granulopoiesis, C/EBP-ε protein is highly expressed in MC/MMs and almost absent in both more immature and more mature cells [16, 25]. The *CEBPE* transcript, on the other hand, is present also in MB/PMs, and to avoid premature translation of C/EBP-ε downregulation by miR-130a is required. This is demonstrated by the untimely expression of C/EBP-ε in MB/PMs transfected with a Locked Nucleic Acid (LNA) oligo against miR-130a and by repression of C/EBP-ε and genes encoding secondary granule proteins, by forced expression of miR-130a [25]. During normal granulopoiesis the level of miR-130a is 8–10 times higher in MB/PMs than in more mature neutrophil precursors in accordance with a role as fine-tuner of C/EBP-ε expression in MB/PMs [13].

Smad4 is an essential component of the TGF-β signaling pathway [29]. Following stimulation of the TGF-β-receptor complex, Smad2 and Smad3 are phosphorylated and form a complex with Smad4 that translocates to the nucleus and acts as a transcriptional regulator [29]. In dividing myeloid cells, stimulation with TGF-β acts as a cell cycle inhibitor by inducing transcription of CDK inhibitor p57Kip2 [30] and inhibiting expression of c-Myc [31]. Furthermore, Smad4 binds to HoxA9 and retains this pro-proliferative transcription factor in the cytosol [32]. Like C/EBP-ε, Smad4 is a miR-130a target and is absent in MB/PMs despite high levels of Smad4 mRNA [33]. This expression pattern ensures that proliferating MB/PM cells are not inhibited by TGF-β while potentially allowing more mature proliferating cells (myelocytes) to respond to TGF-β, which may be of relevance during emergency granulopoiesis to adjust the number of PMNs needed to combat an

infection. The transcript for Smad4 carries two miR-130a MREs, and inhibition of Smad4 by overexpression of miR-130a renders the murine myeloblastic cell line 32Dcl3 nonresponsive to the growth-inhibitory effect of TGF-β [33]. Transcription of miR-130a is governed by the proto-oncogene c-Myc [34, 35], a well-characterized driver of cell proliferation through induction of cyclins and repression of p21Kip1 transcription [36, 37]. Expression of c-Myc is high in MB/PMs and low in more mature cells as seen also for miR-130a further indicating a role for miR-130a as regulator of cell proliferation [25].

Gfi-1 is a transcriptional repressor and targeted mutation of *Gfi1* in mice results in a phenotype sharing many traits with that of *Cebpe*−/− mice with arrested neutrophil development and increased monopoiesis [38]. Reduced expression or mutated forms of Gfi-1 have been observed in an SGD patient and several severe congenital neutropenia (SCN) patients and are responsible for the disorders characterized by a lack of terminally differentiated neutrophils in peripheral blood and recurrent bacterial infections [39]. Four miRNAs (miR-21, miR-196a, miR-196b, and miR-489) were significantly upregulated in bone marrow from *Gfi1*−/− mice compared to wild-type littermates and three of these (miR-21, miR-196a, and miR-196b) were likewise increased in $CD34^+$ bone marrow cells from an SCN patient with a *GFI1* mutation compared to healthy donors [40]. Gfi-1 binds to the promoters of the *Mir21* and *Mir196b* genes and represses their expression. Deregulated expression of either miRNA partly affects myelopoiesis, but if both miRNAs are overexpressed, a complete block of granulopoiesis is observed in vitro [40]. Although no mRNA targets of miR-21 and miR-196a were ascribed to explain their actions on granulopoiesis, the data clearly demonstrate that coordinate action of several miRNAs is important for proper neutrophil development.

Mice with targeted disruption of the *Mir223* gene are characterized by neutrophilia due to an expansion of the granulocyte progenitor pool in the bone marrow [22]. The critical target for miR-223 in these cells is the transcription factor Mef2c that promotes myeloid progenitor proliferation. The neutrophilic condition can be reversed by crossing *Mir223*−/− and *Mef2c*−/− mice thereby restoring normal neutrophil numbers in peripheral blood. A second trait of the *Mir223* knockout mouse is the production of hypermature PMNs that are hypersensitive to activating stimuli causing unwanted tissue damage in, e.g., lungs. This defect was not alleviated by the lack of Mef2c expression demonstrating that another miR-223 target is of importance in more mature neutrophils [22]. These findings, therefore, underscore the necessity of correct miR-223 expression at both early and late stages of neutrophil development in order to ensure accurate neutrophil homeostasis and function.

4 Regulation of Cell Cycle

Cell proliferation ceases and terminal differentiation commences at the MC stage of granulopoiesis [2, 3]. A number of changes in the composition of the cell cycle machinery accompany this process such as increased levels of cyclin-dependent kinase (CDK) inhibitor p27Kip1 and downregulation of CDK2, CDK4, and CDK6 [2]. As a consequence, phosphorylation of Rb does not occur, which is a prerequisite for E2F release from Rb and G1 to S phase progression [2].

Both C/EBP-α and C/EBP-ε are important cell cycle regulators in neutrophil precursors as illustrated by, for example, increased proliferation of immature myeloid cells in *Cebpe−/−* mice [41, 42]. Proliferation is blocked by C/EBP-α through inhibition of E2F1 transcriptional activity [43, 44] and by C/EBP-ε through interaction with both E2F1 and Rb causing the transition from G1 to S phase to be slowed down [45]. Besides direct interaction with the cell cycle machinery, C/EBP-α also induces expression of miR-223 [46] and miR-34a [47], which both affect expression of proteins involved in cell cycle progression. miR-223 targets the *E2F1* transcript and causes translational repression without affecting mRNA stability. As a result, proliferation rate is diminished and a larger proportion of cells accumulate in the G0/G1 phase [46]. Both C/EBP-α and E2F1 bind the promoter of the *MIR223* gene and act as inducers and repressors of transcription, respectively. As the amount of C/EBP-α increases with granulopoiesis, E2F1 is dislodged from the *MIR223* promoter due to C/EBP-α binding and transcription of miR-223 is accelerated. Together, this diminishes the amount of E2F1 available for cell cycle progression, in part as a consequence of E2F1 tethering by C/EBP-α and in part due to translational repression of *E2F1* mRNA by miR-223 [46]. miR-34a is also transcriptionally induced by C/EBP-α and targets E2F3 [47], a second E2F member important for granulocyte differentiation [48]. Expression of miR-34a in an in vitro-based cell system peaked 6 h after introduction of C/EBP-α and then returned to base-line level within the next 24–48 h [47]. This indicates a role for miR-34a at the time where high C/EBP-α expression sets in, which coincides with cell cycle arrest. E2F3, among other targets, transactivates E2F1 expression [49] and, accordingly, an inverse relationship between E2F1 and E2F3 levels and the amount of miR-34a expressed is observed in different AML samples [47]. Myeloid expressed miR-34b, that carries the same seed sequence as miR-34a, has been shown to target CREB [50] that also acts as a promoter of cell proliferation [51]. Besides the above-mentioned experimentally demonstrated targets for miR-34abc, translational repression of mRNAs encoding other cell cycle regulatory proteins in neutrophils, such as CDK4, CDK6, Notch, Cyclin E2, and Cyclin D1, may occur based on the reported effects of miR-34abc in other cell types [52]. E2F7 production is repressed by miR-26a during monocytic differentiation where E2F7 was found to inhibit transcription of p21Cip1 and be required for CDK2, CDK4, and CDK6 synthesis [53]. Expression of miR-26a increases twofold and miR-26b almost fivefold in a stepwise fashion from MB/PMs to BC/SCs [13]. If E2F7 plays the same role in neutrophils as in monocytes, an miR-26ab-mediated reduction of E2F7 might be

involved in the termination of CDK2, CDK4, and CDK6 expression in MC/MMs and their complete disappearance in BC/SCs [2]. CDK6 mRNA is also targeted by miR-29a in HL-60 and NB4 cells when differentiated in vitro towards granulocytes [54]. During normal granulopoiesis the amounts of miR-29a and miR-29b are 3–5 times higher in BC/SCs and PMNs than in MB/PMs and MC/MMs and miR-29c increases twofold at the transition between MC/MMs and BC/SCs [13] indicating a role for these miRNAs in silencing of CDK6 expression.

The membrane receptor and transcriptional regulator, Notch1 is also regulated by a C/EBP-α-induced miRNA, in this case miR-30c [55]. Continued Notch1 signaling inhibits granulocytic differentiation and retains the cells in an immature proliferative state [56]. Deregulation of Notch1 translation, but not *Notch1* mRNA stability, is mediated by miR-30c and is a prerequisite for myeloid differentiation [55].

Expression levels of the CDK-inhibitor p27Kip1 transcript (*CDKN1B*) are similar in MB/PMs and MC/MMs and increase twofold in BC/SCs [2, 57]. In contrast, the p27Kip1 protein is almost undetectable in MB/PMs, weakly expressed in MC/MMs, and highly expressed in BC/SC and PMNs [2] indicating posttranscriptional control of protein synthesis. Two reports demonstrate a role for miR-181a and miR-181b in regulating translation of the *CDKN1B* mRNA during in vitro differentiation of the human myeloid cell line HL-60 [55, 58]. In both cases the level of miR-181 was high in undifferentiated cells and decreased with myeloid development thus relieving repression of *CDKN1B* mRNA and permitting expression of p27Kip1. The miR-181abcd family is highly expressed in MB/PMs during normal granulopoiesis and decreases to half the amount in MC/MMs and remains at this level (miR-181a and miR-181c) or decreases to 15 % (miR-181b) or 33 % (miR-181d) in MC/MMs and further 50 % in BC/SC [13]. This pattern of miR-181 expression fits a role of these miRNAs as fine-tuners of p27kip1 synthesis.

As described above, miR-130a acts as a translational inhibitor of *CEBPE* mRNA to ensure repression of C/EBP-ε synthesis in proliferating MB/PMs and production of the protein in MC/MMs to counteract further cell cycle progression [25]. C/EBP-ε has also been shown to downregulate c-Myc expression which further inhibits cell proliferation [45]. As *MIR130* transcription is induced by c-Myc, it is possible that the emergence of C/EBP-ε in MCs initiates a "c-Myc:miR-130a:C/EBP-ε" feedback loop where C/EBP-ε-mediated downregulation of c-Myc results in reduced miR-130a expression and derepresses C/EBP-ε translation as a consequence. Max, the transcriptional dimerization partner of c-Myc, is targeted by miR-22 in HL-60 cells [59]. A gradual twofold increase of miR-22 is seen from MB/PMs to BC/SC [13], which fits a model where repression of Max synthesis is an additional means to counteract the proliferative effect of c-Myc.

5 Modulation of Intracellular Signaling Pathways by miRNAs

Neutrophil differentiation as well as survival following extravasation is regulated by granulocyte colony-stimulating factor (G-CSF) which by ligation to the G-CSF receptor (G-CSFR), induces activation of JAK1, JAK2, STAT3, and to a minor degree STAT1 [60]. C/EBP-ε expression is also induced by G-CSF stimulation but by a STAT3-independent mechanism [61]. Disruption of either STAT3 or C/EBP-ε activity blocks neutrophil differentiation demonstrating the need for both pathways for proper granulopoiesis [24, 61]. As described, overexpression of c-Myc blocks myeloid differentiation by inhibiting C/EBP-ε expression [61] which, at least in part, may be explained by c-Myc-driven miR-130a gene expression causing translational inhibition of CEBPE mRNA [25]. Interference with the STAT3 signaling pathway by forced expression of miR-125b was seen to block G-CSF-induced differentiation of murine 32Dcl3 cells and continued proliferation of the cells in the presence of G-CSF [62]. miR-125b not only targets *STAT3* mRNA directly but also affects expression of the STAT3 co-factors c-JUN and JUND [62].

During normal granulopoiesis miR-125b demonstrates a very steep (30-fold) decrease in its expression level at the transition from MB/PMs to MC/MMs [13], which nicely fits a role of this miRNA as a guardian against premature termination of cell proliferation. The high levels of miR-130a and miR-125b in MB/PMs may thus act in concert to counteract G-CSF-mediated differentiation of neutrophil precursors by acting on two different mediators induced by G-CSF receptor stimulation. The *CBFB* transcript is also direct target of miR-125b in hematopoietic cells [63]. The protein expression profile of CBF-β protein during normal granulopoiesis is not known but *CBFB* mRNA shows peak expression in MB/PMs, about half the amount in MC/MMs and one third the amount in BC/SCs [57]. As both *CBFB* mRNA and miR-125b expression levels peak in MB/PMs, it is possible that miR-125b acts as a biological "rheostat" to fine-tune CBF-β levels in proliferating precursor cells.

Silencing of TAB2, an intermediate protein in the IL-1-signaling pathway associated with NF-κB activation, has been shown to promote neutrophil maturation in vitro [54]. The *TAB2* transcript is targeted by miR-142-3p [54], a miRNA that increases fourfold between the MB/PM and MC/MM stages of normal granulopoiesis and remains highly expressed throughout neutrophil maturation [13]. The mechanism by which downregulation of TAB2 promotes neutrophil differentiation is not known. Stimulation of PMNs with LPS, IL-1β ,or TNF-α, all activators of NF-κB, but not IFN-γ or IFN-β, that do not induce NF-κB activity, caused a prominent increase of miR-9-1 [64]. The *NFKB1* transcript is repressed by miR-9-1 suggesting that this miRNA is involved in a feedback loop to control NF-κB expression during inflammation [64].

Extravasation from the blood stream and migration of neutrophils to infected tissue are governed by a gradient of chemoattractive agents [1, 5]. Initially PMNs migrate towards "intermediate" chemoattractants like IL-8 and LTB4 produced and

released at intermediary sites such as epithelium or endothelium after which the neutrophils are attracted by stronger signals produced by, or in the vicinity of, the infecting bacteria (e.g., bacterially derived fMLP) which are known as "end-target" chemoattractants [5]. Intermediate chemoattractants activate the phosphatidylinositol-3-kinase (PI3K) signaling pathway in neutrophils, whereas end-target chemoattractants induce a signaling cascade involving p38 mitogen-activated protein kinase (p38 MAPK) [5]. Patients with rheumatoid arthritis were found to have lower levels of miR-451 in peripheral blood neutrophils than healthy persons indicating that miR-451 might play a part in development or progression of this disease [65]. Overexpression of miR-451 significantly reduced migration of neutrophils in an fMLP gradient and the decreased chemotactic ability was ascribed to reduced phosphorylation of p38 MAPK via 14-3-3ζ and Rab5a. The repressive effect of miR-451 was caused by targeting of the 14-3-3ζ and Rab5a transcripts causing degradation of their mRNAs and consequently a reduction in the amount of protein produced [65]. Stimulation of neutrophils with LPS, TNF-α, or IL-1β did not affect the level of miR-451, whereas IFN-γ and GM-CSF both induced downregulation of miR-451 demonstrating that external stimuli may affect neutrophil chemotaxis by altering miR-451 expression levels [65]. Targeted deletion of the gene encoding the phosphatase wild-type p53-induced phosphatase 1 (Wip1) promoted an accelerated differentiation of GMPs to neutrophils with a hypermature and hypersensitive phenotype [66, 67]. The increased maturation rate of neutrophils was mediated through a faster and more intense phosphorylation of p38 MAPK and STAT1 following stimulation of the G-CSF receptor [66]. In addition, the inflammatory response of the neutrophil is also regulated by Wip1 as is its migratory ability [67]. Neutrophils from *Ppm1d* (Wip1 gene) knockout mice produced higher amounts of pro-inflammatory cytokines and migrated more rapidly and in greater numbers to inflamed tissue, in part due to higher expression of CXCR2 on the neutrophils [67]. The increased chemotactic and inflammatory response was due to the inability of phosphatase Wip1 to modulate p38 MAPK and NF-κB activity. Within hours following LPS or TNF-α administration to neutrophils, the amount of Wip1 mRNA and protein was severely reduced in concert with a more than twofold increase of miR-16 [67]. As miR-16 inhibits translation of Wip1 mRNA [68], its induction is believed to be responsible for the reduced Wip1 levels in PMNs stimulated with LPS or TNF-α.

Granulocyte macrophage colony-stimulating factor (GM-CSF) is important for survival of neutrophils extravasated from blood vessels to the surrounding injured/infected tissue. PMNs from peripheral blood stimulated in vitro for 4 h with GM-CSF were found to upregulate six miRNAs [69]. No direct repression of mRNAs was demonstrated and it can be questioned whether the induction of these miRNAs reflects the in vivo situation. In a more physiological setting human neutrophils exudated in vivo to skin chambers were investigated and seven miRNAs found to be differentially regulated [13]. None of the miRNAs were the same as for GM–CSF stimulated PMNs probably reflecting the different physiological conditions to which the cells were exposed.

Resolution, the active termination program of acute inflammation, serves to restrain the duration and magnitude of the inflammatory response in order to protect tissue and reestablish homeostasis [70]. Important mediators of this process are locally produced lipid-derived resolvins that act to reduce leukocyte recruitment, induce neutrophil apoptosis, and promote removal of apoptotic cells by macrophages (efferocytosis) [70]. miR-466l was demonstrated to play a dual role in a zymosan-mediated murine acute inflammation model. Initially miR-466l expression in neutrophils, the first leukocytes to arrive at the site of inflammation, accentuated the inflammatory response and later, as monocytes arrived to the site, miR-466l intrinsic to these cells initiated resolution [71]. The MRE of miR-466l coincides with an AU-rich element (ARE) in the 3′-UTR of many mRNAs encoding inflammatory mediators [72]. The ARE-sequence renders the transcript prone to degradation by RNA-destabilizing proteins but binding of RISC with miR-466l excludes binding of the destabilizing proteins to ARE with the net result that the target mRNA is stabilized [72]. ARE-containing transcripts include those encoding the pro-inflammatory cytokines IL-1β and TNF-α as well as resolution-mediating cytokines like IL-10 and lipid metabolic enzymes as COX-1 and COX-2 needed for biosynthesis of resolvins [71, 72].

6 Conclusions

Over the last decade it has become increasingly clear that microRNAs are important regulators of granulopoiesis. While transcriptional initiation and accumulation of mRNAs are a rapid process, cessation of gene expression is not achieved merely by dismantling of the transcriptional complex as the cell still contains the transcriptional mRNA product, which, unless posttranscriptionally regulated, will continue to be translated. For this reason miRNAs are important as they act as inhibitors of translation that allows the cell to rapidly adjust to altered conditions such as termination of proliferation or external stimuli. miRNAs typically target low abundant transcripts encoding proteins that have to be turned on/off within a short time frame such as transcription factors, cell cycle regulatory proteins, and intracellular signaling proteins. This is also the case in neutrophils where numerous examples demonstrate that malexpression of miRNAs may be detrimental for biogenesis or the physiological response of the neutrophil. The current view is that miRNAs play a major regulatory role at the early stages of granulopoiesis, whereas mature PMNs are less dependent on miRNA-based control. Experiments with neutrophils from peripheral blood are, however, complicated by their propensity to undergo apoptosis in vitro. It is thus possible that a different view on miRNAs as regulators in mature granulocytes will emerge when more sophisticated "miRNA-gene"-targeted animal models become available allowing the in vivo examination of miRNAs in mature neutrophils.

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How Noncoding RNAs Contribute to Macrophage Polarization

Huachun Cui and Gang Liu

Abstract Macrophages are an essential component of the innate immune response and adaptive immunity. These cells display a great deal of phenotypic plasticity in order to fulfill their diverse functions. The functional phenotypes of macrophage have been conceptually divided into two classes, namely the M1 and the M2 subtypes. The regulation of macrophage polarization has been extensively studied at the transcriptional, epigenetic, and translational levels and many critical protein mediators have been identified to take part in this cellular event. However, recent evidence indicates that a new type of molecule, noncoding RNAs (ncRNAs), plays a similarly important role in the differential activation of macrophages. This chapter includes a concise summary of the characterization and core protein mediators of macrophage polarization. Furthermore, it reviews in detail the biology of ncRNAs, including miRNAs and lncRNAs, and how they can participate in this process.

1 Macrophage Activation and Polarization

Macrophages are an essential component of the innate immune response and adaptive immunity and have been well recognized to play a central role in inflammation, host defense, systemic metabolism, hematopoiesis, angiogenesis, oncogenesis, apoptosis, and reproduction [1–4]. Given these many processes that they participate in, it is not surprising that macrophages can assume a wide spectrum of phenotypes. In recent years, the description of the functional phenotypes of macrophage has been conceptually divided into two classes, namely the M1 and the M2 subtypes, a specification that emulates the Th1/Th2 polarization of T cells (Fig. 1) [2–7].

Functional activation of macrophages was initially described in 1970s for the response following stimulation by IFN-γ, a cytokine produced by CD4 T helper (Th)1 cells, CD8 cytotoxic T cells, and natural killer cells [6, 7]. Macrophages activated by IFN-γ display enhanced antigen-presenting activity, increased

H. Cui • G. Liu (✉)
Division of Pulmonary, Allergy, and Critical Care Medicine, Department of Medicine, University of Alabama at Birmingham, Birmingham, AL 35294, USA
e-mail: gliu@uab.edu

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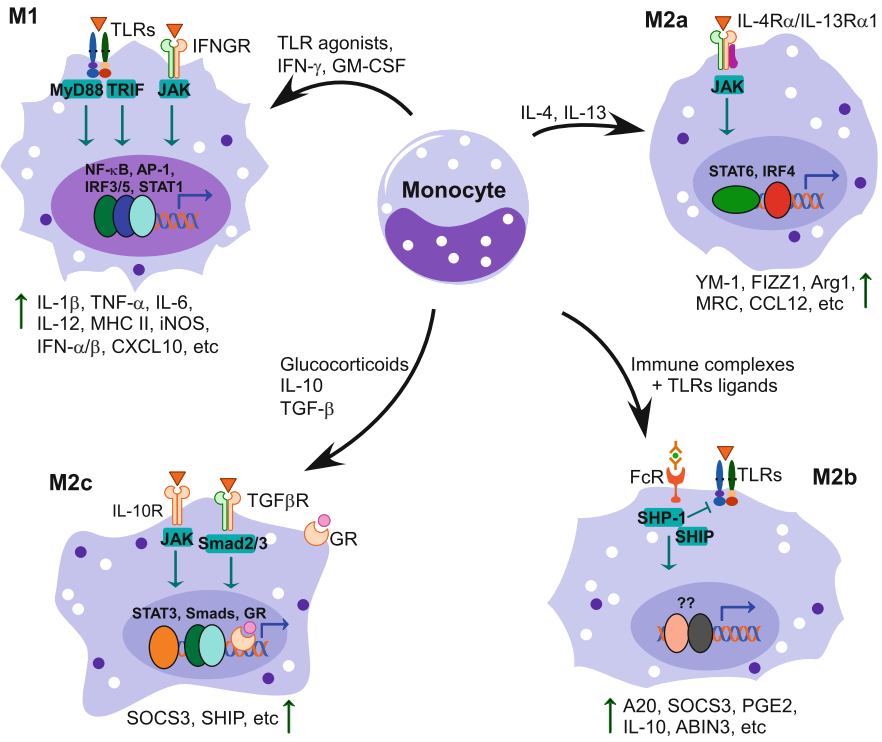

Fig. 1 Categorization of macrophage polarizations and core protein mediators

expression of proinflammatory cytokines and mediators, and augmented phagocytosis of microbial organisms [7, 8]. This type of activation has become known as classical activation or M1. Later on, many microbial products, such as lipopolysaccharide (LPS), intracellular contents from damaged cells, including high mobility group box 1 protein (HMGB1) and mitochondrial DNA, as well as TNF-α were also found to induce M1 macrophage polarization (Fig. 1) [4, 9, 10].

Macrophages with functional phenotypes opposed to those of M1 are termed M2 macrophages. This subtype includes virtually all activated macrophages other than M1 and has thus been recognized as alternatively activated macrophages [3, 11]. In contrast to the homogenous nature of M1 macrophages, M2 macrophages generated from various stimuli demonstrate distinct phenotypes while sharing many properties [11–13]. To better differentiate M2 macrophages, this group of cells have been further divided into three subclasses, namely M2a, M2b, and M2c [13]. The typical M2 macrophages are those activated by Th2 cytokines IL-4 and IL-13 and have become known as M2a [11, 12]. The other two subclasses are immune complex plus Toll-like receptor (TLR) agonist-activated M2b macrophages and IL-10-, TGF-β-, or glycocorticoid-activated M2c macrophages (Fig. 1) [12–14].

M1 macrophages activated by IFN-γ and/or various TLR agonists have quite homogenous phenotypes. These cells express high levels of proinflammatory

cytokines, such as TNF-α, IL-1β, IL-6, and IL-12, produce large amounts of reactive oxygen and nitrogen species, and promote a Th1 response. M1 macrophages participate in clearing bacterial, viral, and fungal infections as well as eradicating cancer cells [2, 4, 5].

IL-4/IL-13-activated M2a macrophages have high expression of markers of alternative activation, such as arginase-1 (Arg1), chitinase 3-like 3 (also called YM-1), found in inflammatory zone 1 (Fizz1) and IL-10. They also demonstrate high levels of scavenger, mannose and galactose-type receptors [2, 3, 11, 13, 15]. M2a macrophages regulate responses to parasite infection, tissue remodeling, angiogenesis, and tumor progression [12, 16, 17]. M2b and M2c macrophages share some but not all defining features of M2a cells. Macrophages activated by immune complex plus TLR ligands, IL-10, or glycocorticoids express high levels of IL-10 as well as angiogenic and mannose receptors. M2b and M2c are recognized to play a central role in immunomodulation [12–14].

The M1/M2 classification is at best to represent the two extremities of macrophage polarization. However, there are many examples that activated macrophages assume a mixed phenotype of both M1 and M2, an observation demonstrating a nature of phenotypic continuum and plasticity of macrophage polarization [2, 18, 19]. Furthermore, it has been also well recorded that macrophages from various diseased states display a great deal of heterogeneity, evidenced by the coexistence of both M1- and M2-polarized cells [2, 20–22].

2 Core Signaling Events Involved in M1 Polarization

IFN-γ was the first cytokine identified to induce M1 macrophage polarization [8]. Binding of IFN-γ to its receptor activates Janus kinases 1/2 (JAK 1/2) to phosphorylate signal transducers and activators of transcription 1 (STAT1), causing dimerization and subsequent nuclear translocation of this transcription factor. The STAT1 homodimer binds to *cis* elements known as γ-IFN-activated sites (GAS) in the promoter of its target genes, such as iNOS and IL-12, and thereby leading to functional activation of macrophages [7, 23, 24].

Microbial and self-cellular products are perhaps the more prevalent inducers of M1 polarization in vivo. Macrophages and other immune cells recognize these products through pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) present in microbial pathogens or as cellular molecules released by damaged tissues [25–27]. Binding of PAMPs or DAMPs to specific cell surface or cytoplasmic receptors, such as TLRs and Nod-like receptors (NLRs), triggers a series of intracellular signaling events that lead to inflammatory activation of immune cells, including macrophages [25–28].

LPS-induced M1 macrophage polarization involves a series of well-defined signaling events [26, 29]. Engagement of LPS with its receptor TLR4 leads to the formation of a complex consisting of adaptor protein myeloid differentiation primary response gene 88 (MyD88), kinases interleukin-1 receptor-associated

kinase 1 (IRAK1) and IRAK4, and TNF receptor-associated factor 6 (TRAF6) [26, 30, 31]. This complex activates TAK1 that mediates the subsequent activation of the IκB kinases (IKKs). IKKs then phosphorylate IκB, resulting in its degradation and liberation of the transcription factor NF-κB [26, 30, 31]. Additionally, the MyD88–IRAK1/4–TRAF6 complex also activates mitogen-activated protein kinases (MAPKs), which ultimately triggers the activation of transcription factor AP-1 [26]. Many M1 genes have κB sites in their promoters, such as *iNOS*, *COX-2*, *CCL2*, and a number of proinflammatory cytokines, underscoring the central role of NF-κB in LPS-induced M1 macrophage polarization [7]. Similarly, AP-1 is able to activate the expression of many proinflammatory genes and mediate M1 macrophage polarization [32, 33]. LPS binding to TLR4 also triggers MyD88-independent, but TIR-domain-containing adapter-inducing interferon-β (TRIF)-dependent signaling cascade that leads to the activation of the transcription factor interferon regulatory factor 3 (IRF3) [34]. IRF3 is known to mediate the LPS-induced type I interferon response. IFN-β stimulates the phosphorylation and heterodimeration of STAT1 and STAT2. Associated with IRF9, STAT1/2 binds to *cis* elements known as IFN-stimulated response elements (ISRE) that are present in many genes involved in the interferon response and thus promotes optimal M1 macrophage polarization [34, 35].

Although the molecules described above are core mediators of M1 macrophage polarization, there are many more positive or negative protein regulators participating in this event, such as transcriptional co-activators, co-repressors, epigenetic modifiers, and ubiquitin ligases [5, 7, 36–38]. The existence of multilayer regulation in M1 polarization indicates the necessity of tight control of this event. The complexity of the modulatory networks also renders this process susceptible to dysregulation that leads to inflammatory disorders, such as sepsis, arthritis, and metabolism-associated diseases [2].

3 Core Signaling Events Involved in M2 Polarization

IL-4- and IL-13-induced M2a polarization has been well characterized. The IL-4 and IL-13 receptors share a key signal transducer, IL-4 receptor-α (IL4Rα) [39–41]. The binding of IL-4 and IL-13 to their receptors leads to phosphorylation of the cytoplasmic tail of IL4Rα, enabling its recruitment of JAK1/JAK3 or JAK1/Tyk2, respectively. These kinases subsequently phosphorylate and activate STAT6 [39–41]. Many of the M2-associated genes are regulated by STAT6, such as Arg1, YM-1, FIZZ1, and mannose receptor, C type 1 (MRC1) [3, 5]. These findings have put STAT6 in the central place in IL-4- and IL-13-induced M2 macrophage polarization.

IL-10-induced M2c macrophages are the major effectors to mediate the immunosuppressive role of this cytokine [42, 43]. Engagement of IL-10 with its receptor triggers JAK1-mediated STAT3 phosphorylation, leading to transactivation of many M2-associated genes, including Arg1, Arg2, and p50 [41].

In addition to the central players described above, there are a number of other mediators that participate in M2 macrophages [5, 7]. Transcription factor CCAAT/

enhancer-binding protein beta (C/EBP-β) has been shown to be a positive regulator of M2 polarization. Impaired C/EBP-β is associated with suppression of M2 but elevation of M1 polarization [44–46]. Kruppel-like factors (KLF) 2 and 4 have also been known to play critical roles in M2 macrophage polarization. KLF2 and KLF4 exert their functions either by directly activating the transcription of M2-associated genes or through the interaction with NF-κB or STAT6 [47, 48]. Peroxisome proliferator-activated receptors (PPARs) are another well-established family of transcription factors that have important roles in M2 macrophage polarization. PPAR-γ can be induced by IL-4 and IL-13 and promotes M2 polarization in concert with STAT6 [49–51]. Similar to PPAR-γ, PPAR-δ is also induced by IL-4 and promotes an anti-inflammatory M2 phenotype [52].

4 Regulation of Macrophage Polarization by Noncoding RNAs

The polarization of macrophages has been under extensive study in recent years [2, 5, 53]. These extraordinary efforts have led to very fruitful knowledge that helped us to gain a deep understanding of how macrophage polarization is regulated at the transcriptional and translational levels by protein mediators. However, there is accumulating recent evidence showing that a new type of molecule, namely noncoding RNAs (ncRNAs), may have similarly important roles in this process.

The mammalian genome is pervasively transcribed to form many types of RNAs. However, only 2 % of them encode a protein [54, 55]. Among the large portion of the noncoding RNAs, transfer RNAs (tRNAs) and ribosomal RNAs (rRNAs) are known to participate in protein synthesis. Other previously undefined ncRNAs, including microRNAs (miRNAs), piwi-interacting RNAs (piRNAs), and long ncRNAs (lncRNAs), have become increasingly recognized to play important regulatory roles in a variety of biological processes [56–58].

Regulatory ncRNAs are divided into two groups based on their lengths. ncRNAs longer than 200 nucleotides (nt) are termed lncRNAs and those smaller than 200 nt are small ncRNAs [59, 60]. Among all small ncRNAs, miRNAs are the most ubiquitously expressed in cells and have also been best characterized [61, 62]. Compared to the well-recognized importance of miRNAs in numerous pathophysiological settings, the roles of lncRNAs in these conditions have just begun to be appreciated.

5 miRNAs

miRNAs are generally 20–25 nt in length. The first miRNA lin-4 was discovered in *Caenorhabditis elegans* by Ambros and colleagues in 1990s after they found that a small RNA encoded by the gene *lin-4* controls larval development by regulating *lin-*

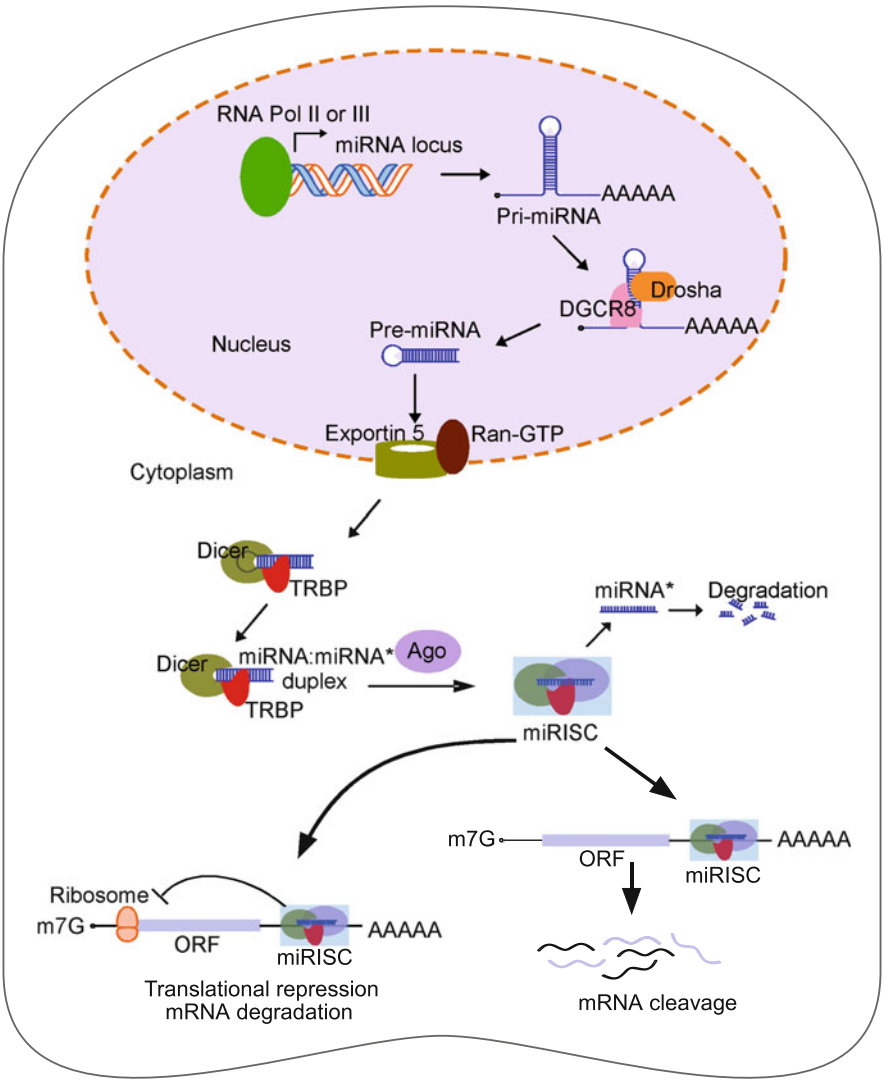

Fig. 2 miRNA biogenesis and mode of functions

14 mRNA translation through its 3′ untranslated region (UTR) [63]. Since then, thousands of miRNAs have been identified in various organisms (Fig. 2) [61, 64].

In general, miRNAs are primarily transcribed by RNA polymerase II. This feature renders miRNA expression subjects to positive and negative regulation at the transcriptional level, similar to that found with protein-coding genes [61, 65]. Canonical miRNA biogenesis begins with the transcription of the miRNA genes, giving rise to primary transcripts (pri-miRNAs) [61, 66–68]. The pri-miRNAs are then cleaved by RNase III endoribonuclease Drosha/DGCR8,

emerging as 60–120 nt hairpin RNAs known as precursor miRNAs (pre-miRNAs) [56, 61, 65, 66]. In the following steps, pre-miRNAs are transported from the nucleus by exportin-5 in a Ran-GTP-dependent manner to the cytoplasm, where they are further processed by the endoribonuclease Dicer to generate mature miRNA duplexes [56, 61, 65, 66]. The mature miRNA duplexes are then loaded onto the miRNA-induced silencing complex (miRISC) consisting of Argonaute proteins, human immunodeficiency virus (HIV) transactivating response RNA (TAR) binding protein (TRBP), protein activator of the interferon-induced protein kinase (PACT), and others. The miRNA duplexes are dissociated in the miRISC, giving rise to mature miRNA strands, while the miRNA* strands undergo rapid degradation. In action, the "seed" sequences of the miRNAs (position 2–8 from the 5′ end) are used as a guide for an individual miRISC to specifically bind to the 3′ UTR of target mRNAs through Watson–Crick base-pairing mechanisms (Fig. 2). It has been previously shown that miRNAs fulfill their functions by promoting target mRNA degradation and/or inhibiting mRNA translation [56, 61, 65, 66]. However, more recent evidence indicated that the primary mechanism by which miRNAs suppress target expression is through inducing target mRNA degradation [69]. Given the overwhelming evidence that miRNAs serve in fine-tuning gene expression in numerous cellular events, one can conveniently assume that the actions of miRNAs in macrophage polarization could simply rely on them controlling the expression of various mediators in this process.

However, in addition to intracellular actions, miRNAs can be secreted into the extracellular milieu by immune cells, including macrophages, as enclosures in exosomes or microvesicles [70]. These exosome miRNAs can act in *trans* to regulate gene expression in other cells through the canonical mode of action. miRNA transfer via exosomes has been recognized as an important way of intercellular communications [70]. Recent studies discovered another new mechanism by which miRNAs can regulate macrophage polarization. In these studies, miR-21 and miR-29a enclosed in exosomes, as well as extracellular synthetic let-7b, were found to induce M1 polarization of microglia and macrophages through binding to TLR7 (or TLR8 in humans) located in the endosomes of these cells [71, 72]. Interestingly, TLR7 only bound a selection of miRNAs because miR-16 or mutant forms of miR-21, miR-29a, and let-7b did not bind to the receptor or stimulated immune response [71, 72]. miR-21, miR-29a, and let-7b share a GU-rich element in their sequences, which possesses a GU content similar to that of ssRNA40, a known TLR7 ligand [71, 72]. The GU-rich element was shown to be indispensable to the binding of these miRNAs to TLR7 [71, 72]. Predictably, other miRNAs with similar GU-rich element can also trigger an immune response and induce M1 macrophage polarization by engaging TLR7. These astonishing findings demonstrate that exosome miRNAs not only act in intercellular communications but also serve as paracrine agonists of TLRs to modulate macrophage polarization.

6 Negative miRNA Regulators in M1 Macrophage Polarization

M1 macrophages are an integrated part of innate immunity and host defense. However, overexuberant M1 activation often leads to collateral tissue damage [73, 74] and many mechanisms have evolved to keep the inflammatory activation of macrophages in check [5, 7]. In the last decade, miRNAs have emerged as one of the most important regulators in M1 macrophage polarization (Table 1) [75–77].

There have been many systemic efforts to identify miRNAs involved in the innate immune response and M1 macrophage polarization [75, 78, 79]. Conceivably, miRNAs that can dampen inflammation are likely to function as negative regulators in M1 macrophage polarization. One of the earliest examples came from Baltimore and colleagues. They found that miR-146a expression is rapidly upregulated in LPS-treated monocytic cells and functions in a negative feedback loop to inhibit TLR signaling, presumably by targeting TRAF6 and IRAK1 [80]. miR-146a upregulation has also been shown to be indispensable to the development of endotoxin tolerance in the innate immune response [81]. These findings suggest that an impairment in miR-146a induction could lead to hyperactivated M1 macrophages and associated prolonged inflammation. Additionally, miR-146a is a negative regulator of the vesicular stomatitis virus (VSV)-induced type 1 interferon response and M1 macrophage polarization by targeting TRAF6, IRAK1, and IRAK2 [82]. Thus, miR-146a participates in the inflammatory response and M1 macrophage polarization during both bacterial and viral infections. The critical role of miR-146a in inflammation and macrophage activation was also highlighted in mice deficient in miR-146a [83, 84]. Macrophages from these mice are hypersensitive to LPS stimulation [83, 84]. One interesting recent study showed that miR-146 is involved in phenotypic alterations associated with immunoaging [85]. Unlike other immune cells, miR-146 expression was not responsive to LPS stimulation in macrophages from aged mice. This led to a defect in the negative feedback regulation associated with miR-146, which presumably contributed to the aggravated LPS-induced inflammatory response in the aged macrophages [85]. This study suggests that aberrant miRNA regulation can be involved in aging-related inflammatory disorders. miR-146 was also found to play a role in M1 macrophage-associated atherosclerosis [86]. The expression of miR-146a decreased under oxLDL stimulation. Furthermore, through targeting TLR4, miR-146a significantly reduced oxLDL-induced M1 macrophage phenotypes, as evidenced by the diminished secretion of IL-6, IL-8, and CCL2 [86].

miR-21 is upregulated in macrophages by LPS stimulation [87]. Elevated miR-21 was found to inhibit M1 macrophage polarization by dampening LPS-induced NF-κB activation, while enhancing IL-10 expression [87]. The anti-inflammatory effect of miR-21 lies in it targeting tumor suppressor programmed cell death 4 (PDCD4), a proinflammatory protein [87]. However, miR-21 was also shown to potentially trigger M1 activation by engaging TLR7 and TLR8 [71]. Therefore, the functional outcome of miR-21 upregulation in M1 macrophage

Table 1 Noncoding RNAs that participate in macrophage polarization

Noncoding RNAs	miRNA/ lncRNA	Targets/ Mediators	Effects
Pro-M1 miRNAs	miR-155	SOCS1, SHIP1	Promotes inflammation through targeting negative regulators of inflammation signaling [102, 109–111]
	miR-125b	IRF4	Enhances costimulatory factor expression and responsiveness to IFN-γ [112]
	miR-27b	PPAR-γ	Enhances response to LPS stimulation [113]
	miR-27a	IL-10	Inhibits IL-10 mediated anti-inflammatory response [115]
	miR-29b	A20	Enhances proinflammatory and IFN response [75, 116]
	miR-21, miR-29a, and let-7b	TLR7/8	Serve as paracrine agonists of TLR7/8 [71, 72]
Anti-M1 miRNAs	miR-146a	TRAF6, TLR4, IRAK1, IRAK2	Inhibits inflammatory response through negative regulation of TLRs signaling [80–86]
	miR-155	TAB2, Pellino-1, IKKε	Negative feedback regulator of TLRs signaling [96, 100]
	miR-21	PDCD4, IL-12p35	Negative regulator of TLR4 signaling; Diminishes inflammation [87]
	miR-9	NF-κB1	Negative regulator of NF-κB activation [95]
	miR-19, 105, 223, and let-7	TLRs (TLR2/ 3/4)	Negative regulators of TLRs signaling [89–93]
Pro-M2 miRNAs	miR-124	C/EBP-α	Promotes M2, while inhibits M1 polarization of microglia [121]
	miR-223	Pknox1; IKKα	Inhibits proinflammatory activation of macrophages [92, 123]
	let-7c	C/EBP-δ	Promotes M2, while inhibits M1 polarization of BMM [127]
	miR-125a-5p	KLF13	Promotes IL-4 induced M2, and inhibits LPS induced M1 polarization of BMM [128]
Anti-M2 miRNAs	miR-155	C/EBP-β, IL-13Rα1	Negatively regulates Akt1-dependent and IL-13 induced M2 polarization [124, 125]
	miR-19a-3p	Fra-1	Inhibits Fra-1/STAT3 signaling and M2 TAM activation in breast cancer [136]
	miR-511-3p	ROCK2	Inhibits expression of extracellular matrix of MRC1+ TAMs, and inhibits tumor growth [133]
LncRNAs	Tmevpg1 (NeST)	WDR5	Enhances IFN-γ expression through modulating H3K4me3 at the *IFN-γ* locus [147, 148]
	lincRNA-Cox2	hnRNPs	Mediates both the activation and repression of distinct immune genes [149]
	THRIL	hnRNPL	Enhances TNF-α expression [150]
	NEAT1	SFPQ	Enhances IL-8 expression [151]
	lnc-DC	STAT3	Promotes DC differentiation [152]

(continued)

Table 1 (continued)

Noncoding RNAs	miRNA/ lncRNA	Targets/ Mediators	Effects
	lnc-IL7R	Unknown	Inhibits the expression of inflammatory genes via modulating H3K27me3 at their promoters [153]

activation may depend on a number of factors, including the timing of the extracellular secretion of miR-21, the extent of TLR7/8 stimulation by miR-21, and miR-21 activity. It is presumable that at early time points miR-21 upregulation is pro-M1 through activating TLR7/8. However, downregulation of PDCD4 later on by intracellular miR-21 may set a limit on M1 macrophage activation and fine-tune the inflammatory response to maintain immune homeostasis. Another recent study discovered a new role of miR-21 in immune homeostasis during wound healing [88]. It is well known that the efficient clearance of apoptotic cells by wound macrophages is a prerequisite for the timely resolution of inflammation. Das et al. found that the expression of miR-21 is increased in post-efferocytotic macrophages and miR-21 upregulation is responsible for the dampened LPS-induced NF-κB activation and TNF-α expression in these cells. Further studies demonstrated that miR-21 achieves this function by targeting PDCD4 and PTEN. This study underlines the significance of miRNAs in the resolution of wound inflammation [88].

There are additional miRNAs that also regulate different aspects of innate immune responses by targeting various inflammatory signaling pathways and thereby potentially modulating M1 macrophage activation. miR-19, miR-105, miR-223, and let-7 family members were found to directly target TLR2, TLR3, or TLR4 [89–93]. Therefore, these miRNAs can impair macrophages engaging with the agonists of these TLRs. miR-147 is induced in macrophages by ligands for TLR2, TLR3, or TLR4 in both MyD88- and TRIF-dependent manners and functions in a negative feedback mechanism to prevent excessive inflammatory activation in macrophages [94]. miR-9 is upregulated by LPS stimulation in both neutrophils and monocytes and diminishes NF-κB-dependent inflammatory responses by targeting the expression of NF-κB1 transcripts [95]. Presumably, miR-9 is able to dampen M1 macrophage polarization. let-7i and miR-125b are downregulated in response to LPS and microbial infections. The reduced expression of these miRNAs was found to promote immune responses and M1 activation [93, 96–98]. Therefore, miRNAs also serve as important components in feed-forward networks to enhance inflammatory amplification and presumably M1 macrophage polarization.

7 Positive miRNA Regulators in M1 Macrophage Polarization

The predominant actions of miRNA in cells are to suppress target expression. Thus, for a miRNA to enhance a signaling pathway or cellular event, it needs to target a negative regulator in the processes in question. This is probably also true for miRNAs that enhance M1 macrophage polarization. There is no shortage of such examples.

A number of studies have shown that miR-155 can be upregulated in macrophages in response to agonists from both bacteria and viruses that activate TLR4, TLR2, TLR3, or TLR9 [80, 90, 96, 99]. Although an early study found that miR-155 is a negative regulator in the response of dendritic cells (DCs) to LPS [100], the majority of evidence indicates that miR-155 is pro-M1. That miR-155 promotes M1 macrophage polarization is likely mediated by its repression of negative regulators in inflammation, such as suppressor of cytokine signaling 1 (SOCS1) and phosphatidylinositol-3,4,5-trisphosphate 5-phosphatase 1 (SHIP1), both of which are directly targeted by miR-155 [90, 101, 102]. By downregulating these anti-M1 pathways, miR-155 is able to promote inflammation and M1 macrophage activation. miR-155 was also shown to prolong the half-life of *TNF-α* transcripts, although the mechanism is unclear [96, 97, 103]. Mice deficient in miR-155 have decreased immune responses, whereas mice having miR-155 overexpression develop a myeloproliferative disorder resembling chronic inflammation and also suffer from hematopoietic cancers [104–108]. miR-155 is involved in B4 (LTB4)-induced M1 macrophage polarization. Its expression is increased by LTB4 and in turn decreases SOCS1 [109]. miR-155 is also a target of 1,25-dihydroxyvitamin D (1,25[OH]2D3). In a recent study, Chen et al. demonstrated that 1,25(OH)2D3 downregulates miR-155 expression. Decreased miR-155 was deemed responsible for the anti-M1 effect of 1,25(OH)2D3 through SOCS1 elevation in macrophages [110]. miR-155 also plays an important role in the formation of atherosclerotic plaques [111]. Macrophages in atherosclerotic plaques generally display M1 phenotypes. Leukocyte-specific miR-155 deficiency reduced plaque size and the number of M1 macrophages after partial carotid ligation in atherosclerotic (Apoe−/−) mice [111]. All of these studies demonstrate a central theme that miR-155 is pro-M1, and miR-155 dysregulation causes lasting inflammation and inflammation-associated disorders. These studies also suggest that miR-155 represents a new therapeutic target to treat pathologies where M1 macrophage activation plays a crucial role.

There are several other miRNAs that target negative regulators of M1 activation and thereby promoting M1 macrophage polarization. For example, the ectopic expression of miR-125b was shown to promote M1 macrophage activation, as reflected by findings that the overexpression of miR-125b in macrophages increases costimulatory factor expression and responsiveness to IFN-γ [112]. This effect was likely mediated by miR-125b-targeting IRF4, a transcription factor that inhibits NF-κB activity [112].

miR-27b can be induced by LPS stimulation in human macrophages, accompanied by a rapid decline in PPAR-γ expression [113]. PPAR-γ is a nuclear hormone receptor that is involved in lipid and glucose metabolism. In macrophages, PPAR-γ has been shown to be able to inhibit the production of proinflammatory mediators or enhance the expression of anti-inflammatory molecules [50, 113, 114]. In that study, PPAR-γ was identified as a novel target of miR-27b in macrophages. The overexpression of miR-27b decreased, whereas the inhibition of miR-27b enhanced the expression of PPAR-γ. Further experiments indicated that the activity of miR-27b to promote M1 macrophage polarization is presumably mediated by PPAR-γ [113]. These data have again placed miRNAs on the center of the stage of metabolic disorders where M1 macrophage activation plays a big role.

In one of our recent studies, we found that miR-27a is downregulated in macrophages following stimulation by TLR2 and TLR4 [115]. Overexpression of miR-27a increased M1 macrophage polarization. In contrast, the knockdown of miR-27a diminished M1 phenotypes. Mechanistically, miR-27a was found to negatively regulate IL-10 expression. Our study suggests that miR-27a downregulation is a negative feedback mechanism that prevents overly exuberant TLR2- and TLR4-driven inflammatory responses and M1 polarization. Conversely, maintenance of a higher miR-27a level may promote proinflammatory M1 phenotypes and enhance inflammatory responses, which could be beneficial in certain immunodeficiency disorders.

Graff and colleagues recently found that miR-29b and its passenger strand miR-29b* are significantly upregulated in M1 macrophages [75]. They demonstrated that overexpression of miR-29b in monocytic cells results in increased expression of M1-associated transcripts, including IL-6, TNF-α, and CXCL9. Furthermore, this study identified A20 (encoded by *TNFAIP3*), a negative regulator of NF-κB signaling, as the target of miR-29b. These data suggest that miR-29b promotes M1 macrophage polarization via A20 [75]. The findings were confirmed by another recent study, which found that miR-29b is significantly upregulated in a Japanese encephalitis virus (JEV)-infected mouse microglial cell line (BV-2) and primary microglia [116]. miR-29b induction was required for microglial activation after JEV infection, as indicated by attenuated expression of proinflammatory mediators, such as iNOS, COX-2, IL-1β, IL-6, and TNF-α, in cells where miR-29b was knocked down. Inhibition of miR-29b also diminished the activation of NF-κB in JEV-infected microglia, presumably via downregulating A20 [116]. Additionally, miR-29b participated in proinflammatory and antiviral responses in cells infected with hepatitis C virus and simian immunodeficiency virus [117, 118]. These data suggest that miR-29b may be a critical component in the defense against various viral infections by promoting M1 macrophage polarization and associated Th1 and Th2 T cell activation.

8 Regulation of M2 Polarization by miRNAs

Similar to how they act in M1 macrophages, miRNAs regulate M2 macrophage polarization primarily via key M2 mediators. Although miRNAs that specifically participate in M2 polarization could exist, it is more often that miRNAs involved in M1 activation are also capable of regulating M2 macrophage polarization, but with opposing effects. These studies support a concept that miRNAs are critical regulators of macrophage plasticity.

The first systemic effort to identify miRNAs that undergo altered expression in differentially polarized macrophages came from Graff and colleagues [75]. However, in that study, only a few miRNAs were found to have more than twofold changes of expression in one of the three M2 subtypes or M1 macrophages [75]. Furthermore, almost all but two identified miRNAs were low abundant passenger strands [75]. Although there have been reports that passenger strands do have regulatory roles [119], they are generally at low levels and excluded from miRISC [120]. Thus, the functional significance of these passenger strand miRNAs in macrophage polarization remains to be determined. Regardless, this study was the first systemic attempt to identify M1- and M2-regulating miRNAs in a cellular model with definitive phenotypes of polarized macrophages.

Other examples with stronger supporting evidence exist on how miRNAs participate in M2 polarization. miR-124, a brain-specific miRNA, was found to be highly expressed in microglia, but not in other tissue macrophages [121]. Expression of this miRNA is decreased in activated microglia from the central nervous system (CNS) of mice with experimental autoimmune encephalomyelitis (EAE), a mouse model of the human disease multiple sclerosis [121]. EAE is characterized by inflammation of the CNS, and is associated with microglial cell activation and infiltration of T cells and leukocytes [122]. Enhancing miR-124 expression promoted M2 macrophage polarization while diminishing M1 phenotypes, as evidenced by attenuated expression of the M1 surface markers CD45, CD11b, F4/80, MHC class II, and CD86, but increased levels of the M2 markers FIZZ1 and Arg1 [121]. Conversely, decreasing miR-124 enhanced the expression of the M1 surface markers in macrophages co-cultured with neural and astroglial cells [121]. More significantly, systemic delivery of miR-124 in vivo reduced CNS inflammation and blunted the development of EAE [121]. This study identified C/EBP-α as the transcription factor mediating the effect of miR-124 on macrophage polarization [121]. Although the role of C/EBP-α in M1 macrophage activation has been established, it remains unclear how C/EBP-α suppresses M2 polarization. Nevertheless, this study provided a good example how an individual miRNA is able to regulate macrophage plasticity.

Another case of miRNA regulation of M2 macrophage polarization was demonstrated by a recent study showing the role of miR-223 in adipose tissue inflammation and insulin resistance [123]. miR-223 was upregulated in M1, while downregulated in M2 macrophages [123]. More importantly, macrophages ablated of miR-223 were hypersensitive to LPS, but displayed retarded responses to IL-4

stimulation [123]. Bearing in mind the effect of miR-223 deficiency on macrophage polarization, miR-223 knockout mice demonstrated increased adipose tissue inflammatory responses and decreased adipose tissue insulin signaling [123]. The importance of macrophage miR-223 was also confirmed by experiments with bone marrow transplantation. Mice that received miR-223-ablated bone marrow were found to have increased adipose tissue inflammation and insulin resistance [123]. The study identified Pknox1 as a bona fide target of miR-223. The expression of Pknox1 is inversely correlated with miR-223 in M1 macrophages and inflamed adipose tissue. Pknox1's role in macrophage polarization was substantiated by gain-of-function and loss-of-function studies where levels of miR-223 and Pknox1 were modulated and tested in macrophages [123]. Although the role of miR-223 seemed apparent, how Pknox1 regulates macrophage polarization remains unclear.

As has been described above, miR-155 is a critical miRNA regulator of M1 macrophage polarization. However, there has been plenty of evidence showing its involvement in M2 polarization. Recent studies addressed the role of miR-155 in Akt-mediated macrophage activation [124]. Akt has two isoforms and it has become known that the two isoforms have opposite roles in macrophage polarization, which could explain some of the conflicting results from previous studies that showed Akt is both pro- and anti-M1 activation [124]. More definite studies found that Akt1 ablation in macrophages promotes the M1 macrophage phenotype and Akt2 ablation leads to the M2 phenotype [124]. Consistently, Akt2−/− mice are more resistant to LPS-induced endotoxin shock and to dextran sulfate sodium (DSS)-induced colitis than wild-type mice, whereas Akt1−/− mice demonstrate the opposite effect [124]. miR-155 appeared to be the key player responsible for the different responses in these mice. miR-155 expression was repressed in naive and in LPS-stimulated Akt2−/− macrophages, suggesting its involvement in the pro-M1 effect of Akt2. C/EBP-β, a well-recognized regulator of M2 macrophages, was upregulated in the absence of Akt2 and was proven to be the target of miR-155. Experiments to manipulate miR-155 expression confirmed its central role in Akt isoform-dependent macrophage polarization [124]. Additionally, miR-155 knockdown had a direct impact on IL-13-induced M2 macrophage polarization by derepressing IL-13Rα1, leading to increased IL-13Rα1-dependent STAT6 phosphorylation and activation [125]. These studies provide a further example that miRNAs play a key role in determining macrophage plasticity, with the ability to skew between different macrophage phenotypes.

Aimed to delineate the role of miRNAs in macrophage polarization, we performed systemic screening studies to identify miRNAs with differential expression in M1 and M2 macrophages. We employed granulocyte-macrophage colony-stimulating factor (GM-CSF)-induced macrophages as M1 and macrophage colony-stimulating factor (M-CSF)-induced macrophages as M2, a macrophage polarization model system that has been used in a number of previous studies [126]. We found that microRNA let-7c and miR-125a-5p are expressed at a higher level in M2 versus M1 macrophages [127, 128]. let-7c expression was decreased when M2 cells were converted to M1, whereas it was increased in the opposite

orientation. Overexpression of let-7c promoted polarization to the M2 phenotype. In contrast, the knockdown of let-7c promoted M1 polarization. C/EBP-δ, a transcription factor that plays an important role in the inflammatory response, was identified to be the target and mediator of let-7c [127, 129]. Similarly, miR-125a-5p overexpression diminished LPS-induced M1 activation but promoted IL-4-induced M2 polarization. In contrast, miR-125a-5p knockdown demonstrated exactly the opposite effect. miR-125a-5p targets KLF13, a transcription factor that has an important role in T lymphocyte activation and inflammation. We found that KLF13 knockdown mirrors the effect of miR-125a-5p overexpression, suggesting KLF13 to be a mediator of miR-125a-5p [128]. These findings proposed that let-7c and miR-125a-5p have important roles in suppressing the classical activation of macrophages (M1), while promoting alternative activation (M2).

In contrast to various types of resident tissue macrophages, tumor-associated macrophages (TAMs) are unique in that they only emerge inside or within the close proximity of cancerous tissues. TAMs are generally considered to assume M2 phenotypes and have been shown to promote tumor growth and progression, adaptive immunity, and angiogenesis [130, 131]. The functional phenotypes of TAMs are determined by the tumor microenvironment (TME). In general, TME is rich in Th2 cytokines, such as IL-4, IL-13, TGF-β, and IL-10, but lacks Th1 signals [132]. The role of miRNAs in the regulation of TAM phenotypes has been increasingly appreciated. Squadrito and colleagues found that miR-511-3p is highly expressed in TAMs [133]. Forced expression of miR-511-3p in TAMs profoundly downregulated the expression of genes involved in synthesis and remodeling of extracellular matrix (ECM), such as collagens, proteases, and scavenger receptors [133–135], suggesting an anti-M2 activity of this miRNA in TAMs. This provides a therapeutic opportunity to enhance the tumoricidal activity of TAMs by increasing miR-511-3p in the cells.

Another miRNA that participates in the regulation of TAM phenotype is miR-19a-3p. miR-19a-3p promotes TAMs to an M1 phenotype in breast cancers [136]. Yang and colleagues found that miR-19a-3p is one of the most downregulated miRNAs in TAMs. Functionally, miR-19a-3p directly targets Fra-1, a key transcription factor involved in the transformation of TAMs. In the established TME, miR-19a-3p downregulation was shown to derepress Fra-1 and its associated STAT3 signaling pathway, thereby promoting M2 polarization and TAM formation [136]. Therefore, miR-19a-3p appears to be another potential therapeutic target for treating breast cancer.

9 Regulation of Macrophage Polarization by lncRNAs

In contrast to the protein-coding mRNAs, most lncRNAs have fairly low cellular abundance. They also lack evolutionary conservation. Partially due to these characteristics, lncRNAs had been always considered to be background transcriptional noise and to be nonfunctional [137, 138]. However, findings from recent studies

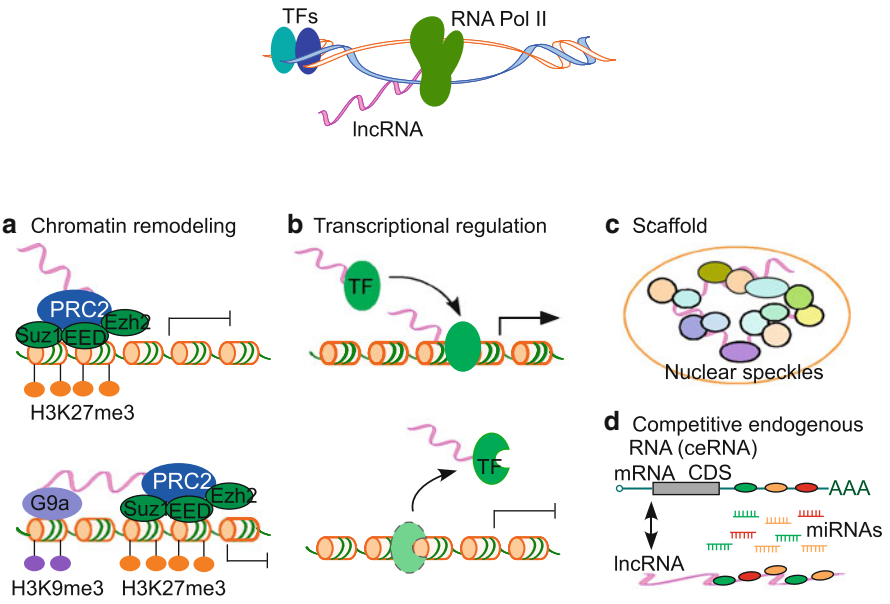

Fig. 3 lncRNA mode of functions

indicate that this class of RNAs actually plays some crucial roles in a number of cellular, developmental, and pathological processes, such as cell apoptosis and differentiation [139, 140], tumorigenesis [141, 142], and X-inactivation [143–145]. Similar to small ncRNAs, lncRNAs function primarily by regulating the expression of other genes. They fulfill their actions through epigenetic, transcriptional, and translational mechanisms. In addition, lncRNAs can also serve as molecular scaffolds, thereby serving to maintain subnuclear structure (Fig. 3) [146]. Despite rapid progress made in other fields, the role of lncRNAs in immune system regulation has just begun to unfold (Table 1).

Studies on Tmevpg1 (also known as NeST) were the first to demonstrate the participation of lncRNAs in the immune response. NeST is transcribed from a locus near the promoter region of the *IFN-γ* gene in both mice and humans and was reported to positively regulate IFN-γ expression through modulating histone H3 methylation at the *IFN-γ* locus [147, 148]. Given that IFN-γ induces M1 macrophage polarization, these findings suggest that NeST may play a role in this process through increasing IFN-γ expression.

In another study to determine the role of lncRNAs in the inflammatory response, Carpenter et al. found that TLR agonists induce numerous lncRNAs [149]. In their initial effort, they characterized one lncRNA, mouse lincRNA-Cox2, which is proximal to the prostaglandin-endoperoxide synthase 2 (*Ptgs2*/*COX-2*) gene. LincRNA-Cox2 mediated both the activation and repression of immune response genes through physical interactions with multiple heterogeneous nuclear ribonucleoproteins (hnRNPs) [149]. Perhaps not by coincidence, Li et al. found that

the long noncoding RNA THRIL regulates TNF-α expression through its interaction with hnRNPL [150]. Collectively, these studies suggest that hnRNPs appear to be the central mediators of the actions of many lncRNAs, presumably including those during macrophage polarization.

Imamura et al. found that nuclear-enriched abundant transcript 1 (NEAT1) is induced by influenza virus, herpes simplex virus infection, and the TLR3 agonist poly I:C, resulting in excess formation of paraspeckles [151]. NEAT1 can facilitate the expression of antiviral genes including cytokines such as interleukin-8 (IL-8). Mechanistically, NEAT1 induction relocates splicing factor proline/glutamine-rich (SFPQ), a NEAT1-binding paraspeckle protein as well as a repressor of IL-8 transcription, from the IL-8 promoter to the paraspeckles, leading to the transcriptional activation of IL-8 [151]. These data suggest that NEAT1 may be able to promote TLR agonist-induced M1 macrophage activation.

A recently identified lncRNA, lnc-DC exclusively expressed in human DCs, functions as a specific regulator of DC differentiation [152]. It does so by activating the transcription factor STAT3. Indeed, lnc-DC binds directly to STAT3 in the cytoplasm, promoting STAT3 phosphorylation on tyrosine-705 by preventing STAT3 binding to and dephosphorylation by SHP1 [152]. Given that STAT3 signaling is the main mediator of IL-10-induced M2c macrophages, these findings suggest that lnc-DC is capable of regulating M2 macrophage polarization.

In our efforts to delineate the roles of lncRNAs in the regulation of inflammation and macrophage activation, we performed lncRNA microarray assays and identified a number of lncRNAs that undergo altered expression in LPS-treated monocytes [153]. Of these lncRNAs, lnc-IL7R, which overlaps with the 3′ UTR of the human IL-17 receptor α-subunit gene (*IL7R*) gene, was markedly upregulated in LPS-treated cells. In action, lnc-IL7R was capable of attenuating LPS-induced inflammatory responses, evidenced by the enhanced expression of LPS-induced inflammatory mediators in lnc-IL7R-depleted cells. Furthermore, we found that suppressing lnc-IL7R diminished trimethylation of histone H3 at lysine 27 (H3K27me3), a hallmark of silent chromatin, at the proximal promoters of the inflammatory mediators [153]. Although we did not directly establish a role of lnc-IL7R in macrophage polarization, the anti-inflammatory actions of this lncRNA in our model system suggest that it may serve as a negative regulator of M1 macrophage polarization.

10 Concluding Remarks

In summary, there is rapidly accumulating evidence showing that ncRNAs play a crucial role in regulating macrophage polarization. These studies have certainly added another layer of complexity into the regulatory mechanisms involved in macrophage activation. More importantly, they also suggest a completely new set of therapeutic targets for developing novel treatments for inflammatory disorders, where aberrant macrophage activation plays a significant role. Although the studies

of lncRNAs in various pathophysiological settings are still in their infancy, there are several miRNA-based therapies that have already advanced into different phases of clinical trials [154]. Giving these encouraging recent examples, it is foreseeable that the studies on ncRNAs and macrophage polarization will transit from bench-side to bed-side in the not too distant future.

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Endogenous Control of Dendritic Cell Activation by miRNA

Sun Jung Kim and Betty Diamond

Abstract Dendritic cells are professional antigen-presenting cells and determine adaptive immune responses. Numerous factors which modulate pro- or anti-inflammatory functional properties of dendritic cells have been discovered. Recently, researchers are beginning to understand the regulatory function of microRNAs (miRNAs) in dendritic cells. miRNAs are small noncoding RNAs that regulate the expression of target genes in a posttranscriptional manner. Expression of miRNAs is tightly regulated in a cell type-dependent and a developmental stage-specific manner, and disease-related miRNA signatures have been observed. In this chapter, we briefly introduce the biogenesis of miRNAs and their expression pattern and regulatory function in dendritic cells. We also summarize the miRNA signature in systemic lupus erythematosus. Because miRNA are key regulators in dendritic cells, which are known to modulate immune homeostasis, they are potential therapeutic targets. miRNA research in dendritic cells should be expanded with this goal in mind.

1 Introduction

The immune system consists of adaptive and innate immune cells, and a proper functioning of the immune system is maintained by the harmonious collaboration of many immune cell types. Dendritic cells (DCs) are key innate immune cells which were discovered by Steinmann and Cohn [1]. One of the major functions of DCs is efficient antigen presentation; accordingly, they constitutively express high levels of co-stimulatory molecules that are required for the activation of T lymphocytes. DCs constantly sense environmental antigens through various pathogen-associated molecular patterns (PAMPs), including toll-like receptors (TLRs). They express

S.J. Kim (✉) • B. Diamond
The Center for Autoimmune and Musculoskeletal Diseases, The Feinstein Institute for Medical Research, Manhasset, NY, USA
e-mail: sjkim@nshs.edu

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multiple TLRs, and TLR activation leads to strong induction of pro-inflammatory cytokines. TLR expressions as well as TLR-activated signaling pathways have been actively investigated, and regulatory molecules within these pathways are being identified [2].

In parallel with this emerging interest in the molecular mechanisms that control the activation of DCs, a new aspect of gene regulation has become apparent in recent years with the discovery of mammalian microRNAs (miRNAs) [3]. There is abundant evidence that various miRNAs are induced during DC activation and actively modulate DC activation. More interestingly, the abnormal expression of miRNAs is often correlated with disease development, underscoring the importance of tight regulation of miRNAs.

2 Biogenesis and Expression of miRNA and Noncoding RNA in Dendritic Cells

The majority of the genome of mammals and other complex organs are transcribed into noncoding RNAs (ncRNAs), many of which are alternatively spliced and/or processed into smaller products. These ncRNAs include microRNAs (miRNAs), small nuclear RNAs (snRNA), and small nucleolar RNAs (snoRNA) as well as other classes of small regulatory RNAs. ncRNAs are involved in numerous cell biological processes. For example, U1 snRNA can regulate transcription initiation by RNA polymerase II through an interaction with the transcription initiation factor TFIIH [4], and H/ACA snoRNA is a component of telomerase and plays a role in chromosome maintenance and segregation [5–7]. The regulatory functions of ncRNAs in most cases are carried out by base-pairing with complementary sequences in other RNAs or DNA, forming RNA:RNA or RNA:DNA complexes. RNA:RNA complexes can be recognized by the RNA-induced silencing complex (RISC) or RNA editing enzymes [8]. The untranslated regions (UTRs) as well as the coding sequences themselves can be the targets of *trans*-acting regulatory RNAs, especially miRNAs through base sequence recognition [9, 10].

There are ~1,255 miRNAs in murine cells and ~2,578 miRNAs in human cells that have been identified and are currently under investigation. The miRNA genes are located throughout the genome. The primary miRNA (pri-miRNA) is generated by the initial transcript. The pri-miRNA is the longest form of three miRNA forms [11]. It is processed by the microprocessor complex, composed of Drosha (an RNase-III enzyme that cleaves dsRNA) and Pasha (dsRNA binding protein) generating a pre-miRNA in the nucleus [12–14]. With the initial pri-miRNA processing being completed in the nucleus, pre-miRNAs are exported from the nucleus into the cytoplasm by exportin 5 [15]. In the cytoplasm, the pre-miRNA is bound by another dsRNA-cleaving enzyme, Dicer, which processes the pre-miRNA into a 22-nucleotide mature miRNA [16]. The single-stranded mature miRNA is incorporated from the dsRNA into the miRNA RNA-induced silencing complex

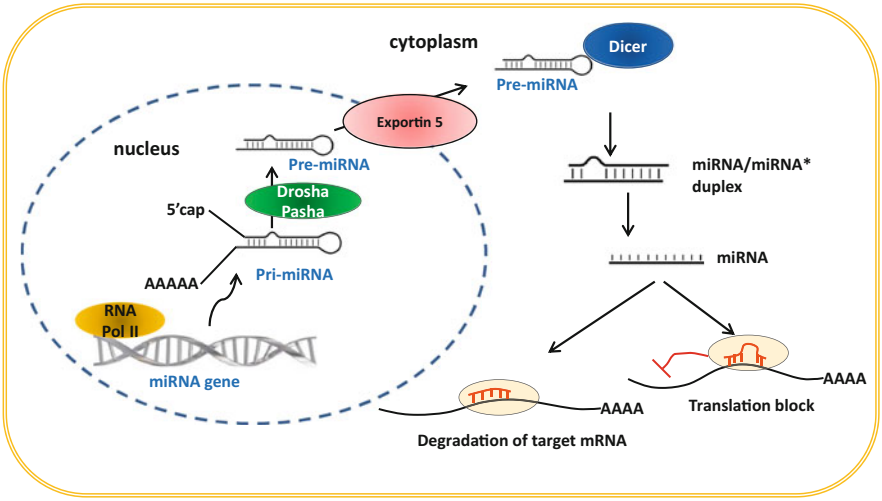

Fig. 1 Biogenesis of miRNA in mammalian cells. RNA polymerase II recognizes and transcribes a miRNA gene as a primary miRNA (pri-miRNA). The Pri-miRNA is recognized by the Drosha/Pasha enzyme complex and processed to a pre-miRNA, which is recognized by exportin 5 and transported to cytoplasm. In the cytoplasm, pre-miRNA is further processed by Dicer, generating a mature miRNA and miRNA* duplex. Mature miRNA can recognize its target mRNA and inhibit translation either by degradation of mRNA or translation block

(miRISC), which then regulates target mRNAs by regulating mRNA translation and/or stability (Fig. 1).

MiRNAs are known to regulate lineage commitment during hematopoiesis [17], and more than 100 miRNAs are expressed in mature immune cells [18]. Early studies identified the role of miRNAs in different immune cells by generating transgenic (Tg) mice carrying a floxed Dicer allele. Mutations in Dicer are associated with multiple human malignancies [19, 20]. Employment of the loxP-CRE technology enabled investigators to study the cell type-specific function of Dicer, and Dicer-deficient mice showed defective immune homeostasis and function in various cell types, including T cell subsets [21, 22], B cells [23], and NK cells [24]. In CD11c-driven Dicer conditional knockout mice, there is no apparent abnormality observed in DCs in spleen and lymph nodes (LNs) [25]. In contrast, Dicer-deficient Langerhans cells (LCs) show an increased turnover and apoptosis, and a failed induction of MHC II, CD40, and CD86 upon activation. These defects render them inefficient at CD4+ T cell priming [25]. Thus, although mice with a general deficiency of miRNA in DCs (Dicer knockout) did not successfully elucidate a function of miRNA in DCs, the study of LCs suggested a crucial role of Dicer in antigen presentation by that cell type.

Studies of miRNAs moved next from Dicer deficiency to selective miRNA deficiencies. A common approach to identifying the potential role of individual miRNAs is to characterize their expression in cells of interest and assess whether manipulation of their expression alters cell function. From these studies, several

miRNAs have been identified as key modulators in DC development in differentiation toward specific DC subsets and in DC homeostasis.

3 Dendritic Cells: Locations and Subsets

3.1 Location

The distribution of DCs in vivo clearly supports the function of DCs as the immune system's sentinel, maximizing antigen capture and subsequently antigen presentation and activation of specific T cells. Lymphoid tissues, both peripheral and mucosal, are the site where primary immune responses develop, and DCs reside primarily in the T cell zone where they can capture antigens transported through blood into spleen, through afferent lymph in lymph nodes (LNs) and from antigen-transporting M cells in mucosal lymphoid organs and subsequently activate T cells. DCs also reside throughout the body in all tissues. Tissue-resident DCs often express different sets of surface markers compared to DCs found in lymphoid organs; an understanding of tissue-resident DCs has lagged behind our understanding of DCs in lymphoid organs. Nonetheless, the finding of integrin E (CD103) surface marker expression together with CD11c on tissue-resident DCs has enabled a more sophisticated analysis [26]. We now appreciate that there are different subsets of DCs in almost every organ, for example, skin, lung, and intestine, which directly interact with the environment.

3.2 DC Subsets

Twenty years after the discovery of DCs, the significance of DC diversity is still being acknowledged. It is now well appreciated that diverse subsets of DCs exist in different tissues (Fig. 2). There are two different types of DCs in lymphoid organs: conventional DCs (cDCs) and plasmacytoid DCs (pDCs). cDCs are professional antigen-presenting cells (APCs) and express high levels of CD11c and MHC II. cDCs are further subgrouped into CD8a+ or CD8a−. PDCs are a recently identified subset of DCs; they morphologically resemble plasma cells [27]. The gene expression profile and functional contribution of pDCs and cDCs are quiet distinct. pDCs express low levels of CD11c, MHC II, and other costimulatory molecules in the steady state. They express a narrow range of pattern-recognition receptors, with a high level of TLR7 and TLR9 and upon exposure to viral stimuli, DNA and RNA, pDCs produce high levels of IFNα [28]. pDCs are differentiated from hematopoietic stem cells in the bone marrow (BM) and reside mainly in the blood and lymphoid organs.

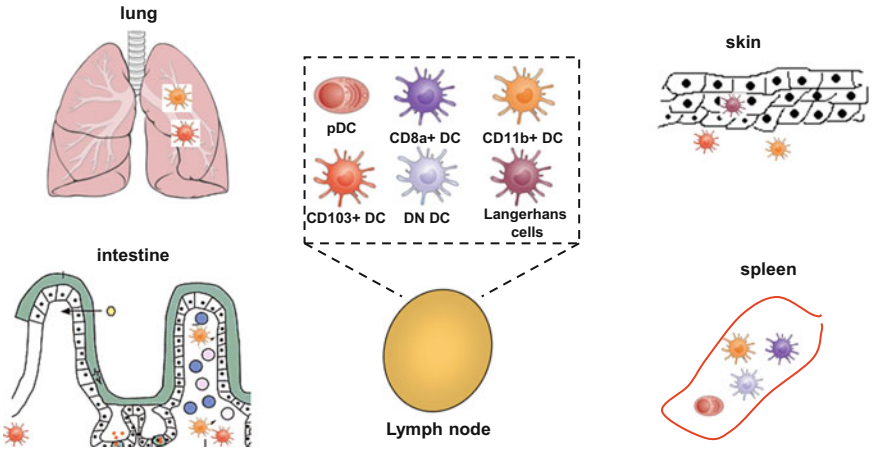

Fig. 2 DC subsets in different locations. There are various DC subsets which have distinctive functions and they can be recognized by different surface markers. Most DC subsets can be identified in lymph nodes and spleen with the exception of CD103+ DCs. DCs also can be found in nonlymphoid organs. In the lung and intestine, CD103+ DCs and CD11+ DCs are the major DC types with minor pDCs. In the skin, in addition to CD103+ DCs and CD11b+ DCs, Langerhans cells are found and play a local immunity

In nonlymphoid tissue, cDCs represent 1–5 % of tissue cells depending on the organ. They consist of two major subsets, CD103+CD11b− (CD103+ cDCs) and CD103−/+CD11b+ (CD11b+ cDCs). In contrast to lymphoid organs, there are few pDCs identified in tissues. In addition to tissue-resident cDCs, there are tissue-migratory cDCs that migrate to tissue-draining LNs through lymphatics [29]. In the skin, LCs populate the epidermis layer of the skin and account for 3–5 % of epidermal cells [30]. LCs differ from other tissue cDCs based on their unique ontogeny and properties [31]. Compared with dermal cDCs, LCs express a lower level of MHC II, intermediate CD11c, and a high level of C-type lectin langerin (CD207). Although LCs are an important component in skin immunity, they will not be discussed in this chapter.

4 miRNAs and Their Role During DC Differentiation and Activation

4.1 Expression and Function of miRNAs During DC Subset Differentiation

Since the exploration of the functions of miRNAs in DC biology is recent, there are many gaps in our understanding of expression patterns of miRNAs in each step of DC development and DC subset differentiation (Table 1). The bifurcation of cDCs

Table 1 miRNAs and their target genes during DC differentiation

miRNA	Target	Expression	Function	References
miR-21	JAG1	Increased expression during differentiation	Regulates monocyte differentiation to MO-DC	[37]
miR-34a	JAG1, WNT1	Increased expression during differentiation	Regulates monocyte differentiation to MO-DC	[37]
miR-22	IRF8 M-CSFR	Increased expression in cDC compared to pDC. Increased expression during BM-DC differentiation by GM-CSF compared to FLT3L	Required for cDC differentiation	[32, 36]

and pDCs is an important checkpoint in DC differentiation, and many studies have focused on the identification of miRNAs that are associated with this step. Although the data are limited, miR-22 is a known key component associated with cDC differentiation in mice [32, 33]. The endogenous level of miR-22 is highly upregulated in splenic cDCs compared to splenic pDCs. Moreover, the level of miR-22 is increased in in vitro-differentiated DCs from bone marrow DC precursor cells stimulated with granulocyte macrophage-colony stimulating factor (GM-CSF) compared to DCs arising from precursors stimulated with Fms (Mcdonough feline sarcoma)-like tyrosine kinase 3 ligand (Flt3L), which acts through the flt3 receptor. The level of miR-22 is also increased significantly in mature compared to immature cDCs. miR-22 binds to the 3′UTR of *IRF8* mRNA which encodes the transcription factor IRF8 and negatively regulates IRF8 expression. IRF8 is a critical factor for myeloid cell differentiation, and studies of IRF8-deficient mice show that the proper expression of IRF8 is required for the development of CD8a+ cDCs. Interestingly, IRF8 is also necessary for the development of pDCs [34]. Thus, the differential role of miRNA-22 in cDCs and pDCs still requires clarification. The critical role of IRF8 expression for DC differentiation is consistent in studies of in vitro GM-CSF-differentiated DCs [35]. This in vitro study supports the requirement for IRF8 in cDC differentiation since the culture system induces cDCs exclusively. Another function of miR-22 in DC differentiation is the downregulation of the macrophage colony-stimulating factor (M-CSF) receptor (CSF1R) which is encoded by the c-fms protooncogene and has recently identified DC differentiation factor [36].

In human DCs, expression of miR-34a and miR-21 is associated with monocyte-derived DC (MD-DC) differentiation [37]. Levels of both miRNAs are increased during differentiation, and inhibition of either miR-21 or miR-34a reduces phenotypic changes, in particular, the ratio of DC-specific intracellular adhesion molecule-1 grabbing nonintegrin (DC-SIGN) to CD14 on the surface of MD-DCs. Both miRNAs regulate MD-DC differentiation by targeting *JAG1* (encoding JAG1 which is a ligand of Notch-1) and *WNT1* (encoding WNT1 protein which is a ligand of Frizzled). The contribution of the Notch pathway to DC differentiation is generally accepted, but the precise mechanisms are still

controversial in both human and mouse DCs [38, 39]. In human MD-DCs, the protein but not mRNA level of JAG1 and WNT1 is decreased during MD-DC differentiation, suggesting that miR-22 and miR-34a act through translational repression of these molecules.

4.2 *Expression and Function of Individual miRNAs During TLR Activation*

Compared to the shortage of knowledge about miRNAs during DC differentiation, there are many studies focused on miRNA expression during DC activation, especially activation through the engagement of TLRs. Multiple subsets of miRNAs are induced in activated DCs depending on which TLR is activated. miR-21, miR-146, and miR-155 are ubiquitously increased by the engagement of multiple TLRs. There is also a downregulation of certain miRNAs following TLR stimulation (Table 2).

4.2.1 Let-7 Family

The lethal-7 (*Let-7*) gene was initially discovered as an essential developmental gene in *C. elegans* [40] and later as one of the first known miRNAs in mammals [41]. The *Let-7* family members and their genomic organization are highly conserved; however, the total number of *Let-7* genes varies among organisms with

Table 2 miRNAs and their target genes during TLR activation in DCs

miRNA	Receptor/ agonist	Target molecules	Cell types	References
Let-7 (Let-7c, Let-7i)	TLR4	SOCS-1	BM-DCs, splenic DCs, human MO-DCs	[42, 43]
Mir-146	TLR2, TLR4, TLR5	TRAF6, IRAK1, p-IkBa, p-p38, CD40L, Notch1	THP-1, HL60, intDCs, human MO-DCs, BM-DCs	[44–47]
Mir-148/ 152	TLR3, TLR4, TLR9	CaMKII	BM-DCs	[61]
Mir-155	TLR4	Pellino-1, TAB2, PU.1, M-CSFR, c-Fos	MO-DCs	[48, 49, 51, 54]
Mir-155/ Mir-155*	TLR7	TAB2/IRAK-M, respectively	Human pDCs	[50]
Mir-21	TLR4	IL-12 p35, PDCD4	BM-DCs, macrophages	[62, 63]
Mir-29	NOD2, oxLDL	ATF2, lipoprotein lipase (LPL)	MD-DCs	[46, 64]

more complex organisms having the greater numbers. There are eight *Let-7* members in humans and mice, and some of them are transcribed from distinct precursors in the genome (annotated in mirBase release 11.0).

Let-7c and Let-7i are expressed in DCs and their functional associations have been characterized [42, 43]. In BM-DCs, the level of Let-7i is increased following stimulation with LPS. Expression and induction of Let-7i is critical for the upregulation of CD86, the expression of proinflammatory cytokines IL-12, IL-23, and IFNγ, and for the downregulation of IL-10. Another Let-7 member, Let-7c, is also increased in activated DCs, in both splenic DCs and BM-DCs in a Blimp-1-dependent manner. Both Let-7c and Let-7i directly target the suppressor of cytokine signaling-1 (SOCS-1) during TLR activation, leading to enhanced activation (Fig. 3).

4.2.2 mir-146

The importance of miR-146 following TLR activation has been appreciated in various innate immune cells including DCs, macrophages, and human monocytic

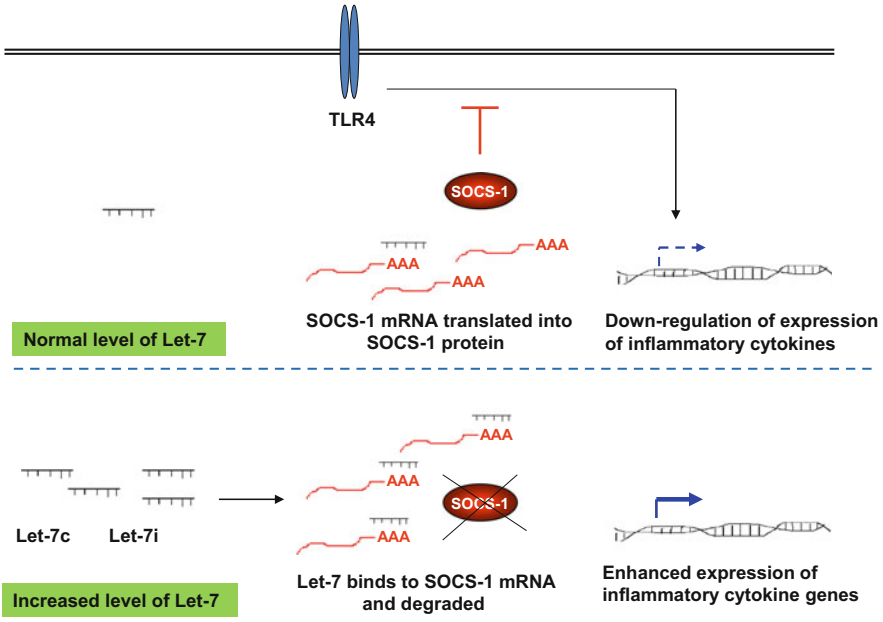

Fig. 3 Let-7 miRNAs regulate SOCS-1 and inflammatory cytokine production in DCs. SOCS-1 expression is induced by TLR4 engagement. The expression of SOCS-1 downregulates TLR4 signaling and inflammatory cytokine transcription. In DCs, the level of miRNA Let-7 is tightly regulated. If there is an increase in the level of Let-7 miRNAs (in this diagram, Let-7c and Let-7i), SOCS-1 mRNAs are recognized by Let-7c and degraded, permitting an enhanced expression of inflammatory cytokine genes

cell lines [44]. In the THP-1 and HL-60 cell lines, the level of miR-146 is rapidly increased by TLR2, TLR4, and TLR5 engagement. There are two nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) binding sites in the miR-146 promoter region, hence the expression of miR-146 is regulated by NFκB. Induced expression of miR-146 targets two key adaptor molecules downstream of TLRs, the TNF receptor-associated factor 6 (TRAF6) and IL-1 receptor-associated kinase 1 (IRAK1), suggesting that miR-146 operates through a negative regulatory feedback mechanism.

Interstitial DCs (intDCs) derived from CD34+ progenitor cells express moderate levels of miR-146a, and the level of miR-146a is not changed by either IFNγ, IL-1β, or LPS stimulation. Ectopic expression of miR-146a interferes with the TLR2 signaling pathway [45]. MiR-146a does not regulate TLR2 itself, but blocks NFκB activation and MAPK pathways through direct targeting of IκBα and p38, respectively. In human MO-DCs, oxidized low density lipoprotein (oxLDL) exposure was shown to increase the expression of miR-146a. An increased level of miR-146a inhibits the production of the proinflammatory cytokines IL-6 and TNFα as well as CD80 and CD40 expression [46]. CD40L is also a direct target of miR-146a.

Induction of miR-146a also plays a key role in cross-presentation of antigens to induce a cytotoxic T lymphocyte (CTL) response against infections [47]. In $CD11b^{ko}$ BMDC differentiated with GM-CSF, IL-12 p70 is upregulated by the stimulation of TLR9, but not by TLR4, subsequently promoting cross-priming. The expression of CD11b is required for the upregulation of miR-146a following TLR9 stimulation in DCs, and miR-146a directly regulates Notch1, which is a negative regulator of IL-12 p70. This study suggests that CD11b expression can negatively regulate cross-priming by DCs through miR-146a.

4.2.3 mir-155

miR-155 is expressed in many immune cells including B cells, T cell, and macrophages [18]. The upregulation of miR-155 is a general feature of DC activation and maturation in response to various TLR ligands [48], TNFα [49], and IFNα [50]. In response to LPS, human MO-DCs upregulate the levels of miR-155 more than 100-fold. The increased miR-155 directly targets Pellino-1 and TAB2 which are important adaptor proteins in the TLR/IL-1 signal transduction cascade [51]. Both Pellino-1 and TAB2 are part of a molecular complex containing TRAF6 and allowing the activation of an inflammatory response upon TLR4 or IL-1 receptor triggering [52, 53]. Increased miR-155 in human MD-DCs also targets the transcription factor PU.1 and PU.1-mediated expression of DC-SIGN, thereby regulating the pathogen-binding capacity of DCs [54]. Dunand-Sauthier et al. reported that LPS-activated miR-155-deficient DCs showed an impaired activation of CD4+ T cells accompanied by the failed upregulation of MHC II, CD40, and CD86 and low levels of secretion of IL-12 and TNF-α [49]. In GM-CSF-derived BM-DCs, there was no significant change in CD40, CD80, CD86, and MHC II expression in

miR-155^{ko} DCs compared to wild type DCs [48]. Although co-stimulatory molecules which are involved in antigen presentation and lymphocyte activation were not affected in miR-155^{ko} BM-DCs, miR-155 can target other surface molecules, in particular M-CSF R. miR-155 also indirectly regulates IL-12 p70 level by targeting SOCS-1 in BM-DCs.

Another verified target of miR-155 in human MD-DCs and mouse BM-DCs is c-Fos [49]. C-Fos is a component of the dimeric transcription factor AP-1 which has numerous functions in various immune cells [55]. The overexpression of c-Fos in activated DCs reflecting the loss of miR-155 led to decreased proinflammatory cytokine expression and decreased T cell activation capacity, suggesting that miR-155-mediated downregulation of c-Fos allows DC activation.

miR-155 regulates type I IFN secretion in human pDCs [50]. Human pDCs upregulate the expression of both miR-155 and its star-form miR-155* following TLR7 stimulation. Star-forms of miRNA are generated during miRNA biogenesis. Although both strands of duplexes are produced in equal amounts, their accumulation is asymmetric at steady state [56]. Depending on the thermodynamic stability of each end of this duplex, one of the strands becomes a biologically active miRNA, and the other is considered as an inactive carrier strand called miRNA* [57]. Generally the miRNA* strand is degraded [58]. Zhou et al. found that the miR-155 and miR-155* play opposite roles in regulating IFNα expression. miRNA-155* is induced and reaches peak expression at 4 h post stimulation and directly targets a negative regulator of the TLR signaling pathway, IRAK-M [59], augmenting IFNα secretion. In contrast, miR-155 is induced at the later stage of activation (at 12 h poststimulation) and targets TAB2, thereby downregulating IFNα expression.

4.2.4 Other miRNAs

mir-148

Expression of miR-148 members, mir-148/152, are increased in BM-DCs following TLR3, TLR4, and TLR9 engagement. Increased miR-148/152 inhibits the expression of calcium/calmodulin-dependent protein kinase II (CaMKII) which is a major downstream effector of Ca^{2+}, resulting in the inhibition of the production of several cytokines including IL-12, IL-6 TNF-α, and IFNα and preventing the upregulation of MHC II [60].

mir-21

Expression of miR-21 is highly induced in the lung of the IL-13-dependent mouse model of allergic airway inflammation [61]. mir-21 expression is high in myeloid cell types including BM-DCs and macrophages compared to lung epithelial cells, and increased miR-21 directly targets IL-12 p35 mRNA. One study has shown that miR-21 expression is induced by TLR4 stimulation, targeting the proinflammatory

tumor suppressor PDCD4 in macrophages [62]. Analogous studies have not been performed in DCs.

mir-29

In a recent study, miR-29 expression was found to be upregulated in human MO-DCs following NOD2 stimulation [63]. An increased level of miR-29 directly and indirectly regulates IL-12 p40 and IL-23 p19 which are key pro-inflammatory cytokines in inflammatory bowel disease (IBD). IL-12 p40 mRNA is directly regulated by miR-29, but IL-23 p19 is not. ATF2 and SMAD3 are both negatively regulated by the overexpression of miR-29 pre-miRNA in DCs, and both act as transcriptional activators in macrophages [64]. siRNA-targeting ATF2, but not SMAD3, downregulates IL-23 p19 in DCs. An interesting observation was that MO-DCs derived from NOD2 variant patients did not exhibit miR-29 upregulation following the downregulation of IL-12 p40 expression. This study suggests that decreased miR-29 contributes to the pathology of Crohn's disease, perhaps by permitting increased IL-12/IL-23 secretion.

In human MO-DCs, miR-29a expression is upregulated upon oxLDL exposure and increased miR-29a directly targets lipoprotein lipase (LPL) [46]. Modification of miR-29a by either miR-29a mimics or inhibitors directly regulates the expression of IL-6 and TNFα, surface scavenger receptor CD36, and lipid uptake in oxLDL-treated MO-DCs.

However, the molecular mechanisms by which the expression of miR-29a regulates cytokine production are not clear, and whether the downregulation of these cytokines is the mechanism for decreased atherosclerosis is also not clear.

4.3 MicroRNA in Lupus Patients

Systemic lupus erythematosus (SLE) is a chronic, multi-organ autoimmune disease characterized by the development of autoantibodies. The disease is more common among Asians, Hispanics, and African Americans [65–67]. Importantly, SLE is more prevalent in women, particularly during their childbearing years [68–70]. Numerous studies show that genetic predisposition significantly contributes to the pathogenesis of SLE, and abnormal expression of miRNAs as important regulators of immune function has been demonstrated in patients with autoimmune diseases, such as SLE.

There have been several attempts to identify aberrant miRNA level as a diagnostic signature of SLE (Table 3). The first study showing that there is a difference in miRNA expression profile between SLE patients and healthy controls dates to 2007 [71, 72]. In this study, 7 miRNAs were identified as being downregulated and 9 miRNAs as upregulated in SLE patients compared to healthy controls. In a subsequent study, 30 miRNAs were shown to be downregulated and 36 miRNAs

Table 3 Aberrant miRNA expression reported in SLE

Study	Source of miRNA	Up-regulated miRNA	Down-regulated miRNA	Method	References
Chinese lupus patients	PBMCs	miR-189, miR-161, miR-78, **miR-21**, **miR-142-3p**, miR-342, miR-299-3p, miR-198, miR-298	miR-196a, miR-17-5p, miR-409-3p, miR-141, miR-383, miR-112, miR-184	Microarray	[72]
African American and European American SLE patients	EVB-transformed B cell lines	**miR-142-3p**, miR-602, miR-371-5p, miR-125a-3p, miR-148a, miR-92b, miR-373, miR-181a, miR-663, miR-638	miR-1224-3p	Microarray and qPCR	[73]
African American SLE patients	PBMCs	miR-675, miR-199 cluster, **miR-371-5p**, miR-18a, miR-150, miR-185, miR-20b, miR-223, miR-663, miR-25, miR-423-3p, miR-93, **miR-638**, miR-301a, miR-27b, miR-361-3p, **miR-92a**, miR-155, miR-145	miR-342-3p	Microarray and qPCR	[73]
African American monozygotic discordant twins	EVB-transformed B cell lines	**miR-638**, miR-149, miR-1228, miR-149a, miR-328-5p, miR-146b-5p, miR-423-3p, miR-34a, miR-29, miR-675, miR-24, miR-663,	miR-720, miR-155	Microarray and qPCR	[73]

(continued)

Table 3 (continued)

Study	Source of miRNA	Up-regulated miRNA	Down-regulated miRNA	Method	References
		miR-222, **miR-371-5p**, **miR-21**, miR-92b, miR-221, miR-30d, miR-23a, miR-342-3p, Let-7i, Let-7g, **miR-92a**, miR-150, Let-7c, miR-125a-3p, Let-7f, miR-483-3p			
Chinese lupus nephritis patients	Kidney	miR-15b, miR-195, miR-608, miR-601, miR-30a-5p, miR-662, miR-185, Let-7a, miR-658, miR-513, miR-494, miR-134, Let-7e, miR-197, miR-184, miR-320, miR-142-5p, miR-516-5p, miR-612, miR-583, miR-198, miR-638, miR-23a, miR-518c	miR-296, miR-150, miR-365, miR-324-3p, miR-518b, miR-346, miR-637, miR-133, miR-557, miR-615, miR-345, miR-642, miR-654, miR-484, miR-611, miR-30d, miR-500, miR-663, miR-381, miR-612, miR-210, miR-486, miR-769, miR-92b	Microarray and qPCR	[74]
Chinese SLE patients	PBMCs	N.D.	miR-146a	qPCR	[77]
Danish and Swedish lupus cohort	Plasma	**miR-142-3p**, miR-181, miR-146a	miR-106a, miR-17, miR-20a, miR-203, miR-92a	qPCR	[75]

(continued)

Table 3 (continued)

Study	Source of miRNA	Up-regulated miRNA	Down-regulated miRNA	Method	References
Chinese SLE patients	Serum and urine	N.D.	miR-200a, miR-200b, miR-200c, miR-429, miR-192	qPCR	[76]

Bold indicates that the data was confirmed by qPCR

upregulated in renal biopsy samples from lupus nephritis patients compared to normal kidney samples [73]. In addition to PBMCs, miRNAs in plasma or urinary samples were determined [74, 75]. Although independent studies discovered that several miRNAs differ significantly between SLE and control PBMCs or plasma, only a few miRNAs showed a consistent pattern of expression in SLE patients compared to healthy controls, including miR-21, miR-142-3p, miR-371-5p, and miR-92a. This might reflect the variance of SLE disease activity in patients who participated in the published studies or differences in genetic background or in medication. There are different frequencies of immune cell types in PBMCs of SLE patients, and this could also influence the variable miRNA patterns. Interestingly, there is no common miRNA pattern between PBMCs and kidneys of SLE patients, suggesting that the source of miRNA-expressing cells is one of the important factors to be considered.

More recent studies have tried to understand a role of specific miRNAs in the pathogenesis of SLE. The level of miR-146a in serum is inversely correlated with SLE disease activity, and low miR-146a might contribute to SLE pathogenesis by regulating IFNα expression [76]. miR-21, which is commonly identified as a miRNA that is increased in SLE patients, may contribute to SLE pathogenesis through the regulation of T cell activation by PDCD4 expression or DNA hypomethylation by direct target of methyltransferase 1 [77, 78].

5 Summary

There is increasing evidence and appreciation of the critical function of noncoding RNAs including miRNAs in the regulation of immune responses. Although numerous studies have been performed, there are many gaps that need to be filled in. The expression of various miRNAs is tightly regulated by cell activation and often an increased or decreased level of miRNA is associated with a natural feedback mechanism during cell activation. Most of the currently available data have been obtained from in vitro-differentiated DCs (BM-DC in mouse or MO-DCs in human), and the functions of miRNAs implicated in DC function in these studies have not been studied in vivo; miRNA expression in blood DCs or DCs derived

from tissues needs to be determined. The specific functions of miRNAs that are discovered in macrophages need to be verified in DCs, as it appears that miRNA function may differ between macrophages and DCs. To understand the role of miRNA in the pathogenesis or as a signature in SLE, more defined cell type-specific or disease activity-specific miRNA studies need to be performed.

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Part II
ncRNA and Human Inflammatory Diseases

Noncoding RNA Expression During Viral Infection: The Long and the Short of It

Laurence Josset, Jennifer Tisoncik-Go, and Michael G. Katze

Abstract New technologies have expanded our view of viral–host interactions with the growing identification of non-coding RNAs (ncRNAs) that act as key regulators of viral infection. In this chapter, we explore novel genomics-based approaches used to characterize ncRNAs involved in viral infection, focusing mainly on microRNAs and long noncoding RNAs. We present recent evidence implicating virally encoded and host-derived ncRNAs in viral replication and pathogenesis regulation, focusing on four different viral diseases (IAV, KHSV, HIV, and HBV). Finally, we discuss the current knowledge of ncRNAs modulation of innate and adaptive immune responses to viral infection. These findings highlight the complexity of host–pathogen networks determining the outcome of viral disease. Understanding the role of ncRNAs in these networks may offer novel antiviral therapy and diagnostic tools.

1 Introduction

Less than 2 % of the mammalian genome encodes protein-coding genes, while the majority of the genome is pervasively transcribed. This suggests that most of the mammalian transcriptome consists of noncoding transcripts. Noncoding RNAs (ncRNAs) comprised of microRNAs (miRNAs), long noncoding RNAs (lncRNAs), and Piwi-interacting RNA (piRNA), to name a few, regulate a variety of diverse cellular and organismal processes. Since the discovery of RNA interference in nematodes 20 years ago [1–3], ncRNAs have garnered a great deal of attention, in

L. Josset (✉)
Department of Microbiology, Laboratoire de Virologie et Centre National de Référence virus influenzae Laboratoire VirPatH EA4610, Faculté de Médecine Lyon Est, Université Claude Bernard Lyon 1, Lyon, France
e-mail: laurence.josset@chu-lyon.fr

J. Tisoncik-Go • M.G. Katze
Department of Microbiology, School of Medicine, University of Washington, Seattle, WA, USA
e-mail: tisoncik@uw.edu; honey@uw.edu

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particular for their role in pathogen–host interactions. To date, there are over 24,000 miRNA loci from 206 species registered with miRBase, a primary miRNA sequence repository that has grown tremendously with small RNA deep sequencing [4]. LncRNAs have been emerging more recently as key regulators of diverse cellular processes, including viral infection. In general, they are defined as transcripts that have at least 200 nucleotides and which lack any positive-strand open-reading frames longer than 30 amino acids. The number of lncRNAs is still a matter of debate, with, in humans, between 9,277 manually annotated lncRNA genes producing 14,880 transcripts present in GENCODE [5], to 56,018 lncRNA genes producing 95,135 transcripts in NONCODEv4 [6]. The nomenclature of lncRNAs is constantly evolving and they have been classified according to their location with respect to protein-coding genes: intergenic (lincRNA), antisense, sense exonic, and sense nonexonic [6]. In contrast to miRNAs that have a well-defined function in the cytoplasmic compartment by translational inhibition and/or degradation of target mRNAs, lncRNAs have a wide range of functions through diverse molecular mechanisms (reviewed in [7]). This chapter aims to summarize our current knowledge of miRNA and lncRNA roles in the context of viral infection in mammals, with a specific focus on ncRNA regulation of viral replication and immune responses.

2 Virus-Encoded ncRNAs

Mammalian viruses are highly diverse etiologic agents that can cause an array of human disease. Virus replication and the cellular responses activated to combat infection coordinately determine virus-induced pathogenesis. Similar to what is found in the host genome, viruses can encode small noncoding transcripts, or virally encoded miRNAs, that are more prevalent in DNA viruses due to their much larger genomes and their ability to replicate in the nucleus and cause persistent infection.

Virus miRNA expression was first reported in the context of Epstein–Barr virus (EBV) infection. Small RNAs cloned from a Burkitt's lymphoma cell line latently infected with EBV were found to originate from the virus including two miRNAs from the introns of the BARTS (BAMHI A rightward transcripts) [8]. Since then, more than 200 viral miRNAs have been identified from large DNA viruses of the *Herpesviridae* family. Similar to host-derived miRNAs, virally encoded miRNAs are approximately 22 nucleotides in length and primarily regulate gene expression by binding to sequences located in the 3′ untranslated region (UTR) of target mRNAs through their "seed" region. Viral lncRNAs, on the other hand, are often smaller than 200 nucleotides that have been described for host lncRNAs. There are several novel classes of viral ncRNAs, including influenza A virus small viral RNAs (svRNA) [9], West Nile virus subgenomic flavivirus RNA (sfRNA) [10], Herpesvirus saimiri small nuclear RNAs [11], and the chimeric HBx-LINE1 viral-human gene fusion transcript, that functions as a hybrid RNA [12]. Retroviral antisense ncRNAs, such as HIV antisense lncRNA, regulate viral transcription by directing epigenetic silencing complexes to the LTR [13]. The HBZ antisense transcript of human T-lymphotrophic

virus 1 (HTLV-1) is consistently expressed in adult T-cell leukemia/lymphoma (ATL) cells and codes for a multifunctional protein that positively correlates with proviral load and disease severity associated with HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) [14]. The roles of these ncRNAs in viral replication and pathogenesis remain poorly understood; however, HBZ antisense transcript, for example, could be a predictive biomarker of disease.

Viral ncRNAs are capable of regulating a variety of viral and cellular processes, including viral latency, apoptosis, and immunity. For instance, Kaposi's sarcoma-associated herpesvirus (KSHV) encodes over 90 genes and has an abundant number of miRNAs, 12 of which are expressed from two latency-specific promoters that regulate the expression of latent viral proteins ORF71, ORF72, ORF73, and KapB controlling KSHV latency [15]. KSHV-encoded miRNAs can regulate cellular transformation and tumorigenesis [16] and target TLR-responsive genes, *IRAK1* and *MYD88*, to reduce herpesvirus-induced inflammation [17]. Virally encoded miRNA mimics of cellular miRNAs have also been discovered. For example, KSHV miR-K12-11 is highly homologous to cellular miRNA-155, known to function as an oncogene, with both miRNAs containing identical seed regions critical for target recognition and downregulating a common set of mRNA targets [18]. The viral miR-155 analog is thought to contribute toward KSHV-associated malignancies in infected patients, a viral mimic strategy similar to herpes simplex virus 1 (HSV-1) $\gamma_1$34.5 protein with homology to cellular GADD34 controlling host protein synthesis [19]. In addition to human gammaherpesviruses, DNA virus families with members known to encode miRNAs include *Polyomaviridae*, *Adenoviridae*, *Ascoviridae*, and *Baculoviridae* (reviewed in [20]). The current knowledge of virally encoded ncRNAs and their cellular targets is summarized in Table 1.

3 Genomics-Based Approach to Identify and Characterize Host-Encoded ncRNAs Involved in Host-Response to Viral Infection

Initial studies of ncRNAs were based on conventional molecular biology techniques including RT-PCR and northern blot, characterizing the expression of single ncRNAs. The landscape of ncRNAs has widely expanded with recent advancements in sequencing technologies and computational biology.

3.1 Methods for Genome-Wide Characterization of ncRNA Expression

Large scale gene expression analysis has been dominated by DNA microarray since its development in the 1990s. Array technologies provide simultaneous

Table 1 Viral-encoded ncRNAs and their cellular target genes and pathways

Virus family or subfamily	Virus	ncRNA type	ncRNA name	Functional role and cellular targets	Reference (s)
Retroviridae	HIV	lncRNA	Antisense transcript	Epigenetic regulation of gene expression	[13]
	MLV		U3 antisense transcripts	Insertional activation of cellular proto-oncogenes *Jdp2* and *Bach2*	[21]
	HTLV-1		*HBZ* transcript	Promotes T-cell lymphoma; CREB-mediated inhibition of cyclin D1; regulates hTERT transcriptional activity	[22–24]
Gammaherpesviridae	EBV	miRNA	ebv-miR-BART6-5p	DICER	[25]
			ebv-miR-BART5	Modulator of apoptosis PUMA	[26]
			ebv-miR-BART5	Pro-apoptotic protein BIM	[27]
			miR-BHRF1	Promotes cell cycle progression and proliferation; inhibits apoptosis	[28]
			miR-BHRF1-3	CXCL-11	[29]
		lncRNA	EBER-1 (166 nt) and EBER-2 (172 nt)	Promotes cellular proliferation and transformation; inhibits apoptosis	[30]
	KHSV	miRNA	miR-K12-10a	TWEAKER	[31]
			miR-K12-1, 3 and 4-3p	Targets CASP3 to regualte apoptosis	[32]
			miR-K12-9-5p and miRK12-7-5p	KSHV RTA	[33, 34]
			miR-K12-11	targets IKKε to modulate IFN signaling	[35]
			miR-K12-1	IkB-alpha	[36]
			miR-K12-1, miR-K12-6-5p, miR-K12-11	MAF	[37]
			miR-K12-4-5p	Targets Rbl2 to regualte global epigenetic reprogramming	[38]
			miR-K12-9 (miR-K9) and miR-K5	IRAK1 and MYD88	[17]
			miR-K1, miR-K3-3p, miR-K6-3p, miR-K11	THBS1	[39]

			miR-K1	p21	[40]
			miR-K12-3, miR-K12-7	C/EBPbeta p20 (LIP)	[41]
			miR-K12-11	BACH1, FOS	[18, 42]
		lncRNA	lncRNA-PAN	Multifunctional transcript regulating viral replication and host response	[43, 44]
	MCMV	miRNA	miR-M23-2	CXCL16	[45]
	HCMV	miRNA	miR-US25-1	CCNE2, H3F3B, TRIM28	[46]
	HCMV, KSHV, EBV	miRNA	miR-UL112, miR-K12-7, miR-BART2-5p	MICB	[47, 48]
			miR-K5; miR-K9; miR-K10a/b	BCLAF1	[49–51]
	HVS	snRNAs	*H. saimiri* U-rich nuclear RNAs (HSURs)	HSUR 1 and 2 bind specific host miRNAs in virally transformed T cells. HSUR 1 downregulates cellualr miR-27a	[11]
Alphaherpesviridae	MDV1	miRNA	miR-M4	PU.1	[52]
			miR-M4	PU.1, GPM6B, RREB1, c-Myb, MAP3K7I, P2 PU.1, C/EBP	[53]
			miR-M3	SMAD2	[54]
Flaviviridae	WNV	lncRNA	Noncoding subgenomic flavivirus RNA (sfRNA)	Required for viral pathogenicity and evasion of the type I interferon response	[10]
Orthomyxoviridae	IAV	miRNA	Influenza A virus-derived small viral RNAs (svRNAs)	Interacts with the viral RdRNP to initiate the switch from viral transcription to replication	[9]

quantification of tens of thousands of transcripts and have been widely used to characterize transcriptomic responses of cells, animal models, and human patients to virus infection (reviewed in [55]). Accurate detection and quantification of miRNAs poses several challenges that include the lack of common sequences for their purification, the high sequence similarity among miRNA within the same family, and the presence of natural variants of miRNAs, called isomiRs, which result from post-transcriptional nucleotide additions or deletions to 3′ and 5′ ends of mature miRNAs (reviewed in [56]). Various microarray-based strategies for profiling miRNA expression have been developed, including different approaches for fluorescent labeling of the miRNA for subsequent hybridization to classical DNA-based probes or locked nucleic acid (LNA)-modified capture probes (reviewed in [56]). LncRNA-specific microarrays offer less technical variations and they mainly differ by the number of lncRNAs targeted by DNA probes present on the chip. At the time of writing, lncRNA microarrays include Agilent® custom arrays by GENECODE targeting 11,880 human lncRNA transcripts [5] (22,001 lncRNAs transcripts in the newest version), Arraystar® LncRNA human and mouse arrays targeting ~30,600 and ~31,423 lncRNA transcripts, respectively, and Affymetrix® Human Gene ST Array covering 11,000 lncRNA RefSeq transcripts. The main limitations of microarray-based characterization of ncRNA are a restricted linear range of quantification and their dependence on the prior knowledge of annotated transcripts for probe design. In addition, microarrays can have imperfect specificity in some cases for miRNAs that are closely related in sequence.

The recent development of next-generation sequencing (NGS) platforms has enabled a novel approach for ncRNA expression profiling by RNA-Seq. RNA-Seq relies on the preparation of a cDNA library from the RNA sample of interest, followed by the "massively parallel" sequencing of millions of individual cDNA molecules from the library. The method of cDNA library construction used determines the type of transcripts to be sequenced: small RNA-Seq for sequencing of miRNAs, small nuclear (sn), small nucleolar (sno), and piwi-associated (pi)RNAs; mRNA-Seq for sequencing various types of polyadenylated transcripts; and total RNA-Seq for sequencing whole transcriptomes (all transcripts after ribosomal RNA (rRNA) removal). Remarkably, as many lncRNA transcripts may not be polyadenylated [57, 58], it is important to use total RNA-Seq approaches for the comprehensive detection of lncRNAs. Compared to microarray, RNA-Seq has a wider dynamic range, higher precision, and reproducibility [59–61], and is able to distinguish ncRNA that differ by as few as one nucleotide. Importantly, because RNA-Seq does not require prior transcript annotation for probes, novel transcripts can be detected, including both protein-coding and ncRNA transcripts, as well as RNA with somatic mutations and alternative splicing forms [62, 63]. The main limitations of RNA-Seq are the computational infrastructure required for data analysis, interpretation, and storage. The cost associated with sequencing can also be a limiting factor, though newer technologies, such as Illumnina NextSeq, are enabling quicker run times to generate up to 400 million clusters with a more cost-effective system that is used to store, analyze, and share genomic data.

3.2 Methods to Computationally Infer ncRNA Function

3.2.1 miRNA Annotation, Analysis, and Target Prediction

A number of tools are available for miRNA annotation, analysis, and target prediction (reviewed in [64]). The miRBase database is the primary online repository for miRNA sequences and annotation [4]. Several software packages predict novel miRNAs from RNA-Seq data, including miRDeep [65] and mirTools [66]. Finally, many miRNA target prediction tools have been published. These tools indirectly predict miRNA function, as miRNAs binding to the 3′ UTR of their mRNA target lead to the degradation or the translational repression of the target mRNA. Target prediction tools are based on sequence comparisons between mature miRNA and 3′ UTRs in candidate mRNA targets. Three widely used algorithms that have enhanced mRNA target prediction include TargetScan [67], miRanda [68], and DIANA-microT [69]. The best algorithms currently identify 60 % of all available targets and provide one valid target in approximately every three predicted targets [64], highlighting the importance of experimental validation.

3.2.2 lncRNA Annotation and Function Prediction

Many online databases are available for lncRNA annotation, some of which include predicted or experimentally validated lncRNA functions [6, 70–75]. Identifying lncRNA functions is especially challenging because of the large number of lncRNAs and the variety of mechanisms driving their functions. With the large amount of transcriptome data created by microarray and RNA-Seq technologies, predicting gene function on the basis of expression has been proposed as an attractive strategy [76]. One approach for predicting function of both coding and noncoding genes is the "guilt by association" approach, which relies on finding which RNAs have similar expression profiles to protein-coding genes of known function. Several methods have been used to infer co-expression networks with the objective of predicting gene function: algorithms using the concept of mutual information (MI) [77, 78]; random matrix theory (RMT) [79]; and correlation analysis such as weighted gene co-expression network analysis (WGCNA) [80, 81]. This strategy was applied to mouse lncRNAs after re-annotation of the Affymetrix Mouse Array using 34 datasets derived from diverse mouse tissues and allowed the functional annotation of 340 lncRNAs mainly involved in development, cellular transport, and metabolic processes [81]. A similar method was also applied for lncRNA function annotation based on RNA-Seq data [82]. Many intergenic long noncoding RNAs (lincRNAs) that were co-expressed with genes in the p53 pathway were further validated as p53 transcriptional targets, including lincRNA-*p21* which serves as a repressor in p53-dependent transcriptional responses [83]. Similarly, Hu et al. inferred potential functions of lincRNAs in T cells by analyzing their co-expression with coding genes and showed that the expression of LincR-*Ccr2-5′*AS was correlated with the expression of genes within a chemokine-mediated

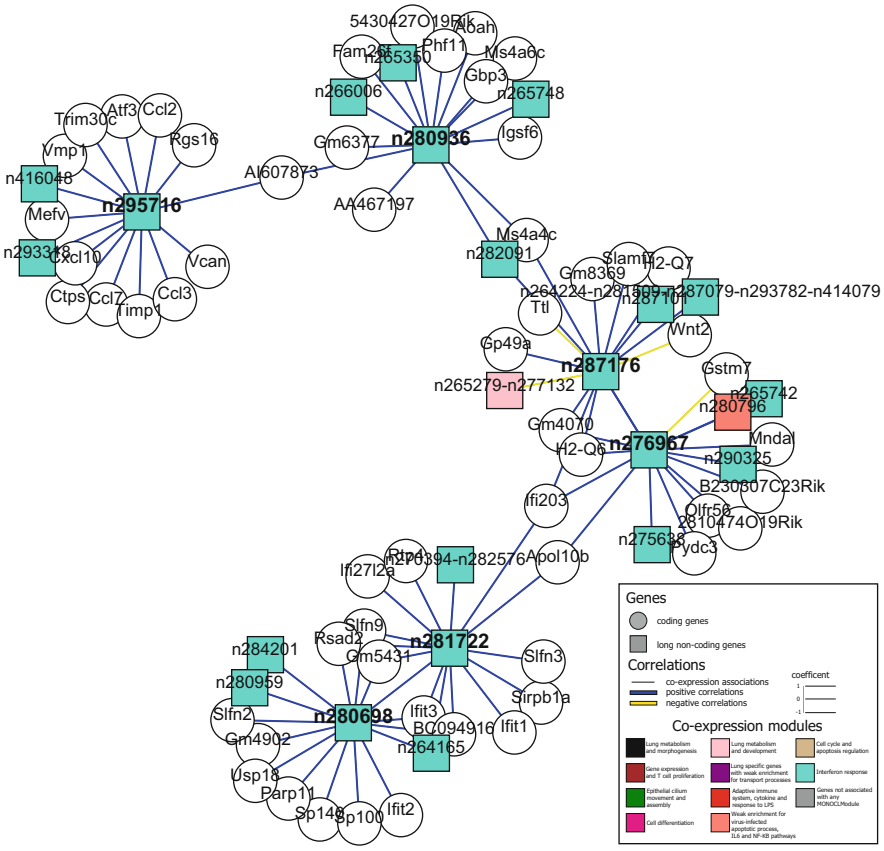

Fig. 1 Example of six lncRNAs induced in mouse lung after respiratory virus infection and co-expressed with interferon response genes. These lncRNAs: n280936, n280698, n281722, n276967, n295716, and n287176, depicted in bold in the figure, also have binding motifs for IRF9 in their promoters. The network shows the co-expression associations between each lncRNA and top 15 most correlated genes. Nodes in the graph represent the coding or lncRNA genes while edges represent the significant co-expression associations. Based on whole network topology, n280936, n280698, and n295716 were classified as hubs of the interferon response module. The lncRNA-coding genes co-expression network was visualized using the Mouse NOnCode Lung database (MONOCLdb) at http://www.monocldb.org/. The MONOCLdb contains annotations, expression profiles, and functional enrichment results of lncRNA expressed in Collaborative Cross founder mice in response to respiratory infection caused by influenza and SARS-CoV [52]

signaling pathway [84]. The knockdown of LincR-*Ccr2-5′*AS decreased the expression of its neighboring chemokine receptor-encoding genes.

Recently, we predicted the function of 5,329 lncRNAs involved in pulmonary responses to influenza A virus or SARS-CoV infection by using WGCNA and two levels of annotation: (1) a coarse annotation based on lncRNA membership within a co-expression module and (2) a finer rank-based annotation method [85]. This analysis identified many lncRNAs induced after viral infection and that were closely associated with interferon (IFN) response genes (Fig. 1).

3.3 Genome-Wide Characterization of ncRNA Expression During Viral Infection

Microarrays have been widely used to characterize miRNA expression in response to viral infections, with more than 100 publications referenced in PubMed to date. Among them, analysis of miRNAs expressed in the lung of mice following infection with influenza A virus found 130 cellular miRNAs were differentially regulated, with distinct expression patterns in response to highly pathogenic 1918 H1N1 virus compared to a nonlethal seasonal H1N1 influenza virus [86]. Some of these miRNAs had predicted mRNAs targets with anti-correlated expression levels, such as miR-200a and *sip1*, that were enriched in immune response and cell death pathways, suggesting that type I IFN singaling and CREB activity linked with the high virulence of 1918 pandemic influenza virus may be regulated by miRNAs.

RNA-Seq analysis has also been used to study the ncRNA response to respiratory virus infection. Peng et al. identified over 1,500 lncRNAs and 200 small RNAs, such as snRNAs and piRNA, expressed in mouse lung in response to SARS-CoV or influenza A virus [87, 88]. Using a large RNA-Seq dataset consisting of a wide-range of pulmonary transcriptional responses during SARS-CoV and influenza infection, we have expanded upon this analysis and identified 5,329 lncRNAs differentially expressed after infection in the lungs of 8 genetically diverse mouse strains [85]. These lncRNAs accounted for about 40 % of total genes differentially expressed upon viral infection. Most of the upregulated lncRNAs were related to the innate immune response and were co-expressed with genes specific to immune cells, suggesting they might be associated with immune cell infiltration of the lung following infection (Fig. 1). Some of these lncRNAs were highly connected (hubs) in the interferon-response co-expression module and could therefore have a role in controlling type I IFN signaling during viral infection [85].

Finally, RNA-Seq analysis of ncRNA expression in a CD4+ T cell line identified over 1,000 lncRNAs and 531 miRNAs differentially expressed upon HIV-1 infection [88, 89]. Integration of mRNA-Seq and small RNA-Seq data identified 5,023 anticorrelated interactions involving 46 differentially expressed miRNAs predicted to target as many as 518 mRNAs, including target genes with transcription regulatory activity, such as P/CAF (P300/CBP-associated factor), and genes within T cell activation-related pathways. The downregulation of miRNAs may have contributed to increased T cell receptor signaling.

4 Role of Host and Viral ncRNA in Regulating Viral Replication and Pathogenesis

Host and viral ncRNAs can impact viral replication and pathogenesis. For example, ncRNAs can influence poliovirus tissue tropism [90], Coxsackie A21 virus-induced myositis [91], hepatotoxicity associated with oncolytic adenoviruses [92], and HIV control of HLA-C expression [93]. Here, we focus on how host and viral ncRNAs

impact the replication or pathogenesis of influenza A virus, KSHV, HIV, and HBV infection associated with hepatocellular carcinoma.

4.1 Influenza A Virus

Influenza virus is a respiratory pathogen that causes significant morbidity and mortality, with over 200,000 people hospitalized from infection each year in the United States alone. Influenza H1N1 and H3N2 viruses circulate within the human population and on occasion new viruses emerge as a result of gene reassortment, the shuffling of viral gene segments from different influenza A viruses. This in turn can give rise to a worldwide pandemic, as demonstrated by the recent 2009 pandemic H1N1 influenza virus that originated in swine due to a reassortment event between swine H1N1 and human H3N2 viruses.

4.1.1 Influenza svRNA

It has been generally accepted that RNA viruses do not transcribe ncRNAs; however, this notion is beginning to change with NGS technologies. For influenza A virus (IAV), a segmented negative-strand RNA virus, deep sequencing has captured small viral RNAs (svRNA) varying between 22 and 27 nucleotides in length originating from all eight genomic segments of the virus. The production of svRNAs is dependent on the IAV RNA-dependent RNA polymerase (RdRp), comprised of polymerase subunits, PB1, PB2, and PA, and corresponds to the shift from IAV transcription to replication. Unlike other virally encoded small ncRNAs, IAV svRNAs do not induce host antiviral defenses (e.g., IRF3 activation or IFNβ induction), but they were found to be important for viral replication. For instance, the use of LNA complementary to segment 4 svRNA (HA svRNA) significantly reduced HA vRNA synthesis resulting in decreased HA protein levels and reduced influenza H1N1 virus replication in cell culture [9].

4.1.2 Host lncRNA Regulation of Influenza Replication

VIN is a nuclear lncRNA, the expression of which is strongly increased 10–60-fold in human lung epithelial cells in response to H1N1, H3N2, or H7N7 IAV infection [94]. Interestingly, this lncRNA is not increased following infection with influenza B virus, IFNβ treatment, or TLR3 stimulation by polyI:C. Silencing of *VIN* decreases influenza virus replication suggesting that VIN could be a proviral factor, but its mechanism of action has not yet been determined. Silencing of another lncRNA named *NRAV* was found to decrease influenza virus replication in vivo and in vitro. However, *NRAV* was downregulated following viral infection, which increasing ISG induction and thereby inhibiting influenza virus replication [95].

4.2 Human Immunodeficiency Virus

There are more than 35 million people living with HIV in the world today. The retrovirus targets CD4+ T cells and their depletion facilitates a patient's progression toward acquired immunodeficiency syndrome (AIDS) if left untreated. Mucosal transmission is one of the primary routes of HIV transmission and mucosal immune responses have been suggested to control systemic infection after virus exposure. Deep sequencing is revealing new insight into mucosal immunity against HIV and in particular, changes in ncRNA expression and their potential role in viral pathogenesis and HIV-associated disease.

4.2.1 Host ncRNAs Involved in Mucosal Immunity Against HIV

MiRNAs regulate intestinal epithelial differentiation, architecture, and barrier function [96], which are disrupted during HIV infection in part due to the rapid depletion of CD4+ T cells in the gastrointestinal tract [97]. In a nonhuman primate model of AIDS, simian immunodeficiency virus (SIV) infection decreased the expression of mucosal miRNAs (e.g., miR-16, -194, and -200c) involved in epithelial homeostasis of the gut and coincided with increased 5′–3′-exoribonuclease 2 (XRN2) protein expression and altered levels of miRNA biogenesis machinery components, DICER1 and Argonaute 2 (AGO2) [98]. In this same study, miRNA profiled from total RNA extracted from jejunal biopsy specimens from HIV-infected and HIV-negative patients also found reduced intestinal miRNA expression in patients chronically infected with HIV. These findings suggest that the gut mucosal response to viral infection involves decreased miRNA expression likely impairing epithelial cell growth and development during SIV and HIV infections. In a separate study, co-expression analysis between differentially expressed lncRNAs and coding genes revealed the downregulation of transcripts in rectal mucosa of rhesus macaques infected with SIV that were associated with wound healing, cell–cell adhesion, and tissue formation during acute SIV infection [99]. Taken together, alteration of the local microenvironment in the small intestine through ncRNA downregulation could lead to the dissemination of virus and intestinal enteropathy.

4.2.2 Viral Antisense lncRNA Involved in HIV Latency

After entering a cell, retroviruses integrate into the host genome where viral genes can be transcribed or remain latent. In addition to viral genomic sense RNA and mRNA, several retroviruses, including HIV, encode antisense RNAs (asRNAs) that are transcribed during infection (Table 1). Kobayashi-Ishihara et al. report the transcription of an apparent major form of HIV asRNAs, *ASP-L*, in HIV-1-infected cells. This transcript is localized in the nucleus and inhibits HIV-1 replication

[100]. The molecular mechanism of HIV asRNA action was recently elucidated [13]. It is involved in the epigenetic regulation of viral transcription through its recruitment of DNMT3a and possibly other chromatin-remodeling proteins, such as enhancer of Zeste 2 (EZH2) and histone deacetylase 1 (HDAC-1), to the viral promoter (5′LTR). This complex induces a repressive chromatin state, which epigenetically silences the transcription of viral genes. Together with viral asRNAs, cellular lncRNAs may also be involved in regulating proviral HIV latency, as many lncRNAs function as epigenetic regulators [101, 102].

4.2.3 Host lncRNA Controlling HIV Replication

A host lncRNA named *NEAT1* was shown to directly control HIV replication. *NEAT1* is an essential structural component of paraspeckles, which are nuclear bodies rich in RNA-binding proteins and splicing factors. Recently, *NEAT1* was found upregulated in human cell lines infected with HIV-1 [103]. Silencing of *NEAT1* decreased nuclear paraspeckle bodies and was associated with enhanced HIV expression and increased levels of unspliced HIV transcripts in the cytoplasm. Zhang et al. hypothesized that *NEAT1* modulates HIV-I replication by promoting HIV mRNA nuclear sequestration in paraspeckles; however, Imamura et al. recently described that silencing of *NEAT1* impairs the induction of numerous genes including antiviral factors following TLR3 stimulation [104]. Therefore, the effect of *NEAT1* on HIV replication may also be indirect and mediated by dysregulation of the antiviral host response.

4.3 Kaposi's Sarcoma-Associated Herpesvirus

Kaposi's sarcoma-associated herpesvirus KHSV is a gammaherpesvirus causing several human cancers and lymphoproliferative disorders, including Kaposi's sarcoma (KS), primary effusion lymphoma (PEL), and a subset of multicentric Castleman's disease (reviewed in [105]). Like other herpesviruses, KSHV infection is characterized by two states: viral latency and lytic growth. During latency, very few viral genes are expressed, reducing the number of viral epitopes available to trigger a host immune response. KSHV latent genomes are bound to host histones and can form either minichromosomes or episomes, which are epigenetically regulated [106]. Upon stimulation by various stress responses, KSHV activates the lytic program of its replication cycle to produce new infectious viral particles. Disruption of the repressive viral chromatin state is essential for KSHV reactivation, and it is mediated by viral proteins and ncRNAs [106].

4.3.1 miRNA Regulation of KSHV Replication

Early and late miRNA expression in primary lymphatic endothelial cells (LECs) infected with KSHV regulates the antiviral response by facilitating viral gene expression. Early-response miR-132 is highly upregulated after infection, which affects viral gene expression by suppressing transcriptional co-activator *p300*. Silencing of *p300* in LECs impairs antiviral responses to KSHV by decreasing *IFNβ*, *ISG15*, *IL-1β*, and *IL-6* mRNA expression. Conversely, the inhibition of miR-132 induction suppresses KSHV replication in LECs, resulting in increased IFNβ mRNA levels. A similar phenomena is observed with herpes simplex virus-1 (HSV-1)- and human cytomegalovirus (HCMV)-infected monocytes where virus-induced host miR-132 inhibits *p300* expression [107].

4.3.2 lncRNA Control of KSHV Replication

KSHV encodes a viral lncRNA known as polyadenylated nuclear RNA (*PAN RNA*), which was discovered 18 years ago as the most abundant transcript produced during the lytic phase [108]. Several reports suggest that *PAN RNA* is a multifunctional regulatory transcript and plays an important role in KSHV replication [43, 44, 109–111]. *PAN RNA* interacts with several host factors, including histones H1 and H2A and chromatin modifiers, such as lysine demethylases UTX and JMJD3, and the histone methyltransferase MLL2 [109]. These interactions remove H3K27me3, a repressive chromatin marker within the KSHV genome [109]. *PAN RNA* also mediates changes in histone modifications by binding the Polycomb repressive complex 2 (PRC2) [110]. In addition, *PAN RNA* interacts with the viral latency-associated nuclear antigen (LANA), which is a transcriptional repressor associated with latent viral episomes. Upon reactivation, *PAN RNA* sequesters LANA away from the viral episome [44]. Finally, *PAN RNA* interacts with the host poly(A)-binding protein C1 PABPC1, which allows late viral mRNA exportation and translation. In addition to its role in regulating viral replication, *PAN RNA* also acts on cellular gene expression, modulating the expression of genes involved in cell cycle, immune response, and production of inflammatory cytokines [43, 110]. To date, there are no known host lncRNAs reported to directly regulate KSHV replication. However, similar to HIV, host lncRNAs that regulate chromatin state could also potentially control KSHV episomal latency [101, 102].

4.4 Hepatocarcinoma Associated with Hepatitis B Infection

Hepatitis B virus (HBV) is an oncogenic virus belonging to the *Hepadnaviridae* family of DNA viruses. HBV infection is the leading cause of acute and chronic hepatitis B, liver cirrhosis, and it is a major risk factor of hepatocellular carcinoma

(HCC). Nearly 2 billion people are infected with HBV worldwide and more than 350 million are reported to be chronic HBV carriers. Many host cellular ncRNAs are involved in HCC (reviewed in [112]) in addition to specific viral ncRNA that can facilitate oncogenesis.

4.4.1 Viral-Human Chimeric lncRNA

After infection, HBV not only replicates as an episome, but HBV DNA can also integrate into the host genome, leading to chromosomal rearrangements and deletions. In most integrated subviral DNAs, X-ORF is maintained and the HBx gene transcribed at low levels during acute and chronic hepatitis (V. Schluter et al., Oncogene 1994). Such integration has been linked to liver cancer formation, with 85–90 % of HBV-associated HCC tumors having at least one HBV insertion [113]. Recently, Lau et al. detected a specific integration site of HBV into a transposable *LINE1* element in chromosome 8 for about 23 % of HBV-HCC cases in a cohort of 90 patients [12]. [114] This integration leads to a chimeric viral-human transcript, HBx-LINE1. Silencing of *HBx-LINE1* inhibits cell motility and Wnt/β-catenin signaling. The HBx-LINE1 fusion transcript encodes for a fusion protein; but unexpectedly, its oncogenic effect is dependent on the *HBx-LINE1* mRNA and not protein expression. It was therefore concluded that *HBx-LINE1* functions as a lncRNA. Moreover, *HBx-LINE1* transgenic mice had increased risk of HCC development proving the oncogenic role of the *HBx-LINE1* transcript.

4.4.2 Host lncRNA Regulating HBV- and HCV-HCC

Several cellular lncRNAs are differentially expressed in HCCs, including *MALAT1*, *HULC*, *H19*, *HEIH*, *HOTAIR*, *MEG3*, *uc002mbe.2*, *lncRNA-LET*, *MVIH*, and *Dreh* (reviewed in [115]). Interestingly, these lncRNA could provide prognostic and diagnostic markers of HCC [112]. Most of these lncRNAs have general oncogene-like effects and only two of them, HULC and Dreh, have been directly linked to HBV infection. Highly Upregulated in Liver Cancer (*HULC*) is upregulated in HBV-HCC and correlated with HBx levels [116]. HBx activates the *HULC* promoter through its interactions with the transcription factor cAMP-responsive element-binding protein (CREB) [117]. Interestingly, a genetic variation in *HULC* (rs7763881) is associated with a low-risk susceptibility to HCC in HBV-persistent carriers [118]. Another lncRNA found to be regulated by HBx is *Dreh*, which is downregulated in HBx transgenic mice and in human HBV-related HCC tissues [119]. *Dreh* acts as a tumor suppressor by changing the normal cytoskeleton structure to inhibit tumor metastasis [119].

5 Role of ncRNA in Immune Responses to Virus Infection

Innate immune sensing is a host's first line of defense against invading pathogens. Cellular membrane-bound Toll-like receptors (TLRs) and cytosolic pathogen recognition receptors (PRRs), such as RIG-I and MDA-5, recognize pathogen-associated molecular patterns (PAMPs) present in genomic viral RNA. These viral molecular motifs initiate signaling cascades that culminate in interferon regulatory factor (IRF)-mediated transcription of *IFNβ* gene expression. IFNβ is then secreted from the cell and in an autocrine and paracrine manner binds to type I IFN receptor (IFNAR) expressed on the plasma membrane to induce hundreds of interferon-stimulated genes (ISGs), many with well-known antiviral effector functions. A host must be able to withstand physiological and oxidative stresses triggered by viral infection, such as the effects of NFκB-regulated pro-inflammatory cytokines, including TNF, IL-1, IL-6, and IL-8. Interferon, while the hallmark of innate immunity, is no longer the only major player in initiating and modulating antiviral defenses. As we have discussed, ncRNAs have widespread functions in a variety of cellular processes and the relationship between ncRNA regulation, innate immunity, and inflammatory responses is becoming increasingly clear. Here, we will discuss the current knowledge of ncRNA induction and modulation of innate and adaptive immunity in the context of pathogen–host interactions (Fig. 2).

5.1 Activation of Innate Immune Responses by miRNAs

David Baltimore was among the first to study TLR-induced miRNA expression in response to a variety of microbial stimuli. Lipopolysaccharide (LPS), a component of the outer membrane of Gram-negative bacteria, is a strong stimulant of innate immune responses and a TLR4 agonist. When used to treat human monocytic THP-1 cells, LPS induces significant differences in miRNA expression [120]. Widespread and transient changes in miRNA expression have also been observed in the lung of mice exposed to LPS, further emphasizing ncRNA changes among transcriptional responses to microbial products. Among the miRNAs profiled in LPS-stimulated THP-1 cells, miR-146a/b, in particular, was significantly induced by pro-inflammatory mediators NFκB, TNF, and IL-1β [120]. Computational analyses identified TRAF6 and IRAK1, downstream kinases of TLR4, as targets for posttranscriptional repression by miR-146a/b based on near perfect complementarity to the miR-146a/b seed sequences (Fig. 2). This early evidence indicated that TLR could induce a potential new class of signaling regulators in response to pathogens and under pro-inflammatory conditions. Indeed, TRL4-responsive miR-146a was largely increased in human airway epithelial cells infected with human H3N2 influenza virus [121]. In a separate study, *Cryptosporidium parvum*, a protozoal species that causes cryptosporidiosis, was found to upregulate TLR4 in

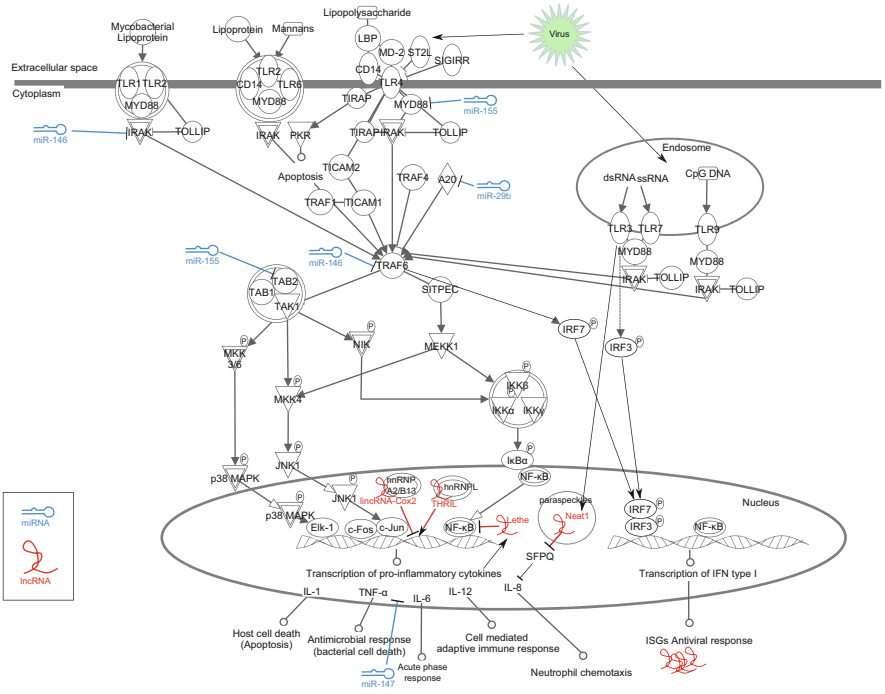

Fig. 2 miRNAs and lncRNAs modulate innate immune response to viral infection. The innate immune-sensing pathway diagram was generated through the use of IPA (Ingenuity® Systems, www.ingenuity.com). MiRNAs are shown in *blue* and lncRNAs are shown in *red*. The cellular targets regulated by these ncRNAs are indicated

infected human biliary epithelial cells, which was concomitant with changes in ncRNA expression, such as decreased *let-7i* expression [122]. While enhancing innate immune signaling, miRNA expression can have the opposite effect on pro-inflammatory mediator production. For example, increases in miRNA expression was accompanied by decreased TNF, KC, a mouse cytokine involved in neutrophil chemotaxis and cell activation, and macrophage inflammatory protein (MIP)-2 expression in vivo, suggesting dual miRNA function in the modulation of host inflammatory responses [123].

In addition to miR-146, changes in the expression levels of several other miRNAs, including miR-155, miR-132, and miR-125b, have been associated with the activation of the innate immune response [124]. Cellular miR-155 is considered a lymphoma-associated oncomir and was recently found to modulate pro-inflammatory activities of different immune cell types, including macrophages [125], monocytes, and dendritic cells (DCs) [126, 127]. In human plasmacytoid DCs, miR-155 expression is increased in an NFκB- and Jnk-dependent manner that resulted in negative regulation of IFNα production [128]. While miR-155 was shown to negatively regulate *IFNα* expression, a separate study argued that

miR-155 promotes type I IFN signaling in antiviral immunity by targeting SOCS1 [129]. Cellular mechanisms aimed at attenuating pro-inflammatory responses also target miR-155-mediated activity, including IL-10, which inhibits miR-155 and acts as a negative regulator of miR-155-mediated pro-inflammatory responses [126]. Taken together, miR-155 not only acts as a regulator of interferon responses but also serves as a target of anti-inflammatory factors that help balance miR-155-mediated enhancement of innate immunity.

Japanese encephalitis virus (JEV) is a mosquito-borne virus and a member of the *Flaviviridae* family that enhances neuroinflammation of the central nervous system. MiR-155 is found to regulate JEV-induced inflammatory responses by targeting Src homology 2-containing inositol phosphatase 1 (SHIP1) 3′ UTR [130]. For example, anti-miR-155 treatment of mice infected with JEV decreases the expression of pro-inflammatory cytokines *TNF*, *MCP-1*, and *IL-6* in the brain. This in turn improves survival and JEV-associated disease, alleviating behavioral symptoms related to body stiffening and hind limb paralysis [130]. Microglial cell activation is a hallmark of neuroinflammation. MiR-29b was found to be significantly upregulated in JEV-infected mouse microglia (BV-2) and primary microglia cells, regulating microglia activation by targeting TNFα-induced protein 3 (TNFAIP3, also known as A20), a negative regulator of NFκB (Fig. 2) [131]. In response to a separate flavivirus, HCV patients with liver fibrosis have downregulated miR-107 and miR-449a expression, with upregulated *CCL2* expression. Both miR-449a and miR-107 were found to regulate IL-6-mediated *CCL2* expression and STAT3 phosphorylation by targeting IL-6R and JAK1 in HCV-infected hepatocytes [132]. Taken together, these studies demonstrate a role for miRNA in regulating inflammation in neurologic and liver diseases caused by flaviviruses.

Inflammatory responses are critical for controlling viral infection. One such mechanism is the inflammasome, an intracellular signaling complex that increases IL-1β when activated. Recently, two miRNAs encoded by EBV with sequence homology to miR-223 were shown to target the miR-223 binding site in the NLRP3 3′ UTR and inhibit inflammasome activity [133]. This demonstrates the diverse strategies of virally encoded miRNAs in mimicking cellular miRNA function regulating inflammatory pathways. Endogenous inflammatory mediators called damage-associated molecular patterns (DAMPs) have been suggested to modulate immune responses and inflammation, though their role in the context of viral infection is poorly understood. DAMP molecule, S100A9 (also known as Calgranulin B or MRP-14), enhances inflammation during acute influenza infection by acting as a non-PAMP activator via the TLR4-MyD88 pathway [134]. Interestingly, a new miRNA class known as damage-associated molecular pattern molecule-induced miRNAs (DAMPmiRs) was recently identified in human peripheral blood mononuclear cells [135]. Future studies will likely unveil the role of DAMPmiRs in virus-induced inflammatory responses.

5.2 Regulation of Inflammatory and Innate Immune Responses by lncRNA

Many studies have shown that lncRNAs have central roles in the control of gene expression during cellullar differentiation in the development of diverse organs and tissue types (reviewed in [136]). In addition to their roles in hematopoiesis and leukemogenesis [137–139], many lncRNAs are involved in immune cell activation (reviewed in [140]). Moreover, some lncRNAs modulate inflammatory and innate immune gene expression following the activation of some TLRs.

Similar to miRNAs, lncRNAs are expressed in response to TLR agonists. In mouse dendritic cells, 20 lncRNAs showed marked upregulation after stimulation with LPS including lincRNA-*Cox2*, which was not upregulated in TLR3-stimulated cells [82]. LincRNA-*Cox2* was also upregulated in mouse macrophages stimulated with TLR2 ligand and following *Listeria monocytogenes* infection [141]. Silencing and overexpression of lincRNA-*Cox2* revealed that the lncRNA regulates distinct classes of immune genes both basally and after TLR stimulation [141]. Using a custom microarray, 159 lncRNAs were found to be differentially expressed following TLR2-stimulation of human macrophages, including *THRIL* (also known as linc1992), which was found to be downregulated [142]. *THRIL* and lincRNA-*Cox2* both interact with different heterogeneous nuclear ribonucleoproteins (hnRNPs) to bind immune gene promoters. LincRNA-*Cox2* forms a complex with hnRNP-A/B and hnRNP-A2/B13 to repress the transcription of some immune genes including chemokine *CCL5* gene [141], while *THRIL* binds hnRNPL to stimulate *TNFα* gene transcription (Fig. 2) [142].

Aside from these TLR2/TLR4-mediated lncRNA regulations, the lncRNA *NEAT1* is induced after TLR3 stimulation and in response to influenza virus, herpes simplex virus 1 (HSV-1) [104], and HIV [103]. Upon infection, viruses detected through TLR3 activate p38 MAPK pathway which leads to *NEAT1* induction (Fig. 2). This relocates the transcriptional repressor, SFPQ, from the promoter region of antiviral genes into paraspeckles, leading to the transcriptional activation of antiviral genes such as *IL-8* (Fig. 2) [104]. Finally, numerous lncRNAs can be induced in response to cytokine stimulation, such as IFNβ [85, 88] and TNFα [143]. Among the 166 mouse lncRNAs induced by TNFα, a pseudogene transcribed into lncRNA named *Lethe* is also expressed in response to IL-1β or glucocorticoid receptor agonist, but it is unresponsive to TLR1-7 agonists. *Lethe* is directly induced by NFκB signaling and acts as a negative feedback inhibitor of NFκB by binding to RelA.

5.3 Regulation of Adaptive Responses by miRNA During Viral Infection

The inflammatory response has been linked to aberrant allergen-specific CD4+ T-helper 2 (T_H2) cell function and the recruitment and activation of eosinophils and

mast cells in the airways. T_H2 cell-induced eosinophilia and airway hyperresponsiveness (AHR), as well as the secretion of a range of cytokines, including IL-4, IL-5, IL-9, IL-10, and IL-13, are hallmark features of allergic asthma, of which the TLR4 signaling pathway is central to the progression of allergic airway disease, as mice deficient in TLR4 signaling have been shown to have an attenuated allergic phenotype [144]. Single miRNAs can have profound effects on the development of pulmonary inflammation. For example, antagonism of miR-126 suppresses the effector function of T_H2 cells, including the recruitment of eosinophils and neutrophils into the airways and the overproduction of airway mucus [145].

MiR-155 is upregulated in primary effector and effector memory CD8+ T cells and it is required for lymphocyte responses and effector T cell memory against influenza virus and *Listeria monocytogenes*. Antiviral CD8+ T cell responses and viral clearance were impaired in miR-155 knockout (KO) mice that exhibited reduced primary and memory responses, whereas CD8+ T cell responses were much more robust when miR-155 was overexpressed in infected mice [146]. MiR-155 has also been investigated for its role in cytotoxic T cell function in response to lymphocytic choriomeningitis virus (LCMV). Mice lacking miR-155 are deficient in primary CD8+ T cell responses following LCMV infection due to impaired Akt signaling that impacts cell survival. After infection, there were reduced numbers of IFNγ-producing T cells found in miR-155 KO spleens compared to wild-type (WT) spleens, as well as differential CD8+ T cell responses in the spleen between WT and KO mice [147].

5.4 Regulation of Adaptive Responses by lncRNA During Viral Infection

Expression of ncRNAs during T cell development was observed almost 30 years ago, with the transcription of the T early alpha (*TEA*) noncoding transcript in the T-cell receptor alpha chain locus [148] required for V(D)J recombination assembling the variable regions of T cell receptors [149]. Several lncRNAs were also found expressed in activated CD4+ T cells, including *NTT* [150], and the proto-oncogene, *BIC* [151].

More recently, systematic characterization of lncRNAs identified over 1,000 lncRNAs dynamically expressed during $CD8^+$ T cell differentiation and activation after exposure to viral antigens [152]. Further exploring lncRNAs involved in T cell function, Hu et al. identified 1,524 regions encoding for lncRNA that were expressed at various developmental and differentiation stages of T cells [84]. The expression of most of them is controlled by T cell-specific transcription factors. Among these lncRNAs, LincR-*Ccr2-5′*AS was specifically expressed in T_H2 cells and its expression was positively correlated with genes involved in chemokine signaling. Silencing of lincR-*Ccr2-5′*AS by shRNA led to deregulation of many genes, including lower expression of the neighboring *Ccr1*, *Ccr2*, *Ccr3*, and *Ccr5* genes. This resulted in reduced migration of T_H2 cells to the lung. While the

mechanism of gene regulation by lincR-*Ccr2-5′*AS is still being elucidated, it is known to be distinct from the modulation of chromatin accessibility or recruitment of RNA polymerase II, a common mechanism for lncRNAs to regulate gene expression [153].

One of the most studied lncRNA in viral infection, *Tmevpg1* (also named *NeST*), was initially discovered for its role in Theiler's virus persistence [154, 155]. Theiler's murine encephalomyelitis virus (TMEV or Theiler's virus) is a picornavirus that causes acute infections in mice. In some mouse strains, such as SJL/J, TMEV causes persistent infection of the spinal cord followed by a late chronic demyelinating disease similar to multiple sclerosis in humans. TMEV infection is mainly controlled by the *H2D* class I gene; however, two other susceptibility loci were mapped on chromosome 10 close to the *IFNγ* locus and named *Tmevp2* and *Tmevp3* [156]. The lncRNA *Tmevpg1* was identified in the *Tmevp3* locus [154] and was expressed in unstimulated T cells and in CNS-infiltrating immune cells of resistant B10.S mice after TMEV infection [155]. While Vigneau et al. initially hypothesized that *Tmevpg1* may downregulate the expression of *IFNγ*, *Tmevpg1* was later found to positively regulate the transcription of *IFNγ* in cooperation with the transcription factor T-bet in CD4+ T_H1 T cells [157]. Recently, Gomez et al. showed that transgenic expression of SJL/J-derived *Tmevpg1* allele in B10. S mice prevents the clearance of TMEV, yet confers resistance to lethal infection with *Salmonella enterica* serotype Typhimurium [158]. Therefore, they renamed *Tmevpg1* as *NeST* for "Nettoie *Salmonella* not TMEV" (clear *Salmonella* not TMEV). The SJL/J-derived *NeST* allele also conferred increased resistance to the lethal inflammatory disease caused by LPS injection. In contrast to the previous report of Vigneau et al. [155], *NeST* RNA was undetectable in T cells from B10.S, but it was expressed in SJL/J mice and its RNA level positively correlated with *IFNγ* transcription. *NeST* was shown to interact with the subunit WDR5 of the H3K4 methyltransferase complex that catalyzes the trimethylation of histone H3 at lysine 4, a mark of active gene expression [158]. Increased *NeST* RNA abundance in CD8+ T cells was associated with increased H3K4 trimethylation at the *IFNγ* locus and increased IFNγ synthesis in splenic tissue. A proposed mechanism is that *NeST* recruits histone H3 lysine 4 methyltransferases to the *IFNγ* gene locus, enhancing IFNγ expression in key T cell subsets. However, the link between NeST-mediated IFN regulation and its opposite effects on TMEV and *Salmonella* control remains unclear. In particular, whether *NeST* may regulate the chromatin state of other genes with key roles in the immune response to pathogens was not tested. It would be interesting to systematically characterize *NeST* functions using systems biology approaches [159].

6 Conclusion

The noncoding transcriptome has been increasingly implicated in regulating human development and disease over the past decade, with several reports suggesting that ncRNAs have enormous clinical potential. MiRNAs are being developed as serum

biomarkers in cancer detection and diagnosis, and their usefulness toward a variety of clinical diseases is being explored. Cell-free miRNAs have been detected in the blood of patients with diffuse large B-cell lymphoma [160], Duchenne muscular dystrophy [161], pediatric Crohn disease [162], cardiovascular diseases (reviewed in [163]), and HBV-positive HCC cases [164]. For example, circulating miR-146a and miR-223 were significantly decreased in patients with sepsis compared to normal control patients. These candidate biomarkers could be used in conjunction with well-known biomarkers of acute systemic inflammation, such as C-reactive protein (CRP) [165]. MiR-122 is highly expressed in the liver, and serum miR-122 has been found to correlate with virologic responses to pegylated IFN therapy in chronic HCV [166], liver injury in patients with chronic HBV [167, 168], and HCC [169]. Therapeutic silencing of miR-122 in nonhuman primates with chronic HCV has been assessed and shown to suppress viremia [170]. In clinical settings, this would be a vast improvement to the current standard of care that combines pegylated IFNα with ribavirin, which is effective in only 50 % of patients and associated with serious side effects. Considering the therapeutic potential of miR-122, Regulus Therapeutics Inc. and GlaxoSmithKline are currently developing miRNA drugs linked to inflammatory diseases for commercialization. LncRNAs, such as *HBx-LINE1*, may also hold great promise as potential biomarkers for diagnosis and prognosis of viral infection [12]. Other host lncRNAs, such as *MALAT1*, could also be used as prognosis biomarkers for several cancers, independently of viral infection. Finally, long noncoding mitochondrial RNAs (ncmtRNAs) were recently reported as deregulated following human papillomavirus (HPV) infection [171]. The expression profile of these transcripts allows researchers to distinguish between normal, pre-tumoral, and cancer cells. One of these transcripts, SncmtRNA-1, has been characterized as a regulator of cell cycle progression, while two others, ASncmtRNA-1 and 2, have been suggested to act as three tumor suppressors. Expression of SncmtRNA-2 might contribute to the screening of early cervical intraepithelial lesions [172].

To conclude, novel sequencing technologies and computational methods have widely expanded the landscape of the mammalian transcriptome. NcRNAs have emerged as key players regulating various biological processes, including viral infection. Among ncRNAs, many miRNAs and lncRNAs have been identified as deregulated following viral infection. Only a few lncRNAs have been functionally characterized, but it is clear this class of ncRNAs is involved in a large variety of biological processes, similar to the ubiquitous importance of miRNAs. Virally encoded and host-derived miRNAs and lncRNAs have been linked to the virulence and pathogenesis of several different DNA and RNA viruses. They regulate viral replication and pathogenesis and modulate innate and adaptive immune responses to viral infection. Research on ncRNAs opens new avenues toward novel therapy and diagnostic tools, as well as development of novel paradigms about transcriptome regulation of biological systems. Most recent advances include the characterization of circular RNAs [173], RNAs acting as competing endogenous RNAs (ceRNAs) or natural microRNA sponges [174], and mRNA with noncoding functions such as *HBx-LINE* [12]. Now, the challenge is to understand their

functions and how these diverse RNAs interact together to form a large interconnected transcriptional network that can shape the outcome of viral infection.

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Wound Inflammation: Emerging Role of miRNA

Amitava Das, Scott Chaffee, Chandan K. Sen, and Sashwati Roy

Abstract Wound inflammation occurs in response to injury. It is a coordinated process involving tissues, cells, and an array of soluble, cell-associated, and intracellular factors. The role of certain microRNAs in wound healing and inflammation, in particular (a key process underpinning wound healing), has emerged in recent years. Here, mechanisms involving inflammatory cells, secreted molecules, and miRNAs that can affect wound healing are reviewed. Of interest miR-155, miR-146a, and miR-21, among others, have been shown to have a number of specialized roles in the inflammatory process, Toll-like receptor signaling, and efferocytosis. Other important miRNAs that regulate inflammation are also discussed.

1 What Is Inflammation?

Inflammation is a self-protecting immune response of the body against harmful stimuli in an attempt to eliminate the stimuli and initiate the healing cascade [1, 2]. The word "inflammation" is derived from the Latin word "*inflammo*," meaning "*I set alight, I ignite*" [3]. More than 2,000 years ago, a Roman encyclopedist Cornelius Celsus, in Book III of his only surviving work "De Medicina," was the first person to record the cardinal signs of inflammation: *Notae vero inflammationis sunt quattuor: rubor et tumour cum calore et dolore* (now the signs of an inflammation are four: redness and swelling with heat and pain) [4, 5]. Though Galen considered inflammation as a beneficial response to injury, Rudolf Virchow considered inflammation as a pathological condition and added *functio laesa* (loss of function) as the fifth cardinal sign of inflammation [1, 6]. The present perception about inflammation defines it as "a complex set of interactions among soluble factors and cells that can arise in any tissue

A. Das • S. Chaffee • C.K. Sen • S. Roy (✉)
Heart and Lung Research Institute, Ohio State University Medical Center, Columbus, OH, USA
e-mail: Sashwati.Roy@osumc.edu

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in response to traumatic, infectious, postischemic, toxic, or autoimmune injury" [7]. The cardinal signs of inflammation can be explicated by altered blood flow, higher cellular metabolism, vasodilatation, discharge of soluble mediators, leakage of fluids, and cellular influx [1].

Even though inflammation and infection are often correlated (the former often being a result of the latter), inflammation is not a synonym for infection and can even occur in the absence of infection as well. Inflammation is one of the several responses of the body towards a pathogen. Being a stereotyped response, inflammation is considered as more of a mechanism of innate immunity than adaptive immunity [8]. Clinically, inflammation can be classified into two main types, acute or chronic [9]. Acute inflammation is the initial response of the body to detrimental stimuli and is accomplished by the amplified movement of plasma and leukocytes from the blood into the injured tissues [3]. Clinical characteristics of acute inflammation include the basic cardinal signs at the site of the injury. A series of biochemical events is initiated which disseminates and develops the inflammatory response, involving the local vascular system, the immune system, and various cells within the injured tissue [3]. However, if the causative agent is not eliminated then the inflammation persists for a prolonged period of time and becomes chronic [10]. Chronic inflammation leads to a progressive shift in the cell population at the site of inflammation [3]. In spite of inflammation being a protective response of the body, in some disorders the inflammatory process, which under normal conditions is self-limiting, becomes continuous and chronic inflammatory diseases develop subsequently, which is specific for each pathogen [1].

2 Wound Healing

Wound healing is a physiological response of the body to injury that is conserved across tissue systems [11]. Wound healing is a well-synchronized event that occurs in response to injury and its microenvironment, which aims at restoring the normal physiological function of the injured tissue [12]. Chronic wounds fail to progress through the normal phases of healing, which leads to prolonged pathologic inflammation [13]. Four specific functional phases exist in the entire process of wound healing, viz: hemostasis, inflammation, proliferation, and remodeling and all of which are necessary to ensure an efficient healing of the wound (Fig. 1). The hemostasis phase is vital to the wound healing process and is the initiation stage of the wound healing process [12–15]. The process of hemostasis is aimed at preventing blood loss from the site of injury through the process of forming the fibrin plug which eventually lays the foundation for the next phases leading to competent wound healing [16]. The process of hemostasis not only helps in the formation of blood clot but also helps in the gathering of necessary growth factors and cytokines to regulate the inflammatory response. Once the necessary inflammatory mediators have assembled, the inflammatory phase begins. This phase is characterized by blood coagulation by infiltration of neutrophils and macrophages

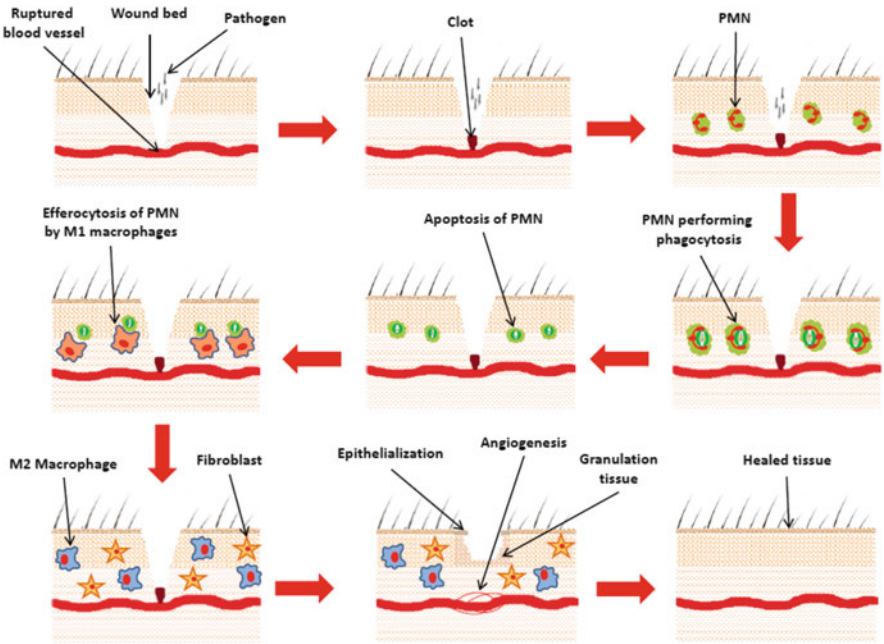

Fig. 1 Wound healing. Four specific functional phases exist in the wound healing cascade: hemostasis, inflammation, proliferation, and remodeling. Injury will result in a ruptured blood vessel, followed by hemostasis or clot formation at the site of injury. Pathogen invasion may occur at the time of injury. Following hemostasis, platelets release growth factors and cytokines at the wound site that initiate the inflammatory response. The first sets of inflammatory cells that arrive at the wound site are neutrophils (PMNs). The PMNs initiate pathogen phagocytosis following which they undergo apoptosis. Macrophage infiltration at the wound site occurs after the PMNs. Macrophages engulf apoptotic PMN at the wound site. This process is known as efferocytosis. Recent studies have shown that following efferocytosis macrophages switch from an M1 to M2 phenotype resulting in resolution of wound inflammation and initiation of the proliferative phase. The proliferative phase primarily involves neovascularization and fibroblast proliferation and granulation tissue formation. Collagen is laid down at the wound site by fibroblasts. Finally at the remodeling phase, collagen remodeling occurs to provide tensile strength to the injured site

at the wound site to release reactive oxygen species (ROS) using an oxygen-consuming respiratory burst [17]. This phase is characterized by phagocytosis, efferocytosis (clearance of dead cells by macrophages), and release of growth, angiogenic, and proliferative factors that pave the way for the proliferative phase [18]. The proliferative phase is marked by the formation of new blood vessels, the infiltration of blood vessels to supply nutrients to the site, and the production of pro-angiogenic cytokines, and within a few days, blood vessels form a network throughout the developing tissue [19]. The phase is regulated by the synthesis and breakdown of extracellular matrix (ECM) components and decides the scar outcome of the healed tissue [20].

3 The Inflammatory Response

Neutrophils Regarded as the first of a number of overlapping processes that constitute wound healing, the inflammatory phase is characterized by infiltration of leukocytes into the wound site, which are the principal cellular components of the inflammatory response [21]. These leukocytes serve not only as effector cells to combat invading pathogens but are also involved in tissue degradation and tissue formation [21]. The first subset of nucleated immune cells to infiltrate a wound is the neutrophils (PMNs) which act as a first line of defense by phagocytosing the invading pathogens, foreign material, and infectious agents, thereby decontaminating the wound [22]. To perform their task, the PMNs secrete a large variety of highly active antimicrobial substances (ROS, cationic peptides, and eicosanoids) and proteases [23]. Once their job is done, the PMNs undergo apoptotic cell death [24].

Monocytes/Macrophages Forty-eight hours after the initial injury, the next lineage of cells, monocytes are recruited via the numerous chemoattractants at the site of injury [25]. Monocytes are highly plastic cells that differentiate into macrophages based on signals at the specific wound microenvironment [26]. Binding to the ECM via integrin receptors, monocytes differentiate into tissue macrophages [27]. At an injury site, efficient clearance of apoptotic cells is a prerequisite for the timely resolution of inflammation [28]. Clearance is attained through the process of efferocytosis, a term coined by deCathelineau and Henson [29], and Gardai et al. [30], referring to phagocytosis of apoptotic cells [31]. At an injury site, efferocytosis is the final destiny of apoptotic PMNs [28]. Successful efferocytosis is reported to drive timely resolution of inflammation [32], while malfunction in the clearance of apoptotic cells has been connected to autoimmunity and persistent inflammatory diseases [33]. Macrophages are dynamic and heterogeneous cells assigned to two groups: pro-inflammatory classically activated or type I macrophages (M1) and anti-inflammatory alternatively activated or type II macrophages (M2) [34]. However it still remains unclear whether the type II macrophages that appear during the healing phase originate from newly attracted monocytes or they are derived as a result of the M1 macrophages switching their phenotype [24]. Studies have shown that a macrophage population which was involved in inflammation may change to a more reparative phenotype [35]. Diabetic wound macrophages were reported to exhibit dysfunctional inflammatory responses which are attributed to the impaired efferocytosis ability by these macrophages [32]. During the reparative process, macrophages are also thought to play a crucial role in fibrosis and scarring [36]. Macrophages are responsible for the transition from the inflammatory to the proliferative phase of healing. By releasing a wide variety of growth factors and cytokines, macrophages recruit fibroblasts which get activated eventually by organizing the new tissue matrix, while others promote angiogenesis [27].

Mast Cells Mast cells are yet another leukocyte subset present in the skin which contribute to a variety of pro-inflammatory mediators and cytokines that can promote inflammation and vascular changes [21].

4 miRNA and Inflammation

Since the discovery of miRNA in 1993, the various roles and effects that they have on gene expression and eventually on development of disease have become an important area of research [37]. Studies have shown that miRNAs play a pivotal role in the innate and humoral immune system response. Due to the importance of miRNA in the activity of the immune systems, overexpressed miRNA levels can lead to the progression of serious physiological disorders [38, 39]. miRNAs are able to regulate gene expression by complementarily binding to the 3′ UTR of targeted mRNA [40], and regulate gene expression in a posttranscriptional manner [41]. miRNAs have the ability to alter the magnitude by which a gene is being expressed as a result of the environment and chemokine signals it is exposed to. Reports have shown that the presence of a long-lasting NADPH oxidase mechanism is the vehicle that miRNAs use to manage the cellular redox environment [11]. The emerging science suggests that miRNAs have a critical role in the regulation of inflammation, in particular the targeting of specific genes and regulatory loops [11]. Dysregulation of pro-inflammatory and anti-inflammatory miRNAs could be the driving force behind an improper wound healing process. Vascular endothelial growth factor (VEGF) and transforming growth factor beta (TGF-β) are two very important growth factors that are critical to initiating an inflammatory response, leading to the repair of the injury [42]. Studies have shown that VEGF and TGF-β seem to be regulated by various miRNAs [14]. Lack of regulation involving inflammatory-related miRNAs can result in devastating physiological effects and human diseases [14].

The large presence of TNF-α and IL-1 in early stages of inflammation can be posttranscriptionally limited by certain miRNAs. Some of the major components of the inflammatory response are toll-like receptors (TLRs) and monocyte chemoattractant protein-1 (MCP-1), and the ability of these components to contribute to the inflammation is mediated by miRNA [43]. For example, MCP-1 and TLRs are essential to generating the inflammatory response, and consequently they are all regulated by specific miRNAs [43]. Reports show that miRNAs are also responsible for regulating the presence and activity of macrophages as they respond to stimuli and signals [44]. Examples of inflammatory-related miRNAs that will be discussed in detail below are miR-155, miR-146, and miR-21.

5 Toll-Like Receptors

Toll-like receptors are important detection and signaling receptors that enable the initial stages of the inflammatory response to begin. TLRs are capable of detecting pathogen particles and damaged tissue in the body [45]. Once these signals are detected, the TLRs trigger the activation of pro-inflammatory pathways, many culminating in the activation of NFκB pathways [45] and many via a signal intermediate called MyD88 [46]. This activation process is what allows TLRs to connect rapid onset innate immunity with adaptive immunity seen later in the wound healing process.

6 MCP-1

MCP-1 is a chemokine that has the ability to induce an inflammatory response. The role of MCP-1 is known because like TLRs, it can control a cascade that activates NFκB-regulated pro-inflammatory pathways [47]. Recent research has shown that MCP-1 induces the production of cytokines and adhesion molecules [48]. Studies show that MCP-1 has the ability to attract inflammatory cells such as monocytes, T cells, and mast cells [49]. Therefore, based on the ability of MCP-1 to recruit these cells, it suggests that the molecule has proinflammatory properties. MCP-1 acts as a pro-inflammatory mediator after activation via LPS [50]. The expression of pro-inflammatory and anti-inflammatory regulators is in part due to the levels of LPS that are present [51]. In certain situations, MCP-1 may exhibit anti-inflammatory behavior by negatively regulating the production of pro-inflammatory cytokines [50].

7 miR-155

miR-155 is a miRNA that has multiple functions ranging from immune responses, pro-inflammatory actions, and even cancer [52]. Many recent studies have shown a strong correlation between chronic, uncontrolled inflammation and the development of cancer [53]. It has been shown that miR-155 operates via the JNK pathway, and that this miRNA is inducible from various sources depending on the type of cell [54]. For example, miR-155 can be induced by TNF-α in wound macrophages and blood monocytes, and LPS also induces miR-155 in monocytes [55]. One of miR-155's most intriguing roles is the one that it plays in regard to inflammation and wound healing. High levels of miR-155 are found in situations involving the innate immune response [56]. An important consequence of high levels of miR-155 is that its overexpression can lead to an increased level of gene mutations which allow the development and progression of tumors [57]. Animal studies involving miR-155 null mice showed impairment of adaptive immune responses as well as influencing the levels of cytokine production [43]. Additionally, miR-155 led to strong responses from the wound macrophages in regard to pro-inflammatory cytokine expression [58]. Therefore, a potential promising therapeutic strategy to avoid chronic inflammation, atherogenesis, and dysregulated wound healing would be to regulate the level and duration of pro-inflammatory miR-155 [59, 60].

8 miR-146a

The expression of miR-146a is regulated by standard inflammatory mediators such as TNF-α and IL-1β [61]. Studies have shown that dysregulated levels of miR-146a over time may lead to chronic inflammation due to a constant pro-inflammatory environment [62]. Toll-like receptors are also capable of altering levels of

miR-146a, specifically TLR2, TLR4, and TLR5 [43]. Research regarding the effects of miRNAs on hemostasis and normal inflammatory responses have shown that increased levels of miR-146a lead to chronic inflammation [63]. The IL-1 pathway is known to upregulate the levels of miR-146a and the production of pro-inflammatory cytokines. miR-146a is responsible for managing the levels of pro-inflammatory cytokines via a negative feedback mechanism, in which it targets two specific proteins TNF receptor-associated factor 6 (TRAF6) and IL-1 receptor associated kinase 1 (IRAK1) [64, 65]. The important role miR-146a plays in the negative feedback loops explains why consistently overexpressed levels of miR-146a would be damaging to the resolution of inflammation (Fig. 2).

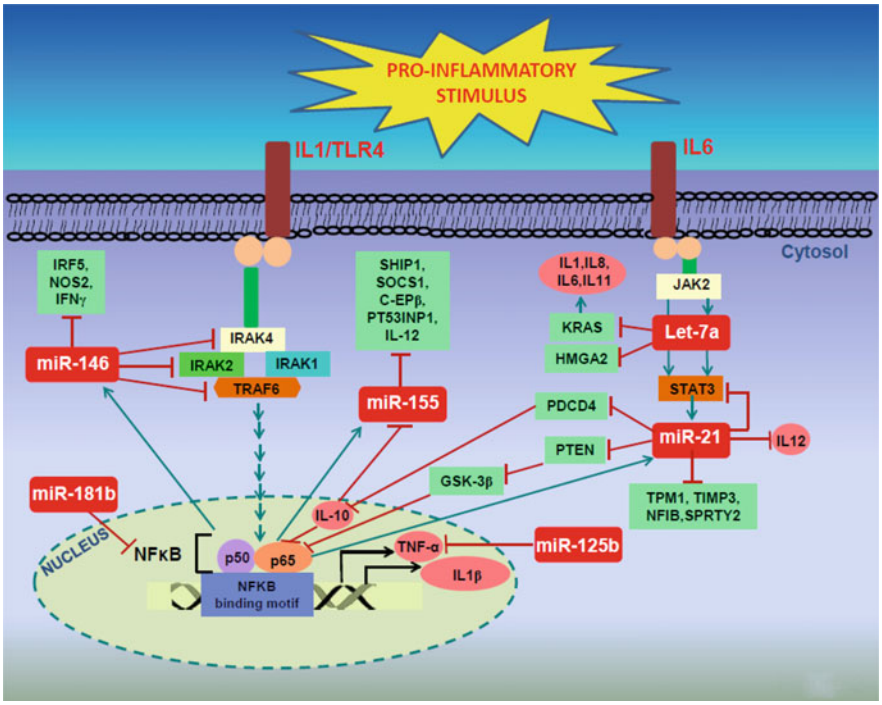

Fig. 2 Regulation of IL-1/IL-6/TLR4 pathways by major miRNA induced as a response to inflammation. Known key targets of miR-146, miR-181b, miR-125b, miR-21, and miR-155 in the TLR4 and IL-6-NFκB pathways are shown. The activation of IL-1/TLR4 triggers the NFκB pathway, a hallmark transduction pathway for innate immune responses leading to production of inflammatory cytokines such as TNFα. miRNAs act on multiple targets in this cascade to fine-tune this response. *TLR* Toll-like receptor

9 miR-21

miR-21 has been shown to have an anti-inflammatory effect on macrophages present at the site of injury, and it appears to play a critical role in the resolution of inflammation [66]. Initial research showed that miR-21 had a repressing effect on IL-12 [67]. Potential anti-inflammatory therapeutic strategies targeting miR-21 would involve the JAK and STAT pathways, which are involved in triggering a response to foreign stimuli [32]. miR-21 levels have been found to be regulated by the TLR/MyD88 pathway leading to NFκB [68]. Dysregulation of miR-21 levels can lead to unresolved inflammation and may have a role in cancer, given that miR-21 regulates tumor suppressor PTEN [69, 70]. Elevated levels of miR-21 provide an environment suitable for the development of chronic inflammatory diseases, by allowing uncontrolled cell growth without programmed cell death [28]. Recent evidence has pointed out the role of miR-21 in the efferocytosis-induced switching of macrophage phenotype [20].

10 Other miRNAs with a Role in Inflammation

miR-378a miR-378a is another miRNA that recent studies have shown plays an important role in the resolution of wound healing and cutaneous wound closure. A recent study showed that miR-378a targets vimentin and β3 integrin, which are integral to the closure of the wound via fibroblasts joining the ECM and angiogenesis, respectively [71]. As we have previously discussed, the dysregulation of miRNAs can lead to severe consequences. In particular, the overexpression of miR-378a can lead to a constant pro-inflammatory and pro-angiogenic environment, which could lead to the development of chronic inflammatory diseases [72].

miR-181b Reports regarding miR-181b show that it has the potential as a therapeutic to treat chronic inflammatory diseases due to its ability to suppress downstream inflammatory response pathways [73, 74]. NFκB is rapidly activated in response to a tissue injury or harmful stimuli [75, 76].

miR-125 Being a highly conserved miRNA throughout diverse species, miR-125 consists of three homologs: hsa-miR-125a, hsa-miR-125b-1, and hsa-miR-125-2 [77]. miR-125 is known to play a critical role in host defense, especially in response to bacterial or viral infections [77]. Following LPS challenge the expression of miR-125b was repressed within 1 h in Raw-264 cells, an event which is linked to increased production of TNF-α [78] as miR-125b is reported to bind to the 3′-UTR of TNF-α mRNA [78]. Both miR-125a and miR-125b, by targeting the tumor necrosis factor alpha-induced protein 3 (TNFAIP3, A20), are reported to constitutively activate the NFκB pathway [79]. Also overexpression of miR-125b in macrophages repressed IFN regulatory factor 4 (IRF4) and elevated responsiveness

to IFN-γ, augmenting the functional role of macrophages in inducing immune responses [80].

miR-223 In silico and experimental approaches have identified miR-223 in the myeloid compartment of the hematopoietic system [81]. miR-223 targets Mef2c, a transcription factor that promotes myeloid progenitor proliferation [82]. Using a knockout mouse model, miR-223 is reported not only to negatively regulate progenitor proliferation and granulocyte differentiation and activation but also these mice exhibited an expanded granulocytic compartment resulting from a cell-autonomous increase in granulocyte progenitors [82]. Granulocytes devoid of miR-223 were found to be hypermature and hypersensitive to activating stimuli and displayed increased fungicidal activity indicating a central role for miR-223 in granulocyte production and the inflammatory response [82].

The miR-17–92 Cluster Residing on chromosome 13 in the human genome, the miR-17–92 cluster encodes six miRNAs (miR-17, miR-18a, miR-19a, miR-19b-1, miR-20a, and miR-92-1), some of which are known to regulate proliferation and autoimmunity [83]. Mice with targeted deletion of this cluster die within minutes of birth, revealing a selective defect in adult B-cell development with a block in the transition from pre-B to pro-B cell and high apoptosis in the pro-B-cell fraction [83], while mice with ectopic expression of the miR-17–92 cluster in the lymphocyte compartment developed high lympho-proliferative disease and autoimmunity, which resulted in premature death [84].

11 Conclusions

Seminal studies published in the past decade have highlighted the central role of miRNAs in the arena of inflammatory responses. These small RNA species add a new layer of complexity in the multi-tiered regulation of inflammation. Although still in its infancy, therapeutic modulation of miRNAs is a promising new approach for treating human inflammatory disorders. The unprecedented rapidity at which miRNAs have moved from discovery to therapy speaks to the extraordinary potential of miRNA technology. Miravirsen is the first microRNA-targeted drug to receive approval from the FDA for hepatitis C, miR-34 against cancer (MiR Therapeutics), and miR-155 against inflammatory diseases (Regulus Therapeutics) [85, 86]. Although there are many challenges for miRNA therapeutics such as targeted delivery, potential off-target effects, and safety, the strategy of miRNA manipulation in regulating disease outcomes is proving to be promising [85–87].

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The Importance of MicroRNAs in Rheumatoid Arthritis

Victoria Saferding, Eliana Goncalves-Alves, and Stephan Blüml

Abstract Rheumatoid arthritis (RA) is a chronic inflammatory disease that is triggered by yet unknown mechanisms, leading to chronic inflammation of the joints. Multiple cell types contribute to the pathogenesis of the disease, especially hematopoietic cells such as macrophages, T and B cells, as well as synovial fibroblasts [1]. Ultimately, synovial inflammation leads to damage to the affected joint, resulting in the fact that RA is the most important cause of disability worldwide. This has spurred tremendous efforts in arthritis research, leading to the development of novel therapies to treat the disease. Despite the advances in the field, there are still a great number of patients not responding to current therapeutic regimens, and, most importantly, the cause of the disease has not been elucidated yet. In this review, we will discuss the possible role of microRNAs in the pathogenesis of RA.

1 Background

MicroRNAs are small noncoding RNAs and as epigenetic regulators of gene expression they are implicated in the control of many vital biological functions, including proliferation, differentiation, the regulation of (the immune system, and many others [2–5]). Their existence has been discovered relatively recently, and the name was established in the early 2000s. During their biogenesis, microRNA genes are transcribed into primary microRNAs, and these pri-microRNAs are next endonucleolytically cleaved by the nuclear microprocessor complex formed by the RNase III enzyme Drosha (RNASEN) and the DiGeorge critical region 8 (DGCR8) protein and then Dicer to generate microRNA duplexes consisting of a mature strand and a passenger microRNA strand (also called miRNA star [miRNA*] strand). To form the active RNA-induced silencing complex (RISC), the double-stranded duplex is separated into the functional guide strand, which is complementary to the target, and the passenger strand, which in the majority of

V. Saferding (✉) • E. Goncalves-Alves • S. Blüml
Division of Rheumatology, Medical University of Vienna, Vienna, Austria
e-mail: victoria.saferding@meduniwien.ac.at

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cases is subsequently degraded. In recent years, it has been suggested that miRNAs contribute significantly to the development of inflammatory arthritis and especially RA. This chapter summarizes the current data available, although the number of papers in the field is expanding rapidly.

2 MicroRNA-155 in Arthritis

Genetic as well as environmental factors are known to contribute to RA pathogenesis; therefore, expression levels of microRNAs as well as single nucleotide polymorphisms (SNPs) were frequently examined in RA. Increased expression of miR-155 was reported in RA synovial tissue, especially in macrophages, T cells, and fibroblasts in comparison with osteoarthritis (OA) [6]. MiR-155 was also demonstrated to be upregulated in human monocytes/macrophages, with higher expression levels in synovial macrophages than in circulating monocytes [7]. It was demonstrated to regulate the production of proinflammatory cytokines in these cells, possibly via regulation of the Phosphatidylinositol-3,4,5-trisphosphate 5-phosphatase 1 (SHIP1) and/or suppressor of cytokine signaling 1 (SOCS1), both of which are known targets of miR-155 [7, 8]. Its expression in peripheral blood mononuclear cells (PBMCs) correlates with tumor necrosis factor (TNF) and interleukin-1 beta (IL-1β) levels in blood, as well as disease activity as measured by the RA disease activity score DAS28.

In animal models of rheumatoid arthritis, this microRNA was found to be essential for the development of collagen-induced arthritis (CIA) [7, 9]. In this model, miR-155 was shown to regulate the generation of autoantibodies, as anti-collagen antibodies, which are a prerequisite for disease development in this model, were greatly reduced in mice lacking miR-155. These findings were in line with earlier reports showing an important role of miR-155 in class switch recombination and affinity maturation [10, 11]. In addition, autoreactive T cells, particularly cells that produce IL-17, were greatly diminished in mice lacking miR-155, suggesting an important involvement of this microRNA in the generation of pathogenic Th-17 cells. Earlier studies have already shown an important contribution of miR-155 in the polarization of T cells into Th17 cells in another disease model [12]. The mechanistic basis of this effect was recently reported when Escobar and colleagues showed that miR-155 targets Jarid2, a DNA-binding protein that orchestrates recruitment of the polycomb repressive complex 2 (PRC2) to chromatin, thereby repressing the transcription of a set of genes associated with Th17 development. Therefore, miR-155 deficient T cells overexpress this inhibitor, thereby showing reduced capacity to differentiate into Th17 cells [13]. The effect on antibody-induced arthritis was less striking, although miR-155 contributes to local bone destruction by partly regulating the generation of bone resorbing osteoclasts. This was due to miR-155 deficiency in bone marrow cells, which were found to show diminished potential for macrophage colony stimulating factor (MCSF) and

receptor activator of nuclear factor kappa-B ligand (RANKL)-mediated differentiation [9]. Furthermore, miR-155 also seems to be important in the biology of regulatory T cells (Tregs), since miR-155 deficient mice show reduced numbers of Tregs. Their function, however, was not found to be regulated by miR-155 [14]. In contrast to the important pro-inflammatory functions that have been attributed to miR-155 in macrophages, T and B cells, functional analysis showed that this microRNA downregulated matrix metalloproteinases (MMPs) in RA-fibroblast like synoviocytes (FLS), pointing towards a rather anti-inflammatory effect in these cells [6].

In rheumatoid arthritis patients, Zhou et al. showed that miR-155, as well as miR-146a, expression levels in regulatory T cells were reduced after in vitro stimulation compared to healthy controls [15]. In addition to this role in RA, miR-155 was also found to be involved in gouty arthritis by inhibiting SH2 (Src homology 2)-containing inositol phosphatase-1 (SHIP-1) and promoting the production of proinflammatory cytokines TNF and IL-1β [16].

3 MicroRNA-146a in Arthritis

Another microRNA that is highly expressed in RA in comparison to OA is the mature miR-146a and primary miR146a/b. Pri-miR-146a is expressed in cells of the superficial and sublining layer of synovial tissue from RA patients. Moreover, stimulation of RA-FLS with TNFα and IL-1β leads to increased miR-146a and pri-miR-146a expression [6, 17]. In addition, miR-146a was also found to be increased in PBMCs of patients with RA [18–20], particularly in CD4+ T cells. Furthermore, the authors demonstrate an involvement of miR-146a in the regulation of T cell apoptosis, as overexpression of this microRNA inhibited FAS-induced apoptosis. They further imply FAS-associated factor 1 (FAF1) as the mediator of this effect, as overexpression of miR-146a decreased and sponging miR-146a increased the expression of this protein [21]. In another study, miR-146a was found to be expressed in IL-17 producing cells and miR-146a was associated with IL17 expression in the synovial membrane of RA patients [20].

MicroRNA-146a/b (miR-146a/b) was also shown to be induced in response to inflammatory stimuli such as lipopolysaccharide (LPS) in myeloid cells. Targets of this microRNA family are TNF receptor-associated factor 6 (TRAF6) and IL-1 receptor-associated kinase 1 (IRAK1), two signal transducers of the NF-κB pathway, which might contribute to the role of miR-146 in initiation and maintenance of chronic inflammation [22]. Further evidence for an involvement of this microRNA in arthritis pathogenesis came from animal models. In CIA, administration of miR-146a to arthritic mice prevented bone and cartilage destruction. In addition, miR-146a injected mice showed less osteoclasts (tartrate-resistant acid phosphatase (TRAP) positive cells) compared to their controls, but infiltration of inflammatory cells was not completely inhibited by miR-146a administration. Moreover, immunofluorescence analysis of TNF, IL-1β, and IL-6 showed reduction of these

proinflammatory cytokines in miR-146a treated arthritic mice. In addition, the authors demonstrated that miR-146a regulates osteoclastogenesis. Osteoclasts are the most important if not the only cells that are able to resorb bone. They are formed as multinucleated giant cells by the fusion of hematopoietic cells of the monocyte and macrophage lineage at or near the bone surface. Overexpression of miR-146a in human PBMCs suppressed osteoclastogenesis in vitro. The expression of c-Jun, NF-ATc1, PU.1, and TRAP in PBMCs was significantly downregulated upon transfection with miR-146a, although it is unclear whether these genes are direct targets of miR-146a or this reduced expression was secondary to the reduced generation of osteoclasts. When the authors analyzed TRAF6, a known target of miR-146a, they also found reduced expression levels suggesting that this molecule might at least partly be responsible for the observed inhibitory effect of miR-146a on osteoclastogenesis [23].

Expression levels of miR-146a in Tregs of patients with rheumatoid arthritis correlated inversely with disease activity parameters such as the global disease activity score DAS28, as well as tender and swollen joint count [15]. Of note, there was no correlation with acute phase parameters C-reactive protein (CRP) or erythrocyte sedimentation rate (ESR). MiR-146a was also shown to be overexpressed in human and experimental OA and also to increase vascular endothelial growth factor (VEGF) in rat chondrocytes in response to IL-1β. MiR-146a increases expression of VEGF via downregulation of SMAD4, an inhibitor of VEGF induction [24, 25]. In an attempt to link single nucleotide polymorphisms (SNPs) in miR-146a to RA, the hsa-miR-146a rs2910164 variant was analyzed in a cohort of 104 Iranian patients compared to 110 healthy controls. However, no association was found between this particular SNP and RA susceptibility [26].

Another miR, miR-182, has been shown to be important in T cell activation and clonal expansion by targeting the cell cycle inhibitor forkhead box protein O1 (Foxo1). Treatment with antagomirs to miR-182 led to a significant amelioration of ovalbumin (OVA)-induced arthritis, which was associated with reduced numbers of OVA-specific CD4+ T cells in the draining lymph nodes after challenge with OVA [27].

A novel and potentially important characteristic of microRNAs is that they can be secreted by cells and subsequently measured in the supernatant of cell cultures or, more importantly, in body fluids such as plasma of patients [28]. MicroRNAs have been demonstrated to be released from cells in exosomes, enabling the exchange of nucleic acids between cells [29]. Although the biological significance of this phenomenon is not entirely clear at the moment, it allows easy measurement of microRNAs in a number of diseases, potentially identifying diagnostic biomarkers or predictors of response to therapy for a given disease. Using a microRNA array, Murata et al. identified miR-24 and miR-125a-5p as potential diagnostic biomarkers, which were confirmed by qPCR in a larger cohort [30].

4 MicroRNAs in Osteoclast Generation

MicroRNAs, in general, have been shown to be important in controlling osteoclast generation also in steady sate, as mice deficient of dicer in osteoclasts show increased bone volume, trabecular thickness, and reduced number of osteoclasts. Dicer deficiency in osteoclasts suppressed RANKL-induced development of TRAP-positive multinucleated cells in culture, showing that dicer intrinsically controls the development of osteoclasts. Analysis of mRNA of osteoclast-specific dicer-deficient mice revealed reduced levels of TRAP and Nfatc1, both important for the development of osteoclasts, while analysis of osteoclast precursor numbers in bone marrow did not show a difference between wild-type and osteoclast-specific dicer-deficient mice, suggesting an important function of microRNAs in RANKL-mediated differentiation of OCs [31].

In addition to miR-155 and miR-146a, miR-31 has also been shown to be involved in osteoclastogenesis. It was demonstrated to be highly upregulated by RANKL stimulation and functionally involved in osteoclast generation, as transfection of bone marrow cells with a miR-31 antagomir inhibited osteoclast generation. Mechanistically, miR-31 inhibition was associated with increased activity of the GTPase RhoA, leading to impaired actin ring formation. Inhibition of RhoA could partially restore osteoclastogenesis after inhibition of miR-31 [32].

Also miR-21 has been shown to be important in the regulation of RANKL-induced osteoclastogenesis. MiR-21 is a RANKL-induced microRNA, and seems to be important in RANKL-induced production of NFATc1 as inhibition of miR-21 decreases NFATc1 and consequently osteoclastogenesis. The function of miR-21 in osteoclast generation seems to be via downregulation of the protein programmed cell death 4 (PDCD4), which the authors show to be an important negative regulator of osteoclastogenesis [33]. Conversely, miR-124 was shown to be downregulated in bone marrow cells upon RANKL stimulation. Transfection of miR-124 inhibited osteoclastogenesis, and this inhibition was accompanied by a reduced induction of NFATc1 by RANKL. NFATc1 could be a direct target of miR-124 since an in silico analysis suggests two target sites of this microRNA in the 3′ UTR, although biochemical proof is missing yet [34].

In an attempt to identify microRNAs that significantly contribute to arthritis, Pandis and coworkers performed a microRNA profiling of synovial fluid (SF) from the human TNF transgenic mouse model, a well-established mouse model of human RA [35], where overexpression of human TNF leads to spontaneous arthritis. The obtained microRNA profiles were intersected with microRNA profiles of biopsies of RA patients. The authors found dysregulation of miR-221/222 and miR-323-3p, which are also dysregulated in RA FLS compared to OA FLS. Bioinformatic analysis suggested Wnt/cadherin signaling as a putative pathway target. Wnt activity was significantly increased in HEK293T cells overexpressing miR-323-3p but not with miR-221/222. β-transducin repeat containing (BTRC) and glycogen synthase kinase 3 beta (GSK3B), known mediators of degradation of β-catenin, show decreased expression in RA compared to OA SF. Overexpression of

miR-323-3p in RA SF leads to decreased expression of BTRC but not GSK3B. Therefore, miR-323-3p overexpression leads to enhanced activation of Wnt/cadherin pathway, partly by targeting BTRC in RA SF [36].

Fulci and coworkers analyzed microRNA 223 in CD3+ T cells from patients with RA and showed marked overexpression of this microRNA in naïve T cells of RA patients. However, they noted no association with disease activity or treatment [37]. MiR-223 was also shown to be expressed in synovial tissue of patients with RA, and expression levels were significantly higher in RA than OA. Measuring miR-223 levels in the joints of animals where CIA was induced, the authors show that also in this mouse model of RA, increasing expression of this microRNA during arthritis development could be observed, and the expression of nuclear factor 1 A (NF-1A), a known target of miRNA-223, decreased during CIA. Silencing of miR-223 in mice with CIA was found to ameliorate CIA symptoms, both clinically and histologically. In this study it was furthermore demonstrated that silencing of miRNA-223 leads to a decrease of osteoclastogenesis and bone erosion in CIA and it is correlated with increased expression of NF-1A [38]. They also show that miR-223 is upregulated during osteoclastogenesis in vitro in response to RANKL stimulation, and that upregulation of miR-223 is accompanied by downregulation of NF-1A. Suppression of miR-223 by a lentiviral construct decreased osteoclastogenesis and increased NF-1A, confirming previous in vitro reports of an important role of miR-223 on osteoclast generation [39]. Of note, another study examining osteoclast generation in human cells reached a different conclusion, as the authors demonstrate inhibition of osteoclast formation after overexpression of miR-223 [40].

5 MicroRNAs and FLS

MiR-346 has been found to be overexpressed in RA FLS treated with LPS and has been linked with the posttranscriptional regulation of IL-18 in these cells, as inhibition of miR-346 increased IL-18 release by LPS-activated FLS. Moreover, transient transfection of miR-346 in THP-1 cells inhibited IL-18 secretion by these cells in response to LPS. Luciferase assay revealed that miR-346 does not directly target IL-18 mRNA, but indirectly inhibits Bruton's tyrosine kinase (Btk) expression at the transcriptional level in LPS-activated RA FLS and inhibition of Btk expression negatively regulates IL-18 release [41]. Inhibition of miR-346 was also shown to be involved in stabilizing mature TNFα mRNA in LPS-activated FLS, resulting in the release of measurable amounts of this cytokine. In parallel, overexpression of miR-346 in LPS-activated THP-1 cells increased the expression of tristetraprolin (TTP) which resulted in the inhibition of TNF-α synthesis. Mechanistically, miR-346 seems to affect TNFα mRNA stability by indirectly targeting TTP expression. Therefore, miR-346 plays an anti-inflammatory role, controlling TNF-α synthesis by regulating TTP expression [42].

In some instances, not only mature miRNAs but also microRNA star strands can have biological functions, as it was shown in RA-FLS for miR-34*. Expression of miR-34* was found to be reduced in RA-FLS compared to OA-FLS and was furthermore demonstrated to target X-linked inhibitor of apoptosis protein (XIAP). The authors conclude that downregulation of this pro-apoptotic microRNA contributes to the known resistance of RA-FLS to apoptosis [43]. RA-FLS have also been described as a target cell type for the actions of miR-203. Its expression has been reported to be higher in RA-FLS than OA-FLS. Its abundance is not altered by various classical proinflammatory cytokines (IL-1, TNF, IL-6). However, treatment with a DNA demethylating agent induced its expression, suggesting that epigenetic mechanisms regulate this microRNA. Furthermore, miR-203 seems to indirectly control MMP1 and IL-6 production through yet unknown mechanisms [44]. MiR-203 has also been measured in serum of patients with RA, especially at an early stage of the disease, and was found to correlate with disease activity (DAS28) as well as markers of systemic inflammation. In addition, patients with early RA in which a treatment with a disease modifying anti-rheumatic drug has been started, levels of miR-203 in serum went down [45].

MiR-20a has been shown to regulate the inflammatory response in RA-FLS by controlling the expression of apoptosis signal-regulating kinase (ASK) 1, a member of the mitogen activated protein 3 kinase (MAP3K) family of protein kinases and involved in the transduction of various proinflammatory signals. Transfection with an miR-20a mimic reduced LPS and bacterial lipoprotein 2 (BLP2) induced proinflammatory cytokine expression in FLS and also LPS induced TNF and IL-1β production in human THP1 cells, further demonstrating an anti-inflammatory role of this microRNA [46].

6 MicroRNAs and TLRs

Other microRNAs have been shown to modulate the inflammatory response by regulating the expression of proinflammatory receptors, as dicer deficiency resulted in higher expression of toll-like receptor 2 (TLR2) after stimulation. This effect seems to be partly mediated by miR-19a and miR-19b, which were shown to directly target TLR2 in FLS [47]. Importantly, IL-6 and MMP3 induction after stimulation with TLR2 but not TLR4 ligands was significantly impaired after transfection with miR-19 mimics, suggesting a selective role of this microRNA in TLR2-dependent inflammatory responses [47].

Also TLR3 has been shown to be regulated by microRNAs, namely miR-26a, as demonstrated by upregulation of TLR3 upon miR-26a inhibition and downregulation after overexpression in rat macrophages. Decrease of miR-26a expression and concomitant upregulation of TLR3 expression was then shown in both pristane-primed rat macrophages and during pristane-induced arthritis (PIA) in bulk extracts of rat spleens. Moreover, decreased miR-26a and increased TLR3 expression were rescued in methotrexate (MTX) treated animals in rat spleens.

Ultimately, the authors show that treatment of rats with PIA with a miR-26a mimic led to depression of TLR3 expression and amelioration of PIA disease severity in the rats. Therefore, miR-26a negatively regulates TLR3 signaling via targeting of TLR3 itself in rat macrophages.

7 Conclusions

Collectively, there are a number of data showing an important contribution of certain microRNAs to the pathogenesis of rheumatoid arthritis and other inflammatory arthritides. MicroRNAs have been shown to be deregulated in human disease and in some cases might even be useful as biomarkers for disease activity or response to therapeutic interventions. Functionally, microRNAs have been demonstrated to be involved in many aspects of arthritis, such as regulation of innate and adaptive immunity leading to autoinflammation and autoimmunity as well as in inflammatory responses in fibroblasts and macrophages and in osteoclast biology. Certainly, in the years to come, many additional functions of microRNAs in arthritis will be uncovered and eventually, microRNAs might even become the target of therapeutic interventions in human disease.

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MicroRNAs with Impact on Adipose Tissue Inflammation in Obesity

Michael Karbiener and Marcel Scheideler

Abstract The past decades have been hallmarked by an unprecedented steady rise in the prevalence of overweight and obesity around the globe. Characterized by excessive expansion of white adipose tissue (WAT), obesity constitutes a major health problem as the primary disease predisposes to a series of follow-up complications including type 2 diabetes mellitus, cardiovascular disease, and certain types of cancer. Interestingly, the majority of obese individuals show signs of low-grade inflammation, and many of the obesity-associated downstream pathologies seem to be amplified—if not even caused—by inflammatory signals emanating from obese WAT. Not only immune cells within WAT but also adipocytes and their precursors actively secrete a wide panel of cytokines—coined "adipokines"—the levels of which frequently change upon weight gain. Their systemic effects and the molecular networks tuning their expression are not completely understood. In this respect, microRNAs, noncoding transcripts that act as negative regulators of protein output, have recently entered the field of adipose tissue physiology. This chapter will provide an overview of inflammatory processes in WAT, as well as their causes and consequences in the obese state. In particular, current knowledge regarding the regulation of adipose tissue microRNAs by inflammatory stimuli will be summarized, as well as the role of microRNAs as mediators of detrimental inflammatory processes in obesity.

1 Obesity

Obesity and overweight are defined as abnormal or excessive fat accumulation, defined by a simple index of weight-for-height (kg/m^2), called body mass index (BMI), that is commonly used to classify overweight and obesity in adults [1]. Based on this method, currently more than 1.4 billion adults, 20 years and older, are overweight, of which over 200 million men and nearly 300 million women are obese. Moreover, more than 40 million children under the age of

M. Karbiener • M. Scheideler (✉)
RNA Biology Group, Institute of Molecular Biotechnology, Graz University of Technology, Graz, Austria
e-mail: marcel.scheideler@tugraz.at

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5 were overweight or obese in 2012. Despite the fact that obesity is preventable in most cases, the worldwide prevalence of obesity has progressively increased over the past decades and nearly doubled since 1980. In 2001, 21 % of U.S. adults, and 6 years later, almost a third of U.S. adults were classified as obese, with another third of the population being overweight [2, 3]. However, the situation is not restricted to North America, as in Europe more than 50 % of adults were overweight or obese, three times more people than 20 years before [4]. Although obesity is classified as a disease itself, the massive effects on public health, i.e., the high contributions to morbidity and mortality rates, are due to the close association of obesity with an increased risk of type 2 diabetes mellitus (T2DM), cardiovascular disease (CVD), metabolic syndrome (MS), and certain forms of cancer. As such, the recent obesity epidemic has imposed significant health, economical, and societal concerns. Moreover, effective preventive and therapeutic strategies are currently lacking, primarily due to a lack of comprehensive understanding of the underlying molecular mechanisms.

Therefore, future studies that are directed to obtain on the one hand a more comprehensive, systems-level understanding of disease mechanisms and to investigate on the other hand novel therapeutic strategies against the obesity-related molecular alterations are in demand.

Although obesity is well recognized as a health hazard, it is a heterogeneous disorder with several possible etiologies. Indeed, obesity assessed by BMI cannot properly estimate whether an obese patient has an elevated risk for follow-up complications such as dyslipidemia, hypertension, T2DM, or cancer. Interestingly, not adiposity per se but rather body fat distribution is an important risk factor for obesity-related disorders. People with excess of intra-abdominal fat around the waist, called central or apple-shaped obesity, rather than people with excess of subcutaneous fat around the hips, called peripheral or pear-shaped obesity, have a higher risk for obesity-associated follow-up complications [5]. Moreover, while the majority of people with obesity are metabolically unhealthy (MUO), as much as 30 % of those with obesity are metabolically healthy (MHO). Thus, the subtypes of obesity are important models for the study of distinct obesity-related risk profiles as it has been shown by several studies that MHO individuals have a reduced propensity to develop cardiovascular disease and mortality [6, 7].

2 Metabolic Health and Inflammation

An intimate interrelationship between metabolic and immune responses can be drawn from an evolutionary perspective. The functional units that control key metabolic and immune functions in higher organisms have evolved from common ancestral structures. For example, the *Drosophila* fat body incorporates homologs of the mammalian hematopoietic and immune system and is the equivalent of mammalian adipose tissue, sharing similar developmental and functional pathways [8]. The fly's fat body exerts crucial functions in energy homeostasis and immunology, including energy and nutrient sensing as well as metabolic response

coordination upon pathogen defense. This enables common or overlapping pathways to regulate both metabolic and immune functions through common regulatory molecules and signaling systems. Although the adipose tissue and hematopoietic system have specialized into distinct functional units or organs in higher organisms, the adipose tissue has an architectural organization in which metabolic cells like adipocytes are in close proximity to immune cells like macrophages with immediate access to blood vessels. Thus, adipose tissue forms a suitable environment for continuous and dynamic interactions between immune and metabolic responses and establishes communications with other sites such as pancreatic islets, muscle, and brain [9–12].

The first substantial evidence for a relationship between obesity and inflammation was indicated by the expression of the proinflammatory cytokine tumor necrosis factor α (TNFα) at higher levels in adipose tissue of obese mice with a linkage to insulin resistance (IR). Administration of recombinant TNFα to cultured cells or to whole animals impairs insulin action, and obese mice lacking functional TNFα or TNF receptors have improved insulin sensitivity compared with wild-type counterparts [13, 14]. Over the last two decades, it has become clear that obesity is characterized by a broad inflammatory response across species ranging from mice to humans [15–17].

Interestingly, a recent association study investigated the inflammatory status in obesity subgroups and revealed that inflammatory markers such as complement component 3 (C3), C-reactive protein (CRP), interleukin-6 (IL-6), TNFα, plasminogen activator inhibitor 1 (PAI-1), and white blood cell counts were significantly higher in MUO compared to MHO subjects, while concentrations of the adipokine adiponectin decreased [18–20]. These results highlight that metabolic health as well as its dysregulation in the metabolic syndrome (MS) seem to be tightly linked to the inflammatory status.

3 Initiation and Progression of Adipose Tissue Inflammation

Obesity is the direct result of a prolonged imbalance between energy uptake and energy expenditure. The excess energy is primarily stored in white adipose tissue (WAT) in the form of triglycerides. This leads to the enlargement of fat cells, also called hypertrophy. Although adipocytes are specifically designed to store energy with great plasticity, their maximal fat load is not unlimited, and in response to reaching or exceeding the maximal loading capacity during obesity, adipose tissue function is compromised [21, 22]. This leads to a number of events that coordinately initiate adipose tissue inflammation [23].

Hypoxia develops in adipose tissue of animals and humans as the tissue mass expands [24]. The resulting reduction in oxygen partial pressure is induced by a diminished blood flow [25–27]. This reduced blood flow velocity is most apparent in capillaries containing adherent leukocytes and platelet aggregates, suggesting

that this slowing is a result of enhanced leukocyte–endothelial interactions. These enhanced interactions occur in visceral, but not in subcutaneous adipose tissue of obese mice, indicating not a systemic but a local event [28, 29]. At the molecular level, hypoxia triggers the expression of hypoxia-inducible factor-1α (HIF-1α), the master regulator of oxygen homeostasis which governs a plethora of genes that are involved in erythropoiesis, angiogenesis, and glycolysis [30, 31]. Moreover, hypoxia modulates adipokine production by decreasing mRNA levels of adiponectin, while elevating those of pro-inflammatory PAI-1, TNFα, interleukin 1 (IL-1), IL-6, monocyte chemoattractant protein-1 (MCP-1), and transforming growth factor beta(TGF-β) [32–34].

Lipotoxicity occurs by a massive release and ectopic accumulation of free fatty acids (FFAs) when the limited loading capacity of adipocytes is exceeded. FFAs are potent ligands that induce toll-like receptor (TLR) signaling with a broad spectrum of TLRs being expressed in adipose tissue [35, 36]. TLRs are a family of pattern-recognition receptors with a crucial role in the activation of pro-inflammatory signaling pathways. For example, TLR4 expression is increased in adipose tissue of obese mice and activates intracellular inflammatory pathways such as c-Jun N-terminal kinase (JNK) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) [37], while mice deficient in TLR4 expression or function are partially protected from fat-induced inflammation [38, 39].

Metabolic endotoxemia defined by increased plasma lipopolysaccharide (LPS) concentrations can occur by high fat diet [40, 41]. LPS is continually produced within the gut by the death of gram-negative bacteria. A high fat diet increases the proportion of gut LPS-containing microbiota and also causes moderate increases in plasma LPS concentrations which result from increased absorption of LPS across the intestinal barrier. Obese patients with T2DM have 76 % higher circulating LPS levels than healthy controls, and these LPS levels decrease significantly after surgical weight loss [42, 43]. In murine adipocytes, LPS is able to initiate inflammation via TLR4 and induces the secretion of pro-inflammatory cytokines via downstream activation of NF-κB, while in human adipocytes, LPS increases the release of TNFα, IL-6, and MCP-1. Moreover, it also upregulates TLR2 expression which further amplifies pro-inflammatory signaling in the adipose tissue [42, 44].

The endoplasmic reticulum (ER) is responsible for protein folding and trafficking in the cell, but it also has an important role in sensing cellular stress [45]. ER stress is triggered by obesity-associated conditions, such as hypoxia and excess of cytokines, lipids, or glucose. This in turn causes an ER stress response, also known as the unfolded protein response (UPR) which is also critical in chronic metabolic diseases such as obesity, insulin resistance, and T2DM [45, 46]. When activated, all three sensors of the UPR, PRKR-like endoplasmic reticulum kinase (PERK), endoplasmic reticulum to nucleus signaling 1 (IRE1), and Activating transcription factor 6 (ATF6) participate in upregulating inflammatory processes [47, 48]. Therefore, the ER and related signaling networks are emerging as a potential site for the intersection of inflammation and metabolic disease. Moreover, it has recently been postulated that sufficient oxidative redox potential at the ER is created during physical exercise (which generates reactive oxygen species (ROS)) and that,

conversely, unfavorable redox states could accelerate or even cause T2DM and CVD due to the accumulation of improperly folded proteins [49].

4 Adipokines: Linking Adipose Tissue to Inflammation in Obesity

Nowadays adipose tissue is considered no longer as a mere energy store, but also as major endocrine organ which releases a wide range of bioactive peptides, proteins, and other signaling factors collectively termed adipokines [50–52]. Adipokines are at the center of adipose tissue communication with other organs, involved in the regulation of cellular and physiological processes such as appetite and satiety, energy expenditure, endothelial function, blood pressure, insulin sensitivity, adipogenesis, fat distribution, and insulin secretion [53].

Thus, the dysregulated production and release of adipokines is tightly linked to the pathogenesis of various diseases. In obesity, which is characterized by a state of chronic low-grade inflammation, circulating levels of pro-inflammatory markers and adipokines are elevated, while those of anti-inflammatory adipokines are decreased. Moreover, elevated levels of inflammation-related adipokines are increasingly considered to be important in the development of follow-up diseases of obesity, particularly of T2DM, CVD, and MS [54, 55].

For example, adipokines that are linked to pro-inflammatory pathways are TNFα, leptin, IL-6, MCP-1, PAI-1, and resistin [23, 52].

TNFα is overexpressed in adipose tissue of obese subjects to a higher degree in visceral than in subcutaneous sites and is more abundant in stromal vascular cells (SVC) than adipocytes [56]. Moreover, circulating levels of TNFα are elevated in obese subjects and fall after weight loss [57]. The multiple actions of TNFα include the inhibition of carbohydrate metabolism, lipogenesis, adipogenesis, thermogenesis, and anti-inflammatory adipokine expression as well as the stimulation of lipolysis and pro-inflammatory adipokine secretion [23, 58, 59]. Thus, TNFα overproduction in obesity may be viewed as an ultimate attempt to limit further weight gain through lipolysis and insulin resistance, impaired adipogenesis, and increased adipocyte apoptosis [60]. However, these effects of TNFα occur at the expense of worsening insulin resistance and inflammation [23].

Leptin is produced and secreted mainly by adipocytes and has structural similarity to other pro-inflammatory cytokines. It links nutritional status with neuroendocrine and immune functions with the control of appetite as its major role [61, 62]. Circulating leptin levels not only parallel adipose tissue mass but also reflect immediate changes in nutritional status as they decrease soon after the beginning of fasting [63]. Similar to other pro-inflammatory cytokines, leptin promotes T helper 1 (T_H1)-cell differentiation and can modulate the onset and progression of immune responses. Moreover, leptin affects the secretion of acute-phase reactants such as TNFα and IL-1 [61]. Leptin stimulates fatty acid oxidation and glucose uptake and

prevents lipid accumulation in adipose and nonadipose tissues by involving the activation of AMP-activated protein kinase (AMPK) signaling [64–66]. However, leptin replacement therapy in patients with severe lipodystrophy and leptin deficiency can reduce insulin resistance, hypertriglyceridemia, and hepatic lipid storage (steatosis); thus, leptin function strongly depends on the metabolic context [67].

IL-6 is produced in adipose tissue, contributing to 15–35 % of the systemic IL-6 in humans, with a higher expression in visceral than subcutaneous sites [68, 69]. Obesity leads to increased circulating levels of IL-6, and increased production by adipose tissue leads to insulin resistance in adipose tissue and liver which is triggered by c-Jun NH2-terminal kinase 1 (JNK1) signaling [70–72]. IL-6 is one of the main factors responsible for the induction of acute phase protein production, such as C-reactive protein (CRP) [73]. Thus, IL-6 leads to the persistence of inflammation in obese subjects.

MCP-1, also known as chemokine (C-C motif) ligand 2 (CCL2), is a potent chemoattractant playing a role in the recruitment of monocytes/macrophages into the adipose tissue that contributes to insulin resistance, hepatic steatosis, and atherosclerosis [74, 75]. MCP-1 levels in adipose tissue and the circulation are elevated in obesity [76]. Although MCP-1 is expressed by both fractions of adipose tissue, like TNFα and IL-6, it is more highly expressed in SVC than in adipocytes and in visceral adipose tissue compared to subcutaneous adipose tissue [76]. Studies in mice and human have demonstrated that MCP-1 production and signaling are crucial for the development of adipose tissue inflammation [75, 77].

PAI-1 plasma levels have been shown to rise with increasing BMI, while weight loss by dietary restriction lowers PAI-1 plasma levels [78–80]. Moreover, it presumably contributes to the increased risk of obese subjects for type 2 diabetes and atherothrombotic events [81]. In line with these findings, pro-inflammatory cytokines which are increased in the obese state, e.g., tumor necrosis factor α (TNFα), are known to induce PAI-1 expression [82]. However, the regulation of PAI-1 is not yet completely understood. Furthermore, although epidemiological studies suggest a connection between PAI-1 and insulin resistance, data on molecular mechanisms by which PAI-1 might impair insulin signaling are sparse.

Resistin, also known as FIZZ3, belongs to the resistin-like molecule (RELM) gene family and is produced and secreted by adipocytes. Circulating resistin levels increase with obesity and have been shown to impair glucose tolerance and insulin action. Administration of antiresistin antibodies improves blood sugar and insulin action thus linking obesity to diabetes [83–85].

Adiponectin (ApN, Acrp30) is an adipokine that is associated to anti-inflammatory functions [86]. It is abundantly produced by the adipose tissue where its expression is restricted to mature adipocytes [87, 88]. In contrast to most other adipokines, circulating levels are negatively correlated with pro-inflammatory markers and the BMI and decreased in obese individuals as well as in patients with CVD and T2DM [89–91]. Adiponectin attenuates inflammatory responses with beneficial effects on cardiovascular and metabolic disorders including atherosclerosis and insulin resistance [92, 93]. In the circulation, it exists in three forms: trimer, hexamer, and high molecular weight (HMW) 12- to 18-mer

adiponectin [94]. However, low HMW rather than total adiponectin levels appear to have a pathogenic role in MS [95, 96].

Altogether, these findings reveal an extensive cross-talk of the adipose tissue with other organs and multiple metabolic systems through adipokines, linking inflammation to obesity and its follow-up complications.

5 MicroRNAs as Novel Regulatory Determinants

For decades, research on the molecular mechanisms of life had been centered around a small part of the human genome: the 1.5 % which directly codes for proteins, the "molecular machines" of our body. However, efforts to understand what regulates this 1.5 % of our DNA brought up surprising findings.

First, precisely the relative amount of nonprotein-coding DNA (ncDNA) correlates well with organismic complexity [97]. Second, pervasive transcription of eukaryotic genomes has been widely described within the last decade, with the prediction that the majority of transcripts do not code for proteins [98–105]. High-resolution analysis of the human genome by the ENCODE consortium has even shown that three-quarters of the human genomic sequences can be found in primary transcripts with a vast majority of novel transcripts corresponding to non(protein-)coding RNA (ncRNA) [106, 107]. Third, multifaceted regulatory roles of various ncRNA species in a broad range of cellular processes have already been discovered. For instance, PIWI-interacting RNAs (piRNAs) appear necessary for transposon silencing in the germ line [108], endogenous small interfering RNAs (endo-siRNAs) are implicated in viral defense mechanisms [109], promoter-associated RNAs (paRNAs) seem to work in transcriptional regulation [110], and long non-coding RNAs (lncRNAs) function as epigenetic regulators [111].

MicroRNAs (miRNAs) are a subclass of small ncRNAs, first identified in 1993, which play a central role in RNA interference (RNAi), a post-transcriptional gene-silencing mechanism existing in many eukaryotes [112–116]. As small RNAs of approximately 19–23 nucleotides, miRNAs interact with partially complementary sites in the 3′ untranslated region (3′UTR) of mRNAs to diminish protein output, predominantly via mRNA destabilization rather than the inhibition of translation [117, 118]. In total, more than 45,000 miRNA target sites with human 3′UTRs are conserved, and more than 60 % of human protein-coding genes have been under selective pressure to maintain pairing to miRNAs [119]. Despite their recent discovery, miRNAs have already been shown to participate in the regulation of almost every cellular process investigated so far [120]. Importantly, fat-selective inactivation of Dicer, a necessary factor for miRNA biogenesis, resulted in mice that were almost devoid of white adipose tissue [121], implicating miRNA regulatory roles in adipocyte development [122–126]. Moreover, miRNAs have also been shown to be involved in human lipid metabolism, obesity, and inflammation [41, 124, 125, 127, 128]. Beyond, miRNAs have also been identified as a novel class of biomarkers circulating in the blood stream, with diagnostic and prognostic impact

for obesity and follow-up complications [129–133]. In addition to the potential use of miRNAs as biomarkers, their impact on diseases is also acknowledged by their deployment as drugs and/or drug targets, with clinical trials having currently reached phases I and II [134].

6 MicroRNA Deregulated upon Adipose Tissue Inflammation

In 2009, Xie et al. for the first time described how the miRnome of mouse epididymal WAT is altered upon obesity. They compared miRNA expression profiles by microarray in ob/ob mice and wild-type mice fed a high-fat diet (HFD; 55 % fat, 3 months treatment) versus lean control mice [135]. Furthermore, they investigated miRNA expression changes during in vitro adipocyte differentiation using the 3T3-L1 preadipocyte cell line. Intriguingly, many miRNAs that were up- or downregulated during adipocyte differentiation showed an opposing expression profile in WAT in the obese state: For example, miR-30c (mmu-miR-30c-5p[1]), miR-143 (mmu-miR-143-3p), and miR-103 (mmu-miR-103-3p) increased in abundance during differentiation; however, the levels of these miRNAs were decreased in WAT upon obesity. It is important to note that, for many miRNAs, treatment of in vitro differentiated adipocytes with TNFα led to a similar deregulation as observed in WAT of obese mice. Thus, as obesity develops, the changes in the miRnome in WAT might largely be the result of inflammatory processes. Furthermore, the downregulation of miRNAs which were demonstrated to enhance adipogenesis, at least in vitro [123, 135], might aggravate prominent downstream complications of obesity, most importantly insulin resistance and T2DM, as it could result in a reduced capacity for de novo adipogenesis.

A more recent microarray study by Chartoumpekis et al. identified 26 miRNAs that were differentially expressed between epididymal WAT from mice fed an HFD (60 % fat, 5 months) as compared to mice fed a standard diet [136]. At least some dynamically expressed miRNAs—miR-146b (mmu-miR-146b-5p), miR-378 (mmu-miR-378a-3p; previously also denoted miR-422b), miR-222 (mmu-miR-222-3p)—had also been identified in the previous study [135].

With respect to humans, Klöting et al. were the first to analyze (by RT-qPCR) the expression of ~150 miRNAs in WAT and to compare expression profiles

[1] The nomenclature for miRNAs has been subject to repeated changes during the last years. As a consequence, names of miRNAs in older publications might not be retrieved in current versions of miRBase, the central database for miRNA sequences (www.mirbase.org). To guarantee for unambiguity, whenever occurring for the first time in the following sections, the name of a particular miRNA as stated in the original publication is followed by the current name (according to miRBase release 20 (June 2013)) in parentheses. If studies have investigated particular miRNAs in mouse and human species, the reader can assume sequence conservation to be existent, unless indicated otherwise.

between (1) obese individuals with T2DM, (2) obese individuals with normal glucose tolerance (NGT), and (3) nonobese controls [137]. As expected and indicative of low-grade inflammation, diabetic subjects had a significantly higher number of macrophages in their omental WAT, while serum adiponectin levels were significantly reduced compared to obese subjects exhibiting NGT. Analysis of abdominal subcutaneous as well as intra-abdominal omental WAT revealed a total of 11 miRNAs with different abundance between T2DM and NGT obese individuals. Furthermore, the combination of miRNA expression data with histological examination of WAT revealed miR-132 (hsa-miR-132-3p) to negatively correlate with macrophage infiltration in omental fat. Additionally, miR-181a (hsa-miR-181a-5p) was found anticorrelated to circulating adiponectin levels, and further reciprocal relationships were also identified for miR-99a (hsa-miR-99a-5p)/miR-325 (hsa-miR-325) and circulating IL-6.

Similar to the currently known plethora of proteins that function as adipokines, a possible systemic effect of WAT-secreted miRNAs appears conceivable, albeit this has not been demonstrated to date. It should be noted that a study examining the expression of 95 miRNAs in subcutaneous and omental WAT from obese and nonobese humans found two miRNAs—miR-17-5p (hsa-miR-17-5p) and miR-132—to be not only downregulated in WAT upon obesity but also similarly decreased in blood samples of obese individuals [138]. Hence, these or other miRNAs might be endocrine players in the context of obesity-associated metabolic dysfunction.

Another human study employed miRNA expression profiling by microarray using subcutaneous WAT from obese individuals with and without T2DM, as well as from nonobese (control) subjects [139]. Interestingly, correlation of the global miRNA expression pattern was higher between nondiabetic obese versus control groups as compared to diabetic obese versus control groups. This observation could reflect the successive impairment of normal WAT function as an individual first gets obese and then also diabetic, which is paralleled by an increase in inflammatory stimuli that might be partly caused by progressive loss of the "healthy WAT miRNome." As for particular miRNAs that could impact on adipose tissue inflammation, this study found miR-30a* (hsa-miR-30a-3p) to be significantly decreased in WAT of obese diabetic compared to obese nondiabetic subjects.

In a recent study by Arner et al., abdominal subcutaneous WAT was obtained from obese and nonobese humans to perform miRNA expression analysis by microarray [140]. As expected, the obese cohort exhibited reduced insulin sensitivity and signs of WAT inflammation due to significant elevation of MCP-1 secretion. In sum, 18 miRNAs were identified as downregulated in the WAT of obese subjects while two miRNAs were found to be upregulated. 17 of these miRNAs were subsequently confirmed to be correspondingly deregulated also in isolated adipocytes of obese individuals (versus isolated adipocytes of nonobese control subjects). Notably, several of these miRNAs have already been demonstrated to impact on adipocyte differentiation in mouse or human [123, 126, 141–144].

To summarize, differences in miRNA expression between WAT with signs of low-grade inflammation (i.e., in the obese, insulin resistant state) and metabolically healthy WAT (either obese or nonobese states) have been addressed by several comparative studies. Although each study has identified interesting candidates for further investigation, the overlap of concordantly deregulated miRNAs between studies is generally poor. Among other factors, this might be related to distinct study designs (e.g., some involving only one gender, some combining both genders), distinct anatomical locations of the WAT biopsies, and also intrinsic differences between human and mouse species. Nevertheless, at least for some of the identified miRNAs, functional studies have already revealed a role in inflammatory signaling within WAT, as reviewed below.

7 MicroRNAs Regulated by Distinct Inflammatory Mediators

Comparative analysis of mouse WAT (in vivo) and adipocytes (in vitro) suggested that a considerable fraction of miRNAs might be deregulated in the obese state due to TNFα, one of the major cytokines involved in adipose tissue inflammation [135]. While miR-148a (mmu-miR-148a-3p), miR-422b, and miR-143 were the miRNAs most strongly downregulated in adipocytes after a 24 h exposure to TNFα, miR-146b (mmu-miR-146b-5p), miR-221 (mmu-miR-221-3p), and miR-222 showed the strongest upregulation. Interestingly, the clustered miRNAs miR-221 and -222 possess an antiangiogenic function via direct targeting of the c-kit receptor [145]. Supported by additional studies that found a downregulation of miR-222 in WAT of obese mice [136] and humans [140], this raises the hypothesis that both miRNAs might act at the molecular interplay between adipose tissue inflammation and hypoxia, a hallmark of WAT in the obese state [146]. However, further studies dissecting the effects of TNFα on different cell types within WAT are in demand, as a recent study by Meerson et al. found decreased levels of miR-221 both upon short-term treatment of human preadipocytes as well as upon long-term treatment of differentiated adipocytes with TNFα [147]. Thus, the impact of TNFα on miR-221/222 expression might be highly cell-type specific.

miR-378 is an miRNA residing in an intron of the PPARGCA1B gene, coding for peroxisome proliferator-activated receptor γ coactivator 1β (PGC-1β), which is highly enriched in BAT versus WAT and a powerful inducer of mitochondrial biogenesis and fatty acid oxidation in adipose tissue and skeletal muscle [148, 149]. While many independent studies have confirmed the expression of miR-378 in WAT and adipocytes, there are conflicting results with respect to the regulation of miR-378 expression by inflammatory cytokines. On the one hand, a downregulation in WAT of obese mice [135] and humans [140] has been shown, which (at least in differentiated adipocytes) might be mediated by TNFα [135]. A possible role in "healthy" adipose tissue metabolism could therefore be envisioned

for miR-378, and indeed, treatment of human in vitro differentiated adipocytes with pioglitazone, an insulin sensitizer of the thiazolidinedione class that has anti-inflammatory effects, led to an upregulation of miR-378 [150]. On the other hand, two recent studies have described miR-378 to increase upon the stimulation of human adipocytes with TNFα, IL-6, and Leptin [151, 152]. A pro-inflammatory function of miR-378 could therefore also be postulated, and indeed, genetically engineered mice lacking pre-miR-378 exhibited ameliorated insulin resistance [153]. However, in this "global" knockout mouse model, the specific function for miR-378 in adipose tissue could have been obscured due to the deletion of pre-miR-378 in other metabolic tissues (liver, heart, and skeletal muscle).

In 2008, a study on different genetically obese mouse models reported a significant upregulation of miR-335 (mmu-miR-335-5p) in WAT of ob/ob, db/db, and KKAy versus wild-type mice [154]. Correspondingly, a recent report described the upregulation of this miRNA in response to the treatment of human adipocytes with several pro-inflammatory adipokines, i.e., leptin, resistin, TNFα, and IL-6 [155]. In conjunction with a reported antiadipogenic effect during differentiation of human mesenchymal stem cells [156], miR-335 can be envisioned to participate in the worsening of insulin resistance during obesity, being induced by inflammatory signals, thereby blunting the WAT capacity for lipid storage, which in turn would result in increased lipotoxicity.

Compared to miR-335, a mirrored picture has emerged for miR-26b (hsa-miR-26b-5p): Short-term treatment of human in vitro differentiated adipocytes with TNFα, leptin, and resistin (but not IL-6) resulted in decreased miR-26b levels [157]. A second report further added free fatty acids and glucose as negative regulators of miR-26b [158], the increase of which is a hallmark of T2DM. While the necessity of miR-26b for adipocyte differentiation has recently been shown [126, 159], the effects of this miRNA on metabolism and secretory profile of mature adipocytes have not been investigated to date.

Effects of the metabolically beneficial adipokine adiponectin on miRNA expression have been addressed by a microarray study comparing inguinal WAT from wild-type mice to mice overexpressing adiponectin selectively in their adipose tissues [160]. The authors identified a total of 51 miRNAs that were differentially expressed between the two mouse groups. They subsequently validated miR-532-5p (mmu-miR-532-5p) and miR-1983 (mmu-miR-1983) to be significantly downregulated, while miR-883b-5p (mmu-miR-883b-5p) and miR-1934 (mmu-miR-1934-5p) were significantly upregulated by adiponectin overexpression in inguinal WAT. Additionally, miR-1983 and miR-1934 also exhibited an inverse expression profile (i.e., upregulation and downregulation, respectively) when wild-type mice were compared to adiponectin knockout mice. Further, miR-883b-5p was also found upregulated by adiponectin treatment of 3T3-F442A preadipocytes and adipocytes derived thereof. While the overall approach to search for adiponectin-regulated miRNAs is highly interesting to obtain candidates with potential anti-inflammatory properties, it should be noted, though, that homologs of mouse miR-1983 and miR-1934 have not been identified in human to date.

8 MicroRNAs That Regulate Distinct Inflammatory Signaling Pathways

In 2009, Strum et al. showed that inflammatory NF-κB signaling is subject to regulation by miRNAs in the context of adipogenesis [161]. When assaying a large set of cytokines in human preadipocytes and adipocytes, the authors found that the secretion of interleukin 8 (IL-8)—known to be increased in the circulation of obese individuals [162] and to be correlated with measures of insulin resistance [163]—and MCP-1 from both cell types was increased upon stressing by serum starvation. The same experimental setting was used for a RT-qPCR-based miRNA screening and identified miR-132 as likewise induced by deprivation of serum. Subsequent functional analysis revealed miR-132 to directly target the mRNA of silent information regulator 1 (SirT1), thereby reducing the deacetylation of p65 (RELA) by SirT1 protein, which in turn evoked the upregulation of IL-8 and MCP-1. In spite of these coherent in vitro results, current in vivo data is unsupportive with regard to a pro-inflammatory role of miR-132, as a negative association between the miRNA and macrophage infiltration was reported [137], and as miR-132 levels were found decreased in WAT of obese versus lean humans [138].

A study by Chang et al. discovered a regulatory influence of miRNAs on heme oxygenase 1 (HO-1) [164], a protein involved in heme breakdown [165]. HO-1 has also anti-inflammatory properties [166] and was shown to reduce WAT mass and ameliorate obesity-associated insulin resistance [167], presumably due to its positive effects on adiponectin [168]. Chang et al. demonstrated that HO-1 was induced, while miR-155 (mmu-miR-155-5p), miR-183 (mmu-miR-183-5p), and miR-872 (mmu-miR-872-5p) were decreased upon the exposure of mouse adipocytes to insulin. Conversely, overexpression of each miRNA reduced HO-1 expression [164]. Although predicted, the direct physical interaction of miR-155, miR-183, and miR-872 with HO-1 mRNA remains to be proven.

MiR-30c, which is downregulated in WAT of obese mice [135] and humans [140], has recently been shown to directly target the mRNA of PAI-1 [123]. While this study found a reciprocal expression pattern of miR-30c and PAI-1 during human adipocyte differentiation, others have also reported the direct, negative influence of miR-30c on PAI-1 production in endothelial cells [169, 170]. Due to these findings and the well-known function of PAI-1 in blood coagulation, an involvement of miR-30c in the increased risk of obese subjects for atherothrombotic events can be hypothesized [81].

Secretion of MCP-1 by adipocytes has been proposed to constitute an early event in obesity-associated metabolic dysfunction as it initiates the entry of inflammatory cells into WAT. Thus, the influence of miRNAs on MCP-1 production is of particular interest and has been addressed by Arner et al. [140]. First, the authors identified a group of miRNAs which were coordinately downregulated in WAT upon obesity, and that reduced MCP-1 secretion from adipocytes when transfected into human adipocytes. Second, miR-126 (hsa-miR-126-3p) was validated to

directly target the MCP-1 3′UTR. Third, by combining global mRNA and miRNA expression data with bioinformatic analysis for transcription factor binding and activity, a network of miRNAs and transcription factors regulating MCP-1 levels in adipocytes was generated. MiR-193b was finally validated to impact on MCP-1 production via direct targeting of the transcription factors ETS1 and MAX, which are known upstream regulators of MCP-1. Recently, a follow-up study has extended this network, showing additional effects of miR-92a (hsa-miR-92a-3p; also downregulated in WAT upon obesity) on MCP-1 secretion via direct targeting of the transcription factor SP-1 [171].

In 2012, Ge et al. discovered a mechanistic link between mouse miR-883-5p and several inflammatory cytokines in preadipocytes and adipocytes [160]. As described above, miR-883-5p was induced in vitro (3T3-F442A cells) and in vivo (inguinal WAT) by adiponectin. Subsequent analysis showed that miR-883-5p directly targets the 3′UTR of lipopolysaccharide-binding protein (LBP), an important upstream regulator of TLR-mediated inflammatory responses. Indeed, while TNFα treatment of preadipocytes and adipocytes heavily induced the expression of IL-6 and TNFα, the ameliorating effect of adiponectin on the induction of these inflammatory markers was shown to be strongly dependent on miR-883-5p. The connection between the miRNA and inflammatory responses was further demonstrated in vivo, as injection of 3T3-F442A cells expressing an anti-miR-883-5p oligonucleotide resulted in upregulated TNFα and IL-6 levels in de novo-formed adipose tissue. However, it should be noted that a human ortholog of mmu-miR-883-5p has not been discovered until now.

MiR-21 is one of the most abundant miRNAs in adipose-derived mesenchymal stem cells, and was among the first miRNAs discovered to stimulate human adipocyte differentiation in vitro [172]. A recent study performed gain- and loss-of-function studies during adipogenesis of 3T3-L1 preadipocytes and found miR-21(mmu-miR-21a-5p) to promote expression of the beneficial adipokine adiponectin [173]. However, this report also corroborated the pro-adipogenic effect of miR-21. Thus, future studies are needed to dissect whether the induction of adiponectin is largely due to the general stimulation of adipocyte differentiation, or whether there are "adipogenesis-independent" stimulatory effects of miR-21 on adiponectin which might also be existent in terminally differentiated adipocytes.

As for TNFα, Li et al. have used porcine primary preadipocytes to show that miR-181a (which is conserved between pig and human) promotes adipocyte differentiation and suppresses TNFα production [174]. Subsequently, a direct interaction of miR-181a with the TNFα 3′UTR was demonstrated, which was also corroborated by a recently demonstrated direct interaction between the closely related family member miR-181c and TNFα [175]. Interestingly, miR-19a (hsa-miR-19a-3p) was shown to strongly regulate TNFα in an esophageal cancer cell line via a direct miRNA–mRNA interaction [176]. As miR-19a is a member of the miR-17~92 cluster of miRNAs, which appears to be expressed in WAT and adipocytes [140, 142], the involvement of miR-19a in TNFα regulation and thus adipose tissue inflammation is conceivable, in principle.

9 Outlook

As the miRNA research field is still very young, a significant amount of groundwork remains to be done in terms of therapeutic applications. However, the past 20 years have greatly expanded our understanding about synthetic oligonucleotide pharmacology and toxicology. To date two approved RNA-based drugs—fomivirsen, an antiviral drug for the treatment of cytomegalovirus retinitis (CMV) and macugen/pegaptanib, an antiangiogenic drug for the treatment of neovascular age-related macular degeneration (AMD)—have already reached the market [177–179]. However, both are delivered locally by intravitreal injection. Therefore, it has been argued that oligonucleotide-based drugs cannot be effective systemically because these large polar molecules do not meet the empirical Lipinski rules stating that, among other characteristics, effective drugs invariably have molecular weights below 500 Da and are fairly soluble in both polar and nonpolar solvents [180]. While oligonucleotides clearly do not meet these criteria, monoclonal antibodies are also far from Lipinski's rules, yet they have now reached a worldwide market for therapeutic applications [181].

Despite the challenge of drug delivery to fully harness the power of RNA-based therapeutics, its potential to reach hitherto undruggable targets and affect untreatable diseases has greatly promoted efforts to turn these compounds into effective drugs. Thus, numerous novel RNA-based therapeutics are in development, including miRNAs serving either as drug target for miRNA inhibition or as drug in miRNA replacement studies which are now in clinical trials phase I and II [134, 182–184].

With this, miRNA-based therapeutic strategies are clearly an exciting and dynamic area of research and development that can be expected to yield effective treatments against a variety of diseases in the near future.

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The Relationship Between miR-29, NOD2 and Crohn's Disease

Oliver Brain and Alison Simmons

Abstract Crohn's disease (CD) is a chronic inflammatory bowel disease with a complex aetiology that includes genetic susceptibility and the gastrointestinal microbiome and results in an aberrant Th17 inflammatory response. NOD2 is an intracellular sensor that responds to bacterial cell wall peptidoglycan and contributes to immune defense. Polymorphisms in the NOD2 gene predispose to Crohn's disease, with the largest effect of any of the known genetic risk factors. We have found that wild-type NOD2 controls the expression of miR-29 in human dendritic cells (DCs). miR-29 regulates the expression of a number of immune mediators including the IL-23 cytokine subunits IL-12p40 and IL-23p19. CD patient DCs expressing NOD2 polymorphisms fail to induce miR-29 and show enhanced IL-12p40 release on exposure to adherent invasive *E. coli*. Moreover in a murine model deficient in miR-29, a more severe Th17-driven colitis is established after DSS administration. Therefore, we suggest that the loss of miR-29-mediated immunoregulation in CD-variant NOD2 DCs contributes to elevated IL-23 and aberrant Th17 response in this disease.

Abbreviations

AIEC	Adherent-invasive *Escherichia coli*
APC	Antigen presenting cell
ATG16L1	Autophagy related 16-like 1
CARD15	Caspase recruitment domain family member 15
CD	Crohn's disease
Chr	Chromosome
CLR	C-type lectin receptors

O. Brain (✉)
Translational Gastroenterology Unit, John Radcliffe Hospital, Oxford OX3 9DU, UK
e-mail: aobrain@googlemail.com

A. Simmons
Translational Gastroenterology Unit, John Radcliffe Hospital, Oxford OX3 9DU, UK

MRC Human Immunology Unit, Weatherall Institute of Molecular Medicine, John Radcliffe Hospital, Oxford OX3 9DS, UK

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DAMP	Damage-associated molecular pattern
DC	Dendritic cell
DSS	Dextran sodium sulphate
F. prau	*Faecalibacterium prausnitzii*
Foxp3	Forkhead box P3
GI	Gastrointestinal
GWAS	Genome-wide association study
HD	Human defensin
IBD	Inflammatory bowel disease
IFN	Interferon
IL	Interleukin
IL-12B	Interleukin 12B/IL-12p40
IL-23R	Interleukin-23 receptor
IRGM	Immunity-related GTPase family, M
JAK2	Janus kinase 2
KO	Knock-out
LPS	Lipopolysaccharide
LRR	Leucine-rich repeat
MDDC	Monocyte-derived dendritic cell
MDP	Muramyl dipeptide
miR	microRNA
MyD88	Myeloid differentiation primary response gene 88
NFκB	Nuclear factor kappa B
NK	Natural killer
NLR	NOD-like receptor
NOD2	Nucleotide-binding oligomerisation domain containing 2
Pam_3CSK_4	Synthetic triacylated lipoprotein—TLR1/2 ligand
PAMP	Pathogen-associated molecular pattern
PCR	Polymerase chain reaction
PGN	Peptidoglycan
PRR	Pattern recognition receptor
qPCR	Quantitative polymerase chain reaction
RIPK-2	Receptor-interacting protein kinase 2
RORγt	RAR-related orphan receptor gamma
STAT3	Signal transducer and activator of transcription 3
T-bet	T-box transcription factor
Th1/17	T helper 1/17
TLR	Toll-like receptor
TNF	Tumour necrosis factor
Wnt	Wingless
WT	Wild-type

1 Introduction

Crohn's disease (CD) is a form of chronic inflammatory bowel disease (IBD). It typically presents in young people (peak onset between years 10 and 20), with the highest incidence in the western world with a rising incidence as nations move towards a western lifestyle and environment [1]. CD usually manifests clinically with a combination of abdominal pain, weight loss, and diarrhoea. The clinical course is variable, from a fairly mild self-limiting illness through to severe disease resulting in multiple surgeries, short gut syndrome and severe malabsorption. Although there have been substantial improvements in medical therapy, there exists a significant therapeutic gap as evidenced by the modest sustained remission rates with anti-TNF therapy [2] (currently the most potent available therapy), plus the requirement for at least one surgery in the majority of patients (up to 80 % in ileal disease [3, 4]). The transmural granulomatous inflammation typical of Crohn's can involve any part of the gastrointestinal (GI) tract from mouth to anus, but usually affects the terminal ileum, colon and/or perianal regions.

The aetiology of Crohn's disease is complex and multifactorial, with the best evidence suggesting that it involves a combination of genetic susceptibility, a disordered GI tract microflora (dysbiosis), and possibly a third as yet poorly characterised environmental factor, or factors.

There is good observational evidence for a genetic component to disease susceptibility, from the documentation of familial clustering by Burrell Crohn himself [5] to the monozygotic twin disease concordance rate of nearly 50 % [6] and the high percentage of patients with a positive family history [7]. Over the past 15 years genetic studies have identified an increasing number of polymorphisms associated with CD, and currently there are 163 loci associated with IBD [8]. The modern era of IBD genetics was initiated by the discovery of mutations in the caspase recruitment domain family member containing 15 (CARD15), or nucleotide-binding oligomerisation domain containing 2 (NOD2), gene in the IBD1 locus on chromosome 16 [9].

CARD15/NOD2 (henceforth NOD2) encodes a cytosolic innate immune receptor and is a member of the NOD-like receptor (NLR) family. NOD2 is triggered by bacterial cell wall muramyl dipeptide (MDP) [10, 11], which is expressed by both gram +ve and gram −ve organisms. This aligns with another central tenet of CD susceptibility—that of the influence of the GI tract bacteria or microbiome. The GI tract is home to a staggering quantity of bacteria—at their most dense the bacteria number 10^{12}/ml. Coupled with this is the startling fact that these bacteria are only physically separated from GI tissue residence/invasion by a single layer of epithelial cells and a covering of mucus (varying in depth from approximately 200 to 700 μm). In many respects, it is interesting that we do not develop overt inflammation of the GI tract more frequently.

Nevertheless, we are dependent on the development and the presence of a normal gut flora for a healthy gut epithelium, for digestion and for the development and function of the immune system [12]. Defining what constitutes a 'normal' gut

flora is still a work in progress and depends in part on what is sampled (faeces or mucosa-associated bacteria) [13] and on other factors such as ones underlying genotype [14], diet [15], age [16] and any recent antibiotic use. The colonisation of the GI tract begins at birth (with commensals from the mother's vagina or skin, depending on delivery via birth canal or C-section respectively), and then slowly develops over 2–3 years into that consistent with an adult faecal signature. Some elements of the commensal bacterial flora, such as *Faecalibacterium prausnitzii* [17, 18] (or *F. prau*) in humans, or certain Clostridial species in mice [19], appear to have crucial anti-inflammatory properties. Conversely, the presence of 'pathobionts' such as adherent-invasive *Escherichia coli* (AIEC) [20–22] correlates with an increased risk of ileal CD.

There is observational and experimental evidence that the presence of the microbiota is necessary in driving, if not definitively initiating, GI inflammation. In humans, antibiotics can ameliorate CD [23], faecal stream diversion is an effective therapy for Crohn's [24, 25], and lamina propria T cells from CD patients are reactive to gut flora [26]. Murine models of colitis are for the most part dependent on the presence of the microbial flora to develop colitis (an exception being the anti-CD40 model). Moreover, under certain conditions, it is possible to transfer a colitogenic flora from one mouse model to another [27].

Patients with Crohn's disease have an altered microbiome or 'dysbiosis', and disease severity corresponds with the degree of difference from healthy controls [28]. The main difference in CD appears to be that the microbial community is more unstable over time and less diverse, for example, with the loss of Bacteroidetes and certain Firmicutes, and an increase in Proteobacteria [29]. It remains unclear how much of this dysbiosis is a primary aetiological event, and how much is secondary to an already established inflammation.

The nature of the aberrant inflammatory response in Crohn's has been demonstrated genetically, in murine models and in human studies. The significance of IL-23 and the Th17 pathway is highlighted by polymorphisms in *IL23R*, *IL12B* (encoding for IL-12p40), *STAT3*, *JAK2* and *TYK2* [30]. Both innate and T cell-dependent models of murine colitis demonstrate a key role for IL-23. In innate colitis, IL-23 directs the expression of IL-17 from innate lymphoid cells [31]. In T cells, IL-23R signalling leads to enhanced Th17 polarisation and reduced $FoxP3^+$ T cell differentiation and IL-10 expression [32]. Finally, in human studies, IL-23 expression is increased in the mucosa of IBD patients [33].

2 NOD2 and Crohn's Disease

The role of NOD2 within this complex GI environment is an area of active research, and some progress has been made in the 14 years since the gene was identified. NOD2 is constitutively expressed in GI epithelial cells [34], as well as in myelomonocytic cells such as dendritic cells (DCs) [35, 36]. NOD2 has two N-terminal caspase-recruitment domains (CARDs), a central nucleotide-binding

oligomerisation domain (NOD) and a C-terminal leucine-rich repeat (LRR) domain. The polymorphisms that predispose to CD occur in (or adjacent to in the case of R702W) the LRR domain that functions as the MDP ligand-recognition domain. Three single nucleotide polymorphisms (one frame-shift: FS1007insC and two missense: G908R and R702W), account for over 80 % of those identified [37]. Homozygosity, or compound heterozygosity, confer a 17-fold increased risk of developing CD, whilst around 40 % of European CD patients carry at least one mutation [38] versus 14 % of healthy controls. Mutations in NOD2 predispose to terminal ileal CD [38], early-onset disease [37, 39] and possibly a stricturing phenotype [40].

NOD2 activation, on recognition of MDP, is thought to result in oligomerisation [41, 42] and recruitment of an adaptor protein RIPK-2 [43] via a CARD–CARD domain interaction. This leads, via an incompletely understood signalling cascade, to NFκB activation [36] and pro-inflammatory cytokine production. NOD2 is just one of a number of innate immune pattern recognition receptors (PRRs) expressed by antigen-presenting cells such as DCs. Others include the membrane-bound toll-like receptors (TLRs), the C-type lectin receptors (CLRs) and the cytosolic Nod-like receptors (NLRs) of which NOD2 is a member. These PRRs typically respond to pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs). Immune activation by whole microbes is therefore complex, and it is the combination of PRR triggering by a given organism, plus the nature of the cellular microenvironment, that will influence the nature of the subsequent immune response.

Although there is now a large literature on the function of NOD2, the key findings can be summarised reasonably succinctly. Firstly, NOD2 induces autophagy on bacterial recognition in both dendritic [44] and epithelial cells [45], helping to clear the invasive bacteria and (in DCs) facilitating MHC class II antigen presentation. Autophagy is an intrinsic cytosolic process in which the formation of double-membrane vesicles facilitates the degradation of intracellular proteins (macro-autophagy) [46], bacteria (xenophagy) [47] or mitochondria (mitophagy) [48]. The role of autophagy in Crohn's pathogenesis only became apparent when genetic studies revealed polymorphisms in both ATG16L1 and IRGM [30]. The subsequent studies linking NOD2 and xenophagy are important in that they coalesce apparently unrelated CD polymorphisms around the same defective pathway.

Secondly, NOD2 is highly expressed in specialised small intestinal epithelial cells, called Paneth cells [34, 49], which are themselves most numerous in the terminal ileum [50]. Paneth cells are located in the crypts of Lieberkühn, and amongst other functions they secrete anti-microbial peptides, such as defensins. NOD2 polymorphisms are associated with decreased alpha defensin production (HD-5 and HD-6) from Paneth cells [51, 52], raising the question as to whether this is a primary defect in CD pathogenesis. However, there is some continued debate as to whether this apparent reduction owes more to inflammation rather than NOD2 expression [53] or whether the reverse is true [54]. In certain cases, the lack of defensins is due to other pathways altogether, such as defective Wnt signalling [55, 56].

Lastly, as already described, NOD2 is only one of a broad range of innate immune receptors. Large-scale gene-expression studies reveal that NOD2 can synergise with other PRRs, and that this synergy is lost in the presence of CD-variant NOD2 [57–59]. Wild-type NOD2, which in isolation has a relatively weak effect [57], has a key role in amplifying the release of certain pro-inflammatory cytokines, particularly interleukin-1β (IL-1β), IL-6 and IL-23 from DCs and macrophages [60, 61]. It is mooted that this NOD2-driven amplification of TLR responses is in keeping with the cytosolic expression of the NOD2 protein, because MDP stimulation may be indicative of invasive bacterial infection.

3 NOD2 and miR-29

One of the questions that arise from the cytokine data is why the CD-variant NOD2 should predispose to a pro-inflammatory condition. PRR signalling pathways that induce effector responses, including cytokines, need to be tightly regulated in order to limit bystander damage and terminate the immune response. microRNAs (miRNAs), the main function of which is to target messenger RNA (mRNA), are key regulators of gene expression in mammalian cells. Prior to our investigation, a number of studies of TLR signalling had established a role for miRNAs in the negative regulation of innate immune responses [62–66]. We hypothesised that wild-type NOD2 triggering would contribute to miRNA expression and that the absence of this in CD-variant NOD2 would lead to aberrant cytokine expression and the immunopathology seen in Crohn's.

We used human monocyte-derived dendritic cells (MDDCs) to explore miRNA expression initially after NOD2 and/or TLR2 stimulation [67]. TLR2 responds to Pam_3CSK_4 that, along with MDP, is a component of bacterial cell wall peptidoglycan, meaning that TLR2 is very likely to be co-triggered with NOD2. An miRNA array identified not only the already-described miR-155 and miR-146 as downstream of TLR2 triggering but also miR-29 expression as a novel miRNA in relation to innate immune signalling. Further investigation established that wild-type NOD2 stimulation, and the presence of the adaptor molecule RIPK-2, is critical for the up-regulation of miR-29 and that this is amplified by the co-stimulation of TLR2 or TLR5. Interestingly, this effect is not dependent on the TLR-signalling adaptor molecule MyD88. miR-29 is part of a miRNA family expressed from two clusters on chromosomes 1 and 7 (mir-29a, b and c), and these miRNAs possess identical seed sequences, therefore targeting the same mRNAs. miR-29 expression in MDDCs is detectable from 12 h after NOD2 + TLR2 stimulation and peaks at around day 3. This delayed expression pattern would be in keeping with a role as a negative regulator of immune response, allowing effector mechanisms to work before appropriate termination.

In order to identify potential miR-29 targets in MDDCs, we transfected NOD2- and TLR2-stimulated DCs with miR-29 premiR to artificially increase miR-29 expression. We used large-scale gene-expression microarrays to subsequently

identify a number of differentially regulated genes and went on to validate these by quantitative PCR (qPCR). These genes included a number of pro-inflammatory and immune pathway mediators, and one of the most strongly down-regulated genes identified by this methodology was IL-12p40. IL-12p40 is a predicted target of miR-29 (Targetscan) and is a cytokine subunit of both IL-12 (with IL-12p35) and IL-23 (with IL-23p19). We established that miR-29 directly targets IL-12p40 mRNA via the 3′UTR and indirectly down-regulates IL-23p19, but not IL-12p35.

We went on to demonstrate that NOD2-directed control of miR-29 is relevant at physiological miRNA expression levels. Firstly, we utilised an IL-12p40 3′UTR seed target protector that was designed for miR-29 binding sites. MDDCs transfected with this protector and then stimulated with NOD2 and TLR2 ligands for 24 h express elevated levels of IL-12p40. Secondly, we identified patients with ileal CD and who are homozygous for CD-variant NOD2 polymorphisms. MDDCs from these patients fail to up-regulate miR-29 when NOD2 is triggered and, perhaps more importantly, they express higher levels of IL-12p40 after AIEC infection than their wild-type counterparts. This discrepancy is rescued by the transfection, or replacement, of miR-29 using a premiR.

We next explored the role of miR-29 in vivo using mice with a targeted deletion of the *miR-29a/b-1* locus (henceforth mir-29 KO mice). We investigated whether a lack of miR-29 in a murine model alters the development or susceptibility to colitis in a DSS model. miR-29 KO mice show an increased susceptibility to colitis (1.7-fold higher compared to WT littermates) and exhibit more severe pathological scores and weight loss. In keeping with the human in vitro data, we demonstrated a marked Th17 transcriptional signature from inflamed colonic tissue. This included elevated expression of the miR-29-targeted *Il12b* and *Il23a*, as well as the mRNA encoding cytokine IL-17A, and the Th17 subset-determining transcription factor RORγt. This contrasts with the transcription factors GATA-3, T-bet and Foxp3 which are essentially unchanged. Moreover, there is no general change in pro- or anti-inflammatory mediators between wild-type and mir-29 KO mice, with similar colonic expression of *Il1b*, *Tnfa* and *Il6* and *Il10*. In other murine models, miR-29 targets IFN-γ in NK cells, $CD4^+$ and $CD8^+$ T cells [68], and through targeting of T-bet and Eomes in T cells influences Th1 bias [69], but in our model we found no difference in IFN-γ expression or Th1 cell numbers in colonic tissue.

4 Conclusion

In summary, our data suggest that wild-type NOD2 has an important role, via the expression of miR-29, in ensuring that the critical balance of pro- and anti-inflammatory responses is maintained within the GI tract (Fig. 1). There is evidence that IL-23 expression is particularly prominent within the terminal ileum [70], which would align with the known phenotype of NOD2-associated Crohn's. We would propose that the defective expression of miR-29 observed in the presence of

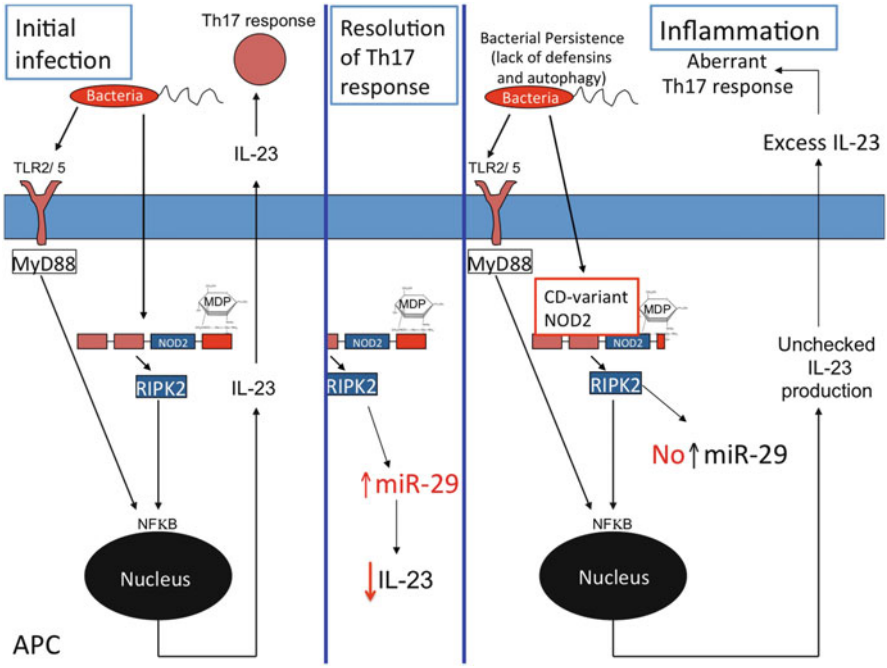

Fig. 1 A representation of the role of NOD2-stimulated miR-29 expression in antigen presenting cells (APCs) such as gastrointestinal DCs. In the *left-hand panel* the cell is challenged by bacterial infection and mounts an appropriate Th17 immune response. The *central panel* details appropriate resolution of this response through delayed miR-29 expression in the presence of wild-type NOD2. The *right-hand panel* depicts the problems that occur in the presence of CD-variant NOD2, with initial bacterial persistence due to defective autophagy, and a subsequently unchecked Th17 response due to lack of miR-29 up-regulation

CD-variant NOD2 should be placed in context of other NOD2 data [71]. In this model excess inflammation in the terminal ileum would result from a dysbiosis (possibly related to defensin deficiency), aberrant bacterial handling and persistence due to defective autophagy and an inability to appropriately arrest the resultant Th17 response due to deficient miR-29 expression.

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The Role of ncRNA in Diabetes

Fatjon Leti, Matthew Taila, and Johanna K. DiStefano

Abstract Diabetes mellitus represents a multifactorial, heterogeneous collection of disorders, characterized by a deficiency or failure to maintain normal glucose homeostasis. In patients with type 2 diabetes (T2D), the most common type of diabetes mellitus, elevated glucose levels arise from multiorgan insulin resistance and/or inadequate insulin secretion from pancreatic β-cells. The etiology of T2D is complex, and genetic predisposition, environmental exposures, and lifestyle factors are known to contribute to defects of glucose homeostasis, primarily in the pancreatic β-cells, liver, muscle, and adipose tissue. A role for small and long noncoding RNAs (ncRNAs) in the pathogenesis of T2D is also beginning to emerge, and there is already strong evidence of their involvement in the development of many of the pathophysiological mechanisms underlying the disease. This chapter summarizes the current state of knowledge of specific ncRNAs, particularly miRNAs, involved in the control of β-cell function and regulation of insulin sensitivity and/or action in peripheral organs. Although much less is known about lncRNAs in T2D, we provide an overview of the current literature in this field. Finally, we discuss the potential value of ncRNAs to serve as biomarkers for T2D development and clinical management of the disease.

1 Introduction

Diabetes mellitus represents a multifactorial, heterogeneous collection of disorders characterized by a deficiency or failure to maintain normal glucose homeostasis. In patients with type 2 diabetes (T2D), the most common type of diabetes mellitus, elevated glucose levels arise from multiorgan insulin resistance and/or inadequate insulin secretion from pancreatic β-cells [1, 2]. The prevalence of diabetes has increased sharply in recent decades and is estimated to currently affect over 300 million individuals worldwide [3]. The number of people with diabetes is expected to surpass 520 million by 2030 [4], and the disease is predicted to become

F. Leti • M. Taila • J.K. DiStefano (✉)
Diabetes, Cardiovascular and Metabolic Diseases Division, Translational Genomics Research Institute, 445 Fifth Street, Phoenix, AZ 85004, USA
e-mail: jdistefano@tgen.org

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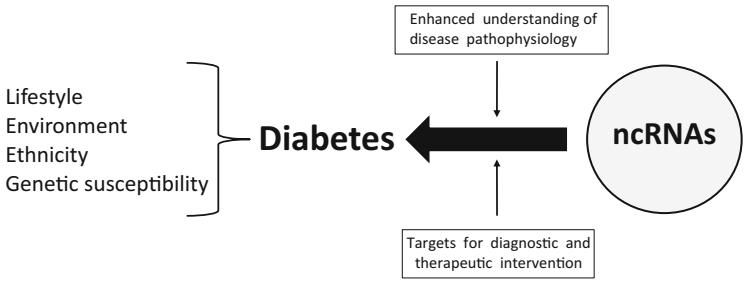

Fig. 1 Relationship between ncRNAs and diabetes pathophysiology

the seventh leading cause of death in 2030 [5]. It is widely recognized that T2D is a major public health concern worldwide.

The etiology of T2D is complex and multifactorial, and genetic predisposition, environmental exposures, and lifestyle factors mediate susceptibility to the disease [6]. Ongoing research to identify specific biological targets and signaling pathways relative to these factors represents an area of active focus, yet, despite these efforts, little is known of the molecular mechanisms by which environmental influences affect the pathogenesis of T2D in susceptible individuals. However, evidence emerging from recent clinical and basic science investigations supports a role for additional factors such as noncoding RNAs (ncRNAs) in the development of T2D, and it is possible that these molecules link environmental exposures with pathological mechanisms underpinning the disease (Fig. 1).

Noncoding RNAs are generally classified on the basis of transcript size, although there are additional differences in both biological function and molecular/cellular effects that also serve to distinguish among them. Small ncRNAs include a number of RNA species, most of which exert effects via association with the 5′ or 3′ regions of genes. The best-characterized members of this species are microRNAs (miRNAs), which are endogenous, single-stranded RNAs (21–25 nucleotides in length) that regulate gene expression either posttranscriptionally, by blocking translation or promoting cleavage of specific target mRNAs, or less frequently, transcriptionally, through targeting of the promoter region [7]. The dysregulation or mutation of miRNAs has been associated with the development of many diseases including neurologic disorders, cardiovascular diseases, and certain cancers [8, 9]. As discussed below, miRNAs are also critical in pancreatic development and play a role in biological processes related to T2D pathogenesis, including glucose-stimulated insulin secretion, glucose uptake in muscle and liver, adipogenesis, and adipocyte function; excellent reviews of the role of miRNAs in these pathophysiological elements of hyperglycemia and T2D have been recently published [10–12]. Altered levels of circulating miRNAs have also been documented in human diseases, including diabetes, and the potential of utilizing these molecules as biomarkers for diagnosis, prognosis, and clinical management is an area of active investigation [13].

The second class of ncRNAs, long ncRNAs (lncRNAs), is comprised of transcribed RNA molecules with much longer lengths than small ncRNAs, typically >200 nucleotides. Like mRNA molecules, lncRNAs are capped, spliced, and

polyadenylated; however, they do not lead to protein synthesis [14–16]. In contrast to miRNAs, which act through specific base pair recognition mechanisms to modulate the expression of target genes [17, 18], lncRNAs exert effects by binding to DNA, RNA, and protein to direct chromatin-modifying complexes to specific genomic loci, provide molecular scaffolds in the maintenance of nuclear infrastructure, modulate transcriptional programs, and regulate miRNA expression [19]. A number of biological functions have been assigned to lncRNAs, including the regulation of gene expression, genomic imprinting, maintenance of pluripotency, nuclear organization and compartmentalization, and alternative splicing [16, 19–21]. Like miRNAs, lncRNAs have been found to be dysregulated in human diseases, although the role these molecules play in the disease process is not well understood [22, 23].

The role of small and long ncRNAs in the pathogenesis of T2D is only recently beginning to emerge, but there is already strong evidence of their involvement in pathophysiological mechanisms underlying the disease. The goal of this chapter is to present an overview of the current state of knowledge of specific ncRNAs, particularly miRNAs, involved in the control of β-cell function and regulation of insulin sensitivity and/or action in peripheral organs. Although much less is known about lncRNAs in T2D, we also summarize the current literature in this field. Finally, we discuss the potential value of ncRNAs to serve as biomarkers for diabetes development and clinical management of the disease.

2 miRNAs Involved in β-Cell Development, Proliferation, and Function

Pancreatic β-cells are highly specialized endocrine cells located in the islets of Langerhans that function primarily to synthesize and secrete insulin in response to glucose stimulation. Loss of β-cell function due to autoimmune destruction or environmental factors leads to the development of diabetes mellitus. In addition to genetic and environmental factors, miRNAs are known to contribute to biological processes in the β-cell, including β-cell differentiation and proliferation, as well as insulin biosynthesis and secretion (Table 1).

The generation of pancreas-specific *Dicer1* knockout mice, which exhibit impaired pancreas development and reduced pancreatic β-cell mass, yielded the first insight into the involvement of miRNAs in β-cell development [35]. β-cell-specific *Dicer1* knockout reduced insulin gene expression and insulin secretion, which preceded the development of progressive hyperglycemia and diabetes [36]. *Dicer1*-depleted animals also showed altered islet cell morphology, reduced β-cell mass, and differential pancreatic islet morphology [36, 37]. Because Dicer1 is a member of the ribonuclease III family, and plays a significant role in the generation of miRNAs [38], these findings suggested that networks of miRNAs orchestrate the development, and regulate the function, of pancreatic β-cells.

Table 1 miRNAs involved in β-cell development and function

miRNA	Target	Effect	Reference
miR-9	OneCut2	Reduces glucose-induced insulin secretion	[24, 25]
miR-24	HNF1a, Neurod1	Increases β-cell proliferation, reduces insulin secretion	[26]
miR-29a/b	MCT1, OneCut2	Reduces insulin secretion	[27]
miR-124a	Foxa2, Creb1, pdx-1, Kir6.2, Sur-1, Preproinsulin	Increases β-cell differentiation, affects pancreatic development, influences glucose metabolism, and insulin secretion	[28]
miR-184	Slc25a22	Regulates insulin secretion	[29]
miR-187	HIPK3	Reduces glucose-induced insulin secretion	[30]
miR-375	PDK1	Reduces insulin gene expression, decreases β-cell mass, leads to β-cell failure	[31–34]

Dicer1 expression was recently shown to exhibit a tissue-specific diurnal pattern that is lost during both aging and diabetes [39]. Loss of Dicer1 rhythmicity resulted in altered circadian patterns of miRNAs, including miR-146a and miR-125a-5p. These findings not only demonstrated that diabetes affects the diurnal rhythmicity of Dicer1 expression, leading to effects on Dicer-controlled miRNAs, but also suggested that restoring Dicer1 activity may ameliorate some of the deleterious consequences of diabetes on specific miRNAs.

To date, one of the best-characterized miRNAs in pancreatic β-cell development and function is miR-375. Overexpression of miR-375 in pancreatic endocrine cells suppressed glucose-induced insulin secretion, while the inhibition of endogenous miR-375 expression led to increased insulin secretion [40]. Myotrophin was identified and validated as a miR-375 target, and inhibition of this gene mimicked miR-375 effects on glucose-stimulated insulin secretion and release. Reduction of another miR-375 target, 3′-phosphoinositide-dependent protein kinase-1 (PDK1), inhibited glucose-mediated effects on insulin gene expression [31]. In that study, glucose negatively regulated miR-375 expression while concomitantly increasing PDK1 levels. Mice deficient in miR-375 (375KO) exhibited hyperglycemia, increased total pancreatic α-cell numbers, increased gluconeogenesis and hepatic glucose output, and higher levels of fasting and fed glucagon [33]. In these animals, impaired β-cell proliferation resulted in decreased β-cell mass. Analysis of mRNA transcripts in 375KO islets identified a number of genes with roles in cellular growth and proliferation. In *ob*/*ob* mice, leptin-deficient animals that are commonly used as a model for T2D, miR-375 expression was increased, and miR-375 deletion in these animals reduced the proliferative capacity of the endocrine pancreas, leading to diabetes. Similarly, in islets of fed diabetic Goto-Kakizaki (GK) rats, a model for nonobese insulin resistance and T2D, miR-375 expression was decreased [31], and targeted knockdown of mature miR-375 resulted in the aberrant migration of pancreatic islet cells and malformation of the endocrine pancreas in zebrafish

[32]. Together, results from animal studies are consistent with a role for miR-375 in the development and function of the pancreatic β-cell.

In humans, T2D patients showed higher miR-375 expression in the pancreas compared to nondiabetic individuals [34]. In patients with T2D, increased miR-375 expression was associated with islet amyloid deposition, decreased β-cell mass, and reduced islet mitochondrial density. Islet amyloid deposition is a histological feature of pancreatic β-cell failure, but the mechanism by which miR-375 contributes to this derangement is not known. In miRNA profiling analyses of primary human islets and enriched β-cell preparations, high expression of several miRNAs, including miR-375, was observed, predominantly in β-cells [41]. Combined, the results reported to date provide strong evidence that miR-375 is essential for maintaining normal glucose homeostasis and contributing to pancreatic β-cell expansion in response to increasing insulin demands associated with insulin resistance and T2D [42].

Like miR-375, miR-187 also regulates genes involved in β-cell function. Expression of miR-187 is increased up to sevenfold in pancreatic islets of T2D patients compared to healthy controls and is inversely correlated with glucose-induced insulin secretion in normoglycemic individuals [30]. In primary cultures of rat islets and INS-1 cells, overexpression of miR-187 decreased glucose-induced insulin secretion without affecting insulin content [30]. Homeodomain-interacting protein kinase 3 (HIPK3), which plays a role in insulin secretion, was validated as a direct target of miR-187, and small reductions in HIPK3 expression via miR-187 interaction significantly increased glucose-stimulated insulin secretion. Levels of HIPK3 were also reduced in islets from patients with T2D, suggesting that miR-187 may exert effects on glucose-stimulated insulin secretion through mechanisms involving HIPK3. Additional functional studies will further clarify the role of this miRNA/mRNA relationship in the pathogenesis of T2D.

In addition to these miRNAs, miR-124a and miR-184 also affect β-cell function. MIN6 cells overexpressing or underexpressing miR-124a exhibited reduced or elevated levels of its target, Foxa2, respectively [28]. The authors observed that Creb1 and miR-124a directly interacted to regulate Foxa2, although the relationship between Creb1 and Foxa2 is not fully understood. Foxa2 protein levels were correlated with reduced expression of genes involved in β-cell function, insulin secretion, and glucose metabolism, including *pdx-1*, *Kir6.2*, *Sur-1*, and *Preproinsulin*, suggesting a regulatory role for miR-124a in diabetes pathogenesis via mechanisms including both direct and indirect regulation of its target mRNA [28]. miR-184, a miRNA showing high expression in pancreatic islets, significantly inhibited glucose-induced insulin secretion in a pancreatic β-cell line [29]. Slc25a22, which functions to export mitochondrial-synthesized glutamate in response to elevated glucose levels, was validated as a miR-184 target. Cytoplasmic glutamate targets insulin granules, promoting insulin exocytosis. miR-184 suppressed Slc25a22 expression, in turn reducing cytoplasmic glutamate and insulin secretion [29]. miRNA cluster miR-29 a/b/c regulates insulin secretion by selectively targeting the OneCut2 transcription factor and membrane monocarboxylate transporter (MCT1) in β-cells [27]. Increased expression of

miR-29 isoforms increased MCT1 transcription in β-cells in primary mouse islets, leading to differential insulin release. Further, insulin secretion was reduced in response to glucose in dissociated islets and MIN6 cells overexpressing miR-29a/b/c, and impaired insulin secretion was associated with downregulated OneCut2. Similarly, miR-9 mediates glucose-induced insulin secretion by inhibiting OneCut2 expression in β-cells [25]. Elevated miR-9 also diminished sirt1 expression in β-cells [24].

As an miRNA already expressed at high levels in β-cells, miR-24, was even further upregulated in islets from *db*/*db* mice and mice fed a high-fat diet [26]. Overexpression of miR-24 correlated with β-cell proliferation and reduced insulin secretion. Two known maturity-onset diabetes of the young (MODY) genes, *Hnf1a* and *neurod1*, were identified as miR-24 targets, and the inhibition of either gene yielded the same cellular phenotype as that seen with miR-24 overexpression, while restoring expression improved β-cell function. These results not only demonstrated that genes involved in the miR-24/MODY pathway may underlie the development of T2D but also suggested that overnutrition and genetic susceptibility may be linked through mechanisms involving this particular miRNA.

A recent microarray analysis to profile miRNAs in pancreatic islets of prediabetic and diabetic *db*/*db* mice and mice fed a high-fat diet identified two distinct categories of differentially expressed molecules [43]. miR-132, miR-184, and miR-338-3p exhibited expression changes in islets well before the onset of diabetes, while miR-34a, miR-146a, miR-199a-3p, miR-203, miR-210, and miR-383 showed differences mostly in diabetic mice. Expression changes in prediabetic animals exerted positive effects on β-cell activity and mass, and those in diabetic mice increased β-cell apoptosis. These findings suggested that obesity and insulin resistance produce changes in miRNAs that initially sustain β-cell function but that further deregulation in additional miRNAs lead to β-cell loss and the development of T2D [43]. These results also indicated that the maintenance of glucose homeostasis, or in contrast, the development of glucose intolerance, might be mediated by alterations in expression patterns of specific miRNAs.

The idea that a different number of miRNAs can interact in a combination to control glucose homeostasis is supported by an investigation of the Let-7 family of miRNAs in transgenic mice [44]. In an elegant set of experiments, the authors first generated transgenic mice with *Cre*-inducible activation of Let-7a, Let-7d, and Let-7f expression, which allowed the overexpression of miRNAs in a tissue-specific manner. Global and pancreas-specific overexpression of Let-7 led to the development of impaired glucose tolerance due to reduced glucose-stimulated insulin secretion from the pancreas. Global knockdown of the Let-7 family was sufficient to prevent and treat obesity-induced glucose intolerance, as well as restore insulin signaling in muscle and liver. Together, the results showed that Let-7 influences different aspects of glucose metabolism in multiple tissues, and suggested that the inhibition of Let-7 may improve pancreatic β-cell function. While it is tempting to speculate upon the potential of Let-7 knockdown as a potential treatment strategy for T2D, the precise role that this family of miRNAs plays in the pathogenesis of the disease remains to be determined.

A similar investigation of glucose-regulated miRNAs in pancreatic islets of nonobese T2D GK rats also found evidence supporting a network of regulatory miRNA molecules in processes involving insulin secretion [45]. In that study, incubation of isolated islets in different glucose concentrations and for different lengths of time showed distinct differences in the magnitude and direction of miRNA expression in hyperglycemic rats versus control animals. These results suggested differences in short- and long-term glucose dependence against the backdrop of genetic susceptibility. In GK rats, the expression of miR-130a, miR-132, miR-212, and miR-335 was regulated by hyperglycemia. Glucose regulation of miR-132 and miR-212 was also reported in MIN6 cells [46], and these two miRNAs were upregulated in pancreatic islets in obese mice [47], suggesting a common pathway for these ncRNAs in disease pathogenesis for both diabetic GK rats and obese mice models.

3 miRNAs and Regulation of Insulin Sensitivity in Peripheral Tissues

Glucose homeostasis is maintained by a balance between the amount of insulin released by pancreatic β-cells and action of the hormone at insulin-sensitive target tissues, including adipose tissue, skeletal muscle, and liver. In insulin resistance, the sensitivity of target tissues to the hormone decreases, leading to the development of hyperglycemia. Although the molecular mechanisms underlying the development of insulin resistance are not fully understood, factors such as age, obesity, diet, and hypertension are known to affect insulin sensitivity of target tissues. Ongoing research also suggests that the dysregulation of miRNA expression or action may contribute to some of the pathology related to the development of insulin resistance (Table 2).

A role for miRNAs in the maturation of human adipocytes was postulated over a decade ago. A pioneering investigation of this relationship identified miR-143 as a promoter of adipocyte differentiation [56]. Subsequent studies verified the role of miR-143 in adipogenesis and also suggested a role for the miRNA in the regulation of lipid metabolism [57, 58]. Overexpression of miR-143 is associated with increased levels of adipocyte differentiation markers, including CCAAT/enhancer binding protein (C/EBP)-β, adipocyte fatty acid-binding protein 4 (FABP4), and leptin in preadipocytes [49, 56, 59], whereas the inhibition of miR-143 expression correlates with reduced levels of adipogenesis and adipocyte differentiation [56, 58, 59]. Recently, miR-143 was found to regulate adipogenesis by directly inhibiting MAP2K5 [48]. Combined, these studies identified miR-143 as a key regulator of adipocyte differentiation and, as discussed below, demonstrated that the dysregulation of its expression contributes to the development of diet-induced insulin resistance.

Table 2 miRNAs and regulation of insulin sensitivity in peripheral tissue

miRNA	Tissue	Effect	Reference
miR-143	Adipose	Promotes adipogenesis and lipid metabolism	[24, 25, 48, 49]
	Liver	Downregulates insulin stimulated AKT activation and impairs glucose metabolism	[50, 51]
miR-29a/b/c	Adipose	Decreases insulin-stimulated glucose uptake	[26]
miR-29a	Liver	Prevents insulin-mediated inhibition of PEPCK gene	[52]
miR-103/107	Adipose	Regulates insulin receptor	[49]
	Liver	Regulates gluconeogenesis and insulin signaling	[49]
miR-320	Adipose	Improves insulin sensitivity	[27]
miR-1/miR-133	Muscle	Promotes differentiation and proliferation of myoblasts	[53–55]

As noted above, miR-29a/b/c plays a known role in insulin signaling through mechanisms involving MCT1 [27, 60]. In diabetic rats, the expression of miR-29a/b/c was elevated in muscle, fat, and liver [61], and in the liver of *fa/fa* rats, an animal model for obesity, and high-fat diet-fed mice [62]. Overexpression of miR-29a/b/c in 3T3-L1 adipocytes decreased insulin-stimulated glucose uptake by inhibiting p85alpha and AKT activation [61, 63], and led to insulin resistance, but through mechanisms not involving AKT as the direct target of the miRNA [61]. Overexpression of miR-29a in HepG2 cells prevented insulin-mediated inhibition of phosphoenolpyruvate carboxykinase gene (PEPCK) by repressing the p85alpha, the upstream intermediate of AKT [52].

miR-320 has also been shown to play a role in insulin sensitivity. In insulin-resistant 3T3-L1 adipocytes, miR-320 expression was 50-fold greater than in normal adipocytes [64]. Conversely, the inhibition of miR-320 in insulin-resistant adipocytes resulted in improved insulin sensitivity. The p85 subunit of phosphatidylinositol 3-kinase (PI3-K) was identified as a potential miR-320 target, and the inhibition of the miRNA resulted in increased p85 expression, insulin-stimulated glucose uptake, phosphorylation of AKT, and expression of the glucose transporter type 4 (GLUT-4) [64]. Interestingly, these changes were only observed in insulin resistant adipocytes, suggesting that the dysregulation of insulin action leads to alterations in the miR-320/p85 pathway that further exacerbate the condition of insulin resistance.

In a comparison of miRNA expression profiles in skeletal muscle biopsies from healthy individuals before and after a 3-h euglycemic–hyperinsulinemic clamp, expression of miR-1, miR-133a, and miR-306 was downregulated by insulin [54]. miR-1 and miR-133 are located in the same chromosomal locus, but while miR-1 inhibits myoblast proliferation, miR-133 enhances myoblast growth, resulting in differentiation and proliferation [53]. Both miRNAs are directly activated by myocyte enhancer factor 2C (MEF2C), a major regulator of muscle development [55], and in human cells, insulin downregulates miR-1 and miR-133

via the repression of MEF2C and the activation of sterol regulatory element binding protein 1c [54]. In streptozotocin-treated mice, a model of diabetes, levels of miR-1 and miR-133 were significantly higher than those in control animals [54]. However, miR-133 expression was not altered in healthy human subjects during a hyperglycemic–euglycemic clamp, showing that hyperglycemia, in the absence of hyperinsulinemia, did not affect the levels of this miRNA [54]. In contrast, skeletal muscle expression of miR-133a was found to be significantly different among individuals with normal glucose tolerance, impaired glucose tolerance, and T2D, and an association between higher fasting glucose levels and lower miR-133a expression was also observed in these patients [65].

In a systematic analysis of ncRNAs in human muscle insulin resistance, 62 out of 171 miRNAs showed differential expression in muscle from individuals with T2D, and approximately 15 % of upregulated and downregulated miRNAs were altered early in the disease process [65]. Six canonical signaling pathways, including ones related to insulin resistance and/or muscle metabolism, were identified based upon the genes ranked most strongly as potential targets. The authors postulated that it is the combinatorial nature of miRNA action in vivo that produces significant changes in target protein levels, thereby contributing to the development of insulin resistance and T2D. In another comprehensive study, the expression of 283 miRNAs was measured in adipose tissue, skeletal muscle, and liver from hyperglycemic (GK), intermediate glycemic (Wistar Kyoto), and normoglycemic (Brown Norway) rats [66]. Out of 49 differentially expressed miRNAs across the three tissues, only five exhibited levels that correlated with the degree of glycemia. Specifically, miR-222 and miR-27a were upregulated in adipose tissue, miR-195 and miR-103 were upregulated in liver, and miR-10b was downregulated in muscle. Similar patterns of expression for miR-222, miR-27a, and miR-29a were also observed in 3T3-L1 adipocytes cultured under hyperglycemic conditions, suggesting that altered miRNA expression may occur early in the pathogenesis of T2D.

In the body, the liver is the main site for gluconeogenesis, which is suppressed by insulin under normal conditions and becomes dysregulated in individuals with insulin resistance and T2D. Several studies have recently demonstrated that not only is miRNA expression altered in the livers of animal models of obesity, hyperglycemia, and insulin resistance but also that restoring miRNA levels may improve glucose homeostasis and insulin sensitivity. An examination of liver-specific miRNA expression in *ob*/*ob* and diet-induced obese mice identified miR-103/miR-107 as two of the most significantly upregulated miRNAs [49], validating the findings from hyperglycemic GK rats [66]. Of note, the sequences of mature miR-103 and miR-107 differ only at one nucleotide and are thus nearly indistinguishable from each other.

The expression of miR-103 and miR-107 was increased in liver biopsies from individuals with nonalcoholic fatty liver disease, which is often part of the metabolic derangement seen in patients with T2D; further, the levels of these miRNAs were positively correlated with homeostatic model assessment (HOMA) index, a measure of insulin resistance [49]. Hepatic overexpression of miR-107 produced excess glucose output through a mechanism involving increased gluconeogenesis. Global silencing of miR-103 and miR-107 resulted in increased insulin signaling in

both liver and adipose tissue, although liver-specific inhibition of expression in obese and insulin-resistant conditions was not able to compensate for the observed metabolic abnormalities. Caveolin-1, which regulates the insulin receptor, was identified as a direct target of miR-103/miR-107, and its expression was upregulated by the inhibition of the miRNAs in adipocytes, which was also accompanied by the stabilization of the insulin receptor, increased insulin signaling, decreased adipocyte size, and enhanced insulin-stimulated glucose uptake. Further studies to characterize the precise mechanism by which the miR-103/107–caveolin interaction affects insulin signaling will have important implications for the development of these miRNAs as targets for T2D treatment [49].

Sequencing studies of liver ncRNAs have also identified miRNAs important for insulin sensitivity and impaired glucose metabolism. Sequencing of small RNA molecules from the livers of mice that had varying levels of access to food (i.e., free access, food-restricted, or fasted/refed) found that out of 32 confirmed miRNAs, only miR-143 varied with feeding conditions [50]. Expression of miR-143 was similarly upregulated in livers of *db*/*db* mice and mice fed a high-fat diet. miR-145, which is located in the same gene cluster as miR-143, was also upregulated in the livers of these two animal models. Conditional overexpression of miR-143 in transgenic mice disrupted insulin-stimulated AKT activation and impaired glucose metabolism, while the inhibition of miR-143 protected mice from diet-induced insulin resistance and AKT activation [50]. Oxysterol-binding protein-related protein 8 (ORP8) was identified as a miR-143 target, and decreased ORP8 expression in liver impaired insulin-induced AKT activation. Sequencing of liver ncRNA in *ob*/*ob* and control mice identified 37 differentially expressed hepatic miRNAs [51]. Although miR-122 showed the greatest alteration in expression between the two groups, miR-24, miR-195a, miR-106b, miR-15b, miR-802, miR-185, miR-214, miR-378, and Let-7c were also significantly upregulated. In contrast, levels of miR-224, miR-126, miR-7a, miR-128, miR-455, miR-452, miR-135b, miR-145, miR-18a, and miR-196a were significantly downregulated.

4 lncRNAs and T2D

The extent of investigation of lncRNAs in T2D, in contrast to miRNAs, is relatively limited; however, the cell-specific expression patterns of these molecules may yield deeper insights into defects in specialized cellular functions. A recent genome-wide search for human β-cell lncRNAs identified more than 1,000 intergenic and antisense islet-cell lncRNAs of which 55 and 40 % were islet-specific [67]. Nearly all of the examined lncRNAs were silent or expressed at low levels in pancreatic progenitors, but active in adult islets, suggesting that lncRNAs play a role in pancreatic endocrine differentiation. Similarly, during in vivo differentiation of human embryonic stem cells, six lncRNAs were expressed at very low or undetectable levels throughout all in vitro differentiation steps and were only activated during the in vivo maturation step [67]. In a comparison of 14 islet-specific lncRNAs,

KCNQ1OT1 and *HI-LNC45* were significantly increased or decreased in T2D islets, respectively, and out of 55 T2D susceptibility loci, nine contained islet lncRNAs within 150 kb of the reported lead marker, six of which have been linked directly to β-cell dysfunction [68–72].

Other lncRNAs have been found to harbor genetic variants associated with T2D, the most notable of which is the *ANRIL* locus [22]. This lncRNA maps to the *INK4* locus, which encodes three tumor suppressor genes, including $p15^{INK4B}$. *ANRIL* is required for the silencing of this tumor suppressor gene [73], and it is possible that variants that disrupt the expression or function of this lncRNA may affect compensatory increases in pancreatic β-cell mass in response to increasing demands for insulin in the pre-diabetes state [74].

The discovery of dysregulated islet-specific lncRNAs adds a new layer of complexity to the molecular etiology of T2D. The studies reported thus far, although limited in number, not only point to a role for lncRNAs in the regulation of β-cell identity and function, but also suggest that variants in islet-specific lncRNAs contribute to β-cell physiology and T2D. Functional characterization of islet-specific lncRNAs is underway [67], although a substantial amount of work is needed to understand the relative importance of these molecules in the pathogenesis of T2D. These findings, in combination with emerging results, are expected to yield new insights into the complex pathogenesis of T2D and may eventually lead to the identification of novel islet-specific therapeutic targets with limited effects in other cell types.

5 ncRNAs as Biomarkers for Diabetes

T2D is a progressive disease with a long, asymptomatic development, resulting in delayed diagnosis and early morbidity and mortality [2]. For those individuals at high risk for developing T2D, early identification would enable lifestyle and/or pharmacological interventions to delay or prevent disease development [75, 76]. Characteristics such as age, family history, body mass index, and waist circumference are already used to predict the development of T2D and facilitate the identification of individuals at risk for developing the disease [77–79]. However, the clinical utility of models based upon classical risk factors to predict disease development is somewhat limited [77], and other methods for improving the prediction of T2D risk are currently being explored. Emerging evidence is beginning to show that miRNAs underlie, at least in part, many of the biological mechanisms that lead to β-cell dysfunction and defects in insulin secretion and action, which presents opportunities to not only augment our understanding of the pathophysiology of T2D, but also lead to the identification of novel diagnostic biomarkers of the disease.

Chen et al. [80] provided the first evidence that individuals with T2D have an altered serum miRNA profile compared to healthy individuals. In this study, serum miRNA was sequenced in Chinese individuals with lung cancer, colorectal cancer,

and diabetes, and specific expression patterns for each disease, compared to normal, healthy controls, were identified. Interestingly, an overlap of 23 miRNAs was observed between lung cancer and T2D patients, suggesting that a change in expression of these miRNAs may represent a general inflammatory response shared between the two diseases. In a comparison of miRNAs derived from serum or blood, the authors identified 84 common miRNAs; however, 17 and 27 miRNAs were identified only in serum or blood, respectively, suggesting that serum-derived miRNAs more accurately represent T2D. Although this study was the first investigation of miRNAs as potential classifiers of T2D, no specific miRNAs emerged as potential biomarkers of disease; this information would come later as a result of three pioneering studies of circulating miRNA profiling for the identification of disease biomarkers. The first study identified a plasma miRNA profile for T2D comprised of five mRNAs: miR-15a, miR-29b, miR-126, miR-223, and miR-28-3p [81]. In 19 individuals who developed T2D over the 10-year follow-up period, baseline levels of miR-15a, miR-29b, miR-126, and miR-223 were lower, while those of miR-28-3p were higher, compared to controls. Using this miRNA signature, 92 % of controls and 70 % of T2D cases could be correctly classified, while 52 % of normoglycemic individuals who developed T2D over the follow-up period were already classified as diabetic prior to the onset of the disease.

The second study focused exclusively on seven miRNAs negatively associated with the expression, production, secretion, or effectiveness of insulin [82]. Levels of miR-9, miR-29a, miR-30d, miR-34a, miR-124a, miR-146a, and miR-375 were measured using RT-PCR in 18 individuals with newly diagnosed T2D, 19 individuals with impaired glucose tolerance and/or impaired fasting glucose, and 19 T2D-susceptible individuals with normal glucose tolerance. Serum levels of all miRNAs were elevated in T2D patients compared with normoglycemic individuals, while five were upregulated compared to the pre-diabetes group; however, miRNA levels were not significantly different between the pre-diabetes and the normoglycemic groups. Hierarchical clustering analysis showed that ~70 % individuals with diabetes could be clustered together, which indicated slightly better recognition than single miRNA analysis. However, the miRNA panel could not discriminate between pre-diabetic and normoglycemic individuals, thereby limiting the clinical applicability of this profile in the differential diagnosis of the two groups.

The third study compared miRNA expression in blood, pancreas, liver, adipose tissue, and skeletal muscle from male Wistar rats treated with low dose streptozotocin and high fat diet with similar untreated animals fed a normal fat diet and found miR-146a, miR-182, miR-30d, miR-144, miR-150, miR-192, miR-29a, and miR-320a to be among the most significantly dysregulated miRNAs across all five sources [83]. In humans, these eight miRNAs showed similar expression patterns in blood from patients with T2D and impaired fasting glucose. Of these, miR-192, miR-29a, and miR-144 expression was linearly correlated with increasing glycemic status. Elevated circulating miR-144 levels corresponded with decreased levels of a putative target, insulin receptor substrate 1, at both the mRNA and protein levels [83].

More recently, serum levels of over 700 miRNAs were assessed in pooled samples of 13 individuals with T2D, 20 obese patients, 16 obese patients with T2D, and 20 healthy controls, and of these, miR-138, miR-15b, and miR-376a were found to distinguish obese patients from normal, diabetic, and obese diabetic individuals, while levels of miR-503 and miR-138 could discriminate between diabetic and obese diabetic individuals [84]. Because not all obese patients develop T2D, these results may be useful for identifying those individuals who are at greater risk of developing metabolic disease as a result of obesity. Another study identified ten circulating miRNAs in six men with normal glucose tolerance and six with T2D, and validated levels in an extended sample of 45 individuals with normal glucose tolerance and 48 diabetic patients [85]. Levels of miR-140-5p, miR-142-3p, and miR-222 were increased, while levels of miR-423-5p, miR-125b, miR-192, miR-195, miR-130b, miR-532-5p, and miR-126 were decreased in individuals with T2D. Decreased and increased plasma levels of miR-140-5p and miR-423-5p, respectively, accounted for approximately 49 % of fasting glucose variance in nonobese individuals after controlling for age and BMI. These miRNAs along with miR-195 and miR-126 were specific for T2D with a diagnostic accuracy of ~89 % [85]. In addition, in seven healthy volunteers, insulin infusion during clamp reduced miR-222 levels by ~62 %, while insulin plus intralipid/heparin infusion significantly increased circulating concentrations of miR-222 (163 %), miR-140-5p (67 %), and miR-195 (165 %). To determine whether these miRNAs were modified by insulin sensitization, the authors compared levels of miRNAs at baseline and after 3 months of treatment with metformin. In the individuals treated with metformin, levels of miR-140-5p and miR-222 decreased, while those of miR-142-3p and miR-192 increased [85]. These longitudinal findings suggest that circulating T2D-related miRNAs may be modulated by pharmacological strategies aimed at improving insulin sensitivity.

A recent study investigating a panel of 14 miRNAs previously associated with metabolic measures in Swedish or Iraqi patients with T2D found that plasma levels of miR-24 and miR-29b were significantly different between cases and controls [86]. Following stratification by ethnicity, miR-144 expression was found to be significantly associated with T2D in Swedish, but not Iraqis [86], providing evidence for population-specific effects.

A number of studies have also focused on individual circulating miRNAs. For example, basal levels of miR-155 and miR-146a in peripheral blood mononuclear cells were found to be decreased in 20 patients with T2D relative to 20 unaffected controls and in these individuals, both miRNAs were significantly correlated with glucose, HbA1c, and BMI [87]. In contrast, plasma miR-146a levels showed elevated expression in 90 patients newly diagnosed with T2D compared with 90 age and sex-matched controls [88]. These results are concordant with those reported earlier [82]. Notably, individuals in the highest tertile of miR-146A levels also showed a much higher risk for T2D relative to patients in the lowest tertile. A third study investigated a plasma miRNA signature comprised of miR-29b, miR-28-3p, miR-15a, miR-223, and miR-126 (previously reported by [81]) in 30 T2D patients, 30 T2D-susceptible individuals, and 30 unaffected controls

Table 3 Changes in circulating miRNA levels associated with T2D

Population	Phenotype	Source	Major miRNAs identified	Reference
Chinese	T2D	Serum	Not known	[80]
Italian	T2D	Plasma	miR-15a, miR-28-3p, *miR-29b*, *miR-126*, miR-223	[81]
Han Chinese	IGT/IFG[a], T2D, s-NGT[b]	Serum	miR-9, *miR-29a*, *miR-30d*, miR-34a, miR-124a, *miR-146a*, miR-375	[82]
Singapore	IFG, T2D	Blood	*miR-29a*, *miR-30d*, *miR-144*, *miR-146a*, miR-150, miR-192, miR-192, miR-320	[83]
Spanish	T2D, obese	Serum	miR-138, miR-503	[84]
Spanish	T2D	Plasma	miR-125b, *miR-126*, miR130b, miR-140-5p, miR-142-3p, miR-192, miR-195, miR-222, miR-423-5p, miR-532-5p	[85]
Iraqi, Swedish	T2D	Plasma	miR-24, *miR-29b*, *miR-144*	[86]
Mexican	T2D	PBMCs	*miR-146a*, miR-155	[87]
Han Chinese	New-T2D	Plasma	*miR-146a*	[88]
Han Chinese	IFG, T2D	Plasma	*miR-126*	[89]

Italic font represents miRNAs identified in more than one study
[a]Impaired glucose tolerance; impaired fasting glucose
[b]T2D-susceptible, normal glucose tolerance

[89]. Of these miRNAs, only miR-126 showed altered levels between T2D and T2D-susceptible individuals compared to the control group. Interestingly, neither miR-29b nor miR-28-3p was detected, and miR-15a and miR-223 showed comparable levels among groups.

As shown in Table 3, there is only minimal overlap among study findings of circulating miRNAs in individuals with T2D. Differences in study design, such as serum vs. plasma, pools vs. individual samples, sample size, ethnicity, clinical variability between case and selected controls, statistical evaluation, and experimental approaches may underlie most of the discrepancies among these studies. Among the identified miRNAs, miR-126 and miR-146a were validated across at least two different studies. Decreased levels of miR-126 were corroborated between studies [81, 85], and were found to be the only miRNA that showed significantly decreased expression in T2D-susceptible and T2D patients compared to healthy controls [89]. Likewise, increased plasma levels of miR-146a were found in patients with newly diagnosed T2D [88], while decreased levels of changes in miR-146a in peripheral blood mononuclear cells were associated with T2D [87], insulin resistance, poor glycemic control, and subclinical inflammation [90]. Many of the other miRNAs identified in these profiling studies have also been previously implicated in T2D-related conditions. For example, altered levels of miR-142-3p, miR-140-5p, and miR-222 have been observed in morbid obesity [91]. Similarly, miR-222 shows increased expression in response to stress [92] and in internal

mammary artery segments from patients with T2D [93]; in these individuals, miR-222 levels correlated inversely with metformin dose in T2D, consistent with downregulatory effects of the drug on this miRNA [93]. More recently, miR-222 was identified as a potential regulator of estrogen receptor alpha in estrogen-induced insulin resistance in gestational diabetes, making it a candidate biomarker and therapeutic target for this disease [94].

Although the current findings are promising, these investigations relied upon relatively small cohorts, and validations in larger study samples with diverse ethnic representation using standardized study designs are necessary before conclusions about clinical relevance can be drawn. It is also worth noting that while circulating miRNAs may have clinical utility as biomarkers, they do not provide information with regard to miRNA deregulation inside cells, so the functional roles and the significance of miRNAs deregulated in T2D still need to be determined. Despite this limitation, the findings obtained to date may have potential applications for diabetes classification, prognosis, and assessment of therapeutic efficacy.

6 Conclusions

In T2D, a significant number of miRNAs have emerged as key players in the regulation of biological processes relevant to the disease; however, much less is known of the specific targets of candidate molecules, and how, in fact, they affect disease development in susceptible individuals. Studies aimed at delineating specific miRNA/mRNA networks will enhance our understanding of the complex pathogenesis of T2D and enable the exploitation of relevant miRNAs as novel targets for therapeutic interventions. Notably, the identification and validation of circulating miRNA signatures may facilitate the development of improved methods for diagnosis and clinical monitoring of disease progression. At present, current findings, combined with the rapidly expanding field of ncRNA research, are expected to yield new insights into the complex pathogenesis of T2D and may eventually lead to the identification of novel biomarkers for the disease.

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ncRNA as Diagnostics and Prognostics for Hepatocellular Carcinoma

Jun Zhao and Matthew W. Lawless

Abstract Hepatocellular carcinoma (HCC), the most common form of primary liver cancer, is the sixth most common cancer in the world with an estimated over half a million new cases annually. Due to the difficulty in early diagnosis, poor prognosis and lack of effective treatment options, HCC is currently ranked as the second most common neoplastic-related death with a significantly low 5-year survival rate of 6–11 % worldwide. Non-coding RNAs (ncRNAs) are genes that are frequently transcribed without protein-coding ability. Two major subsets of ncRNAs, microRNAs (miRNA) and long non-coding RNAs (lncRNA), are considered essential components at multiple levels in gene regulation processes including transcription, post-transcription and translation. The aberrant expression of ncRNAs has been shown to play an important role in many diseases including HCC. ncRNAs are abundant and stable; these fundamental characteristics make them candidates as diagnostic and prognostic biomarkers with wide reaching potential. Here we review the current status of diagnostic, prognostic and therapeutic biomarkers for HCC.

1 Introduction

Liver cancer is one of the top six cancers in the world with 782,000 (5.5 % of all cancers) new cases diagnosed in 2012 ([1], IARC). Hepatocellular carcinoma (HCC) is the most common form of liver cancer. HCC can develop under the influence of one or a combination of several risk factors such as infectious hepatitis B virus (HBV) [2] or hepatitis C virus (HCV) [3], obesity [4], alcoholic and non-alcoholic fatty liver disease (AFLD and NAFLD) [5, 6], genetic components (hereditary haemochromatosis and Z-alpha-1 antitrypsin deficiency) [7–13] and aflatoxin [14]. Due to the difficulty in early diagnosis and lack of effective treatment options, liver cancer ranks as the second most common neoplastic-related

J. Zhao • M.W. Lawless (✉)
Experimental Medicine, School of Medicine and Medical Science, University College Dublin, Catherine McAuley Centre, Mater Misericordiae University Hospital, Dublin 7, Ireland
e-mail: matthew.lawless@ucd.ie

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death with a generally low 5-year survival rate of less than 11 % worldwide. While the incidence of HCC in children is low (0.5 % of all paediatric carcinomas), the 5-year survival rate and event-free paediatric survival rates are 28 % and 17 %, respectively [15]. Therefore, identification of novel approaches for HCC diagnostics and prognostics with effective treatment options is imperative.

It has been 20 years since the first small non-coding RNA (ncRNA) (lin-4) was discovered [16, 17]. Today, it is accepted that throughout the human genome, the majority of genes lack protein-coding ability. Genes that are frequently transcribed without protein-coding ability are defined as ncRNAs and can be categorised into several subsets consisting of microRNA (miRNA), small nucleolar RNA (snoRNA), piwi-interacting RNA (piRNA), small interfering RNA (siRNA) and long non-coding RNA (lncRNA) (Fig. 1). Mainly due to the development of current technologies (e.g. high throughput RNA sequencing techniques), scientists now have the capacity to simultaneously identify and investigate a multitude of ncRNAs in great detail at a functional level and in so doing identify their specific contribution to the pathogenesis of disease [18]. ncRNAs are now considered as key players in human diseases including neurological disorders, cardiovascular malfunction and carcinogenesis. Accumulating evidence has demonstrated a role for ncRNAs in HCC [19], principally miRNAs and lncRNAs. However, other ncRNAs are also likely to have significance in the pathogenesis of cancer and should not be underestimated (Table 1); for example, snoRNAs and piRNAs can have telomerase activity and epigenetic modification function, while recently it was reported that some small ncRNAs 60–300 nucleotides in length derived from snoRNAs have similar functions to miRNAs [20]. In this chapter, the diagnostic and prognostic potential of miRNAs and lncRNAs for HCC are reviewed.

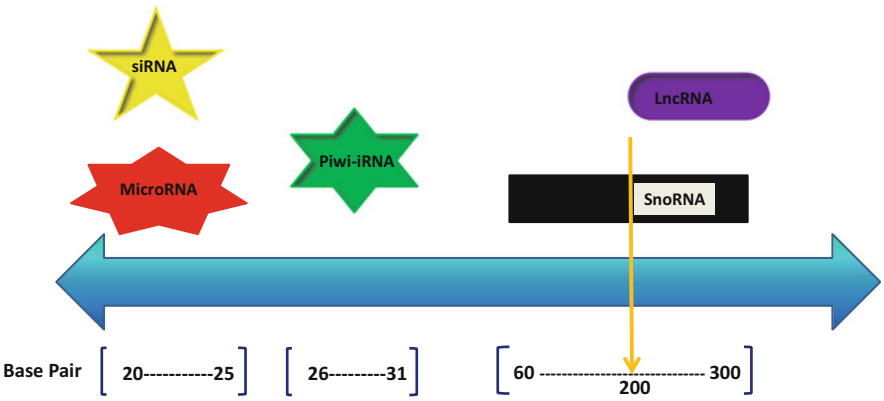

Fig. 1 Size scale overview of ncRNA families. MicroRNAs, siRNAs, piwi-iRNAs, snoRNAs and lncRNAs are defined in a size scale map in a small to large bidirectional fashion

Table 1 Examples of ncRNAs

ncRNAs	Functions
snoRNA	Mainly regulates target genes via post-transcriptional modification including 2′-O-methylation and pseudouridylation. Telomerase regulation
siRNA	Post-transcriptional gene silencing to repress and activate target genes
piRNA	Transcription and post-transcriptional regulation and epigenetic modification
microRNA	Gene regulation at post-transcriptional and translational level
lncRNA	Epigenetic regulation, gene regulation and protein modification

2 miRNAs and HCC

miRNAs are evolutionarily conserved small non-coding transcripts (~22 nucleotides in length) that are matured during a complex biogenesis process. Following their initial transcription by RNA Polymerase II, primary miRNAs (pri-miRNAs) are processed by a protein complex (Drosha-DGCR8) in the nucleus to become pre-miRNAs. They are then exported to the cytoplasm by exportin 5. Pre-miRNAs are further cleaved by the protein complex Dicer-TRBP (transactivation-response RNA-binding protein) to form miRNA–miRNA duplexes. Subsequently, one strand of the duplex is removed interacting with Argonaute proteins to form a miRNA-induced silencing complex (miRISC).

While the first miRNA was discovered in 1993, their significance was not fully appreciated until 10 years later [21]. miRNAs are now known to be fundamental elements that are involved in gene regulation both at the post-transcriptional and translational level. miRNAs can regulate gene expression during post-transcription by repressing the translation of messenger RNA (mRNA). Notwithstanding, the aberrant expression of miRNAs has been demonstrated in a range of human diseases including cancer [22]. Numerous experimental studies have revealed mechanistic roles for miRNAs and their potential therapeutic targeting in HCC [23–26]. The role of miRNAs in the onset and progression of HCC including invasion, metastasis and apoptosis has been recently reviewed [27, 28]. The detectable abnormality of miRNAs in human disease makes them perfect candidates for the prediction and management of treatment outcomes for HCC patients.

3 Diagnostics: From the Big World to the Small World

The history of diagnosis can be rooted as far back as 300 BC. Hippocrates, ‘The Father of Medicine,’ known as the inventor of disease diagnosis examined patients’ disordered body fluids. The use of ‘Uroscopy (using urine as an aid in diagnosis)’ became a central concept in European medicine by 1300 AD. By 1600, the microscope as a technology became part of the diagnostic arsenal as a powerful diagnostic tool right up to today. The explosion of modern technology has elevated the diagnostic world to the next level, whereby sample requirement has shrunk,

hand in hand with faster highly sensitive techniques that can yield a plethora of clinical biochemistry data. For example, ncRNAs analysis in cancer is a landmark technology in the continuous development of diagnostics.

4 Modern Clinical Biochemistry Diagnostics

Currently cancer biochemistry analysis includes blood chemistry, along with a complete blood cell count. Typically cytogenetic analysis with genetic testing for specific mutations (e.g. single nucleotide polymorphism (SNP)), hereditary cancer risk and counselling are also performed. Immunophenotyping, sputum cytology, tumour marker tests, urinalysis and urine cytology are also available as diagnostic aids. Molecular diagnostics form a large part of the day-to-day clinical decision, where most of these clinical tests require biopsy and blood/urine samples from patients. Improving on the battery of tests that are available on non-invasive samples will no doubt lead to better diagnostic and prognostic information enabling an improvement in clinical cancer care.

miRNA multiplex profiling can detect and quantify the expression of multiple miRNAs simultaneously, with data showing its usefulness in lung cancer, prostate carcinoma and colon adenoma diagnosis [29–31]. Recently, a DNA-modified gold nanoparticle-based bio-barcode assay was developed to detect miRNA expression at ultra-low levels without the need for polymerase chain reaction amplification for cancer diagnosis. This and other highly sensitive methods will no doubt revolutionise diagnostic and prognostic analyses [32].

While genetic testing solely on DNA may provide us with specific information regarding the potential risk factors for developing certain type(s) of cancer, the data obtained is not always useful for the detection and validation of cancer. Recent studies have shown that mutations in miRNAs at the very early maturation stages are independent biomarkers for cancer even in patients without the typical carcinogenic gene mutations [33, 34]. ncRNAs are potentially useful biomarkers as their expression can be variable depending on various carcinogenic associated microenvironments, for example, elucidating the phenomenon underlying cell–cell communication and drug response. Furthermore, considering the rise in ncRNAs as stand-alone factors in carcinogenesis, sequencing of the epigenome in order to identify mutations in multiple stages of disease may provide a better picture for accurate clinical determination.

5 Diagnostic Role of miRNAs in HCC

The common diagnostic methods for HCC include detection of serum α-fetoprotein (AFP) and imaging techniques such as abdominal ultrasonography, magnetic resonance imaging (MRI), computed tomography (CT) and angiograms (Fig. 2a, [35]). However, a limited sensitivity of the AFP test and the high cost of imaging

techniques can limit the possibility of early diagnosis of HCC. An early profiling study has revealed that among 200 precursor and mature miRNAs tested in HCC tissue and adjacent benign liver tissue samples, 16 mature miRNAs were at least twofold upregulated or downregulated in HCC tumour compared with adjacent benign liver tissue [36]. Furthermore, recent retrospective clinical studies have revealed other novel biomarkers for HCC. For example, miR-16 expression was significantly lower in patients with HCC compared to chronic liver disease patients and healthy controls [37]. Moreover, 69.2 % of HCC-positive patients with negative results from conventional markers showed positive results with miR-16, including many with a tumour size of less than 3 cm. The combination of miR-16, AFP, lens culinaris agglutinin-reactive AFP (AFP-L3%) and des-γ-carboxyprothrombin (DCP) had an overall sensitivity of 92.4 % and specificity of 78.5 % for HCC diagnosis. Thus miR-16 is a strong candidate biomarker for the diagnosis of HCC even at an early stage. Therefore, this may suggest its use as a second line of testing increasing the accuracy of the results obtained. Tables 2 and 3 list diagnostic and prognostic miRNA biomarkers for HCC.

6 LncRNAs in HCC

While miRNAs are well studied in the context of their roles in human disease, our knowledge of lncRNAs is less detailed. LncRNAs are larger ncRNAs (>200 base pair) that are expressed in various species. LncRNAs can be subcategorised into four groups based on their location of expression [52, 53]: (1) sense/antisense lncRNAs—with the same or complementary sequence on the same or opposite strand of a transcript. (2) Bidirectional lncRNAs—located at the opposite strand near the transcription initiation site of a transcript. (3) Intronic lncRNAs—embedded within an intron of a transcript. (4) Intergenic lncRNAs—located between the genomic intervals of two transcripts. Currently, there are estimated to be over 30,000 lncRNAs detectable in the human genome.

LncRNAs were initially discovered as transcriptional noise. Interestingly, recent breakthroughs have identified their key biological roles in regulating many essential target genes that are involved in many human diseases [54, 55]. Mounting evidence has suggested a critical role for lncRNAs in the onset and progression of HCC [56].

Highly upregulated in liver cancer (HULC) was the first liver specific lncRNA identified to be associated with HCC [57]. Since then, accumulating studies have revealed dysregulation of several lncRNAs including Hox transcript antisense intergenic RNA (HOTAIR), H19, maternally expressed gene 3 (MEG3), high expression in HCC (HEIH), downregulated expression by HBx (Dreh), microvascular invasion in HCC (MVIH), low expression in tumour (LET) and metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) that correlate with the development and progression of HCC [25, 26, 58–65]. In fact, many of these newly identified lncRNAs have been demonstrated as having great potential as diagnostic

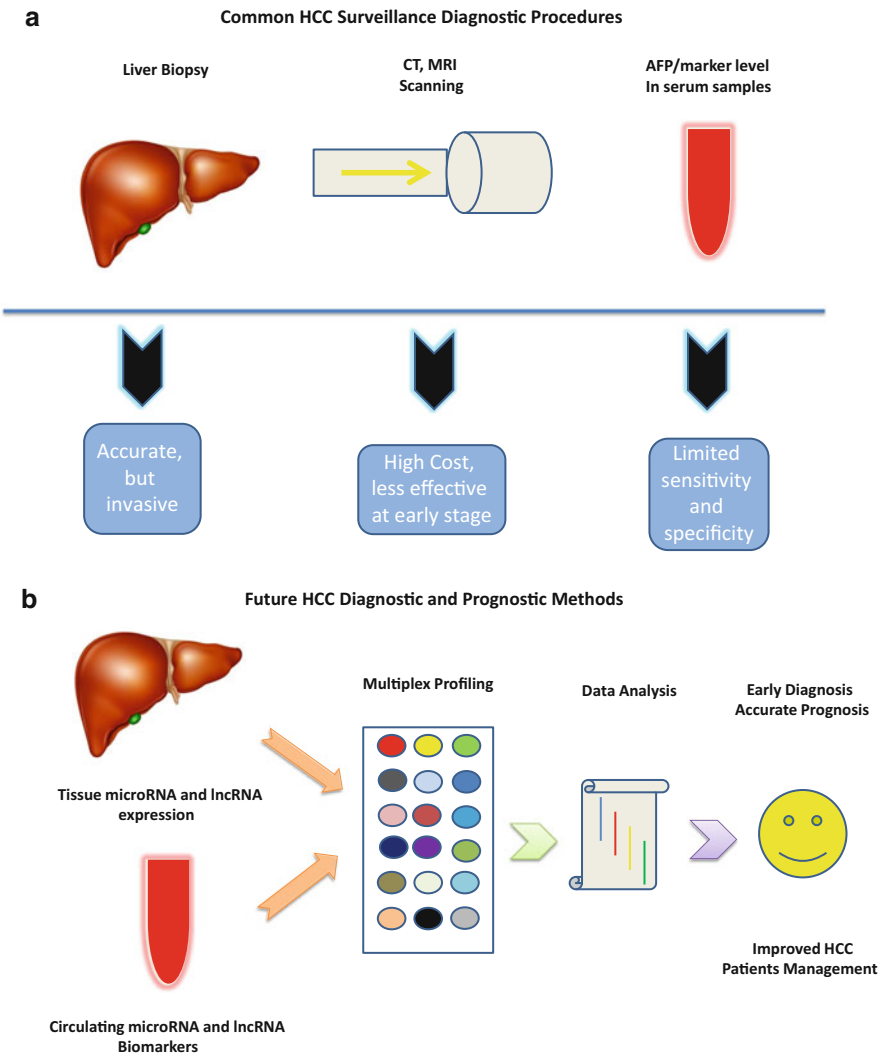

Fig. 2 Current common HCC versus future diagnostic technology. (**a**) The current common HCC diagnostic procedures include liver biopsy, imaging technology (CT, MRI) and AFP/marker level in blood sample. These standard diagnostic methods are invasive, costly, time consuming and can lack sensitivity and specificity with difficulty for early HCC diagnostics. (**b**) ncRNAs such as microRNAs and lncRNAs are stable biomarkers expressed in both serum and tissue samples giving them the capacity to be the next generation of diagnostic, prognostic and drug responsive markers for HCC

Table 2 Diagnostic miRNAs for HCC

miRNA	Diagnostic role	Reference
miR-122	Upregulated in HCC and chronic HBV compared with healthy subjects	[38, 39]
miR-222	Higher in HCC compared with healthy subjects	[38, 39]
miR-223	Levels increased in HCC compared with healthy subjects	[38]
miR-224	Upregulated 3.5-fold in HCC patient tumour tissue	[40]
miR-125b-5p	Upregulated 2.85-fold in chronic HBV, 2.46-fold in HBV cirrhosis, 1.89-fold in HBV-HCC	[41]
miR-15b	Highly expressed in HCC tissue and markedly reduced in serum after hepatectomy	[42]
miR-130b	Highly expressed in HCC tissue and markedly reduced in serum after hepatectomy	[42]
miR-16	Serum levels significantly lower in HCC than patients with chronic liver disease and healthy controls	[37]
miR-885-5p	Significantly higher in patients with HCC, liver cirrhosis and chronic HBV	[43]
miR-139	Downregulated in both cancerous tissue and plasma from HCC patients	[44]
miR-183	Significantly higher in tissue from HCC than chronic viral hepatitis, liver cirrhosis, non-tumourous tissues and healthy controls	[45]
miR-500	Significantly higher in HCC cancer tissue than non-cancerous tissue and in serum of HCC patients. Levels returned to normal after surgery	[46]

Table 3 Prognostic miRNAs for HCC

miRNA	Prognostic role	Reference
miR-122	Post-operative serum levels significantly reduced to similar level of healthy subjects compared with pre-operative samples in HCC	[38]
miR-1 and miR-122	Higher levels of both miRNAs correlated with longer overall survival of HCC. miR-1 was independently associated with overall survival	[47]
miR-101	Downregulated in 90 % HBV-HCC tumours compared with adjacent non-cancerous tissue. Expression level of miR-101 was decreased in HBV-HCC tissue compared with healthy controls. Interestingly, serum miR-101 level was significantly elevated in HBV-HCC patients	[48]
miR-139	Downregulation of miR-139 in plasma correlated with low 1-year survival rate	[44]
miR-183	Increased with stage of HCC. Not correlated with survival rate	[45]
miR-146a	Lower in HCC tissue than non-cancerous tissue. Correlated with more advanced stages of HCC, metastasis, multiple tumour nodules and portal vein tumour embolus	[49]
miR-185	Lower expression associated with rapid recurrence and remarkably poor survival rate	[50]
miR-20a	Downregulation associated with significantly poor recurrence-free survival and overall survival in HCC patients underwent liver transplantation	[51]

Table 4 Diagnostic and prognostic lncRNAs in HCC

LncRNA	Diagnostic/ prognostic evidence in HCC	Details	References
HULC	Diagnostic	HULC expression in HCC patients' blood samples were between 10 and 30 times higher than healthy and cirrhotic individuals	[57]
HOTAIR	Prognostic	3-Year recurrence-free survival rate in patients with high levels of HOTAIR that underwent hepatic resection was significantly lower compared to patients with low level of HOTAIR	[62, 66]
H19	Prognostic	Patients with lower ratio of intratumoural tissue/non-tumour tissue less than 2 cm from HCC tissue were estimated to have shorter disease-free survival	[67]
HEIH	Prognostic	HEIH was significantly associated with HCC tumour recurrence after 18 months. Multivariate analysis revealed that HEIH was an independent prognostic factor for overall survival	[61]
hDREH	Prognostic	Kaplan–Meier analysis illustrated that HCC patients with low levels of hDREH were markedly correlated with reduced recurrence-free survival and overall survival	[63]
MVIH	Prognostic	HCC patients with high levels of MVIH that underwent hepatectomy were estimated to have poorer recurrence-free survival and overall survival. Early stage HCC patients with high levels of MVIH who underwent hepatectomy had shorter recurrence-free survival	[64]

and prognostic biomarkers. Table 4 summarises the diagnostic and prognostic role of some lncRNAs in HCC.

7 Conclusion

Current tumour profiling methods include detection of cancer mutation genes, abnormal expression of proteins, RNAs and ncRNAs. ncRNAs are stable biomarkers making them useful candidates in cancer diagnostic and prognostics. Moreover, the expression level of ncRNAs can vary dependent on different environmental situations thus making them useful for therapeutic studies. To date, most studies have shown aberrant regulation of miRNAs as potential biomarkers in several liver disorders including chronic liver disease and chronic HBV associated with end stage liver cirrhosis and HCC. While these observations are promising, it has become apparent that a unique tested and validated biomarker capable of predicting the likelihood of an HCC episode in a patient is urgently required.

LncRNA HULC shows promise as a biomarker to predict HCC onset and progression. Considering the specificity of lncRNAs in HCC, ncRNAs may hold the answer in identifying a unique signature. Multi-centred studies are now necessary to provide useful validated clinical biochemistry data for diagnostic, prognostic and treatment plans that can be tailored to patients with HCC.

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