

Advances in microRNAs: implications for immunity and inflammatory diseases

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Abstract

Since their discovery in 1993 and the introduction of the term *microRNA* in 2001, it has become evident that microRNAs (miRNAs) are involved in many biological processes, including development, differentiation, proliferation and apoptosis. The function of miRNAs is the control of protein production in cells by sequence-specific targeting of mRNAs for translational repression or mRNA degradation. Interestingly, immune genes are apparently preferentially targeted by miRNAs compared to the average of the human genome, indicating the significance of miRNA-mediated regulation for normal immune responses. Here, we review what is known about the role of miRNAs in the pathogenesis of immune-related diseases such as chronic inflammatory skin diseases, autoimmunity and viral infections.

Keywords: microRNA • biogenesis • inflammation • immunity • psoriasis • atopic eczema/dermatitis • autoimmunity • rheumatoid arthritis • viral infection • systemic lupus erythematosus • primary biliary cirrhosis • vascular inflammation

Introduction

Since the discovery of the first microRNA (miRNA) *lin-4*, as a regulator of developmental timing in *Caenorhabditis elegans* [1], it has become evident that this short non-coding RNA was only the first member of a huge gene family with potentially enormous importance in the regulation of fundamental biological processes (Fig. 1). In 2000 there were only two miRNAs known (*lin-4* in *C. elegans* and *let-7* in *H. sapiens*), and even the term 'microRNA' was introduced only in 2001 in a set of three articles in *Science* (October 26, 2001) [2]. Barely 8 years later there are 6396 miRNA entries (as of July 2008), out of which 678 were discovered in

H. sapiens (Fig. 1). Indeed, miRNAs are implicated in virtually every biological process in multicellular organisms – including the onset and progression of many diseases.

MiRNAs are small, single-stranded RNA molecules (~22 nucleotides in length) that are encoded in the genomes of animals, plants and viruses. MiRNAs are one of the largest known gene families, consisting of hundreds or, in higher life forms, more than a 1000 genes. MicroRNAs play important roles in regulating the amount of proteins in the cells in order to maintain homeostasis. This is achieved by their negative influence on the synthesis of

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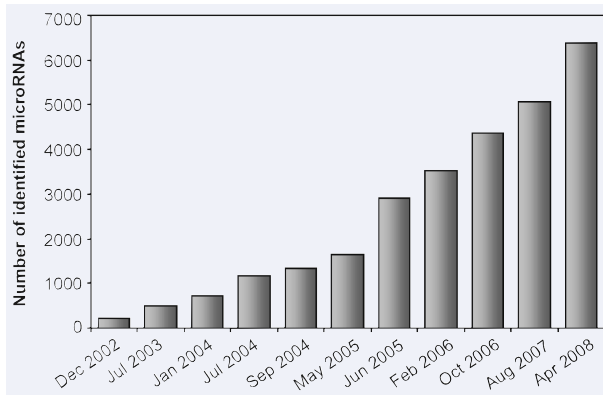


Fig. 1 The number of identified microRNAs (miRNAs) based on the number of entries listed in the miRBase sequence database, which is the primary repository for published miRNA sequence and annotation data. Each column represents new public releases of the database, from v1.0 to the latest version, v11.0.

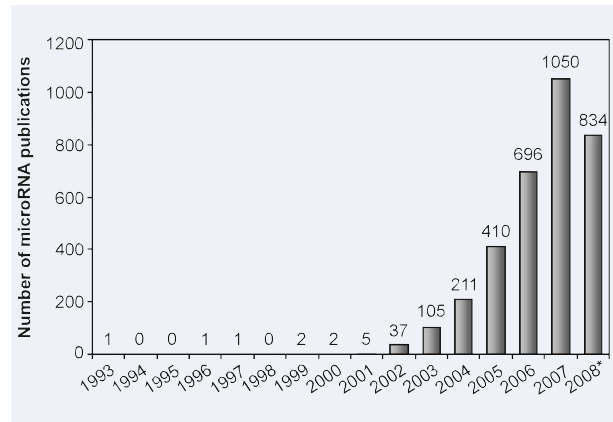


Fig. 2 The number of microRNA publications each year from 1993 to present based on search for scientific articles by the keyword 'microRNA'. Note that the term 'microRNA' was introduced in 2001, and publications before the introduction of the official term were retrieved by manual search for articles about lin-4 and let-7 the 'prototype' miRNAs that were described first. Source: NCBI PubMed. *, as of July 2008.

corresponding proteins by targeting mRNAs for translational suppression or degradation. The sequences of many of the miRNAs are highly conserved among organisms, suggesting that miRNAs represent a relatively old and important regulatory pathway [3]. Their importance is demonstrated by the fact that mice lacking the Dicer enzyme, which is required for the processing of the precursor miRNA into the mature form, are not viable [4–6].

Since the discovery of miRNAs, the number of publications about miRNAs is increasing exponentially (Fig. 2) and significant progress has been made in dissection of biogenesis and functions of miRNAs. To date, more than 600 individual miRNAs have been identified in the human genome, which are estimated to regulate the vast majority of protein coding RNAs (mRNAs) and each mRNA is likely to be regulated by several miRNAs simultaneously [7, 8]. In total, miRNAs may comprise 5% of all human genes making them the most abundant class of regulators. Experimental evidence suggested that most miRNAs are present at very high steady-state levels – more than 1000 molecules per cell, with some exceeding 50,000 molecules per cell [9].

Although we are only at the beginning of understanding the specific roles of individual miRNAs in cellular functions, several reports have provided evidence that miRNAs act as key regulators of processes as diverse as early development [10], proliferation, differentiation, cell fate determination, apoptosis, signal transduction and organ development [5, 11–20]. These known functions probably represent only a small part of a much bigger picture. With the continuing discovery of new miRNA functions, it is possible that miRNAs will be associated with the regulation of almost every aspect of cell physiology.

Abnormal miRNA expression may lead to the development of diseases; compelling studies have implicated miRNAs in various human diseases such as cancer [17, 18, 21–24], developmental abnormalities [5], muscular [25, 26] and cardiovascular disorders [27, 28], schizophrenia [29] and most recently inflammatory

diseases [30–32]. In this review, we aim to summarize about our knowledge about miRNAs related to inflammatory and immune-mediated diseases. Since the role of miRNAs in the normal immune system (*i.e.* development of immune cells and regulation of innate and acquired immune responses) have been extensively discussed elsewhere [31–36], in this review we will not discuss it in detail, but focus on what is currently known about the role of miRNAs in the pathogenesis of immune-related diseases such as chronic inflammatory skin diseases, autoimmunity and viral infections.

The biogenesis of miRNAs

Similar to ordinary protein-coding RNAs, miRNAs are encoded in the genomic DNA. To date, more than 600 miRNAs have been identified in the human genome and computational predictions suggest that there are more than 1000 miRNA genes in the human genome [37]. Approximately half of all human miRNA genes are contained within the introns of protein-coding genes or in the exons of untranslated genes, while others reside apart from known genes in intergenic regions [38]. Some miRNA primary transcripts encode only a single mature miRNA (*e.g.*: mir-203), while other loci contain clusters of miRNAs that appear to be produced from a single primary transcript (*mir-17-92* cluster) [39].

The generation of functional single-stranded miRNAs from miRNA genes occurs through a multi-step process that involves several different enzymes first in the cell nucleus and finally in the cytoplasm (Fig. 3). The genes encoding miRNAs are much longer than the processed, biologically active, mature miRNA molecule and transcribed by the RNA polymerase II as long primary transcripts termed primary miRNAs (pri-miRNAs) [40], which can be several hundred or thousand nucleotides long. Like other pol II

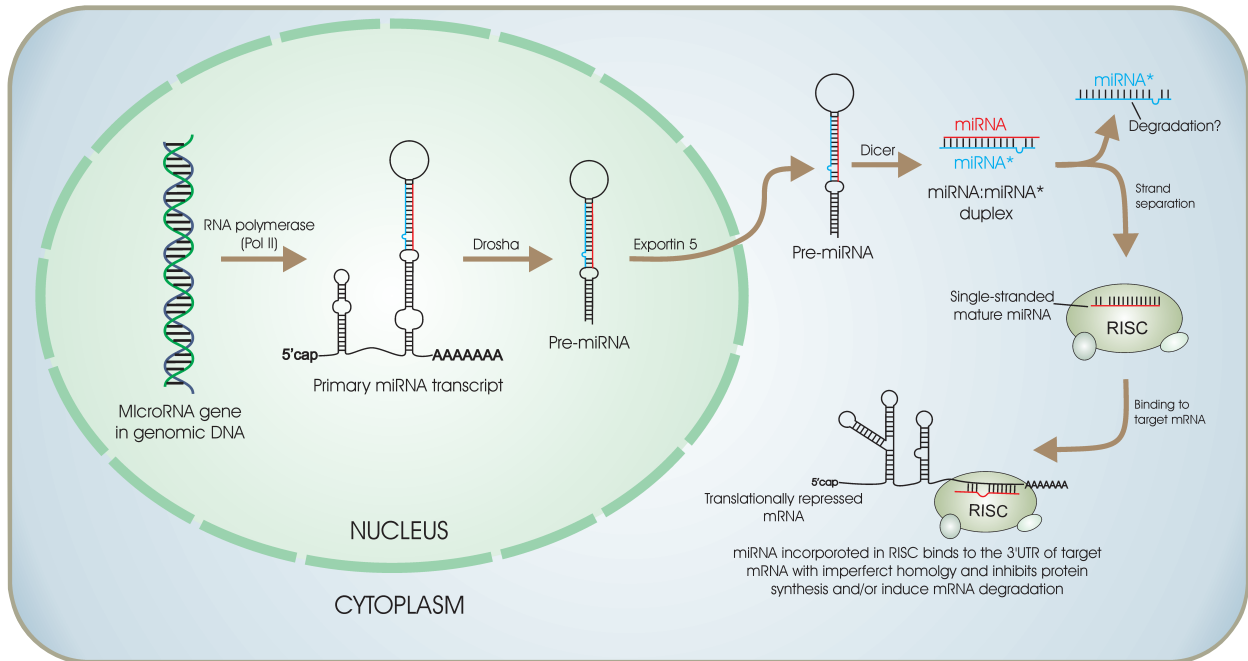


Fig. 3 The biogenesis of miRNAs. Most miRNAs are transcribed by RNA polymerase II from the genomic DNA as long (several 100–1000 nt) primary transcripts (pri-miRNA) that are polyadenylated and capped. Next, the RNase III-type enzyme Drosha processes pri-miRNAs in the nucleus, yielding a hairpin precursors (pre-miRNA) consisting of approximately 70 nt. The pre-miRNA hairpins are exported to by a process that involves Exportin 5. In the cytoplasm, pre-miRNAs are further processed by the RNase III protein Dicer into an unstable, 19–25 nt miRNA duplex structures consisting of the ‘guide strand’ (miR) and the ‘passenger strand’ (miR*). The less stable of the two strands in the duplex will become the mature. Single stranded miRNA and it is incorporated into a multiple-protein nuclease complex, the RNA-induced silencing complex, which regulates protein expression.

transcripts, the primary transcripts (pri-miRNAs) are capped and polyadenylated (Fig. 3).

Pri-miRNAs contain a local stem–loop structure that encodes miRNA sequences in the arm of the stem. This stem–loop structure is cleaved in the nucleus by the dsRNA-specific ribonuclease Drosha in a process known as ‘cropping’ [41, 42] to release the precursor miRNA (pre-miRNA) [43] (Fig. 3). Pre-miRNAs are approximately 70 nt RNAs with 1–4 nt 3’ overhangs, 25–30 bp stems and relatively small loops. Drosha generates either the 5’ or 3’ end of the mature miRNA, depending on which strand of the pre-miRNA is selected by RNA-induced silencing complex (RISC) [43, 44]. The pre-miRNAs are then actively transported from the nucleus to the cytoplasm *via* a mechanism that involves exportin-5 (Exp5). Exp5 has been shown to bind directly and specifically to correctly processed pre-miRNAs [44, 45].

Pre-miRNAs are subsequently cleaved by the cytoplasmic RNase III Dicer into ~22-nt miRNA duplexes [43, 44] (Fig. 3). One strand of the short-lived miRNA duplex is degraded (miRNA* or ‘passenger’ strand), whereas the other strand (miR or ‘guide’) will be incorporated into the RISC and serve as a functional, mature miRNA (Fig. 3). Selection of the active strand from the dsRNA appears to be based primarily on the stability of the termini of the two ends of the dsRNA [46, 47]. The strand with lower stability

base pairing of the 2–4 nt at the 5’ and 3’ end of the duplex preferentially associates with RISC and thus becomes the functionally active miRNA [46]. The potential function – if any – of the miRNA* strand of the miRNA duplex is unclear at present (Fig. 3). After the mature, single-stranded miRNA is selectively loaded into the RISC, this miRNA-programmed RISC (miRISC) carries out the repression of gene expression (Fig. 3).

Interestingly, the complex containing active miRNAs and the RISC involved in RNA interference mediated by siRNAs are similar if not identical, as endogenous miRNAs can cleave mRNAs with perfect complementarity [48], and exogenously introduced siRNAs can translationally repress mRNAs bearing imperfectly complementary binding sites [49–51].

Mechanism of action of miRNAs

MiRNAs bind to complementary sequences in the 3’UTR of target mRNA (s) and can prevent protein synthesis by two modes of action: (1) in cases where the miRNA is only partially complementary to its corresponding mRNA, translation inhibition occurs [7] and (2) in cases where there is a near-perfect complementarity between the miRNA and the mRNA, deadenylation and subsequent

degradation of the target mRNA occurs [52, 53]. In the first case, miRNA represses translation without affecting mRNA levels, while in the second the miRNA prevents protein synthesis by an siRNA-like effect resulting in the degradation of its target mRNAs. The first mechanism (translational repression) is thought to be more general in the animal kingdom, although animal miRNAs can also act *via* the degradation of their target mRNA, as demonstrated by findings of Lim *et al.* showing that transfected miRNAs induced the degradation of a large number of mRNAs containing the binding sites [8]. However, the inhibition of translation is probably sufficient to account for the majority of the repression of protein production observed in mRNAs that harbour miRNA target sites, and it was recently shown that miRNA repression occurs before mRNA destabilization [54–56]. Comprehensive studies analysing global changes in protein production after overexpression or inhibition of a miRNA resulted in the conclusion that miRNAs alter the expression of most of the proteins at a relatively low extent and that miRNA action involves detectable changes at the mRNA levels in approximately 70% of all regulated proteins [57, 58]. However, it is not entirely clear whether the alteration in the mRNA level is a cause or a consequence of miRNA-mediated translation inhibition. Results from studies conducted in different systems and different laboratories have often been contradictory regarding the mechanistic details of the function of miRNAs in repressing protein synthesis. At present it is unclear which mechanisms or pathways miRNAs choose to silence their targets. It could depend on the specific miRNA, specific targets, other RNA binding proteins, or specific tissue and cell types and be under the control of signalling pathways [59].

Each miRNA is thought to regulate multiple genes, and since hundreds of miRNA genes are predicted to be present in higher eukaryotes [20, 60, 61] the potential regulatory circuitry affected by miRNA is enormous. Moreover, instead of ‘randomly’ targeting several hundred genes for suppression, miRNA-target genes are frequently part of a gene network, or pathway regulating a biological process such as proliferation, cytokine signalling or differentiation. Interestingly, many miRNA targets are transcription factors [61, 62]. By regulating both transcription factors and ordinary protein coding genes, miRNAs can exert a massive regulatory effect in cells in which they are expressed.

MiRNAs, similar to transcription factors, can act in combination (or cooperatively) by binding to the same mRNA in a concentration-dependent manner [7]. Computational predictions as well as experimental evidence shows that a protein-coding gene is potentially regulated by several if not several dozen miRNAs simultaneously which may act in combination and exert synergistic effect [7]. Thus, predicted miRNA : mRNA interactions must be viewed in the context of other potential interactions.

Identification of miRNA targets

The function of a miRNA can be interpreted as the sum of the function of the genes it regulates within a cell. Thus, a central goal

for understanding miRNA functions has been to understand how they recognize their target mRNAs and identify miRNA targets.

As a general rule, the ability of an miRNA to translationally repress a target mRNA is largely dictated by the free energy of binding of the first eight nucleotides in the 5’ and 3’ region of the miRNA (from base 2 to base 8 or 9) often referred as ‘seed nucleotides’ [7]. The 3’ region of the miRNA can modulate activity in certain circumstances but is considered to be less critical [7]. Many target prediction algorithms rely on the presence of the complementary match between the seed nucleotides of the miRNA and the corresponding targets sites in the mRNA. However, these seed matches are not always sufficient for repression, indicating that in addition to an exact Watson-Crick base pairing other characteristics help specify targeting. To explore these mechanism, Grimson *et al.*, combined computational and experimental approaches, and uncovered five general features of site context that boost site efficacy: (1) AU-rich nucleotide composition near the target site, (2) proximity to sites for coexpressed miRNAs (which leads to cooperative action), (3) proximity to residues pairing to miRNA nucleotides 13–16, (4) positioning within the 3’UTR at least 15 nt from the stop codon and (5) positioning away from the centre of long UTRs [63]. In addition to providing insights and constraints for mechanistic models, these context features provide valuable information for the researcher who is facing the difficult task of trying to select the most promising miRNA-target genes for experimental follow-up [63] (<http://www.targetscan.org>).

Despite the rapid progress in the understanding of miRNA–mRNA interactions and computational target predictions, the currently available miRNA target-prediction algorithms are likely to have high false positive and false negative discovery rates. Some of the verified miRNA targets do not fulfil the criteria of the above-listed criteria, while others fulfil them but cannot be experimentally validated. In fact, in most miRNA articles authors identified only a single miRNA target and used that target as an entry point into the understanding of miRNA function. Unfortunately, this approach implies that a miRNA exerts its function *via* regulating a single gene (or few genes) only, which is probably far from being true.

Since miRNAs may regulate their target proteins without changing the mRNA level, cDNA microarray studies with cells transfected with miRNA precursors (although widely used in the search for potential targets) are not ideal for identifying the whole range of targets for a miRNA. Sensitive and specific large-scale protein expression analyses methods could facilitate experimental identification of miRNA targets. The first example for such a systematic approach appeared at the time of writing this review (July 2008). Two compelling studies published in the same issue of *Nature* demonstrated that miRNAs cause widespread but mild changes in protein synthesis [57, 58]. These studies suggest that miRNA may act as rheostats to make fine-scale adjustments to protein output acting on several hundred genes simultaneously. The new proteomic approach (SILAC quantitative mass-spectrometry) used in these studies can reveal important insights into the function of miRNAs by identifying their global impact on the proteome.

Multi-level control of miRNA expression

Similar to regular mRNAs, in principle, miRNA expression could be controlled at any step of its processing. The level of transcription, enzymatic processing of the pri- and pre-miRNAs, subcellular localization, stability and finally the selective loading of the mature miRNA strand onto the RISC complex all may be subject to some kind of regulation though as yet not all of them have been observed [64].

Many miRNA genes are located within introns of protein-coding genes and are transcribed by RNA polymerase II as part of their hosting transcription units. Hence, the regulation of transcription of these miRNAs is dependent on the same signalling pathways and transcription factors as the host gene. It is generally believed that intronic miRNAs are released by Drosha from excised introns after the splicing reaction has occurred and thereby both mRNA and miRNA can be coexpressed from a single primary transcript molecule [65].

Most miRNA genes that are located in intergenic regions are also transcribed by RNA polymerase II [42, 66, 67], the same RNA polymerase that transcribes protein-coding genes. Therefore it is reasonable to think that many of the signalling pathways and transcription factors that regulate protein-coding genes may regulate the transcription of miRNAs. Although the precise transcriptional control of most miRNAs has been unknown there are more and more examples for the transcription factor mediated regulation of miRNA expression. An interesting example for immunologists is the NF- κ B-mediated induction of several immune-related miRNAs (miR-155 and miR-146) that have been recently described in monocytes and macrophages [68].

For many miRNAs, the level of the mature miRNA in the cell is not simply determined by the level of transcription. Comparison of tumour-specific mature miRNA expression with pri-miRNA transcript levels showed poor correlation indicating that also the maturation of the pri-miRNA transcript is a regulated process [64].

In addition to regulation at the transcriptional and post-transcriptional levels, several independent studies have shown that epigenetic mechanisms also affect the expression of miRNA genes [69–72]. In the human genome, DNA methylation can occur at CpG dinucleotides and methylation of CpGs in the promoter of a gene can induce stable changes in gene expression by affecting chromatin structure and the accessibility of the promoter to transcription factors. In order to identify miRNA genes that are controlled by epigenetic alterations Saito *et al.* have treated human bladder cancer cells with the DNA methyltransferase inhibitor 5-aza-2'-deoxycytidine followed by miRNA expression analysis [69]. Expression profiling revealed that seventeen out of 313 human miRNAs became up-regulated more than threefold demonstrating that many miRNA genes are methylated (inactivated) in tumour cells. Since these first investigations, a number of articles demonstrated that aberrant methylation of miRNA genes leads to deregulated miRNA expression in cancers [73, 74]. Aberrant DNA methylation may contribute to the deregulated expression of miRNAs also in inflammatory diseases.

The regulation of the biogenesis any given miRNA is likely to be different for each miRNA and may depend on the specific tissue, developmental stage or cell type and be under the control of signalling pathways. Investigation of the regulation of miRNA expression represents an important subject of study since the activation or down-regulation of miRNAs by drugs could be a novel strategy for the prevention and treatment of diseases associated with deregulated miRNA expression.

Interestingly, mRNAs can and do avoid miRNA-mediated repression of translation by the selective usage of alternative, shorter 3'UTR isoforms. This phenomenon was identified recently by Sandberg *et al.* who found that activation of T lymphocytes resulted in increased abundance of mRNA isoforms with shorter 3'UTRs relative to those with longer 3'UTRs as measured 48 hours after activation [75]. Shorter 3'UTRs lacked regulatory elements, such as miRNA target sites, through which translation is inhibited. The production of mRNAs with shorter 3'UTRs to avoid miRNA-mediated suppression in activated cells may serve as a global cellular mechanism to increase protein abundance as the cells begin to proliferate. This mechanism apparently is a characteristic of gene expression during immune cell activation and correlates with proliferation across diverse cell types and tissues [75]. It is likely that we will see an increased attention on the selective usage of alternative 3'UTRs in diseases characterized by abnormal proliferation, including immune-mediated diseases.

Strikingly, statistical analysis of all transcript expression patterns reveals that genes containing miRNA target sequences seem to avoid being expressed in tissues that express those particular miRNAs [76]. This finding also raises basic questions about the real function of miRNAs: are miRNAs regulating gene expression similar to transcription factors in order to maintain the physiological concentration of proteins in the cell or are they part of a kind of 'noise-removal' machinery in the cell to prevent the synthesis of proteins from transcripts that are not needed? The answer probably depends on the specific miRNA, its targets and specific tissue and cell types [76].

How can miRNAs contribute to diseases?

Malfunction of miRNA regulation is associated with a wide variety of human diseases, including cancer, diabetes, inflammation and viral infection. In principle, miRNAs may contribute to or cause diseases, developmental abnormalities by the following ways [76]:

- (1) Loss or down-regulation of miRNA expression due to mutation, epigenetic inactivation, aberrant processing or transcriptional down-regulation. Down-regulation of miRNAs is a common phenomenon in cancers. For example, miR-15a and miR-16-1 can target the anti-apoptotic *BCL2*, and they are often down-regulated in chronic lymphocytic leukaemia and other tumours [77]. Members of

the human let-7 family of miRNAs are also frequently lost in solid tumours, allowing the uncontrolled expression of their targets, including Ras oncogene, which, in turn promotes tumour growth. Many miRNAs are down-regulated in inflammatory diseases. For example, miR-125b is down-regulated in psoriasis. miR-125b is a known regulator of the NF- κ B pathway, whose activation is a characteristic of psoriasis [30, 78].

- (2) Overexpression of a miRNA due to gene amplification or mutations in its promoter region, or due to transcriptional up-regulation may result in the suppressed production of its target proteins. Overexpression of oncogenic miRNAs (oncomirs) suppresses the expression of tumour suppressor genes and contribute to the increased survival and proliferation of tumour cells. For example, the overexpression of miR-155, miR-21 and the miR17-92 cluster has been described in a wide variety of solid tumours and leukaemias [79, 80].
- (3) A mutation in the 3'UTR of an mRNA may affect a miRNA binding site and the miRNA may no longer be able to bind to it. This would result in the overexpression of the target gene. For example, an SNP in the 3'UTR of HLA-G, a known asthma-susceptibility gene, disrupts the binding sites of three miRNAs (*e.g.* miR-148) targeting this gene [81] and thus might contribute to susceptibility to asthma.
- (4) A mutation in the 3'UTR of a gene may generate a new miRNA binding site. The function of this gene may be suppressed in the cells or tissues in which the miRNA whose target site was accidentally created happens to be expressed constitutively.

In fact, many natural sequence polymorphisms (SNPs) result in the creation or disruption of a miRNA binding site [82], suggesting this as a general phenomenon that could be relevant to many disease states. Recently Saunders and coworkers identified approximately 250 SNPs that potentially create novel target sites for miRNAs [82]. If some variants have functional effects, they might confer phenotypic differences among human beings and contribute to common diseases. Thus, the investigation of SNPs in miRNA genes as well as in the 3'UTR of miRNA target genes represents an upcoming field of research aiming to explore the genetic background of common diseases.

miRNAs in immunological and inflammatory disorders

With the discovery of miRNAs, intensive research focused on delineating their roles in the immune system. These investigations revealed that different haematopoietic organs and cell types have distinct miRNA expression profiles and that the expression of specific sets of miRNAs is dynamically regulated during immune cell development (reviewed in: [31–36]). In addition to haematopoiesis, miRNAs are also involved in the regulation of innate as well as

acquired immune responses (as reviewed in: [31–33, 36]). Given the role of miRNAs in the development of immune cells, the fine tuning of T-cell sensitivity to antigens and antibody response, it is conceivable that deregulation of one or more miRNAs may lead to reduced tolerance against self-antigens and the development of autoimmune diseases. Moreover, deregulation of miRNAs fine-tuning the immune response may lead to sustained inflammation which is a hallmark of common chronic inflammatory diseases. Some examples for miRNAs that have been implicated in immune-related and inflammatory disorders are listed in Table 1.

Psoriasis and atopic eczema

The first report implicating miRNAs in human inflammatory diseases appeared in 2006 and identified miRNAs deregulated in the two most common chronic inflammatory skin diseases, psoriasis and atopic eczema [30]. A systematic, genome-wide analysis of miRNA expression revealed that psoriasis and atopic eczema display miRNA expression profiles distinct from healthy skin as well as from each other. While miRNAs specifically deregulated in psoriasis (*e.g.* miR-146a) or atopic eczema may be associated with the specific type of immune reaction in these diseases, miRNAs that are deregulated in both diseases (*e.g.* miR-125b) might have a more general role in inflammation. Some of the inflammation-associated miRNAs seem to be involved in the regulation of inflammatory pathways and cytokine signalling (unpublished data and [30]). For example, both miR-146a, a miRNA specifically up-regulated in psoriasis, and miR-125b, a miRNA down-regulated in both psoriasis and atopic eczema, have been shown to regulate the activity of inflammatory pathways in mouse macrophages [36, 78]. Of note, miR-125b has been implicated in the negative regulation of the TNF- α pathway [78], which is of crucial importance in the development of psoriasis, as demonstrated by the effectiveness of anti-TNF- α therapies [68]. Further studies are in progress to identify the specific roles of these miRNAs in the altered immune responses in psoriasis and atopic eczema.

In addition to miRNAs previously implicated in immune functions, the study identified several inflammation-associated miRNAs whose function and expression was unknown, such as miR-203. MiR-203 showed a strikingly specific expression pattern, being expressed exclusively in skin and oesophagus, and within cells types of the skin, exclusively in keratinocytes [30]. The authors identified suppressor of cytokine signalling-3 (SOCS-3) as a potential target of miR-203, showing mutually exclusive expression pattern with miR-203 and showing suppressed expression in psoriasis lesional skin compared with healthy skin. SOCS-3 is a negative regulator of the interleukin (IL)-6 and interferon (IFN)- γ induced signalling pathways [30] and SOCS-3 deficiency leads to sustained activation of STAT3 in response to IL-6 [83]. Therefore, the suppression of SOCS-3 by miR-203 may result in an increased or elongated inflammatory response in the skin. Direct regulation of SOCS-3 by miR-203 was also confirmed using luciferase 3'UTR assays (unpublished data). In a recent study, SOCS-3 protein levels were not suppressed

Table 1 MiRNAs implicated in inflammatory and immune-mediated diseases

MicroRNA	Disease	Expression	Targets	References
miR-155	Rheumatoid arthritis (RA), vascular inflammation	↑	AT1R, suggested MMP-3; FADD, IKK ϵ , and Ripk1; c-maf	[78, 86, 96, 114, 115]
miR-146a	RA, psoriasis	↑	TRAF6, IRAK1	[30, 68, 86, 87]
miR-125b	Psoriasis, atopic eczema	↓	N/A	[30]
miR-21	Psoriasis, atopic eczema	↑	N/A	[30]
miR-203	Psoriasis	↑	SOCS-3, p63	[30, 84, 85]
miR-17-92 cluster	Primary biliary cirrhosis (PBC), systemic lupus erythematosus, idiopathic thrombocytopenic purpura	↓	PTEN and BIM; suggested TNFRSF21	[89, 90]
miR-296	Idiopathic thrombocytopenic purpura	↓	N/A	[88]
miR-198	Systemic lupus erythematosus	↑	N/A	[88]
miR-342	Systemic lupus erythematosus, idiopathic thrombocytopenic purpura	↑	N/A	[88]
miR-383	Systemic lupus erythematosus, idiopathic thrombocytopenic purpura	↓	N/A	[88]
let-7b	PBC	↓	suggested IL-6	[89]
miR-346	PBC	↓	N/A	[89]
miR-20a	PBC	↓	N/A	[89]
miR-451	PBC	↑	N/A	[89]
miR-129	PBC	↑	N/A	[89]
miR-126	Vascular inflammation	↓	VCAM-1	[95]

in primary mouse keratinocytes overexpressing miR-203 [84]; this may reflect a difference between human and mouse, although it is more likely that detection of SOCS3 by Western blotting is less reliable due to the low level of SOCS-3 expression in unstimulated cells. Suppression of SOCS-3 by miR-203 may lead to sustained STAT-3 activation and an increased and elongated inflammatory response in the skin. Interestingly, the murine orthologue of miR-203 has been demonstrated to play an important role in epidermal morphogenesis [85]. Transgenic mice overexpressing miR-203 are characterized by thinner epidermis and impaired proliferative potential (repression of 'stemness') [85], whereas psoriasis is characterized by epidermal hyperproliferation. These seemingly conflicting findings may reflect the dysfunction of miR-203 in psoriatic patients, for example by mutations that affect some of its target genes.

In conclusion, miRNAs deregulated in psoriasis and atopic eczema have the potential to become targets for future therapeutic interventions to treat chronic skin inflammatory diseases, and potentially even inflammatory diseases affecting other organs.

Asthma

Recently, an indirect evidence for the potential involvement of miRNAs in asthma appeared, in a report describing that an SNP in the 3'UTR of HLA-G, a known asthma-susceptibility gene, disrupts the binding sites of three miRNAs (miR-148a, miR-148b and miR-152) targeting this gene [81]. Although direct evidence is missing, it is likely that earlier observations on the association of the HLA-G gene to asthma susceptibility

may, at least in part, be due to the allele-specific regulation of this gene by miRNAs [81].

Rheumatoid arthritis

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by irreversible joint damage, caused by the inflammation of synovial tissue. Synovial fibroblasts are thought to have a critical role in joint destruction in RA patients. Two recent studies investigated the expression of miRNAs in RA [86, 87]. Stanczyk *et al.* identified miR-155 as a miRNA regulated by TNF- α in synovial fibroblasts from RA patients (RASFs) [86]. Similar to TNF- α , other inflammatory mediators involved in joint destruction such as the TLR ligands LPS, poly (I-C) and bLP markedly induced miR-155 in RASFs. Although miR-146a was not among the TNF- α -regulated miRNAs, the authors included this miRNA in their analyses intrigued by previous observations showing the regulation of miR-146a by TLR ligands in monocytes [68]. In synovial fibroblasts, miR-146a was up-regulated by IL-1 β and LPS but not by TNF- α or other TLR ligands. Both miR-155 and miR-146a were found to be overexpressed in synovial tissues of RA patients, compared with patients with osteoarthritis. Interestingly, both miRNAs were already overexpressed in untreated synovial fibroblasts from RA patients, suggesting either permanent changes (epigenetic regulation) of miRNA expression by inflammatory mediators, or the presence of genetic alterations in RA patients leading to increased miRNA expression. Among the markers of joint destruction, MMP-3 was suppressed by enforced expression of miR-155 [86]. An independent group also identified miR-146a as a miRNA overexpressed in synovial tissue of RA patients in comparison to both osteoarthritis and normal synovium [87]. MiR-146b showed a similar expression pattern [87]. Double *in situ* hybridization/immunohistochemistry showed that miR-146a was primarily expressed in CD68⁺ macrophages, CD3⁺ T cell subsets and CD79a⁺ B cells.

These findings suggest that miR-146a/b and miR-155 may have a role in the inflammatory process in the joints of RA patients. However, it is still not clear exactly how these miRNAs are regulated and what is the functional consequence of their up-regulation in the different cells of the inflamed joint. Nevertheless, these findings suggest that miRNAs have the potential to become biomarkers for the inflammatory process and therapy response, as well as targets for RA therapy.

Systemic lupus erythematosus

The first indication that miRNA deregulation might play a role in autoimmunity was a report by Dai *et al.* in 2007 [88], analysing miRNA expression profiles in the peripheral blood cells from patients with systemic lupus erythematosus (SLE), a systemic autoimmune disease and ITP (idiopathic thrombocytopenic purpura), an organ-specific autoimmune disease, as well as healthy controls. The study identified 16 SLE-related miRNAs and 19 ITP-related miRNAs. 13 miRNAs showed a similar expression

pattern in SLE and ITP (among others miR-17-5p, miR-196a, miR-383, miR-409-3p were down-regulated, while miR-142-3p and miR-342 were up-regulated). These miRNAs may be generally associated with autoimmunity, while miRNAs specifically regulated in SLE (*e.g.* miR-184, miR-198, miR-21) or ITP (*e.g.* miR-214, miR-296, miR-513) may be involved in systemic inflammation/organ-specific tissue destruction. Interestingly, several miRNAs had different expression between two groups of SLE patients with different disease activity (SLEDAI < 12 or SLEDAI > 15). Although these results are interesting and implicate miRNAs in the pathogenesis of SLE and ITP, a limitation of this study is that RNA was pooled in each sample group, not allowing for comparison of different groups by statistical methods; moreover, functional studies demonstrating the involvement of these miRNAs in immune functions are lacking (with the exception of miR-17-5p, see below). MiRNAs specifically associated with SLE may potentially serve as biomarkers useful in differential diagnosis and follow-up of disease activity, and as targets for therapy.

Primary biliary cirrhosis

A very recent study implicated miRNAs in primary biliary cirrhosis (PBC), an autoimmune disease characterized by lymphocytic infiltrates in the portal tracts and anti-mitochondrial antibodies [89]. Comparison of miRNA expression profiles using oligonucleotide arrays in peripheral blood cells of PBC patients and healthy controls revealed down-regulation of 23 miRNAs (*e.g.* let-7b, miR-17-5p, miR-346 and miR-20a) and up-regulation of 2 miRNAs (miR-451 and miR-129). The functional significance of the miRNA deregulation in PBC was demonstrated by transfection of anti-let-7b, anti-miR-346, anti-miR-17-5p (inhibition of miRNAs) and pre-miR-129 (overexpression of miRNAs) into autoantigen-specific T cells. The transfected cells showed a significant increase in proliferation and IFN- γ production. Hence, aberrant expression of these miRNAs may contribute to autoimmunity by affecting the proliferation and activation of autoreactive immune cells. Overexpression of miR-17-5p in PDC-E2-specific T cells led to decreased protein (but not mRNA) expression of one of its predicted targets, TNFRSF21 (death receptor 6 (DR6)), while inhibition of miR-17-5p increased TNFRSF21 expression, suggesting that miR-17-5p might target TNFRSF21 for translational repression. In the same setting, the authors showed that let-7d might target IL-6. However, it has not been confirmed whether these genes are targeted directly by miR-17-5p and let-7d.

miR-17-5p and miR-101 in autoimmunity

Interestingly, down-regulation of miR-17-5p was observed in peripheral blood cells from patients with SLE, ITP and PBC, three autoimmune diseases targeting different organs, pointing to an important role of this miRNA in human autoimmune disorders [88, 89]. miR-17-5p is part of the miR-17-92 polycistronic miRNA gene cluster, encoding 6 miRNAs. The sequences of these miRNAs and their organization is highly conserved in all vertebrates. The

miR-17-92 cluster has been linked to carcinogenesis as well as tumour angiogenesis [39, 80], and recent data point to a functional role in the development of the immune system. Surprisingly, transgenic overexpression of the miR-17-92 cluster in T and B cells of mice resulted in lymphoproliferative disease and in autoimmunity, with increased IgG and IgM levels, increased titres of anti-ds-DNA and anti-ss-DNA and massive infiltration of lymphocytes into non-lymphoid tissues [90]. The authors identified PTEN and Bim as direct targets of miRNAs in the miR-17-92 cluster, both proteins important in maintaining central and peripheral tolerance; down-regulation of these proteins could therefore cause breakdown of these tolerance mechanisms. However, these results contrast with the finding that miR-17-5p was down-regulated in peripheral blood cells in three human autoimmune diseases; therefore, the proposed mechanism is not likely to be relevant in autoimmune diseases in human beings. At present it is not clear whether the autoimmune syndrome is a direct consequence of the overexpression of miR-17-92 cluster, or a by-product of the lymphoproliferative effect. An alternative explanation for the discrepancy between these observations can be the differences between the human and the murine immune system.

Another recent report using a mouse model for autoimmunity revealed a novel pathway that prevents autoimmunity by limiting the levels of a co-stimulatory receptor, the inducible T-cell stimulator (ICOS) on T lymphocytes, [91]. The novel pathway involves the gene called *Roquin*, which can undergo a specific mutation (M199R) that results in instructing T cells to react against the self, as previously shown by the same group in 2005 [92]. The *sar-roque* mice homozygous for the M199R mutation develop a lupus-like autoimmune syndrome, with lymphadenopathy, splenomegaly and accumulation of T cells. In their new study, the authors show that in these mice, ICOS overexpression is an essential contributor to the phenotype, indicating that tight regulation of ICOS by *Roquin* is critical to prevent T- and B-cell accumulation [91]. The authors demonstrate that binding of miR-101 (and potentially other miRNAs) to the unusually long 3'UTR of ICOS is essential for this regulation as disruption of the miR-101 binding site disrupts the repressive activity of *Roquin*. However, it is still unclear whether *Roquin* binds to the target mRNA or to the complementary miRNA(s). It will be interesting to see whether natural sequence polymorphisms in miR-101 or in the 3'UTR of ICOS can be associated with autoimmune diseases.

Vascular inflammation

Inflammation is thought to contribute to multiple acute and chronic vascular diseases, including atherosclerosis, which is considered as a chronic inflammatory disease. In the formation of early lesions (fatty streaks), leucocyte activation and their infiltration to the vascular wall is an important step [93]. To date, only a few studies assessed the role of miRNAs in vascular inflammation and diseases [94].

A recent study provided evidence that miRNAs control vascular inflammation [95]. Harris *et al.* identified miR-126 as a miRNA

highly and selectively expressed in endothelial cells. MiR-126 was found to regulate the expression of vascular cell adhesion molecule 1 (VCAM1), which is an intracellular adhesion molecule mediating leucocyte adherence to endothelial cells. Resting endothelial cells do not express VCAM-1, but pro-inflammatory cytokines such as TNF- α activate the transcription factors NF- κ B and IRF-1, which induce VCAM-1 transcription. Inhibition of miR-126 increased TNF- α -stimulated VCAM-1 expression. Moreover, functional experiments measuring the adhesion of labelled leucocytes to HUVEC cells demonstrated that TNF- α -induced leucocyte adherence was decreased when HUVEC cells overexpressed miR-126. Conversely, antisense inhibition of miR-126 in HUVEC cells increased leucocyte adherence. Since miR-126 is constitutively expressed in endothelial cells, its function may be the suppression of leucocyte adhesion and inflammation in resting endothelial cells.

One of the first studies indirectly implicating miRNAs in cardiovascular disease [96] showed that the angiotensin II type I receptor (AT1R) is translationally repressed by miR-155. AT1R is the main receptor for angiotensin II and its activation initiates a cascade of pathological events, including altered vascular tone, endothelial dysfunction, structural remodelling and also vascular inflammation, all of which may significantly contribute to the development of cardiovascular and kidney disease [97]. Therefore it is conceivable that decreased levels of miR-155 might contribute to these disorders, including vascular inflammation. Although there is no evidence showing decreased miR-155 levels in these diseases, decreased miR-155-mediated translational repression by another mechanism has been implicated in cardiovascular diseases. Another study from the same group showed that a silent polymorphism (+1166 A/C) in the 3'UTR of the AT1R gene that has been associated with cardiovascular disease disrupts the binding site for miR-155 [98]. Hence, the presence of this polymorphism leads to decreased translational repression, and thereby increased AT1R densities in endothelial cells and vascular smooth muscle which in turn might lead to vascular inflammation and cardiovascular disease.

MiRNAs in antiviral immunity and viral immune escape

Given the broad regulatory roles of miRNAs in cellular functions, it is not surprising that these tiny molecules are also part of the cat-and-mouse game known as virus-host co-evolution. The usage of miRNAs encoded in the viral genome is advantageous from the viral perspective: they can specifically down-regulate host cell genes, they occupy less than 200 nucleotides of the viral genome and they are not antigenic [99]. One can predict that viral miRNAs probably interfere with various aspects of the host's antiviral immune responses, including antigen presentation, or innate immune responses, including the IFN system [99]. The first study showing that miRNAs can be encoded in the viral genome was published in 2004 [100], and since then several DNA viruses

Table 2 MiRNAs involved in virus-host interactions

MiRNA	mRNA target	Virus	Function	References
<i>Viral</i>	<i>Viral</i>			
miR-S1-5p and miR-S1-3p	T antigen	SV40	Decreased susceptibility to CTL killing	[107, 108]
miR-UL-112-1	IE72	hCMV	Unknown (may promote transition to latent infection)	[109]
miR-H2-3p	ICP0	HSV-1	May facilitate the establishment and maintenance of viral latency	[107]
miR-H6	ICP4	HSV-1	May facilitate the establishment and maintenance of viral latency	[107]
<i>Viral</i>	<i>Host</i>			
miR-UL-112	MICB	hCMV	Decreased susceptibility to NK cell killing	[101]
10 miRNAs in a cluster	Thrombospondin-1 (THBS-1)	KSHV	Reduced TGF- β activity	[102]
miR-K12-11	BACH-1 and other genes	KSHV	B-cell lymphoma development	[103, 104]
<i>Host</i>	<i>Viral</i>			
miR-122	5'UTR of HCV genomic RNA	HCV	Facilitates HCV replication	[110]
miR-196, miR-448	HCV genomic RNA	HCV	Inhibits HCV accumulation in hepatocytes	[113]

have been shown to encode viral miRNAs, mostly from the herpesvirus family. Viral miRNAs can regulate viral or host genes to manipulate immune responses and cellular functions for the benefit of the virus. Additionally, viruses can regulate and use host miRNAs to facilitate their own replication. Host miRNAs may on the other hand also limit viral replication or modulate cellular processes to the disadvantage of the virus; however, viruses have their countermeasures to evade such restriction. Examples for viral and host miRNAs affecting host-viral interactions are listed in Table 2. Studying the intricate relationship between viruses and their host is not only important for understanding viral pathogenesis but may also contribute to our understanding about the role of miRNAs in immune functions.

Viral miRNAs regulating host genes

A recent study demonstrated that viral miRNAs can suppress host cell genes to evade antiviral immune response. miR-UL112, a miRNA encoded in the human cytomegalovirus (hCMV) genome suppresses the expression of the host protein major histocompatibility complex class 1-related chain B (MICB) [101]. Since MICB is a ligand of the NK cell activating receptor NKG2D, its suppression by miR-UL112 leads to reduced killing of the virus-infected cell by NK cells. Another example for viral miRNAs regulating host genes is a cluster of miRNAs encoded in the Kaposi's sarcoma herpesvirus (KSHV) genome that regulate thrombospondin

(THBS1) [102]. THBS1 is a multifunctional extracellular matrix glycoprotein that activates TGF- β ; hence reduced THBS1 levels might lead to reduced TGF- β activity, and reduced apoptosis as a consequence. KSHV-1 also encodes a miRNA, miR-K12-11, that uses an existing system of regulatory networks in the host: this miRNA has a seed sequence identical to that of miR-155, as demonstrated by two independent groups [103, 104]. Accordingly, several targets are shared between the two miRNAs. It is tempting to speculate that miR-K12-11 might contribute to Kaposi sarcoma tumourigenesis, since miR-155 has been implicated in many types of haematological malignancies.

Another viral miRNA that was suggested to protect infected cells from apoptosis by regulating the TGF- β pathway is the HSV-1 encoded miR-LAT processed from the latency-associated transcript (LAT) [105]. Although the mechanism of viral miRNA-mediated apoptosis inhibition seemed logical this report was recently retracted since other groups could not detect miR-LAT [106]. Interestingly, a recent study demonstrated that the assumption that LAT serves as a precursor for miRNAs was right; however, LAT-derived miRNAs seem to regulate viral genes instead of host genes (see below) [107].

Viral miRNAs regulating viral genes

Viral miRNAs can also target viral genes and thereby interfere with host antiviral responses [108]. The polyoma virus simian

virus 40 (SV40) encodes miRNAs accumulating at late times in infection which target early viral mRNAs for cleavage. By down-regulating the early transcripts encoding large and small T antigens, which are dominant targets of cytotoxic T-cell (CTL) response, these viral miRNAs switch the virus into 'stealth mode' to avoid detection of infected cells by CTLs [99, 108]. Ironically, the existence of these viral auto-regulatory miRNAs indicates that viruses can hijack the host RNAi machinery, which is often speculated to have evolved as an anti-viral mechanism, to generate small RNAs for their own purposes.

In addition to contributing to viral immune escape, viral miRNAs can also facilitate latent infection. Umbach and colleagues reported the discovery of five latently expressed HSV-1-derived miRNAs processed from the LAT, two of which (miR-H2-3p and miR-H6) targets genes expressed during productive infection [107]. MiR-H2-3p targets the immediate-early gene ICP0, which promotes viral replication and may facilitate reactivation from latency, while another miRNA, miR-H6 suppresses the expression of ICP4, a gene required for the expression of most HSV-1 genes during early infection [107]. A hCMV miRNA miR-UL-112, has also been suggested to promote viral latency by suppressing the immediate early gene IE72 (and other genes involved in viral replication) during the later stage of viral replication [109]. Hence, late expressed miRNAs may facilitate establishment and maintenance of viral latency.

Host miRNAs regulating viral genes

In the evolution of host-virus interactions, not only viral miRNAs have evolved to regulate host genes to their benefit, but viral mRNAs have also appeared that can be targeted by miRNAs constitutively expressed in the target cells of the virus. In 2005, a striking observation was made by Jopling *et al.* that a liver-specific miRNA known as miR-122 is actually required for hepatitis C virus (HCV) replication, without known beneficial effect for the host [110]. Mutational analysis of a predicted miR-122 binding site in the HCV genome provided evidence for the direct role of miR-122 for HCV replication. The effect of miR-122 on HCV seems to be a novel mechanism of miRNA action; however, the mechanism is still unknown. MiR-122 might facilitate HCV replication by aiding RNA folding or RNA accumulation into replication complexes, and an indirect effect can also not be excluded [111]. These findings demonstrating the requirement of miR-122 for HCV replication are not only of special interest because of the novel and unusual action of a miRNA but also because these provided the first link of miRNAs to a major infectious disease. Pharmaceutical development of drugs interfering with miR-122 is underway as it is hoped that inhibition of miR-122 expression in the liver could interfere with the replication of HCV [112].

Host miRNAs are not always beneficial for the virus but can also inhibit viral replication. A recent report demonstrated that IFN- β , an antiviral cytokine, induces numerous cellular miRNAs (such as miR-196, miR-296, miR-351, miR-431 and miR-448),

with sequence-predicted targets within the HCV genomic RNA [113]. Overexpression of these miRNAs in infected liver cells substantially attenuated viral replication demonstrating the importance of miRNAs in anti-viral immunity. Elegantly, miR-122, which has a positive effect on HCV replication in liver cells, was suppressed by IFN- β , thereby contributing to the antiviral effect of this cytokine [113]. Thus, host miRNAs may have evolved to target viral genes and thereby inhibit viral replication and represent a part of the host's antiviral immune response.

Understanding how cellular and viral miRNAs influence viral-host interactions have important implications not only for infectious diseases but also for carcinogenesis, since several viruses are associated with carcinogenesis. Therefore, miRNAs have the potential to become novel drug targets in virally induced infectious or malignant diseases.

Perspectives

Even in 2000 there were only two miRNAs described. Barely 8 years later there are 6396 miRNA entries, out of which 678 were discovered in *H. sapiens* (Fig. 1). Computational analysis of the human genome suggest that the total number of miRNAs may be more than 1000 not considering other short, non-coding RNAs that were discovered only recently. Although the number of miRNA publications is increasing rapidly (Fig. 2), the functions are still unclear for the majority of identified miRNAs.

Computational prediction of miRNA targets has become an important tool in the investigation of miRNA functions but its reliability needs further improvements. Although it is now known that certain factors – seed match, evolutionary conservation – may be important for the miRNA : mRNA binding, their relevance to the repression of protein production is largely unknown. Understanding of miRNA functions has been hindered by the lack of high-throughput protein expression analysis. However, the recent appearance of new, high-throughput proteomic approaches can in the future aid the identification of targets on a global scale.

Deregulation of miRNAs in diseases have been only partially understood and can be explained by genetic (mutations, deletions, translocation) and epigenetic mechanisms (methylation) or by aberrations in the miRNA-processing machinery. Most studies investigating the role of miRNAs in diseases have been focusing on cancer, and still little is known about the effect of miRNAs on normal immune functions and immune-related diseases. However, miRNAs are undoubtedly required for the development of the immune system as well as for normal innate and acquired immune response. An increasing body of evidence shows deregulation of miRNAs in autoimmune, chronic inflammatory and other immune-mediated diseases, although their role in the pathogenesis of these diseases still awaits discovery.

The emergence of miRNAs will not make the understanding of regulatory networks easier. Each miRNA acts by suppressing the

expression of hundreds of protein-coding genes and each gene is regulated by more than one miRNA that may act in a cooperative fashion. The complexity of the full picture about gene regulation in health and disease apparently exceeds our wildest dreams. However, regulation of whole pathways instead of single genes

also makes miRNAs excellent candidates for gene therapy. There are promising results from the first experiments with drugs based on, or targeting miRNAs which can give rise to radically new therapies for the treatment of common immune-mediated and malignant diseases in the next decades.

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