Human- and Virus-Encoded microRNAs as Potential Targets of Antiviral Therapy

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Abstract: It has recently been demonstrated that short RNA molecules, called microRNAs, are one of the major factors regulating the expression of human genes. There are several lines of evidence that microRNAs also play a key role in host-virus interactions. It is believed that both human- and virus-encoded miRNA will, in the nearest future, become very attractive targets of antiviral therapy.

Key Words: microRNA, host-virus interaction, antiviral therapy, antagomiR, miRNA mimetic, target protector, artificial miRNA.

INTRODUCTION

For a very long time, practically until the end of the twentieth century, proteins were commonly considered the main products and regulators of the gene expression process. In this context, the results of the Human Genome Project came as a surprise. They indicated that less than 5% of the human genome encodes proteins. Another breakthrough in our understanding of mechanisms regulating the expression of genetic information was the discovery of RNA interference (RNAi) by Fire and Mello [1]. RNAi can be defined as a sequence-specific and evolutionarily conserved mechanism of gene silencing. It is mediated by 20-24 nucleotide (nt) RNA molecules (small interfering RNA – siRNA) generated from a long double-stranded precursor homologous to the silenced gene or its fragment [2]. During subsequent studies several other classes of short regulatory RNA (srRNA) were identified in eukaryotic cells, e.g.: microRNAs (miRNAs), tiny non-coding RNAs (tncRNAs), small modulatory RNAs (smRNAs) and piwi-interacting RNAs (piRNAs) [3]. As a result, it became clear that RNA, especially 20-30 nt-long molecules should be placed among the most important factors controlling the expression of genetic information.

However, one may ask how it is possible that such simple molecules can efficiently and specifically regulate these complicated processes. srRNA are, effectively, no more than probes. They selectively target multienzymatic complexes to the respective gene or transcript. Depending on the enzymatic properties of the ribonucleoprotein complex, a DNA or RNA molecule can be modified or degraded. Alternatively, the process in which it participates can be inhibited or intensified. srRNA thus ensure specific recognition between enzymes and nucleic acids. Recent observations indicate that srRNAs are capable of controlling the release and flow of genetic information in many different ways. They can induce changes in the genome structure, inhibit transcription, mediate mRNA degradation and repress translation, Fig. (**1**). Interestingly, in different organisms, different pathways are used to regulate gene expression.

It seems that a vast majority of srRNAs occurring in human cells can be classified as members of the microRNA (miRNA) family. All of them are encoded in the human genome; they are also all generated from longer precursors. miRNAs target partially or fully complementary mRNAs and negatively modulate gene expression at posttranscriptional or translational levels. During the last years, over 500 miRNAs have been identified in humans (*Homo sapiens* miRNAs, hsa-miRNAs). It has been shown that genes encoding miRNAs (*MIR*) can be located within intergenic regions as well as within other genes, both protein-coding and non-protein-coding. It is suggested that, in mammals, 35% of miRNA loci overlap with other genes, and over 90% of these are located in introns [4-6].

It has recently been estimated that, in humans, the expression of 30-40% of genes is controlled by miRNA [7]. More detailed studies revealed that miRNAs play important roles in many biological processes, including developmental timing, growth control, and differentiation. They may also control signaling pathways, apoptosis and metabolism [6]. Furthermore, it has been shown that *MIR* expression is tissue-specific and its deregulation is associated with many types of cancers [8, 9]. It is also beyond doubt that srRNA (in mammals, especially miRNA) can play a very important role in host-virus interactions. First of all, it has been shown that plants and some animals use RNAi as a specific antiviral mechanism [10]. The RNAi machinery can produce virus-specific siRNA by digesting either double-stranded regions of viral RNA or double-stranded intermediates generated

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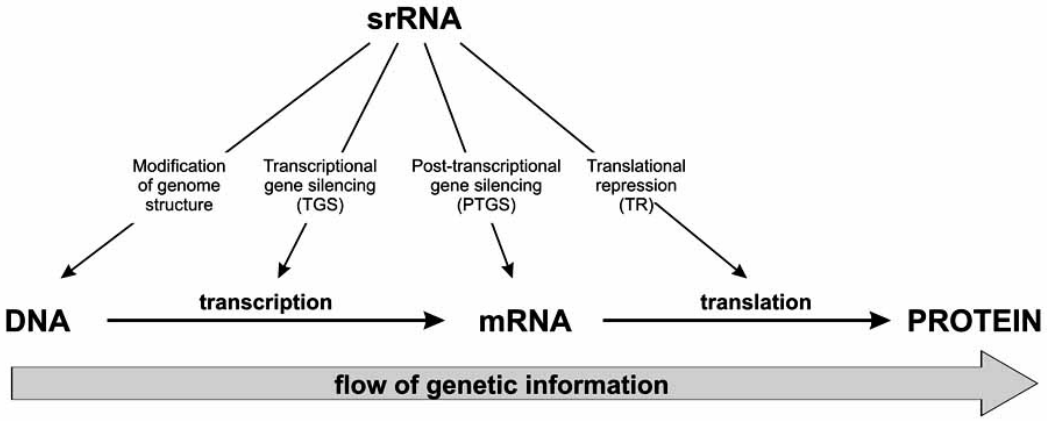

Fig. (1). srRNAs as regulators of eukaryotic gene expression. In general, srRNAs function as probes, which bind with protein complexes to ensure a specific recognition of target RNA or DNA. The resultant ribonucleoprotein complex can interact with genomic DNA and by its modification influence genome structure or inhibit transcription. Otherwise it can also bind with mRNA and mediate its degradation or inhibit its translation.

during RNA virus replication. Moreover, it has been found that host-encoded miRNAs are also capable of participating in host-virus interactions; they can stimulate as well as suppress viral infections [11, 12]. Finally, it has been shown that miRNAs which affect either virus or host gene expression are also encoded in viral genomes [13]. All the above finds have caused miRNA to be considered a very important factor shaping host-virus interactions [14].

In this review we briefly present current knowledge about miRNA biogenesis and mechanisms of miRNA-mediated gene regulation. Further, we discuss the role of miRNA in host-virus interactions and perspectives of using miRNAs as targets of antiviral therapy.

miRNA BIOGENESIS AND FUNCTION

In mammals, miRNAs are generated in a multi-step process. They are formed from hairpin-shaped transcripts (pri-miRNAs) synthesized usually by polymerase II, Fig. (**2**). In the first step, nuclear RNase Drosha excises from pri-miRNA a 50-70 nt stem-loop precursor, called pre-miRNA. It is transported to the cytoplasm by the nuclear transport receptor, exportin-5. In the second step, the Dicer uses pre-miRNA as a substrate and generates a short 19-23 nt asymmetric duplex, with 2 nt overhangs at the 3'-ends and phosphorylated 5'-termini. The short duplex is incorporated into a multi-protein complex referred to as the RNA-induced silencing complex (RISC) [15]. Next, during the RISC activation process, one strand of the duplex is released and degraded [16]. This strand is always the one whose 5′ end is more tightly paired to its complement. The second, called miRNA, remains in the active complex (RISC*) and acts as a sequence-specific probe which targets RISC* to the complementary mRNA. If the miRNA is perfectly complementary to the mRNA, the latter is cleaved within the target site. This process is generally called posttranscriptional gene silencing (PTGS) and frequently occurs in plants and insects. However, if the mRNA is only partially complementary to the miRNA, it is not degraded. Instead, its translation is repressed (a process referred as translational repression; TR), Fig. (**2**).

Data collected in the last 15 years suggest that miRNAs function as mRNA-specific suppressors of protein synthesis at the transcriptional or translational level. However, knowledge of the mechanisms by which miRNAs repress gene expression is still very limited. It is suggested that in animals, miRNAs recognize their targets mainly through limited base-pairing between their 5'-end (a seed region, comprising nucleotides 2–7) and a complementary sequence located within the 3' untranslated region (3'-UTR) of the silenced mRNAs [7]. Since interactions between miRNA and a target 3'-UTR are often restricted to as few as 6 base-pairs, it is predicted that a single miRNA can bind and regulate many different mRNA targets. Conversely, several different miRNAs can bind and cooperatively control a single mRNA [17]. In this way miRNAs and their targets constitute complex regulatory networks.

The presented mechanism implies that miRNAs, which partially base-pair with the target mRNA, repress its translation without affecting its stability. However, recent findings suggest that miRNA may also mediate mRNA degradation, although it does not form a perfect duplex with the targeted mRNA [18, 19]. There is a hypothesis suggesting that degradation of the target mRNA is preceded by translational inhibition [20]. It was proposed that RISC* binds the target mRNAs while they are being translated. Next, the ribonucleoprotein complex is directed to specialized centers of mRNA degradation called processing bodies – P bodies (alternatively called cytoplasmic bodies or GW bodies) [21, 22]. Moreover, several miRNAs have recently been identified which destabilize their target mRNAs through deadenylation followed by decapping [18, 19, 23-25]. It was also shown that, whereas poly(A) tail removal is essential for miRNA-mediated mRNA degradation, it is not required for translational repression by miRNAs. There are some reports indicating that in human cells non-polyadenylated mRNAs can also be inhibited at the translational level by miRNA-dependent mechanism [19]. Results collected by Wu *et al*. suggested that these two regulatory mechanisms: (i) miRNA-mediated deadenylation and degradation, and (ii) translational repression operate independently. Thus, miRNAs

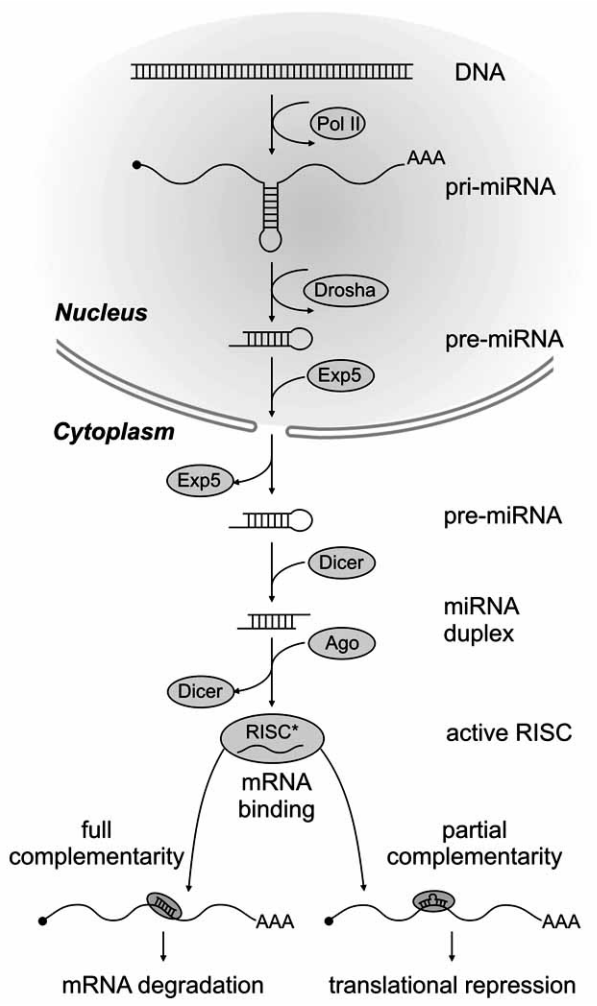

Fig. (2). Biogenesis and function of miRNA (detailed description in text). Functional miRNAs are generated in a multi-step process. During the first stage, the 3'-polyadenylated and 5'-capped transcript (pri-miRNA) containing local double stranded regions (hairpin structures) is synthesized by RNA polymerase II. Next, a 50-70 nt hairpin-shaped precursor (pre-miRNA) is excised from pri-miRNA by RNase Drosha and transferred from the nucleus to the cytoplasm by exportin-5. Then ribonuclease Dicer cuts pre-miRNA and releases a short (19-23 nt) miRNA duplex, which enters RNA-induced silencing complex (RISC). One strand of the duplex is removed and degraded and the second, called miRNA, serves as a probe targeting RISC to specific mRNA. If the miRNA is fully complementary to the target molecule, mRNA is degraded, if base-paring is not perfect mRNA translation is repressed.

seems to have much greater overall impact on gene expression than previously expected [19].

ROLE OF CELLULAR miRNAs IN HOST-VIRUS INTERACTIONS

More detailed studies of dsRNA-mediated gene silencing suggest that RNAi can function as a specific antiviral mechanism. Indeed, it was shown that in plants and invertebrates the RNAi machinery can recognize local double-stranded regions present in the viral genome or dsRNA intermediates forming during virus replication and produce siRNAs targeting viral RNAs. Unlike simpler organisms, vertebrates developed an interferon-based system which is induced by dsRNAs forming upon viral infections. However, interferon-response is activated by long dsRNA. It is not sensitive to short dsRNA (< 30bp). Recently, it has been demonstrated that short dsRNA can induce RNAi in human cells, which strongly suggests that this phenomenon can also play an important role in antiviral defense in mammals [14]. It was shown that in humans, cellular miRNAs can modulate virus replication. However, viruses have also evolved very sophisticated strategies for evading the host cell response, even miRNA pathways. Human viruses encode proteins and/or RNA molecules that suppress the miRNA-based antiviral mechanism and modulate the expression of specific host genes [26].

There are several documented examples of human miRNAs that act on viruses, Fig. (**3A**). For instance, hsa-miR-32 effectively limits replication and accumulation of the primate foamy virus type 1 (PFV-1) in human cells [12]. A potential target sequence for the hsa-miR-32 was found in the PFV genome; the proposed mechanism of viral gene silencing assumes the translational repression of PFV transcripts. Further, it was shown that human-encoded liver-specific hsa-miR-122 is required for effective replication of the HCV genome [11]. In addition, it was demonstrated that interferon beta (IFN-beta) treatment leads to a significant reduction of the hsa-miR-122 accumulation, which results in decline of HCV load in the liver cells [27]. There are also reports showing that IFN-beta may induce the expression of numerous cellular miRNAs, and that some of these miRNAs may inhibit HCV replication [27]. The presented findings strongly support the assumption that mammals can use cellular miRNAs to combat viral infections, and that miRNA-mediated pathways cooperate with the mammalian protein-based immune system.

Other examples of human-encoded miRNAs that influence virus replication were provided by Huang *et al.* [28]. They demonstrated that cellular miRNAs contribute to HIV-1 latency and potently inhibit HIV-1 production in resting CD4+ T cells. Huang *et al*. found that the 3'-ends of HIV-1 mRNAs are targeted by cellular miRNAs, including hsa-miR-28, hsa-miR-125b, hsa-miR-150, hsa-miR-223 and hsa-miR-382, which are abundant in resting CD4+ T cells. Unfortunately, the latency of HIV-1 in these cells is beneficial for the virus, but not for the host. The latent virus, stably integrated with the host genome, is not producing any viral proteins. In this way, it is invisible for the host immune system. Furthermore, the latency of HIV-1 in resting primary CD4+ T cells is the major barrier for the eradication of the virus in patients subjected to antiretroviral therapy. Based on these data, Huang *et al*. suggest that targeting cellular miRNAs could be a new way of purging the HIV-1 reservoir.

VIRAL GENOMES AS THE SOURCE OF miRNAs

The discovery of the first virus-encoded miRNAs (vmiRNA) opened a new perspective of exploring complex host-pathogen interactions. The first report concerning this

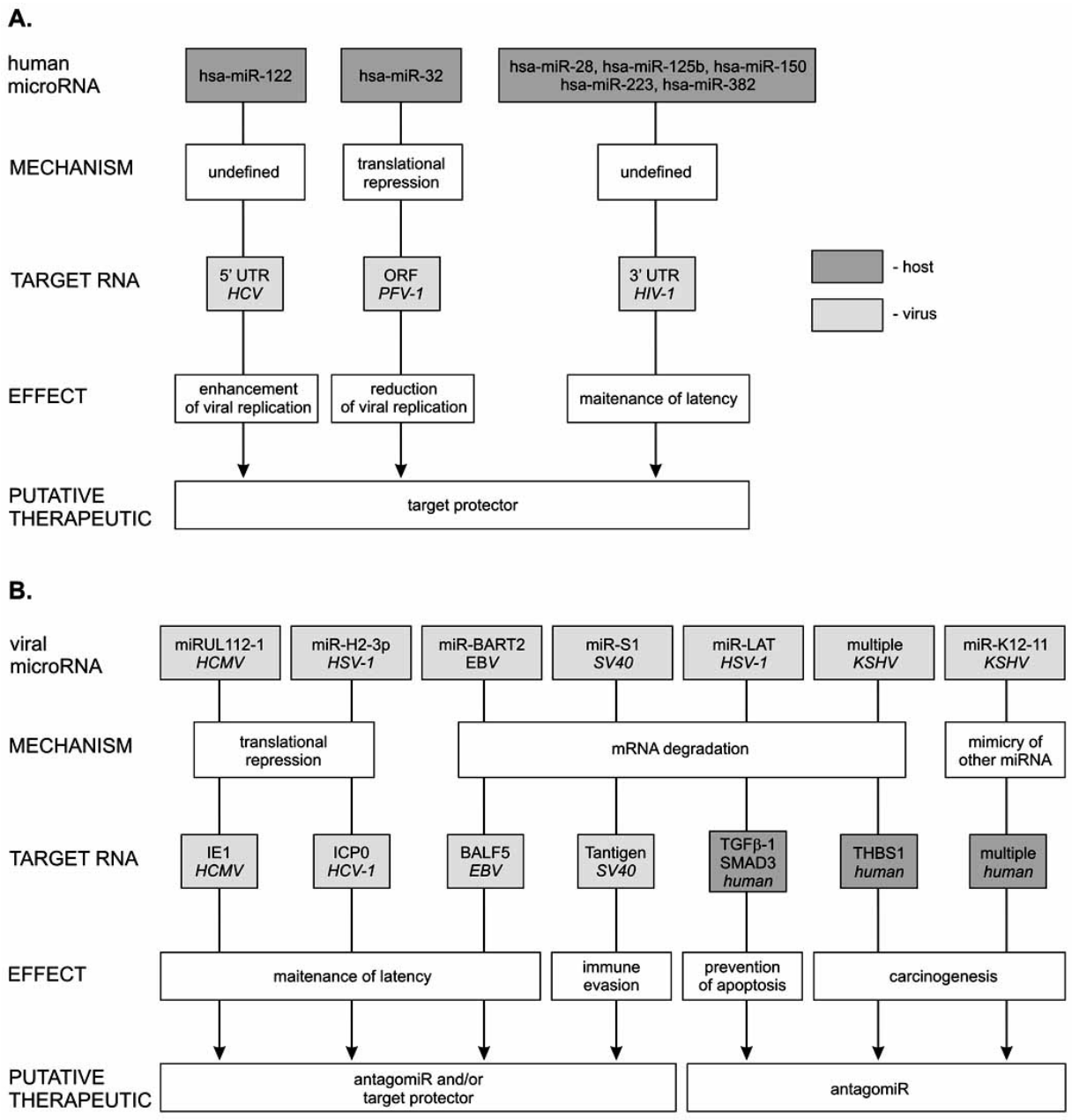

Fig. (3). Selected examples of human (A) and viral (B) miRNA-dependent regulation of virus/host gene expression. For each miRNA its target RNA, proposed mechanism of action and observed effect are indicated. In addition, a putative therapeutic RNA which can interfere with host-virus interaction is proposed.

problem showed that Epstein-Barr virus (EBV) encodes five miRNAs in its large DNA genome. In addition to modulating EBV replication cycle, vmiRNA proved to interfere with human gene expression [13]. In the last four years, further vmiRNA have been identified [26, 29-37]. They all are encoded by DNA viruses. These pathogens utilize a host machinery to transcribe their genomes. Therefore their replication cycle requires a nuclear stage. Considering the fact that miRNA precursors are formed in the nucleus, one has to assume that, by definition, vmiRNAs cannot be produced by viruses replicating in cytoplasm (e.g. RNA viruses). The only RNA-based viruses that could possibly possess vmiRNA coding sequences are retroviruses, which reverse-transcribe and integrate their genetic material with host DNA. Indeed, vmiRNAs derived from the nef RNA and TAR element have recently been found in human immunodeficiency virus 1 (HIV-1) infected cells [38-42]. Another four hypothetical precursors of HIV-1 vmiRNAs remain to be confirmed experimentally [43].

Like precursors of cellular miRNAs, those of vmiRNAs are most frequently encoded within untranslated regions (introns and intergenic regions) and transcribed by host polymerase II. However, there exist vmiRNA coding sequences

which are located in open reading frames. This is the case for EBV, human cytomegalovirus (HCMV) and Kaposi's sarcoma-associated herpesvirus (KSHV) [13,32,34]. As shown by the example of adenovirus and mouse herpesvirus 68 (MHV68), vmiRNAs can also be derived from polymerase III transcripts [34, 44]. Generally, the processing of vmiRNAs precursors proceeds as described for cellular miRNAs. Nevertheless, it remains unclear whether vmiRNAs originating from polymerase III transcripts are generated in the same way [45].

One of the primary functions of vmiRNAs is the regulation of virus gene expression, Fig. (**3B**). It seems that miRNA-dependent regulation is typical for viruses which need to switch between latent and lytic phases during their replication cycle. In latency, the virus remains quiescent and only a limited portion of its genome is expressed. Protein synthesis is arrested but RNAs, including vmiRNAs and their precursors, are regularly detected.

srRNA-mediated RNA degradation appears to operate in the first discovered vmiRNA-target pair (EBV vmiRNA - EBV mRNA) [13]. EBV was shown to encode miR-BART2, which perfectly matches mRNA encoding viral DNA polymerase (BALF5; BamHI left reading frame 5 protein). It was demonstrated that miR-BART2 targets 3'UTR of BALF transcript, triggering its cleavage [13]. The expression of viral T antigen is regulated during simian virus 40 (SV40) infection in an analogous way [46]. In this case, vmiR-S1 originates from one of the late transcripts and is fully complementary to early T antigen mRNA. As a result, the latter is cleaved. Although this interaction does not affect the replication cycle per se, it prevents SV40-infected cells from lysis and thereby enhances infection.

vmiRNA-mediated translational repression is also frequently exploited by viruses. For example, it was observed that HCMV miR-UL112-1 negatively regulates the synthesis of immediate-early protein (IE1), a pivotal factor inducing the lytic phase. Detailed analysis showed that IE1 mRNA level did not differ significantly compared to control, which pointed to translational repression as the regulatory mechanism [47]. Another example of this kind of regulation is the interaction of herpes simplex virus-1 (HSV-1) miR-H2-3p with ICP0 transcript, encoding a viral immediate-early transcriptional activator, proposed to have a role in HSV-1 reactivation from latency [48]. miR-H2-3p is able to down-regulate ICP0 gene expression, without its mRNA degradation.

Interestingly, the gene expression control provided by vmiRNAs may not be restricted to the two mechanisms mentioned above. Another hypothetical model was postulated based on the observation that some vmiRNAs (e.g. KSHV miR-K10 or three EBV vmiRNAs) are encoded inside open reading frames (ORFs) [30, 35, 49]. In these instances, vmiRNA-mediated control of viral gene expression would be directly connected with their biogenesis. The processing of such transcripts by Drosha would exclude them as mRNAs. Naturally, there might exist an equilibrium between vmiRNAs and mRNA production. However, it is also plausible that under some circumstances the balance is shifted towards one of the processes.

VIRUS-ENCODED miRNAs MODULATE HOST GENE EXPRESSION

Theoretically, there are at least three strategies by which vmiRNA can affect host gene expression: (i) vmiRNA-dependent host mRNAs degradation; (ii) vmiRNAs-mediated translational repression of host mRNA and (iii) host miRNA mimicry, Fig. (**3B**).

Until now, computational screening has been the predominant method of recognizing vmiRNA targets in the human genome. These investigations do not provide much insight into the molecular mechanism of the studied process. They are, however, necessary to select host genes whose expression can be controlled by vmiRNAs. It was demonstrated that EBV vmiRNAs can potentially regulate several genes encoding proteins involved in apoptosis, cell proliferation, signal transduction, transcription regulation and immune response [13]. Similar screening conducted for ten putative HIV-1 vmiRNAs resulted in the identification of about 800 targets, e.g.: mRNAs of ion channels, DNA-binding protein and kinases [43]. In addition, another theoretical study demonstrated that HIV-1 potentially encodes miRNAs capable of regulating the production of some specific receptors and interleukins, which accumulation declines during infection [50]. Although computational predictions have obvious inherent limitations, they provide important prerequisites pointing towards the considerable role of vmiRNAs in the modulation of host gene expression.

Empirical evidence for vmiRNA-driven regulation of human genes expression came from several recent studies conducted in cell cultures. They suggest that the first mentioned above strategy, vmiRNA-dependent host mRNA degradation, underlies the anti-apoptotic effect exerted by one of HSV-1 miRNAs. Latency-associated transcript *LAT*, previously known to inhibit cell death by an unidentified protein-independent mechanism, was suspected to encode miRNA (miR-LAT) [51]. It was hypothesized that putative miR-LAT substantially decreases the accumulation of two target cellular mRNAs (TGFβ-1 and SMAD3). Since both of them encode proteins involved in apoptosis, their decline prevents this process. As a result, infected neurons are not eliminated but contribute to the increase of virus reservoir. However, this hypothesis needs to be verified since other researchers have not confirmed the formation of miR-LAT [52]. KSHV was also shown to encode vmiRNAs able to mediate the degradation of host mRNAs (e.g THBS1; thrombospondin-1). DNA microarray analysis of cells stably expressing KSHV-encoded vmiRNAs revealed changes in the expression of about 80 genes, eight of which were down-regulated between 4- and 20-fold [53].

So far, there is lack of direct evidence that vmiRNAs mediate translational repression of host mRNA. The third strategy, vmiRNAs mimicry of cellular miRNAs has been described only for KSHV. This pathogen encodes miR-K12-11, which displays significant homology, including the entire seed region, to cellular oncogenic miRNA (hsa-miR-155). Both these miRNAs can target a common set of cellular transcripts, including those connected to metastasis, cell cycle regulation, differentiation, carcinogenesis and apoptosis [54, 55]. Interestingly, an analysis of other known viral miRNAs

showed that the seed region homology observed for miR-K12-11 and miR-155 is not exceptional. In view of this observation, it is imaginable that vmiRNAs mimicry of human microRNAs may turn out to be a more common phenomenon.

HUMAN- AND VIRUS-ENCODED miRNAs AS POTENTIAL TARGETS OF ANTIVIRAL THERAPY

Human- or virus-encoded miRNAs can be potential targets of antiviral therapy at three different levels: (i) at the genomic level, in the nucleus - epigenetic modifications of the genome, both host and viral, (ii) at the level of miRNA precursors: pri-miRNAs and pre-miRNAs (in the nucleus or cytoplasm) and finally (iii) at the level of mature miRNAs, present in the cytoplasm. Proteins taking part in the processing of miRNA precursors can naturally also be targets of antiviral therapy. However, this strategy does not provide specific treatment against viruses, but rather shuts down overall miRNA biogenesis pathways.

With respect to the availability of targets, delivery of therapeutics to the cytoplasm appears to be a much easier strategy than delivery to the nucleus. Thus far, a vast majority of trials has been focused on mature miRNAs and their mRNA targets present in the cytoplasm. To the best of our knowledge there are no reports describing therapeutic agents directed strictly to the nucleus. The next question is: what is a better target, host miRNA, vmiRNA or mRNA? Since the profiling of miRNA accumulation in healthy and virus-infected cells does not provide information about their targets, it seems much easier to direct a treatment to host miRNA or vmiRNA rather than to unidentified mRNAs. It also has to be taken into account that an individual miRNA can target roughly 100-200 transcripts, so inhibition of even a single miRNA may lead to many unintended effects [56]. Thus, it seems more reasonable to target vmiRNAs, present only in the infected cell.

POTENTIAL AGENTS MODULATING miRNA PATHWAYS

At present, RNA and DNA molecules, their derivatives and analogues are used mainly to inhibit or enhance miRNA activity. Depending on the mode of action, they can be divided into four classes: (i) miRNA mimetics which restore reduced miRNAs levels, (ii) antagomiRs that counteract specific miRNAs, (iii) target protectors which shield transcripts, precluding base-pairing between miRNA and its target and (iv) artificial microRNAs [57, 58], Fig. (**4**).

The first class, miRNA mimetics, are intended to increase the level of under-represented miRNAs, Fig. (**4A**). They have already been successfully applied as therapeutics

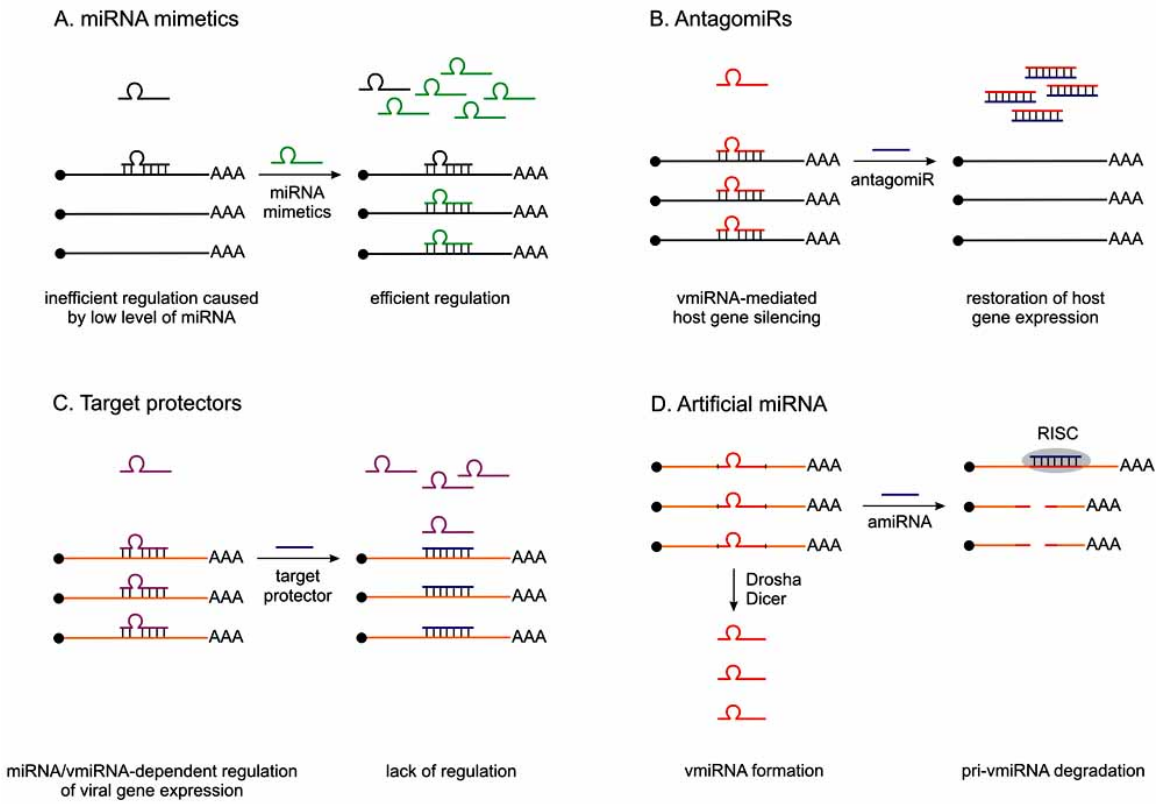

Fig. (4). Modulators of miRNA pathways and their potential applications as antiviral agents. miRNAs are depicted with bulged lines; black – human, red – viral, purple – human or viral. mRNA targets are represented by straight lines with caps (black dots) and polyA tails; black – human, orange – viral. (A) miRNA mimetics (green) enlarge the pool of under-represented miRNA, thereby restore efficient regulation. (B) AntagomiRs (blue) decoy vmiRNA, and in this way restore a normal gene expression. (C) Target protectors (blue) shield transcripts, precluding miRNA-mediated regulation. (D) Artificial miRNAs (blue) enter RISC and mediate the degradation of vmiRNA precursors.

against cancers [59, 60]. Assuming viral, but not host, miRNAs as potential targets, no antiviral application for miRNA mimetics can be proposed so far. Nonetheless, future findings concerning host-virus interactions may reveal some pathways where this kind of therapeutics could be employed.

In contrast, antagomiRs seem to be very promising antiviral agents. These molecules can efficiently and selectively block virus-encoded miRNAs, Fig. (**4B**). AntagomiRs are antisense oligonucleotides capable of acting in two ways. First, they are able to bind miRNA precursors in the cytoplasm and disturb their biogenesis [61]. Second, they can base-pair with mature miRNAs, diminishing their active pool [57]. High therapeutic potential of antagomiRs was demonstrated in mice [62]. In addition, antagomiR-mediated inhibition of KSHV oncogenic miR-K12-11 was reported [55]. Since this field quickly develops, new such antivirals are expected in the nearest future. AntagomiRs can be used to block vmiRNAs that regulate viral latency [13, 47, 48]. This approach may reduce viral persistence and increase the efficiency of current therapy, which is only effective during the active phase of a virus replication cycle. Similarly, antagomiRs targeted against vmiRNAs, which down-regulate the expression of viral antigens [46], can facilitate the host immune response. Interestingly, antagomiR has also been used to inhibit cellular hsa-miR-122, leading to changes in lipid metabolism [63]. Considering the fact that hsa-miR-122 enhances HCV replication [11], the same antagomiR could possibly exert an antiviral effect in infected cells.

Although antagomiRs can efficiently impair miRNA-mRNA interaction, in some situations the use of target protectors could be more beneficial. Target protectors compete with miRNA for the same binding sites present within mRNA, Fig. (**4C**). Their specificity is accomplished by base-pairing with a seed region and with sequences flanking it. Whereas seed regions are identical or highly similar within various targets of one miRNA, flanking sequences are usually unique to a specific transcript. In this way, one can use target protectors to inhibit miRNA binding with one specific mRNA, leaving interactions with other targets unaffected. These modulators were first applied to study gene expression in zebrafish [64]. They may become potent antivirals in the future. Target protectors can be especially beneficial in cases where host miRNA positively influences viral replication as it is observed for hsa-miR-122 and HCV. A protector of the target sequence located within the 5' UTR of HCV genome would inhibit pathogen replication without abolishing hsa-miR-122 interactions with other cellular targets.

Artificial miRNAs (amiRNAs) are produced *in vivo* from engineered precursors, which are delivered into a cell by using vectors, Fig. (**4D**). Experiments conducted in human cell lines show that amiRNAs can efficiently inhibit expression of target genes [58]. As antiviral agents, they were shown to successfully mediate virus resistance in plants [65]. Theoretically, artificial miRNA can be designed for any target, including viral transcripts. Of course, many factors have to be carefully considered in order to ensure their proper processing and specificity. To simplify the construction of amiRNAs, specific modification are introduced into vectors containing sequences encoding natural miRNA precursors.

DELIVERY OF AGENTS MODULATING miRNA PATHWAYS

Efficient and tissue-specific delivery of agents modulating miRNA pathways is the most difficult problem to overcome before their practical application. Various strategies of oligonucleotide-based drug administration (aptamers, rybozymes, antisense RNA and DNA) have already been developed [57, 66]. The same approaches could be exploited in miRNA-targeted antiviral treatment. Because naked oligoribonucleotides are subjected to degradation at physiological conditions, their stability is frequently improved by modifications which increase RNA resistance to cleavage by nucleases, Fig. (**5A,B**). In addition, the modifications can provide higher affinity to the target and optimize the thermodynamic stability of drug-target interactions. Thus, tested agents often include one or more 2'-O-methyl, 2'-O-methoxyethyl and 2'-fluoro nucleotides [63, 67, 68]. Additionally, some of them are hybrids composed of RNA and DNA or locked nucleic acid (LNA) which has an extra linkage between 2' and 4' carbon atoms [69-71]. Moreover, the natural phosphodiester backbone can be substituted by phosphorothioate [62] or replaced with N-(2-aminoethyl)-glycine- or morpholine-based chain [64, 71]. The former is linked by peptide bonds (peptide nucleic acids; PNA) the latter by phosphoamide group, Fig. (**5B**). Another problem connected with therapeutic oligonucleotides application is their transmembrane transport. As polyanions, they are not able to diffuse across negatively charged cellular membranes. The simplest solution is to enclose them in nanoparticles, like liposomes or polyethylenimines, Fig. (**5C**). Unfortunately, liposomes, usually created from cationic lipids, turned out to be toxic in some cases [72]. This effect can be overcome by enriching liposomes with neutral lipids [73]. Alternatively, polyethylenimines (PEIs) or virus-like particles (VLP) can be applied. PEIs are synthetic polymers which possess a high positive charge density due to a protonable amino group in every third position. They have been broadly used as DNA transfection reagents. However, it has been shown that not all PEIs offer the same transfection efficiency – novel low molecular weight PEIs seem to be most promising [66]. Therapeutic agents can also be packed into VLPs. The latter are formed by viral coat proteins in a self-assembly process *in vitro* or *in vivo*. Having positively charged interior, VLPs are capable of transporting RNA molecules into the target cells [74]. Other cationic carriers used for oligonucleotide transport are cell-penetrating peptides (CPPs). They bind oligonucleotides covalently or through electrostatic interactions and transfer them to target cells independently from membrane receptors. This feature could be used to target CPP-RNA complexes to various types of tissues [75]. A cholesterol moiety has also been successfully attached to the oligonucleotides to aid their delivery to various organs [62, 76]. Efficient uptake of the cholesterol group is most probably accomplished by low-density lipoprotein (LDL) receptors, present on many cell types. In addition to assisting transmembrane transport, conjugation with cholesterol probably further improves oligonucleotide intracellular activity [77].

Many methods enabling successful administration of regulatory RNAs *in vivo* have also been elaborated. In ani-

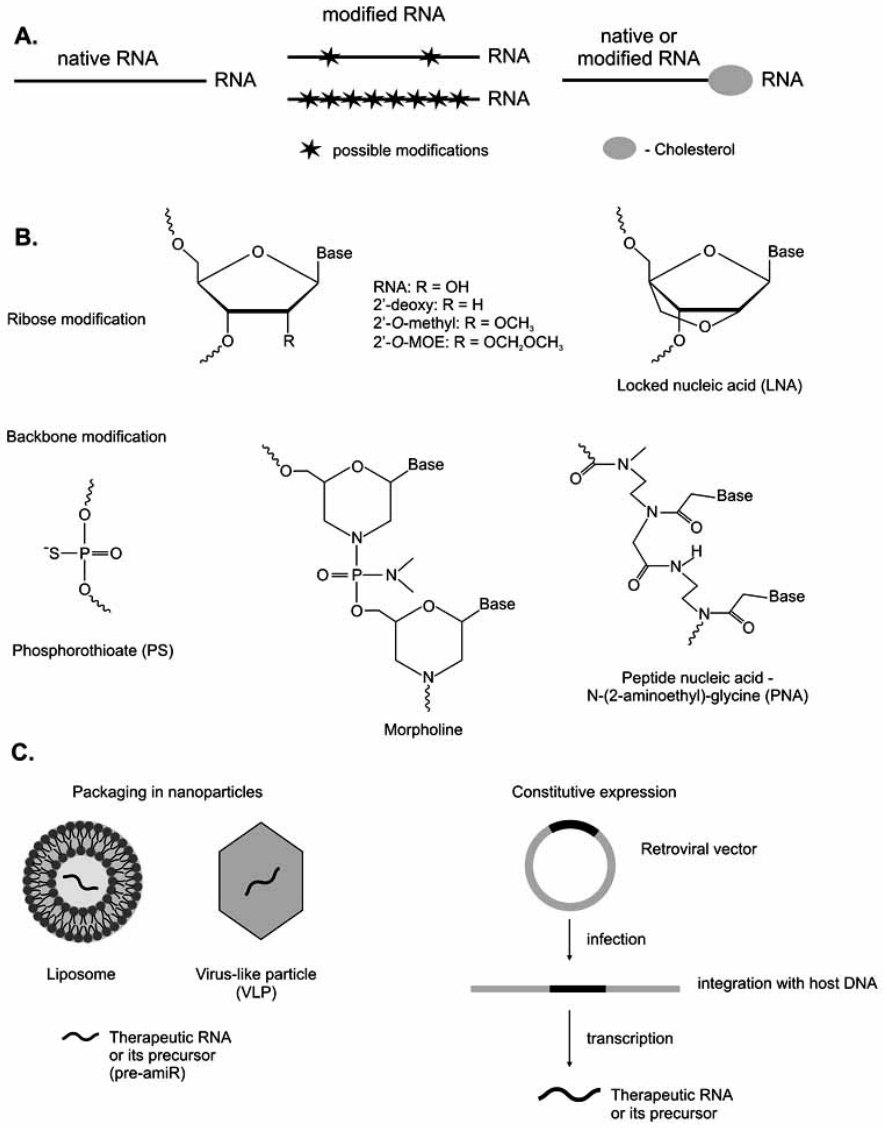

Fig. (5). Modifications of therapeutic oligonucleotides and their delivery. (**A**) Therapeutic oligonucleotide can be administrated in native or modified form (the latter can contain one or more modified nucleotides). In addition, it can be conjugated with cholesterol aiding its uptake by target cells. (**B**) Selected modifications frequently used to improve the stability and cell internalization of therapeutic oligonucleotides. (**C**) Selected methods of oligonucleotides delivery into human cells.

mal models, unmodified nucleic acids are usually applied to target organs (e.g. liver, kidney, lung) by hydrodynamic injection. This strategy is, however, unusable in humans. At present, it is only possible to target unmodified RNAs directly to certain organs, like eyes and the respiratory tract. Nucleic acids enclosed in nanoparticles are mainly delivered intravenously. The main problem in this kind of delivery is the dilution of the therapeutic in the bloodstream [66].

No matter which carrier is employed, the nucleic acid has to be delivered in sufficiently high amounts to exert its effect *in vivo*. Obviously, the concentration of therapeutic agent decreases after each cell division. That is why a stable effect could only be achieved by repeated administration of the compound. Alternatively, cells can be transfected with different types of vectors, from which therapeutic molecules are produced [78, 79]. Recently, several viral systems ensuring either transient or constitutive expression of artificial miRNA genes in mammalian cells have been elaborated. Among them, retroviral vectors seem to be especially promising as they allow for the integration of miRNA coding sequences with a host genome [80].

Minimizing unintended effects of agents modulating miRNA pathway strongly depends on selective targeting them to specific cells. Liver-targeting is easiest and does not need special signals. Intravenously administrated compounds will be carried to the liver with the blood. Specific delivery of nucleic acids to other tissues and organs requires the application of ligands binding particular cell receptors. For example, transferrin receptor is often present on the surfaces of tumor cells. Consequently, siRNAs coupled to transferrin

receptor-targeting cyclodextrins have been designed as antiproliferative therapeutics [57]. Virus infected cells also expose specific proteins. Thus, a similar strategy could possibly be employed as an antiviral approach. Otherwise, protamine-antibody carriers can be used. Protamine is a natural positively charged protein that efficiently packages nucleic acids. The antibody component specifically recognizes target cell surface antigens. These constructs have already been demonstrated to effectively target cells and suppress hepatitis B virus (HBV) gene expression in mice [81]. In addition, combinatorial methods can be employed to obtain molecules specifically interacting with target cells. This approach has been used to modify so-called tryptophan cages. Tryptophan cages are small, naturally occurring protein folds of hydrophobic amino acid residues surrounding tryptophan in central position. Several amino acid residues can be substituted without disturbing the cage fold. Accordingly, randomized peptide libraries of tryptophan cages with diverse cell-type affinities have been obtained by phage display technology [57, 82].

CONCLUSIONS

MicroRNA pathway seems to be widely exploited in complex host-virus interactions. Since the discovery of short dsRNA-induced gene silencing in mammals, it has become clear that this phenomenon can play a role in antiviral response. It has been shown that cellular miRNAs can modulate virus replication. On the other hand, it has been found that viruses encode miRNAs capable of altering the expression of specific host genes. Because both pathogen- and host-encoded miRNAs can function as regulators of the viral replication cycle, agents interfering with this process (miRNA mimetics, antagomiRs, target protectors, and amiRNAs) are considered new promising antiviral drugs [83]. Unfortunately, our knowledge about miRNA-mediated gene regulation and miRNA biogenesis is still very limited. Thus, further intensive studies are needed to develop new effective therapeutic methods/agents restricting viral infections by interfering with miRNA pathways.

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