SURVEY AND SUMMARY

Sequence-non-specific effects of RNA interference triggers and microRNA regulators

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ABSTRACT

RNA reagents of diverse lengths and structures, unmodified or containing various chemical modifications are powerful tools of RNA interference and microRNA technologies. These reagents which are either delivered to cells using appropriate carriers or are expressed in cells from suitable vectors often cause unintended sequence-non-specific immune responses besides triggering intended sequence-specific silencing effects. This article reviews the present state of knowledge regarding the cellular sensors of foreign RNA, the signaling pathways these sensors mobilize and shows which specific features of the RNA reagents set the responsive systems on alert. The representative examples of toxic effects caused in the investigated cell lines and tissues by the RNAs of specific types and structures are collected and may be instructive for further studies of sequence-non-specific responses to foreign RNA in human cells.

THE LANDSCAPE OF CELLULAR TRANSCRIPTS AND RNA-BINDING PROTEINS

To review the non-specific responses of human cells to various RNA interference triggers and microRNA regulators, we begin with a brief presentation of the action scene on which both the sequence-specific silencing and non-specific side effects take place, and focus on the cellular repertoire of the transcripts and RNA-binding proteins named jointly the ribonome (1). The elements of this complex system are either targeted by foreign RNA or are implicated in generating the appropriate cellular responses.

Recent studies show that most of the human genome is transcribed (2), and only ~1% is translated. Besides the abundant RNAs such as rRNAs, tRNAs and snRNAs a plethora of mature cellular transcripts include mRNA variants from about 20 500 human protein-coding genes

(3), and it is still difficult to estimate the numbers of long and short non-coding RNAs (ncRNAs). The long ncRNAs encompass species overlapping with protein coding transcripts, *cis*-antisense, bidirectional and intronic transcripts (4), whereas short ncRNAs include microRNAs (miRNAs), endogenous short interfering RNAs (siRNAs) and PIWI-interacting RNAs (piRNAs) (5).

Considering the structural features of cellular RNAs these also come in many varieties (6,7). The sense and antisense transcripts from numerous genes or genomic regions may form long stretches of double-stranded structures in the cell nucleus but most of the nuclear and cytoplasmic transcripts remain single-stranded. These transcripts typically form intrinsic higher order structures, which depending on their nature and stability, may or may not interfere with normal cellular processes. For example, short RNA hairpins containing stems composed of up to 10 bp are very common RNA structures both in the nucleus and cytoplasm and they are harmless to human cells. On the other hand, hairpins that are excessively long (8), very stable pseudoknots (9) and quadruplexes (10) are less frequent and potentially harmful.

The cellular complement of RNA-binding proteins is very rich in comparison to other protein superfamilies. At least 2500 different human proteins contain RNA-binding domains (1) and among these proteins there are already identified and yet unknown cellular sensors of foreign RNA. The most commonly occurring domains include: the RNA recognition motif (RRM) found in about 2% of human proteins, and the K-homology domain (KH) both recognizing primarily the single-stranded RNA, and the double-stranded RNA-binding domain (dsRBD) and zinc fingers (ZnF) showing a high affinity to dsRNA fragments. These domains bind RNA in a structure-dependent manner (11–13). For example, the dsRBD binds any dsRNA provided the base-paired region extends to at least 12 bp and is not interrupted by too many bulges or internal loops (13).

The dynamic associations of proteins containing RNA-binding domains with cellular RNAs define all the steps of the lifecycle of transcripts starting from their synthesis,

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processing and modifications, *via* intracellular transport and localization, to the stages at which transcripts perform their functions, and are finally degraded (14). The high functional diversity of RNA-binding proteins contrasts with the much fewer number of different types of RNA-binding domains. To accompany the multitude of transcripts in their cellular journey and participate in their functions, these proteins have developed modular structures in which the repeated RNA-binding domains are often separated by peptide spacers of diverse lengths (12). This structural organization enables the binding of different RNAs with different affinities and allows finding the cognate transcripts in a crowded cellular milieu.

It is also becoming apparent that the issue of the cellular localization of transcripts needs to be more appreciated when considering the efficiency of various RNA-targeting technologies and their side-effects. This question is no longer relevant to only a handful of mRNAs performing specialized local functions in the polarized asymmetric cells such as fibroblasts or neurons but may apply to many other mRNAs as well (15). What is very likely is the emerging scenario in which mature transcripts after leaving a cell nucleus are assembled together with ribosomal subunits and translation factors into the large RNA granules that are transported by motor proteins along cytoskeletal elements to their destination sites (16). At these specific sites the messages are translated, and targeted by endogenous or exogenous regulators of translation.

Human cells are also equipped with efficient transcript turnover and quality control systems designed to degrade the products of excessive transcription, the by-products of RNA processing, normal transcripts that ended their life-cycle, and defective transcripts having impaired processing, translation, folding or protein assembly (17). Along with these systems the RNA interference (RNAi) pathway exists and shares cellular resources with a functionally relevant system of posttranscriptional regulation of gene expression by miRNAs.

MicroRNA AND RNA INTERFERENCE PATHWAYS AND THE CORRESPONDING TECHNOLOGIES

At the beginning of this decade an entirely new level of gene regulation by miRNAs was uncovered in the cells of eukaryotic organisms (18–20). Since then more than 700 miRNAs have been identified in human cells (21) and it is estimated that the majority of human protein coding genes is under the control of these ~20 nt RNAs (22). The main steps of miRNA biogenesis and activity as well as the sites of potential intervention with the tools of RNAi and miRNA technologies are shown in Figure 1. The majority of the primary transcripts of human miRNA genes (pri-miRNAs) are generated by RNA polymerase II (23). The pri-miRNAs, which harbor a long stem and loop structure are processed in the nucleus to shorter hairpin precursors (pre-miRNAs) which are ~60 nt in length. The nuclear processing enzyme is ribonuclease Drosha (24) which forms together with the DGCR8 protein (25) the core of the Microprocessor complex

(26,27). Drosha which is the RNase III enzyme usually leaves a 2 nt overhang at the 3′-end of pre-miRNA and defines one end of mature miRNA. The pre-miRNA is then exported to cytoplasm by Exportin-5/Ran-GTP (28) where it is intercepted by the RISC loading complex (RLC) composed of another RNase III enzyme Dicer, TAR RNA binding protein (TRBP) and a member of the argonaute protein family (AGO1–4). The pre-miRNA cleavage by Dicer excises an imperfect duplex from its hairpin stem and defines the other miRNA end. The presence of structure imperfections within this duplex facilitates the non-miRNA strand to be disposed from the RLC to form an active miRISC complex (29). The miRISC then uses the miRNA strand as a guide to search for mRNAs having in their 3′-UTR complementary sequences to its seed region (30) and target mRNAs are translationally inhibited or deadenylated and degraded.

The first reagents of RNAi technology were developed before the details of miRNA biogenesis were revealed. The technology we use today has been fine-tuned by taking lessons from the more advanced knowledge of the miRNA biogenesis process. The exogenous silencing reagents enter the miRNA pathway at its different stages and take advantage of the same processing and efector proteins to evoke a sequence-specific mRNA cleavage (Figure 1). Although the process of RNAi can be induced by the single-stranded siRNAs (ss-siRNAs) (31–33) it is more efficiently triggered by ~20 bp siRNA duplexes (ds-siRNAs) mimicking the cleavage products of miRNA precursors by Drosha and Dicer, having typically the 2 nt 3′-overhangs. The ds-siRNAs (34,35) and ss-siRNAs (36) are also effective in transcriptional gene silencing (TGS) by targeting gene promoters. Longer RNA duplexes (27–30 bp) that have to be first processed by Dicer to generate siRNAs are also very efficient RNAi inducers (37–39). Synthetic RNA hairpins transfected to cells were also shown to be potent RNAi triggers (40). The vector-based RNAi triggers such as short hairpin RNAs (shRNAs) (41,42) and long hairpin RNAs (lhRNAs) (43) or pri-miRNA-based expression cassettes (sh-miRs) (44) enter the miRNA biogenesis pathway prior to the Dicer or Drosha processing steps, respectively, and confer the long-lasting silencing effects as compared to synthetic reagents.

The more recently developed miRNA technology (Figure 1) is aimed at either blocking or overexpressing cellular miRNAs for different purposes. To downregulate the overexpressed miRNAs, the antisense oligonucleotides known as antimirs (45) or antagomirs (46,47) are used. Another approach to inhibit the overexpressed miRNAs is the use of miRNA sponges (48) or decoys (49,50) that are either plasmid or viral vector-encoded mRNA fragments designed to sequester the overproduced miRNAs. In still another strategy, the abnormally increased levels of specific miRNAs remain unchanged but the antisense RNA oligomers named 'target protectors' are used (51) to protect the miRNA binding sites in the regulated mRNAs. On the other hand, in cases of miRNA under-expression, such miRNA may be transfected to cells either as an antisense strand of siRNA-like duplex known as miRNA mimetic (52) or in the form of synthetic pre-miRNA (40). Alternatively,
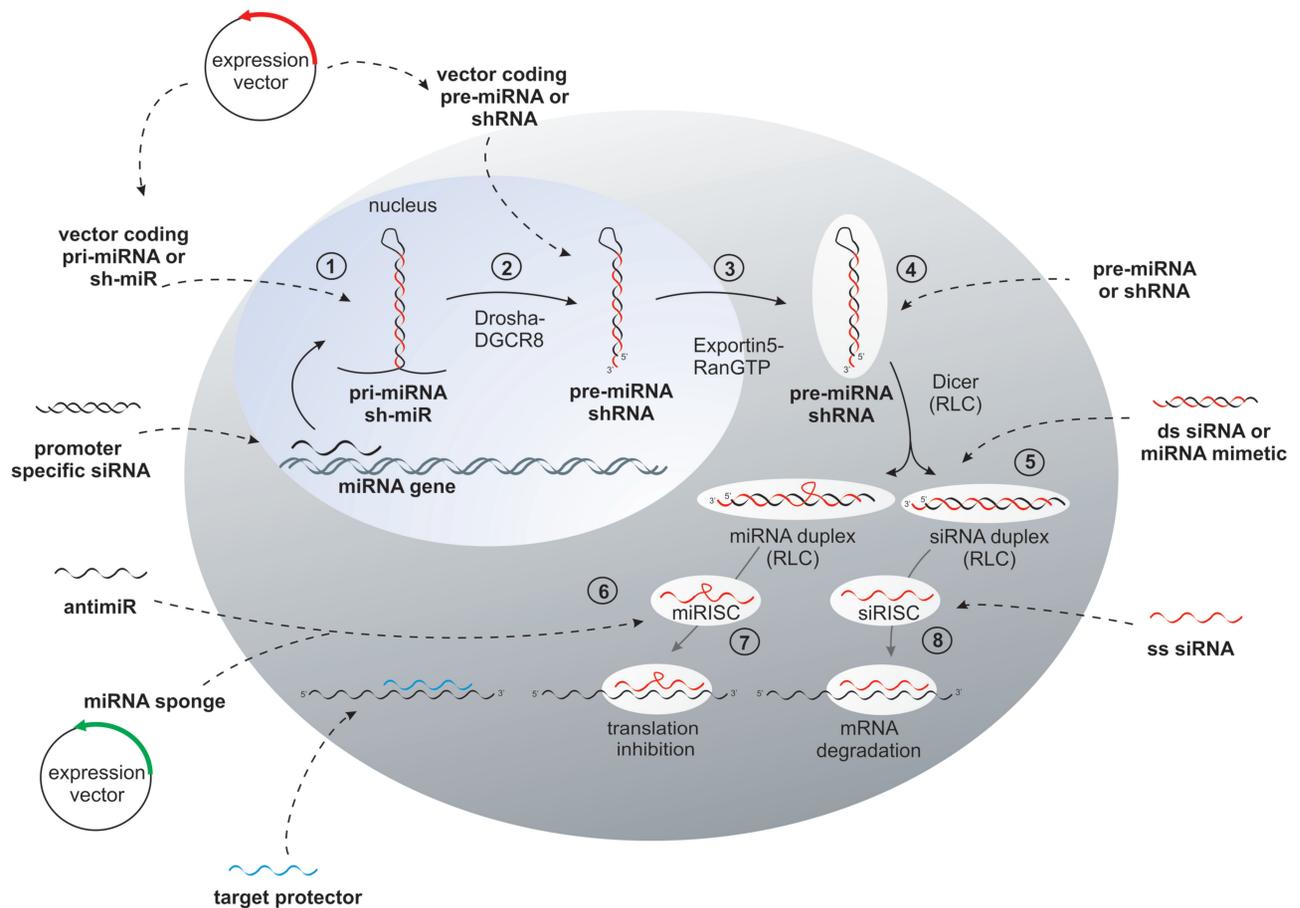
Figure 1. The steps of miRNA biogenesis and functioning as well as sites of intervention with the tools of RNAi and miRNA technologies. The miRNA biogenesis includes: 1, transcription of most of the miRNA genes by RNA Pol II to produce the pri-miRNA precursor; 2, nuclear processing of pri-miRNA by the Microprocessor complex, to generate the 50–70 nt long hairpin-shaped pre-miRNA; 3, the export of pre-miRNA to cytoplasm guided by the Exportin-5/Ran-GTP complex; 4, the pre-miRNA takeover by RISC loading complex; 5, excision of the imperfect miRNA/antimiRNA duplex from the pre-miRNA; 6, the antimiRNA strand release resulting in the formation of active miRISC, and finally finding the target mRNA by the miRISC and binding to its 3′UTR with either a partial or complete base complementarity leading, respectively, to 7, mRNA translational inhibition or 8, mRNA deadenylation and degradation. RNAi and miRNA technology essentials delivered either by transfection (ds-siRNA, ss-siRNA, shRNA, pre-miRNA, promoter-specific siRNA, target protector, antimiR, miRNA sponge and mimetic) or released from expression vectors (shRNA, sh-miR, pri-miRNA and pre-miRNA).

specific miRNAs may be expressed in cells from suitable vectors (53,54) in the way the shRNAs and sh-miRs are expressed.

To be effective, the RNAi triggers and miRNA regulators have to first enter the cell and reach their targets. The nucleic acids delivery methods fall into two major categories viral and non-viral. Among the non-viral methods, the use of various synthetic and natural carriers is very popular. Numerous positively charged carriers have been shown to facilitate the crossing of a negatively charged cellular membrane by the negatively charged nucleic acids reagents. The major types of synthetic carriers include cationic lipids, polymers and cell penetrating peptides that easily traverse a cellular membrane and protect their cargo from rapid degradation by nucleases (55,56). The cellular uptake of nucleic acids reagents occurs *via* the natural portals of entry to cells by which various macromolecules and particles are imported. This process known as endocytosis includes a number of diverse pathways and mechanisms (57,58) that may

deliver complexes of reagents and their carriers to specific cellular compartments (59,60). A common theme in these mechanisms is that cargo is internalized in transport vesicles derived from fragments of the plasma membrane. Cellular uptake of the frequently used cationic lipid complexes with small RNA often referred to as lipoplexes, is thought to occur *via* clathrin-mediated endocytosis. The use of this transportation pathway results in lipoplex localization in endosomes/lysosomes as it was shown for DNA complexes with cationic lipids (61). To elicit its activity, the small RNA has to be released to cytosol from the endosomal trap. The process of endosomal escape being a critical barrier of effective siRNA delivery (59) is facilitated by endosome acidification that occurs as endosome matures into a lysosome (62). Also, the ion pair interactions between carrier cationic lipids and anionic lipids in endosome ultimately destabilize the endosomal membrane and facilitate the endosome escape by lipoplex (55). Then, in the low pH endosome environment small RNAs also dissociate

themselves from the carrier (63) and are ready to enter the RNAi pathway. The delivery issues have been discussed in more detail in several excellent recent reviews (63–65).

Both the RNAi and miRNA technologies are widely used in basic research to study the functions of specific proteins and miRNAs. These technologies may also provide new therapeutics, rationally designed by taking advantage of the human genome sequence knowledge. On the way to achieving this objective several obstacles have to be overcome (66) and avoiding or reducing the toxic effects caused by foreign RNA belongs to the most serious challenges (67–69).

CELLULAR SENSORS AND RESPONDERS TO FOREIGN RNA

The toxicity of reagents of RNAi and miRNA technologies may have different sources: (i) saturation of cellular proteins involved in RNAi and miRNA pathways (70,71), (ii) sequence-specific targeting of other, unintended transcripts (off-target effect) (72,73) and (iii) sequence-non-specific activation of cellular sensors of foreign RNA and their downstream effects leading to interferon (IFN) induction and cell death (67,74). The latter effect depends mostly on the length, structure, chemical modification and cellular localization of the reagent rather than on its sequence features. The question of cellular responses to foreign RNA was addressed a long time before the appearance of RNAi and microRNA technologies in the context of viral infections. The first identified cellular sensors of foreign RNA were the IFN-inducible dsRNA-activated protein kinase (PKR) (75) and 2′-5′-oligoadenylate synthetase (OAS) (76). Later, the first RNA-responsive toll-like receptor 3 (TLR3) was described (77), and the main components of the downstream signaling pathways from these RNA sensors were identified (Figure 2).

PKR

This 68 kDa protein is composed of the C-terminal kinase domain (KD) and N-terminal dsRNA-binding domain (dsRBD) containing two dsRNA binding motifs (dsRBMs), each capable of binding the 11–13 bp RNA (78–81). The protein is localized predominantly in the cytoplasm associated with ribosomes (82), but its fraction is also found in the cell nucleus (83). PKR is perhaps the best studied RNA sensor in the aspect of the activation mechanism and induced downstream effects. It has been established that upon dsRNA binding protein dimerization and autophosphorylation occurs and is followed by the phosphorylation of α-subunit of a translation initiation factor eIF2 (79,84) and IκB protein (85). This results in the general inhibition of protein synthesis and nuclear translocation of NF-κB respectively (Figure 2). PKR is also involved in the regulation of other signaling pathways. One of these pathways involves the p38, MAP/INK and STAT1 proteins leading to the enhanced transcription of IFN-stimulated genes, and the other pathway results in the activation of transcription factors ATF2 and c-Jun leading to the

expression of inflammatory cytokines. Recently, the PKR was shown to be engaged in the modulation of transcription *via* interaction with RNA helicase A (RHA) (86). It was shown by simple tests such as filter-binding, gel retardation and kinase assay that the kinase domain of recombinant PKR is optimally activated by long ∼80 bp dsRNAs (81) and that the length of ∼30 bp is the lower limit of dsRNA that activates PKR (81,87,88). It was also reported that even shorter dsRNAs containing bulges and internal loops are able to activate the enzyme, suggesting the importance of RNA structure in this activation process (89,90). For example the RNA composed of the 16 bp hairpin stem flanked by 10–15 nt single-stranded tails was shown to activate PKR (91). Moreover, it has been demonstrated that PKR can be activated by relatively unstructured transcripts containing 5′-triphosphate, recognized as a pathogen-associated molecular pattern (PAMP) (92). In a more relevant cell culture study that used T98G cells and PKR kinase activity assay it was shown that the transfection of 21 bp siRNA activates PKR in a concentration-dependent manner (93). On the other hand, a microarray study (94) demonstrated that the level of PKR mRNA was not influenced by 19 bp siRNA transfection to HEK293 and HeLa S3 cells but it was up-regulated after 27 bp siRNA transfection. Taking into account that up-regulation of the PKR mRNA may result from the secondary effect of its activation by IFN, we consider the results of protein phosphorylation tests to be more relevant.

OAS

Three members of the OAS family: OAS1 (40 kDa), OAS2 (69 kDa) and OAS3 (100 kDa) are products of different genes and contain one, two and three OAS units, respectively, encoded by five translated exons (95,96). The fourth member the OASL (OAS-like IFN-induced protein) is devoid of the 2′-5′ OAS catalytic activity. The OAS enzymes contain two non-contiguous domains that together form a platform for dsRNA binding (97). The three forms of OAS are associated with different subcellular fractions, including membranes, cytoplasm and nucleus. The activated OAS converts ATP to the 2′-5′-oligoadenylates of various sizes and these activate RNase L (76,98) which is an endonuclease involved in RNA degradation (Figure 2). This sequence of events leads to the general inhibition of protein synthesis and ultimately to cell apoptosis (99). OAS activation takes place in human cells upon strong IFN induction after a viral infection and among the known OAS inducers are the TAR RNA of HIV-1 (100) and VAI RNA of adenoviruses (101). Like other IFN-inducible genes, the promoter regions of OAS1, OAS2 and OAS3 contain an IFN-stimulated response element (ISRE). The information regarding OAS activating RNAs that was gathered prior to the appearance of RNAi technology is inconsistent. In an earlier study using 2′-5′-oligo(A) syntethase and protein kinase assays in HeLa cell extract the poly (I:C) of the length >40 bp showed an activatory effect but >65 bp RNAs were required for maximum activity (102). Two decades later using the same assay the length
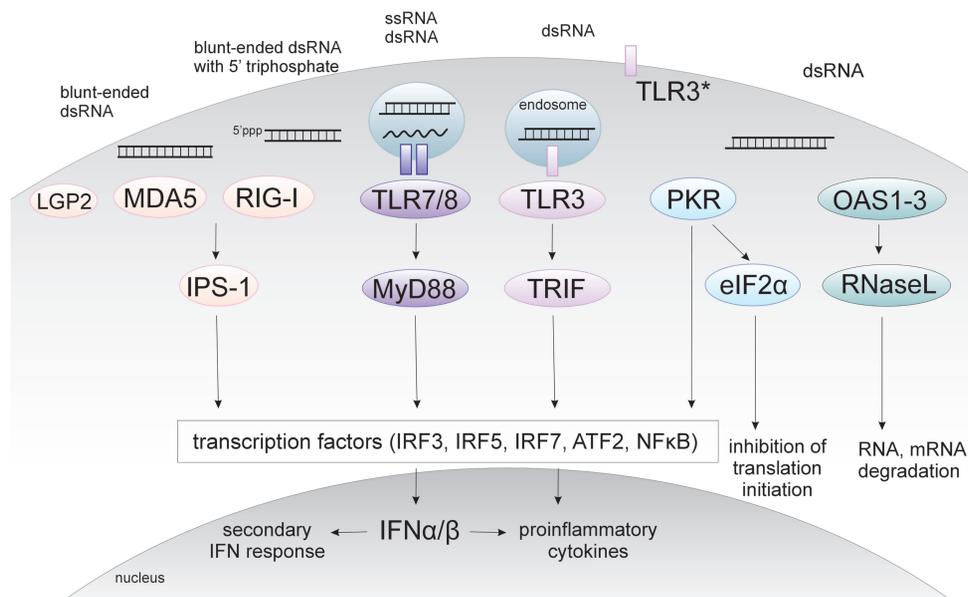
Figure 2. Cellular sensors and responders to dsRNA and ssRNA. After the activation of cytoplasmic (MDA5, RIG-I, PKR, OAS1-3) and endosomal (TLR3, 7, 8) sensors, a signal is transduced *via* specific adaptor proteins to transcription factors that stimulate proinflammatory cytokines and IFN production. TLR3 localization denoted by asterisk is cell type dependent. Endosomal TLR7/8 are expressed mainly in the immune system cells.

limit of dsRNA, that activated recombinant OAS2, was shown to be lower >15 bp (103). This discrepancy can be explained by the differences in the experimental conditions used in these two studies. The structure imperfections within dsRNA were shown to inhibit the activation of OAS proteins (102). In a more relevant RNAi experiment in which the silencing of GAPDH was evaluated by microarrays, the 21 bp siRNAs were shown to activate OAS2 and to a lower extent OAS1 and OAS3 in a concentration-dependent manner (93).

TLRs 3,7,8

The TLR3 belongs to a family of proteins composed of 11 members that recognize different PAMPs (104). The cellular role of 116 kDa TLR3 is to detect viral dsRNA either in the cytoplasm or at the cell surface and activate the pathways involved in the transcriptional response (77,105). The RNA recognizing activity resides in the leucine-rich region (LRR) projecting towards the cell exterior (fibroblasts, macrophages) or endosome interior (CD11c cells), while the Toll/IL-1 receptor domain (TIR) that is the signaling domain projects towards the cytoplasm. TLR3 activates transcription factors IRF3, NFκB and ATF2-c-Jun *via* the TRIF adaptor protein and promotes the expression of IFNβ and pro-inflammatory cytokines such as IL-6 and TNFα (106,107). It has been shown that the endothelial cell surface TLR3 is activated *in vivo* by exogenous siRNA of at least 21 nt in length, leading to the production of IFNγ and IL-12 cytokines (108). Among the known TLR3 inducers is synthetic poly(I:C) frequently used as a positive control in experiments testing cell responses to natural activating dsRNAs or

exogenous RNAi triggers. The TLR3 induction similarly to the activation of the earlier discussed dsRNA sensors is sequence independent. In contrast, the toll-like-receptors TLR7 and TLR8 which are presumably expressed in the endosomes of immune cells, recognize the RNAs trapped in these vesicles in a sequence-dependent manner (109). It has been shown for example, that these receptors are sensitive to specific sequence motifs that are U- and G-rich (110–112). It was also demonstrated that the presence of ribose backbone and multiple uridine residues in close proximity were the elements required for ssRNA recognition by TLR7 (113). The TLR7 and TLR8 primarily bind the single-stranded viral RNA but have also the potential to recognize short dsRNAs. These receptors were shown to be activated by both the siRNA duplexes and single-stranded siRNAs (74,114–115). The TLR7 and TLR8 signal *via* the MyD88 adaptor (Figure 2). Depending on the cell type and TLRs expressed their signals are transmitted either to the IRF5, IRF7 or to NF-κB transcription factors, responsible for the upregulation of IFNα and inflammatory cytokines.

RIG-I, LGP2, MDA5

More recently, three different PAMP helicases: the retinoic acid inducible gene I (RIG-I) (116), as well as the LGP2 (117) and melanoma differentiation associated gene-5 (MDA5) (118) also known as RIG-I like helicases (RLHs) have been shown to be the primary cytoplasmic sensors of foreign RNA in human cells. The RIG-I and MDA5 contain at their N-terminus two caspase recruitment domains (CARD) followed by a DExD/H

box helicase domain and a C-terminal regulatory domain (CTD) involved in RNA recognition (119–121). LGP2 lacking the CARD domains exhibits no signaling capability, but it can negatively regulate the signaling of RIG-I or enhance the response of MDA5 to poly (I:C) (122,123). After the CARD domains of RIG-I and MDA5 are activated they specifically interact with another CARD-containing adaptor protein, localized on the mitochondrial membrane and named either IPS-1 (IFNβ promoter stimulator 1) (124), or MAVS (mitochondrial antiviral signaling) (125), or VISA (virus-induced signaling adaptor) (126) or Cardif (127). The signal is transmitted further to transcription factors IRF3, IRF5 and IRF7 that stimulate cytokine and IFN production (128,129) (Figure 2). Upon dsRNA recognition the RIG-I and MDA5 also activate the transcription factor NFκ-B, that mounts a cellular response by inducing a synthesis of proinflammatory cytokines and type 1 IFNs (123). RIG-I and MDA5 expression is IFN-inducible and is rapidly upregulated following the IFN response initiation.

The RIG-I and MDA5 helicases were shown to be sensitive to such features of dsRNA as its length, end-structure and phosphorylation status. RIG-I and MDA5 selectively recognize short and long dsRNAs, respectively (130). The full activation of RIG-I requires the blunt-ended dsRNA containing triphosphate at the 5′-end (131–133), whereas MDA5 is activated by dsRNA lacking the 5′-triphosphate. The length of the blunt-ended dsRNA that activates RIG-I is >20 bp (131), whereas the lower length limit of MDA5 activating dsRNA still needs to be determined. The MDA5 and LGP2 might also recognize the blunt ends of dsRNA (121). RNAs containing 3′ overhangs at the 5′-triphosphate end suppress RIG-I activity and any 5′ overhang at the 5′-triphosphate end abolishes the activity. These results are consistent with a previous report which showed that the 21–23 bp RNA duplexes containing the 2 nt 3′ overhangs bind RIG-I but do not induce its helicase activity (134). Several studies have reported the activation of RIG-I by ssRNAs bearing 5′-triphosphates (120,135–138). However, a recent study demonstrated that ssRNA is not a ligand for RIG-I. This discrepancy could be explained by the fact that all previous studies used RNAs from *in vitro* transcription with T7 RNA polymerase which were likely contaminated by dsRNA species (131). It was also recently shown that the RIG-I is an RNA-specific translocase working either on dsRNA or RNA–DNA duplex (139).

The first-line cellular sensors of foreign RNA presented briefly above have been identified using different approaches during more than three decades of research. Notable progress in understanding the molecular basis of RNA toxicity of this kind which has been achieved in this decade has benefited considerably from its importance for today's rapidly developing RNAi technology. In the next section, we will present the selected results of these efforts and discuss what is known about cellular responses to various types of reagents used by the RNAi and miRNA technologies.

SEQUENCE-NON-SPECIFIC RESPONSES INDUCED BY RNAi TRIGGERS AND MicroRNA REGULATORS

Early attempts to study RNA interference in human cells turned out to be unsuccessful due to the strong toxicity of long dsRNAs resulting in cell death from IFN activation (140,141). The discovery that short RNA duplexes (siRNA) having the 2 nt 3′-overhangs that resemble Dicer cleavage products could abrogate the toxic effects (142) gave rise to widespread applications of RNAi technology in mammalian systems. Thousands of different siRNA duplexes have been tested for the silencing of human genes and rules of how to design the effective siRNAs have been published (143–145) and are available in the web sites of academic institutions and commercial companies (http://jura.wi.mit.edu/bioc/siRNAext/, http://katahdin.cshl.org:9331/RNAi_web/, https://rnaidesigner.invitrogen.com/sirna/, www.ambion.com/techlib/misc/siRNA_finder.html, http://bioinfo.clontech.com/rnaidesigner/, http://www.dharmacon.com/DesignCenter/). Yet, the toxicity issue was continuously addressed as many siRNAs still triggered the sequence-non-specific responses which were dependent on siRNA length, structure, specific sequence content and concentration (Supplementary Table S1).

siRNAs

It has been demonstrated that short 19 bp siRNAs do not activate the PKR and TLR3 and do not show IFN response even when used at high 100 nM concentration (94). The 21 bp siRNAs were toxic in HeLa S3 cells at 100 nM, whereas the toxicity of 23 bp siRNAs was observed already at 10 nM (94). The transfection of 21 bp siRNAs to T98G cells resulted in the dose-dependent upregulation of STAT1 expression, as detected by Western blot and semi-quantitative RT–PCR analysis (93). The authors have also shown that several IFN-stimulated genes (ISGs) were activated by 21 bp siRNA in RCC1 cells (93). Some ISGs were activated even at the 10 nM concentration (OAS2, OAS3, IFITM1, IFITM2), whereas others at higher concentrations (OAS1). Among the immunostimulatory sequences found in siRNAs were the GUCCUUCAA (111), UGUGU (112), UGGC (73) and GU (113). These sequences were shown to activate endosomal TLR7/8 sensors. This type of immunostimulation was abrogated when naked siRNAs were delivered by electroporation, skipping the endosomal acidification and maturation, required for siRNA recognition by TLR7/8 (115). These observations are in accordance with a notion that increased siRNA length and concentration are immunostimulatory factors. However, specific sequences and other factors may influence the relationship between siRNA length, structure, concentration and toxicity.

The unmodified siRNAs work well in cell culture but for *in vivo* applications they need to have a higher stability which can be achieved by chemical modification. Again, numerous modification sites and chemistries were tested and some recommendations as to what types of modifications may be introduced to different siRNA

positions have been proposed (145–148). The most successful applications used chemistries that resemble naturally occurring modifications such as 2′-O-methyl RNA (2′-O-Me), 2′-deoxy. The modification of only one siRNA strand (sense strand) was shown to be sufficient to block the immunostimulatory potential of the duplex and to minimize the risk for attenuating RNAi (149,150). It was reported that 2′-O-Me substitutions, in the selected guanosine or uridine residues of synthetic siRNAs may be sufficient to prevent immune activation (148,150–153). However, the activation of TLR3 on the surface of vascular endothelial cells by naked siRNA was not inhibited by 2′-O-Me modification (109). The effects of 2′-deoxy (2′-H) and 2′-fluoro (2′-F) nucleotides on the immunostimulatory activity of siRNA are difficult to explain and depend on the siRNA sequence and the number and positions of the modified nucleotides (67). Locked nucleic acids (LNAs) are known to enhance the stability of siRNAs and do not have a negative impact on RNAi activity (145,154). Also, different siRNA variants such as small internally segmented interfering RNA (sisiRNA) (155) and siRNA with short sticky overhangs (ssiRNA) (156) were successfully used offering several advantages, e.g. increased strand specificity and efficiency of silencing.

Dicer-substrate siRNAs

The RNA duplexes known as Dicer-substrate siRNAs were demonstrated to be efficient RNAi triggers that were active at lower concentrations than typical siRNAs (39). The >23 bp dsRNAs used at 10–100 nM were shown to mount an IFN response in a cell type-dependent manner (94). For example, toxic effects were observed in MCF7 cells whereas HEK293 were found insensitive. The results of another study are in line with the above observations and show that 25–30 bp siRNAs neither activate the PKR nor induce an IFN response in HEK293 cells (39). These results emphasize how important is the right choice of cell lines used for *in vitro* toxicity studies.

The Dicer-substrate siRNAs activated also the p56 protein encoded by the *IFIT1* gene and resulted in IRF-3 activation and enhanced IFN production (134,157). It was also shown that blunt-ended siRNAs ranging from 21–27 nt in length activated the IFN system *via* RIG-I helicase (134) and the introduction of overhangs (DNA) reduced RIG-I activity. The chemical modifications of 47 bp dsRNA such as 2′-deoxyuridine (2′dU), 4-thiouridine (s4U), and 2-thiouridine (s2U) abrogated its recognition by PKR (92).

ss-siRNAs

With regard to the single-stranded RNAi inducers, several studies have shown that it is possible to skip the ds-siRNA stage of RNA interference and assembly RISC using only the antisense siRNA strand (31–33). The 'guide strand siRNAs' were, however, less effective and required about 10–100 times higher concentrations to achieve silencing effects comparable to those of ds-siRNAs (31–33). The use of ss-siRNAs that formed partially complementary strands (158) and ss-siRNAs with boranophosphate modifications (146) substantially improved their silencing efficiency which was likely due to the more efficient formation of active RISC. In spite of that the importance of ss-siRNAs for RNAi technology is much lower than that of ds-siRNAs. The ss-siRNAs containing immunostimulatory sequence motifs were shown to be more toxic than their ds-siRNA counterparts and the replacement of the 2′-hydroxyl of uridine residues in ss-siRNA with either 2′-F, or 2′-H, or 2′-O-Me groups abrogated the activation of TLR7 and TLR8 (110,112,152). Finally, the ss-siRNAs bearing 5′-triphosphates synthesized by phage polymerase were earlier thought to be immunostimulatory by the activation of the RIG-I pathway (134–136). However, according to a recent report the ssRNA is not sufficient to support the recognition of 5′-triphosphate by RIG-I as was earlier explained in section Cellular sensors and responders to foreign RNA (131).

shRNAs

The shRNAs expressed in cells from Pol III promoters typically H1 promoter of RNase P or U6 snRNA promoter are the most commonly used alternatives to siRNAs when longer-lasting silencing effects are required (41,42,159–163). The price paid for the prolonged expression may be a lower silencing specificity caused by the length heterogeneity of siRNAs excised from shRNAs by Dicer (our unpublished data). The shRNAs themselves have a heterogenous 3′-end (the U-tail length) caused by the nature of the Pol III transcription termination signal (usually 4–6 T residues) (164). The shRNA vectors are introduced to cells either by transfection with cationic lipids or within non-integrating adenoviral vectors (AV), adeno-associated vectors (AAV) or lentiviral vectors having the ability to integrate with genome. In the less frequently used approach, shRNAs are delivered to cells as synthetic molecules *via* transfection (40,42,159). It was reported that transcripts synthesized by T7 phage polymerase containing 5′-triphosphates induce strong immune responses (135,165); however, it is possible to overcome this problem by transcript 5′-end modification (166). The shRNA expression is considered more toxic to cells than siRNA delivery and the overall toxicity may be split into effects resulting from vector DNA and effects caused by transcripts usually expressed at high levels. The DNA-related effects include cell responses to the delivery of non-methylated DNA sequences. It is known that introducing foreign DNA that contains non-methylated CpG sequences to human cells stimulates a strong immune response (167), and that such sequences are recognized by TLR9 (168) which is localized like TLR7 and TLR8 on endosomes. Another known cytoplasmic DNA sensor is the DNA-dependent activator of IFN-regulatory factors (DAI; DLM-1/ZBP1) which activates the IFN type-1 production (169). Also, the AA dinucleotide present near the transcription start site of the U6 promoter was reported to induce OAS and other IFN-responsive genes (170). The prototypical shRNA designs contain either a 19 bp or 29 bp stem and loop of various sizes and sequences (171,172). Their long and fully

base-paired stems were also shown to trigger immune responses (173,174). Another serious problem results from the usually high level of shRNA expression. The abundance of transcripts which resemble the natural pre-miRNAs was shown to overload the endogenous pathways of miRNA biogenesis and be lethal (175). The most sensitive to saturation *in vivo* is the step of the Exportin-5-mediated transport of pre-miRNA from nucleus to cytoplasm (71,175). Other studies also reported the accumulation of miRNA precursors in cells expressing high levels of shRNAs (173). Among the ways to reduce such toxic effects is the use of the lowest effective doses of shRNA expressing vector (41,174,176) and less efficient or regulated promoters (160,177). Another way to diminish the immune responses caused by the long stretch of regular dsRNA in the shRNA hairpin stem is to introduce irregularities into this structure to resemble more the natural pre-miRNAs (178). The toxicity of shRNAs is also cell type dependent and according to our experience the transfection of plasmid vectors coding different shRNAs (complexed with Lipofectamine 2000) into human fibroblasts results in strong toxicity and cell death, whereas the same constructs are non-toxic to HeLa and HEK293 cells (M. Olejniczak *et al.*, unpublished data).

sh-miRs

As not all disadvantages of the shRNA constructs could be eliminated by simple means, the second generation of siRNA expressing vectors was developed (44,179,180). The sh-miRs mimic the pri-miRNAs, are expressed from either Pol II or Pol III promoters and siRNAs are expressed typically at lower levels which do not cause saturation problems (161,181). The pri-miRNA-30 is the most often used shuttle for siRNA expression (44,178) known to abrogate OAS1 induction and cell toxicity caused by the use of the shRNA vector (182). It would be wise to test more of such pri-miRNA shuttles for the efficiency and specificity of siRNA excision and subject them to more rigorous toxicity testing.

miRNA delivery

Similar vector-based approaches may be used in miRNA technology to overexpress specific miRNAs for research or therapeutic purposes. The only difference is that the miRNA expression cassettes are the wild-type pri-miRNA sequence fragments of sufficient length to be efficiently processed in cells by the Microprocessor complex. From the perspective of DNA-induced toxicity the miRNA expressing systems, although not reported to be tested in this respect, will suffer from the same limitations as the siRNA expressing systems do. However, the processed transcripts will be recognized by the cell as self since they have identical sequences and structures as the corresponding host transcripts. Another approach used to deliver miRNAs to cells is to transfect cells with miRNA mimetics, that are the 21–23 bp double-stranded RNAs, mimicking natural Drosha and Dicer products. However, reports describing the applications of these synthetic miRNA regulators (47–54,183–185) do not provide any information regarding their sequence-non-specific side-effects (Supplementary Table S1).

miRNA inhibition

Of the reagents used to inhibit the overexpressed miRNAs the antimirs are most frequently used. These single-stranded antisense reagents that inhibit the RISC-bound miRNAs (45) differ in their length ranging from 16 nt (186) to 31 nt (183,187). To increase their nuclease resistance and the stability of their interactions with target miRNAs the antimirs were subjected to various chemical modifications. The most efficient turned out to be the ribose 2′-hydroxyl substitution with one of the following: the 2′-*O*-Me, 2′-*O*-metoksyetylo (2′-MOE), 2′-F or the use of LNA, morpholino or phosphorothio (PS) chemistries (54,183,188,189). The stability of the miRNA-antimir interaction was strongly enhanced after extending the antimir sequence with the flanks forming a double-stranded structure (190). Such antimirs were shown to be functional at sub-nanomolar concentrations. For *in vivo* applications, the cholesterol-conjugated and thio-modified antimirs were successfully used (47,191). Certain types of chemical modifications (2′-*O*-Me-PS and 2′-F-PS) were shown to be toxic to cells when the antisense reagents were used at high 100 nM concentrations (188). According to these authors, it was impossible to transfect by lipofection >500 nM antisense oligonucleotides of any chemistry without inducing non-specific toxicity (188). Taken together, still very little is known about the toxicity of various reagents used by miRNA technology. Yet, the relevant knowledge may be gained from more numerous applications of the RNAi and antisense technologies that use reagents of similar types and lengths and which are stabilized with the same or similar chemistries. One general conclusion that could be drawn from these studies is that RNAs recognized by cells as self (that resemble cellular transcripts in terms of structure and localization) are rather tolerated, while those recognized as foreign induce various responses depending on their structural features, the way they entered the cell and the cellular compartment they localize (128,134,192).

TESTING NON-SPECIFIC CELLULAR RESPONSES TO FOREIGN RNA

Like viral invasion, cell transfection with RNAi or miRNA reagent often results in the increased expression levels of primary sensors of non-self RNA and other proteins of the innate immune system. The increased levels of proteins have typically their source in upregulated transcripts. Thus, the predominant part of a known system of sensors and responders to foreign RNA can be analyzed for its activation using simple tests detecting changes in RNA or protein levels such as RT–PCR or Western blot. By now, the most frequently used tests rely on the measurement of systemic cytokine and type-1 IFN release into the blood of treated animals, supported by cytokine release assays from primary immune cell cultures *in vitro* (67,68). A survey of relevant literature shows, however, that the question of the activation of

the immune system by various RNAi triggers and miRNA regulators is rarely addressed in a comprehensive way, some studies address it only fragmentarily and most studies do not address it at all (Supplementary Table S1). This situation will likely change with the increased number of *in vivo* applications of the RNAi and miRNA technologies in which the safety issues need to be thoroughly considered.

The common theme is also the misuse of tests for immune response activation. Examples of their typical misuse include: analyses of inappropriate cell lines which either do not represent properly the target tissue, are hardly immunostimulatory or both, analyses of one or a few sensors and/or responders only, often not the right ones and not in the right time, as recently discussed in (67,193,194).

While testing non-specific cellular responses to foreign RNA one should also carefully analyze the effect caused by the delivery system itself. It has been shown with the use of microarrays that both Lipofectin and Oligofectamine altered the expression of many genes in human A431 epithelial cells (195). The affected genes are functionally involved in various cellular processes, including cell proliferation, differentiation and apoptosis. Another study demonstrated that Lipofectamine 2000 is toxic to the HeLa and IGROV-1 cells both alone and after complexation with different siRNAs into lipoplex particles (196). It has been also shown that the transfection of human blood cells with Lipofectamine or polylysine complexes with siRNA results in inflammatory cytokine release, whereas siRNA encapsulated with stable nucleic acid-lipid particles (SNALPs) or complexed with polyethyleneimine (PEI) induce a response dominated by IFNα secretion (111). The issue of carrier toxicity earlier ignored or marginalized is now seriously taken into account and carriers such as Lipofectamine™ LTX are offered to meet the RNAi community expectations for an efficient and safe carrier.

The identified sources of sequence-non-specific toxic effects presented and commented in this and previous sections gave rise to a set of guidelines that may help to use the RNAi technology more safely. These guidelines referring mainly to the different features of RNAi reagents are listed below:

siRNA sequence – avoid the immunostimulatory sequences such as GU, UGGC, UGUGU, GUCCU UCAA that activate TLR7/8,

siRNA structure – avoid blunt ends known to activate RIG-I and MDA5 helicases and terminal 5′-triphosphates activating RIG-I, make 2 nt 3′ overhangs for optimum activity,

siRNA length – use siRNAs of standard length 20–22 nt resembling the length of miRNA/miRNA* duplexes, or not much longer Dicer-substrate siRNAs devoid of blunt ends,

siRNA modification – select chemical modification types known to be efficient and safe, e.g. 2′-*O*-Me, 2′-deoxy, LNA,

siRNA concentration – use the lowest effective concentrations/doses of siRNAs as the strength and

scale of toxic side-effects clearly depend on siRNA concentration,

siRNA delivery – use carriers that do not cause by themselves large changes in gene expression in target cells and do not activate immune responses,

shRNA – avoid unmethylated CpG sequences activating TLR9, design the shRNA structure to resemble the pre-miR structure, use weak tRNA or H1 promoter,

sh-miR – use pre-miRNA flanking sequences of sufficient length to ensure Drosha processing occurs at natural sites and use weaker Pol II promoters, sequence and structure features as for shRNA,

target cells – while planning *in vitro* toxicity tests keep in mind that some cells, e.g. MCF7, HeLa S3, PBMC are more sensitive and some other, e.g. HEK293, HeLa are less,

toxicity test – besides testing for IFNs and cytokines levels the cellular pathways leading to toxicity, including the primary RNA sensors and responders, should be identified (Figure 2).

SEARCH FOR OTHER RNA SENSORS AND IMMUNE RESPONDERS USING TRANSCRIPTOMICS AND PROTEOMICS APPROACHES

To gain a more complete insight into the changes in gene expression that occur upon the delivery of RNAi triggers, miRNA mimetics and miRNA inhibitors functional genomics and proteomics approaches were used in several studies. These efforts were primarily aimed at revealing the scale of sequence-specific target-off effects in various RNAi applications (72,73) and identifying messenger RNAs and proteins regulated by specific miRNAs (197) rather than on finding new sequence-non-specific sensors and responders to foreign RNA. In spite of that, in several studies the latter aspect was also investigated. For example, the microarray analysis of cellular transcripts after the 27 bp siRNA transfection resulted in the identification of immunosensitive cells (MCF7, HeLa S3) mounting stronger innate immunity and less sensitive cells (HEK293, HeLa) (94). In HeLa S3 cells, the Dicer-substrate siRNA induced the expression of more than 300 genes that included transcripts associated with the detection of foreign RNA as well as IFN and apoptotic signaling pathways (the TLR3, RIG-I, MDA5, IFITM1, OAS3, OASL, STAT1-2). In other studies, the toxic sequence motif UGGC was identified in siRNAs (73), and shRNAs expressed from the U6 promoter were shown to be more toxic than those expressed from the H1 promoter (198).

The first proteomic analysis of HeLa cells transfected with the miR-1 mimetic revealed a down-regulation of 12 out of 504 investigated proteins (199). The analysis was performed using stable isotope labeling by the amino acids (SILAC) method (200). More recently, the same method and its modified version were used along with microarray analysis to study the effects of other miRNA mimetics on the levels of nuclear (201) and whole-cell proteins (202) in HeLa cells. The analyses have shown that the group of repressed proteins corresponds well to that of transcripts

undergoing degradation. This would mean that transcript analysis alone may be sufficient to reveal the identity of most of the proteins regulated by miRNAs (203). The relationships between the cellular levels of specific miRNAs, mRNAs and proteins found in these studies were shown to be functional as the same groups of transcripts and proteins responded to miRNA inhibition by specific antisense LNA oligonucleotide (189). Neither study considered the problem of cell defense system activation by miRNA mimetics and inhibitors that were delivered to cells on different carriers and at different concentrations. This aspect therefore needs to be addressed in further analyses of proteomes perhaps derived from more immunoresponsive cells than HeLa treated with the tools of RNAi and miRNA technologies. The search for further cellular sensors and responders to foreign RNA using functional genomics and proteomics approaches and basing on the simple criterion of transcript or protein upregulation may not be, however, a simple task. A recent report shows that the transfections of numerous different miRNAs and siRNAs to cells may result besides the downregulation of intended and unintended transcripts also in the upregulation of a myriad of transcripts that are otherwise suppressed by endogenous miRNAs in untreated cells (204). Thus, the overlapping effects of the saturation of cellular RNAi/miRNA machinery by exogenous RNA reagents and cell immune responses (both resulting in transcript upregulation) need to be distinguished. Recent developments in technology and progress in reading out the *in vivo* RNA–RNA and RNA–protein interaction maps (205) and identifying the RNA recognition specificities of various RNA-binding proteins (206) may speed up the deciphering of the human ribonome code.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

The available information on the sequence non-specific, toxic effects of various RNAi reagents strongly outnumbers the regarding reagents of miRNA technology (Supplementary Table S1). This disparity is caused at large by a time delay with which the development of miRNA technology has begun and by the lower number of its published applications. In spite of being far from satisfactory, information on the toxic effects of RNAi triggers that has been gathered thus far has provided some useful guidelines on how to use RNAi more specifically and more safely. Much of the successful research has been focused at unrevealing the sequence and structure features of RNAi reagents that cause immunostimulation. Also, chemistry made a substantial contribution in developing less toxic and more stable reagents by placing specific chemical modifications at well selected siRNA positions. Less attention has been paid to the toxic effects of various carriers used to deliver the RNA reagents to cells. However, the already existing data shows that the carriers should no longer be considered neutral delivery vehicles. The number of available RNA carriers is large and their effects on gene expression need to be more widely and more carefully analyzed. There are also other issues relevant to sequence-non-specific responses that need to be addressed in further research. These issues include the need for: clarifying still controversial facts regarding the immunostimulatory siRNA features, reducing strong toxic effects caused by genetic vectors used to express siRNA and miRNA in cells, and a wider application of functional genomics and proteomics approaches to find new foreign RNA sensors and responders as well as safer RNA carriers. It would be also advisable to establish a database of the toxic effects caused by RNAi and miRNA reagents for which the data presented in Supplementary Table S1 might serve as an inspiration. We anticipate that both the RNAi and miRNA technology will benefit, in the short term, from more widespread applications of well-designed simple toxicity tests. Such tests should be dedicated to provide answers which of the presently known RNA sensors and immune responders are activated by specific reagents in specific cells and tissues. In the long run, the next generation of such tests should be developed and based on more complete information on toxic RNA sensors and responders gained from transcriptomics and proteomics approaches.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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