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SURVEY AND SUMMARY

Dicer-independent processing of small RNA duplexes: mechanistic insights and applications

Elena Herrera-Carrillo and Ben Berkhout*

Laboratory of Experimental Virology, Department of Medical Microbiology, Academic Medical Center, University of Amsterdam, the Netherlands

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ABSTRACT

MicroRNAs (miRNAs) play a pivotal role in the regulation of cellular gene expression via the conserved RNA interference (RNAi) mechanism. Biogenesis of the unusual miR-451 does not require Dicer. This molecule is instead processed by the Argonaute 2 (Ago2) enzyme. Similarly, unconventional short hairpin RNA (shRNA) molecules have been designed as miR-451 mimics that rely exclusively on Ago2 for maturation. We will review recent progress made in the understanding of this alternative processing route. Next, we describe different Dicer-independent shRNA designs that have been developed and discuss their therapeutic advantages and disadvantages. As an example, we will present the route towards development of a durable gene therapy against HIV-1.

RNA INTERFERENCE

RNAi is an evolutionarily conserved mechanism that uses a double-stranded RNA (dsRNA) to regulate gene expression in a sequence-specific manner at the post-transcriptional level (1–3). Canonical miRNAs are transcribed in the nucleus as primary miRNAs (pri-miRNAs) and subsequently processed by the 'Microprocessor' complex, composed of Drosha ribonuclease and DGCR8. This endogenous RNAi pathway is illustrated in Figure 1A. The resulting ∼70 nucleotides (nt) pre-miRNA is exported to the cytoplasm by Exportin-5 that recognizes the 2-nt 3′-overhang (4,5). In the canonical pathway (Figure 1A, canonical pathway), the pre-miRNA is cleaved in the cytoplasm near the terminal loop by the RNAse III-like enzyme Dicer in collaboration with the cofactors TRBP and PACT (6–10). This results in an imperfect ∼22-nt miRNA duplex with characteristic 2-nt 3′-overhangs (Figure 2A). Mature miRNA duplexes are subject to 3′-end modification by addition of a few tailing nt, specifically U or A, which typically stimulate miRNA decay (11,12).

Processed miRNA duplexes are loaded into the RNA-induced silencing complex (RISC) through interaction with a member of the Argonaute protein family (Ago1–4) in human cells (Figure 1A) (13). The Dicer-independent miR-451 is unique in binding preferentially to Ago2, as will be described below (14). The miRNA duplex is subsequently unwound to release the mature form of the active miRNA strand (guide strand), while the passenger strand is degraded or removed. Which strand of the duplex is incorporated as guide into RISC is determined by thermodynamic properties (15,16).

The mature miRNA strand directs the activated RISC complex to mRNAs with an imperfect complementary target sequence(s) within the 3′-untranslated region (Figure 1A). RISC association with the mRNA results in translational suppression (17,18), mRNA destabilization or a combination of both (19–21). Perfect or near-perfect miRNA pairing results in Ago2 cleavage-mediated inactivation of the mRNA (22,23). The RNase H-like domain of Ago2 cleaves the complementary RNA opposite nt-10/11 counted from the 5′-end of the guide (24). Specificity is dictated by the 'miRNA seed sequence' (nt 2–8 from the miRNA 5′-end that initiates mRNA basepairing) and the number of 3′-UTR target sites (25–28). Targeted mRNAs are translocated to cellular RNA processing stations (P-bodies) where storage, deadenylation, de-capping and degradation takes place (29).

NON-CANONICAL miRNA PROCESSING ROUTES

We will focus on a Dicer-independent pathway that was described for miR-451, which instead uses Ago2 for maturation (Figure 1A, non-canonical pathway) (30,31). The miR-451 regulates erythroid development and is characterized by an unusually short basepaired stem of 17 basepairs

*To whom correspondence should be addressed. Tel: +31 20 566 4822; Fax: +31 20 691 6531; Email: b.berkhout@amc.uva.nl

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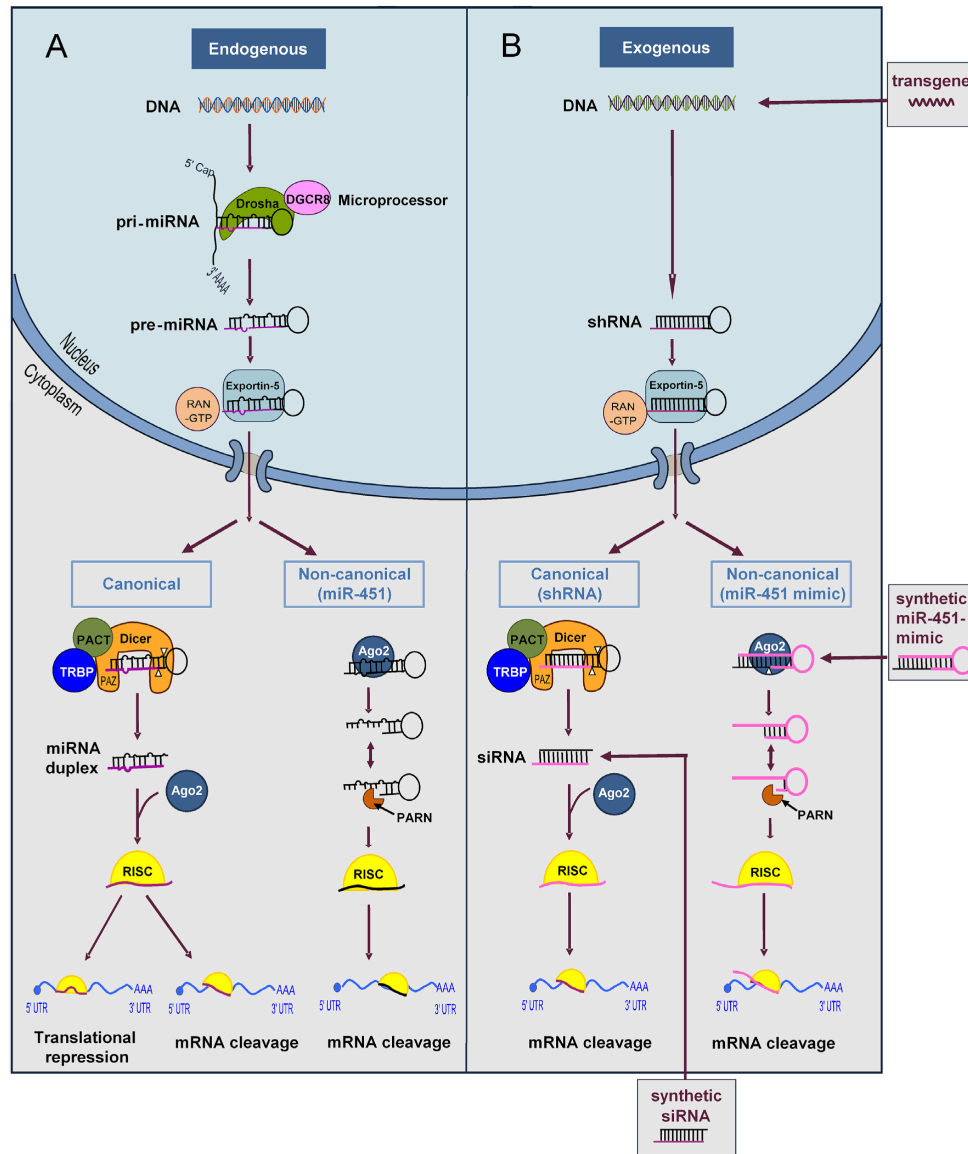

Figure 1. Endogenous miRNA (**A**) and exogenous shRNA (**B**) processing pathways. The intracellular processing pathways are depicted starting from the endogenous miRNA gene in the cell or the transduced shRNA transgene cassette. The canonical Dicer-dependent and non-canonical Dicer-independent pathways are depicted for both molecules. Ago2 plays an essential role in the Dicer-independent pathways. See the text for further details.

(bp), which seems too small for Dicer recognition (Figure 2B) (32). The 30-nt active strand of miR-451 is derived from one side of the stem, the single-stranded loop and part of the complementary stem region, which seems incompatible with regular Dicer-processing. Indeed, normal levels of the mature miR-451 are present in Dicer-minus cells. Drosha cleaves the miR451 precursor, but miR-451 bypasses Dicer and is loaded directly into Ago2. Ago2 cleaves opposite nt-10/11 of the guide and produces the typical 30-nt fragment (33). This product is further trimmed by the Poly(A)-specific ribonuclease (PARN) to the mature 22/26-nt miR-451 (34,35). The Ago2-processed miR-451 may be subject to oligo-uridylation at the 3′-end before trimming (33,35,36), but oligo-uridylation occurs independently of miR-451 maturation (34). Trimming of the Ago2-cleavage derived hairpin seems dispensable for *in vivo* activity (34).

The Dicer-independent miR-451 pathway opened the search for other miRNAs that depend on Ago2 for maturation. Potential Ago2-cleaved pre-miRNAs from the let-7 family were described based on a deep sequencing analysis, which showed cleavage in the 3′-strand of the pre-miRNAs (37). An indication that these deep sequence reads depict true RNA processing intermediates instead of degradation products comes from the presence of 3′-end uridylation, exactly as was described for miRNAs (38). A considerable number of canonical Dicer-dependent miRNAs can - at least to some extent - also enter Ago2 to become processed (31).

shRNA EXPRESSION STRATEGIES

RNAi has been used for fundamental research, biotechnology applications and therapeutic purposes (39,40). RNAi

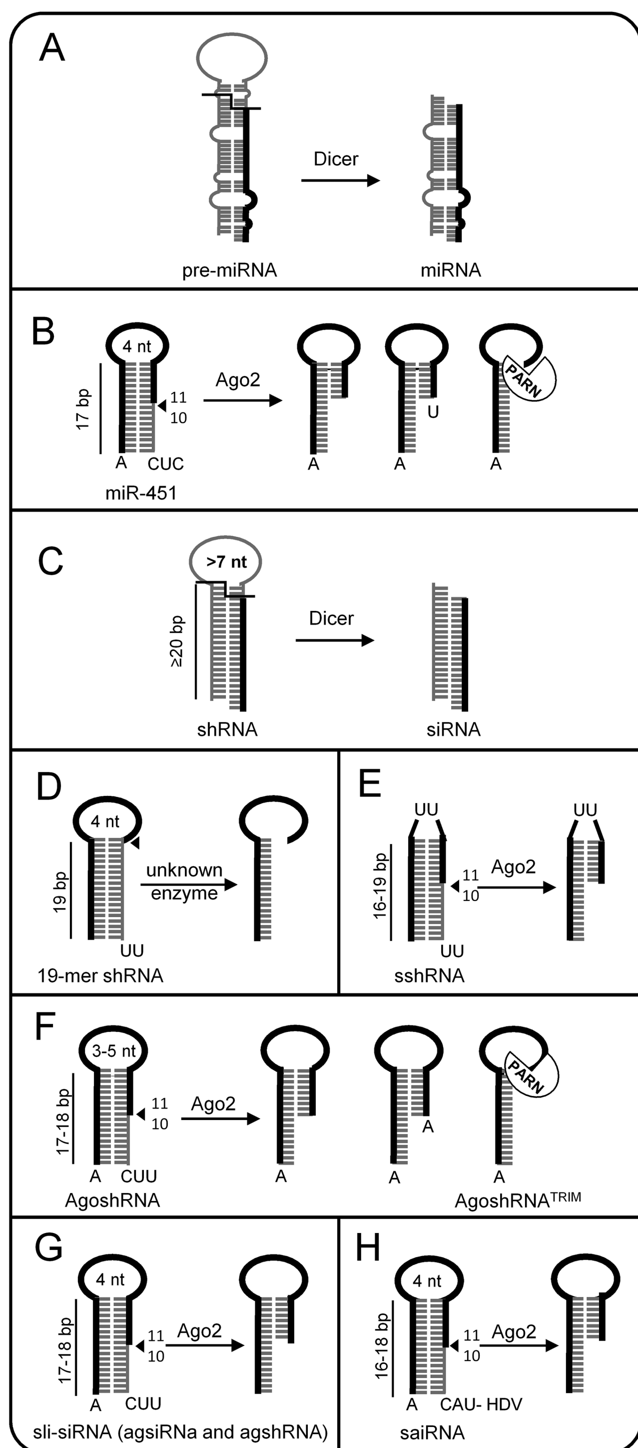

Figure 2. Cartoon of the key features of a regular microRNA (miRNA, **A**), a Dicer-independent miRNA (miR-451, **B**), a Dicer-processed short hairpin RNA (shRNA, **C**), and several Dicer-independent shRNA designs in panels **D–H**: sshRNA: small shRNA, AgoshRNA: Ago2-dependent shRNA, sli-siRNA: Ago2-sliced siRNA agsiRNA: synthetic Ago2-sliced siRNA, agshRNA: Ago2-sliced shRNA and saiRNA: Ago2-processed interfering RNA. See the text for further details.

can also be programmed with a variety of artificial miRNA-like substrates in the exogenous pathway as illustrated in Figure 1B. Synthetic small interfering RNA (siRNA) duplexes that are ∼21-nt complementary RNA strands with 2-nt 3′-overhangs can be transfected into cells. Such siRNAs are designed to be perfectly complementary to the mRNA and cause site-specific cleavage via Ago2. However, the use of siRNAs has several disadvantages. They cause transient silencing because the intracellular concentration drops upon cell division and their intracellular half-life is short due to degradation by RNase A-type nucleases. It is also difficult to transfect the RNA into certain cells types, e.g. primary cells, which hinders siRNA delivery to the desired target tissues (41–44).

Alternatively, gene cassettes can express shRNAs that are processed by Dicer into active siRNAs (Figure 1B, canonical pathway) (45–47). These molecules are expressed in the nucleus and Exportin-5 facilitates entry into the cytoplasm, where they enter the RNAi pathway at the Dicer-processing step, thus avoiding Drosha-cleavage. The shRNAs usually have perfect target-complementarity, thereby inducing mRNA-cleavage. A conventional shRNA consists of a 20/29-bp stem, a loop of at least 5-nt and dinucleotide overhang at the 3′-end (Figure 2C). Conventional shRNAs are designed with the guide on the 3′-side of the duplex. The exact positioning of the guide in the hairpin can affect shRNA activity. Recent studies suggested that Dicer may determine the siRNA orientation such that the proper strand is selected as guide (48).

ALTERNATIVE RNAi STRATEGIES, INCLUDING miR-451 MIMICS

In this review, we will focus on the non-canonical RNAi processing routes that use Ago2 instead of Dicer. We will describe mechanistic details when available and discuss how this pathway can be induced by a variety of miR-451 mimics (Figure 1B, non-canonical pathway). Several groups reported a unique class of short RNA duplexes that demonstrate good silencing activity in a Dicer-independent manner, but each research group named these miR-451 mimics differently (Table 1), which may confuse the field and was the primary reason for writing this review. We will distinguish chemically synthesized and transgene expressed miR-451 mimics.

Chemically synthesized miR-451-mimics like 19-mer shRNA, small shRNA (sshRNA) and Ago2-sliced siRNA (agsiRNA) are transfected into the cell and enter the RNAi pathway at Ago2-processing, thus avoiding Dicer cleavage. Alternatively, miR-451 mimics can be expressed from a transgene in the nucleus as Ago2-dependent shRNA (AgoshRNA), Ago2-sliced shRNA (agshRNA) or Ago2-processed interfering RNA (saiRNA). These molecules are transported to the cytoplasm via Exportin-5, where they enter the RNAi pathway at the Ago2-processing step. We will describe these six miR-451 mimics and the molecular RNA characteristics that facilitate optimal Ago2-processing. Note that well-known design algorithms for siRNA molecules cannot be applied to shRNAs and the Dicer-independent shRNA design (49). Recently, Pelossof *et al.* presented the SplashRNA algorithm to pre-

Table 1. Molecular features of Dicer-independent shRNAs

Name[a]	Source	Optimal stem length (bp)	Loop size (nt)	First described	References
19-mer shRNA	Chemically synthesized	19	4	2005	(32)
sshRNA	Chemically synthesized	16–19	2	2010	(53,54)
AgoshRNA	Transgene expressed	17–18	3–5	2013	(55,56,60)
agsiRNA	Chemically synthesized	17–18	4	2015	(58)
agshRNA	Transgene expressed	17–18	4	2015	(58)
saiRNA	Transgene expressed	16–18	4	2015	(59)

[a]sshRNA: small shRNA, AgoshRNA: Ago2-dependent shRNA, agsiRNA: Ago2-slice siRNA, agshRNA: Ago2-sliced shRNA and saiRNA: Ago2-processed interfering RNA.

dict microRNA-based shRNAs (50). However, this algorithm cannot be used to design Dicer-independent shRNAs, which therefore remains a difficult and time-consuming trial-and-error process.

The first evidence for Dicer-independent shRNA-processing came from studies on synthetic shRNAs (32,51,52). Siolas *et al.* suggested in 2005 that a 19-mer shRNA with a 3′-overhang is efficiently cleaved by a cellular endonuclease of unknown origin (Figure 2D) (32). They compared the efficiency of silencing by synthetic shRNAs with a different stem length. The shRNA stems were either 19- or 29-bp long and each shRNA was tested with or without a 2-nt 3′-overhang, identical to that of Drosha-processed pri-miRNAs. Only shRNAs with the 2-nt 3′-overhang were efficiently processed and yielded 21/22-nt products, while the 19-mer shRNAs generated products of 21/23-nt. Although this group did not disclose the mechanistic basis for this observation, they suggested that it may reflect Dicer-independent cleavage in the loop of the 19-mer shRNA by a cellular single-strand specific ribonuclease.

Five years later, Ge *et al.* reported that small synthetic siRNAs can be potent inhibitors of the hepatitis C virus (HCV) as measured in the replicon system (53). They described a unique subclass of short shRNAs (sshRNAs) with a stem of only 19-bp, but sometimes as short as 16/17-bp, a small UU loop and a 3′-terminal UU-overhang, which are not cleaved by Dicer *in vitro* (Figure 2E). The sshRNAs exhibited anti-HCV activity via RNAi-mediated target RNA cleavage, but their processing route remained unclear. Two years later, this group suggested that Ago2 was involved in the processing of sshRNAs (54). Introduction of mismatches in the putative Ago2 cleavage site resulted in decreased processing and knockdown activities. Around the same time, similar results were obtained for small RNA duplexes expressed from transgenes, either plasmids or viral vectors. We identified a specific shRNA design called AgoshRNA with a short stem length (17/19-bp) and small loop (3/5-nt) that triggers this alternative processing route (Figure 2F) (55). As described above, an activity switch from the regular 3′- to the 5′-side of the stem was observed, coinciding with differential RNA processing as detected on Northern blots. In other words, the 5′-strand is effectively converted from passenger to guide strand. Deep sequence analysis of cellular small RNAs indicated that cleavage occurred half-way the 3′-side of the duplex, suggesting a role for Ago2 that is predicted to cleave the duplex between bp 10 and 11 from the 5′-end of the guide (Figure 2F) (56). Production of these typical 30-nt RNA fragments - derived from the 5′-strand, the single-stranded loop and part of the 3′-strand - was abolished in cells that encode a catalytically-defective Ago2 mutant. This new design was termed AgoshRNA because both processing and silencing are mediated by Ago2. It seems likely that AgoshRNA processing and silencing are functionally coupled and executed by the same Ago2 molecule. If the processed AgoshRNA would leave Ago2 prematurely, it is less likely to be rebound because the duplex is disrupted (57). Similarly, Dallas *et al.* described that 'pre-sliced' synthetic sshRNA molecules are inactive, consistent with a coupled two-step mechanism (54).

More recently, Sun *et al.* designed 'Ago2-sliced siRNA' (sli-siRNA) termed either agsiRNA when chemically synthesized or agshRNA when expressed in the cells from a transgene (Figure 2G) (58). They based the design on the sshRNA and AgoshRNA molecules, but modified the sequence of the hairpin loop. Sli-siRNAs use 4-nt of the antisense guide to form a loop, whereas AgoshRNAs were initially designed with the fixed 5-nt loop sequence CAAGA, and sshRNA use UU to connect a duplex of 19-bp. Around the same time, Shang *et al.* designed small RNA duplexes termed saiRNA that consist of a 16/18-bp stem and a loop complementary to the target transcript (Figure 2H) (59). This group introduced a self-cleaving ribozyme derived from hepatitis delta virus (HDV) to the 3′-end of the saiRNA to enhance the activity.

DESIGN RULES FOR DICER-INDEPENDENT shRNAs

We will review the molecular insights gained on the optimal composition of Dicer-independent shRNAs (Figure 2D–H), which includes the stem length, loop size, nature of the base pairs, and 5′- and 3′-terminal nucleotides. This survey allows us to propose some general rules for the design of active Dicer-independent shRNAs.

Optimal stem length

Due to differences in the structural requirements for silencing by Dicer-dependent versus Dicer-independent shRNAs, several groups including ours were prompted to investigate the shRNA characteristics that either hinder Dicer recognition or stimulate Ago2-mediated processing and the subsequent silencing activity. A detailed mutational analysis indicated that the length of the basepaired stem is the major determinant for shRNA activity via the regular Dicer

route versus the non-canonical Ago2 route (55,60). Regular Dicer templates are hairpins of at least 20-bp. Dicer and Ago2 will compete for processing of shRNAs of 19/20-bp, but shRNAs of 18-bp or less lose the ability to be processed by Dicer, which opens up the possibility for the alternative Ago2-processing route for these unconventional shRNAs. AgoshRNAs remain active down to 17-bp, but shorter hairpins lose all activity (60). Similarly, Ge *et al.* reported that 19-bp sshRNAs are highly potent, yet independent of Dicer-processing, whereas 16/17-bp sshRNAs exhibit reduced silencing activity (53). A distinction between 'Left-hand loop' (L) and 'Right-hand loop' (R) sshRNA was made based on whether the antisense strand is positioned 5′ or 3′ to the loop, respectively. L-sshRNAs are highly potent compared to the R counterparts. These results are consistent with the observation that Ago2 cleaves half-way the 3′-side of the duplex, which will disrupt a 3′-side guide. Ma *et al.* reported very similar results in tests with Ago2-specific shRNAs with stem lengths ranging from 15/21-bp (61). Variants with a 16/18-bp stem demonstrated excellent gene silencing activity, whereas 15/19-bp variants exhibited reduced silencing activity and 20/21-bp forms lost most activity. Even shorter hairpin constructs have been described in literature, e.g. guide hairpin RNA (ghRNA) of just 11-bp (62). Chemically synthesized ghRNAs act in a Dicer and Ago2-independent manner, but their processing route remains unknown. Recently, Sun *et al.* studied the structural parameters required to design active agsiRNA and agshRNA (58). Again, the results suggest that the stem length is the major determinant for the choice of processing route and consequently the downstream activity of these molecules. These combined studies indicate that there is a narrow window for activity of Ago2-dependent molecules in the size range of 16/20-bp, with optimal activity for molecules with a stem-length of 17/18-bp.

Optimal loop size

The loop is an important determinant in the design of regular miRNA and shRNA molecules. The sequence and structure of a regular shRNA influences the activity and the loop sequence has an effect on Dicer recognition and processing efficiency (55,63). Likewise, mutation of the loop of a miRNA can affect or even abrogate its processing (64,65). Our group observed that large loops (>7 nt) cause a partial return from alternative Ago2 to regular Dicer processing, possibly due to steric hindrance with the PAZ domain of Ago2 (66). Therefore, small loops (≤5 nt) are required for optimal Ago2-mediated processing (55). The loop sequence may also influence the intracellular stability and - most importantly - silencing activity. Different loops have been used to design miR-451 mimics, but how the loop nucleotide composition and/or sequence influences the silencing activity remains unknown (53,58,60).

It has been proposed that the guide strand of miR-451 mimics can be extended 'over the loop' (58), but we measured no increased knockdown potency for AgoshRNA molecules with such extended guide strands (67). Inclusion of the evolutionary conserved miR-451 loop (AGUU) or the particularly stable CUUG tetraloop in AgoshRNA molecules did also not result in enhanced silencing activity (68). Surprisingly, a high pyrimidine content of the loop was shown to impair AgoshRNA efficacy, and a high purine content correlated with good silencing activity (68). However, no underlying molecular reason for this purine-preference was disclosed.

Influence of the basepairs

Dueck *et al.* suggested that the top G-U bp in miR-451 is important for activation of the Dicer-independent processing route (14). Mutation to G-C reduced the Ago2-mediated processing efficiency, suggesting that miR-451 was evolutionary optimized for this alternative pathway. To study whether the presence of the weak G-U/U-G as loop-closing bp influences the shift from regular shRNA activity to AgoshRNA activity, we introduced a weak bp (G-U or U-G) at the top of AgoshRNA molecules. Ago2-mediated processing efficiency could be enhanced, but only for some of the AgoshRNA molecules tested (60).

It was also suggested that multiple strong G-C bp in the top half of the hairpin result in poor potency of Dicer-independent shRNAs (69). Upon Ago2 cleavage, the 3′-end of these hairpins will remain basepaired, which may potentially block the subsequent trimming by PARN or the availability of the guide for pairing with the matching mRNA, and may thus limit the silencing activity. Several groups have tested whether the introduction of mismatches, G-U/U-G wobble bp or bulges in different parts of the stem - obviously without changing the guide sequence - have an effect on the silencing activity (58). Sun *et al.* mutated each position along the 3′ strand of agsiRNA and agshRNA molecules. In general terms, mutants were processed more poorly than the original. In contrast to a miR-451 report, a G G mismatch at bp-6 counting from the 5′-end of the guide did not enhance agsiRNA and agshRNA activity and produced less mature product (69). Molecules with mismatches at bp-8/13/14/15, a wobble G-U at bp-8 or a bulge at bp-7 were also processed poorly (58,67,70). It was observed that the seed domain is more tolerant for mismatches and wobble bp than the top half of the hairpin, and bulges are not favoured. Similarly, we described that introduced bulges result in a loss of activity, consistent with the disappearance of the processed AgoshRNAs on Northern blot (70). We also introduced wobble bp to destabilize the hairpin, but no increased activity was scored for any of the mutants compared to the original hairpins. In fact, some of the AgoshRNA lost nearly all activity (67). Thus, the introduction of mismatches, G-U/U-G wobble bp or bulges does not improve, but rather hamper the activity of miR-451 mimics, although one cannot exclude the possibility that certain not-yet-tested mutations at specific positions of the hairpin will be beneficial.

The 5′-terminal nucleotide

There is accumulating evidence that the Ago2-mediated processing route mimics miR-451 biogenesis (71). Interestingly, miR-451 is special among the many miRNAs because of the 5′-terminal A instead of the more common U, which creates a bottom mismatch (A C) at the hairpin. The creation of a bottom bp (G-C) in the pre-miR-451 stem substantially impaired miR-451 processing (72).

We recently reported that the bottom bp is also important for AgoshRNA activity (73,74). This may relate to the observation that the MID domain of the human Ago2 protein has a binding preference for small RNAs with 5′-terminal U or A during RISC loading (75,76). Superior AgoshRNA activity was scored for the 5′-terminal A and G variants, which seems to argue against this scenario. However, the situation is more complex as the 5′-terminal nt also represents the +1 position in the RNA polymerase (Pol) III H1 promoter used for transcription of these small RNAs. In fact, a purine preference at this position has been linked to optimal H1 transcription (56,77). We described that the H1 promoter initiates transcription at multiple start sites, but most frequently at the –1 position. Initiation inaccuracy of the commonly used H1 and U6 promoters was described previously (77,78). Thus, active AgoshRNA molecules will most frequently have a 2-nt 5′-overhang. This overhang may facilitate anchorage to the MID domain of Ago2 or binding into the Ago2 groove to trigger its slicer activity. Another possibility is that a strong bp at the bottom of the hairpin affects the stability of the folded transcript to cause a shift from Ago2 to Dicer processing as observed for AgoshRNA variants with a bottom bp G-C (74). Similarly, agsiRNA are slightly more potent with an additional A at the 5′-end (79). Thus, an additional nt is recommended at the 5′-terminus, A when a RNA Pol III promoter is used for optimal expression of the transcript and U when the RNA is chemically synthesized to optimize the Ago2 interaction.

The 3′-terminal nucleotides

When researchers designed miR-451 mimics, the general assumption was that the 3′-terminal two nt (UU) - encoded by the transcriptional termination signal T6 - would not be required for gene-silencing. When combined with a bottom A C mismatch in the stem of the hairpin, this will result in the 3′-overhang CUU. We wondered whether this 3′-overhang affects the uptake by Ago2 and the silencing activity. By comparison, miR-451 has a 3′-terminal CUC overhang due to Drosha processing that is not likely to sterically hinder RNA uptake by the Ago2 enzyme. An AgoshRNA molecule with 3′-CUU overhang was chemically synthesized and analysed for processing efficiency and silencing activity upon transfection into cells (74). This variant was slightly more active than the original AgoshRNA with a regular 2-nt overhang. Similarly, Sun *et al.* created 3′-terminal agsiRNA variants by attaching U, UU or UUUUU to the 3′-terminal C residue (58). The U and UU variants were efficiently produced, but the UUUUU variant produced less mature products, indicating increased degradation of this agsiRNA. Similarly, Shang *et al.* designed an saiRNA as miR-451 mimic with a HDV ribozyme at the 3′-terminus. This generates a short 3′-terminal overhang that enhanced the silencing activity compared to a miR-451 construct with the regular U stretch 3′-termination signal (59). It was suggested that the short 3′-overhang generated by HDV cleavage facilitates efficient binding of the saiRNA to Ago2. In contrast, saiRNAs with an extended 3′-overhang were unable to bind Ago2, which results in reduced accumulation of processed saiRNA molecules, thus reducing the silencing activity. Thus, the length of the 3′-overhang of miR-451 mimics has no impact on its association with Ago2 in the 1/3-nt range, but larger overhangs can reduce Ago2 binding.

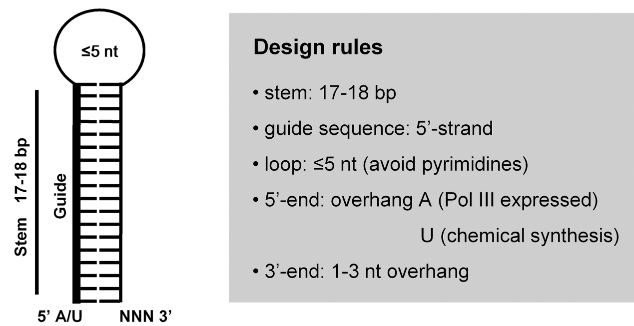

Figure 3. Cartoon of the key features of a Dicer-independent shRNA. See the text for further details.

Figure 3 summarizes the rules for the design of Dicer-independent shRNAs. The stem length is the major determinant with optimal activity in the 17/18-bp range. The guide sequence, preferably fully complementary to the target mRNA, must be located in the 5′-strand of the hairpin. Small loops (≤5 nt) are advised and pyrimidines in the loop are not recommended. Mismatches and bulges in the stem are not allowed, but G-U/U-G wobble bp have little or no effect on Dicer-independent shRNA activity. A bottom mismatch is recommended with A as 5′-terminal nt when an RNA Pol III promoter is used for expression of the transcript and U when the RNA molecule is chemically synthesized for optimal Ago2 interaction. A short 3′-overhang of 1/3-nt is also required for optimal Ago2 uptake.

MECHANISTIC INSIGHTS ON DICER-INDEPENDENT shRNAs

Ago2 cleavage and subsequent processing

Recent studies indicated that processed shRNA molecules have imprecise 5′ and 3′ ends, which can have a serious impact on the silencing activity and target specificity (80–82). We speculated that Ago2-mediated cleavage may be more precise than Dicer-mediated cleavage and therefore investigated by deep sequencing the shRNA versus AgoshRNA products. It was observed that Dicer-mediated cleavage produced shRNA-derived reads ranging from 19/22 nt (expected cleavage site ± 1 nt), whereas Ago2-mediated cleavage occurred exactly at the predicted position between bp 10 and 11 for AgoshRNAs of 17/18/19-bp (56,70). These results demonstrate that AgoshRNAs are cleaved more precisely than regular shRNAs.

A requirement for perfect basepairing around the Ago2 cleavage site of miR-451 mimics can be expected based on the requirements for regular Ago2/siRNA-mediated cleavage of complementary mRNAs. To test this, we introduced single and multiple mismatches around the expected cleavage site (bp 9–12) in AgoshRNA molecules. AgoshRNA mutants with 2–4 mismatches in the centre of the duplex were not processed by Ago2 and lost all activity. An

AgoshRNA with a single mismatch at bp-11 was partially active and produced a slightly longer RNA product due to a shift in the cleavage site from bp position 10 to 9, whereas a variant with a mismatch at bp-10 was not processed by Ago2 and consequently inactive. Two or more mismatches at the cleavage site seriously hindered Ago2-processing and subsequent silencing (70). This mimics the exquisite dependence on perfect basepairing for Ago2 cleavage of regular siRNA-mRNA duplexes (83–85). Dallas *et al.* described similar results for an sshRNA variant with four mismatches at the cleavage site (54). The unprocessed sshRNA molecule was found in association with Ago2, which indicates that the mismatches block Ago2-slicing rather than Ago2-binding. In conclusion, Ago2-processed shRNAs are precisely processed and require a perfect complementarity with the target mRNA for full silencing activity.

The Ago2-processed miR-451 of around 30-nt is trimmed at the 3′-end by PARN to a mature product of 22/26-nt (34,35). We wondered whether tailing and trimming also occurred for AgoshRNAs. This was analysed by deep sequencing of intracellularly processed AgoshRNA molecules. Ago2-processed AgoshRNAs initially acquire a short 3′-tail of 1–3 A nt, but RNA fragments without 3′-tails were also abundant (56). Ago2-processed AgoshRNA can indeed subsequently be trimmed by PARN.

The mRNA cleavage site

Natural miRNAs cause cleavage or translational suppression of complementary mRNAs (3). Regular shRNAs are designed with full complementarity and are supposed to activate mRNA cleavage. To study the class of Dicer-independent shRNAs, we compared regular shRNA and AgoshRNA molecules in their attack on a luciferase mRNA reporter (74). AgoshRNAs did trigger site-specific cleavage of the luciferase mRNA at the expected site. Interestingly, the site of cleavage was more precise than that triggered by the corresponding shRNA. In fact, AgoshRNA-mediated cleavage occurs predominantly at the ‘11/10 position’ (96.2%), whereas a regular shRNA achieved only 77.3% cleavage efficiency at this position, with a significant 10/9 side product (13.7%). Similar to miRNA-mediated mRNA cleavage, we found that the 3′-end of the AgoshRNA-cleaved mRNA is prone to U-addition (occasionally A or C), which may mark the RNA for degradation (38,86). Similarly, Ge *et al.* reported that sshRNA molecules trigger cleavage of the targeted mRNA precisely at 10-nt from the 5′-end of the guide strand (53). In general, no major differences were observed between synthetic and transgene expressed Dicer-independent shRNAs.

Loading of small shRNAs in Ago1-4

Four Ago proteins are present in human cells (Ago1, 2, 3 and 4). It is known that miRNA duplexes are bound and unwound by all Ago proteins. However, although regular shRNAs are loaded into all Ago proteins with the same efficiency, siRNA duplexes are only efficiently unwound and sliced by Ago2 (87–90). Thus, conventional shRNA molecules may compete with endogenous miRNAs for binding to Ago proteins, which may compromise the miRNA-silencing function (91). In addition, shRNA loading into all Ago proteins will reduce the effective concentration of the therapeutic molecules available for Ago2, the only protein with slicing activity that is needed for mRNA silencing. The miR-451 is selectively loaded into Ago2 (14), but how miR-451 mimics are loaded and processed by the different Ago proteins remains unclear despite the fact that this issue was addressed by several groups (54,57,59,61). Dallas *et al.* performed pull-down experiments with antibodies specific for Ago1 and Ago2 and reported that both are capable of sshRNA-processing in a Dicer-cleavage independent mechanism. This result is in accordance with a previous report in which miRNA and siRNA-slicing by Ago1 was reported in a cell-free system (92), but the results are in stark disagreement with subsequent studies that did not observe siRNA-slicing with Ago1 (93). Ma *et al.* demonstrated that miR-451 mimics (16/19-bp stem length) load exclusively in Ago2 (61). Shang *et al.* studied the association of different saiRNA molecules with Ago proteins and reported a binding selectivity for Ago1/2/3 proteins (59). However, Ago2 overexpression enhanced saiRNA processing, whereas Ago1 and Ago3 overexpression resulted in accumulation of unprocessed saiRNA precursors. Similarly, Börner *et al.* demonstrated an enhancement of AgoshRNA activity by Ago2 overexpression in mammalian cells (94). We explored Ago1–4 loading by co-transfection of cells with synthetic AgoshRNAs and a plasmid encoding one of the Flag-tagged Ago forms (57). Flag-tag immunoprecipitation showed enrichment of processed RNAs in the Ago2 sample, whereas Ago1 and 3 yielded a minor RNA enrichment over the unbound fraction and Ago4 did not show any RNA enrichment. Multiple lines of evidence suggest that AgoshRNAs are preferentially loaded in Ago2 over Ago1/3/4. However, one should be cautious because the results may be influenced by the different experimental conditions, e.g. differences in the expression level of the Ago1–4 proteins. For instance, Ago3/4 are expressed at a much reduced level compared to Ago1/2 (94–97) and therefore a more quantitative analysis will be required.

Potential advantages of Dicer-independent shRNAs

Several advantages of Dicer-independent shRNAs over classical shRNAs have been suggested in literature (54,61,98,99) and are listed in Table 2. Dicer-independent shRNAs produce only a single RNAi-active guide strand, which is an important property to restrict RNAi-induced off target effects caused by the passenger strand of regular shRNAs. Dicer-independent shRNAs will likely be the silencing method of choice for certain cell types, e.g. monocytes that lack Dicer (100).

The novel Dicer-independent shRNA design may yield safer therapeutics than regular shRNAs for several reasons. Saturation of Dicer as critical component of the cellular RNAi pathway will not occur for Dicer-independent shRNAs. Innate immune mechanisms will be triggered less likely by these shorter RNA duplexes (101). Greater accuracy is achieved at several levels. Ago2-mediated processing of shRNAs yields more precise ends compared to Dicer processing, which is notoriously inaccurate (56,81). Dicer-independent shRNAs trigger more accurate cleavage

Table 2. Properties of Dicer-dependent versus Dicer-independent shRNAs

Dicer-dependent shRNA	Dicer-independent shRNA
Dicer requirement / Inactive in Dicer-minus cells	No Dicer requirement / Active in Dicer-minus cells
Guide and passenger strand activity	Guide strand activity only
PKR / interferon induction	Less PKR / interferon induction
Less precise processing	More precise processing
Less precise mRNA cleavage	More precise mRNA cleavage
Loading in Ago1–4	Preferential loading in Ago2
Design algorithm available since recently	Lack of design algorithm
Potent activity after trial-and-error tests	Modest activity after trial-and-error tests

of a complementary mRNA than a regular shRNA (74). Dicer-independent shRNAs may load preferentially into Ago2, thus avoiding off target effects via Ago1, 3 and 4 (14,54,57,59,61).

Several potential disadvantages of the novel Dicer-independent shRNA design can also be listed. A restriction for massive Dicer-independent shRNA use is that design algorithms cannot be applied, which calls for a laborious trial-and-error or design-and-test process (16,102–104). A new algorithm for shRNA prediction was developed recently (50), and it remains important that similar algorithms are developed for the design of potent Dicer-independent shRNAs. Furthermore, although Dicer-independent shRNAs have proven to be active *in vitro* and *in vivo*, their RNAi activity is generally somewhat reduced compared to the matching shRNAs (99).

FUTURE PERSPECTIVE

Research performed over the past few years has provided ample evidence for the existence of a Dicer-independent RNAi pathway that is critically involved in the processing of the natural miR-451. Discovery of this alternative pathway has opened the search for a novel type of shRNA-design that also relies on Ago2 instead of Dicer for its maturation. The novel Dicer-independent shRNA design constitutes an interesting, new platform for gene silencing that may outperform current miRNA and shRNA technologies.

Among the many potential applications, antiviral approaches take a prominent place. Ge *et al.* synthesized sshRNA molecules for treatment of hepatitis C virus infection (HCV) by targeting the internal ribosome entry site (IRES) of the HCV RNA genome (105,106). The 19-bp sshRNAs were chemically modified to incorporate 2′-OMe nucleotides to avoid activation of the innate immune system. Optimized sshRNAs were administered intravenously in an HCV-infected immunodeficient mouse model as lipid nanoparticles. Specific and durable inhibition of HCV replication was reported in these mice without significant hepatocyte toxicity during sshRNA treatment and follow-up.

We used the new design rules to generate optimized AgoshRNAs against the human immunodeficiency virus 1 (HIV-1). AgoshRNA molecules were synthesized from the H1 Pol III promoter and A was selected as 5′-terminal nt for optimal Pol III transcription and Ago2-binding (99). Anti-HIV-1 guide sequences that trigger 'self'-targeting of the vector were avoided as a HIV-based lentiviral vector was selected as delivery vehicle for the AIDS gene therapy. Optimized AgoshRNA molecules were designed with a small 5 nt loop (CAAGA) and a duplex of 18 bp with a bottom A C mismatch. The guide sequence that is fully complementary to the HIV-1 RNA genome was located in the 5′-strand of the hairpin. Stretches of four or more Us in the AgoshRNA sequence were avoided because a polyU tract acts as a termination signal for Pol III transcription.

Alternatively, cellular mRNAs encoding protein cofactors that support HIV-1 replication can be targeted (107). We were able to design AgoshRNAs that potently downregulated CCR5 expression on human T cells and peripheral blood mononuclear cells (PBMC), without adverse effects on T cell development as assessed in an ultra-sensitive competitive cell growth assay (107). CCR5 knockdown significantly protected T cells from infection by CCR5-tropic HIV-1 strains. In addition, we demonstrated that a very potent shRNA, which was removed from the therapeutic cocktail because of mild adverse effects on cell growth and physiology, can be reconfigured into an active and non-toxic AgoshRNA that targets the same HIV-1 RNA sequence (99,108). We also demonstrated that AgoshRNA-based antivirals - unlike shRNA-based inhibitors - remain active in Dicer-minus monocytic cells that are susceptible to HIV-1 infection. These combined results suggest that the future for AgoshRNA therapeutics is promising.

RNAi has also proven to be a very powerful screening method, e.g. to determine the function of a cellular protein and RNAi is very useful for experimental validation of putative drug targets. A major problem is that these methods work efficiently only in organisms that possess the basic RNAi machinery. For instance, in protozoan parasites the RNAi machinery is not always present, e.g. the malaria parasite Plasmodium lacks both Dicer and Ago2 (109,110). This impedes the analysis of gene function using RNAi methods and therefore hampers drug and vaccine development. The discovery of the alternative RNAi pathway that requires only a small miR-451 mimic and Ago2 (for processing and silencing) may facilitate the introduction of a minimal RNAi machinery into such organisms (Dirk Grimm, personal communication). This will facilitate the analysis of gene functions and target modulation at selected life cycle stages of the parasite, with the final goal of development of new drug and vaccine candidates.

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