

RESEARCH PAPER



Boosting AgoshRNA activity by optimized 5'-terminal nucleotide selection

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ABSTRACT

RNA interference (RNAi) can be triggered by synthetic small interfering RNAs (siRNAs) or transgene-expressed short hairpin RNAs (shRNAs). Recent evidence indicates that shRNA molecules, with a relatively short stem and small loop, are processed by Argonaute 2 protein (Ago2). We named these molecules AgoshRNA as Ago2 is involved in both the processing and the subsequent mRNA-silencing reaction. This alternative processing route yields only a single guide strand, which thus avoids potential off-target effects induced by the passenger strand of a regular shRNA. We recently described that the introduction of a 5'-terminal purine (A or G) and a mismatch at the bottom of the hairpin enhances the AgoshRNA activity. The critical 5'-terminal nucleotide (nt) represents the +1 position of the transcriptional promoter, which influences the transcriptional efficiency and initiation accuracy as demonstrated for the H1 RNA polymerase (Pol) III promoter. These findings highlight the necessity of considering Pol III requirements in the design of optimized AgoshRNA cassettes. In this study, we report the design and expression of potent AgoshRNAs by two other popular Pol III promoters: U6 and 7SK, which were recently reported to have a distinct transcription profile compared to the H1 promoter. We propose general rules for the design and expression of potent AgoshRNA molecules using Pol III cassettes, which should augment the application of novel AgoshRNA reagents for basic research and therapeutic purposes.

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Introduction

RNA interference (RNAi) is an evolutionarily conserved post-transcriptional gene-silencing mechanism that uses microRNA (miRNA) as effector molecule [1,2]. Cellular primary miRNAs are typically processed by the Drosha endonuclease in the nucleus and subsequently by Dicer in the cytoplasm. This canonical route yields a mature miRNA duplex of 20–24 base pair (bp), of which one strand is preferentially loaded into Ago protein to form the RNA-induced silencing complex (RISC) that facilitates messenger RNA (mRNA) degradation or translation repression. The guide strand selection is determined by the thermodynamic properties of the miRNA duplex, of which the passenger strand is cleaved and degraded [3,4]. The RNAi mechanism can also be triggered by artificial shRNAs that are processed by Dicer into small interfering RNAs (siRNAs) that subsequently programme RISC [5,6]. Although Dicer is required for processing of the majority of miRNAs, some exceptions have been reported. Notably, miR-451 with a short 17 bp stem and 4 nt loop bypasses Dicer and instead is processed by Ago2, generating an ~30 nt guide [7–9]. Subsequent 3' end processing by poly(A)-specific ribonuclease (PARN) produces the 22–26 nt mature miR-451 [10].

More recently, Dicer-independent shRNAs have also been reported [11–14], and we proposed that hairpins with a short stem and small loop can enter this alternative pathway [15]. We termed those molecules AgoshRNAs as Ago2 is involved

in both the processing and silencing steps [13]. The length of the base-paired stem is a major determinant for the selection of the regular shRNA versus alternative AgoshRNA processing route. Fig. 1A depicts the two shRNA processing pathways. Regular shRNAs are processed by Dicer, but AgoshRNAs with a 19 bp or shorter stem avoid Dicer recognition and shift to alternative Ago2-processing. The Dicer-processed regular shRNA generates a duplex siRNA consisting of two candidate guide strands of ~21 nt (Fig. 1A, left). The Dicer-independent AgoshRNA is processed by the Ago2 nuclease that cleaves halfway the 3'-side of the stem (Fig. 1A, right). As a result, an extended ~33 nt guide RNA (marked as grey arrow) and ~12 nt RNA by-product (marked as black line) are generated, the activity of which can be scored by silencing of Luc-antisense and Luc-sense reporters (Fig. 1B).

There is accumulating evidence that the AgoshRNA pathway mimics miR-451 biogenesis. Inspired by features of the natural miR-451 molecule, we recently investigated the effect of the identity of the 5'-terminal nt and its base-paired status on AgoshRNA activity. The introduction of a 5'-terminal purine (A or G) and a bottom mismatch enhanced the AgoshRNA activity, with a 5'-terminal A being moderately better than G. The critical 5'-terminal nt of the AgoshRNA transcript also represents the +1 position of the H1 promoter used and was reported to affect the transcriptional efficiency and transcription start site selection [15]. This result indicates that a promoter effect at the DNA level should also be

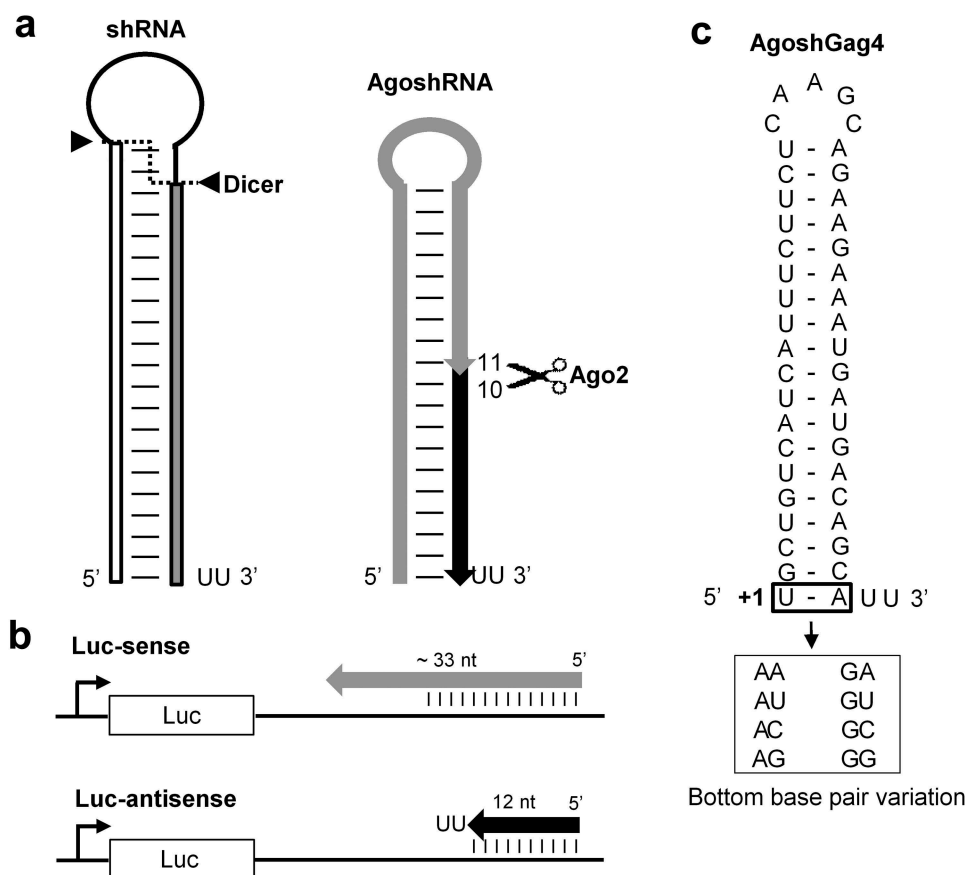


Figure 1. Schematic of the canonical (Dicer-dependent) shRNA and non-canonical (Ago2-dependent) AgoshRNA design. **(a)** Secondary structure of a shRNA that is processed by Dicer into a siRNA duplex of ~21-bp with 2 nt 3'-overhangs that is loaded into RISC. The predicted Dicer cleavage sites are marked as black triangles. The passenger strand (5'-strand, white line) is degraded and the 3'-strand (grey line) acts as guide for RNA silencing. The AgoshRNA is processed by Ago2 on the 3'-side between bp 10 and 11 into an extended RNA molecule of ~33 nt (grey arrow) and a ~12 nt RNA molecule (black arrow) with 3'-UU overhang. **(b)** Luc reporter constructs to evaluate AgoshRNA activity. The Luc-sense reporter scores the AgoshRNA activity of the extended ~33 nt RNA and the Luc-antisense reporter can potentially measure the activity of the ~12 nt RNA product. **(c)** The AgoshGag4 with a 19 bp stem and 5 nt loop was used as backbone for variation in the bottom bp as indicated.

considered for improvement of the AgoshRNA design. We recently reported that the two widely used Pol III promoters U6 and 7SK differ from the H1 promoter in transcription initiation site usage, which may affect the expression of precise RNA molecules such as AgoshRNAs [13,15]. In this study, we investigated the influence of the 5'-terminal nt and bottom base-pairing status on AgoshRNAs production and activity when made from U6 and 7SK expression cassettes. This study enabled us to propose general rules for the optimal expression of potent AgoshRNA molecules from Pol III cassettes.

Results

Design of AgoshRNA variants for the U6 and 7SK cassettes

Previous studies demonstrated that the nucleotide (nt) identity around the +1 position affects the accuracy of transcription start site selection [16,17] and one should thus be careful when using modified promoters with sequence changes around the +1 position. The H1 promoter initiates transcription promiscuously from multiple sites in the -3/-1 window regardless of the nt identity at the +1 position [16]. In contrast, the U6 and 7SK

promoters with a purine (A/G) at the +1 position produce small RNAs with a precise +1 start site. We therefore reasoned that these two promoters may be more suitable for the expression of exact AgoshRNA molecules, potentially with increased specificity and potency. To explore the optimal AgoshRNA design in the context of the U6 and 7SK Pol III cassettes, we systematically altered the +1 nt and bottom bp in the AgoshGag4 backbone (Fig. 1C). The wild type (WT) AgoshGag4 has a U-A bottom bp and a 5'-terminal U that also represents the +1 position of the U6 and 7SK promoters. We tested A or G as the +1 nt because of the precise start site usage and generated all eight dinucleotide combinations at the bottom of the AgoshRNA stem, thus forming a bp (AU, GC and GU) or mismatch (all others). We named these mutant AgoshRNA molecules accordingly, e.g. the AU variant.

The 5'-terminal nucleotide is critical for optimal AgoshRNA activity from the U6 cassette

We first evaluated the knockdown activity of the WT and mutant AgoshGag4 molecules expressed from the U6 promoter. The Luc-sense and Luc-antisense reporters were used to score activity of the 5' and 3'-strand of AgoshGag4, respectively (Fig. 1B). To do this, AgoshGag4 constructs were

titrated (1, 5 and 25 ng) during transfection into HEK293T cells, together with a fixed amount of one of the Luc reporters. A Renilla reporter plasmid was co-transfected to control for variation in the transfection efficiency. Luciferase expression was measured two-days post-transfection and the relative luciferase activity (Firefly/Renilla) was determined. An irrelevant shRNA (shNef) was used as negative control, for which the luciferase activity was arbitrarily set at 100%. The WT and all variant AgoshGag4 molecules exhibited inhibition of the Luc-sense reporter with a clear dosage effect, but to a variable degree (Fig. 2A). Surprisingly, the inhibitory capacity of WT is significantly boosted for all four AN variants, but not the GN set. This result indicates that the 5'-terminal A is important for optimal AgoshRNA expression and/or activity. The four AN variants were similarly strong, indicating that the presence of a bottom bp or mismatch is not an important determinant for AgoshRNA activity. This conclusion is confirmed by the GN set, where a similar activity is apparent for

three variants (GA, GC and GG) that have either a bottom bp or mismatch. We noticed a bit increased silencing activity for the GU variant at all three concentrations tested, which may be due to the fact that this U becomes part of the T-stretch transcription termination signal. A -1 shift in termination site may thus occur, which will result in an AgoshRNA with a shorter 3' overhang that may be favoured in AgoshRNA pathways. However, this 'U' advantage was not apparent for the AN set, where it may be masked by the dominant 5'-A effect. No or little knockdown activity was scored for most AgoshGag4 molecules on the Luc-antisense reporter (Fig. 2B), consistent with the notion that no active 3' guide is generated by AgoshRNA molecules. However, we observed weak inhibitory activity for the four AN variants at the high dose. The combined results for U6-expressed AgoshGag4 molecules indicate that the 5'-terminal nt is a critical determinant for AgoshRNA expression and/or activity, whereas the status of the bottom bp/mismatch has little effect.

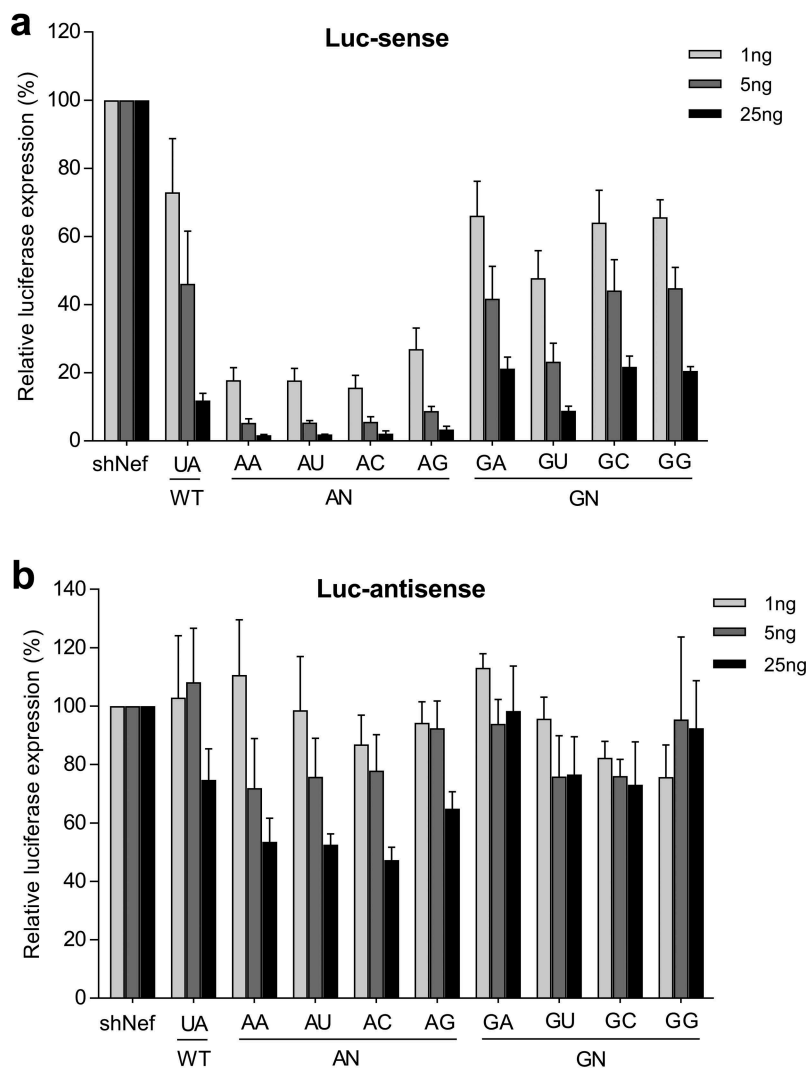


Figure 2. Knockdown activity of AgoshRNA expressed from the U6 promoter. The knockdown activity of the AgoshRNA constructs was determined by targeting a Luc reporter containing either sense (a) or antisense (b) target sequence in a co-transfection experiment. HEK293T cells were co-transfected with 100 ng of the respective Luc reporter plasmid, 1 ng renilla luciferase plasmid as internal control and the corresponding AgoshRNA constructs with a serial titration (1, 5 or 25 ng). The unrelated shNef served as negative control, the activity of which was set at 100% luciferase expression. Three independent transfections, each in duplicate, were performed and the standard deviation was calculated.

Increased AgoshRNA activity with a 5'-terminal A is not due to variation in expression level or processing

We next analysed the AgoshGag4-processing products by Northern blotting using probes that detect the 5' and 3'-side of the AgoshRNA (Fig. 3A, B, respectively). A fixed amount of the AgoshRNA constructs was transfected into HEK293T cells. Total cellular RNA was extracted 2 days post-transfection and a fixed amount was subjected to Northern blotting. Using the 5'-side probe, an RNA band of ~33 nt was apparent for WT and all

AgoshGag4 variants (Fig. 3A, marked with a star). This signal corresponds with the typical AgoshRNA-processing product, but with minor variation in size among the constructs, which may be caused by differential cleavage by Ago2 or differential 3'-end trimming by the PARN enzyme. Interestingly, an apparent ~21 nt RNA signal that usually reflects Dicer-processing was detected for the variants with a bottom bp (AU, GU and GC), especially the latter with the strongest G-C Watson-Crick bp. These results suggest a partial shift from Ago2 to regular Dicer processing, which indeed requires an extended hairpin stem. Close inspection

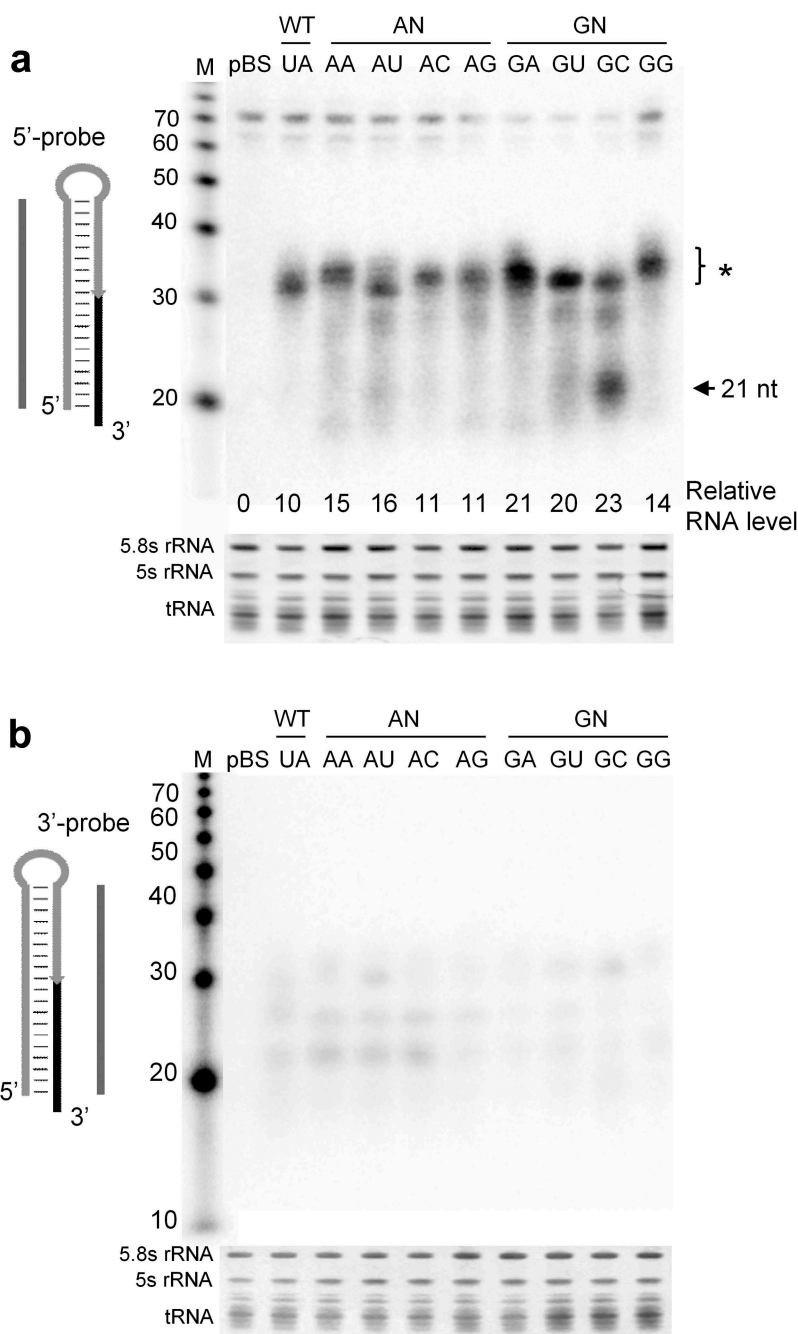


Figure 3. Detecting the processing products of AgoshRNA expressed from the U6 cassette. HEK293T cells were transfected with the indicated constructs. Two days post-transfection, total cellular RNA was extracted and a fixed amount was subjected to Northern blotting. The 5'-side and 3'-side probe were used to detect the 5'- and 3'-strand products of AgoshRNA constructs (**3a** and **3b**, respectively). The RNA markers (indicated in nt) were included for transcript size estimation. The shNef was included as negative control. The ~33 nt signal (*) and the regular ~21 nt product are marked. The 5' strand RNA signal of ~20–30 nt was quantitated and the relative RNA level is listed at the bottom of the lanes. The WT (UA) value was arbitrarily set to 10. Ethidium bromide staining of small rRNAs and tRNAs is shown as loading control below the blots.

of the processed AgoshRNA molecules (Fig. 3A, marked with a star) indicates a slight difference between constructs with or without a bottom bp. The AU, GU and GC variants produce transcripts that are less diffuse and seemingly a bit shorter than the non-base-paired variants (Fig. 3A, 5A). The current analyses do not provide a more detailed resolution. Quantitation of the RNA signals indicates that the GN variants yield slightly more active strand than the WT and AN variants, suggesting that +1G induces most efficient transcription of the AgoshGag4 precursor. Nevertheless, the GN variants exhibited reduced silencing activity. We thus conclude that the intrinsic activity of AgoshRNAs with 5'-G is much reduced compared to the 5'-A set.

With the 3'-side probe only weak signals in the ~20–30 nt range were detected (Fig. 3B), consistent with the absence of knockdown activity (Fig. 2B). These weak signals likely include the 21-nt product of regular Dicer cleavage and the 30 nt AgoshRNA products that are only sub-optimally detected by the 3'-probe due to partial complementarity. We did not detect the predicted ~12 nt 3'-strand processing product (Fig. 1A), which is likely rapidly degraded, as previously suggested [13,18].

The AgoshRNA set expressed from the 7SK promoter

We next inserted the same AgoshRNA set behind the 7SK promoter and assessed the AgoshRNA activity and expression/processing. The Luc-sense reporter revealed that the WT construct is not active (Fig. 4A), and is quite different from the U6 results (Fig. 2A). This may be caused by the different transcription requirement of these two promoters when T is at the +1 position [16]. But activity is profoundly rescued for the AN variants and, albeit to a lesser extent, by the GN variants (Fig. 4A). This pattern is very similar to that observed for the U6 constructs (Fig. 2A). Again, the bottom bp/mismatched status does not seem important for AgoshRNA activity, as is apparent for both the AN and GN sets. GU is most potent among the GN variants, exactly as was observed in the U6 context. All AgoshRNAs show little or no inhibitory activity on the Luc-antisense reporter (Fig. 4B).

Northern blotting of the 7SK promoter constructs largely confirmed the results obtained for the U6 constructs. Prominent ~33 products were detected for all AgoshGag4 molecules by the 5'-side

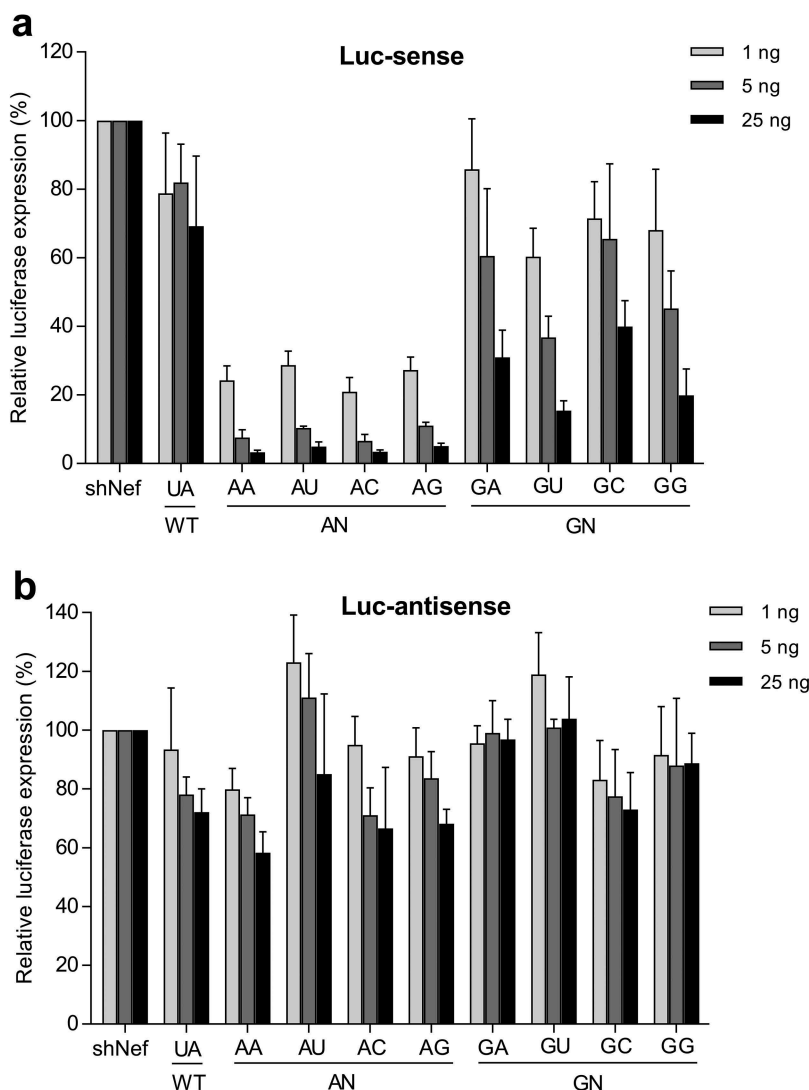


Figure 4. Knockdown activity of AgoshRNA expressed from the 7SK promoter. The knockdown activity of the individual AgoshRNA construct was determined by co-transfecting Luc-sense (a) or Luc-antisense (b) reporters as described for Figure 2. Three independent transfections, each in duplicate, were performed and the standard deviation was calculated.

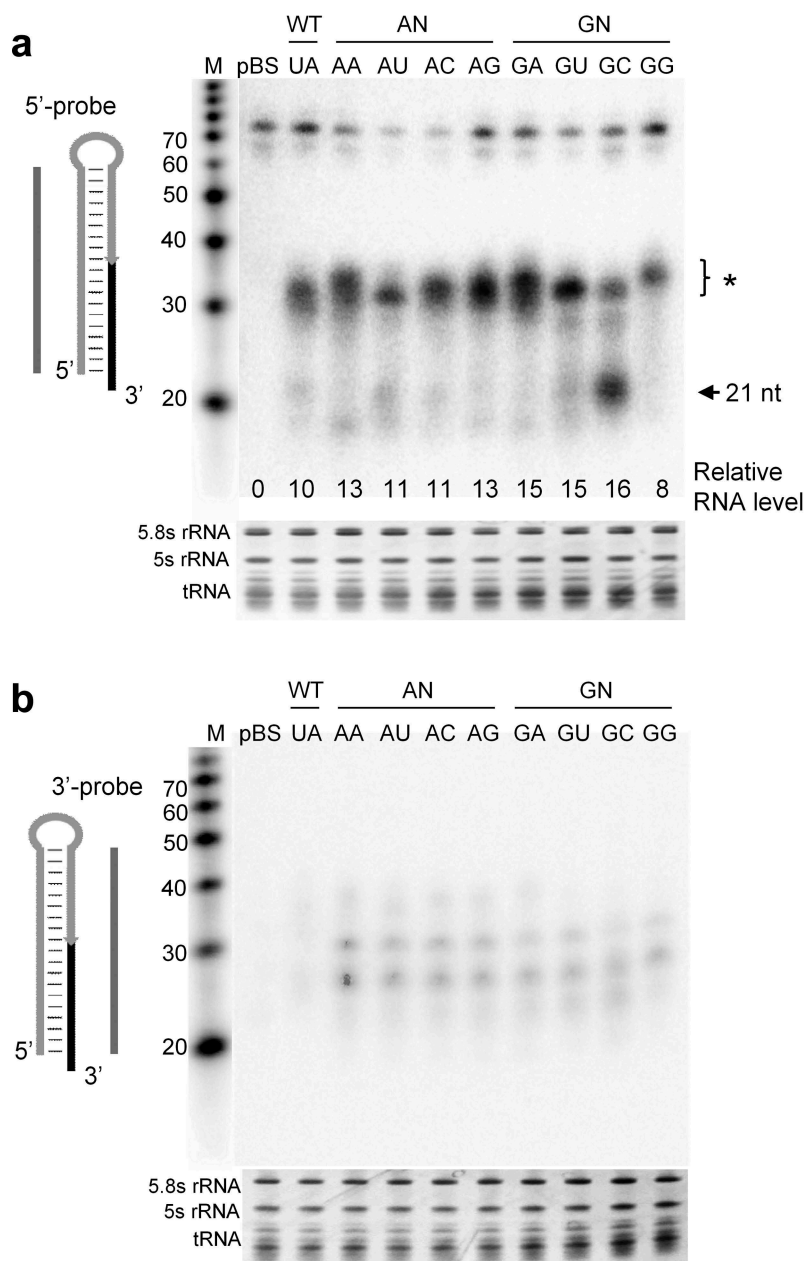


Figure 5. Probing the processing products of AgoshGag4 molecules expressed by the 7SK promoter. Northern blotting assay was performed as described for Figure 3. See the legend of Figure 3 for further details.

probe. A ~ 21 nt RNA signal that indicates a partial shift from Ago2 to Dicer processing was apparent for the GC variant and to a lesser extent for the AU and GU variants (Fig. 5A). Therefore, three variants share the property of forming an additional bottom bp. Quantitation of the RNA signals is difficult as some products are more discrete in size (e.g. AU) than others (e.g. GA), but indicates a roughly similar product level for the AN and GN variants. Using the 3'-side probe, weak RNA signals of a size between 20 and 30 nt were detected for all AgoshRNA constructs, but less for WT (Fig. 5B). The absence of strong RNA signals correlates with the poor silencing activities on the Luc-antisense reporter (Fig. 4B). The combined results for 7SK-expressed AgoshGag4 molecules indicate that the 5'-terminal nt is a pivotal determinant for AgoshRNA activity, whereas the bottom bp/mismatch status only has a minor effect.

Taken together, the set of AgoshRNA variants exhibited quite similar activity profiles in the U6 and 7SK cassettes. We conclude that the introduction of 5'-terminal A can profoundly enhance the AgoshRNA activity, an effect that is not caused by variation in RNA abundance or Ago2-mediated processing efficiency.

Discussion

Recent evidence indicated that one can design shRNAs with a short stem and small loop that avoid Dicer recognition and instead are processed by Ago2 [11–13]. This new design was termed AgoshRNA, with as major advantage over regular shRNAs that no active passenger strand is generated to

avoid off-target effects. We previously revealed that the 5'-terminal nt and the base-pairing status are important for AgoshRNA activity and that AgoshRNA optimization can be achieved by introducing a 5'-terminal A or G with a bottom mismatch in H1 expression cassettes [15]. In order to further optimize the AgoshRNA portfolio, we attempted to modulate AgoshRNAs expression/activity for the other two popular Pol III promoters U6 and 7SK. These promoters have a transcription profile distinct from H1 and have the potential to generate more specific and potent AgoshRNA molecules [16]. Therefore, we designed eight dinucleotide combinations at the bottom of the AgoshGag4 stem. Very similar AgoshRNA activity and biogenesis results were obtained for these two promoters, but the results differ significantly from those previously reported for the H1 promoter. The AgoshRNA design with 5'-terminal A is significantly more active than the 5'-G variant and the bottom base-pairing status only subtly affects the silencing activity.

Several possibilities can be envisaged to interpret the profound +1 effect on AgoshRNA activity made from U6 and 7SK constructs. First, the +1 position of the AgoshRNA transcript also represents the +1 position of the Pol III promoter, which could affect the transcription initiation efficiency and start site selection. Second, +1 nt and bottom bp variation may affect AgoshRNA stability, processing and intrinsic silencing activity.

An 'early' effect at the transcriptional level is possible due to +1 nt variation in Pol III promoters. The AN and GN

variants exhibited similar activity in the H1 cassette [15], but profound differences were scored in the 7SK and U6 cassettes. This AN/GN variation coincides with different transcription profiles of the promoters, especially with respect to the transcription start site usage. U6 and 7SK use a precise +1A/G start, but H1 with +1A/G starts from multiple sites in the $-3/-1$ window [16]. Accordingly, the H1 promoter – due to promiscuous transcription initiation – will generate AgoshRNA transcripts with unwanted 5'-overhangs. As the 5'-terminal nt contacts the MID domain of Ago2 [19,20], the 5'-extension may interfere with Ago2 binding, but may also affect target recognition due to the change in seed sequence. On the other hand, the U6 and 7SK promoters with precise +1A/G usage will produce the exact designed AgoshRNA molecule. Consistent with previous findings [13], the precursor AgoshRNA transcript was never observed for the WT and variant constructs, arguing that there is no significant difference in the Ago2-processing efficiency, but there may be minor differences in the actual site of Ago2-cleavage.

To interpret the profound +1 effect for AgoshRNA biogenesis, we schematically plotted the AgoshRNA-mediated mRNA cleavage pathway according to what we know about regular shRNAs (Fig. 6). The 5'-terminal nt is indicated as red dot. Three steps are illustrated: shRNA/AgoshRNA expression (step 1), processing to generate the guide (step 2) and target mRNA cleavage (step 3). The shRNA and AgoshRNA pathways differ mainly in step 2, where the shRNA is processed by

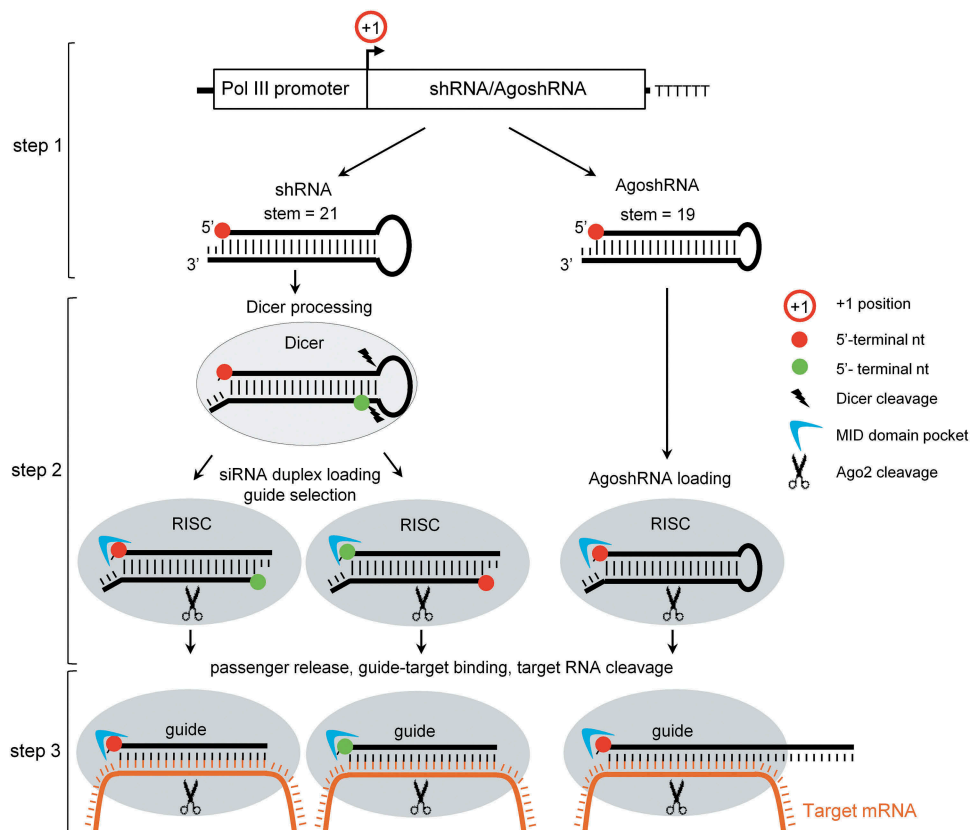


Figure 6. Pathways for shRNA versus AgoshRNA-mediated mRNA cleavage. Step 1: expression of shRNA/AgoshRNA molecules from a Pol III promoter. Pol III transcription starts at +1 position (red open dot) and terminates at the T-stretch. The red closed dot of the expressed shRNA/AgoshRNA represents the 5'-terminal nt. Step 2: shRNA/AgoshRNA processing for guide generation. Step 3: target mRNA cleavage. This step involves base pairing with the target mRNA, which nucleates in the seed region (position 2–8 from 5'-end) and then propagates to the 3'-end of the guide, accompanied by extensive conformational changes in the Ago2/guide complex.

Dicer into a siRNA duplex that can be loaded – in two orientations – in Ago2-containing RISC, whereas the AgoshRNA is loaded directly – in a fixed orientation – into RISC. In both routes, the 5'-terminal nt of the guide (red and green dot for the two shRNA-derived strands, red dot for the single AgoshRNA-derived guide strand) of the guide is anchored at the MID domain pocket of Ago2 (marked in blue) and the passenger strand is released upon Ago2-cleavage (scissor). Previous studies on canonical shRNA (miRNA)-mediated RNA cleavage revealed that the MID domain pocket prefers to bind A and U over C and G¹⁹. It was proposed that the tight interaction with the 5'-terminal A or U is required for maintenance of the Ago2/guide complex once the 3' end of guide is released upon duplex formation with the target RNA (step 3), and hence for efficient target silencing [19,21]. Similarly, this 5'-A or U property should benefit AgoshRNA-mediated silencing as demonstrated by our results that AgoshRNAs with 5'-terminal A is more potent than the 5'-G variant (Fig. 2A, 4A). For siRNA duplex loading into Ago2 (step 2), the guide strand 5'-terminal nt needs to be unpaired before it can enter the MID domain pocket, consistent with the fact that the selected guide strand of an siRNA prefers a thermodynamically unstable 5'-terminal [22]. Similarly, one can expect that AgoshRNAs require an accessible 5'-terminal nt for anchoring. This hypothesis may explain why a mismatch at the bottom of the AgoshRNA stem improves the activity [15,23].

The 5'-terminal nt of the guide strand binds to the MID domain pocket and is not used for target RNA binding [24]. Therefore, the 5'-terminal nt of the guide strand can be any nt without affecting target recognition, but an U or A with superior Ago2 binding affinity is preferred because of the increased silencing activity. This 5'-terminal A or U criterion should also apply to AgoshRNA molecules and this was confirmed in tests with synthetic AgoshRNA with 5'-nt variation [15]. On the other hand, Pol III promoters prefer +1A/G to support efficient transcription and precise +1 initiation. Therefore, +1A would satisfy both requirements for a potent AgoshRNA reagent. As AgoshRNA requires an accessible 5'-terminal nt for anchorage, a mismatched bottom bp would be preferred. AgoshRNA with a mismatched bottom bp also reduces Dicer-processing compared to that with a base-paired bottom bp (Fig. 3A, 5A). Combined with our previous results [13,15,25], we now establish the following rules for the design and expression of AgoshRNA under Pol III promoters: a hairpin with a 5 nt loop and a duplex length of 18 bp with a bottom AV (V = A, C or G) mismatch; an A at +1 position of the Pol III promoters.

The AgoshRNA design exhibits several advantages over regular miRNA/shRNA molecules. AgoshRNAs produce only a single guide strand, thus avoiding any adverse effects induced by the passenger strand. Ago2-mediated AgoshRNA processing yields more precise molecules than Dicer cleavage, which creates imprecise ends [26]. The shorter AgoshRNA duplex is less prone to trigger innate immune response and thus may exhibit an improved safety profile [27]. AgoshRNA may also mimic miR-451 by exclusive loading into Ago2, thus avoiding interference with endogenous miRNAs and competition for Ago1, 3 and 4 [28]. Another advantage is that

AgoshRNAs are active in Dicer-deficient cells, e.g. monocytes that lack Dicer expression [29]. Therefore, the AgoshRNA provides an alternative silencing platform with some unique features compared to current miRNA and shRNA technology.

Materials and methods

Vector construction

DNA constructs used in this study were made by annealing complementary oligonucleotides and insertion in the pSilencer 2.0-U6 vector (Ambion) opened with the BamHI and HindIII enzymes and psiRNA-h7SK G1 hygro vector (Invivogen) digested with Acc65I and HindIII. The Luc reporter plasmids were constructed by insertion of a 50–70 nt HIV-1 sequence, with the Gag4 target region in the centre, into the EcoRI and PstI sites of the pGL3 plasmid [30]. The luciferase reporters with the sense and antisense target sequences were previously described [13].

Cell culture and dual-luciferase reporter assays

Human embryonic kidney (HEK) 293T cells were maintained in Dulbecco's modified Eagle's medium (Life Technologies, Invitrogen, Carlsbad, CA, USA) supplemented with 10% foetal calf serum (FCS), penicillin (100 U/mL) and streptomycin (100 µg/mL). One day prior to transfection, 0.5 mL DMEM/10% foetal bovine serum with 1.5×10^5 [5] cells was seeded per well in 24-well plates. AgoshGag4 constructs were titrated (1, 5 or 25 ng) and transfected with Lipofectamine 2000 (Invitrogen) into HEK293T cells together with 100 ng pGL3-control Luc reporters (sense or antisense) and 1 ng Renilla luciferase plasmid according to the manufacturer's instructions. Two days post-transfection, luciferase activity was measured with the dual-luciferase reporter assay system (Promega, Madison, WI, USA). The ratio of Firefly to Renilla was calculated to control for variation in transfection efficiency. Three independent transfections were performed, each in duplicate. The resulting six values were corrected for between session variations as described previously [31].

Northern blotting analysis

Northern blotting was performed as previously described [16]. Briefly, 1.5×10^6 [6] HEK293T cells were transfected with 5 µg AgoshRNA construct using lipofectamine 2000 (Invitrogen). Total cellular RNA was extracted 2 days post-transfection with the mirVana miRNA isolation kit (Ambion). The RNA concentration was measured with NanoDrop 2000 (Thermo Fisher Scientific). Five µg of total RNA was electrophoresed in a 15% denaturing polyacrylamide gel (Precast Novex TBU gel, Life Technologies). [γ -³²P]-labelled decade RNA marker (Life Technologies) was run alongside for size estimation. To check for equal sample loading, the gel was stained in 2 µg/mL ethidium bromide for 20 min and visualized under UV light. The RNA in the gel was electro-transferred to a positively charged nylon membrane (Boehringer Mannheim, GmbH) and cross-linked to the membrane using UV light (1200uJ \times 100). Locked nucleic acid (LNA)

oligonucleotides (5'-probe: 5'-ATTACTACTGCCCTTCAC-3', 3'-probe: TGCTGTCATCATTCTTCT) were 5' end-labelled with the kinaseMax kit (Ambion) in the presence of 1 μ L [γ - 32 P]-ATP (0.37 MBq/ μ L, Perkin Elmer). Sephadex G-25 spin columns (Amersham Biosciences) were used to remove the unincorporated nucleotides. The membrane was incubated in 10 mL ULTRAhyb hybridization buffer (Ambion) at 42°C for 30 min, after which the labelled LNA probe was added. After overnight hybridization at 42°C, the blot was washed twice for 5 min at 42°C with 2 \times SSC/0.1% SDS and twice for 5 min at 42°C with 0.1 \times SSC/0.1% SDS. The signals were captured by Typhoon FLA 9500 (GE Healthcare Life Sciences) and quantified using ImageQuant.

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Disclosure of potential conflicts of interest

No potential conflict of interest was reported by the authors.

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