MicroRNA

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An Aptamer Targeting the Apical-Loop Domain Modulates pri-miRNA Processing**

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MicroRNAs (miRNAs) are short noncoding RNAs that recognize complementary bases on target mRNAs, thereby triggering either inhibition of translation initiation or mRNA degradation.[1,2] They have unique expression patterns and are involved in almost every important biological process, including cell proliferation, differentiation, and apoptosis.[3] In turn, deregulation of miRNA expression patterns is a key condition in the onset and progression of tumor development.[4,5]

Following the synthesis of the primary transcript (pri-miRNA), the maturation process of miRNAs comprises several steps.[6] First, the pri-miRNA is hydrolyzed by the microprocessor complex, consisting of Drosha/DGCR8, to release hairpin-shaped precursor RNAs (pre-miRNAs). Subsequently, the pre-miRNA is exported into the cytoplasm and further processed by the type III ribonuclease Dicer to produce mature miRNAs.

Owing to the prominent role of miRNAs in regulating gene expression, considerable efforts have been made to develop selective tools that will allow the direct targeting of miRNAs affecting either their biogenesis or function. One such class of tools is represented by the so-called antagomirs, short single-stranded 2′-methoxy-modified oligonucleotides.[7] Antagomirs recognize mature miRNAs by complementary bases, thereby preventing miRNA–mRNA association. Here, we introduce another class of nucleic acid based molecular tools to interfere with miRNA activity, namely, RNA aptamers that specifically recognize the loop domains of a pri-miRNA and modulate its processing.

Aptamers are short single-stranded nucleic acids that fold into well-defined three-dimensional structures that facilitate specific target recognition.[8,9] Aptamers can be isolated by an in vitro selection process, and a wide variety of target molecules, such as proteins, cells, small molecules, and nucleic acids have been already applied for aptamer identification. Especially in the latter case, the interaction between the aptamer and the target RNA has been proven to rely on fitting three-dimensional shapes, going beyond mere recognition through complementary base pairing.[10–13]

We sought to elucidate whether RNA aptamers could be used as an alternative nucleic acid based molecular tool to specifically interfere with the biogenesis of individual miRNAs. Here we describe the isolation and characterization of an RNA aptamer that specifically targets the pri-miRNA polycistron 17 ~ 18a ~ 19a ~ 20a ~ 19b-1 ~ 92. We show that the aptamer binds inter alia to the apical-loop domain of pri-miR18a and thereby inhibits the biogenesis of all miRNAs 17-19b-1 within this cluster. Our results show that aptamers can be applied as agents that modulate pri-miRNA processing and as tools for elucidating mechanisms of this process. Furthermore, the ability to modulate the maturation of miRNA by targeting the apical-loop domain supports the importance of these domains during pri-miRNA processing.

To obtain aptamers that specifically target pri-miR17 ~ 18a ~ 19a ~ 20a ~ 19b-1 ~ 92, we applied an in vitro selection scheme in which the 791 nucleotide (nt) miRNA polycistron comprising *pri*-miR17 ~ 18a ~ 19a ~ 20a ~ 19b-1 was biotinylated at its 5′-end and immobilized on streptavidin-coated magnetic beads (Figure 1).[14,15] The beads were incubated with an RNA library comprising a 25 nt random region. To avoid the participation of the constant regions of the RNA library in pri-miRNA binding, we sequestered these regions by hybridization to complementary oligodeoxynucleotides (ODNs), leaving the random nucleotides free for independent folding. After removal of all unbound RNA sequences, the retained RNAs were eluted by adding EDTA. This step essentially favors the release of those molecules that require Mg^{2+} ions for RNA binding over those that exclusively rely on complementary base pairing. After seven selection cycles, enhanced pri-miRNA binding was detectable, which could be further improved by five succeeding cycles of selection and amplification (Figure S1 A in the Supporting Information).

The RNA library obtained from selection cycle 12 was cloned and sequenced. Amongst 17 analyzed sequences, nine revealed the consensus motif I, 5′-AACACCUC, comple-

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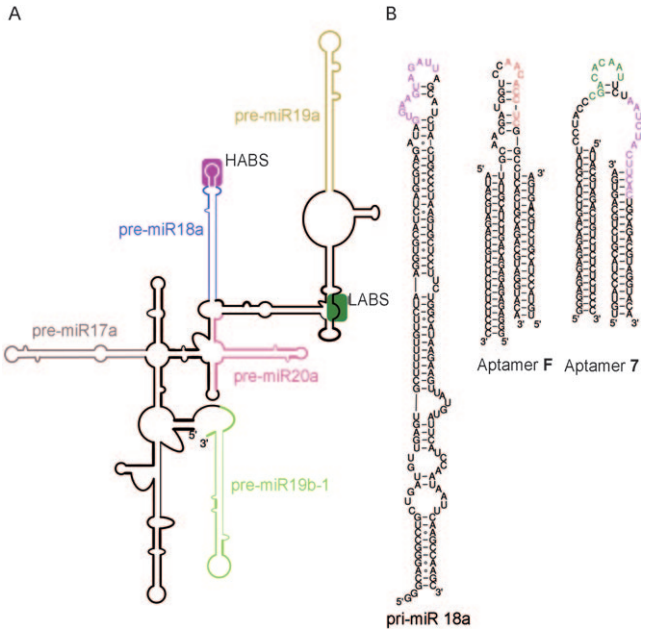

Figure 1. A) Secondary structure of the pri-miR17~18a~19a~20a-~19b-1 cluster; the colors indicate the different pre-miRNA domains. B) The pri-miR18a and the selected aptamers **7** and **F** (including the hybridized blocking oligonucleotides). The highlighted nucleotides of **F** and **7** in (B) bind the pre-miRNA shown in (A). Domains in the pri-miRNA targeted by aptamer **7** are highlighted by colored boxes in (A). HABS: high-affinity binding site (magenta); LABS: low-affinity binding site (green). The magenta nucleotides in (B) of the pri-miR18a interact with high affinity with aptamer **7**.

mentary to a region of the pre-miRNA cluster located at its very 5′-end (Figure S2 in the Supporting Information). Two other motifs, motif II (5′-AUCGACAC) and motif III (5′-CCGCAC), which were complementary to regions located outside of pre-miRNA coding sequences, similarly to motif I (Figure S2), were also enriched. Sequence **15** bears two motifs, motifs I and II. In addition, two orphan sequences were identified, namely aptamer **7** and aptamer **17**; the former comprises the sequence 5′-AUCUAC complementary to the apical-loop domain of pri-miRNA18a and the sequence 5′-CGACACAAU complementary to a region located between pri-miR19a and pri-miR20a (Figure 1 and Figure S2 in the Supporting Information), which thus indicates two potential binding sites. Aptamer **17** did not bear any obvious sequence complementarity to sites in the target pri-miRNA and was not analyzed further.

Surface plasmon resonance (SPR) revealed that binding to the pri-miRNA cluster occurred with a dissociation constant of 9 nM for aptamer **7**, whereas the motif I representative aptamer **F** bound with a K_D of 13 nM (Table 1 and Figure S3 in the Supporting Information). Consistent with the selection procedure, the interaction of **7** with the pri-miRNA strongly depends on the presence of Mg^{2+} ions and the complementary oligodeoxynucleotides used during the selection (Table S1 A in the Supporting Information). Furthermore, **7** also showed remarkable, albeit 20-fold decreased, affinity towards the isolated pri-miR18a domain, whereas the RNA construct pri-miR18a~19a, bearing both potential

Table 1: Dissociation constants of aptamer **7** in the presence of blocking ODNs determined using surface plasmon resonance analysis.[a]

pri-miRNA	k_a [$M^{-1}s^{-1}$]	k_d [s^{-1}]	K_D [M]	Chi2
pri-miR17~18~19a~20~19b-1	6.4×10^4	1.2×10^{-3}	9×10^{-9}	4
pri-miR18a~19a	2.8×10^4	1.1×10^{-3}	40×10^{-9}	2
pri-miR18a mut~19a	1.7×10^3	5.7×10^{-4}	–[a]	–[a]
pri-miR18a~19a mut	3.3×10^4	1.3×10^{-3}	40×10^{-9}	6

[a] K_D values cannot be determined because of very low off rates (k_d) and a nonsaturated curve.

binding sites, revealed a dissociation constant of 40 nM (Table 1).

The isolate pri-miR19a, comprising the second binding site, was also recognized by the aptamer, but the affinity was considerably lower than that of the pri-miR18a~19a construct (Table S1 B in the Supporting Information). To further locate the binding regions of **7** we performed interaction studies with pri-miR18a apical-loop domain mutants as well as with mutants of the pri-miR18a~19a molecule (Figures S4 and S6 in the Supporting Information). The apical-loop domain mutants were thought to have a rather marginal, if any, influence on the formation of the extended stem structure; this was confirmed by secondary-structure determination by mfold.[16] None of the mutants of the pri-miR18a apical-loop domain revealed measurable interaction with aptamer **7**, indicating that the wild-type apical-loop region is indeed the interaction domain of this aptamer (Figures S3 and S4 in the Supporting Information).

This finding is supported by SPR studies with the pri-miR18a~19a construct in which the putative second binding domain has been mutated (pri-miR18a~19a mut) and which still displays high affinity binding (40 nM, Table 1). In contrast, mutating the apical-loop of the pri-miR18a within the pri-miR18a~19a molecule (pri-miR18a mut~19a) induces a loss of affinity (Table 1). These data indicate that aptamer **7** possesses two potential interaction sites: the apical-loop domain of pri-miR18a represents a high-affinity binding site (HABS), whereas the region located between pri-miR19a and pri-miR20a constitutes a low-affinity binding site (LABS; Figure 1 A).

These findings were further verified by footprinting analyses. We performed chemical probing experiments of pri-mi18a in the presence and absence of aptamer **7** and with aptamer **F** as a control.[17] As shown in Figure 2 and Figure S5 in the Supporting Information, the presence of **7** results in the protection of the apical-loop domain modification by the chemical probes whereas **F** had no effect. The two studies—the interaction analysis with the apical-loop domain mutants and the footprinting analysis—support the conclusion that aptamer **7** recognizes the apical-loop domain of pri-miR18a.

Recently, evidence has emerged that conserved apical-loop domains play an important if not essential role during miRNA maturation.[18] Moreover, 2′-methoxy-modified oligonucleotides perfectly complementary to conserved pri-miRNA terminal loops (termed looptomiRs) were shown to inhibit the Drosha-mediated processing of the targeted miRNAs. We therefore analyzed whether aptamer **7** influen-

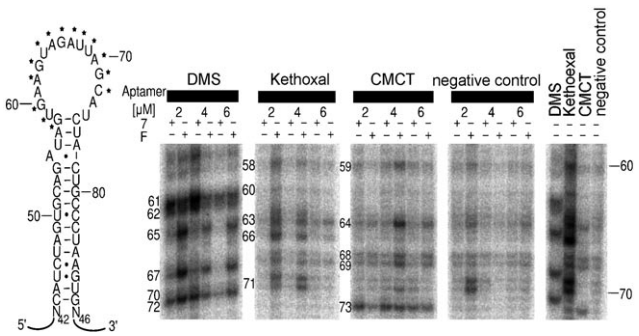

Figure 2. Footprinting analysis of the binding of aptamer **7** on pri-miR18a. Chemical probing was conducted in the presence of **7** or **F** at the indicated concentrations. Left panel: Representation of the pri-miR18a apical-loop domain. The sites that are protected by binding to aptamer **7** are indicated by dots. Middle panels: Sections representing the apical-loop domain of pri-miR18a visualized by polyacrylamide denaturing gel analysis and autoradiography after chemical probing assays with chemicals DMS (dimethyl sulfate), kethoxal, and CMCT (1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide-metho-*p*-toluolsulfonate) in the presence of either **7** or **F** (2 μM, 4 μM, and 6 μM) at increasing concentrations. The negative control was conducted with aptamers and without chemicals. Right panel: Chemical probing in the absence of **7** and **F**.

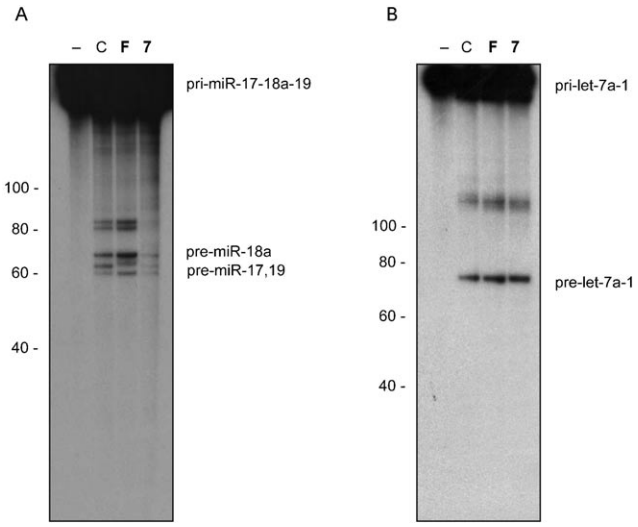

Figure 3. A) In vitro processing of pri-miR-17-18a-19a (pri-miR-17-19). Radioactive labeled primary microRNAs (100×10^3 cpm) were incubated in the presence of HeLa nuclear extracts. RNA substrates were incubated in the presence of aptamers **7** or the control aptamer **F** (6 μM). Lane (–) shows negative controls with no extract added and lane (C) shows the control reaction in the absence of aptamers but in the presence of extract. Products were analyzed on an 8% polyacrylamide gel. Numbers at the left-hand side of the panel were derived from an RNA size marker. B) In vitro processing of pri-let-7a-1. Radioactive labeled primary RNA sequences (100×10^3 cpm) were incubated in HeLa nuclear extracts. RNA substrates were co-incubated with the aptamers **7** (6 μM) or **F** (6 μM). Lane (C) shows HeLa extract incubation control with no aptamers added. Lane (–) shows negative controls with no extract added. The analysis conditions are the same as that in (A).

ces the processing of pri-miR18a. We found that aptamer **7** inhibits the formation of pre-miR18a in a concentration-dependent manner without affecting the processing of pri-Let-7a-1. Interestingly, addition of this aptamer also inhibits the processing of both pri-miR17 and pri-miR-19 (Figure 3). This could reflect a role for this aptamer in altering the correct two- and three-dimensional architecture of the whole cluster and may be caused by additional interaction with the low-affinity binding site located in close proximity to pri-miR19a.

Intriguingly, looptomiR against pri-miR18a was shown to inhibit pre-miR18a production, at the same time slightly increasing the processing of both pri-miR17 and pri-miR-19.[18] This most likely reflects mechanistic differences on how these two reagents differentially affect miRNA processing.

It should not be disregarded that the addition of artificial RNAs (aptamer **F**) causes a nonspecific enhanced processing of pri-miR18a (Figure 3A). However, increasing concentrations of aptamer **7** clearly resulted in an inhibition of miRNA processing to background levels, whereas the control aptamer **F** had no inhibitory effect (Figure 4). These data underline the efficiency of aptamer **7** for pri-miR18a recognition and inhibition of microRNA processing.

It has been shown previously that hnRNP A1 associates with the apical-loop domain of pri-miR18a and promotes its processing.[18] To further elucidate the effects of aptamer **7** on miRNA processing, we investigated whether this aptamer can compete with hnRNP A1 in binding to the apical-loop domain. RNA chromatography experiments revealed that aptamer **7** reduces the amount of hnRNP A1 protein bound to pri-miR18a, whereas the control aptamer **F** had almost no effect (Figure 5). Notably, the association of hnRNP A1 with the control pri-miRNA pri-let7a-1 was not affected by the aptamer, thus demonstrating the specificity of this aptamer.[19]

In conclusion, we have identified an RNA aptamer that mainly interacts with the apical-loop domain of pri-miR18a

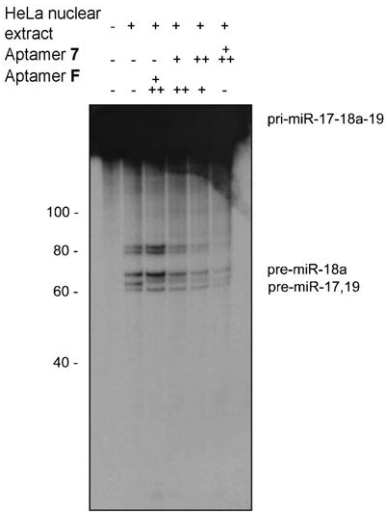

Figure 4. In vitro processing of pri-miR-17-18a-19a (pri-miR-17-19). Radioactive labeled primary microRNAs (100×10^3 cpm) were incubated in the presence of HeLa nuclear extracts. RNA substrates were incubated in the presence of aptamers **7** and the control aptamer **F** (+: 1.5 μM, ++: 3 μM, +++: 6 μM). The first lane from left shows negative controls with no extract added. The second lane shows the control reaction in the absence of aptamers but in the presence of extract. Products were analyzed on an 8% polyacrylamide gel. Numbers at the left-hand side of the panel were derived from an RNA size marker.

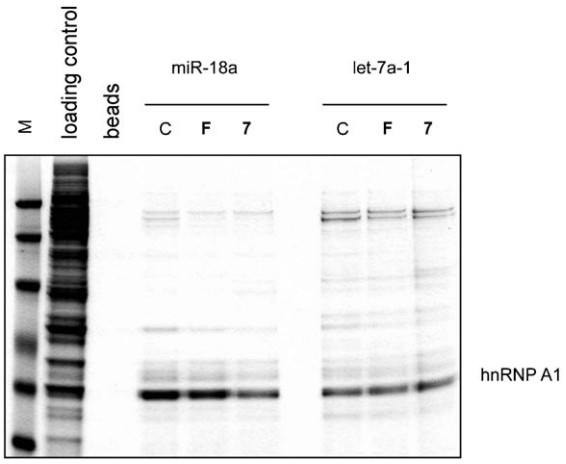

Figure 5. Aptamer **7** specifically displaces hnRNPA1 from the miR-18a terminal loop. RNA chromatography of pri-miR-18a and pri-let-7a-1 terminal loops performed in HeLa nuclear extracts in the presence of aptamer **F** (6 μM), aptamer **7** (6 μM), or without the addition of any aptamer (C). Samples resulting from RNA chromatography were resolved on SDS gel and visualized using GelCodeBlue (Pierce). The hnRNPA1 protein was identified by mass spectrometry analysis.

within a pri-miRNA cluster. Mutational studies together with chemical footprinting analyses and RNA chromatography verified the binding site of the aptamer. More importantly, we demonstrate that the application of the aptamer in vitro interferes with accurate miRNA maturation and its function is distinct from that of previously described antagomirs and looptomiRs. This is the first study to show that aptamers can be used as efficient tools to interfere with miRNA processing and have an impact on miRNA function. This approach opens a route for the generation of novel specific regimens to address miRNA processing and maturation especially at an early stage of the processing pathway. It will be of utmost interest to determine how the in vitro data presented here translate into a complex cellular environment. Moreover, our data show that small structured RNA motifs are, in principle, capable of modulating pri-miRNA processing specifically. At this stage, there is no reason to exclude the possibility that natural RNA motifs exist which harness similar mechanisms for regulating pri-miRNA processing. We are currently setting up genomic SELEX experiments to explore this hypothesis.

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