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Antagomirzymes: Oligonucleotide Enzymes That Specifically Silence MicroRNA Function**

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MicroRNAs (miRNAs) are a recently discovered class of small (ca. 22 nucleotides in length), single-stranded non-coding RNA molecules with enormous regulatory potential.[1,2] The miRNAs bind to transcripts with near-perfect complementarity into the 3′ untranslated regions (UTRs) and mediate posttranscriptional gene silencing.[3] These miRNAs are now thought to be crucial regulators of gene expression and to influence a large spectrum of physiological and pathological processes ranging from host–pathogen interaction[4] to cell differentiation and oncogenesis.[5] The relationship between abnormalities in miRNA expression and several diseases, notably cancers, has been well established.[6] These small RNA molecules are now known to be crucial regulators of important cellular processes. An understanding of the role of miRNAs in fundamental processes associated with diseases such as cancer, chronic infections, and immune disorders may aid in disease diagnosis and prognosis and potentially lead to the identification of new therapeutic targets. However, the precise molecular function of many miRNAs is largely unknown; different types of loss-of-function methods are required to gain insight into miRNA biology. The silencing or deactivating of miRNAs has been an active field of interest, not only for the design of therapeutics, but also as a tool for gaining an understanding of the precise molecular functions of a particular miRNA molecule. Recently, several approaches have been put forward for the specific targeting of miRNAs: The reverse complement of modified oligonucleotides has been used to block miRNA function in cell-based systems,[7] and oligonucleotides modified with locked nucleic acids (LNAs) have also been used to silence miRNAs in cultured cells.[8] Specialized antisense oligonucleotides against miRNA molecules termed "antagomirs" have also been developed.[9,10] Herein, we establish oligonucleotide enzymes, antagomirzymes, as valuable tools for the specific knockdown of miRNA in vitro and in vivo.

DNAzymes are synthetic DNA analogues of catalytic ribozyme motifs with the potential to cleave practically any RNA substrate in a sequence-specific manner.[11–13] The 10–23 DNAzyme has a central conserved motif containing the catalytic core and two variable flanking regions on either end of the core that are complementary to the RNA-substrate sequence. The Watson–Crick base pairing of the variable flanking regions confers specificity and selectivity of substrate recognition, whereas the catalytic core determines the cleavage activity. The 10–23 DNAzyme cleaves RNA between any purine–pyrimidine (R–Y) junction in the presence of divalent cations. These features prompted us to apply DNAzymes to the silencing of miRNA. Herein, we show that this class of enzymes, referred to as "antagomirzymes", are efficient and specific miRNA silencers in vitro and in vivo. Two miRNAs were silenced, the human miRNAs hsa-miR-372 and hsa-miR-373, which are known to be specifically dysregulated and directly involved in the patho-

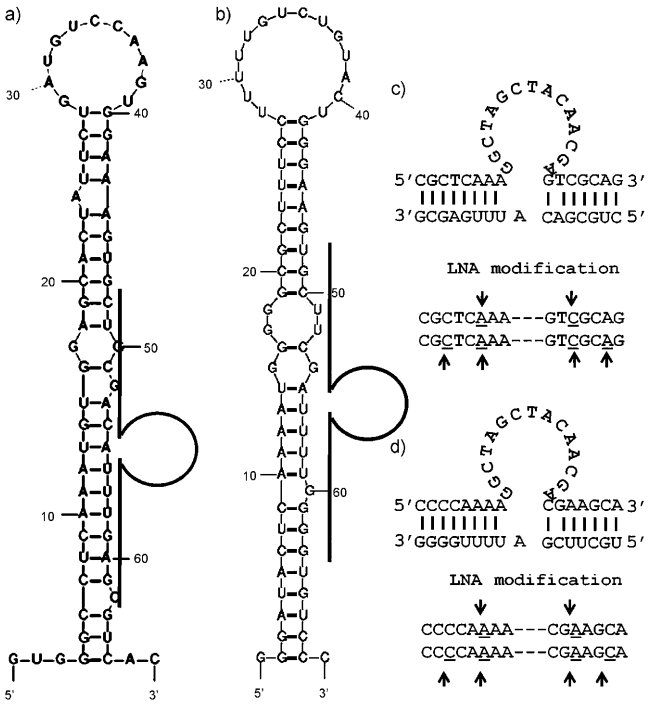

Figure 1. The design of DNA and LNA antagomirzymes. The secondary structures of hairpin precursors of hsa-miR-372 (a) and hsa-miR-373 (b) are presented on the left-hand side. The regions targeted by the DNAzyme/LNAzyme are marked by straight lines joined by a curve. The regions of hsa-miR-372 (c) and hsa-miR-373 (d) that are targeted by the antagomirzymes are magnified on the right-hand side: The bottom strand depicts the miRNA sequence, and the top strand represents the DNA or LNA antagomirzyme. The LNA antagomirzymes were designed to include two or four LNA modifications, which are represented by underlined nucleotides in the binding arms shown below the respective miRNA–antagomirzyme interaction.

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genesis of germ-cell tumors.[14] We also show that LNA modifications in the variable flanking regions of the DNAzyme enhance its activity.

The design and sequences of DNA and LNA antagomirzymes are shown in Figure 1. The catalytic domain was chosen on the basis of the previously well-studied 10–23 DNAzyme sequence motif,[15] whereas flanking arms were designed to be complementary to the miRNA region of precursors (Figure 1). Precursor miRNAs were chosen, as such a targeting strategy offered the advantage that it would be possible to cleave both the precursor and mature miRNA sequences simultaneously. The 30-mer DNA and LNA antagomirzymes comprised a central 15-nucleotide catalytic domain (5′-GGCTAGCTACAACGA-3′) flanked by eight- and seven-nucleotide arms (Figure 1). A sequence in which the catalytic domain was shuffled was also used as a negative control. LNA modifications were incorporated in the variable flanking regions complementary to the substrate RNA to enhance substrate-binding affinity and thus the catalytic activity of the enzymes.[16] Two and four LNA modifications were introduced into the binding arms. Thus, for each miRNA, one DNA antagomirzyme (DZ) and two LNA antagomirzymes (LZ2 and LZ4) were evaluated in vitro and in cultured cells.

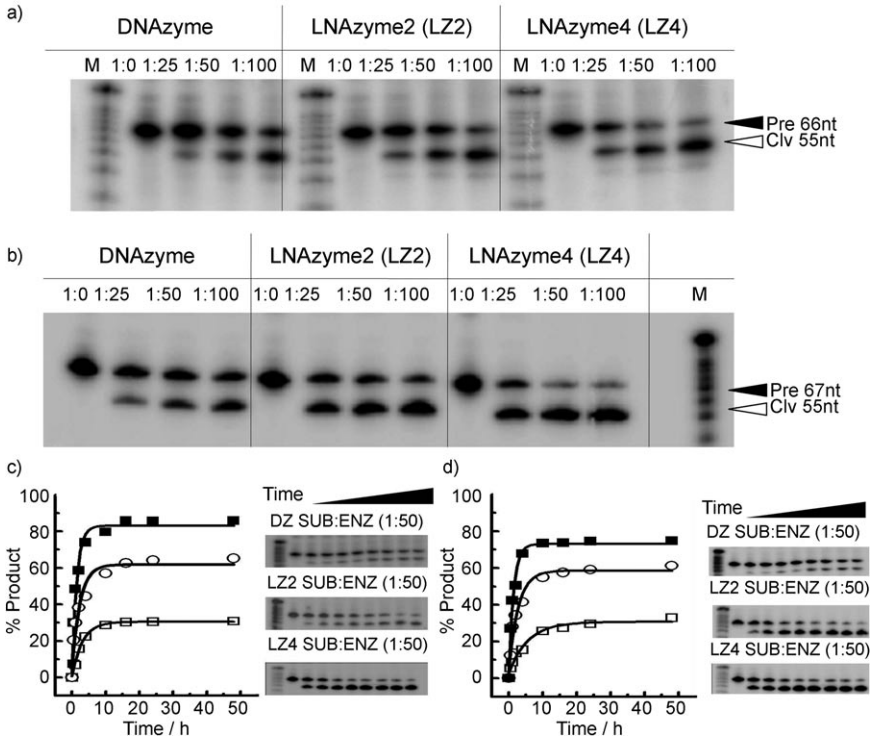

Figure 2. In vitro cleavage of a) pre-miR-372 and b) pre-miR-373 by DNA and LNA antagomirzymes. RNA labeled at the 5′ end (20 nmol) was incubated with a DNAzyme or LNAzyme (20 nmol–2 μmol) for 2 h. The antagomirzymes designed to target a specific miRNA site cleaved the miRNA primarily at the expected site to yield a 5′-end-labeled fragment of 55 nucleotides (unfilled arrow). Substrate bands of 66 and 67 nucleotides for pre-miR-372 and pre-miR-373, respectively, are shown by filled arrows. M = Marker. c,d) Kinetics of cleavage of hsa-mir-372 (c) and hsa-mir-373 (d) by DNA- and LNAzymes. End-labeled RNA was incubated with antagomirzymes in a 1:50 ratio (i.e. 20 nmol:1 μmol) for 48 h, and an aliquot of the reaction mixture was taken at each time point indicated (open rectangles: DNAzyme, ellipses: LZ2, filled rectangles: LZ4). The cleavage efficiencies, (cleaved RNA/uncleaved RNA) 100%, were calculated, and kinetic parameters were calculated after fitting of the data with an exponential decay function.

Initial in vitro characterization showed that all three antagomirzymes (DNA and LNA) designed for each miRNA molecule were capable of cleaving the specific target miRNA in vitro. Figure 2a and b show the cleavage of target miRNA by each enzyme, and the expected cleavage products are matched with the observed fragment sizes after cleavage. There was no cleavage of miRNAs observed in the control reactions in which the catalytic domain was shuffled (see Figure SI-1 A in the Supporting Information). These observations indicate that an intact catalytic domain is necessary for cleavage activity. Under single-turnover conditions with RNA/enzyme ratios of 1:25, 1:50, and 1:100, much more efficient cleavage was observed with the LNA antagomirzymes than with the unmodified DNA antagomirzyme (see Figure SI-1 B in the Supporting Information). Moderate cleavage (50–60%) was observed with the DNA antagomirzyme when present in 100-fold molar excess, whereas the two LNA antagomirzymes cleaved greater than 75% of the RNA substrate under the same conditions. There was no marked improvement when the degree of modification of the arms of the enzymes was increased (see Figure SI-1 B in the Supporting Information). Kinetic analysis under single-turnover conditions with an RNA/enzyme ratio of 1:50 showed that both the efficiency of cleavage and the rate of cleavage were higher for the LNA antagomirzymes than that for the DNA antagomirzyme (Figure 2c; see also Table SI-1 in the Supporting Information).

To check the ability of oligonucleotide enzymes to cleave miRNA in vivo, we cloned precursors of hsa-miR-372 and hsa-miR-373 into pSilencer 4.1 vectors (pSilencer-miR-372/3) and transfected these plasmid vectors transiently into HEK-293 cells (Figure 3a). First, we verified the expression of both miR-372 and miR-373 by pSilencer-miR-372 and pSilencer-miR-373 vectors, respectively, and then compared the results with the level of expression of miRNAs when these vectors were cotransfected with oligonucleotide enzymes (100 nmol) by northern blot analysis (Figure 3b). In all cases, miRNA-cleavage activity was observed. However, better cleavage activities were found for LNA antagomirzymes than for the respective DNA antagomirzyme.

The effective use of this technique for selective miRNA cleavage is possible only if the technique is efficient in cell-culture systems or animal models. We used a known target of hsa-miR-372 and hsa-miR-373 to monitor the efficacy of the

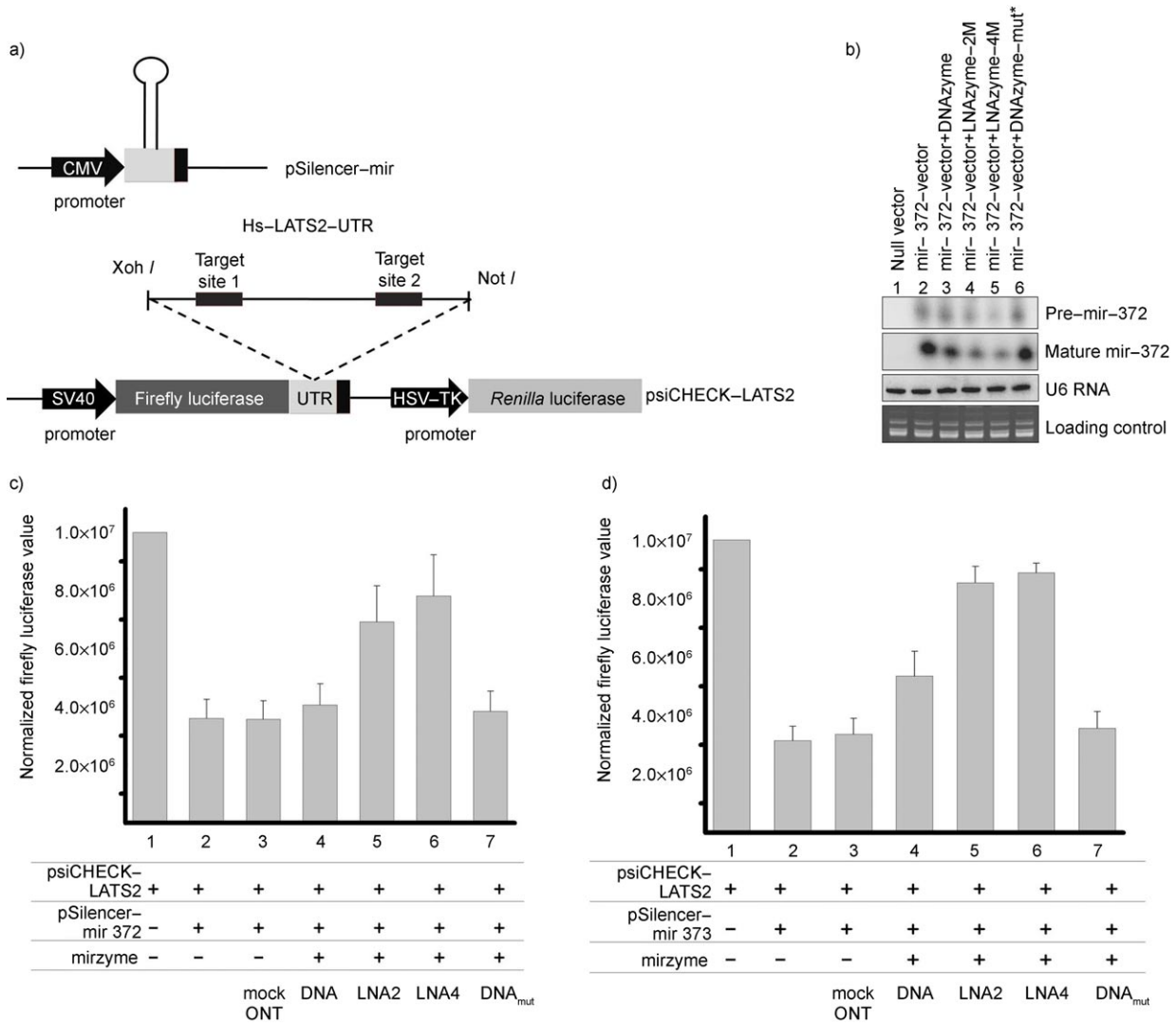

Figure 3. a) The plasmid vectors used in the study. For the expression of microRNAs, pre-hsa-miR-372 or pre-hsa-miR-373 (pSilencer-mir-372/3) was cloned into the vector pSilencer 4.1 (Ambion). The dual-luciferase reporter vector psiCHECK-2 (Promega) was used to clone the target site, LATS2 3′ UTR (psiCHECK-LATS2). b) In vivo cleavage of microRNA by DNA and LNA antagomirzymes. HEK-293 cells were cotransfected with pSilencer-mir-372/3 and a DNA or LNA antagomirzyme (100 nmol), as indicated. pSilencer 4.1 neo was used as a negative control. After 36 h, the total RNA (20 μg) was isolated and expression was checked by northern blot analysis. c,d) Dual-luciferase reporter assay for validating the in vivo function of DNA/LNAzymes. The indicated vectors and DNA or LNA antagomirzymes were transfected into the HEK-293 cell line. The relative firefly luciferase levels (normalized to a *Renilla* control and compared to psiCHECK-LATS2) are shown. Error bars represent the standard deviation for three independent experiments.

oligonucleotide enzymes in cultured cells. The LATS2 gene has been shown previously to be a direct target of hsa-miR-372 and hsa-miR-373. The oligonucleotide enzymes were transiently cotransfected with two plasmids that encode the miRNA and its corresponding target in the 3′ UTR of LATS2 cloned downstream of the luciferase reporter gene into HEK-293 cells (Figure 3c,d). The expression level of the reporter LATS2 UTR fusion construct (column 1 in Figure 3c,d) was decreased by 65–70% in the presence of the clones expressing miRNA 372 and 373 (column 2 in Figure 3c,d). Down regulation of the reporter gene was observed in both cases, which indicates that miRNAs bind to the target and suppress the expression of the target gene by a factor of up to 2.7 (P value > 0.005, Student t test). We tested the effect of the enzymes by cotransfecting them with the reporter LATS2 UTR and the miRNA-overexpression construct (columns 4–6). The expression of the target gene was restored marginally by the DNA antagomirzyme against hsa-miR-372 and hsa-miR-373. However, the LNA antagomirzymes LZ2 and LZ4 against hsa-miR-372 restored the reporter level to 65 and 80%, respectively. Similarly, the LNA antagomirzymes LZ2 and LZ4 against hsa-miR-373 restored the reporter level to 90 (P value > 0.005, Student t test; columns 4–6 in Figure 3c) and 95% (P value > 0.005, Student t test; columns 4–6 in Figure 3d) of the level in the absence of the miRNA. Suppressed expression was observed when nucleic acid enzymes were replaced either by mock oligonucleotides (ONTs) or by the oligonucleotides in which bases in the catalytic domain had been shuffled (columns 3 and 7 in Figure 3c,d). The reduction in the steady-state level of the

miRNA and the resulting increase in target expression clearly shows that the antagomirzymes can deregulate targets by specifically cleaving regulatory miRNAs. Furthermore, silencing activities were enhanced in the case of LNAzymes with two and four additional LNA modifications.

In conclusion, we have demonstrated that oligonucleotide enzymes, antagomirzymes, can be designed to cleave miRNAs effectively and specifically and thus silence their respective functions both in vitro and in vivo. This technique can be used effectively to study biological functions of specific miRNAs in vivo. It may also find application in therapeutics as a method for the specific silencing of miRNAs and inhibition of the pathophysiological processes caused by the overexpression of miRNAs. The very high concentrations of antagomirzymes required in the present study for in vivo activity could be a limiting factor for such applications. However, this limitation could be overcome by targeting efficient cleavage sites, such as loop regions of pre-miRNA. Another potential area of application is the design of synthetic oligonucleotide switches for protein-free modules constructed by synthetic biology.

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[1] a) L. He, G. J. Hannon, *Nat. Rev. Genet.* **2004**, *5*, 522; b) P. D. Zamore, B. Haley, *Science* **2005**, *309*, 1519; c) C. Zhang, *Physiol. Genomics* **2008**, *33*, 139; d) W. Filipowicz, S. N. Bhattacharyya, N. Sonenberg, *Nat. Rev. Genet.* **2008**, *9*, 102.
[2] V. N. Kim, *Nat. Rev. Mol. Cell Biol.* **2005**, *6*, 376.
[3] K. Chen, N. Rajewsky, *Nat. Rev. Genet.* **2007**, *8*, 93.
[4] a) V. Scaria, M. Hariharan, S. Maiti, B. Pillai, S. K. Brahmachari, *Retrovirology* **2006**, *3*, 68; b) V. Scaria, M. Hariharan, B. Pillai, S. Maiti, S. K. Brahmachari, *Cell Microbiol.* **2007**, *9*, 2784; c) V. Scaria, M. Hariharan, S. K. Brahmachari, S. Maiti, B. Pillai, *ChemMedChem* **2007**, *2*, 789; d) M. Hariharan, V. Scaria, B. Pillai, S. K. Brahmachari, *Biochem. Biophys. Res. Commun.* **2005**, *337*, 1214; e) V. Scaria, V. Jadhav, *Retrovirology* **2007**, *4*, 82.
[5] G. A. Calin, C. M. Croce, *Nat. Rev. Cancer* **2006**, *6*, 857.
[6] a) P. Y. Chen, G. Meister, *Biol. Chem.* **2005**, *386*, 1205; b) C. Sevignani, G. A. Calin, L. D. Siracusa, C. M. Croce, *Mamm. Genome* **2006**, *17*, 189; c) M. Jovanovic, M. O. Hengartner, *Oncogene* **2006**, *25*, 6176; d) H. S. Soifer, J. J. Rossi, P. Saetrom, *Mol. Ther.* **2007**, *15*, 2070.
[7] J. Weiler, J. Hunziker, J. Hall, *Gene Ther.* **2006**, *13*, 496.
[8] J. Elmén, M. Lindow, S. Schütz, M. Lawrence, A. Petri, S. Obad, M. Lindholm, M. Hedtjärn, H. F. Hansen, U. Berger, S. Gullans, P. Kearney, P. Sarnow, E. M. Straarup, S. Kauppinen, *Nature* **2008**, *452*, 896.
[9] J. Krützfeldt, N. Rajewsky, R. Braich, K. G. Rajeev, T. Tuschl, M. Manoharan, M. Stoffel, *Nature* **2005**, *438*, 685 – 689.
[10] J. Krützfeldt, S. Kuwajima, R. Braich, K. G. Rajeev, J. Pena, T. Tuschl, M. Manoharan, M. Stoffel, *Nucleic Acids Res.* **2007**, *35*, 2885.
[11] J. C. Achenbach, W. Chiuman, R. P. Cruz, Y. Li, *Curr. Pharm. Biotechnol.* **2004**, *5*, 321.
[12] D. A. Baum, S. K. Silverman, *Cell. Mol. Life Sci.* **2008**, *65*, 2156.
[13] W. H. Pan, G. A. Clawson, *Expert Opin. Biol. Ther.* **2008**, *8*, 1071.
[14] P. M. Voorhoeve, C. le Sage, M. Schrier, A. J. Gillis, H. Stoop, R. Nagel, Y. P. Liu, J. Van Duijse, J. Drost, A. Griekspoor, E. Zlotorynski, N. Yabuta, G. De Vita, H. Nojima, L. H. Looijenga, R. Agami, *Cell* **2006**, *124*, 1169.
[15] S. W. Santoro, G. F. Joyce, *Proc. Natl. Acad. Sci. USA* **1997**, *94*, 4262.
[16] a) B. Vester, L. B. Lundberg, M. D. Sørensen, B. R. Babu, S. Douthwaite, J. Wengel, *J. Am. Chem. Soc.* **2002**, *124*, 13682; b) J. Wengel, B. Vester, L. B. Lundberg, S. Douthwaite, M. D. Sørensen, B. R. Babu, M. J. Gait, A. Arzumanov, M. Petersen, J. T. Nielsen, *Nucleosides Nucleotides Nucleic Acids* **2003**, *22*, 601; c) B. Vester, L. B. Lundberg, M. D. Sørensen, B. R. Babu, S. Douthwaite, J. Wengel, *Biochem. Soc. Trans.* **2004**, *32*, 37; d) H. Kaur, B. R. Babu, S. Maiti, *Chem. Rev.* **2007**, *107*, 4672.

