Supplementary Material (ESI) for Molecular BioSystems
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# Modulation of microRNA Function by Synthetic Ribozymes

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## Supplementary Data

## Methods and Materials

### Design and modifications of the hammerhead ribozyme

The Ribozyme was primarily designed according to the rules of hammerhead ribozyme design suggested by Haseloff *et al.*[1] The hammerhead ribozyme designed was comprised of 38 nucleotides, including 22nt catalytic core and 16nt (7nt in its 5' hybridizing arm and 9nt in 3' arm) flanking antisense sequence (Fig. 1). The catalytic domain of anti-miR-21 ribozyme was chosen on the basis of the previously well-studied hammerhead ribozyme sequence motif, whereas flanking arms were designed to be complementary to the miRNA region of precursors. The cleavage site was selected such that the ribozyme can simultaneously cleave both mature and precursor miRNA. The sequences of wild type (Wt R) and modified (Mod R) ribozymes designed to target pre-miR-21 were 5'-UCAGUCUCUGAUGAGUCCGUGAGGACGAAAUAAGCUAC-3' and 5'-uca gucucUGAUGaguccgugaggacGaaAuaagcuacX-3' respectively. The *uppercase* depicts ribonucleotides, *lowercase* signifies 2'-O-methyl nucleotides, 'U' represents 2'-amino nucleotides and 'X' designates 3'-3' inverted T. These modifications provide nuclease resistance to ribozyme motif without hindering its catalytic activity.[2] The sequence of scrambled ribozyme used as negative control (Scr R) was 5'-UCAGUCUGUAGAGUGCCAGUGCACGAUAGAUAAGCUAC-3'. The chosen target site for cleavage in pre-miRNA-21 sequence 5' UGUCGGGUAGCUUAUC*AG ACUGAUGUUGACUGUUGAAUCUCAUGGCAACACCAGUCGAUGGGCUGUCUGACA 3' (miRNA ID:

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hsa-mir-21, http://www.mirbase.org) is shown by asterisk. All the ribozymes were PAGE purified and procured from Ocimum biosolutions.

### *In vitro* transcription of pre-miR-21

The pre-miR-21 RNA substrate was prepared from template oligonucleotides using Megascript® High yield transcription kit (Ambion, Inc. cat # AM1334). Briefly, forward 5'-TAATACGACTCACTATAGGGTGTCGGGTAGCTTATCAGACTGATGTTGACTGTTGAATC-3' and reverse 5'-TGTCAGACAGCCCATCGACTGGTGTTGCCTTGAGATTCAACAGTCAACATCAGTCTG-3' primers, 2 µM each, were subjected to primer extension using Taq polymerase (5 U), dNTPs (0.2 mM), Taq polymerase buffer (1X) and $MgCl_2$ (2 mM). The reaction mixture was denatured by heating at 95° C for 5 minutes followed by snap-chilling on ice for 10 minutes, followed by primer extension incubation at 72° C for 30 minutes. The hybrid template with T7 promoter (underlined) was used for *in vitro* transcription following manufacturer's instructions (Ambion, Inc.). The pre-miR-21 substrate was purified by NucAway™ Spin Columns (Ambion, Inc.) and checked on 12 % denaturing PAGE.

### *In vitro* cleavage reaction

The *in vitro* transcribed pre-miR-21 substrate (72-76 nucleotides) was 5'-$^{32}$P-labeled with KinaseMax™ 5' End-Labeling Kit (Ambion Inc.). The reaction mixture containing 2 µM of pre-miR-21, Kinase buffer (1X), T4 Polynucleotide Kinase (10 U), [$\gamma$-$^{32}$P] ATP (2500 Ci/mmol) was incubated at 37° C for 1 hour. The T4 polynucleotide Kinase was heat inactivated at 95° C for 2 minutes. The end labeled RNA substrate was then purified by NucAway™ Spin Columns (Ambion, Inc.) and checked on 15 % denaturing PAGE.

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The ribozyme and labeled pre-miR-21 substrate were pre-heated separately at 85° C for 5 minutes and mixed together in equal concentration (1 μM each) in ribozyme buffer (100 mM Tris–HCl, pH 7.5, 1 U RNasin) following incubation at 37° C for 30 minutes. The cleavage reaction was started by addition of $MgCl_2$ (final concentration 25 mM). After 2 hours of incubation at 37° C, the reaction was stopped by adding equal amount of Gel Loading Buffer II (Ambion, Inc.) and snap-cooling on ice for 10 minutes. These samples were denatured at 85° C for 5 min, resolved on 15 % denaturing PAGE and analyzed using Typhoon Trio phosphorimager. The 5'-$^{32}$P-labeled Decade™ Marker (Ambion Inc,) was used as a reference to determine the size of RNA substrate and cleavage products.

**Cell culture and Transfection**

MCF-7 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) procured from Sigma-Aldrich® and supplemented with 10% fetal bovine serum, 5000 mg/L glucose, 1X Antibiotic-Antimycotic (Gibco, Invitrogen). The cells were incubated at 37° C in a humidified chamber supplemented with 5% $CO_2$. The MCF-7 cells were seeded ($1x10^6$ cells/well) in 6-well plate and transfected following day with 0.5 μM of each of the ribozymes using Lipofectamine™ 2000 (Invitrogen) using manufacturer's instructions. After 48 hours of transfection, cells were washed with phosphate-buffer saline and total RNA was isolated using TRizol® Reagent (Invitrogen).

**Stem-loop Real-Time PCR**

Expression of mature microRNA-21 was determined by Stem-loop Real Time PCR strategy as previously described.[3,4] The sequence of primers used were,

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Stem-loop RT primer-

5'-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGTCAACATC-3'

Forward Primer-

5'-ACACTCCAGCTGGGTAGCTTATCAGACTGA-3'

Reverse primer-

5'-GTGTCGTGGAGTCGGCAATTC-3'

The microRNA-21 expression was detected using SYBR-green I PCR master mix (Applied biosystems) on Roche Lightcycler LC 480 and normalized with respect to β-2-Microglobin as a reference gene. The Real Time data were analyzed by method described by M. Pfaffl.[5]

**Western Blotting**

Total protein was isolated from MCF-7 cells transfected with appropriate ribozyme using cell lysis RIPA buffer (Pierce Chemical Co., Rockland, IL). Protein concentration was estimated using BCA™ protein assay (Pierce Chemical Co., Rockland, IL). Equivalent amount of protein from each treatment was resolved on 10% SDS-PAGE. Following transfer to nitrocellulose membrane and blocking with 5% nonfat milk, the blot was incubated with primary antibody (1:500) specific for PDCD4 protein (Santa Cruz Biotechnology, Inc.) After washing the blot with 1X TBST, it was probed with Alkaline Phosphatase Conjugated secondary antibody (1:3000 dilutions). The blot was developed using AP developing solution (B genie) followed by densitometric analysis (AlphaEaseFC software, Alpha Innotech Corporation).

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**Cell viability assay**

MCF-7 cells were seeded in 96 well-plates and transfected the following day with 50nM appropriate ribozyme (wild type, modified or scrambled ribozyme) using Lipofectamine™ 2000 (Invitrogen). After 3 days of growth, cells were incubated for 4 hr at 37 °C with 1 mg/ml 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (Sigma-Aldrich). After incubation, supernatants were discarded and formazan crystals were dissolved in DMSO solution. Sample absorbance was measured at 570 nm with a reference wavelength of 630 nm. Data was normalized with untreated control set.

**Statistical Analysis**

Statistical analysis was performed where values were expressed as mean ± S.E. Differences between groups were calculated with Student's *t* test. A *P*-value < 0.05 was defined as significant.

**References**

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