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Antagonism of microRNA Function in Zebrafish Embryos by Using Locked Nucleic Acid Enzymes (LNAzymes)

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MicroRNAs (miRNAs) have crucial functions in many cellular processes, such as differentiation, proliferation and apoptosis; aberrant expression of miRNAs has been linked to human diseases, including cancer. Tools that allow specific and efficient knockdown of miRNAs would be of immense importance for exploring miRNA function. Zebrafish serves as an excellent vertebrate model system to understand the functions of miRNAs involved in a variety of biological processes. We designed and employed a strategy based on locked nucleic acid enzymes (LNAzymes) for in vivo knockdown of miRNA in zebrafish embryos. We demonstrate that LNAzyme can efficiently knockdown miRNAs with minimal toxicity to the zebrafish embryos.

Introduction

MicroRNAs (miRNAs) are small noncoding RNAs of 19–24 nucleotides, and their implication in vast array of biological process, such as development, cell differentiation, senescence makes them a key regulatory factor.[1–3] Typically, miRNA biogenesis starts with the formation of a long primary miRNA (pri-miRNA) transcript that includes a stem-loop precursor (pre-miRNA). Drosha (an RNase III enzyme) and its partner, DGCR8/Pasha, cleave the base of the stem loop, and the pre-miRNA is exported out of nucleus by exportin 5. In the cytoplasm a second RNase III, Dicer, cleaves the pre-miRNA stem-loop to create an miRNA/miRNA* duplex that is loaded onto a RISC complex. Subsequently, miRNA* is selectively degraded, thereby leaving an miRNA:RISC complex (miRISC).[4] Binding of an miRISC complex to the 3′-untranslated region (UTR) of an mRNA silences its expression.[5] To date, more than 1000 human miRNAs have been identified and annotated, and these are predicted to regulate the activity of around 50% of all protein-coding genes.[6] Furthermore, mounting evidence indicates a strong association of aberrant miRNA expression with various diseases, including cancer, heart diseases, and neurological disorders.[1–3,7–9] Thus, tools that allow specific and efficient knockdown of miRNA are of immense importance for exploring miRNA function and for therapeutically silencing disease-associated miRNAs. To this end, antisense techniques have been widely explored to silence miRNA. Different modified anti-miRNA compounds, such as phosphorothorate, 2′-*O*-methyl (2′-OMe), 2′-*O*-methoxyethyl, locked nucleic acid (LNA), and peptide nucleic acid (PNA), have been successfully shown to inhibit miRNAs.[10–13]

Zebrafish (*Danio rerio*) has emerged as a powerful, genetically tractable model for studying different aspects of vertebrate development. It has also been successfully used as a model organism for various human diseases.[14] The major advantage of the zebrafish model is the rapid and ex utero (external) development of transparent embryos, which permits direct access at all developmental stages. It has widely been explored for deciphering the role of miRNA in the regulation of several key biological processes, such as brain morphogenesis, vascular homeostasis, erythropoiesis, and heart development.[15,16] So far, approximately 350 miRNAs have been discovered in zebrafish (miRBase, Release 18; http://microRNA.sanger.ac.uk/sequences), and the functions of many miRNAs have been elucidated by using miRNA overexpression and miRNA knockdown studies. Currently, morpholino (MO), an antisense oligonucleotide with morpholine rings, is the only tool widely used to knockdown miRNA in zebrafish embryos.[17]

Recently we have shown the utility of nucleic acid enzymes (ribozymes, DNA enzymes (DNAzymes), and LNA enzymes (LNAzymes)) as efficient anti-miRNA tools in cell-culture-based systems.[18–20] DNAzymes are antisense oligonucleotides that consist of a catalytic motif along with two variable flanking regions (arms) on either end of the motif. The arms are complementary to the RNA substrate, and the catalytic core cleaves a purine–pyrimidine (RY) junction at the cleavage site of the substrate. DNAzyme has been successfully employed for cellular and in vivo knockdown of target genes.[21–23] It has also been well documented that incorporation of LNAs in both arms of DNAzyme significantly enhances its cleavage activity.[24–28] Here, we report for the first time the use of an LNAzyme for in vivo downregulation of miRNA expression in a zebrafish model. In

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addition, we show that LNAzyme effectively silences miRNA at relatively low concentrations with minimal toxicity in zebrafish embryos.

Results and Discussion

In vitro miRNA cleavage assay

In order to assess the anti-miRNA activity of the LNAzyme tool, we selected three candidate miRNAs: dre-mir-451, dre-mir-144 and dre-mir-21. The roles of dre-mir-451 and dre-mir-144 have been well studied, and the miRNA loss-of-function phenotype is well characterized.[27–29] Thus, these miRNAs provide a phenotype-based assay system to investigate the efficiency of anti-miRNA tools. Dre-mir-21 is known to be expressed in cardiac valves and in the otoliths of ears. No loss-of-function study has yet been reported for dre-mir-21.[30,31] Anti-miRNA DNAzymes/LNAzymes were designed against these candidate miRNAs by using previously described rules for DNAzyme design (Figure 1 and Table S1),[24–26] and pre-miRNA were synthesized with an in vitro transcription kit. The designed LNAzymes were then tested by an in vitro cleavage assay with [^{32}P]ATP-labeled pre-miRNA substrates. Single-turnover in vitro cleavage reactions were performed at 1:10 (pre-miRNA/LNAzyme) molar ratios. Incubation of pre-miRNA with the corresponding LNAzyme in the presence of divalent cation resulted in the cleavage of miRNA to generate a cleaved product of the expected size (from the 5′-end of pre-miRNA to the cleavage site), thereby confirming in vitro anti-miRNA activity (Figure 2).

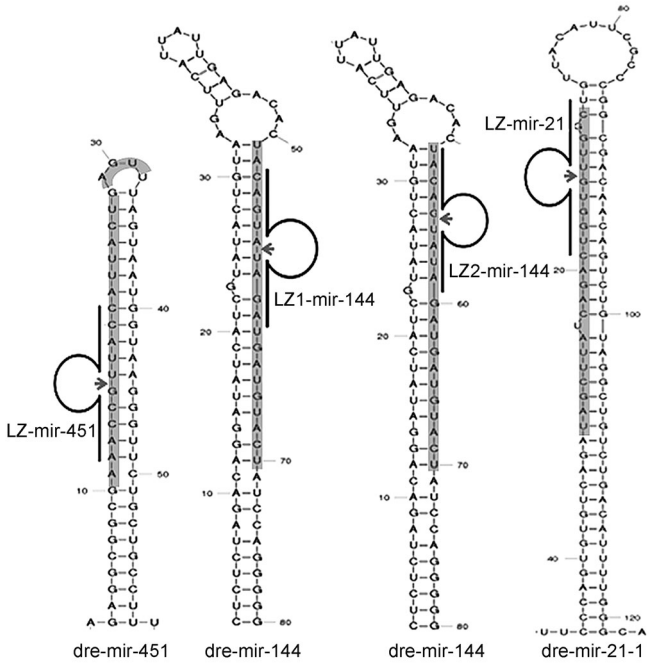

Figure 1. LNAzymes binding to target pre-miRNAs. Mature miRNA sequences are highlighted in gray; arrows indicate cleavage sites.

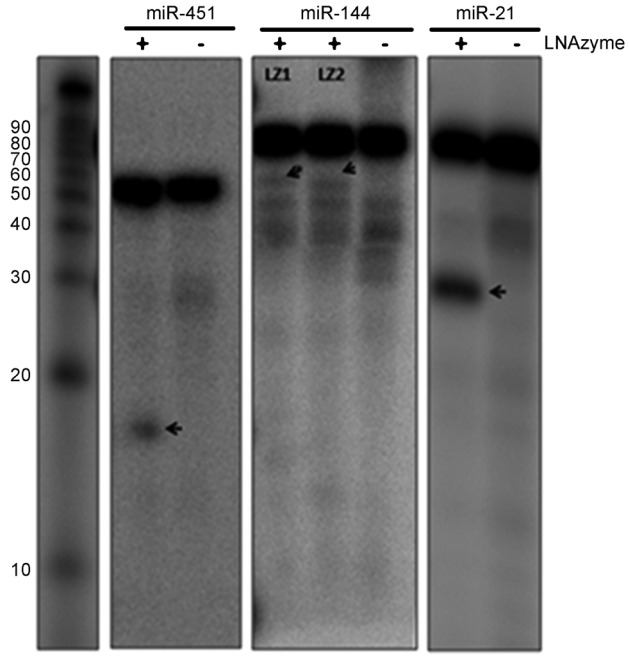

Figure 2. Cleavage activity of anti-miR LNAzyme. In vitro cleavage reactions by LNAzymes (5 mM) and ^{32}P-labeled pre-miRNAs (0.5 mM) in ribozyme buffer (Tris·HCl (50 mM, pH 7.5), $MgCl_2$ (25 mM)) at 37 °C. The cleavage products were resolved on 15% denaturing PAGE. Arrows indicate cleaved products

Effect of dose-dependent LNAzyme injections on embryo survivability

These LNAzymes were then used for in vivo miRNA knockdown experiments in zebrafish embryos. In order to assess toxicity of LNAzymes in the zebrafish embryos we initially tested over a wide concentration range (5–50 μM). The survivability of embryos was substantially reduced beyond 10 μM (Figure 3). As MO is a standard tool for miRNA downregulation in zebrafish embryos, we also tested MO oligonucleotides (10 μM–1 mM) that targeted the respective miRNA. (The upper limit of MO concentration range was based on previous reports.)[27,28] Both LNAzymes and MOs were assessed for their toxicity and ability to deplete miRNA (Figure 3 and Figure S1). From these dose–response experiments we chose 10 μM and 1 mM as optimal concentrations of LNAzyme and MO, respectively, in further experiments. For all in vivo experiments, 2–3 nL of LNAzyme or MO was injected into one- or two-cell-stage zebrafish embryos.[29]

In vivo inhibition of dre-mir-451

Erythropoiesis, the process that generates red blood cells (erythrocytes), is a well-conserved cellular mechanism orchestrated by a specific set of genes. During erythropoiesis, the cell undergoes extensive changes in gene regulatory networks, and several miRNAs are known to be differentially expressed.[17] Recently, it has been demonstrated that in zebrafish, miR-451 plays a crucial role in promoting erythroid maturation and is required for either maintenance/survival or late-stage matura-

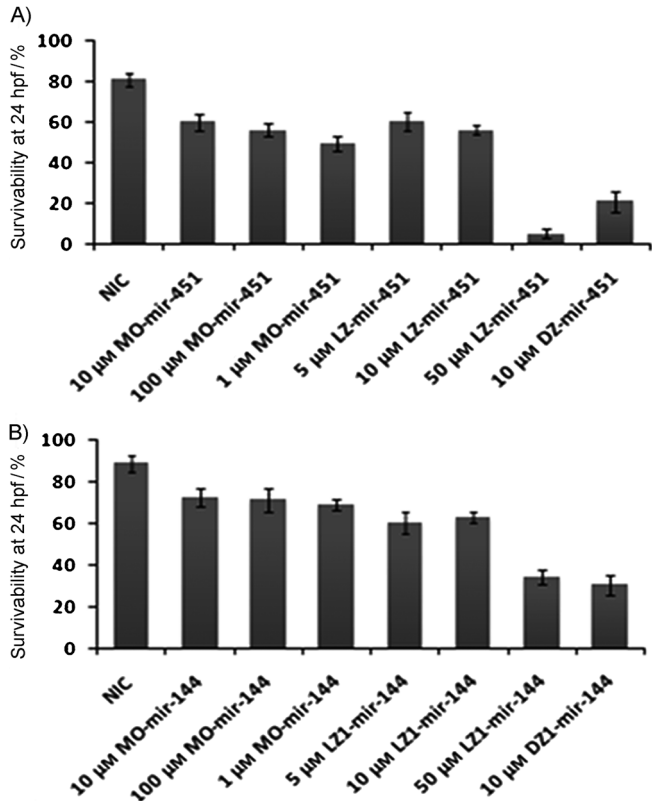

Figure 3. Survival of zebrafish embryos following microinjection at 24 h post fertilization of 2–3 nL of DNAzyme (DZ), LNAzyme (LZ) or morpholino (MO) designed against A) mir-451, or B) mir-144. NIC, noninjected control.

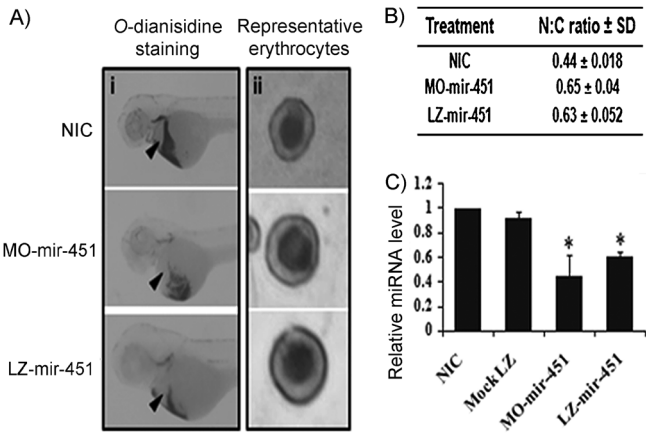

Figure 4. A) Inhibition of miR-451 function i) expression of hemoglobin as demonstrated by *O*-dianisidine staining and ii) morphology of representative erythrocytes upon MO/LZ/Dz injection compared with NIC (noninjected control). Arrows indicate *o*-dianisidine stained erythrocytes. B) Quantitative data of erythrocyte N:C ratio. C) Quantification of miR-451 level using real-time PCR (vs NIC, * $p < 0.05$).

tion of committed erythrocytes.[27] MO knockdown of miR-451 was shown to hamper the erythrocyte maturation process, thereby resulting in reduced numbers of red blood cells with hemoglobin. Repression of miR-451 has also been shown to cause an increase in nuclear/cytoplasmic (N:C) ratio, thus indicating immature morphology of circulating erythrocytes. We decided to use the well-studied miR-451 as one of our target miRNAs, to determine the efficiency of anti-miR LNAzymes.

We injected LNAzyme (LZ-mir-451), unmodified DNAzyme (DZ-mir-451), and MO (MO-mir-451), each designed against miR-451, into one- or two-cell-stage zebrafish embryos. We observed that survivability of DNAzyme-injected zebrafish embryos was significantly reduced 24 h post-fertilization (hpf), so we did not use DNAzyme in further studies (Figure 3). At 48 hpf, embryos injected with LZ-mir-451 (10 μM) or MO-mir-451 (1 mM) displayed significantly reduced numbers of *o*-dianisidine stained cell, 42.6%, ($n = 78$) and 49% ($n = 70$; Figure 4A; *o*-dianisidine stains cells with hemoglobin). Circulating erythrocytes showed marked increase in N:C ratio upon LZ-mir-451 or MO-mir-451 injection, thus indicating immature morphology of circulating erythrocytes (Figure 4A, B). These erythrocyte phenotypes indicate efficient inhibition of miR-451 function.[27] In order to measure miR-451 levels, we performed real-time PCR. MiR-451 levels were reduced by approximately 55 and 65% upon MO-mir-451 and LZ-mir-451 injection, respectively, thereby confirming efficient knockdown of miRNA (Figure 4C). The difference in levels of miRNA between mock LNAzyme (Mock LZ) injected and noninjected control (NIC) embryos was minimal. Notably, the concentration of LZ-mir-451 (10 μM) required for functional inhibition of miR-451 and to bring about a visible phenotypic change in zebrafish embryos was less than the concentration of MO-mir-451 (1 mM) required for a similar phenotype.

In vivo inhibition of dre-mir-144

We also investigated the in vivo anti-miRNA activity of LNAzyme designed against miR-144 (LZ-mir-144). During zebrafish embryogenesis, the erythroid-specific miR-144 negatively regulates embryonic α-E1 *globin* gene expression by targeting krüppel-like transcription factor d (*klfd*).[32] We designed and tested two LNAzymes (LZ1-mir-144 and LZ2-mir-144) that target different sites of pre-mir-144 (Figure 1) and compared their performance with that of a published MO designed against the mature form of miR-144.[28] In vivo inhibition of miR-144 was assessed by injecting LZ-mir-144 (10 μM) and MO-mir-144 (1 mM) into one- or two-cell-stage zebrafish embryos. The survival of DNAzyme-injected (DZ-mir-144, 10 μM) embryos was significantly reduced 24 hpf, and these embryos were not pursued for further experiments (Figure 3). In order to assess in vivo modulation of miR-144 function, we performed whole-mount in situ hybridization (WISH) for the α-E1 *globin* gene. Zebrafish embryos (22 hpf) showed significant increases in α-E1 *globin* gene expression upon injection with LZ1-mir-144 (88%, $n = 36$), LZ2-miR-144 (61%, $n = 54$) and MO-mir-144 (80%, $n = 44$), thereby indicating effective inhibition of miR-144 function (Figure 5A). The direct effect on miR-144 levels upon LZ-mir-144 and MO-mir-144 injection into one- to two-cell stage embryos was assessed by real-time PCR. Effective knockdown of miR-144 was observed upon injection of 10 μM LZ1-mir-144, 10 μM LZ2-mir-144 or 1 mM MO-mir-144 in zebrafish embryos (Figure 5B). Notably, for miR-144 the concentration of LNAzyme (10 μM) required for functional inhibi-

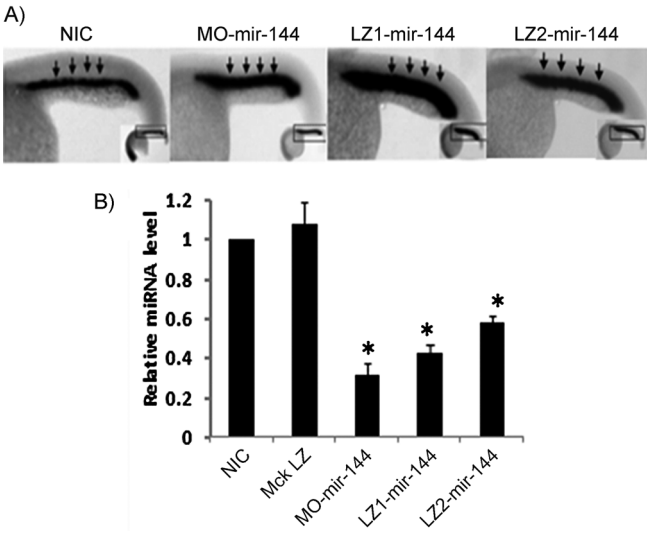

Figure 5. Inhibition of miR-144 function by MO-mir-144, LZ1-mir-144, and LZ2-mir-144. A) WISH assay for α-*E1* globin gene. Arrows indicate α-E1 globin expression. B) Quantification of miR-144 by real-time RT-PCR. NIC, noninjected control; Mock LZ (vs NIC, * $p < 0.05$).

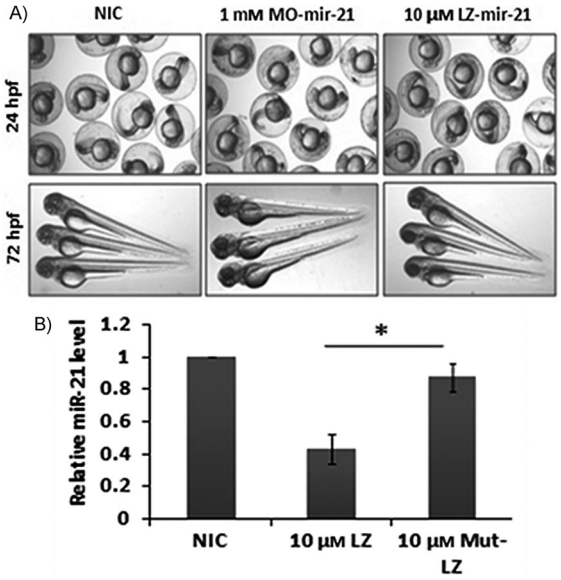

Figure 6. A) Phenotype screening (using standard morphological criteria) following MO-mir-21 or LZ-mir-21 injection. B) Contribution of catalytic core of LNAzyme in depleting the miRNA as determined by real-time PCR. NIC, noninjected control. * $p < 0.05$.

tion and for producing a visible phenotype was less than that required for MO (1 mM).

In vivo inhibition of dre-mir-21

During zebrafish embryogenesis, miR-21 is expressed in cardiac valves and otoliths, however no specific function of miR-21 has been established in developing zebrafish.[33] LNAzyme (LZ-mir-21, 10 μM) and morpholino (MO-mir-21, 1 mM) were injected into one- to two cell-stage embryos and then embryos were observed at 24 and 72 hpf. There was no distinct visible phenotype upon either MO-mir-21 or LZ-mir-21 injection, by morphological criteria, thus indicating a likely dispensable function of miR-21 in early development (Figure 6A). These observations indirectly suggest that LNAzyme does not affect other miRNAs to the extent of giving a distinct phenotype. However further studies with more LNAzymes are required to address nonspecific targeting by LNAzymes. A general observation in all DNAzyme-injected cases was reduced survivability. Next, we investigated whether the miRNA knockdown effect was due to the catalytic activity of LNAzyme or a result of antisense inhibition (probably by binding to pre-miRNA and thereby blocking the maturation process). For this purpose we used a mutated LNAzyme (Mut-LZ) against dre-mir-21 in which the catalytic core was shuffled (thus making it catalytically inactive) without altering the binding arms. We observed that a catalytically active core significantly enhanced the anti-miRNA effect of LNAzyme at 10 μM (Figure 6B). This indicates that although there was depletion of miRNA by Mut-LZ (~15%), the extent of knockdown was dramatically higher with the catalytically active LNAzyme (~55%). This finding highlights the important contribution of the catalytic core of the LNAzyme and hence its cleaving activity in downregulating miRNA levels.

Conclusions

These results demonstrate that LNAzyme can efficiently inhibit miRNA function in vivo, and reproducibly result in miRNA-specific phenotypes, with minimal off-target effects and associated toxicity.

In conclusion we propose a new LNAzyme-based tool for in vivo modulation of miRNA function in zebrafish. We have designed, in vitro tested, and experimentally validated the in vivo functionality of LNAzymes against three zebrafish miRNAs (miR-144, miR-451, and miR-21). Our data suggest that the overall performance of LNAzyme is comparable with that of the widely tested MO oligonucleotides used for transient knockdown of miRNAs in zebrafish. In addition, the concentration of LNAzymes required for ablation of miRNA function was significantly less than that of the respective MOs. The LNAzymes were designed to bind both mature miRNA and precursor miRNA (Figure 1). Therefore, the overall anti-miRNA effect could be the result of LNAzyme action at both mature and precursor level. With precursor molecules, the LNAzyme might form interactions (probably more stable because of the LNA-modified arms) with few unpaired bases on the target RNA, which would promote more efficient disruption of local structure and faster completion of binding (a process known as "nucleation").[34] As LNAzyme employs an enzyme-based mechanism for its anti-miRNA function, the effective concentration required for efficient knockdown of miRNA is much lower. In an animal model system, this might be of great importance for reducing the toxicity, off-targeting, and side-effects of anti-miRNA molecules. As miRNAs play key roles in many cellular processes and are overexpressed in many pathological conditions, LNAzyme-based silencing of miRNAs in animals might find new therapeutic applications.

Experimental Section

In vitro cleavage assay: DNAzymes/LNAzymes were designed as described previously.[25] The miRNA precursor transcripts were synthesized by using an Ambion MEGAscript in vitro transcription kit (#AM1334, Life Technologies). Forward (ex. IVTmiR-144_FP) and reverse (ex. IVT-miR-144_RP) overlapping primers were used for template preparation. The in-vitro-transcribed pre-miRNAs were 5′-^{32}P-labeled by using an Ambion KinaseMax 5′ End-Labeling Kit (Life Technologies). Labeled pre-miRNA (0.5 mM) and LNAzymes (5 mM) were heated separately to 85 °C for 5 min; the remaining cleavage protocol was exactly as previously described.[18] All DNA and LNA oligonucleotides used in this study were purchased from Ocimum Biosolutions (Hyderabad, India); morpholinos were procured from GeneTools LLC (Philomath, OR).

Zebrafish husbandry: The wild-type zebrafish used in this study were housed at the Institute of Genomics and Integrative Biology (GIB), Institute of Council of Scientific and Industrial Research (CSIR) Zebrafish Facility, following standard husbandry practice.[35] Zebrafish embryos were obtained by pairwise mating of adults. Zebrafish embryos were treated with phenyl thiourea (PTU, 0.003 %) for depigmentation.

Microinjections: MO and LNAzyme injections were performed as described previously.[36] Glass capillary micropipettes (World Precision, Sarasota, CA) were pulled by using a Sutter (Novato, CA) instrument and clipped appropriately to deliver solutions (2–3 nL) of LNAzymes/DNAzymes (10–25 μM) and MO (0.5–1 mM) into one- or two-cell-stage zebrafish embryos.

Morphometry: Embryonic zebrafish erythrocytes were collected by transecting tails of 6–8 embryos with a surgical blade and suspended in Hank's solution (600 μL) with ethylenediaminetetraacetic acid (EDTA, 5 mM) and fetal calf serum (4 %). After centrifugation, the supernatant was filtered through 40 μm filter (BD Biosciences). Erythrocyte smear slides were prepared, stained with May-Grünwald/Giemsa stain, and examined at 40×–60× magnification. Nuclear and cytoplasmic areas of 20 to 25 randomly selected erythrocytes were measured by using the outline tool of AxioVision software (Carl Zeiss) and the N:C area ratios were calculated.[27]

WISH and *o*-dianisidine staining: Paraformaldehyde-fixed embryos were examined by in situ hybridization according to standard zebrafish protocols (http://zfin.org/ZFIN/Methods/ThisseProtocol.html). The *α-hemoglobin (α-E1)* coding region was amplified by using primers as described by Fu et al.[32] and cloned into Topo TA vectors (Invitrogen). The *α-E1* clone was linearized with SpeI, and in situ probe was prepared by using T7 polymearse (Roche). *o*-dianisidine staining was performed as described previously.[37]

RT-PCR and real-time PCR: Total RNA was isolated using trizol (Invitrogen). After isolation, total RNA (1 μg) was used for reverse transcription (RT) with QuantiMir kit (#RA420A-1; SBI, Mountain View, CA), following the manufacturer's protocol. RT products were then used for real-time PCR with mature miRNA sequence as the forward primer and the universal (reverse) primer provided with the kit. The expression of all miRNAs was normalized to that of miR-26a. Kapa SYBR Mastermix (Kapa Biosystems, Boston, MA) was used for real-time PCR, and detection was by a Lightcycler LC 480 (Roche).

Imaging: Zebrafish wild-type embryos were injected as described above and incubated at 29±1 °C. The injected and noninjected embryos were observed and imaged by an upright Axioscope 40 fluorescent microscope (Carl Zeiss).

Statistics: Statistical analysis was performed with Microsoft Excel. Values are expressed as mean±S.E. Differences between groups were calculated with a Student's *t* test. A p value <0.05 was defined as significant.

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Keywords: enzymes · locked nucleic acids · microRNA · morpholino · zebrafish

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