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Research Article

In Vivo Testing of MicroRNA-Mediated Gene Knockdown in Zebrafish

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The zebrafish (*Danio rerio*) has become an attractive model for human disease modeling as there are a large number of orthologous genes that encode similar proteins to those found in humans. The number of tools available to manipulate the zebrafish genome is limited and many currently used techniques are only effective during early development (such as morpholino-based antisense technology) or it is phenotypically driven and does not offer targeted gene knockdown (such as chemical mutagenesis). The use of RNA interference has been met with controversy as off-target effects can make interpreting phenotypic outcomes difficult; however, this has been resolved by creating zebrafish lines that contain stably integrated miRNA constructs that target the desired gene of interest. In this study, we show that a commercially available miRNA vector system with a mouse-derived miRNA backbone is functional in zebrafish and is effective in causing eGFP knockdown in a transient *in vivo* eGFP sensor assay system. We chose to apply this system to the knockdown of transcripts that are implicated in the human cardiac disorder, Long QT syndrome.

1. Introduction

The zebrafish has become an attractive model for human disease modeling as there are a large number of orthologous genes in the zebrafish genome [1], and the encoded proteins exhibit similar functions to those expressed in humans. The methods that are available to manipulate zebrafish gene expression are limited, but principal among these methods is morpholino-based antisense technology that enables transient downregulation in order to study the effects of reduced gene expression during early vertebrate development. To create stable mutant fish lines, chemical mutagenesis (ENU) is the method of choice; however, this forward genetics approach is driven by phenotypic outcomes and is not able to target and knock down specific genes. In contrast, zinc finger nucleases (ZFNs) are an emerging technology that has proved successful in targeted gene knockdown in zebrafish [2–4]. Unfortunately, the design and production process is arduous and requires specialised knowledge, thus restricting its use in the wider research community. ZFNs also do not offer the option of causing gene knockdown in a tissue-specific manner or of providing conditional gene knockdown (the ability to turn on or off gene knockdown). This latter outcome would provide a more subtle disease model that is suited for studying late onset disorders, and it is in this context that RNA interference may offer a way forward.

The use of RNA interference (RNAi) technology has proven to be a very useful tool in disease modelling in mammalian systems [5]. The use of different promoters has allowed for tissue-specific expression of miRNAs, therefore restricting gene knockdown to targeted areas/organs, and also conditional knockdown with the use of Tet-on promoters (the Cre/loxP system is used in mice) [6]. In brief, small noncoding RNAs enable fine-tuning gene expression at the posttranscriptional level during development, apoptosis, and metabolism [7, 8]. These noncoding RNAs cause cleavage/degradation, or the stalling of translation, of a target mRNA

(refer to [7] for a detailed review) and can be synthetically created to cause gene knockdown. miRNAs can be expressed by a DNA-based vector system in which miRNA transcripts, consisting of a double-stranded hairpin structure, are processed by several endonucleases to form 22 nucleotide mature miRNAs, which then form a complex with the RNA-induced silencing complex (RISC) to carry out targeted gene silencing.

The use of RNAi in zebrafish has been controversial as there are varying opinions regarding its effectiveness, and specificity, in causing gene knockdown. It has been suggested that the expression of miRNAs in zebrafish embryos leads to off-target/toxic phenotypes due to flooding the endogenous miRNA pathway [9, 10]. However, transgenic zebrafish lines have been constructed that express miRNAs designed to target desired genes of interest with no apparent toxic effects. Dong et al. and Ho et al. successfully used miRNAs to mediate gene knockdown in zebrafish and have created a conditional knockdown model [11] and a heritable gene knockdown fish line [12].

Given the above background, we have considered that RNAi-based technology might allow the development of zebrafish models of heritable cardiac disorders such as Long QT syndrome (LQTS). This syndrome is a congenital cardiac disorder, which is characterized by a prolonged QT-interval that can lead to fatal arrhythmias. Of the many genes (encoding for cardiac ion channels/scaffolding proteins) implicated in LQTS, we have focussed on the *KCNH2* gene, which encodes for the α-subunit of the rapid delayed inward rectifier potassium channel and is vital for the repolarisation phase in the cardiac cycle. The dominant mutations that have been detected in LQTS type 2 patients encompass nonsense mutations, as well as exon deletions and duplications, which suggest that haploinsufficiency of KCNH2 plays a role in the manifestation of disease [13, 14].

Two KCNH2-type genes have been identified in zebrafish, termed *zerg-2* and *zerg-3* [1, 2]. The former appears to be an orthologue of the human *KCNH2* gene, while the latter appears to encode a protein with KCNH2 function but is an orthologue of the human *KCNH6* gene, which is another potassium channel that belongs to the same family as KCNH2. Critically, the (unregulated) knockdown of both zebrafish genes leads to a cardiac phenotype (bradycardia, atrioventricular block, and prolonged QT interval) that mimics the phenotype seen in patients with LQTS [1, 3]. Here, we show that a commercially available miRNA vector system is capable of mediating gene knockdown in zebrafish. We evaluate this approach in a transient *in vivo* method that targets various regions of the zebrafish *zerg-2* and *zerg-3* gene transcripts as a prelude to mimicking the haploinsufficiency of *zerg-2* and *zerg-3* in zebrafish in a stable transgenic fish line and to ensure that the custom-designed miRNAs are able to cause the knockdown of their intended targets.

2. Materials and Methods

2.1. Zebrafish Husbandry. Wild-type zebrafish (*Danio rerio*) were bred and maintained as described by Tang et al. [15].

2.2. Creation of pcDNA 6.2 GW/DsRed Express-miR. Linearised pcDNA 6.2 GW/EmGFP-miR was recircularised by the insertion of synthesised double-stranded oligonucleotides (dsOligo) containing the same cohesive ends as the miRNA cloning site. Two *BsmBI* restriction enzyme sites were included at both 5′ and 3′ ends. Single-stranded DNAs (ssOligos) were synthesised (Invitrogen) and annealed and cloned into the pcDNA 6.2 GW/EmGFP-miR vector as described below. The DsRed Express coding region was amplified by PCR from the pTRE-Tight-BI-DsRed Express vector (Clontech). The EmGFP coding region was removed from the pcDNA 6.2 GW/EmGFP-miR vector by *DraI* restriction enzyme digestion, treated with calf intestinal phosphatise, and purified. The amplified DsRed Express coding region was cloned into the pcDNA 6.2 GW vector and bidirectionally sequenced to confirm the correct orientation of DsRed Express.

2.3. miRNA Design and Construction. miRNA target sites were designed using the BLOCK-iT RNAi Designer (Invitrogen) under the miR RNAi design option. The entire *zerg-2* (Genbank ID: HM209079) and *zerg3* (Ensembl ID: ENSDARP00000085242) gene transcript sequences were used for the design process, and targets were chosen based on location (close to or within the 3′ UTR) and the score the designer programme gave to each target site. The top and bottom ssOligos (Table 1) of the mature miRNA were synthesised (Invitrogen) and cloned into the pcDNA 6.2-GW/dsRed-miR expression vector according to the manufacturer's instructions.

2.4. Construction of eGFP Sensor Vector. As the pCS2+ vector does not contain an eGFP reporter gene, one had to be inserted into the vector before the eGFP sensor vector could be used. The Kozak sequence and the eGFP coding region was excised from pT2AL200R150G (a gift from Dr Koichi Kawakami, National Institute of Genetics) using *Bam*HI and *Cla*I restriction enzymes. The pCS2+ vector was restriction enzyme digested with *Bam*HI and *Cla*I and the eGFP coding region was cloned into the linearised vector (Figure 1).

The 22 nucleotides that form the mature miRNA (Table 1) were used as the target site and the top and bottom ssOligos were synthesized (Invitrogen). The ssOligos were annealed according to the manufacturer's instructions for the construction of the miRNA vectors, and this was ligated downstream of the eGFP reporter gene in the pCS2+ eGFP vector. The pCS2+ eGFP vector was linearized with *Xho*I and *Xba*I to create sites for ligation.

2.5. Microinjection of Zebrafish and In Vivo Assay. Both miR RNAi and the target vectors were linearised using *Eag*I and *Xho*I (miRNA vector) and *Bss*HII (eGFP sensor vector). The digested products were gel-purified using MinElute Gel Extraction Kit (Qiagen). *In vitro* transcription was carried out using the mMESSAGE mMACHINE kit (Ambion, Inc), and transcribed mRNAs were purified using the LiCl precipitation method (according to manufacturer's instructions). RNA quality was determined by gel electrophoresis and

Table 1: Sequences of miRNAs and their respective targets.

Name of miRNA	Target exon	miRNA sequence
zerg2 miRNA 1 (top)	9	TGCTGAACCCTTGAAAGCTTTACAGCGTTTTGGCCACTGACTGACGCT GTAAATTTCAAGGGTT
zerg2 miRNA 1 (bottom)	9	CCTGAACCCTTGAAATTTACAGCGTCAGTCAGTGGCCAAAACGCTGT AAAGCTTTCAAGGGTTC
zerg2 miRNA 2 (top)	14	TGCTGTTCTGTAGGAGACGTCACTGAGTTTTGGCCACTGACTGACTCA GTGACCTCCTACAGAA
zerg2 miRNA 2 (bottom)	14	CCTGTTCTGTAGGAGGTCACTGAGTCAGTCAGTGGCCAAAACTCAGT GACGTCTCCTACAGAAC
zerg3 miRNA 1 (top)	8	TGCTGATCAGAGAGCCAATAAGCATGGTTTTGGCCACTGACTGACCA TGCTTAGGCTCTCTGAT
zerg3 miRNA 1 (bottom)	8	CCTGATCAGAGAGCCTAAGCATGGTCAGTCAGTGGCCAAAACCATGC TTATTGGCTCTCTGATC
zerg3 miRNA 2 (top)	15	TGCTGTGGACAGAGAGTCTGGAGACTGTTTTGGCCACTGACTGACAG TCTCCACTCTCTGTCCA
zerg3 miRNA 2 (bottom)	15	CCTGTGGACAGAGAGTGGAGACTGTCAGTCAGTGGCCAAAACAGTCT CCAGACTCTCTGTCCAC
zerg2 miRNA 1 target (top)	9	TCGAGGCTGTAAAGCTTTCAAGGGTTcT
zerg2 miRNA 1 target (bottom)	9	CTAGAgAACCCTTGAAAGCTTTACAGCc
zerg2 miRNA 2 target (top)	14	TCGAGTCAGTGACGTCTCCTACAGAAcT
zerg2 miRNA 2 target (bottom)	14	CTAGAgTTCTGTAGGAGACGTCACTGAc
zerg3 miRNA 1 target (top)	8	TCGAGCATGCTTATTGGCTCTCTGATcT
zerg3 miRNA 1 target (bottom)	8	CTAGAgATCAGAGAGCCAATAAGCATGc
zerg3 miRNA 2 target (top)	15	TCGAGACCAGGATGACGGCTGATATAcT
zerg3 miRNA 2 target (bottom)	15	CTAGAgTATATCAGCCGTCATCCTGGTc

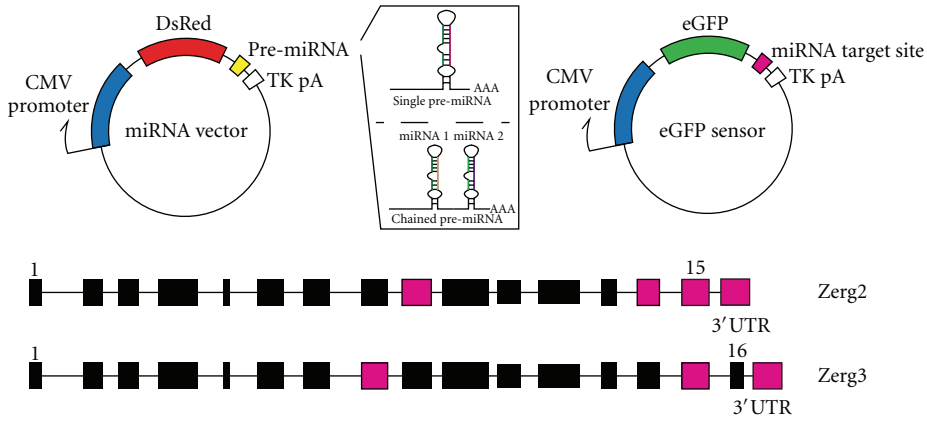
quantified using a Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, Inc).

1 nL of solution containing 300 pg of miRNA and 50 pg of eGFP sensor mRNA was microinjected into 1-2-cell stage embryos (or 300 pg of scrambled sequence miRNA, which was designed not to target any known zebrafish transcript, with 50 pg of eGPF sensor mRNA). 24 hours post-fertilization (hpf) embryos were observed and imaged using the Zeiss Axiovert S100 microscope. All images were taken from Nikon Eclipse T*i* inverted microscope. To quantify the raw fluorescence output, each group of embryos were loaded into a 96-well optical bottom plates in triplicate and the fluorescence was quantified using an EnVision Multilabel Microplate Reader (PerkinElmer, Inc). The settings were as follows: 485 nm excitation filter and 535 nm emission filter with a constant incubation temperature of 28°C. The raw fluorescence was averaged and the difference in fluorescence was compared to determine the efficiency of each miRNA.

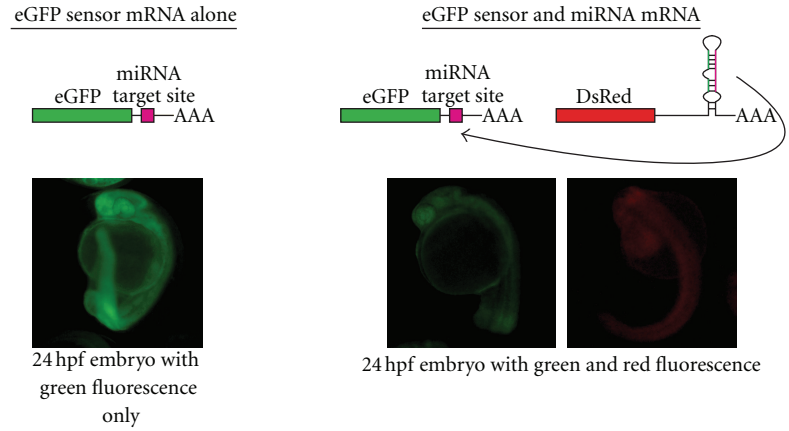
2.6. Data Analysis. Fluorescence readings were expressed in arbitrary fluorescence units. Wells with only E3 medium were used as a measure of background fluorescence. The mean background fluorescence was subtracted from the mean of the embryo containing wells. The readings were then normalized against the negative control embryos and expressed as a percentage. The normalized fluorescence percentages were analysed using one-way ANOVA.

3. Results and Discussion

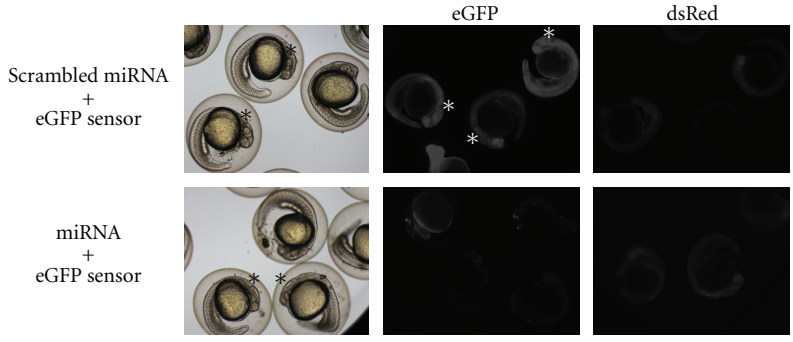
3.1. miRNA Design. The mechanism behind miRNA-mediated gene knockdown is still widely discussed (mechanisms ranging from translation stalling to mRNA cleavage), so it was considered prudent to design several miRNAs that targeted different regions in the two genes of interest. To mediate gene-specific knockdown in zebrafish, four different miRNAs were designed against each of the two zebrafish *kcnh2* genes (*zerg-2* and *zerg-3*). Each target site had a five-star ranking based on the ranking system using the Invitrogen Block-iT RNAi Designer, which ensured that the designed miRNAs would form the most effective RNAi molecules. At least one of the miRNA targets was located within the 3′ untranslated region (3′ UTR), while the others were located at various locations in the open reading frame



(a)



(b)



(c)

Figure 1: miRNA constructs and their effects in zebrafish embryos. (a) Schematic of the miRNA and the eGFP sensor vectors showing the miRNA target sites as well as the qRT-PCR primer locations. The miRNA vector contains a CMV promoter for ubiquitous expression in all tissues, and a DsRed Express reporter gene and the pre-miRNAs. The pre-miRNAs can be single (only one miRNA) or chained (containing more than one miRNA under the same promoter). The eGPF sensor miRNA also contains a CMV promoter, an eGFP reporter gene, and the mature miRNA target at the 3′ UTR of the eGFP gene. The miRNA target sites for *zerg-2* are located in exons 9 (miRNA 1), 14 no. 1 (miRNA 2), 14 no. 2 (miRNA 3), and 3′ UTR (miRNA 4). The miRNA targets for *zerg-3* are located in exon 8 (miRNA 1), exon 15 (miRNA 2), and two targets located on the 3′ UTR. (b) The injection of eGFP sensor mRNA alone produces embryos that exhibit green fluorescence only, and embryos injected with both eGFP sensor and miRNA mRNA exhibit both green and red fluorescence. (c) An example of embryos injected with either miRNA with eGFP sensor or scrambled miRNA (negative control) with eGFP sensor. The ∗ indicates the presence of the midbrain hindbrain boundary.

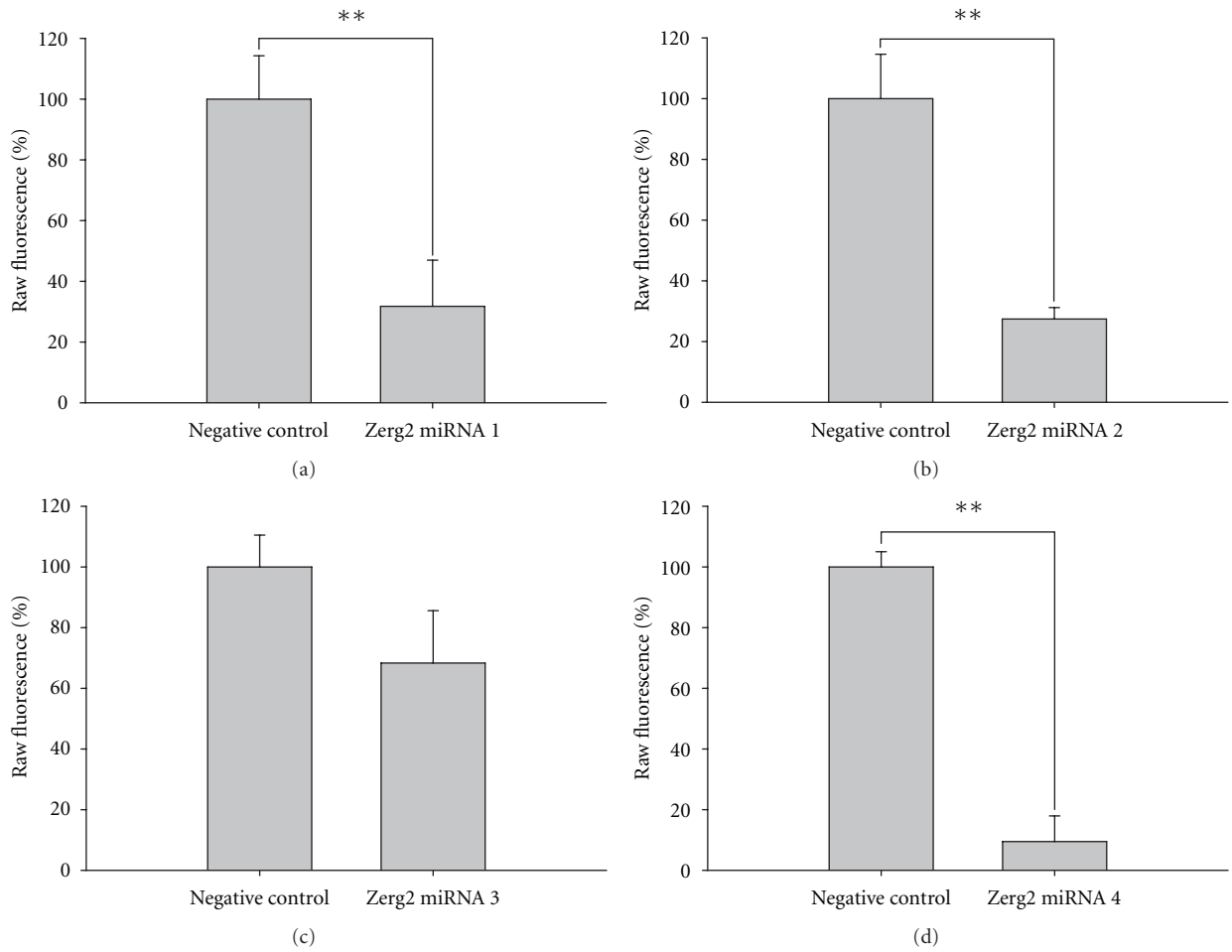


Figure 2: *In vivo* eGFP sensor assay for *zerg-2* miRNAs. (a) *zerg-2* miRNA 1 with eGFP knockdown at approximately 69%. (b) *zerg-2* miRNA 2 with eGFP knockdown at approximately 73%. (c) *zerg-2* miRNA 3 with eGFP knockdown at approximately 32% (the results are not statically significant). (d) *zerg-2* miRNA 4 with eGFP knockdown at approximately 88%. $^{**}P < 0.01$. All statistical analysis was carried out using one-way ANOVA. The percentage of fluorescence emitted by the injected embryos was determined by comparison to negative control embryos (those injected with scrambled miRNA).

(ORF; Figure 1(a)). Dong et al. have shown that miRNA target sites located at the 3′ UTR are more effective at causing gene knockdown compared to miRNA sites in the middle of an ORF [12], and our own laboratory has shown that it is also the case with the Block-iT system (data not shown). It has been suggested that this bias in efficiency is due to the translational machinery displacing bound miRNAs in the ORF regions as the machinery moves from the cap-binding complex to the ORF [7, 16]. Despite this, it has also been found that many miRNA target sites are found within ORFs [7]. Therefore, it was thought that gene expression knockdown could be maximized by targeting four different locations within each transcript.

3.2. Transient In Vivo Testing of the Efficacy of Individual miRNAs. The use of miRNA in mediating gene knockdown in zebrafish has been successfully achieved by Dong et al. and Ho et al. [11, 12]. The miRNA backbone used by Dong et al. was an endogenous zebrafish miRNA that is an orthologue of the mammalian miR-30e [12]. Here we have used a commercially available miRNA backbone that is based on the endogenous mouse miR-155, which has previously been shown to be effective in causing gene knockdown in mammalian cell lines [17]. Despite the fact that Dong et al. [12] found the efficiency of the zebrafish orthologue of miR-155 to be less than miR-30e, the data did not suggest that the mouse miR-155 backbone would not be effective in causing gene knockdown in zebrafish.

It was decided to validate the Block-iT miRNA system in the zebrafish and also to test the effectiveness of the custom-designed miRNAs by using a transient *in vivo* sensor assay. If the custom-designed miRNAs effectively caused knockdown in the transient *in vivo* sensor assay, then the miRNAs could be stably incorporated into the zebrafish genome to create transgenic fish lines. The transient *in vivo* sensor assay involved cloning the putative miRNA target sites into the 3′ UTR of a reporter/sensor gene [9, 18, 19] (Figure 1(a)). Each miRNA target was inserted downstream of an eGFP reporter gene (eGFP sensor), and both the miRNA and their respective sensor were coinjected into

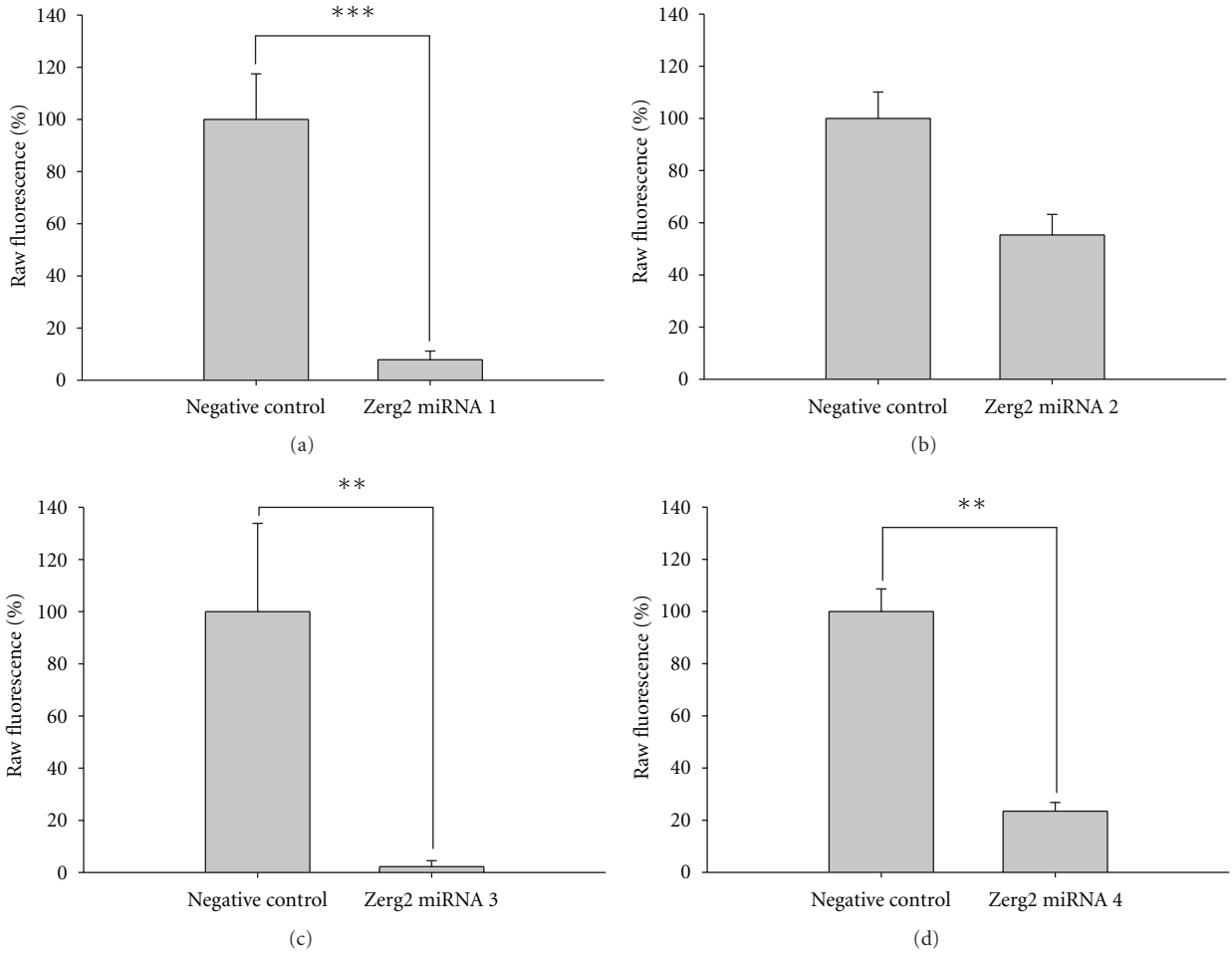


Figure 3: *In vivo* eGFP sensor assay for *zerg-3* miRNAs. (a) *zerg-3* miRNA 1 with eGFP knockdown at approximately 90%. (b) *zerg-3* miRNA 2 with eGFP knockdown at approximately 45% (the results are not statically significant). (c) *zerg-3* miRNA 3 with eGFP knockdown at approximately 98%. (d) *zer-g3* miRNA 4 with eGFP knockdown at approximately 77%. $^{**}P < 0.01$; $^{***}P < 0.001$. All statistical analysis was carried out using one-way ANOVA. The percentage of fluorescence emitted by the injected embryos was determined by comparison to negative control embryos (those injected with scrambled miRNA).

one-cell stage embryos. The duration of this assay was restricted by the half-life of the capped miRNA transcripts, and the embryos were screened for eGFP expression at 24 hours post-fertilization (hpf). At this stage, eGFP and DsRed expression could be detected and the effect of the miRNAs assessed. In the event of targeted knockdown, eGFP fluorescence would be expected to decrease (Figure 1(b)). As the miRNA target site was linked to eGFP, the emGFP reporter gene in the Block-iT expression vector was replaced with the DsRed Express reporter gene. The assessment of the assay was based solely on the level of eGFP expression produced by the injected embryos, and any phenotypic outcomes (i.e., heart rate, electrocardiogram) were not scored.

All eight miRNAs were able to decrease eGFP expression (Figures 2 and 3). *Zerg-2* miRNA 4 caused the greatest level of eGFP expression knockdown (approximately 88%) with *zerg-2* miRNA 1 and 2 causing approximately 69–73% eGFP knockdown (Figure 2). *Zerg-2* miRNA 3 was only able to cause 32% knockdown (Figure 2). *Zerg3* miRNA 3 caused the greatest level of eGFP knockdown (approximately 98%) with *zerg3* miRNA 1 and 4 causing approximately 77–90% knockdown (Figure 3); the extent of knockdown caused by *Zerg-3* miRNA 2 was not statistically significant. The results from the *in vivo* sensor assay demonstrate that the Block-iT miRNA backbone is functional in zebrafish and the custom-designed miRNA is capable of causing gene knockdown as assessed by the *in vivo* sensor system.

3.3. Assessment of Off-Target Effects. One of the main issues in using RNAi knockdown technology is the possibility of causing off-target gene knockdown. In zebrafish, this has proven to be one of the main downfalls of using RNAi as it has been found that the miRNA is vital for early development and the introduction of exogenous miRNA might cause competition for miRNA processing machinery [9, 10]. This in turn leads to a decrease in endogenous miRNA which is needed to clear maternal mRNA during development. Zhao et al. have reported phenotypes that are associated with toxic effects from the use of RNAi in zebrafish, which

included the loss of the midbrain-hindbrain boundary in siRNA-injected embryos [10]. In the current study there has been no evidence of the loss of the midbrain-hindbrain boundary; however, it cannot be ruled out that there is tissue-specific toxicity that is not readily apparent (Figure 1(c)).

4. Conclusions

The transient *in vivo* eGFP sensor assay has shown that the Block-iT is functional in the zebrafish system and also that the designed miRNAs are capable of causing knockdown at the protein level. These pilot data lay the foundation for constructing transgenic zebrafish expressing the miRNAs used here in order to model LQTS.

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