Short interfering RNA-mediated gene targeting in the zebrafish☆

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Abstract Short interfering RNAs (siRNAs) have proved to be a useful tool in studying gene function in plants, invertebrates and mammalian systems. Here we report the use of siRNAs for targeting the zebrafish dystrophin gene. This study demonstrates the efficacy of siRNA-based gene silencing in this vertebrate model species, and illustrates the potential of this approach for determining the roles of multiple protein products expressed by a single gene during the early stages of development. In addition this study illustrates the usefulness of zebrafish as a model for muscle disease, and highlights the potential of siRNA-based gene targeting for disease analysis in this model organism.

Key words: Short interfering RNA; Zebrafish; Duchenne muscular dystrophy; Quantitative real-time polymerase chain reaction; Dystrophin; Sarcoglycan

1. Introduction

Gene silencing via double-stranded RNAs (dsRNAs) has had a significant impact on the ability to suppress gene expression in a wide range of species encompassing mice, human cells, plants, *Drosophila melanogaster*, *Caenorhabditis elegans* and yeast [1,2]. To date, some studies of gene silencing in the zebrafish, which is an emerging model for disease analysis, have shown non-specific effects on gene expression following the microinjection of long (>30 bp) dsRNAs into embryos [3]. This outcome probably reflects significant differences in how vertebrates react to long dsRNA compared to invertebrates. To combat this problem, gene silencing approaches in mammalian systems are now based on using short interfering RNAs (siRNAs) to avoid generalised non-specific effects due to the induction of interferon and the activation of interferon-response genes by long dsRNA [4]. Currently, the use of siRNAs for gene silencing in the zebrafish has not been documented, although a recent report has shown the effectiveness of siRNA gene silencing in rainbow trout embryos [5].

We decided to investigate the use of siRNAs in targeting the zebrafish *Duchenne muscular dystrophy* (*dmd*) gene. This approach was considered to offer potential in dissecting the roles of multiple proteins expressed by this gene. Importantly, the zebrafish offers significant advantages compared to other model systems in the analysis of muscle-related diseases and early developmental effects [6].

In humans, the *Duchenne muscular dystrophy* gene contains eight promoters and expresses multiple transcripts with varying tissue specificity and developmental regulation [7,8]. These transcripts, comprising alternatively spliced variants, encode proteins ranging in size from 427 kDa to 71 kDa (Fig. 1). The full-length transcript expressed in muscle gives rise to a 427 kDa dystrophin protein (Dp427m) that links cytoskeletal actin to the extracellular matrix via the sarcolemmal dystrophin-associated glycoprotein complex (DGC) [8,9]. The role of Dp427m ranges from muscle stabilisation during contraction to localisation of signalling molecules [10,11]. The functional significance of those transcripts of the dystrophin gene encoding other 427 kDa proteins, and the smaller proteins (Fig. 1) that are expressed in the retina and central nervous system during development, remains unclear [11]. Importantly, the zebrafish *dmd* gene expresses a number of isoforms that are similar in size to those found in human tissues [12]. In addition, zebrafish dystrophin (Dp427m) and the DGC are found in the sarcolemma with strong localisation to transverse myosepta [12–15].

We describe here the use of siRNAs directed against the carboxy-terminal coding region of the zebrafish dystrophin gene to effect restricted temporal targeting of the family of dystrophin gene transcripts. This study involved assessing the impact of siRNAs on the expression and localisation of dystrophin and selected members of the DGC, as well as muscle architecture, in order to determine the temporal window of effect and specificity.

2. Materials and methods

2.1. siRNA design

Potential siRNA target sites in the zebrafish were determined using Oligoengine (DNA Engine), and Qiagen siRNA design (Qiagen) programmes. Synthesised siRNA duplexes were purchased from Xeragon in the case of the zebrafish dystrophin gene, and Dharmacon (Dallas, TX, USA) in the case of the siRNA against the green fluorescent protein (catalogue number D-001300-01-05; see Table 1). Stock concentrations of 50 μM were injected into single cell zebrafish embryos with an average injection volume of 0.3 nl.

2.2. Quantitative real-time polymerase chain reaction (PCR)

Zebrafish embryos were collected (10–20 embryos per time point), and RNA was extracted using Trizol (Invitrogen) and further purified using RNeasy columns (Qiagen). 1 μg total RNA from each extraction was reverse transcribed using Superscript III (Invitrogen) and random primers in a 20 μl reaction volume according to the manufacturer's instructions. cDNA samples were diluted 1:5 before use in quantitative PCR (Q-PCR) assays, and templates were pipetted in a minimum volume of 4 μl per 20 μl Q-PCR to minimise variation.

Q-PCRs were performed using an Applied Biosystems model 7900HT platform and a mixture of Taqman probe and Sybr green

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dye chemistries. All reactions (20 μl) were performed in triplicate. Taqman universal mastermix (Applied Biosystems) was used with Taqman probes and a standard PCR mix using Platinum Taq DNA polymerase (Invitrogen) supplemented with 0.2 μl/20 μl reaction of a 1:2000 dilution of Sybr green dye (Molecular Probes) in the case of Sybr assays. All primers and probes were designed to conform to a universal cycling programme using the Primer Express programme (Applied Biosystems). Three control genes were used in all experiments (GAPDH, β-actin, 18S rRNA; the primers and probe for the latter were supplied by Applied Biosystems). The two least variable control genes were determined for each experiment and normalisation factors were calculated based on the geometric mean of these two genes using the program geNorm [16]. Relative expression was calculated using a modified comparative CT method with correction for different amplification efficiencies [17]. See Table 1 for sequences of primers and probes.

2.3. Light microscopy

Images of zebrafish embryos were recorded using differential interference contrast (DIC) optics on a Zeiss Axiovert S100 microscope and a Zeiss Axiocam digital camera.

2.4. Wholemount immunohistochemistry

Embryos were manually dechorionated and fixed in methanol overnight at −20°C then washed in phosphate-buffered saline (PBS), incubated 10 min in 1×PBS with 0.1% Triton X-100, washed in PTWs (1×PBS, 0.1% Tween 20, 0.3% saponin) and blocked for at least 1 h in PBTs (1×PBS; 1% bovine serum albumin, 0.1% Tween 20, 0.3% saponin). Following blocking, embryos were incubated with monoclonal antibodies against: dystrophin (MANCHO 12) (http://www.newi.ac.uk/morrisge/mabs.htm#DMD), β-sarcoglycan (NCL-b-SARC) and β-dystroglycan (NCL-b-DG) (Novocastra Laboratories, http://www.novocastra.co.uk/md.htm), at 1:3 dilution in PBTs for at least 3 h at room temperature. The primary antibody was removed, then samples were washed six times in PTWs and were again blocked for at least 1 h in PBTs before incubation with secondary antibody. The secondary antibody, goat anti-mouse IgG lissamine-rhodamine conjugate (Jackson ImmunoResearch, USA), was pre-adsorbed against a crude zebrafish protein extract in PBTs before use then diluted at 1:50 in PBTs and incubated with samples for at least 1 h at room temperature. Antibody was removed and samples were washed with six PTWs washes then resuspended in 90% glycerol/10% PBS and rocked gently for 2 h prior to mounting. The rhodamine label was detected by scanning confocal imaging of embryos mounted in 1% low melting point agarose using a Leica TCS SP2 laser scanning confocal microscope [15].

2.5. Electron microscopy

Embryos were manually dechorionated and fixed overnight at 4°C in 2% paraformaldehyde, 2% glutaraldehyde in 0.1 M sodium cacodylate buffer pH 7.2 (SCB). After three washes in 1×SCB samples were postfixed in 1% osmium tetroxide for 1 h. Embryos were washed twice in 0.5×SCB, dehydrated in a graded ethanol series, and embedded in EM bed 812 resin (Electron Microscopy Sciences, USA). Ultrathin (60 nm) longitudinal sections were cut, counter-stained with 2% aqueous uranyl acetate for 20 min then stained with Reynolds lead citrate for 3 min and visualised using a Philips Tecnai 12 transmission electron microscope (FEI, The Netherlands) [18].

3. Results and discussion

siRNAs were designed against carboxy-terminal exons 53 and 68 of the zebrafish *dmd* gene, and each was introduced into zebrafish embryos by microinjection. The effect of siRNA-68 was initially assessed by Q-PCR of zebrafish dystrophin gene transcripts through the amplification of the exon targeted by this siRNA. This assessment involved normalisation against two reference genes (β-actin and 18S rRNA). Fig. 1 shows a temporal reduction in the level of the dystrophin family of transcripts, while there was no inhibitory effect of siRNA-68 on the relative expression level of the β-sarcoglycan gene transcript. The protein encoded by this transcript is a member of the DGC complex to which Dp427m binds. Microarray-based studies of Duchenne muscular dystrophy patient muscle have shown that the levels of transcripts encoding the sarcoglycans are not decreased despite reduced levels of Dp427m transcripts in these patients, possibly due to nonsense-mediated decay [19]. As an additional control, siRNA against the transcript of the green fluorescent protein (siRNA-GFP) was also microinjected into zebrafish embryos; this siRNA was used in the studies described by Novina et al. [20]. Q-PCR analysis showed no significant changes in the level of the dystrophin family of transcripts between injected and uninjected embryos at 12 h post fertilisation (hpf) and 24 hpf (see Supplementary Fig. 1).

Confocal microscopy of wholemount zebrafish embryos showed that by 24 h of development, dystrophin, β-sarcoglycan and β-dystroglycan are localised to myosepta (Fig. 2). In contrast, siRNA-68 and siRNA-53 injected embryos showed delayed localisation of dystrophin and β-sarcoglycan (28 hpf), with no apparent delay in terms of β-dystroglycan (24 hpf, Fig. 2). Interestingly, zebrafish treated with a morpholino targeted to the translational start site of Dp427m [21], and the *sapje* zebrafish mutant that carries a nonsense mutation in exon 4 of the *dmd* gene [22] exhibit a lack of localisation of dystrophin and members of the sarcoglycan sub-complex of the DGC at zebrafish myosepta.

Electron micrographs of zebrafish muscle showed disorgan-

Table 1
Sequences of siRNAs, primers and probes

Description	Type	Sequence (5′ to 3′)
Zebrafish *dmd* gene siRNA target sequences	siRNA-53	AAGTTTCGCACAGCGTTGAAG
	siRNA-68	AAACCGGAGTTAGAGGCTTCA
Green fluorescent protein siRNA target sequence	siRNA-GFP	GGCTACGTCCAGGAGCGCACC
PCR primers for the zebrafish *dmd* gene transcript (Sybr green assays)	Exon 53	*FOR* AAAAGGGTGGAGCCTCTCATTAAG
		REV TTAGAAAGTTGCTTGACATCTGCAT
	Exon 68	*FOR* GCTGCTTTCAGTTCGCCAAT
		REV TCGACTGAGGTTCTAAACGCATC
PCR primers for the zebrafish β-*sarcoglycan* gene transcript		*FOR* GGGTCGCAAAGGCATCTTC
		REV ACTGTCCATATCACCAGAGTTATAATGAG
PCR primers and probe for the zebrafish β-*actin* gene transcript	PCR primers	*FOR* CGAGCTGTCTTCCCATCCA
		REV TCACCAACGTAGCTGTCTTTCTG
	Taqman probe	CCCATACCAACCATGACACCCTGATGT
PCR primers and probe for the zebrafish *GAPDH* gene transcript	PCR primers	*FOR* CGCTGGCATCTCCCTCAA
		REV TCAGCAACACGATGGCTGTAG
	Taqman probe	CCAAATTCGTTGTCATACCAGGAGATGAGCT

Zebrafish sequences were identified from the zebrafish genome and whole genome shotgun traces (http://www.sanger.ac.uk/Projects/D_rerio).

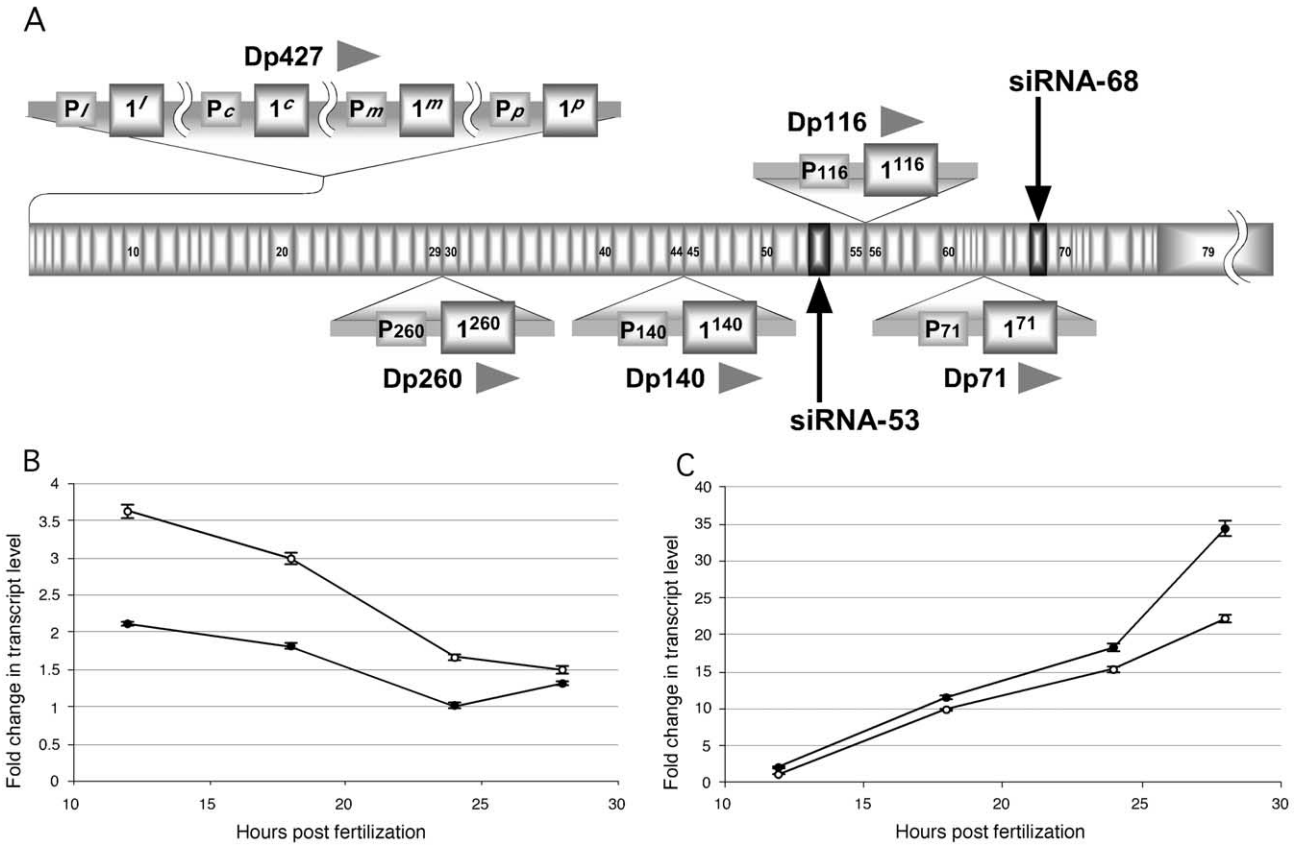


Fig. 1. Dystrophin gene transcript family and effect of siRNAs on relative transcript levels. A: The family of transcripts expressed by the human dystrophin gene. The organisation of the transcripts is depicted schematically. The arrows indicate transcription start sites for dystrophin isoforms expressed in lymphoid, cortical, muscle and Purkinje cells (Dp427 isoforms), and the smaller isoforms expressed in the retina (Dp260) and the central nervous system (Dp140-Dp71). B,C: Quantitative PCR analysis of transcripts expressed from the dystrophin and β-sarcoglycan genes, respectively; (○) control and (●) siRNA-68 injected. The fold change in transcript level is based arbitrarily on the lowest transcript level detected in the samples measured in each experiment (24 h siRNA injected embryos and 12 h control embryos in B and C, respectively). Vertical bars indicate standard deviations.

ised sarcomeres in siRNA-68 and siRNA-53 injected embryos at 36 hpf (Fig. 2). Light microscopic DIC images of siRNA-53 and siRNA-68 injected embryos showed the delayed development of myotubes, with disruption of the characteristic chevron shape of myosepta yielding U-shaped somites (Fig. 3). In addition, the larvae of siRNA-68 injected embryos exhibited significant curvature and reduced motility with an impaired touch response (data not shown). These features are shared to varying extents with those found in zebrafish due to morpholino-based translational knockdown of Dp427m [21] and dystroglycan [18]. The *sapje* mutant is less affected with the detachment of muscle fibres from myosepta, which is accompanied by the collapse of sarcomeres [22].

Taken together, our data support the use of siRNAs for gene targeting in the zebrafish, albeit in a brief temporal manner, and thus demonstrate the effective use of gene silencing via siRNAs in the last major model organism where this has not been successfully demonstrated previously. The work described here supports the use of siRNAs as a tool to achieve targeted loss of function in a vertebrate model system in which multiple promoters complicate the expression of the targeted gene.

Our conclusion of the specificity of gene silencing in zebrafish via siRNAs rests on several observations. First, the functional readout of siRNA-68 is at the transcript level, thus negating the possibility of a micro-RNA role for siRNA-68. Secondly, titration experiments have been undertaken to achieve a minimum effective dose of siRNA-68; four-fold higher doses yielded more severe phenotypes (data not shown). Thirdly, siRNA-68 injected embryos provided a similar readout to those injected with siRNA-53 in terms of muscle phenotype. Finally, the data mirror to a large extent the confocal and muscle morphological analyses reported for morpholino-based translational knockdown of the zebrafish dystroglycan and dystrophin genes [18,21], and the *sapje* zebrafish mutant [22].

The work described here adheres to the precepts of what constitutes the best controls in gene silencing by RNAi, albeit without the virtue of rescue controls [23]. We have deliberately not used mismatched siRNAs because of their limited usefulness and unanticipated effects [23]. We have assessed specificity through Q-PCR analysis that provides evidence supporting a transcriptional effect on the gene we have targeted, with no evidence of non-specific reduction of transcript levels based on the use of three unrelated reference genes. The immunohistochemical and ultrastructural data, and the comparison with morpholino-treated and mutant zebrafish reported in the literature, serve to support the transcriptional

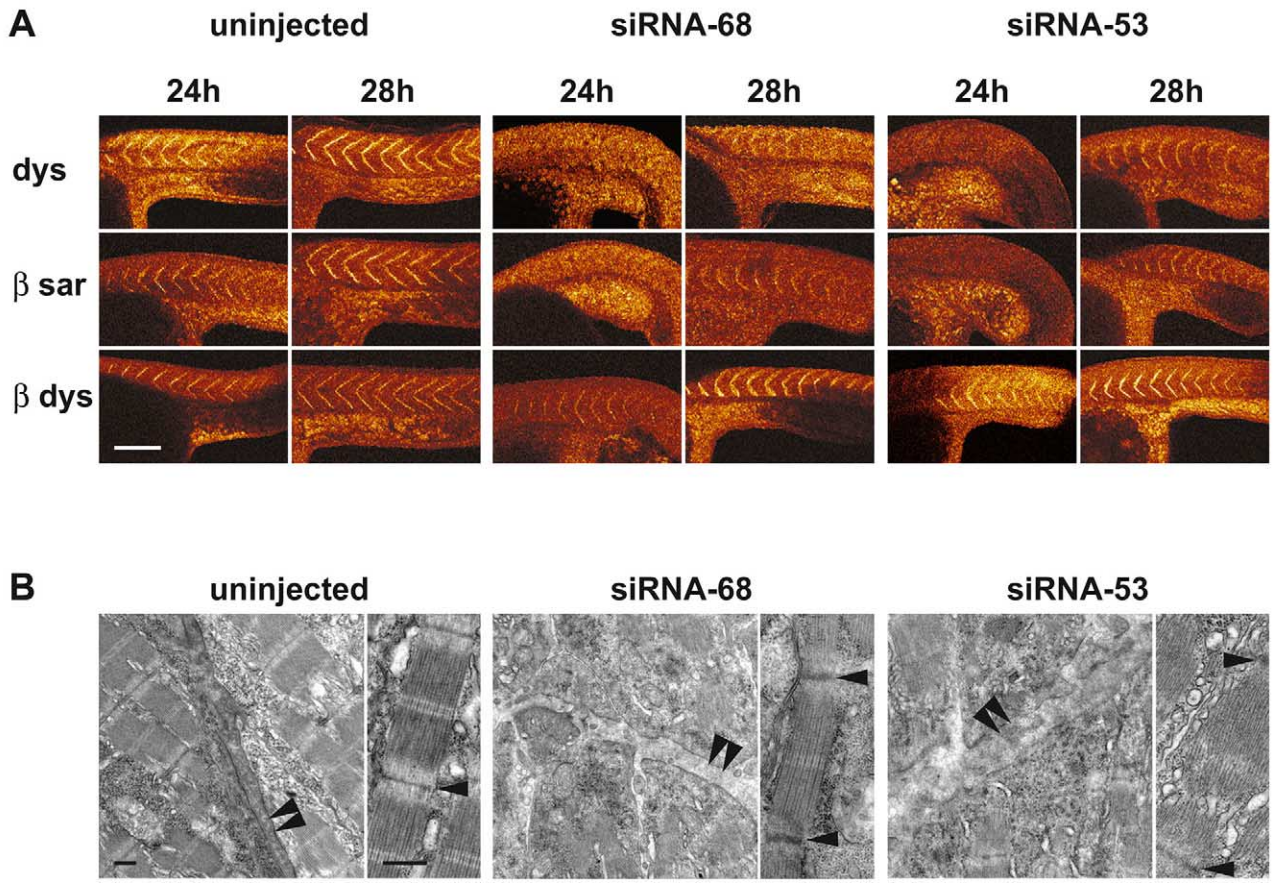
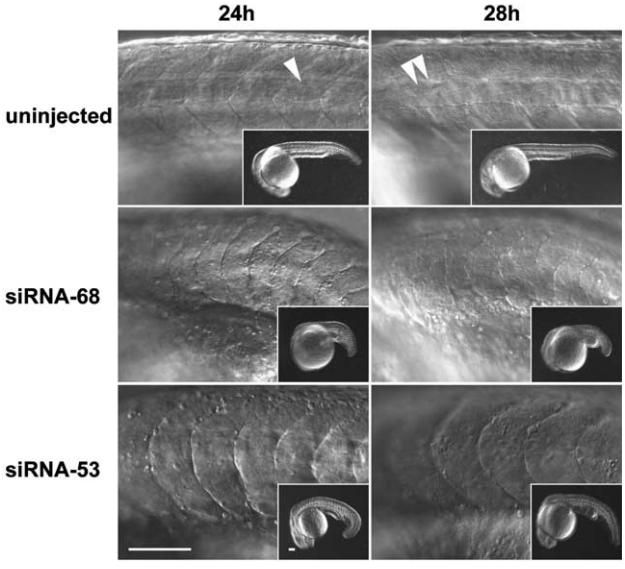


Fig. 2. Histochemical and ultrastructural analysis of siRNA injected zebrafish embryos. A: Immunolocalisation of DGC proteins to the myosepta in zebrafish embryos at 24 hpf and 28 hpf. Embryos were incubated with anti-dystrophin (dys), anti-β-sarcoglycan (β-sar) or anti-β-dystroglycan (β-dys) monoclonal antibodies (scale bar equals 200 μm). At 24 hpf the injected embryos are negative for dystrophin and β-sarcoglycan, whereas β-dystroglycan was detected. Control embryos were all positive for these peptides. At 28 hpf injected embryos begin to show localisation of dystrophin and β-sarcoglycan peptides at their myosepta. B: Ultrastructural analysis of ultrathin longitudinal sections of 36 hpf zebrafish embryos injected with siRNAs showing disruption of myosepta (double arrowheads) and fibre organisation compared to control embryos (scale bars equal 500 nm). Detailed examination of fibre reveals that the z-lines (single arrowheads) and other structures are irregular in injected embryos compared to controls.



data. Finally, the lack of any detectable *dmd* gene transcriptional effects using siRNA-GFP as a negative control underscores the conclusion of siRNA specificity.

The outcome of microinjecting siRNAs into zebrafish embryos offers similarities to the use of pre-mRNA splice site targeting by morpholinos [24]. The distinction to be drawn here, however, is that the creation of transgenic zebrafish lines expressing siRNAs in a tissue-specific or regulated manner could offer the means of effecting controlled changes in gene expression in order to ablate as well as to rescue. In this way, siRNA expression cassettes in zebrafish have the potential to overcome the limitations of relying on transient unregulated gene targeting, while opening up the possibility of using the

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Fig. 3. Light micrographs of siRNA injected embryos. Control embryos exhibit chevron-shaped myosepta at 24 hpf and the nuclei of myoblasts (single arrowhead) are visible in the somites where myotubes are formed by fusion of myoblasts. Myotubes can be observed maturing to striated fibres throughout the somites in 28 hpf uninjected embryos (double arrowhead). In contrast, nuclei of condensing myotubes are less evident in siRNA injected embryos at 24 hpf but are observed at greater frequency at 28 hpf and are particularly visible about the midline of the embryo (scale bar equals 100 μm).

zebrafish for functional vertebrate gene analysis in a disease context, allowing in vivo targeting of multiple transcripts, whether encoded by one gene or several genes.

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