Efficient shRNA-Mediated Inhibition of Gene Expression in Zebrafish

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Abstract

Despite the broad repertoire of loss of function (LOF) tools available for use in the zebrafish, there remains a need for a simple and rapid method that can inhibit expression of genes at later stages. RNAi would fulfill that role, and a previous report (Dong et al. 2009) provided encouraging data. The goal of this study was to further address the ability of expressed shRNAs to inhibit gene expression. This included quantifying RNA knockdown, testing specificity of shRNA effects, and determining whether tissue-specific LOF could be achieved. Using an F0 transgenic approach, this report demonstrates that for two genes, \textit{wnt5b} and \textit{zDisc1}, each with described mutant and morphant phenotypes, shRNAs efficiently decrease endogenous RNA levels. Phenotypes elicited by shRNA resemble those of mutants and morphants, and are reversed by expression of cognate RNA, further demonstrating specificity. Tissue-specific expression of \textit{zDisc1} shRNAs in F0 transgenics demonstrates that conditional LOF can be readily obtained. These results suggest that shRNA expression presents a viable approach for rapid inhibition of zebrafish gene expression.

Introduction

\textit{P}aramount to utility of the zebrafish is the ability to assay loss of function (LOF) phenotypes, and a wide array of techniques to perturb gene expression is available. Forward genetic screens have been very productive, using both chemical and insertional mutagenesis.\textsuperscript{1,2} Gene trapping has allowed tagging and mutagenesis,\textsuperscript{3,4} including an elegant conditional “protein trap” strategy.\textsuperscript{5} Inducible gene expression is possible using the Cre/lox system, Gal4/UAS lines, as well as doxycycline or tamoxifen-responsive promoters.\textsuperscript{6–8} Targeted LOF is achieved through TILLING,\textsuperscript{9} using zinc-finger nucleases\textsuperscript{10,11} or TALEN technology.\textsuperscript{12–14} In the embryo, stabilized morpholino-modified antisense oligonucleotides (MOs) can inhibit translation or splicing.\textsuperscript{15}

Despite this battery of LOF techniques, there remains no reliable method to rapidly test LOF effects after embryonic stages, and in a tissue-specific fashion. Mutants have been identified only for a fraction of fish genes, and stage or tissue-specific loss of function is not generally possible. Antisense MO oligonucleotides (MOs) are quick to use, by injection into early embryos. However, MOs are diluted as the embryo grows, and their efficacy fades as they are diluted with cell division and embryonic growth. Further, MOs are expensive, may have poor solubility, and often give off-target effects.\textsuperscript{16} Photoactivatable MOs can be used later in development; however, they are very expensive, and the frequency with which they can target a gene is not clear.\textsuperscript{17,18} Thus, there is a need for a rapid, inexpensive, tissue-specific method that can target specific genes in zebrafish beyond embryonic stages.

RNAi is an antisense-mediated process present in eukaryotic cells, whereby short hairpin RNAs (shRNAs), derived from longer double-stranded (ds) precursors, act post-transcriptionally to prevent expression of complementary RNA.\textsuperscript{19} Since long dsRNAs elicit an antiviral response and inhibit total translation, in order to use RNAi as a tool to inhibit gene expression, short hairpin RNAs (shRNAs) that do not elicit the antiviral response are employed. These are processed like endogenous miRNAs (Fig. 1), and can be expressed from DNA templates, to drive tissue-specific LOF. Thus, the Drosha enzyme processes double-stranded RNA to yield shRNAs, which are then cleaved into 21–22 bp fragments (siRNAs) by Dicer endonuclease. siRNAs enter the RNA-inducing silencing complex (RISC), which mediates interaction of the antisense (guide) siRNA strand with the target. RISC includes Argonaute endonucleases that cleave target RNA if there is a complete match with the siRNA. RNAi prevents gene expression in many animals, including \textit{Caenorhabditis elegans}, mouse, and \textit{Drosophila}.\textsuperscript{20–22} Conditional RNAi in mouse, after injection of lentiviral constructs or using the Cre-lox system to control RNAi expression, is a popular

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FIG. 1.  shRNA expression from miR30-backbone vectors. (A) Schematic showing promoter-driven expression of shRNAs, where the hairpin sequence is cloned into the miR30 backbone. After transcription, the priRNA is cleaved by Drosha to form an shRNA, which is further processed by Dicer into an siRNA and loaded into the RISC complex. The “guide” (antisense) strand then inhibits gene expression, leading to RNA cleavage if there is a complete match with the target. (B) Schematic of one construct employed. The b-actin promoter drives an exon-intron construct including a miR30/shRNA cassette that is linked to a CFP reporter (pTolDest-bactin-actin/intron-miR30-CFP-pA). Nascent RNA is spliced and cleaved to yield shRNA and CFP, whose expression reflects extent of shRNA expression. (C) Extent of CFP expression in an F0 Tol2 transgenic embryo, using the construct shown in B. Lateral view, 24 hpf, head to top.

Materials and Methods

shRNA design and purification

Hairpins for zDisc1 and wnt5b were designed using Invitrogen Block-IT RNAi Designer software. Hairpin oligonucleotide pairs were purified by UREA-PAGE, annealed by heating to 95°C and slow cooling to 10°C. We have previously detailed these methods on ZFN. At least four hairpins per gene were tested. The guide strand siRNA sequence was designed to be a complete match to the target, which is anticipated to lead to cleavage of the target RNA after siRNA binding. When annealed, each hairpin contains a BbsI compatible site, for insertion into the miR30 backbone.

Short hairpin sequences used

wnt5b ORF268for: 5’GGCTAGCCAACCTCGTGGTTGTCATTTCTGGTGCACTAGTGATGAAATGGACACCAAGTGGTTGCC3’
wnt5b ORF268rev: 5’GCGTGCCAACCTCGTGGTTGTCATTTCTGGTGCAAGATTGGACACCAAGTGGTTGCC3’

wnt5b 3’UTR1299for: 5’GCTTAGGACAGACGGCACTTTCTTGTGTTGCGACTCTGTAATGACCAAACGCAGGTTGCT3’

wnt5b 3’UTR1299rev: 5’GCACCAGTAATGACCAAACGCAGGTTGCTTGCCTGCCT3’

wnt5b 3’UTR1800for: 5’GCTTAGGACAGACGGCACTTTCTTGTGTTGCGACTCTGTAATGACCAAACGCAGGTTGCT3’

wnt5b 3’UTR1800rev: 5’GCTTAGGACAGACGGCACTTTCTTGTGTTGCGACTCTGTAATGACCAAACGCAGGTTGCT3’

wnt5b MO 7 (see Materials and Methods). (a, b, c, d), lateral view of the head; (a’, b’, c’, d’), dorsal view of the head after brain ventricle injection; (a″, b″, c″, d″) lateral view of whole embryo. (D) Whole mount in situ hybridization analysis of myoD expression in 12 hpf embryos expressing the following RNAs: YFP + mcherry RNA (a’), wnt5bh + mcherry RNA (b’), wnt5bh + wnt5b RNA (c, c’) or injected with wnt5b MO (d-d’). Dorsal views. Black bracket: bent tail; white bracket: compressed somites.

FIG. 2.  Inhibition of endogenous wnt5b expression. The 3’UTR1956 shRNA targeting wnt5b was expressed from an intronic miR30 backbone, under the b-actin promoter, using the vector pTolDest-bactin-actin/intron-miR30-CFP-pA (see Materials and Methods). Transient transgenesis was induced using Tol2 transposase. (Aa) Vector schematic. (Ab) Relative expression of wnt5b mRNA after inhibition by shRNA. Expression was quantified by qPCR in 24 hpf embryos expressing the wnt5b 3’UTR1956 shRNA (wnt5bh) relative to embryos injected with a control hairpin (YFPh). Data were normalized to endogenous b-actin RNA. (Ac) Northern blot analysis of RNA isolated from 24 hpf embryos demonstrating expression of the 21 base wnt5bh (antisense) guide strand (lane 1). The control is a synthetic wnt5b antisense strand DNA, run in parallel on the Northern blot (lane 2). (B) Whole mount in situ hybridization analysis of wnt5b expression in 12 hpf embryos expressing YFPb (a, a’) or wnt5bh (b, b’) and in 24 hpf embryos expressing YFPb (c) or wnt5bh (d, d’). (a), dorsal views, anterior up; (a”b)” lateral views, anterior up; (c, d) lateral views. (C) Phenotype of shRNA-expressing embryos and wnt5b morphants. (a-a”) 24 hpf control YFPb embryos coinjected with mcherry RNA. (b-b”) 24 hpf wnt5bh embryos coinjected with mcherry RNA. (c-c”) 24 hpf wnt5bh embryos coinjected with wnt5b RNA. (d-d”) 24 hpf embryos injected with wnt5b MO together with the p53 MO (see Materials and Methods). (a, b, c, d), lateral view of the head; (a’, b’, c’, d’), dorsal view of the head after brain ventricle injection; (a”, b”, c”, d”) lateral view of whole embryo.
FIG. 1.

FIG. 2.
wnt5b 3'UTR1965for:
5'GGCTAGACATAGAACCTTGTAGTGACATAGCTCTTGTGCTGCTGCT3'

wnt5b 3'UTR1965rev:
5'GCATGACATAGAAGCCGCTCTTGTGCTGCTGCTGCT3'

zDisc1 ORF2247for:
5'GGCTTGGCAGTGCTATTATAGGCTTGGAGTGAGCGCTCTGCTC3'

ORF2247rev:
5'GGCTTGGCAGTGCTATTATAGGCTTGGAGTGAGCGCTCTGCTC3'

zDisc1 ORF2461for:
5'GGCTTGGCAGTGCTATTATAGGCTTGGAGTGAGCGCTCTGCTC3'

ORF2461rev:
5'GGCTTGGCAGTGCTATTATAGGCTTGGAGTGAGCGCTCTGCTC3'

zDisc1 ORF2654for:
5'GGCTTGGCAGTGCTATTATAGGCTTGGAGTGAGCGCTCTGCTC3'

ORF2654rev:
5'GGCTTGGCAGTGCTATTATAGGCTTGGAGTGAGCGCTCTGCTC3'

miR expression vector preparation

Two vectors were employed. The first, pTolDest-bactin-actin/ intron-miR30-CFP-pA, included an actin-exon + intron-miR30 fragment. This fragment includes the first exon, including initiator ATG, and the first intron of the fragment. This fragment includes the first exon, including the miR30 sequences and hairpin region is processed to yield the shRNA and then the siRNA (Fig. 1A). The actin-exon + intron-miR30-CFP fragment was cloned into the middle element (pme-MCF) plasmid from the Tol2 kit. The middle element and the pSE-bactin2 containing the b-actin promoter were assembled together with pTolDestR4R2pA (that includes an SV40 poly A addition site) by LR reaction to create the expression GATEWAY vector pTolDest-bactin-actin/intron-miR30-CFP-pA.

Two other plasmids were used, I-SceI-mir124-miR30-GFP-pA and I-SceI-ntl-miR30-CFP-pA, containing I-SceI (meganuclease) target sites. In these, the CNS specific mir124 promoter or the meso-endoderm specific no tail (ntl) promoter was cloned upstream of a GFP reporter followed by the zebrafish miR30 backbone, and of the SV40 poly A site. Hairpin oligonucleotides were inserted into the BbsI site of the miR30 backbone. Expression of GFP reflects transcription of the priRNA that can yield an shRNA.

Transgenic preparation

Transgenic analysis was performed in transient (F0) transgenics. For Tol2 constructs, 1 nl (25 pg) of a 25 ng/µl solution of each transgenesis vector was combined with 1 nl (25 pg) of a 25 ng/µl Tol2 mRNA as described and injected into one-cell-stage eggs derived from the AB zebrafish strain. For meganuclease-based vectors, each construct (1 nl (30 pg) of a 30 ng/µl stock solution), together with 0.5 U of fresh I-SceI meganuclease (New England BioLabs), was injected into 1-cell fertilized embryos.

Quantitative RT-PCR (qPCR)

Total embryo RNA was extracted using Trizol (Invitrogen) followed by chloroform extraction and isopropanol precipitation and DNAase treatment or RNeasy kit (Qiagen). cDNA synthesis was performed with Super Script III Reverse Transcriptase (Invitrogen) and random hexamers. Primers for qPCR are:

zDisc1-for: 5'ATGAGACCCACTCACAGCC3'
zDisc1-rev: 5'GGTTCCTCTTATCGTCCAGTCC3'

As control we designed a hairpin targeting YFP (which is not expressed in the embryos used): YFP ORF481for:
5'GGCTAGACATAGAAGCCGCTCTTGTGCTGCTGCTGCT3'

ORF481rev:
5'GGCTAGACATAGAAGCCGCTCTTGTGCTGCTGCTGCT3'

YFP ORF481rev:
5'GGCTAGACATAGAAGCCGCTCTTGTGCTGCTGCTGCT3'

YFP ORF481for:
5'GGCTAGACATAGAAGCCGCTCTTGTGCTGCTGCTGCT3'

Northern blot analysis

Northern blot analysis was performed as described. RNA from 30 embryos was loaded for each lane. A synthetic oligonucleotide complementary to the guide strand was 5' end-labeled with γ-32P was used as probe. 100 pg of synthetic wnt5b or zDisc1 antisense DNA was used as control.

Whole mount in situ hybridization analysis

For analysis of transcripts in the whole embryo, embryos were fixed in 4% PFA and in situ hybridization performed on 12 hpf or 24 hpf embryos, as described. The zDisc1 antisense
probe was prepared as published. The wnt5b antisense probe was prepared digesting pCS2-wnt5b plasmid, a kind gift from Dr. Diane Slusarski (University of Iowa), with BamHI and transcribing with T7 polymerase. The myoD antisense probe was prepared digesting pBlueScript-myoD plasmid (a kind gift from Dr. Eric Weinberg [University of Pennsylvania]) with EcoRI and transcribing with T7 polymerase.

**Microscopy**

Methods for brightfield and fluorescence microscopy have been described previously. Confocal imaging was performed on a Zeiss LSM710, after fixation in 2% TCA (acetylated α-tubulin) or 4% PFA (phalloidin).

**Immunohistochemistry**

Whole-mount immunostaining used mouse anti-acetylated α-tubulin (Sigma, 1:1000). Goat anti-mouse Alexa Fluor 488 (Molecular Probes, 1:500) was used as a secondary antibody. Staining by phalloidin Texas Red was performed as previously described.

**Brain ventricle injection and imaging**

Brain ventricles were injected with Texas Red dextran, and superimposed brightfield and fluorescence imaging were performed as described previously.

**RNA rescue assays**

Capped RNA was synthesized using the Message Machine kit (Ambion), and injected at one-cell-stage. hDisc1, wnt5b and mcherry mRNAs were transcribed from pCS2-hDisc1, pCS2-wnt5b (very kindly provided by Dr. Diane Slusarski) and pCS2-mcherry (kindly provided by Dr. Frank Gertler [Massachusetts Institute of Technology]), respectively. For rescue, 200 pg (zDisc1) and 2 pg (wnt5b rescue) were injected, with an equivalent amount of mcherry RNA injected. Results obtained with or without injection of mcherry mRNA were indistinguishable.

**Morpholino oligonucleotide injections**

Antisense morpholino oligonucleotides (MOs; Gene Tools, LLC, Philomath, OR) were as follows: zDisc1MO, 5′-TCG CAGTTTTGTCTTACCTGTCCTC-3′; wnt5b MO, 5′TGTTTAT TTCTTACCATTCCCTCG-3′; wnt5b MO, 5′TGTTTAT TTCTTACCATTCCCTCG-3′; zDisc1MO, 5′TGTTTAT TTCTTACCATTCCCTCG-3′; wnt5b MO, 5′TGTTTAT TTCTTACCATTCCCTCG-3′. A similar phenotype was obtained in both cases, namely, a bent tail, and associated abnormal convergence and extension. Four short hairpin sequences were designed to target wnt5b mRNA, that would bind both of the alternately spliced transcripts arising from this gene (Zv5–Zv9 releases, www.ensembl.org). shRNAs were cloned into the zebrafish miR30 backbone, with the cassette driven by a general or tissue-specific promoter, and tested in a transient (F0) transgenic assay. The first gene targeted was wnt5b, considered a non-canonical wnt, which is expressed in the mesendoderm and tail bud. The LOF phenotype for wnt5b has been reported using antisense MOs and in the pipetail mutant wnt5b; wnt5b. A similar phenotype was obtained in both cases, namely, a bent tail, and associated abnormal convergence and extension. Four short hairpin sequences were designed to target wnt5b mRNA, that would bind both of the alternately spliced transcripts arising from this gene (Zv5–Zv9 releases, www.ensembl.org). shRNAs were cloned into the zebrafish miR30 backbone, in the pTolDest-bactin-actin/ intron-miR30-CFP-pA vector, which is ubiquitously expressed from the b-actin promoter (Fig. 2Aa) (see Materials and Methods). F0 transgenic embryos were prepared using the Tol2 method, and examined at 24 hpf for a bent tail. One hairpin, mapping to 3′UTR in position 1956 gave a phenotype and this was used in subsequent assays. ORF269 and 3′UTR1299 hairpins also gave weaker phenotypes. F0 transgenic embryos were sorted for high CFP expression and harvested at 24 hpf for qPCR analysis, which showed an average of 71% (n = 5 independent experiments, 82 total control and 78 total experimental embryos examined) decrease in wnt5b RNA after expression of the 3′UTR1956 wnt5b shRNA relative to a control YFP shRNA (Fig. 2Ab). Expression of the guide strand of the siRNA, which would bind to the wnt5b RNA target, was confirmed by northern analysis (Fig. 2Ac). A strong decrease in wnt5b RNA observed by qPCR was also seen by whole mount in situ hybridization analysis at 12 hpf and 24 hpf (Fig. 2B). Some RNA remained in both assays, possibly reflecting incomplete expression of the shRNA due to mosaicism, or targeting efficiency of the siRNA.

At 24 hpf, the effects of the wnt5b shRNA were monitored by examining embryos after injection of the brain ventricles with Texas Red dextran (Fig. 2Ca-b). Brightfield and fluorescence images were superimposed to allow morphological examination of the brain, body and tail. While expression of a control YFP shRNA did not significantly affect development (n = 81, 86% normal embryos) (Fig. 2Ca-a’), after expression of the wnt5b shRNA, most embryos showed a short and bent tail (n = 74, 19% normal embryos) (Fig. 2Cb’, black bracket). This phenotype appeared identical to that observed after injection of wnt5b antisense MOs (Fig. 2Cd-d’, n = 35, 3% normal and in morphants (i.e., after antisense morpholino-modified oligonucleotide injection). These phenotypes provide a basis for comparison with an shRNA-mediated phenotype. In these studies, shRNAs were expressed from a zebrafish miR30 backbone, with the cassette driven by a general or tissue-specific promoter, and tested in a transient (F0) transgenic assay (Fig. 1A and B). The assay was employed to rapidly test shRNA efficacies, and has the advantage that effects can be examined in the F0 (Fig. 1C). This type of analysis has the disadvantage of mosaicism, where fewer than 100% of cells become transgenic and express the shRNA, reducing the magnitude of shRNA effects. In order to overcome this disadvantage, we chose embryos expressing CFP extensively, and scored these for phenotypes. Each shRNA expression construct was assayed in at least two independent experiments, with total numbers of embryos examined indicated in the relevant section below.
embryos), and in the pipetail mutant, although the tail is shorter and the somites more compressed in morphant than in the mutant. In order to further test specificity of the shRNAs-mediated phenotype, we asked whether injection of RNA encoding wnt5b could prevent the tail phenotype observed after shRNA expression, relative to injection of control mcherry mRNA. This RNA could not bind to the wnt5b RNA since it lacked the 3′UTR that the siRNA targeted, and prevented a tail phenotype in the siRNA-expressing fish. This indicated that the phenotype observed was specific and not due to off-target effects (n = 29, 62% normal embryos) (Fig. 2Cc–c′). The wnt5b LOF phenotype and specificity was further explored by examining expression of myoD RNA that marks the developing somites, using in situ hybridization at 12 hpf (Fig. 2Da–c′). While expression of the FYP control shRNA did not alter somite formation (Fig. 2Da,a′, n = 15, 100% normal), after wnt5b shRNA expression, the somites were compressed (Fig. 2Db,b′, white bracket, n = 15, 20% normal embryos). This indicates a convergence and extension abnormality, and was prevented by simultaneous injection of wnt5b RNA and the shRNA construct (Fig. 2Dc,c′, n = 15, 73% normal embryos). A similar myoD phenotype was observed in wnt5b morphants, although compression of the somites was more pronounced than after shRNA expression (Fig. 2Dd,d′, white bracket, n = 15, 13% normal embryos).

Together, these data indicate that expression of zebrafish wnt5b can be inhibited by shRNAs. Specificity of the phenotype was indicated by similarity to the published pipetail mutant and wnt5b morphant, and by the ability of cognate mRNA to prevent shRNA-associated phenotypes.

shRNAs target zDisc1 effectively and specifically

The second gene targeted was zDisc1, for which we previously reported phenotypes of a morphant and a mutant, Disc1h, demonstrating both brain, muscle segment, and

FIG. 3. Inhibition of endogenous zDisc1 expression. The ORF2654 shRNA (zDisc1h) targeting zDisc1 was expressed in Tol2 F0 transgenic embryos, under control of the b-actin promoter, using the pTolDest-bactin-actin/intron-miR30-CFP-pA vector (see Materials and Methods). (Aa) Vector schematic. (Ab) Relative expression of zDisc1 mRNA after inhibition by shRNA. Expression was quantified by qPCR in 24 hpf embryos expressing zDisc1h relative to embryos expressing a control shRNA, YFPh. Data was normalized to endogenous b-actin RNA. (Ac) Northern blot analysis of RNA isolated from 24 hpf embryos demonstrating expression of the 21 base zDisc1 (antisense) guide strand (lane 1). The control is a synthetic zDisc1 antisense strand DNA, run in parallel on the Northern blot (lane 2). (B) Whole mount in situ hybridization analysis of zDisc1 expression in 24 hpf embryos expressing YFPh (a, a′) or zDisc1h (b, b′). (a-b′) Lateral views, anterior to the left; (a′, b′) higher magnification views. (C) Phenotype of shRNA-expressing embryos and zDisc1 morphants and mutants. (a-a′) 24 hpf control YFPh embryos co-injected with mcherry RNA. (b-b′) 24 hpf zDisc1h embryos co-injected with mcherry RNA. (c-c′) 24 hpf zDisc1h embryos co-injected with hDisc1 RNA. (d-d′) 24 hpf embryos injected with zDisc1 MO together with the p53 MO (see Materials and Methods). (e-e′) 24 hpf zDisc1h291 mutant embryos. The phenotype of embryos expressing shRNA from the zDisc1h promoter. (a-a′) lateral view of the head; (a′, b′, c′, d′, e′) dorsal view of the head after brain ventricle injection; (a′, b′, c′, d′, e′) lateral view of whole embryo. (D) Forebrain neurons stained for acetylated tubulin in 36 hpf embryos expressing the following RNAs: YFPh + mcherry RNA (a), zDisc1h + mcherry (b), zDisc1h + hDisc1 (c), injected with zDisc1 MO together with the p53 MO (d) or zDisc1h291 (e). Lateral views, anterior to left. (E) Muscle segments stained with phalloidin in 36 hpf embryos expressing the following RNAs: YFPh + mcherry RNA (a), zDisc1h + mcherry (b), zDisc1h + hDisc1 (c), injected with zDisc1 MO together with the p53 MO (d) or zDisc1h291 (e). Shape of muscle segments is indicated by dotted white lines. Black bracket: narrow brain ventricles; black asterisk: bent tail; black dotted lines: region between dotted lines was used for muscle segment analysis by phalloidin staining.

FIG. 4. Tissue-specific inhibition of zDisc1 expression. The ORF2654 shRNA targeted to zDisc1 (zDisc1h) was expressed in I-SceI (meganuclease)-derived F0 transgenic embryos, under control of the mir124 or ntl promoter (see Materials and Methods). (Aa) Diagram of the I-SceI-mir124-miR30-GFP-pA vector, where shRNA expression is driven by the CNS-specific mir124 promoter. (Ab) Relative expression of zDisc1 mRNA after inhibition by shRNA. Expression was quantified by qPCR in 24 hpf embryos expressing zDisc1h relative to embryos expressing a control shRNA, YFPh. Data was normalized to endogenous b-actin RNA. (Ac) Whole mount in situ hybridization analysis of GFP expression in in 48 hpf embryos expressing zDisc1h. (B) Phenotype of embryos expressing shRNA from the zDisc1h promoter. (a-a′) 24 hpf control YFPh embryos. (b-b′) 24 hpf zDisc1h embryos. (a, b), lateral view of the head; (a′, b′), dorsal view of the head after brain ventricle injection; (a′, b′), lateral view of whole embryo. (C) Differentiated neurons stained for acetylated tubulin in 36 hpf embryos expressing YFPh (a) or zDisc1h (b) under control of the mir124 promoter. (a, c) lateral view of the head; (b, d) dorsal view of the hindbrain. (D) Muscle segments stained with phalloidin in 36 hpf embryos expressing YFPh (a, b) or zDisc1h (c, d) under control of the mir124 promoter. (a, c) lateral view of the head; (b, d) dorsal view of the hindbrain. (Ea) Diagram of the vector I-SceI-ntl-miR30-CFP-pA, driven by the mesendoderm specific ntl promoter. (Eb) Relative expression of zDisc1 mRNA after inhibition by shRNA. Expression was quantified by qPCR in 24 hpf embryos expressing the zDisc1h relative to embryos expressing the control shRNA, YFPh. RNA levels were normalized to endogenous b-actin RNA. (Ee) Whole mount in situ hybridization analysis of CFP expression in in 24 hpf embryos expressing zDisc1h (F) Phenotype of embryos expressing shRNA from the ntl promoter. (a-a′) 24 hpf control YFPh embryos. (b-b′) 24 hpf zDisc1h embryos. (a, b), lateral view of the head; (a′, b′), lateral view of the head after brain ventricle injection; (a′, b′), lateral view of whole embryo. (G) Differentiated neurons stained for acetylated tubulin in 36 hpf embryos expressing YFPh (a, b) or zDisc1h (c, d) under control of the ntl promoter. (a, c) forebrain neurons, lateral view, anterior to left; (b, d) dorsal view of hindbrain, anterior to top. (H) Muscle segments stained with phalloidin in 36 hpf embryos expressing YFPh (a) or zDisc1h (b) under control of the ntl promoter. Shape of muscle segments is indicated by a dotted white line. Black bracket: narrow brain ventricles; black dotted lines: region between dotted lines was used for muscle segment analysis by phalloidin staining; black asterisk: bent tail; ac, anterior commissure; poc, postoptic commissure; so, supraoptic tract; tpc, tract of posterior commissure; rh, rhombomeres; white asterisk: defective supraoptic tract.
FIG. 3.

FIG. 4.
convergence and extension phenotypes. Similar to the strategy used to inhibit wnt5b expression, four short hairpins targeting \( z\text{Disc1} \) mRNA were designed and tested by expression from the \( b\text{-actin} \) promoter, using the \( p\text{TolDest-bactin-actin/intron-miR30-CFP-pA} \) vector (Fig. 3Aa; see Materials and Methods). Expression of two shRNAs targeting the open reading frame (ORF265 and ORF2911) in F0 transgenics resulted in a phenotype, while the others tested did not. The effect of the ORF2654 shRNA, which gave the strongest phenotype, was examined further. As shown by qPCR at 24 hpf, levels of \( z\text{Disc1} \) RNA were decreased by 70% on average (\( n = 8 \) independent experiments, 138 total control and 129 total experimental embryos examined) (Fig. 3Ab). This decrease may reflect mosaic expression of the shRNA, or targeting efficiency of the siRNA. Consistent with shRNA-mediated targeting, the \( z\text{Disc1} \) siRNA guide strand was detected, by northern analysis (Fig. 3Ac). A decrease in \( z\text{Disc1} \) RNA levels was confirmed by in situ hybridization analysis at 24 hpf (Fig. 3B).

In comparison to embryos expressing the control YFP shRNA (Fig. 3Ca,a) (\( n = 124, 79\% \) normal), embryos expressing the \( z\text{Disc1} \) shRNA ORF2654 showed narrow brain ventricles and a truncated forebrain (Fig. 3Cb,b’, black bracket) as well as a bent tail (Fig. 3Ca,b”, black asterisk) (\( n = 84, 19\% \) normal embryos). This phenotype was prevented by injection of human \( h\text{Disc1} \) mRNA, which does not contain the siRNA target sequence, together with the Tol2 constructs (Fig. 3Cc–c”) (\( n = 34, 73\% \) normal embryos), but not by injection of control \( m\text{cherry} \) mRNA. This result further demonstrates specificity of shRNA effects. The phenotypes observed in the \( z\text{Disc1} \) shRNA expressing embryos were similar to those we previously observed in the \( z\text{Disc1} \) morphant (Fig. 3Cd–d’; \( n = 159, 6\% \) normal) and mutant TILLED allele Disc1fh291 (Fig. 3Ce–e”). This conclusion was further confirmed by examining forebrain axon tracts at 36 hpf, after staining with anti-acetylated \( z\text{-tubulin} \). The supraoptic tract was reduced after \( z\text{Disc1} \) shRNA expression (Fig. 3Da–d”, white asterisk) (\( n = 10, 0\% \) normal embryos), but not after control YFP shRNA expression (\( n = 10, 100\% \) normal). This tract is similarly thin in \( z\text{Disc1} \) mutants or morphants (Fig. 3Dd,e, white asterisk).

The axon tract phenotype was prevented by injection of \( h\text{Disc1} \) RNA, but not by injection of \( m\text{cherry} \) mRNA, confirming specificity of the shRNA-mediated phenotype (Fig. 3Dc) (\( n = 10, 90\% \) normal embryos). Another characteristic phenotype after \( z\text{Disc1} \) LOF is presence of U-shaped muscle segments, likely linked to abnormal convergence and extension. Relative to YFP shRNA controls (Fig. 3Ea) (\( n = 10, 100\% \) normal), U-shaped muscle segments were observed at 36 hpf after expression of \( z\text{Disc1} \) shRNA, when segments were visualized by phalloidin staining (Fig. 3Eb) (\( n = 10, 100\% \) normal embryos). Abnormal muscle shape was prevented by injection of \( h\text{Disc1} \) mRNA together with the shRNA construct, but not by injection of \( m\text{cherry} \) mRNA (Fig. 3Ec), confirming specificity of the shRNA-mediated phenotype (\( n = 10, 90\% \) normal embryos). Similar phenotypes are observed for \( z\text{Disc1} \) mutant and morphant (Fig. 3Ed,e) [31].

Together, the data demonstrate that shRNAs can effectively mediate \( z\text{Disc1} \) LOF, resulting in a brain phenotype indistinguishable to that seen in the mutant, with a weaker brain phenotype than seen in the morphant. Muscle segment phenotypes were identical after shRNA expression to mutant and morphant. The rescue of these phenotypes by injected \( h\text{Disc1} \) RNA demonstrates specificity of shRNA effects and indicates that minimal off-target effects are associated with \( z\text{Disc1} \) shRNA expression.

**Tissue-specific targeting of \( z\text{Disc1} \) expression**

A key use for shRNAs in the zebrafish is to drive tissue-specific LOF, and so define the regional activities of a gene. Previous data, using \( z\text{Disc1} \) RNA specifically expressed in the CNS in a MO rescue assay, suggested that the axonal phenotype was associated with \( z\text{Disc1} \) activity in the neurectoderm. In contrast, the muscle segment phenotype was associated with mesendodermal and, less so, with neurectodermal activity also required for convergence and extension. We therefore tested the tissue-specific activities of \( z\text{Disc1} \) by expressing the \( z\text{Disc1} \) ORF2654 shRNA from the \( z\text{Disc1} \)-specific miR124 promoter [36] or the \( n\text{tl} \) promoter [37] that is active in the early mesendoderm and axial mesoderm (Fig. 4Aa, 4Ea).

After expression of \( z\text{Disc1} \) shRNA from the miR124 promoter, \( z\text{Disc1} \) mRNA levels decreased by an average of 75% at 24 hpf (Fig. 4Ab) (\( n = 3 \) independent experiments, 46 total control and 39 total experimental embryos examined). GFP expression, reflecting mir124 promoter activity, was uniform in the CNS with no detectable ectopic expression (Fig. 4Ac). Relative to controls expressing YFP shRNA from the mir124 promoter (Fig. 4Ba,a”) (\( n = 84, 93\% \) normal), after expression of \( z\text{Disc1} \) shRNA, a brain and weak tail phenotype was observed (Fig. 4Bb-b”) (\( n = 78, 21\% \) normal embryos). Forebrain and hindbrain axon tracts were thin when visualized with anti-acetylated \( z\text{-tubulin} \) at 36 hpf (\( n = 10, 0\% \) normal embryos) relative to normal tracts seen after YFP shRNA expression (\( n = 10, 100\% \) normal) (Fig. 4Ca-d). This is similar to the phenotype seen in the mutant and morphant, and after ubiquitous \( z\text{Disc1} \) shRNA expression (Fig. 3). These data are consistent with the majority of \( z\text{Disc1} \) expression in the brain and spinal cord. In contrast, muscle segments were normally shaped after neurectodermal LOF (Fig. 4Da,b) (\( n = 10, 100\% \) normal embryos; as also observed after YFP shRNA expression: \( n = 10, 100\% \) normal).

Conversely, when \( z\text{Disc1} \) shRNA was expressed from the \( n\text{tl} \) promoter, a 50% average decrease in RNA levels, relative to total \( b\text{-actin} \), was observed at 12 hpf, when \( n\text{tl} \) expression is maximal (Fig. 4Eb) (\( n = 3 \) independent experiments, 51 total control and 49 total experimental embryos examined). This lower level of knockdown likely reflects residual expression of \( z\text{Disc1} \) in the developing brain that was not targeted by the \( n\text{tl} \) promoter. CFP in situ hybridization revealed expression specifically in the notochord, indicating localized \( n\text{tl} \) promoter activity (Fig. 4Ec). After \( z\text{Disc1} \) expression from the \( n\text{tl} \) promoter, brain morphology and axon tracts were indistinguishable from controls (\( n = 10, 100\% \) normal embryos; after expression of the YFP shRNA: \( n = 10, 100\% \) normal) (Fig. 4Fa,a”,b,b’ and 4F). In contrast, the tail was truncated (\( n = 63, 22\% \) normal embryos) relative to embryos after YFP shRNA expression (\( n = 75, 87\% \) normal) (Fig. 4Fa,a”,b”). Consistently, thin and U-shaped muscle segments were observed (Fig. 4H) (\( n = 10, 0\% \) normal embryos) relative to effects of YFP shRNA expression (\( n = 10, 100\% \) normal). The data thus show a specific effect of the \( z\text{Disc1} \) shRNA on muscle segments when expressed in the mesendoderm.
These results indicate that zDisc1 shRNAs can be successfully targeted to specific tissues to elicit local LOF phenotypes.

Discussion

We have demonstrated efficient targeting of two endogenous zebrafish genes using shRNAs, at mid- to late embryonic stages. The genes targeted are of interest in our laboratory, with described mutant and morphant phenotypes. The findings demonstrate that RNAi methodology works efficiently and specifically in the zebrafish, and add new shRNA reagents for functional analyses.

The results show for the first time in zebrafish, that shRNA expression can decrease RNA levels significantly, at least by 70% in mosaic F0 transgenic embryos. In mammalian cells, a 70% knockdown of endogenous RNA levels by transfected shRNAs is considered reasonable (J. Doench and D. Root, Broad Institute, personal communication), and this LOF is clearly achievable in zebrafish. The siRNAs produced from the shRNAs most likely led to RNA cleavage, since the perfect match between siRNA guide strand and target used predicted RNA cleavage. Consistently, endogenous RNA levels decreased significantly. Additionally, qPCR using primers near the 3’ end of the zDisc1 gene or flanking the siRNA target site gave similar results, indicating that the entire RNA had been degraded (not shown). The F0 transgenic approach used to express the shRNAs is rapid and effective, but does give some mosaicism that may result in less than maximal LOF. In order to obtain uniform shRNA expression, inducible lines can be prepared. These may be necessary to allow viability after extensive LOF, and could use the Gal4/UAS strategy, or the various inducible Cre/lox approaches available. It seems very likely that inducible expression of shRNA can be achieved, but this remains to be tested.

The ability to prevent a phenotype by injection of cognate mRNA that does not bind the targeting siRNA, provided, for the first time, a stringent demonstration of specificity. The data further indicated that off-target effects of the shRNAs tested are small. In mammalian systems, rescue assays are generally not performed for shRNA-mediated LOF but are considered the “gold standard”. The zebrafish community established this stringent criterion to confirm MO specificity and we chose to require it of the shRNAs tested. Adopting this criterion for shRNAs would set a very high standard by the zebrafish community. For both wnt5b and zDisc1, phenotypes observed with the shRNAs were not as severe as those seen in the morphant, which required p53 to suppress necrosis, but more like the mutant phenotype. This suggests that toxic effects of shRNAs may be generally lower than those observed with MOs.

shRNAs that were able to target the genes tested mapped to both the 3’UTR, which shRNAs traditionally target, and to the open reading frame (ORF). This indicates that in zebrafish, shRNAs can be designed to either part of the gene. While four hairpin sequences per gene tested yielded 2 per gene that elicited a phenotype, testing more (perhaps 6) per gene may give more efficient knockdown or may be necessary for some genes.

Many tissue-specific promoters have been identified in zebrafish and our demonstration of tissue-restricted effects of shRNAs suggests that assaying shRNA effects in this way will be most useful. In these initial assays, we did not examine effects of shRNA past 24 hpf, into later stages of organogenesis and function, and persistence of shRNA effects in larvae, juveniles, and adults, remains to be addressed.

What else has not been done in this analysis? We have not looked for phenotypes at blastula stages, because our test genes do not show a phenotype before the end of gastrulation. It is not clear whether shRNA processing activity (particularly Ago2) is limiting in the early zebrafish, as it appears to be in Xenopus, and this will need to be tested. However, since MOs are effective in the early embryo, this activity is less critical than effectiveness of shRNAs in older embryos.

We do not have a large-scale measure of how frequently genes can be efficiently targeted by shRNAs. However, in our lab, we have successfully targeted 5 genes (of 6 tested) by shRNA expression (wnt5b, zDisc1, aldoa, kf22, and myoIIA), although the extensive assays presented in this article have not yet been performed on some of these (HLS, unpublished, constructs available on request). In the Dong et al., 2009 study, the chordin gene was targeted by shRNA injection and the gata-1 gene was targeted by shRNA expression from the CMV promoter, but RNA quantification and rescue assays were not performed. Together, these data suggest that shRNAs may be able to target many genes in the zebrafish. However, a more extensive assessment of targeting efficiency and off-target effects will need to come from a future study.

In sum, we have presented results that support usefulness of shRNAs for zebrafish LOF analysis, suggesting that this approach could be used to develop an RNAi resource that would benefit the large group of researchers in the community.

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No competing financial interests exist.

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