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Manipulating Galectin Expression in Zebrafish (*Danio rerio*)

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Abstract

Techniques for disrupting gene expression are invaluable tools for the analysis of the biological role of a gene product. Because of its genetic tractability and multiple advantages over conventional mammalian models, the zebrafish (*Danio rerio*) is recognized as a powerful system for gaining new insight into diverse aspects of human health and disease. Among the multiple mammalian gene families for which the zebrafish has shown promise as an invaluable model for functional studies, the galectins have attracted great interest due to their participation in early development, regulation of immune homeostasis, and recognition of microbial pathogens. Galectins are β -galactosyl-binding lectins with a characteristic sequence motif in their carbohydrate recognition domains (CRDs), that constitute an evolutionary conserved family ubiquitous in eukaryotic taxa. Galectins are emerging as key players in the modulation of many important pathological processes, which include acute and chronic inflammatory diseases, autoimmunity and cancer, thus making them potential molecular targets for innovative drug discovery. Here, we provide a review of the current methods available for the manipulation of gene expression in the zebrafish, with a focus on gene knockdown [morpholino (MO)-derived antisense oligonucleotides] and knockout (CRISPR-Cas) technologies.

Keywords

galectins; zebrafish; morpholino; CRISPR-Cas; gene expression; microinjection

1. Introduction

The zebrafish (*Danio rerio*) is a widely used model organism to study a broad spectrum of human normal and pathological processes, including development, autoimmune, neoplastic and infectious disease (1–3). The growing interest in using zebrafish as a genetically

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tractable model system is due to its multiple advantages relative to the mammalian models, which include high fecundity, external fertilization, rapid development, transparent embryo, low maintenance cost, easy genetic manipulation and an extensive collection of mutants currently available (4–6). Zebrafish and human genomes have been shown to share highly conserved structural and functional features (7). Hundreds of zebrafish genes have been identified and its full genome sequence is now available online (<http://www.ncbi.nlm.nih.gov/genome/guide/zebrafish/>). Moreover, *D. rerio* exhibits some higher-level behaviors previously observed only in mammals, such as memory, conditioned responses and schooling (8,9). In recent years, the zebrafish model has proven useful to gain new insight into the functional aspects of protein-carbohydrate interactions, such as those mediated by galectins (10–12). Galectins are β -galactosyl-binding lectins, which share primary structural homology in their carbohydrate-recognition domains (CRDs) (13). They are classified into three major structural types: (i) proto-type galectins, which contain one carbohydrate recognition domain (CRD) and form homodimers; (ii) chimera-type galectins, which have a single CRD and can oligomerize forming trimers and pentamers; (iii) tandem-repeat-type galectins, which are comprised of two CRD joined by a linker peptide (14). Galectins participate in a multitude of biological processes, such as development, apoptosis, tumor metastasis and regulation of immune responses (15–19). All three structural types of galectins have been identified and characterized in various tissues, plasma and mucus of teleost fish, such as zebrafish (12,20–23). Among the various methodologies for elucidating the biological role(s) of a particular protein of interest, disruption of gene expression represents a useful approach. Multiple strategies have been developed to modulate gene expression at a genetic or epigenetic level (Table 1). Several of these methodologies have been recently applied to functional studies of galectins (10,24,25).

Morpholinos (MOs) are antisense oligonucleotides derivatized with morpholine rings to increase stability, and designed to anneal close to the start codon of the selected gene and disrupt its translation, or to the splice acceptor sequence to induce incorrectly spliced mRNA (26). The MOs do not degrade their mRNA targets, but (i) block mRNA translation by targeting the 5'-UTR through the first 25 bases of coding sequence (10), or (ii) alter the translation modifying pre-mRNA processing by targeting splice junctions or regulatory sites (Fig 1) (27,28). Thus, the “morphant” phenotype results from disrupted protein expression levels. Although their effects are only transient, MOs are relatively long-lived inside the cell upon delivery, with the effect on pre-mRNA splicing or translation lasting for up to five days following microinjection. This technique allows for the rapid manipulation and interrogation of complex processes such as embryonic development, organ formation, innate immunity, and host-pathogen interactions (3,10,28). However, since the effects of mRNA suppression by MO oligos are temporary and not inheritable, and there is no suitable way to deliver them systemically in adult fish, this system is not suitable for more integrated approaches. For inheritable genetic modulation, a set of other techniques has been introduced in the last 15 years (Table 1).

CRISPR-Cas is a genome editing approach based on the prokaryotic immune system. By using a segment of virus-derived DNA from its CRISPR array and processing to crRNA targeting the viral genome, the system leads to the inactivation or degradation of targeted

DNA by the CAS-crRNA complex (29). Cas9 nuclease transgenically expressed in vertebrates is active and able to cleave target DNA when directed by a short guide RNA (gRNA), which at its 5'-end contains 20 base pairs of complementary target DNA (30–32).

Here we describe in detail the use of MOs and CRISPR-Cas to modulate galectin expression in the zebrafish model to elucidate their functions in development and immunity. The MOs Drgal1-L1-MO and Drgal1-L2-MO were designed to block translation initiation based on 5'-UTR sequences of Drgal1-L1 and Drgal1-L2, respectively (Table 2). MOs are usually validated by *in vitro* blockade of the corresponding protein expression using the TNT SP6 Coupled Rabbit Reticulocyte Lysate System. Following MO-oligo injection of zebrafish embryos, expression of the corresponding protein is inspected by whole mount antibody staining. Potential phenotypes are inspected under microscope and by whole mount antibody staining of a specific marker. Zebrafish embryos injected with validated Drgal1-L2-MO (Fig. 2A) show dramatically reduced Drgal1-L2 expression as observed by whole mount antibody staining (Fig. 2C). Drgal1-L2 is strongly expressed in the notochord during early embryogenesis and Drgal1-L2 knock-down results in a characteristic phenotype with a short and bent tail (Fig. 2C). Microscopically, the phenotype exhibits disrupted muscle fiber organization as observed by whole mount immunostaining with the F59 antibody (monoclonal anti-myosin antibody), a marker for slow muscle (Fig. 2E).

2. Materials

2.1. Special Instruments

1. Microinjection system: Pico-injector PLI-100 (Harvard Medical Systems Research Products, U.S.A). It is connected to compress nitrogen gas and to adjust the air pressure for reagent delivery. Modulator M152 (Narishige, Japan).

2.2. General Instruments

1. Microscope Stemi 2000 (ZEISS, USA).
2. Microcentrifuge (Beckman Coulter, Germany).
3. Incubator (Fisher Scientific, USA).

2.3. General Materials

1. Crossing Tank Kit (Thoren Aquatics Housing Systems, USA).
2. Dissecting forceps.
3. Micropipettes and disposable tips.
4. PBS (Bio-RAD, USA).
5. 15 ml and 50 ml Conical tube (BD, USA).
6. Eppendorf tube (Thermo Scientific, USA).
7. ECL Plus detection kit (Amersham Biosciences, USA)

8. Polyclonal antibodies to Drgal1, 3 and 9 were custom-produced (Thermo Scientific, USA).

2.4. Reagents for disruption of gene expression

2.4.I. Morpholino (MO) oligos

Synthesis of MO oligos: MOs for each gene are designed based on the gene sequence to obtain **translational blocker** (designed to bind close to start codon disrupting the translation) or **splice blocker** (designed to bind to splice acceptor sequence to induce incorrectly spliced mRNA). For example, Drgal1-L1-MO and Drgal1-L2-MO shown in Table 2 are translational blockers of the Drgal1-L1 and Drgal1-2, respectively. These MOs were custom synthesized by Gene Tools (www.gene-tools.com) (Note a).

Validation of gene-specific MOs by *in vitro* specific blocking of protein expression

1. Prepare an expression construct for a particular gene for *in vitro* protein expression. For example, we cloned Drgal1-L2 with 5'-UTR sequences into a pCS2⁺ vector (a gift from D. Turner, R. Rupp, J. Lee, and H. Weintraub, Fred Hutchinson Cancer Research Center, Seattle, WA) to obtain the pCS-Drgal1-L2 construct. In this construct, a 27-nucleotide untranslated 5' leader (derived from the *Xenopus* β -globin mRNA 5'-end) is introduced between the SP6 promoter and the Drgal1-L2 insert. The pCS-Drgal1-L2 construct is expected to generate protein when added to a cell-free protein synthesis system that is initiated by SP6 RNA polymerase.
2. Perform *in vitro* direct translation of Drgal1-L2 from pCS-Drgal1-L2 plasmid DNA (0.5 μ g) in the presence or absence of the Drgal1-L2-MO using TNT SP6 Coupled Rabbit Reticulocyte Lysate System (Promega, Madison, WI) according to manufacturer's instructions. To detect the translated product, use [³⁵S]methionine with the methionine free amino acid mixture.
3. After completion of the reaction, analyze the translated product on 15% SDS-PAGE followed by autoradiography. As shown in Fig 2A, Drgal1-L2-MO blocked translation of Drgal1-L2 protein.

2.4.II. sgRNA Construction for CRISPR-Cas

Materials

1. CRISPR-Cas system: Single guide RNA (sgRNA) expression vector pDR274 and Cas9 nuclease expression plasmid pMLM3613 (Addgene, USA). Selected 20 variable nucleotides to base pair with a target genomic DNA sequence will be inserted at its 5' end of pDR274 to produce a short 102 nucleotides guide RNA (See below).
2. Gene-specific oligos: One pair of oligos for each target genes is designed using ZiFiT Targeter (Note b). The single strand oligos are synthesized (Sigma, USA), mixed at 95°C for 10 min, then cool down gradually at room temperature to allow annealing. The annealed oligos contain unique overhangs on each site for directional cloning into BsaI-digested pDR274 (See *Preparation of sgRNA*).

3. QIAprep Spin Miniprep Kit/QIAfilter Plasmid Midi Kit (Qiagen, USA).
4. MinElute Gel Extraction Kit (Qiagen, USA).
5. Rapid DNA Ligation kit (Roche, Germany).
6. DH5 α Competent cells (Invitrogen, USA).
7. SOC medium (Sigma, USA).
8. Restriction enzymes: BsaI, PmeI, DraI (New England Biolab, USA).

Preparation of sgRNA

1. Digest the pDR274 with BsaI for 1 hour at 37°C. The linearized fragment is separated in an agarose gel and recovered using Gel Extraction kit.
2. Mix the 50 ng of linearized vector with annealed targeted sequences (1:3 molar ratio) in 20 μ l of ligation solution from Quick Ligation kit and incubate for 5–15 min at room temperature. The ligates can be used immediately or stored at –20°C for future use.
3. Add 2 μ l of the above ligate in 50 μ l of DH5 α competent cells and incubate on ice for 20 min. Heat-shock the mixture for 45 sec in 42°C water bath, and incubate on ice for at least 2 min. Afterward, add 500 μ l of SOC medium and incubate the bacteria for 1 hour at 37°C. Spread the bacteria onto LB agar with 50 μ g/ml of kanamycin for overnight incubation at 37°C.
4. Inoculate each colony into one 15 ml tube with 3 ml of LB broth supplemented with 50 μ g/ml of kanamycin for overnight incubation at 37°C. Extract plasmid using Mini-prep kit for sequence analysis. Stock colonies in 30% glycerol for future use. Select the colony with expected sequence for Midi-prep.
5. Prepare sgRNA using DraI-digested sgRNA expression vector and the MAXIscript T7 kit as manufacturer's recommendation; prepare Cas9 mRNA using PmeI-digested Cas9 expression vector and mMESSAGE mMACHINE T7 ULTRA kit. Purify both RNA by LiCl precipitation and use for microinjection.

3. Methods

3.1. Preparation for microinjection

1. Morpholino oligo: Aliquots of 5 mM MO stock solution are prepared by adding RNase-free dH₂O (or Danieau's solution) and stored at –20°C. Before use, mixed with Danieau's solution and 1% phenol red dye (final 0.1%, Sigma P-0290) to make 0.5–2 mM working solution (Note c).
2. Danieau's solution: 58 mM NaCl, 0.7 mM KCl, 0.4 mM MgSO₄, 0.6 mM Ca(NO₃)₂ 5.0 mM HEPES, pH 7.6.
3. Phenol red dye: 1% Phenol red (Sigma) in dH₂O.
4. Agarose injection bed: Prepared 1% agarose (Sigma, USA) into 90 mm Petri dish, lay carefully several glass micropipettes parallel onto agarose surface to make the

troughs. After cooling down, discard micropipettes and the injection bed is ready for use or store at 4°C.

5. Glass microinjection needles (Tritech Research, USA) (Note d and e).

3.2. Embryo Preparation

1. The day before spawning, separate adult male and female zebrafish into spawning tanks with divider. Set up 2–4 pairs of animals for each experiment.
2. In the morning of the day for injection, remove the divider to allow mating. Usually the females will begin to lay eggs within 15–45 min.
3. Within 30 minutes of spawning, collect embryos into a fish tray, wash thoroughly several times with fresh water.
4. Place embryos onto the injection bed. Carefully arrange the eggs on the troughs of injection bed.

3.3. Microinjection

1. Prepare the working solution [e.g. morpholino solution (see section 2.4.1.)]. Keep working solution at room temperature. Centrifuge for 30 sec at room temperature and use the supernatant for microinjection.
2. Set up the microinjector: Attach the microinjection needles to the needle holder of the microinjector. Place the injection bed on the stage, and adjust the modulator to appropriated position for injection. Load the microinjection needle with 2 µl of working solution on it. (Optional: Test injection in a few embryos. Adjust pressure values if needed – Note f).
3. Penetrate the needle to the yolk, and deliver 1 nl of working solution. The flow is visible due to the phenol red added to the working solution (Note g). Retract the pipette and repeat on remaining embryos.
4. After microinjection, wash the embryos into a Petri dish with fresh water and place them in incubator at 28°C.
5. Evaluate the injected embryos under the microscope a few hours after injections, and remove the damaged embryos. If the water in the dish is cloudy, transfer the embryos to a new dish with fresh water.
6. After 24 hours, dechorionate the embryos under microscope using forceps.
7. Visually inspect embryos for any potential phenotypes. Further investigation to verify the effect of MO injection on protein expression are done by Western blot and whole mount antibody staining (see below). Following Drgal1-L2-MO injection, we performed whole mount immunostaining with Drgal1-L2 antibody (to validate the Drgal1-L2-MO by in vivo blocking of Drgal1-L2 protein expression) and F59 antibody (to observe potential defects in muscle fiber organization). Once we confirmed the phenotype, we then rescued the defect by injection of embryos

with Drgal1-R2 cRNA (for ectopic expression of Drgal1-L2) along with Drgal1-L2-MO.

3.4. Whole mount antibody staining

1. Fix embryos with 4% paraformaldehyde for 1 h at RT, wash twice with PBS-Tween for 5 min each, and soak in cold acetone for 10 min at -20°C .
2. Wash embryos twice with PBS-Tween 5 min each followed by washing once with 0.1% BSA/1% dimethyl sulfoxide/PBS (BDP) for 5 min.
3. Incubate with avidin (Vector Laboratories, Burlingame, CA) (4 drops/ml) in blocking buffer (10% of goat serum in BDP) for 30 min at RT.
4. Wash the embryos twice with BDP for 5 min each and incubate with 1:10,000 dilution of anti-Drgal1-L2 antibody in blocking buffer containing biotin (Vector) (4 drops/ml) overnight at 4°C .
5. Wash the embryos three times for 30 min with BDP, followed by incubation with diluted (1:1000) biotin-labeled secondary antibody (goat anti-rabbit IgG) (Vector) in BDP for 1 h at RT.
6. Wash the embryos three times for 30 min with BDP and incubate with 1:1 diluted avidin-biotin complex solution (Vector) for 30 min at RT.
7. Wash the embryos three times for 30 min in BDP and develop color with DAB substrate (Vector) according to the manufacturer's protocol.

3.5. Rescuing the phenotypes by co-injection of embryos with Drgal1-L2 cRNA and Drgal1-L2-MO (Ectopic expression of native Drgal1-L2 on Drgal1-L2-MO injected embryos)

1. To determine if the ectopic expression of native Drgal1-L2 rescues the phenotypes observed, Drgal1-L2 mRNAs were synthesized from a pCS-Drgal1-L2 construct using an *in vitro* transcription kit (mMESSAGE mMACHINE SP6, Ambion, Austin, Texas). In this construct, 27 nucleotides derived from the *Xenopus* β -globin 5'-UTR were used to replace the Drgal1-L2 5'-UTR as the Drgal1-L2-MO was specifically targeted to the Drgal1-L2 5'-UTR. Thus, the Drgal1-L2-MO would only inhibit expression of the endogenous Drgal1-L2, but not of the injected Drgal1-L2 mRNA.
2. The integrity of the transcribed RNA was examined on formaldehyde 1.5% agarose gels.
3. For microinjection, mRNA was dissolved in distilled water to a final concentration of 100 $\mu\text{g}/\text{ml}$. The transcribed RNA solution (approximately 2 nl) was microinjected into the cytoplasm of zebrafish embryos at the one- or two-cell stage, and subsequently, the Drgal1-L2-MO was microinjected into the yolk sac.
4. After 24 h, visually inspect embryos for any phenotypes and perform whole mount immunostaining with Drgal1-L2 and F59 antibodies as described before.

4. Notes

- a. GeneTools, LLC (Philomath, OR; <https://oligodesign.gene-tools.com/request/>) provides a free design service that does not require prior knowledge of the start codon of the mRNA target. Despite being very user-friendly and not being held to a mandatory order online from the same site, this software provides very limited sequence design and analysis option to the user. In that regard, Vector NTI® software (Life Technologies: Grand Island, NY) offers an excellent alternative to MO oligo design provided the user has previous knowledge of the target sequence. Additionally, MOorpholino DataBase (<http://www.morpholindatabase.org/>) is a public web-based database with more than 700 morpholinos to date against zebrafish genomic sequences.
- b. The ZiFiT Targeter website (<http://zifit.partners.org/>) offers an online option to identify potential target sites for the described CRISPR-Cas system. By default, the sequences that meet the following criteria: 5'-GG- (N)18-NGG-3' will be identified. ZiFiT Targeter will analyze the user-input sequences and returns a list of recommended target sites and sequences of oligonucleotides that need to be synthesized for cloning into the pDR274 vector.
- c. The first step in using a new MO is to determine the optimum delivery dose. Thus, MOs can be initially injected at different doses and the dosages are increased or decreased to optimize the phenotype to toxicity ratio. Working stocks of MOs were prepared to use as near-isotonic solutions for the zebrafish (i.e., Danieau solution).
- d. Break off the tip of the microinjection needle with your forceps under the microscope, so that they have an open tip.
- e. Prepare the microinjection needles by heating and pulling borosilicate glass capillary tubes (Narishige, Japan) in a micropipette puller (Sutter Instrument CO., model, P-97). Store the needles in a Petri dish on top of small amount of clay or adhesive tape.
- f. Alternatively, “freehand” injection can be practiced without a micromanipulator in a normal Petri dish. It is more robust but time-consuming as proper embryo orientation and microinjection technique is required.
- g. The phenol red color from the injected sample will not change if sample delivered into the embryonic cells. A shift to pink in phenol red color will indicate the microinjection was delivered outside the embryo.

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Figure 1.

Genetic modulation of galectins by morpholinos and CRISPR-Cas microinjection. MO-oligos may be designed to target an exonic sequence, thus blocking mRNA translation. Alternatively, MO oligos may be designed to target an exon-intron junction, preventing the maturation of the targeted pre-mRNAs. MO-oligos and CRISPR transcripts are microinjected into the embryo yolk. In the case of morpholinos (orange arrows), the microinjection will result in a mutant phenotype either/both in embryo and/or adult. When injecting CRISPR transcripts, the screening of the microinjected embryos will result in the selection of the mutant genotypes (usually heterozygous). After crossing individuals with mutant genotypes, fish with mutant phenotypes (homozygous) will be selected from the resultant progeny.

Figure 2. Validation of Drgal1-L2-MO

(A) *In vitro* blocking of Drgal1-L2 protein in a rabbit reticulocyte system. SDS-PAGE/autoradiography analysis of the *in vitro* labeled Drgal1-L2 translation products. Lane 1: translation reaction in the absence of Drgal1-L2-MO (positive control); lanes 2 and 3: reactions in the presence of 170 ng and 340 ng of Drgal1-L2-MO, respectively. (B) Whole mount immunostaining of an uninjected embryo probed with anti-Drgal1-L2 antibodies (positive control). Lateral and dorsal view of a 27 hours post fertilization (hpf) embryo. Drgal1-L2 was seen expressed in the notochord. (C) *In vivo* blocking of Drgal1-L2 protein as shown by whole mount immunostaining of an embryo injected with Drgal1-L2-MO, and probed with anti-Drgal1-L2 antibodies. Lateral and dorsal view of a 27 hpf embryo. (D&E) Whole mount immunostaining of an uninjected embryo (D) and an embryo injected with Drgal1-L2-MO (E) probed with F59 antibody. Lateral and dorsal view of 48 hpf embryos. In E, muscle fibers were found disorganized.

Table 1

Reverse genetic tools and transgenic methodologies for disruption of gene expression

Procedure	Mechanism	References
Morpholinos Photomorpholinos	Custom-designed MO-oligonucleotides block translation or pre-mRNA processing of the targeted gene.	Summerton 2009 (33) Nasevicius et al., 2000 (26) Tallafuss et al., 2012 (25)
TILLING (Targeting Induced Local Lesions in Genomes)	Formation of DNA hetero-duplexes between multiple alleles amplified by PCR and cleaved by single stranded nucleases. Mutagenized products are screened by size.	McCallum et al., 2000 (34) Wienholds et al., 2003 (35)
Heat Shock Promoters	Targeted gene is engineered to be under inducible control of a heat shock promoter. Transgene expression induced by heat shock.	Halloran et al., 2000 (24) Hardy et al., 2007 (36)
Targeted Cell Ablation	Expression of bacterial nitro-reductase (NTR) under a promoter that controls the proliferation and survival of a particular cell type.	Curado et al., 2007 (37)
ZNFs (Zinc-Finger Nucleases)	Engineered DNA-binding endonucleases to break in the targeted double stranded-DNA for specific site mutagenesis.	Ekker 2008 (38)
Gal4/UAS	Targeted gene is engineered to be under inducible control of UAS promoter. Transgene expression induced by tissue-specific Gal4/UAS system.	Scheer et al., 1999 (39) Scott 2009 (40)
TALEN (Transcription Activator-Like Effector Nuclease)	Engineered TAL effector DNA domain fused to a DNA restriction site binds and cleaves targeted gene, introducing a custom-designed mutagenesis site.	Clark et al., 2011 (41)
CRISPR-Cas (Clustered Regularly Inter spaced Short Palindromic Repeats)	Engineered CRISPR system with custom-designed Cas9 nuclease directed by "guide RNA" to introduce single break in the targeted double stranded DNA sequence.	Jinek et al., 2012 (29) Hwang et al., 2013 (32)

Table 2

Verified morpholino-modified antisense oligonucleotides.

Designation	Sequence	Reference
DrGal1-L1-MO	5'- TCTATAAGCACAGTCTCATGCA -3'	Ahmed et al. (2009) (10)
DrGal1-L2-MO	5'- ATAAGCACACCGGCCATTTGACGT -3'	