Zebrafish Functional Genomics Development at UW-Stout

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Abstract
Since the completion of the human genome sequencing project, morpholino phosphorodiamidate oligonucleotide (MO) knockdown in zebrafish has been increasingly used to elucidate human gene function. As part of the effort to expand the functional genomics screening capacity at the University of Wisconsin-Stout, this project investigated MO microinjection techniques, embryo production, and brine shrimp survival. One- to two-cell embryos injected with the pigment-inhibiting tyrosinase MO were observed at 48 hours post fertilization for pigmentation. Injection efficiency was calculated by dividing the number of zebrafish lacking pigment by the total number of injected zebrafish. To obtain sufficient embryos for MO experiments, the effects of a dry food diet and live (brine shrimp) food diet on embryo production were analyzed. Effects of a yeast diet on brine shrimp survival were also studied to provide zebrafish with healthy brine shrimp. Results included a 92% injection efficiency, greater embryo production with the dry food diet, and increased brine shrimp survival with a yeast diet. This work provided an important foundation in the development of a reverse-genetic screen for future students.

Introduction

The sequencing of the human genome has yielded thousands of potential genes with unknown function (HGMIS, 2005). Due to the similarities in genetic composition across species and technological advancements, the functions of these genes can be determined relatively quickly by screening them in model organisms, such as the zebrafish (Pickart et al., 2004; Dahm & Geisler, 2006). Common screening tools for deciphering gene function include mutagenesis and forward- and reverse-genetic screens. Mutagenesis screens mutate genes and determine gene function based on what is observed (the phenotype). Forward-genetic screens generally consist of exposing the organism to a chemical, observing the phenotype, and ascertaining the gene affected. Reverse-genetic screens utilize agents such as morpholino phosphorodiamidate oligonucleotides (MOs) to alter specific gene expression and phenotype and provide insight to gene function. All three of these methods have been used in zebrafish.

With the genome of the zebrafish approximately 70% sequenced (TDRSP, 2006), MO technology is becoming a popular antisense tool (targets the matching nucleic acid sense strand). MOs are synthetically-made, neutral, nucleic acid analogs that can be ordered online from Gene Tools, LCC (Philomath, OR) (Pickart et al., 2006; Deiters & Yoder, 2006). Structurally, MOs have a morpholine group in place of the ribose sugar and a phosphorodiamidate (not phosphodiester) backbone. As a result of these properties, MOs are soluble, bind to specific messenger ribonucleic acid (mRNA) sequences via Watson-Crick base pairing, and are not degraded as easily as some antisense mechanisms. Functionally, MOs work by binding to and

preventing mRNA from translating into protein, thereby inhibiting gene expression. The ~25 nucleotide-long sequence of a MO is designed to bind to the 5' end of the mRNA (to block cellular translational machinery) and, thus, generally contains the translational start codon AUG. Alternatively, MOs can be designed to bind to splice sites to alter or prevent intron splicing, which inhibits the production of normal protein. Gene sequence information for MO design can be obtained from such websites as The Zebrafish Information Network (www.zfin.org), Ensembl Zebrafish (www.ensembl.org/Danio_rerio), and others at Zebrafish Genome Resources (www.ncbi.nlm.nih.gov/genome/guide/zebrafish). Once designed, MOs can be microinjected into an embryo (typically at the one to two-cell stage) and the effects analyzed hours to days afterward. The ability to quickly assess gene function is one of the many advantages of using zebrafish.

A native to the Ganges River in India, the ~2-inch black-striped zebrafish is an emerging model organism (Badman et al., n. d.). Named *Danio rerio* in 1822 by Francis Hamilton-Buchanan, the zebrafish's potential in genetic research was not recognized until the 1970s by the viral geneticist and fish hobbyist George Streisinger, who highlighted the advantages of zebrafish in the journal *Nature* (Dahm, 2006). Indeed, the zebrafish has many favorable attributes (Badman et al.). They are easy to maintain at low labor and cost. Each female can produce hundreds of eggs per week, which allows for plentiful data collection. The embryos (eggs fertilized by sperm) develop outside the females in transparent sac-like chorions, so their development can be readily observed. Easily manipulated embryos allow such technologies as MO knockdown to determine gene function. Compared to a human gestation of nine months, zebrafish's development time of 48 hours equates to quick analysis. Importantly, the conservation of genes and biological processes between humans and zebrafish increases the relevancy of data collected. Research fields utilizing the zebrafish are very diverse, from the study of organ development (organogenesis), nerves, blood vessels (angiogenesis and vasculargenesis), and bone (osteogenesis) to cancer research, toxicological assays, and therapeutic drug screening (Pickart et al., 2004; Badman et al.). Today, over 500 laboratories utilize the zebrafish (Dahm), including the $10-million, 5000-square foot facility in Bethesda, Maryland that has over a half million zebrafish capacity (Agres, 2003).

In spring 2005, the University of Wisconsin-Stout's Zebrafish Laboratory was started by Assistant Professor Michael A. Pickart as part of the Genomics Technology Access Core (GTAC) facility to enable students to conduct hands-on, cutting-edge biological research. Methods for chemical screening were established that fall (Hoage, 2005). The following spring, techniques for using MO microinjection technology were developed and tested (this article).

Initiated in support of UW-Stout's growing Biotechnology curriculum and the GTAC, this project was the first student-lead effort to develop the functional genomics capacity of the Zebrafish Laboratory. As diagramed in Figure 1, the project consisted of three parts: MO microinjection techniques, embryo production, and brine shrimp (*Aretemia*) survival. Microinjection techniques referred to the manipulation of and efficient MO injection into zebrafish embryos. To establish microinjection techniques and measure injection efficiency, the tyrosinase MO was injected into 1- to 2-cell embryos. Similar to the human disease condition oculocutaneous albinism (OCA1) characterized by a lack of pigment in the eyes, hair, and skin, the tyrosinase MO prevented the formation of the tyrosinase protein and the production of black pigment (melanin) in zebrafish (Pickart et al., 2004). Injection efficiency was easily measured by dividing the number of zebrafish lacking pigment by the total number of injected zebrafish at 48 hours post fertilization (hpf). The effects of a dry food diet and live (brine shrimp) food diet on

embryo production were analyzed to obtain sufficient embryos for injection experiments. To provide adequate amounts of healthy brine shrimp, the effects of a yeast diet on brine shrimp survival were studied. Ideas for the live food diet and yeast diet came from Westerfield (2000) and Cleveland et al. (1998), respectfully.

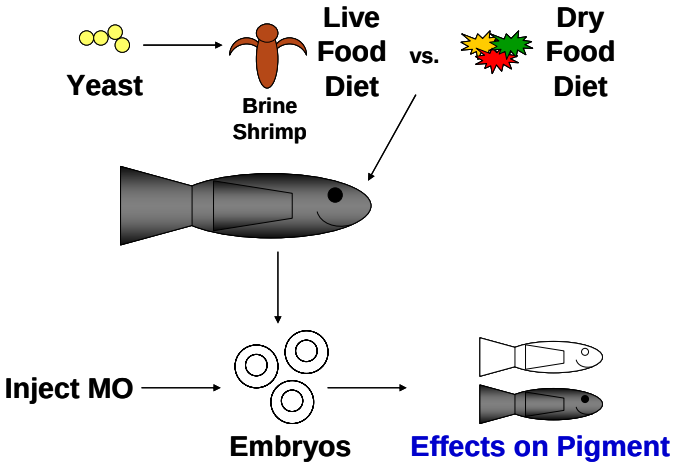

Figure 1. Overview of project.

Methods

Brine Shrimp Survival

Sanders brine shrimp eggs (1.5 g; 85% hatch, Ogden, UT) were grown in ProAquatica funnel hatchers (Gotha, FL) containing 1.6 L of 28.5°C, pH 8, 20-parts per thousand (ppt) Instant Ocean salt (Aquarium Systems, Mentor, OH) water and aerated at a medium flow rate. They were fed 0, 20, or 40 drops of 2 mg/mL Fleischmann's active dry yeast (Fenton, MO) solution on Days 0, 1, and 2. Two to six 1-mL samples of brine shrimp were counted on Days 1, 2, and 3 in Petri dishes with a microscope. The “day’s count” was the average number of live brine shrimp. Percent survival was calculated by dividing the day’s count by Day 1’s count. For detailed protocols on hatching, growing, and harvesting brine shrimp, contact the author at hoaget@uwstout.edu for a copy of *Brine Shrimp Husbandry* (2006).

Embryo Production

Dry Food Diet

One batch of 50 zebrafish was fed a dry food diet consisting of TetraMin flake food (Tetra, Blacksburg, VA). Males and females were combined into one 10-gallon tank every other week on Monday evening of “collection week.” Fifteen minutes before the automatic lights turned on at 9 AM (“dawn”), nine randomly selected fish were placed in each of four breeding boxes containing fish water (28.5°C, 59.9 mg/L Instant Ocean salt).

Live Food Diet

The other batch of 50 zebrafish was fed a live food diet consisting of dry flake food and 6 mL (1:4 diluted) brine shrimp daily. Males and females were combined as described above. Fifteen minutes before dawn, six females and three males were placed in each of four breeding boxes. The methods were similar to those used by Westerfield (2000).

All fish were on a 14 hour light: 12 hour dark cycle.

Embryo Collection

At 9:00 AM, 9:15 AM, 9:30 AM, and 12:00 PM, embryos were collected with a sieve, transferred with salt water to a labeled Petri dish, and manually counted. Designed and constructed by Dr. Pickart, the breeding boxes allowed for easy and inexpensive embryo collection. Consisting of two 8-cup, deep-dish containers and lid (The Glad Products Company, Oakland, CA), each breeding box had an inner compartment and outer compartment with cover (i.e., lid). The inner compartment had the bottom one inch removed and a 5.5 inch x 7.75 inch rectangular piece of polyethylene mesh (3 mm x 3 mm squares; InterNet, Inc., Minneapolis, Minnesota) stapled in it to form a barrier through which only embryos and water could pass. This inner compartment was placed into the outer compartment and filled with salt water. Fish were transferred to the inner compartment for embryo production. To collect the embryos, the inner compartment containing the fish was transferred to another outer compartment containing salt water. Water in the outer compartment containing the embryos was poured through a sieve. A squirt bottle was then used to transfer the embryos to a Petri dish containing salt water. Having a diameter of ~ 1.5 mm, the embryos were counted with the unaided eye. The analysis was conducted for one month. For detailed methods of embryo collection and breeding box construction, contact the author for a copy of *Basic Zebrafish Husbandry for the Classroom* (2006).

MO Microinjection Techniques

Injection needles were made from 1-mm diameter capillary tubes (Fisher Scientific, Raleigh, NC) using a P-97 Flaming/Brown micropipette puller (Sutter Instruments, Novato, CA). Microloader pipette tips (Fisher Scientific) were used to load the MO into the needles. After the end of the needle had been snipped with a Jewelers forceps (Fisher Scientific), it was calibrated with a capillary microloader pipette tip (Fisher Scientific) as described in *Where Art Thou Stripes, Zebrafish?* (Hoage[3], 2006). Briefly, the capillary was placed over the end of the needle, and MO was injected twice at one second each. The volume distance was measured in millimeters and divided by two. That number and the *Injection Time Standardization Table* were used to determine time (in milliseconds x 10) of injection. For injection, embryos were transferred to an agar-coated injection chamber plate. Tyrosinase MO was injected at 3, 6, or 9 nanograms (ng) into thirty 1- to 2-cell stage embryos with a pico-injector (Warner Instruments, Hamden, CT). The tyrosinase MO was made by Gene Tools, LLC and contained the sequence 5'-GAGACATGATGATGAAGAGTCGAGG-3' (Pickart et al., 2004). Non-injected embryos were used as controls. Unfertilized and dead embryos were removed before counting. Injection efficiency was calculated by dividing the number of reduced-pigment embryos by the total number of living embryos at 48 hpf. Reduced pigment was defined as at least 75% reduction in overall black pigment as compared to the control.

Results

Increasing Brine Shrimp Survival with Yeast

The highest brine shrimp percent survival (97%) was obtained using 20 drops of yeast on Day 0, 40 drops on Day 1, and 0 drops on Day 2 (see Figure 2). In general, brine shrimp survival increased with increasing amounts of yeast, whereas a yeast-free diet was characterized by a decrease in brine shrimp survival by Day 3. Within the same yeast-positive conditions, the brine shrimp percent survival increased slightly from Day 2 to Day 3.

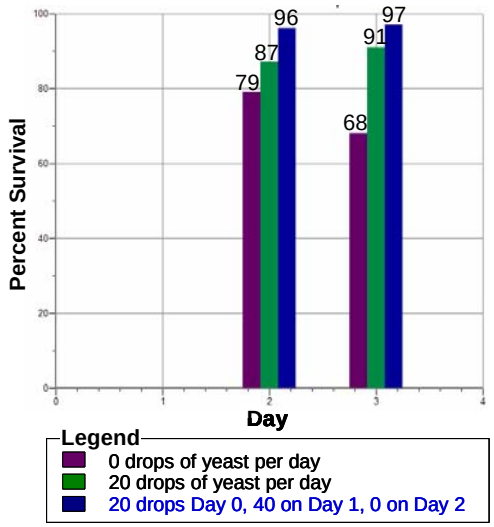

Figure 2. Effects of yeast on brine shrimp survival.

Effects of Diet on Embryo Production

Fish on the dry food diet yielded more embryos than those on the live food diet (see Table 1). The number of embryos produced on Tuesday was similar in both groups, decreased the following day, and increased as the week progressed, with the exception of the live food diet batch on Friday. Both conditions resulted in fewer embryos per week than expected.

Table 1

Average Embryos Produced Per 36 Fish Per Collection Week

Diet	Monday	Tuesday	Wednesday	Thursday	Friday	Total
Dry	NA	181	40	118	228	567
Live	NA	156	20	61	0	237

Note. Fish were not set up for a Monday collection.

Injection Efficiency

The 3-, 6-, and 9-ng tyrosinase MO conditions yielded zebrafish larvae having at least 75% overall reduced black pigment at 48 hpf (see Figure 3). In general, melanin was not visually observed in the head, eyes, and yolk and was reduced at the top and bottom of the tail. The injection efficiency increased to 92% after four practice injection experiments.

Figure 3. Top: Tyrsonase-MO injected larvae. Bottom: Wild-type larvae. (~3-mm length)

Discussion and Conclusion

MO knockdown in zebrafish is a powerful functional genomics tool for deciphering gene function. However, to be able to conduct a MO screen, efficient microinjection techniques must be established and sufficient embryos produced. Thus, this project focused on developing microinjection techniques, maximizing embryo production, and increasing brine shrimp survival. As described below, some results supported and others opposed the literature. All results are springboards for future research.

Cleveland's (1998) advice of sustaining brine shrimp with yeast was accurate according to the results obtained in this study. Brine shrimp survival increased as the amount of yeast increased. It would, however, be expected that surpassing the yeast concentration that sustains brine shrimp could lead to what Cleveland warned as a foul culture (i.e., brine shrimp death). The slight increase within the same yeast-positive conditions from Day 2 to 3 could have resulted from the hatching of additional brine shrimp after Day 2's count. Possible future brine shrimp research can focus on hatcher type, light, water temperature, pH, salt water concentration, aeration, yeast concentration, and duration of analysis.

The embryo production data contradict the literature (Westerfield, 2000; Badman et al., n. d.) and should be validated by switching the diets of the fish and comparing the results. Normally, a female zebrafish can produce at least a hundred eggs per week. However, the fish on the live food diet produced only 237 embryos a week per 24 female zebrafish. Furthermore, instead of the expected increase in embryo production, fewer embryos were collected from fish on the live food diet than those on the dry food diet. The previous fall, approximately a thousand embryos were obtained per week from fish on the dry food diet (data not shown) and seemed to decrease the following months to reported levels. Factors to analyze and possibly increase embryo production include water temperature, water quality, time of set up, breeding frequency, seasons, number of fish in the main tanks and breeding boxes, ratio of males/females in the breeding boxes, age, quantity of food, and hormone supplementation.

The tyrosinase MO-injected zebrafish had at least 75% overall reduction in black pigment at 48 hpf, which supports the observations noted by Pickart et al. (2004) in which tyrosinase pigmentation can be knocked down up to 50 hpf, with some pigment formation increasing after Day 2. A future project could analyze the anatomical locations and time points of melanin formation in control zebrafish and tyrosinase-MO injected zebrafish. The 92% injection efficiency will continue to improve with practice. Additionally, dyes such as phenol red can be combined with the morpholino to better visualize the liquid being injected into the yolk. An alternative method of measuring injection efficiency is to tag the MO with a green fluorescent protein (GFP) and use a fluorescent microscope to count the fluorescing embryos at 4 hpf.

In summary, valuable techniques and methods have been developed to increase UW-Stout's functional genomics screening capacity. A 97% brine shrimp percent survival was obtained using 20 drops of yeast on Day 0, 40 drops on Day 1, and 0 drops on Day 2. The batch of zebrafish fed the dry food diet produced more embryos compared to those on the live food diet; however, the diets must be switched and results compared to validate the results. A 92% injection efficiency is adequate to begin screening other MOs and should continue to increase with practice. The MO microinjection techniques were compiled into a lab module (Hoage[2], 2006) that has been incorporated into the Applied Science, Biotechnology concentration curriculum. A brine shrimp husbandry booklet (Hoage[2], 2006) was also created as a template for future research and for the Zebrafish Laboratory personnel. With more undergraduates learning

these microinjection techniques and increased funding to further develop UW-Stout's GTAC facility, the Zebrafish Laboratory will be in the position to begin reverse-genetic screening and elucidate gene function.

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