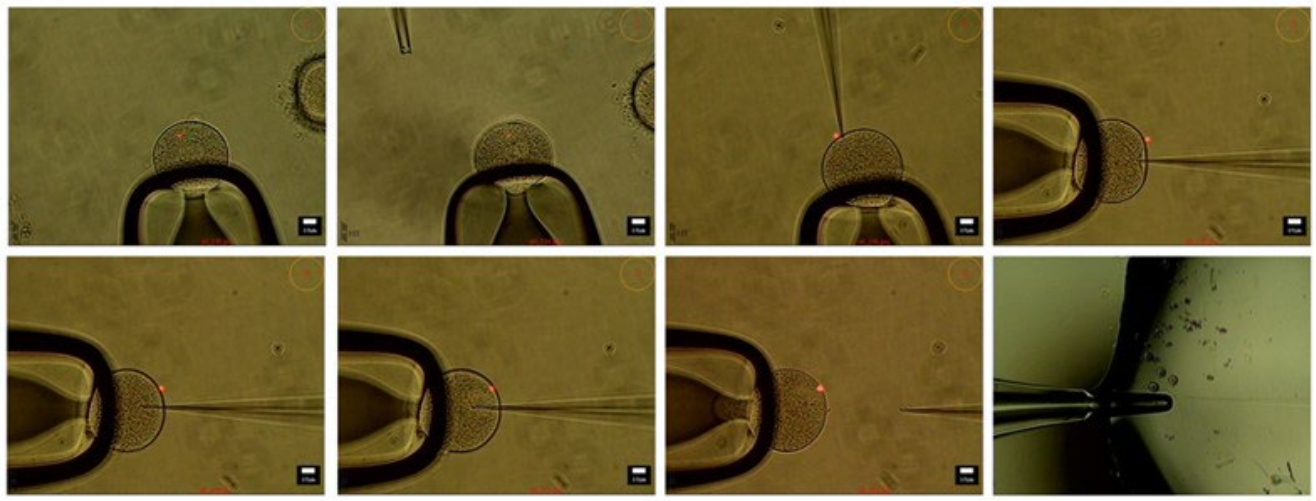
BUREAU OF RECLAMATION

Development of Dreissenid Mussel Engineered Disseminated Neoplasia

Science and Technology Program
Research and Development Office
Final Report No. ST-2022-19006-01



U.S. Department of the Interior

September 2022

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
30-09-2022	Research	2019-2022

4. TITLE AND SUBTITLE
Development of Dreissenid Mussel Engineered Disseminated Neoplasia

Dreissenid Disseminated Neoplasia

5a. CONTRACT NUMBER
RR4888FARD1904401/ F052A

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER
1541 (S&T)

6. AUTHOR(S)
[1]Steve Suhr, Biologist
[1]Marie-Claude Senut, Biologist
[2]Sherri Pucherelli, Biologist
[2]Yale Passamaneck, Ecologist
[2]Jacque Keele, Biologist

5d. PROJECT NUMBER
Final Report No. ST-2022-19006-01

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
[1] Biomilab LLC
4209 S. Pennsylvania Ave.
Lansing MI, 48910

[2] Bureau of Reclamation
1 Denver Federal Center
6th Avenue and Kipling, Building 67 RM 152
Denver, CO 80225

8. PERFORMING ORGANIZATION REPORT NUMBER

EcoLab-F052A-2022-11

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Science and Technology Program
Research and Development Office
Bureau of Reclamation
U.S. Department of the Interior
Denver Federal Center
PO Box 25007, Denver, CO 80225-0007

10. SPONSOR/MONITOR'S ACRONYM(S)
Reclamation

11. SPONSOR/MONITOR'S REPORT NUMBER(S)
Final Report No. ST-2022-19006-01

12. DISTRIBUTION/AVAILABILITY STATEMENT
Final Report may be downloaded from https://www.usbr.gov/research/projects/index.html

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Biomilab LLC has been working for three years on a project aimed at developing, validating, and manufacturing an invasive mussel eradication agent that mimics a natural pathogenic mechanism found in marine bivalves called a disseminated neoplasia (DN). A DN is a form of cancer where the cancer cells themselves travel between organisms in a specific-species manner causing toxicity in the host. Biomilab has established invasive mussel aquaculture in their laboratory and determined methods for the prolonged survival of dissociated mussel cells in culture. They have utilized sequence data provided by Reclamation, and plasmid DNA vectors were constructed for transgene expression and tested in insect cell lines as a proxy for mussel cells. Biomilab tested more than a dozen methods of transduction on mussel cells and determined that because mitotic cells are quite rare, transduced foreign DNA cannot enter the nucleus and is not expressed. In the third year, the focus was on overcoming issues of transduction by exploring infection with recombinant viruses and by establishing methods for the controlled spawning and generation of quagga and zebra mussel embryos by in vitro fertilization.

15. SUBJECT TERMS
Invasive mussels, quagga mussels, zebra mussels, eradication, disseminated neoplasia

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**THIS PAGE**			Sherri Pucherelli
U	U	U		54	**19b. TELEPHONE NUMBER** *(Include area code)* **303-445-2015**

Mission Statements

The Department of the Interior (DOI) conserves and manages the Nation's natural resources and cultural heritage for the benefit and enjoyment of the American people, provides scientific and other information about natural resources and natural hazards to address societal challenges and create opportunities for the American people, and honors the Nation's trust responsibilities or special commitments to American Indians, Alaska Natives, and affiliated island communities to help them prosper.

The mission of the Bureau of Reclamation is to manage, develop, and protect water and related resources in an environmentally and economically sound manner in the interest of the American public.

Disclaimer

Information in this report may not be used for advertising or promotional purposes. The data and findings should not be construed as an endorsement of any product or firm by the Bureau of Reclamation, Department of Interior, or Federal Government. The products evaluated in the report were evaluated for purposes specific to the Bureau of Reclamation mission. Reclamation gives no warranties or guarantees, expressed or implied, for the products evaluated in this report, including merchantability or fitness for a particular purpose.

Acknowledgements

The Science and Technology Program, Bureau of Reclamation, sponsored this research. Thank you to Ashley Baldridge Elgin (NOAA), Seth Herbst, Lucas Nathan, Tom Goniea (MI Department of Natural Resources), Sarah LeSage (MI Department of Environment, Great Lakes, and Energy), and Jose Cibelli (MI State University) for their contributions to this project.

Development of Dreissenid Mussel Engineered Disseminated Neoplasia

Final Report No. ST-2022-19006-01

EcoLab-F052A-2022-11

prepared by

Biomilab LLC
Steve Suhr, Biologist, Co-director, Biomilab LLC
Marie-Claude Senut, Biologist, Co-director, Biomilab LLC

Technical Service Center
Sherri Pucherelli, Biologist
Yale Passamaneck, Ecologist
Jacque Keele, Biologist

Peer Review

Bureau of Reclamation
Research and Development Office
Science and Technology Program

Final Report No. ST-2022-19006-01

Development of Dreissenid Mussel Engineered Disseminated Neoplasia

SHERRI PUCHERELLI Digitally signed by SHERRI PUCHERELLI Date: 2022.09.19 14:01:43 -06'00'

Prepared by: Sherri Pucherelli
Biologist, TSC, Hydraulic Investigations and Laboratory Services Group

DIANE MENCH Digitally signed by DIANE MENCH Date: 2022.09.19 14:56:36 -06'00'

Peer Review by: Diane Mench
Biologist, TSC, Hydraulic Investigations and Laboratory Services Group

"This information is distributed solely for the purpose of pre-dissemination peer review under applicable information quality guidelines. It has not been formally disseminated by the Bureau of Reclamation. It does not represent and should not be construed to represent Reclamation's determination or policy."

Contents

Page

Executive Summary

Efforts to block the transport of invasive mussels among waters of the western U.S. are critical to slowing the infiltration of zebra and quagga mussel from infested waterways to unaffected waters. But what can be done about large, open waterways that are the source of invasive mussels such as Lake Powell, Lake Mead, and Lake Mohave, where infestations are established, and eradication is considered neither logistically nor economically realistic? Biomilab LLC has been working for three years on a project aimed at developing, validating, and manufacturing an agent theoretically capable of eliminating invasive dreissenid mussels from large water bodies safely, cost-effectively, and most importantly, without damaging the delicate ecosystems of native U.S. waterways. If successful in manufacturing this anti-dreissenid agent, the threat of invasive mussels could be eliminated completely.

The eradication agent proposed mimics a natural pathogenic mechanism found in marine bivalves called a disseminated neoplasia (DN). A DN is a form of cancer where the cancer cells themselves travel between organisms in a species-specific manner causing disease and mortality in the host. One distinct advantage of a DN-based eradication agent is that it poses no threat to animals or plants other than the host species from which the DN is derived. Another advantage is that when the last host organism dies, the DN dies with it, leaving no remnant in the environment. Engineered DNs are not the same as genetically modified organisms (GMOs) that can survive and reproduce autonomously. Instead, like other cells of zebra or quagga mussels, they can only survive and thrive within the body of the species of origin. The project proposes to engineer a DN for dreissenid mussels that can infiltrate them, induce toxicity, and eliminate them from a large water body such as Lake Mead over the course of several years while leaving native species unaffected and healthy.

In the first three years of the project, Biomilab has made great strides towards development of the DN eradication agent. In the first year, Biomilab has established invasive mussel aquaculture in their laboratory and determined methods for the prolonged survival of dissociated mussel cells in culture. In the second year, with genomic sequence data provided by Reclamation, plasmid DNA vectors were constructed for transgene expression and tested in insect cell lines as a proxy for mussel cells. Biomilab tested more than a dozen methods of transduction on mussel cells and determined that because mitotic cells are quite rare, transduced foreign DNA cannot enter the nucleus and is not expressed. In the third year, the focus was on overcoming issues of transduction by exploring infection with recombinant viruses and by establishing methods for the controlled spawning and generation of quagga and zebra mussel embryos by in vitro fertilization. Quagga mussel embryos and embryonic cells may be more amenable to transduction using methods of plasmid DNA transfection and viral infection than are adult cells. In addition, microinjection of 1-2-cell early embryos will be explored as a method of stable DNA delivery to provide us with DN cells.

With the data collected and the tools developed in the first three years of this project, Biomilab is more confident than ever that the transformed cell lines that will serve as the foundation of the engineered DN will be produced. Furthermore, the scientists of both Biomilab and Reclamation are making many new discoveries about the basic biology of dreissenid mussels that will help us continue to refine strategies for the biological control of these organisms for many years to come.

1. Introduction and Background

It is generally accepted that once zebra or quagga mussels are established in water bodies such Lake Mead, Lake Havasu, or Lake Powell, eradication is neither logistically nor economically realistic. In a 2008 case study of Otsego Lake in central New York examining preventative measures for zebra mussel invasion, the author Dr. Thomas Horvath states "If the goal is to preserve the natural community and function of a lake, once zebra mussels establish in the lake, no logistically feasible means to eradicate the population exists". More recently (2019), a document from the National Park Service detailing invasive mussel infestation of the Lake Mead Recreational Area states "Once quagga mussels have been established in a water body, there is no economically feasible method of eradication" (https://www.nps.gov/lake/learn/quagga-mussel.htm).

To address this apparently intractable problem, Biomilab LLC has been working with the Bureau of Reclamation (Reclamation) since 2019 on a safe and cost-effective invasive mussel eradication agent with potential to cause no damage to the native organisms and ecosystems of U.S. waterways. This project was the winning theoretical solution of a Prize Challenge conducted by Reclamation in 2018. The agent to be manufactured is theoretically capable of eliminating invasive dreissenid mussels from both smaller water bodies such as the San Justo Reservoir near Hollister, CA and larger open waters associated with the Colorado River such as Lake Mead and Lake Powell. If the anti-dreissenid agent proposed is successful, it would be capable of eliminating invasive zebra and quagga mussels in essentially any infested water body in North America including the Great Lakes.

1.1 Disseminated neoplasias are uncommon but effective pathogenic agents

The Biomilab/Reclamation eradication agent under construction is an engineered form of a transmissible cancer known as a disseminated neoplasia (DN). Unlike most cancers where cells of the body are transformed (or "made cancerous") by genetic mutations introduced by heredity or exposure to mutagens (i.e., sunlight, radiation or viruses (Epstein-Barr virus, Hepatitis B virus)), with DN, the cancer cells themselves are physically transmitted from one animal to another causing disease (Carballal et al., 2015).

Although the immune system generally prevents the survival of cells exchanged between individuals (even of the same species), there are two well-known types of disseminated cancer in mammals: canine transmissible venereal tumor (CTVT) and Tasmanian devil facial tumor disease (DFTD). CTVT (Murgia et al., 2006, Murchison et al., 2008 and 2014) is a DN in dog populations that was first described by an English veterinarian in 1810, has spread across continents, and was recently genetically determined to have originated in a dog living more than 11,000 years ago (Murchison et al., 2008 and 2014). DFTD, first reported in 1996 and which has come extremely close to eliminating the wild Tasmanian devil population, has only recently been determined to also arise from the spread of live cancer cells from one devil to another through direct contact (reviewed in Bender et al., 2014).

DN in mollusks was first described in the late 1960's and has since been studied extensively by marine biologists concerned with preservation of mollusk populations with commercial importance (Carballal et al., 2015). Mollusks lack a major histocompatibility complex (MHC) system and exchange of cells between proximal individuals may be natural and common in mollusks (discussed in Weiss and Fassati, 2015). In the laboratory, DN transmission can be induced experimentally by injecting hemocytes from an infected animal into uninfected animals using a syringe, but in nature it seems clear that DN is transmitted from individual-to-individual by simple proximity. Animal-to-animal transfer has been experimentally reproduced by co-culture of healthy and cancerous mollusks within a shared tank (Elston et al., 1988 and Mateo et al., 2016). A natural DN for freshwater bivalves has not yet been reported, but it would not be surprising to already exist in discrete bivalve populations in isolated waterways.

1.2 Creation of a dreissenid DN is induced by loss of cell-cycle control genes or gain of oncogenes

Cellular transformation, or the conversion of a normal healthy cell to a cancer cell, is a well-studied phenomenon in the biological sciences. Stable transformation generally arises from the incorrect production of cellular factors that control cell division. Mutations compromising the activity of factors that limit the cell cycle (analogous to releasing the brakes) or genetic changes that lead to over-production of factors that stimulate cell division (analogous to pressing the accelerator) can result in the creation of a cancer cell.

To release the mitotic "brakes", it has been shown that one common mutation found in many molluscan DNs is alteration to the cell-cycle and cell death master-regulating protein p53 (Walker et al., 2011; Diaz et al., 2010; Vassilenko et al., 2010; Murtray et al., 2010). p53 is the subject of thousands of studies for its role in cancer in many organisms, and mutations in p53 are widely considered to be the most common mutation in human cancers (Duffy et al., 2017). Based on published reports linking changes in p53 to molluscan/mussel DN and the known role of p53 in neoplasia of mammals from mouse to man, it is predicted that mutation of p53 within the mussel genome also has a high probability of producing cancer. One area of focus over the last three years has been to characterize the quagga mussel p53 gene and test reagents for its functional knock-out or suppression.

To accelerate mitosis, introduction of oncogenes derived from cancer-causing viruses have proved very effective at inducing transformation of cells from a variety of species. One such factor is the Large-T-Antigen (Tag) protein from Simian Vacuolating Virus 40 (SV40) (see review, Ahuja et al., 2005). The SV40 Tag protein has been shown to work through multiple cellular pathways to induce cellular transformation, most notably through inhibition of p53 and another tumor suppressive factor Retinoblastoma-1 (Rb). Temperature-sensitive forms of the SV40 Tag (tsTag) have been discovered that allow control over cellular immortalization by shifting cells containing the factor from a low temperature that induces transformation (usually 32ºC) to a non-permissive temperature that allows the cell to revert to normal growth and growth arrest (usually 37ºC). Scientists in our group have used tsTAG to control mitosis and differentiation of skeletal muscle cells in vitro (MacPherson et al., 2004). We hypothesize that Tag would have the same properties of cellular

transformation in quagga and zebra mussel cells that it has in mammalian, reptile, and amphibian cells.

The primary objective of this project is to knock-out cell cycle control genes such as p53 or to introduce oncogenes such as SV40 Large-Tag to convert normal zebra and quagga mussel cells into cancer cell lines that can then be adapted for use as the disseminated neoplasia eradication agent in waterways infested with these invasive species.

2. Material and Methods

2.1 Establishment of a live quagga and zebra mussel aquaculture station

An approximately 300 ft^2 area of the second floor of our building was designated for mussel culture and related activities. Rubber anti-skid floor protection was put down covering approximately 200 ft^2. An 8x8 ft section of the East wall was covered in water-proof paneling and GFI adaptors and power strips were introduced in the Northwest corner of the room to ensure that electrical current is shut off in case of an electrical short. A double tub sink was installed on the East wall for dishwashing and processing of waters used for aquaculture. Drying racks and lighting was installed above the double tub sink. A catch tub with a sump pump was installed below the tub sink and a 15 ft hose was attached to the sump pump to allow transfer of wastewater to a nearby closet with a deep utility sink. A Y-connector was installed on the faucet of the closet sink and a 15 ft hose to allow transfer of tap water to the tub sinks and the area of the aquaculture system.

For aquaculture of live mussels, two 2x6 ft benches were moved close to the tub sinks and parallel to each other. The benches were topped with 2-in thick Styrofoam planks to create a 5x5 ft insulating foam pad. This surface was covered by heavy plastic sheeting to prevent water infiltration. Two vertical 4x4-in, 7-ft long boards were strapped onto the legs of the benches to provide a stable platform for possible future installation of lighting, tubing, etc., as needed. A 5-ft diameter child's wading pool was placed on top of the Styrofoam sheets and insulated around the exterior circumference with 5-in thick fiberglass insulation wrapped in plastic sheeting. The pool was then filled with 12-15 cm of tap water.

A TECO 150 aquarium chiller was purchased and installed directly below the pool on a shelf. A SICCE aquarium water pump was then inserted into the pool with tubing running from the aquarium pump to the inlet of the chiller. A tube was then run from the chiller outlet to the opposite end of the pool to allow continuous circulation of the water in the pool through the chiller unit. The chiller was set at 15ºC and the water temperature in the pool was verified to be always 15-16ºC. Five-gallon glass aquaria were placed in the chilling pool and filled to 2/3 max capacity with tap water that had set for 24-hours or more to allow inactivation of chlorine. Plastic grids cut to size were placed in the bottom of the aquaria and overlayed with a thin black plastic perforated platform to act as a substrate for live mussels. This plastic platform can be transferred from tank-to-tank

without disturbing mussels when changing aquarium water. For each tank, an aquarium air supplier with tubing and bubbling stone were added to continuously oxygenate water (Figure 1).

A scientific collector's permit for collection and transport of invasive mussels was obtained by Biomilab from the Michigan Department of Natural Resources – Fisheries division (MI DNR) on January 10, 2019, and is renewed yearly. Zebra (*Dreissena polymorpha*) and quagga (*Dreissena rostriformis bugensis*) mussels originating in Saginaw Bay within Lake Huron or more frequently, Lake Michigan, are obtained from Dr. Ashley Elgin, NOAA (Great Lakes Environmental Research Laboratory), Muskegon MI, on several occasions between June-October and transported back to our laboratory by Biomilab personnel.

After setting up the aquaculture station, live zebra and quagga mussels were placed in the tanks on the plastic platforms for extended live culture and are fed a suspension of phytoplankton (Reed Mariculture, Inc.) (1-ml Rotigrow Nano, Reed Mariculture Inc. concentrate in 1 L of conditioned water) (Figure 2). Ten to 12 ml of this solution is added drop-wise to the mussel tanks 5 days a week. As needed, mussel tanks are changed by lifting the platform and any loose mussels from their tank to a new clean tank with fresh water. Any dead mussels, shells, or debris are removed or left behind.

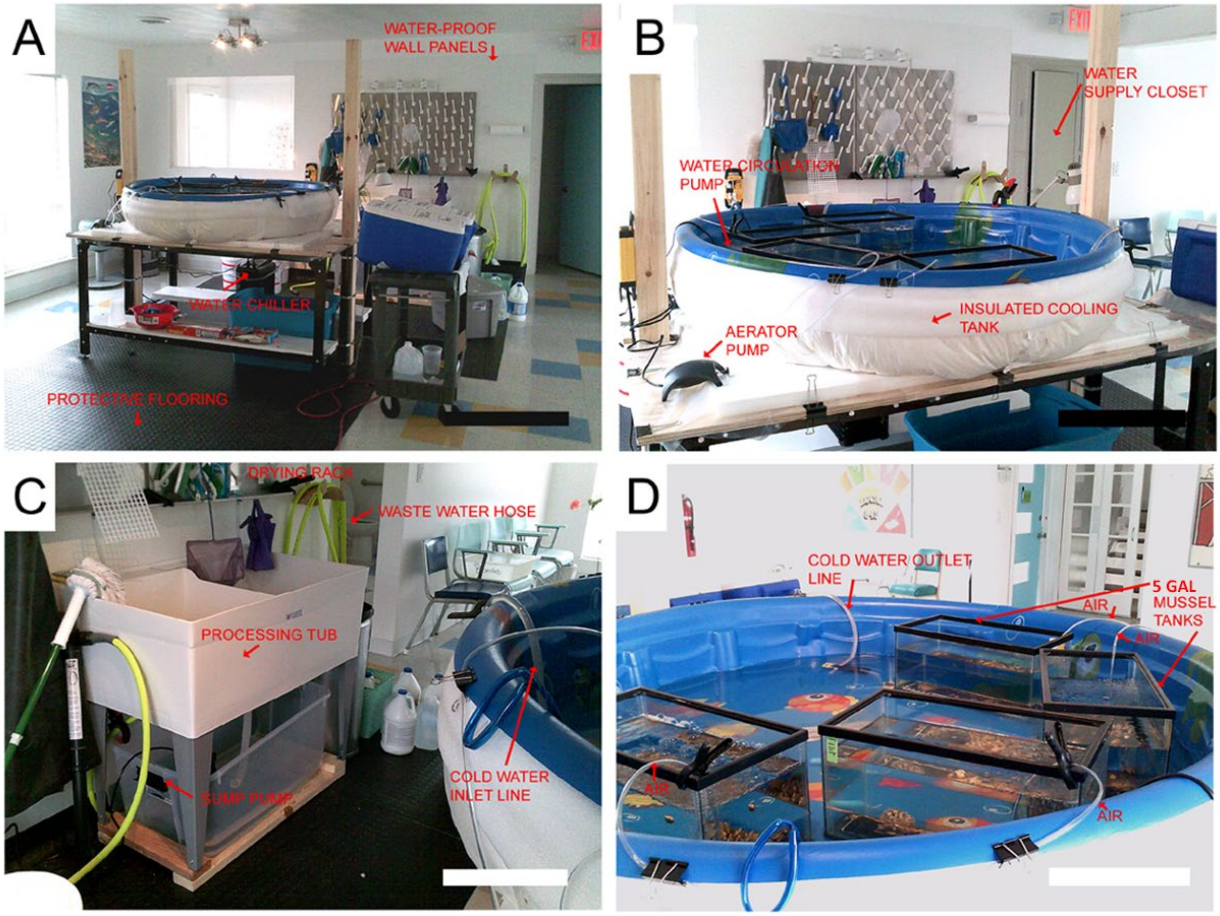


Figure 1 The Biomilab invasive mussel aquaculture facility. A-D) Images of the facility constructed in Biomilab for live culture of zebra and quagga mussels.

Figure 2 Quagga mussels collected from Lake Michigan housed in Biomilab Aquaculture Facility.

2.1 Culture of adult mussel cells

Establishment of culture procedures for zebra and quagga mussel cells built upon previously published methods (Quinn et al., 2009; references in Yoshino et al., 2013). Most experiments were performed with quagga mussels since they make up most of the mussel population maintained in our aquaculture facility. Cultures of mussel cells were derived from various tissues, primarily gills and mantle. Following a 5 minute-bleaching in 0.03% sodium hypochlorite solution (Wright et al., 1996) and multiple rinses in sterile filtered aquarium water, mussels were transferred to a laminar flow hood where subsequent work was performed under sterile conditions. Using a dissecting microscope (Amscope), mussel tissues were carefully dissected out using fine forceps and washed several times in antibiotics-supplemented buffer water (Quinn et al., 2009) or culture medium.

We tested three cell culture paradigms: 1) passively dissociated cells. When placed in cell culture medium, dissected gills can survive for several days, as assessed by the visible regular and synchronized movements of their lateral ciliated cells. During this process, cells detach or exude from the gills into the culture medium, allowing within days the accumulation of a large pool of gill cells that can subsequently be used for cell culture; 2) enzymatically-dissociated cells. For this experimental design, minced tissues of interest were dissociated into single cells by incubation for 1-2 hours into a 0.025% pronase solution at room temperature. After serum-induced pronase inhibition, filtration, centrifugation and rinsing, dissociated cells were re-suspended in cell culture medium and plated in wells of a 96-well plate; 3) explants. In this model, several minced tissue pieces were directly placed in wells of a 96-well plate in a small amount of cell culture medium to favor cellular adhesion. After 1 hour, fresh culture medium was added to reach a final 100 µL volume. Mussel cells were cultured and maintained in a Frigidaire 22 inch-wide wine cooler at 15°C without CO_2 equilibration.

Appropriate coating substrates and cell culture media are key requirements for the successful maintenance and growth of healthy cells in vitro. Thus, we tested the effectiveness of 13 different commonly used cell culture substrates (Figure 3) for promoting in vitro attachment and survival of mussel cells. To this end, cell culture-treated plastic wells were either not coated (control) or coated for several hours or overnight with the candidate substrates before rinsing. Primary mussel cells were cultured on the control and coated wells for 1-3 days, at which time the medium was removed and replaced by fresh medium. Qualitative analysis of cells attached to the substrate was performed before and after medium change.

Cell culture medium consisted in 10-15% in sterile cell culture water (Sigma) of either of the media listed in Figure 3 supplemented with 2-10% fetal bovine serum and the antibiotics Penicillin, Streptavidin, Gentamycin and Kanamycin (Gibco, Sigma). Because it is well established from mammalian cell cultures that supplementation of the culture medium with growth factors/supplements/small molecules/enzymes is crucial for the maintenance and long-term culture of healthy cells, we added to either vehicle (control) or one of the factors listed (Figure 3 and Table 1) to the medium. Concentrations were based on published literature.

Supplementation of the culture medium with fish-specific factors has proven efficient for promoting cell survival and growth of zebrafish primary cells in vitro (Collodi et al., 1992). Likewise, addition of mollusk-specific factors could be beneficial to mussel cell cultures. Thus, we dissected tissues from 6 quagga mussels, placed them in sterile freshwater PBS and subsequently homogenized them using a Polytron homogenizer (8,000 rpm for a few seconds). The resulting mussel extract was then successively filtered on sterile gauze and a 0.45-μm filter before being aliquoted and stored frozen. Gill-derived cells were cultured for 3 days in L15 or Schneider's-based mussel medium alone or supplemented with mussel extract (20 μL/mL).

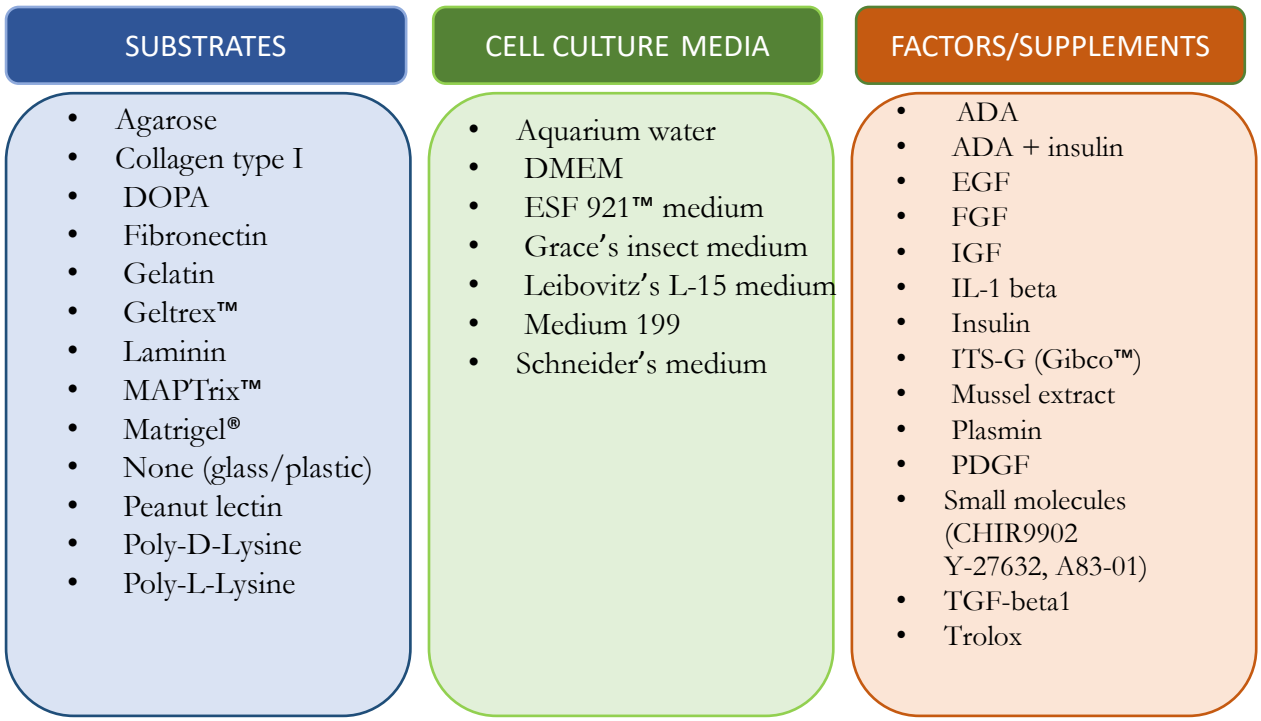


Figure 3 List of coating substrates, cell culture media and factors/supplements/small molecules tested for the in vitro culture of quagga and/or zebra mussel cells.

Table 1 List of factors, supplements, small molecules tested for their effects on the in vitro viability and proliferation of cells dissociated from gills or whole body tissues. (No) indicates no statistical significance ($p>0.05$); NT: not tested yet).

FACTORS	CELL TYPES	DOSE TESTED	DURATION	CELL VIABILITY EFFECTS	CELL DIVISION EFFECTS
ADA	Whole body cells	8.4 ng/mL	5-7 days	No	No
ADA + Insulin	Whole body cells	8.4ng/10µg/mL	5-7 days	No	No
LIF (mouse)	Gill cells	100 ng/mL	1 day	No	No
EGF (rat)	Gill cells	20 ng/mL	3 days	No	NT
FGF (rat)	Gill cells	20 ng/mL	3 days	No	NT
IGF (human)	Gill cells	10 ng/mL	3 days	No	NT
IL-1 beta	Gill/Whole body cells	10 ng/mL	3-5/7 days	No	No
Insulin (bovine)	Whole body cells	10 µg/mL	7 days	No	NT
ITS-G (Gibco™)	Gill/Whole body cells	1/100	3-9 days	No	NT

Mussel extract	Gill cells/Whole body cells	1/50	3 days	No	No
Plasmin	Gill cells	2-16 µg/mL	2-5 days	No	No
PDGF-AB	Gill/Whole body cells	10 ng/mL	3-5/7 days	No	No
Small molecule (Y-27632)	Gill/Whole body cells	10 µM	7-9 days	No	No
Small molecule (A83-01)	Gill/Whole body cells	0.5 µM	7-9 days	No	No
Small molecule (CHIR99021)	Gill/Whole body cells	3 µM	7-9 days	No	No
TGF-beta1	Gill/Whole body cells	1-10 ng/mL	3-5/7 days	No	No
Trolox	Gill cells	500 µM	1 day	No	NT

2.2 Cell survival assessment

For all experimental conditions, we performed live/dead assays as described below to assess cellular viability.

2.2.1 Trypan blue exclusion assay

Mussel cells were harvested and stained with 0.4% Trypan blue (Gibco). Trypan blue is a dye that can enter cells when the integrity of their membrane is jeopardized. Thus, sick or dead cells let the dye in and become blue whereas healthy live cells appear clear. Using a hemocytometer, the number of viable cells was obtained by subtracting the number of dead cells (blue) to the total number of cells (clear + blue). Data were expressed as percentages.

2.2.2 Live/dead fluorescent assay

To determine cell viability, we also used a combination of the live cell fluorescent marker calcein AM (green)(Biotium) and the dead cell DNA marker Ethidium Homodimer-1 (EthD-1) (red) (Biotium). Cells to be tested were incubated for 15 minutes in a mixture of calcein AM/EthD-1 and examined on a VWR inverted fluorescence microscope.

2.2.3 Phenotypic identification of cultured cells

To determine the identity of cells surviving in culture, we fixed cultured mussel cells in 4% paraformaldehyde in freshwater PBS (Nogueira et al., 2013) for 20 minutes. Following multiple rinses, we performed immunofluorescence staining (Senut et al., 2014) for 1) the microtubule marker acetylated tubulin (1:2000, Sigma); 2) the neurotransmitter serotonin (1:500, Sigma) and 3) the

smooth and skeletal muscle marker myosin (1:500, Sigma). Cells were incubated in the various primary antibodies overnight at 4°C, rinsed several times and incubated in secondary antibodies conjugated to fluorescent markers (1:500, Jackson Immunoresearch Laboratories Inc.) for 1-2 hours at room temperature in the dark. At the end of incubation, cell nuclei were stained with 10 ng/mL of the DNA marker 6-diamidino-2-phenylindole (DAPI) and rinsed several times before being examined with a fluorescence microscope. Control experiments consisted in omitting one step of the immunodetection procedure and replacing the primary antibody with serum solution.

2.2.4 Identification of dividing cells

To identify cells replicating their DNA (phase S of the cell cycle), live mussels were kept at 15°C in aquarium water supplemented with the thymidine analog 5-ethynyl-2'-deoxyuridine (EdU) for 18 hours. At the end of exposure, mussel tissues were dissected, fixed in 4% paraformaldehyde in freshwater PBS and staining for EdU was revealed using the Click-IT™EdUAlexaFluor™488 imaging kit from Invitrogen, according to the manufacturer's protocol. In addition to EDU labeling, we also processed mussel tissues and cells for the immunofluorescence detection of histone 3 phosphorylated on serine 10 (PHH3; 1:1000, Abcam), a marker of actively dividing cells.

2.2.5 Cryostorage of mussel cells

Pronase-dissociated quagga mussel cell were collected and re-suspended in either one of the freezing media shown in Figure 4 at a concentration of 1x106 to 1x107 cells/mL. One mL of the cell suspension was then added to previously labeled cryopreservation tubes (USA Scientific). Cryotubes were placed in a cooled Mr. Frosty™ freezing container that was stored at -70°C overnight before being stored in a liquid nitrogen container. One to two weeks later, vials were removed from the liquid nitrogen container and immediately thawed by gentle agitation in a 37°C water bath (about 1 to 2 minutes). The cell suspension was diluted in 5 mL of cell culture medium, centrifuged and the supernatant discarded. The pelleted cells were then re-suspended in fresh cell culture medium and transferred into cell culture plates. Cell viability was assessed by Trypan blue assay as described above.

CRYOPROTECTANTS	CELL VIABILITY (THAW)
Aquarium water + 5% DMSO	NO
Cell culture medium + 5% DMSO	NO
FBS + 10% DMSO	NO
Cell culture medium + 30% DMSO + 10% DMSO	YES (17%)
Cell culture medium + 15% glycerol	YES (5%)
Cell culture medium + 30% FBS + 10% glycerol	YES (7.9%)
Cell culture medium + 10% FBS + 20% DMSO	YES (22.8%)

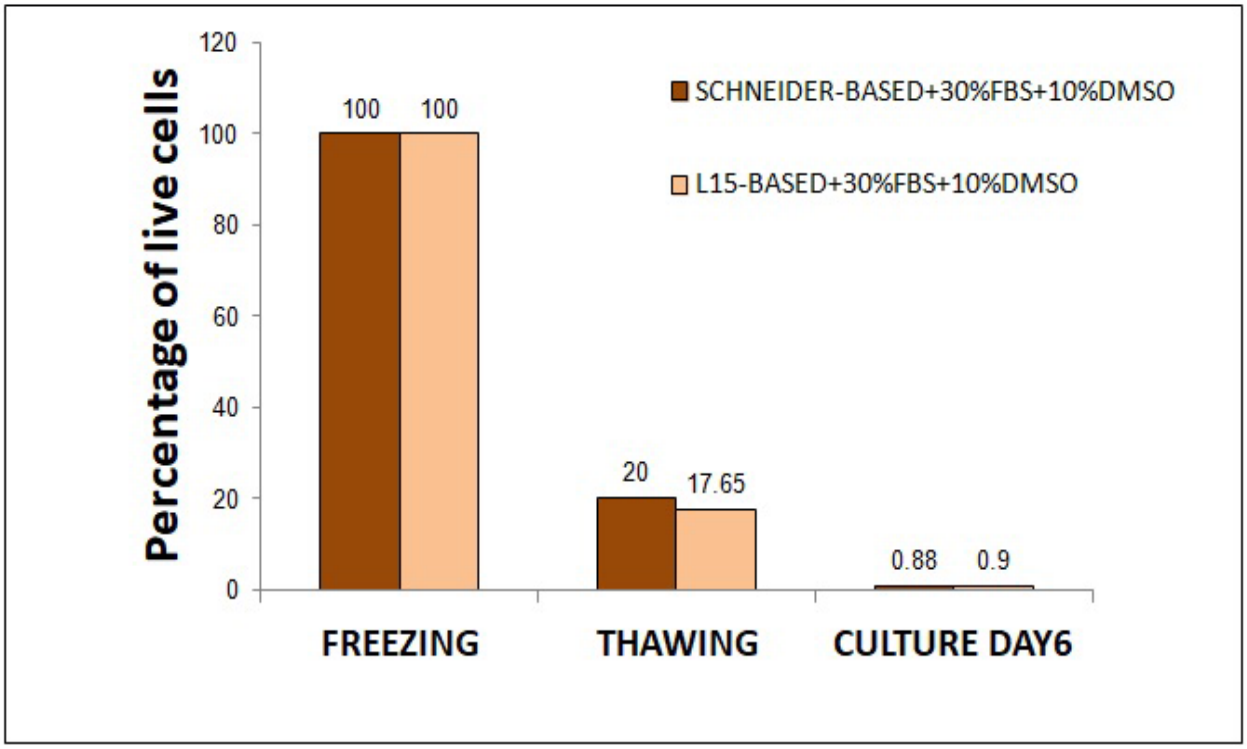


Figure 4 Producing frozen stocks of mussel cells. (A) List of cryoprotectants tested for the cryostorage of mussel cells. (B) Preliminary data illustrating the effects of freeze/thaw procedures on quagga gill-derived cell viability (Trypan blue assay). Suspensions of quagga gill cells in Schneider- or L15-based medium supplemented with 30% FBS and 10% DMSO were stored for 1 week in liquid nitrogen (LN2), thawed for 1 minute at 37°C and cultured for 6 days in their respective medium.

2.2.6 Imaging and data analysis

All experiments were performed in triplicates. Mussel tissues/cells stained with live/dead markers and immunostained were examined on a VWR inverted fluorescence microscope equipped with an INFINITY5-5 Teledyne camera and software (Lumenera). Five fields per well (minimum of 3 wells per experiment) were randomly chosen and imaged under fixed exposure settings. The resulting images were then coded and quantified by an operator blind to the experimental conditions. For live/dead assays, live (green) and dead (red) cell data were expressed as a percentage of the total number of cells (red+green) analyzed. For immunostainings, the ratio of immunopositive cells to the total number of nuclei (DAPI) gave us the percentage of cells positive for each phenotypic marker analyzed.

2.2.7 Spawning and fertilization

Spawning of quagga and zebra mussels was induced according to the well-established protocol of Ram et al. (1993). Following a 5 minute-bleaching in 0.03% sodium hypochlorite solution and multiples rinses in aquarium water (Wright et al., 1996), mussels were placed in glass scintillation vials (one mussel per vial) containing aquarium water supplemented with 1 mM serotonin creatinine sulfate monohydrate (Sigma), a transmitter known to regulate spawning in bivalves (see references in Ram et al., 1993). Vials were kept at room temperature and checked for the presence of gametes every 20 minutes for 4 hours. For fertilization, mature oocytes (eggs), and sperm cells were mixed in aquarium water containing Gentamycin (Sigma) and Penicillin/Streptavidin (Gibco). Water was changed daily and food (Iso 1800 Isochrysis microalgae, Reed Mariculture, Inc.) was added on days 4-5.

2.2.8 Cell trackers

Passively or enzymatically dissociated mussel cells were stained with either Vybrant™DiI cell labeling solution, FM™1-43FX or CellTracker™GreenCMFDA according to the manufacturer's protocols (Invitrogen). In some experiments, tracking dyes were combined to the dead cell marker EthD-1 to verify the viability of stained cells. Cells were then examined at different times post-staining on a VWR inverted fluorescence microscope.

2.2.9 Tracers and beads injections in live mussels

Injections were performed in a laminar flow hood under sterile conditions. Using a dissecting microscope, the shells of quagga mussels were gently pried open, and a sterile pipette tip was inserted in the gap to prevent closure. Five hundred microliters of Vybrant™DiI (1:50 dilution, Invitrogen) or 2 μm red fluorescent latex beads (Sigma) in sterile freshwater PBS (Nogueira et al., 2013) were tentatively injected in the adductor muscle region using a 1-mL syringe fitted with a 25-gauge needle. Following injection, the pipette tip was carefully pulled out and the mussel placed in a separate aquarium in fresh sterile aerated aquarium water at 15°C. Mussels were analyzed 24 and 72 hours following injection, at which time, gills, gut, muscle, foot and mantle were dissected and examined on a VWR inverted fluorescence microscope.

2.4 Statistical analysis of the data

Experiments subjected to statistical analysis were performed with a minimum of 3 biological replicates. SigmaStat 12 (Systat Software Inc.) was used for statistical analysis, with significance set at $p \leq 0.05$. Comparisons between control and experimental groups was performed using student's t-

test, analysis of variance (ANOVA) followed by post-hoc tests or, if the samples were not normally distributed, Kruskal Wallis rank sum tests as appropriate. For pilot/preliminary experiments, or experiments in progress, statistical analysis has not yet been performed and representative data is shown.

2.5 Invasive mussel genes, mRNAs, and expression vectors

DNA/mRNA sequences and gene structure information for the quagga mussel were provided by Dr. Yale Passamaneck at Reclamation. Zebra mussel genome information was generated by direct sequencing of PCR products produced by Biomilab or sourced from the annotated zebra mussel database (Reference genome UMN_Dpol_1.0) recently provided to the NCBI by the University of Minnesota (McCartney et al., 2022).

Gene expression regulating elements, open reading frames (ORFs), and other genetic elements from the quagga and zebra mussel or for other vector components (i.e., reporter genes) were produced for subcloning into plasmid vectors by either direct PCR amplification of mussel genomic DNA/cDNA or by direct synthesis. Primers or synthesized DNA gBlocks® were purchased from IDT, Coralville, IA. Genomic DNA was isolated using the Wizard® Genomic DNA Purification Kit and PCR products were cleaned up using the Wizard® SV Gel and PCR Clean-Up System (Promega, Fitchburg, WI). All other reagents for nucleic acid purification, PCR, ligation, restriction digest, reverse transcription, or other DNA/RNA manipulation were purchased from New England Biolabs (Ipswich, MA). All PCR, ligation, restriction analysis, and other DNA/RNA manipulation were performed using standard methods and procedures provided by the reagent manufacturer.

2.5.1 Modeling mussel expression vectors in human and insect cell types

Since transduction of invasive mussel cells was an uncertain prospect and continues to be problematic, we elected to test expression cassettes for general functionality using the readily transfectable human cell line HEK293. In addition, to model vector function in something closer to freshwater mollusk cells, we also obtained and cultured the commonly used drosophila cell line S2 (ATCC, Manassas, VA) and the *Spodoptera frugiperda* cell line Sf9 (Expression Systems, Davis, CA). Insect cells were cultured at 25ºC in Schneider Medium (S2) or ESF921 (Sf9) and passaged every 4-5 days to maintain healthy cultures. HEK cells were cultured in DMEM with 10%FBS+pen/strep/antifungal at 37ºC/5%CO2 and passaged weekly.

For transfection, cells were passaged to 24-well plates at 1x105 cells/well in their appropriate culture medium. For HEK cells, calcium phosphate co-precipitation using standard methods was used as a carrier of DNA and lipofectamine 3000 (ThermoFisher, Ann Arbor, MI) was used for transduction of RNAs following manufacturer guidelines. Typically, 0.2-2 μg of nucleic acid was delivered per well.

For insect cells, Mirus Transit-insect® or Qiagen Effectene® reagent was used for transduction of DNA and RNA. For the Mirus reagent, a ratio of DNA/RNA (μg) to Transit (μl) ratio of 2:1 was found to be the most effective. For Effectene, results were more variable, but in general, 2 μg of nucleic acid in 98 μl of EC buffer was complexed with 16 μl of enhancer, incubated at RT for 10 min, then mixed with 25 μl of Effectene. After an additional 10 min, 50 μl of this mix was added drop-wise to each well of cells.

Typically, cells were imaged or harvested for analysis of reporter expression at ≥3 days after transduction. For fluorescent reporters such as eGFP, eYFP, or mCherry, cells were imaged on an inverted stage fluorescent microscope. For luciferase reporters, cells were lysed in 250 µl of mild detergent solution for 10-20 min. and 10 µl of the lysate analyzed for light emission after injection of luciferin using a Berthold Lumat 9507 photometer. For gaussia luciferase (gLuc) assay, 10 µl of conditioned medium from each well of live transduced cells was analyzed on the Lumat 9507 photometer after injection of 3 µM coelenterazine in PBS.

2.5.2 Transduction and viral infection of adult and embryonic quagga mussel (QM) cells

Methods of transduction and viruses used in efforts to move foreign RNA and DNA into adult and embryonic (2-3 day) QM cells are shown in the Results. 96- and 24-well plate wells with 1-5X105 cells/well were generally used. Calcium phosphate (cal-phos) co-precipitation was performed using 500-1000ng DNA/well and standard methods. In mussel medium, the cal-phos precipitate did not form sufficiently for transduction. All lipid-based reagents were transduced using manufacturer protocols. Electroporation was performed using a BioRad GenePulser (Hercules, CA) with electroporation media ("zap" medium) and settings shown (see Results). AAV serotypes and Adenovirus was sourced from Vector Biolabs (Malvern, PA). Recombinant MMLV and Lentiviruses were produced by 3-way transfection of HEK cells with vector, Gag-Pol and VSVg envelope plasmids to produce viral supernatants (Suhr and Gage, 1999), that were concentrated by 4-hour/4ºC centrifugation over a 10% sucrose cushion at 10,000 xg followed by resuspension of the pellet in 200-300 µl of mussel culture medium (roughly 100x concentrated). Recombinant viruses were tested by introduction of 1-10 µl of virus concentrate directly into wells of cultured mussel cells and assayed for fluorescence or luciferase expression for up to 1 week.

2.5.3 Production and validation of QM p53 CRISPR/Cas9 reagents for knock-out of QM p53

Potential gRNA targets were identified within the QM p53 gene using the online engine CHOPCHOP (Labun et al., 2021). Since we could not transduce CRISPR reagents efficiently into quagga mussel cells themselves, we elected to create a "model" of QM p53 in mammalian cells to test the efficiency of gRNA targets as a pre-validation pilot experiment.

To do this, a roughly 450-bp region of DNA encoding presumptive exons 4-6 of QM p53 mRNA was synthesized and this gBlock subcloned into the MMLV-based retroviral vector NIT. The NIT plasmid was packaged into an infectious MLV-based virus using 3-way transfection and used to infect mouse NIH3T3 cells. Infected cells were selected with the antibiotic G418 and individual resistant colonies picked for expansion as clonal lines. G418-resistant lines were tested to confirm an integrated viral genome using quantitative PCR of isolated genomic DNA. Two lines (G4 and G5) with readily detected QM p53 transgene fragment were selected for test targeting by CRISPR/Cas9 reagents.

The target gRNA sites located within the 435bp QM p53 fragment were as follows:
TGTTTGAGAGTCCGGGGACATGG (gRNA1, in Exon 4),
TGGCAACCACTTGTCCGGTGAGG (gRNA2 in Exon 6), and
CGGGCGGTCTTGAACCTCACCGG (gRNA3 in Exon 6). Each target was synthesized as a gRNA and complexed with trcRNA and Cas9 protein using the Alt-R system from IDT.

Ribonucleoprotein complexes were introduced into the G4 and G5 3T3 lines using Lipofectamine 3000 following manufacturer directions. Five days post transduction, cells from triplicate wells were harvested, genomic DNA extracted, and the whole 435bp QMp53 region amplified by PCR. The amplified PCR DNA was analyzed for mutation of the individual targets by restriction digest with Msp1 and electrophoresis on a 2-3% agarose gel. Uncut PCR product was indicative of successful CRISPR/Cas9 mutation at the individual loci.

2.5.4 Pilot testing of microinjection with fertilized QM embryos

Immediately following mixture of sperm and eggs, glass vials containing the fertilized eggs were placed in a cooler to hold the temperature relatively constant and transported 5 miles to Michigan State University to the laboratory of Dr. Jose Cibelli for pilot microinjection sessions. The time delay for transport and set-up sometimes resulted in many embryos already in or nearing 2-cell stage, however, variability within zygotes generally provided some 1-cell stage embryos for microinjection. The microinjection station used was composed of a Kinetic Systems Vibraplane (Boston, MA) antivibration table, a Nikon Eclipse Ti microscope with dual micromanipulators for use with a holding needle (left) and a microinjection needle (right) and a FemtoJet Microinjector. Microneedles were sourced from WPI (Sarasota, FL). Following needle poke or injection of mussel water containing phenol red dye (for visualization), embryos were transported back to our facility and kept at 15-17ºC for up to 18 hours for analysis.

3. Results

3.1 Establishment of a live quagga and zebra mussel aquaculture station

Mussels have been cultured continuously in our laboratory system for 3 years (Figures 1 and 2). On occasion, newly arrived mussels exhibit poor survival most likely due to transient warming during the transport process, but recently we have established methods that appear to favor near 100% survival of transition into our system. Additional mussels will be obtained from the NOAA Field Station in Muskegon MI for the foreseeable future. It is our expectation that this quagga and zebra mussel colony will provide us with sufficient material for development and testing of the disseminated neoplasia anti-mussel reagent for the duration of the project.

3.2 Establishment of adult mussel cell cultures

The goal of this research is to establish cultures of quagga and zebra cells to serve as a basis for the development of our disseminated neoplasia model. Studies on the culture of tissues/cells derived from marine and freshwater mollusks including bivalves are quite limited (Yoshino et al., 2013). As a result, maintenance in culture of mussel-derived primary cells remains a challenge. Building upon previously described procedures (references in Yoshino et al., 2013; Quinn et al., 2009), we were able to successfully maintain quagga and zebra cells/tissues in culture, while gaining new understanding

on the factors key to their in vitro survival by manipulating three cell culture parameters: substrate, cell culture medium and factors/supplements. Mussel gill-derived cell cultures were particularly useful in this case since we could directly monitor the movement of ciliated cells. Cell viability was assessed with Trypan blue (Figure 5A) and/or fluorescent live/dead (Figure 5B) assays.

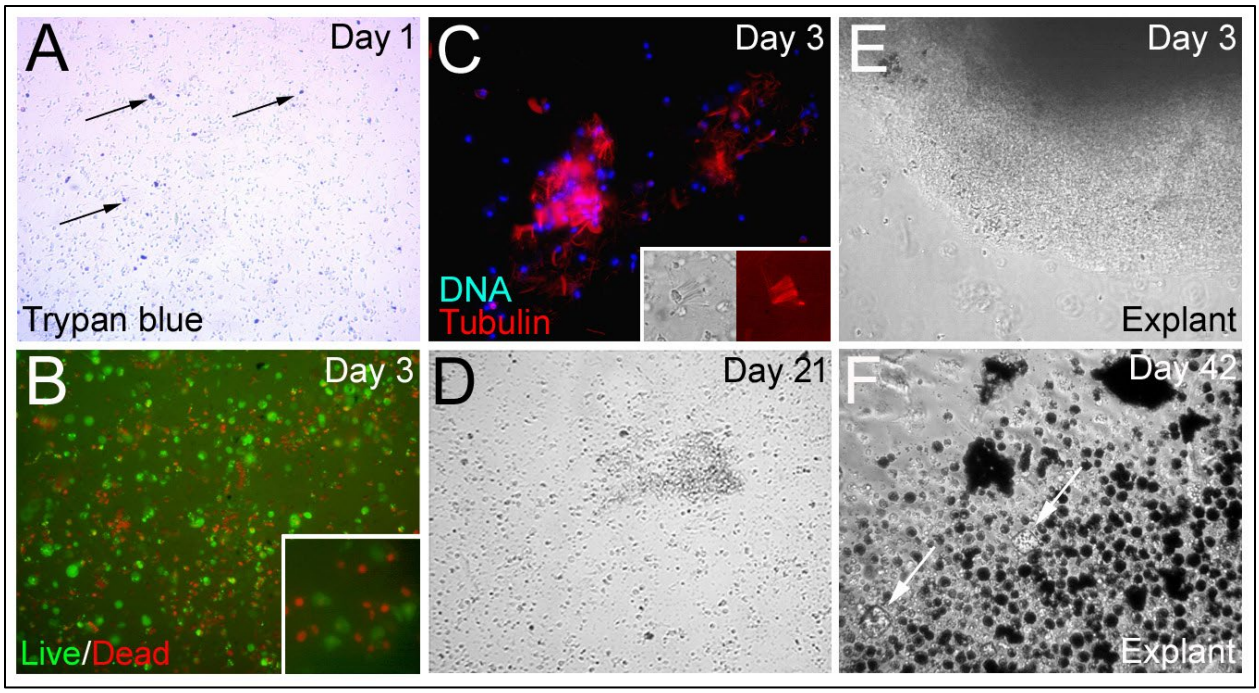


Figure 5 Cultures of primary mussel cells. (A): Trypan blue assay on mussel cells cultured for 1 day in L15-based medium. Arrows indicate dead cells stained in blue. (B): Fluorescence live (calcein AM, green) / dead (EthD-1, red) assay on quagga-derived gill cells cultured for 3 days in Schneider-based medium. Inset illustrates red fluorescent dead nuclei and green, fluorescent live cells; (C): Immunofluorescence staining for the microtubule marker acetylated tubulin (Tubulin) of quagga cells maintained for 3 days in L15-based culture medium on collagen. Inset illustrates a ciliated gill cell; (D): Bright-field image of quagga cells cultured for 3 weeks in L15-based medium on collagen. (E): Bright-field image of a 3 day-old cultured tissue explant derived from quagga mussel foot. (F): Calcium carbonate precipitations (arrows) in 6-week old cultures derived from quagga mantle explants.

3.2.1 Effects of coating substrates

In our cell culture conditions, we have observed that mussel cells do not attach to regular tissue-culture treated plastic of glass substrates, behaving rather as suspension cell cultures. Of the 13 coating substrates we tested so far (Figure 3), only collagen type I favored some attachment of quagga and zebra primary cells in vitro (Figure 5). However, we noticed that mussel cells were only loosely attached to the substrate, resulting in some cell detachment and cell loss during culture medium changes. Even coating wells with 0.2 mg/mL MAPTrix™ (Kollodis Biosciences Inc., Sigma), a commercially available hybrid recombinant of Mytilus edulis adhesive proteins, did not improve mussel cell adhesion. Therefore, we are routinely culturing mussel cells on uncoated or collagen-coated cell culture-treated plastic wells until we identify better substrates.

3.2.2 Influence of cell culture media

Culture media are keys to the survival of primary cells in culture since they provide the nutrients necessary for their maintenance and growth. To date, we have tested the effects of 7 different culture media on the viability of mussel cells in vitro (Figure 3). As assessed by live/dead assays, three culture media proved efficient at maintaining cellular survival. These media were based on L15 (Quinn et al., 2009), Schneider's medium (a medium designed for the culture of fruit flies) and Grace's insect medium (designed for the culture of moth cells) and contained 2-10% of fetal bovine serum (FBS) and antibiotics (gentamycin, penicillin, streptavidin and kanamycin). Similar observations were made when culturing mussel explants. For example, cultured quagga mussel derived gill cells showed a 2.3 fold increase (Figure 6A) in viability when cultured for 24 hours in Schneider's-based medium compared to L15-based medium. In addition, compared to other tested media, ciliated gill cell motility was increased when cultured in Schneider-based culture medium. We are routinely using this latter medium in our cell culture protocols.

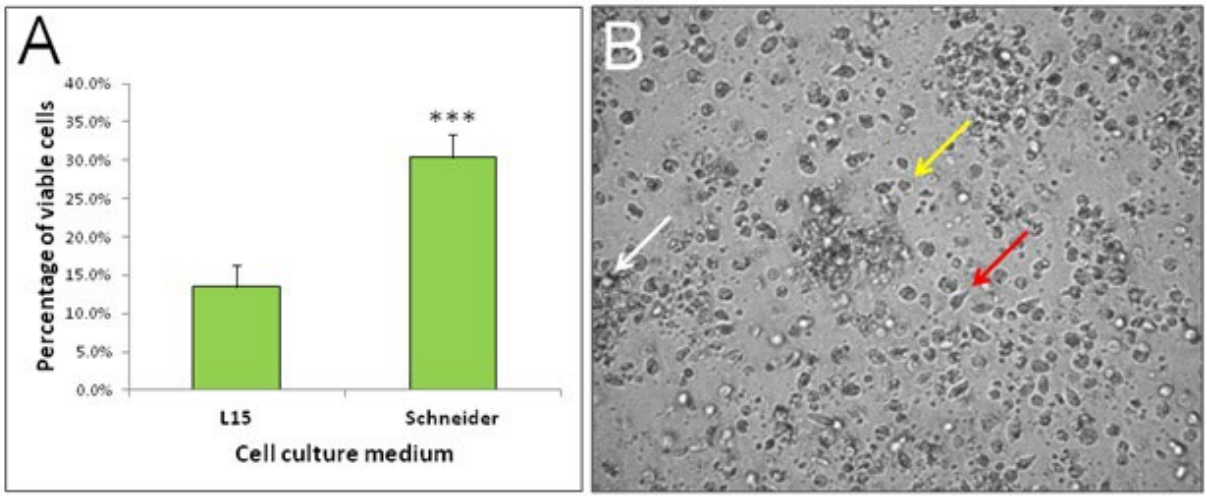


Figure 6 Cultures of mussel cells. (A) Percentage of live quagga gill-derived cells, 24 hours after culture in L15- or Schneider-based medium. (B) Two week-old cultures of mantle explant-derived cells are made of pigmented cell (white arrow), round cells (yellow arrow) and elongated cells (red arrow).

3.2.3 Influence of factors/supplements

It is well established from mammalian and fish cell cultures that supplementation of the culture medium with growth factors such as fibroblast growth factor 2 (FGF2), epithelial growth factor (EGF) or insulin-like growth factor (IGF) are crucial for the long-term culture of healthy differentiated cells. Figure 3 and Table 1 summarize the factors, molecules, supplements so far evaluated for their effects on cell survival. To date, and at the concentrations tested, none of the factors/supplements significantly improved mussel cell viability or growth in vitro. We will keep testing various combinations of coating substrates, cell culture media and factors on mussel cell viability. As new factors become available, we will include them in our culture protocols.

3.2.4 Phenotypic identification of cultured mussel cells

As shown in Figures 5 and 6, we were able to maintain mussel cells in culture. Mussel-gill derived cell cultures were mostly heterogeneous, consisting of round cells, fibroblast-like cells, pigmented cells and ciliated epithelial cells (Figures 5 and 6B) (Gomez-Mendikute et al., 2005). Our main challenge has been to identify antibodies raised against various cells markers that are cross-reacting

with quagga and zebra mussel proteins. Based on the bivalve literature and tests in our own laboratory, we have been able to observe that quagga mussel-derived cells maintained in culture exhibit robust staining for the microtubule marker acetylated tubulin and largely correspond to ciliated epithelial cells (inset in Figure 5C). In cell cultures derived from the whole mussel body, we also observed a small number of cells positive for serotonin, a transmitter involved in various physiological functions in bivalves (Canesi et al., 2022). Those serotonin-positive cells could correspond to neurons, which we will need to confirm with staining for additional neuronal markers. Finally, many cultured cells displayed strong immunoreactivity for smooth and skeletal myosin suggesting the presence of muscle cells in some cultures. We continue looking for commercially available mollusk-specific antibodies to test in our cultures, specifically antibodies that recognize hemocytes, a cell type we believe to be abundant in our preparations.

3.2.5 Long-term survival of cultured cells

Successful long-term cultures of cells derived from mollusks, including quagga and zebra mussels, have been difficult to achieve due to early entry in a quiescent state (see Yoshino et al., 2013 and our data below). The longest time that we have been able to maintain dissociated cells in culture was 3-4 weeks (Figure 5D). Observation of the cells cultured for several weeks did not show any qualitative differences in their size and/or morphology compared to few days-old cultures. Cell viability, however, was greatly variable from one culture to the other. Fluorescence live/dead assay showed that about 85-90% of cells were viable after 1 day in culture, 30-53% by 1 week and 17-20% by 3-4 weeks. To determine if cultured explants could be used as an alternative in vitro model to support long-term mussel-derived cell viability, we cultured small explants derived from a variety of mussel tissues such as gills, foot, mantle and muscle on collagen-coated plastic in L15- or Schneider-based media. Within 3 days, we observed a thin layer of cells developing at the edge of some of the explants, suggesting that some outgrowth was occurring (Figure 5E). However, in most cases, no further cellular layer extension was observed the following days. In a few cases, we were able to maintain mussel explants-derived cells for up to 10 weeks. Cultured explants exhibited slightly better cell viability than cultured dissociated cells with 79.6% at 2 weeks, 38.8% at 4 weeks and 26.4% at 10 weeks. These differences could be due to the type of tissue cultured. In one instance, mantle explants cultured for 6 weeks exhibited a 62.8% cell viability. Interestingly, this culture displayed crystal-shaped formations (Figure 5F) that could correspond to calcium carbonate precipitations required for shell formation (Xiang et al., 2014).

Viable quagga- and zebra-derived cells of various phenotypes can be maintained in vitro up to 10 weeks in our culture conditions. Part of our future effort will focus on further characterizing the time-dependent changes in cell viability and identifying the phenotypes of long-term surviving cells.

3.2.6 Produce frozen stocks of mussel cells

A requirement to the production of stocks of disseminated neoplasia cells is the establishment of frozen storage procedures for quagga and zebra mussel cells. Exposure to sub-zero temperatures is a stress factor that can result into osmotic deregulation, cell membrane breakage and ice crystal formation in cells from many species. Whereas some cryopreservation protocols have been developed with various degrees of success for bivalve gametes, their exploration for adult somatic cells remains limited. We began by evaluating the efficacy of dimethyl sulfoxide (DMSO), a commonly used cryoprotectant (Cheng et al., 2001), to produce frozen stocks of cells. Quagga mussel cell cryostored for 2 weeks in either aquarium water or L15-based culture medium supplemented with 10% FBS and 5% DMSO did not survive the freezing and/or thawing

procedures. We then tried a freezing medium consisting of 90% FBS and 10% DMSO designed for the cryopreservation of the Bge snail cell line (Hansen, 1976). Once again, no cells survived the freeze/thaw procedure a week later. In another series of experiments, we repeated a similar procedure with the difference that quagga gill-derived cells were re-suspended in L15- or Schneider-based medium supplemented with 30% FBS and 10% DMSO. When thawed a week later, cell viability was 17.7% and 20% respectively. These numbers, however, dropped 6 days later to 0.88% and 0.90% (Figure 4). Finally, preliminary experiments using glycerol as a freezing agent suggest lower performance compared to DMSO (7.6% versus 25.3%, cells in cryostorage for 11 days). We will keep testing additional cryoprotectants and freeze/thaw protocols to identify the procedures that will allow long term storage and survival of mussel cells.

3.3 Cell division status in cultured mussel cells

3.3.1 In vivo cell division in adult quagga gill tissue/cells

One option to introduce new genetic material is to use cells that are dividing or induced to divide. In mussels, cell division has been shown to persist in a variety of adult tissues such as gills (Tomasovic and Mix, 1974; Neumann and Kapps, 2003). Cells need to copy or "replicate" their DNA before they can divide. Thus, to analyze the DNA replication status of gill cells in vivo, we maintained live quagga mussels in aquarium water supplemented with EdU, a thymidine analog that incorporates DNA during replication. A few days later, analysis of EdU staining in the gills showed the presence of many positive cell nuclei (Figure 7). EdU-stained cells mostly populated the central region of the gills, which is characterized by an abundance of pigmented cells (Figure 7A and 7B). A subset of positive cells was also noticed in the peripheral regions of the gill (Figure 7C). Phosphorylation on serine 10 of histone 3 (PHH3) is a marker of cells actively undergoing mitosis. As shown in Figure 7D, divided cells were present in the central region of the gill but at much smaller numbers than Edu-positive cells. This pattern of EdU staining distribution in the gill was like that observed in gills from quagga mussels freshly collected from Lake Michigan (data not shown).

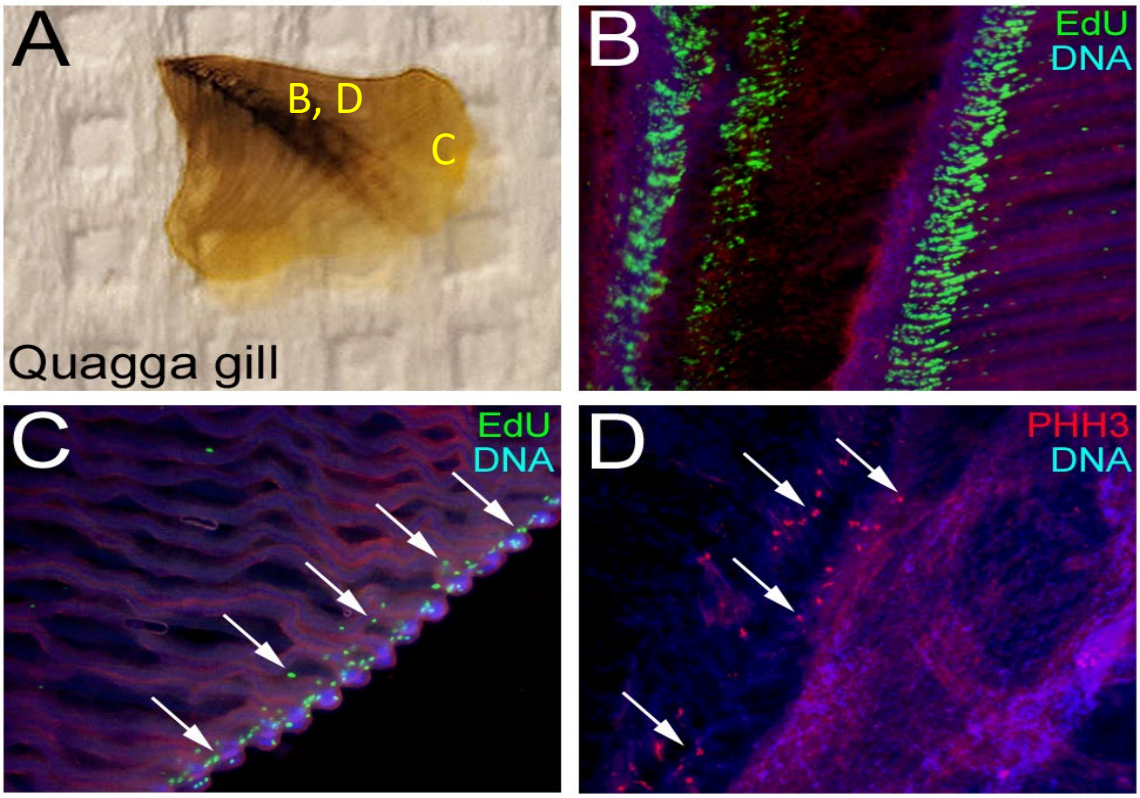


Figure 7 In vivo cell division in quagga gills. EdU staining (cells in S-phase of the cell cycle) and nuclear DAPI (blue) labeling. (A) Bright-field photograph of a quagga mussel gill flattened on a glass slide. Central (B,D) and lateral (C) regions are indicated. (B, C) Fluorescence imaging illustrating the presence of EdU-positive cells in the central (B) and lateral (arrows in C) regions of the gills from a mussel maintained for 18 hours in aquarium water supplemented with EdU. (D) Immunofluorescence staining for histone 3 phosphorylated at ser10 detecting the presence of actively dividing cells in the gill central region (arrows).

We next examined what would be the cell division status of gills maintained in vitro. To this end, we analyzed the staining's for EdU and PHH3 in dissected gills maintained for 3 and 7 days in cell culture medium. As shown in Figure 8 A-C, we observed very few EdU- or PHH3-positive cells at 3 days and none at 7 days, suggesting that in vitro culture of quagga mussel gills and other tissues (not shown) negatively impact their cell division capabilities. We performed similar studies on in vitro cultures of dissociated mussel gill cells and observed a total absence of EdU- and PHH3-positive cells (Figure 8 D-F). These data confirm previous observations (discussion in Yoshino et al., 2013) that mollusk-derived cells enter quiescence in culture, posing thereby a major challenge for their long-term maintenance in vitro. This explains why only one molluscan cell line - the Bge embryonic cell line derived from the snail *Biomphalaria glabrata* (Hansen et al., 1976) – is available at this time.

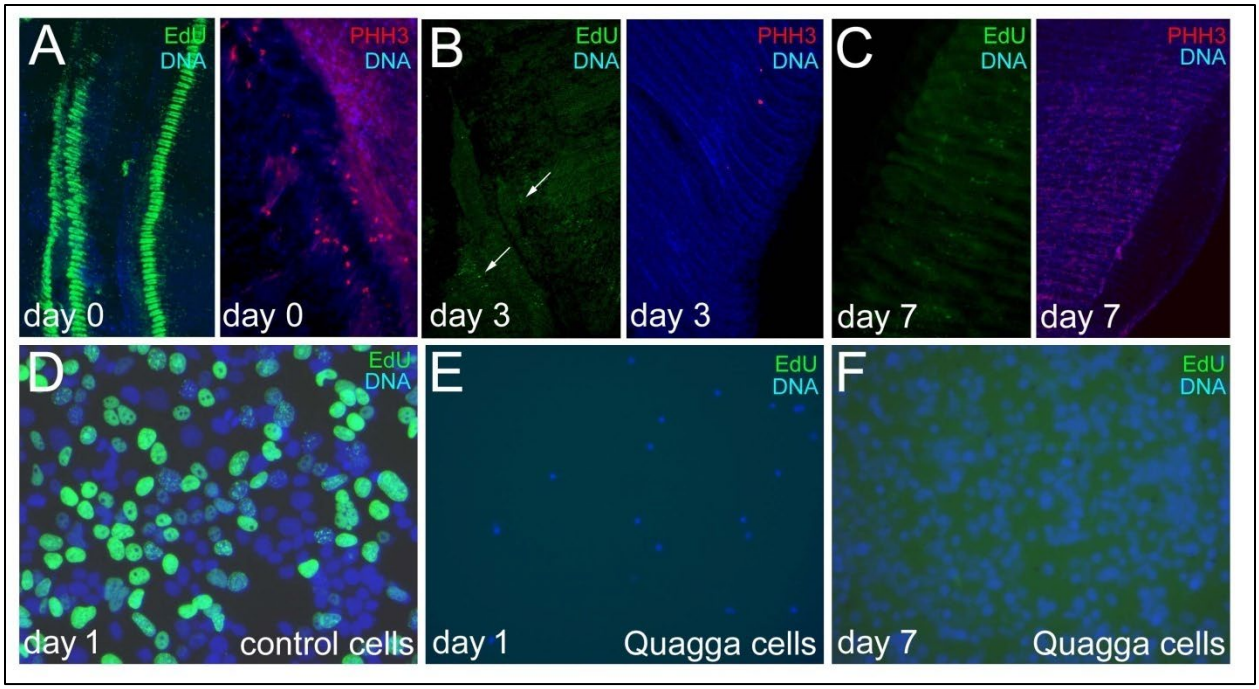


Figure 8 In vitro cell division in quagga gills. EdU staining (green), PHH3 immunofluorescence labeling (red) and nuclear DAPI (blue). Cellular DNA replication (green) and cell division (red) rapidly stop when intact (A-C) and dissociated (D-F) Quagga gills are cultured in vitro. Control cells are actively dividing mammalian cells.

3.3.2 Culture of embryonic cells

A hallmark of early development is the rapid proliferation of cells that will ultimately differentiate into specialized phenotypes. Consequently, embryonic cells are often easier to maintain in culture than cells derived from adult tissues. To explore this further, we started a year ago to induce spawning of quagga and more recently zebra mussels from our aquaculture colony (Figure 9A). On average, over a year, we were able to induce spawning in 57.2% of quagga mussels. Highest spawning efficiency was observed in the 3-4 months following mussel collection, followed by a progressive decrease (Figure 9B). In the peak months, we also determined that approximately 45.2% of the spawning mussels in our colony were female. Under serotonin exposure, motile sperm started to be released within 15 minutes, whereas it took at least 1 hour to observe spawning of eggs (Figure 9 C-E) (Ram et al., 1993). The mixing of sperm and eggs (Figure 9F) resulted in successful fertilization and embryo formation (Figure 10 A and C). In a few instances, embryos failed to develop beyond a few cells, probably due to polyspermy. Fluorescence detection of EdU and PHH3 in 24 hours post-fertilization embryos showed cells replicating their DNA (Fig. 10B) and actively dividing (Fig. 10D). Embryos developed into trochophores and veligers that we were able to maintain in culture for 10 days. Preliminary data suggest that as observed for primary adult cells, dissociated embryonic cells can be maintained in vitro (Fig. 11). Studies evaluating the in vitro effects of various substrates, cell culture media and factors on the survival and proliferation of mussel embryonic cells are ongoing.

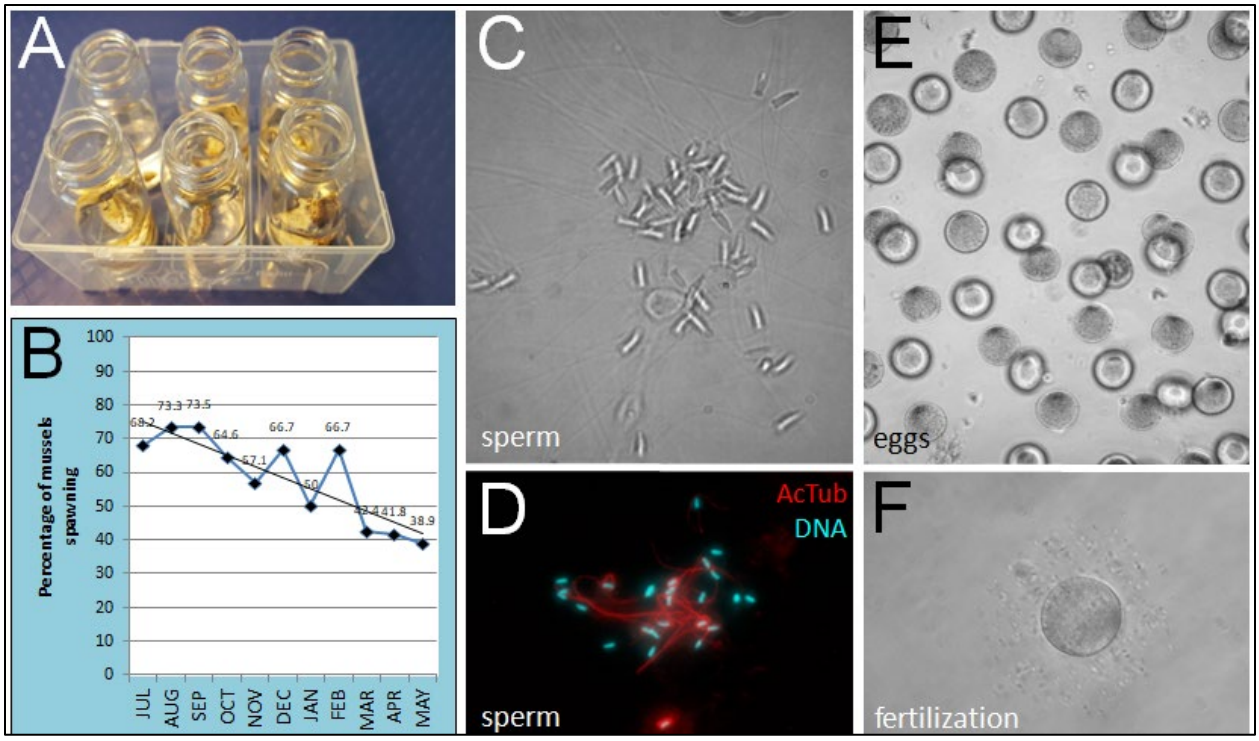


Figure 9 Spawning of quagga mussels. (A) Setting for inducing mussel spawning. (B) Percentage of serotonin-induced spawning for each month. (C, D) Sperm released in aquarium water (C) and after immunofluorescence staining of the flagella (Acetylated Tubulin, red) and nuclear regions (DAPI staining). (E) Mature oocytes spawned in aquarium water. (F) Mixing of sperm and eggs for fertilization.

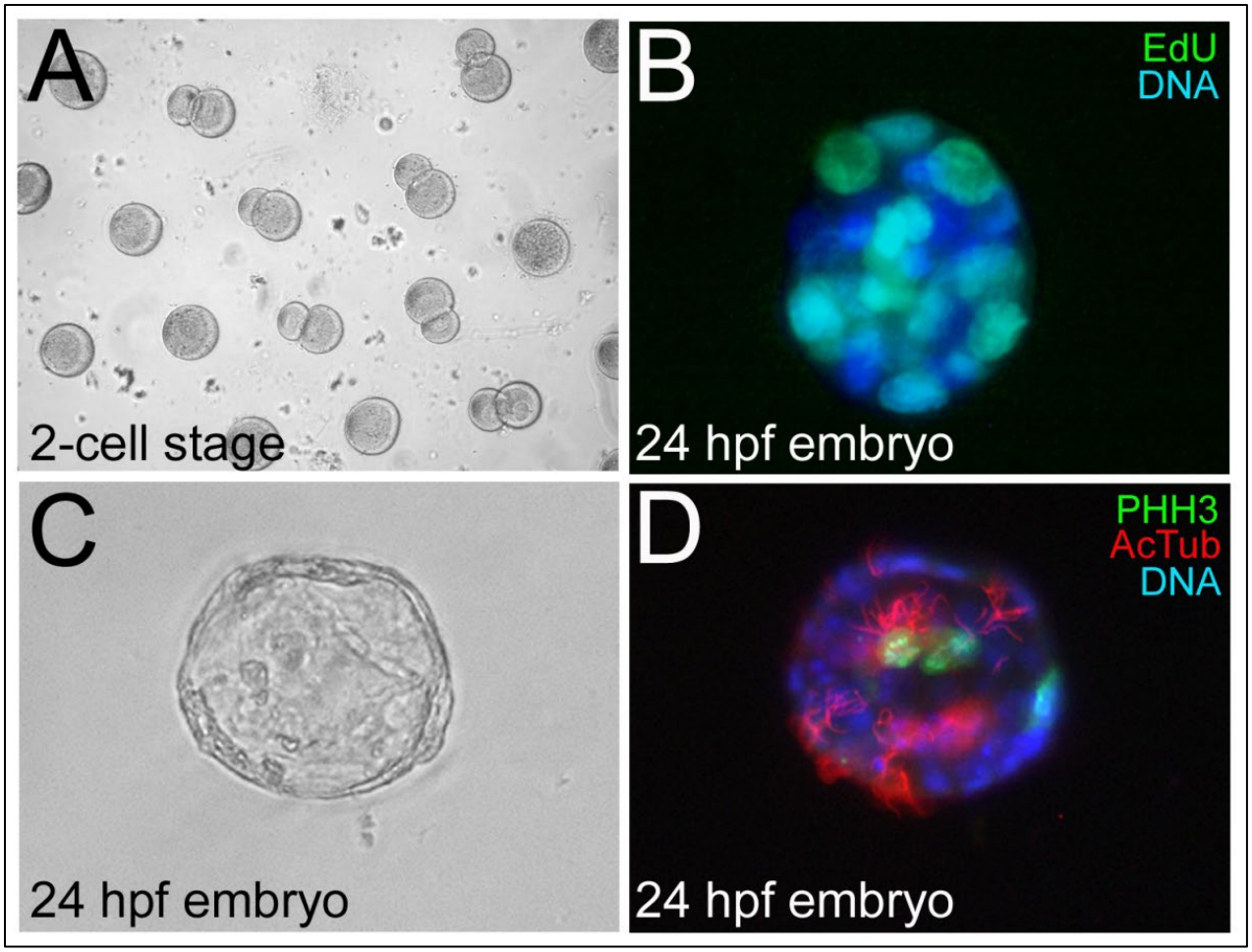


Figure 10 Serotonin-induced spawning in Quagga mussels. A) Embryos at the 2-cell stage, (1 hour post-fertilization, hpf). B) Twenty-four hpf embryo displaying many labeled nuclei (green fluorescence) after 14-hour exposure to the DNA replication marker EdU. C, D) Bright-field (C) and fluorescence (D) imaging of the mitotic marker PHH3 (green) and microtubule marker acetylated tubulin (AcTub, red) in a 24 hpf embryo. DNA is counterstained with DAPI (blue) in B and D.

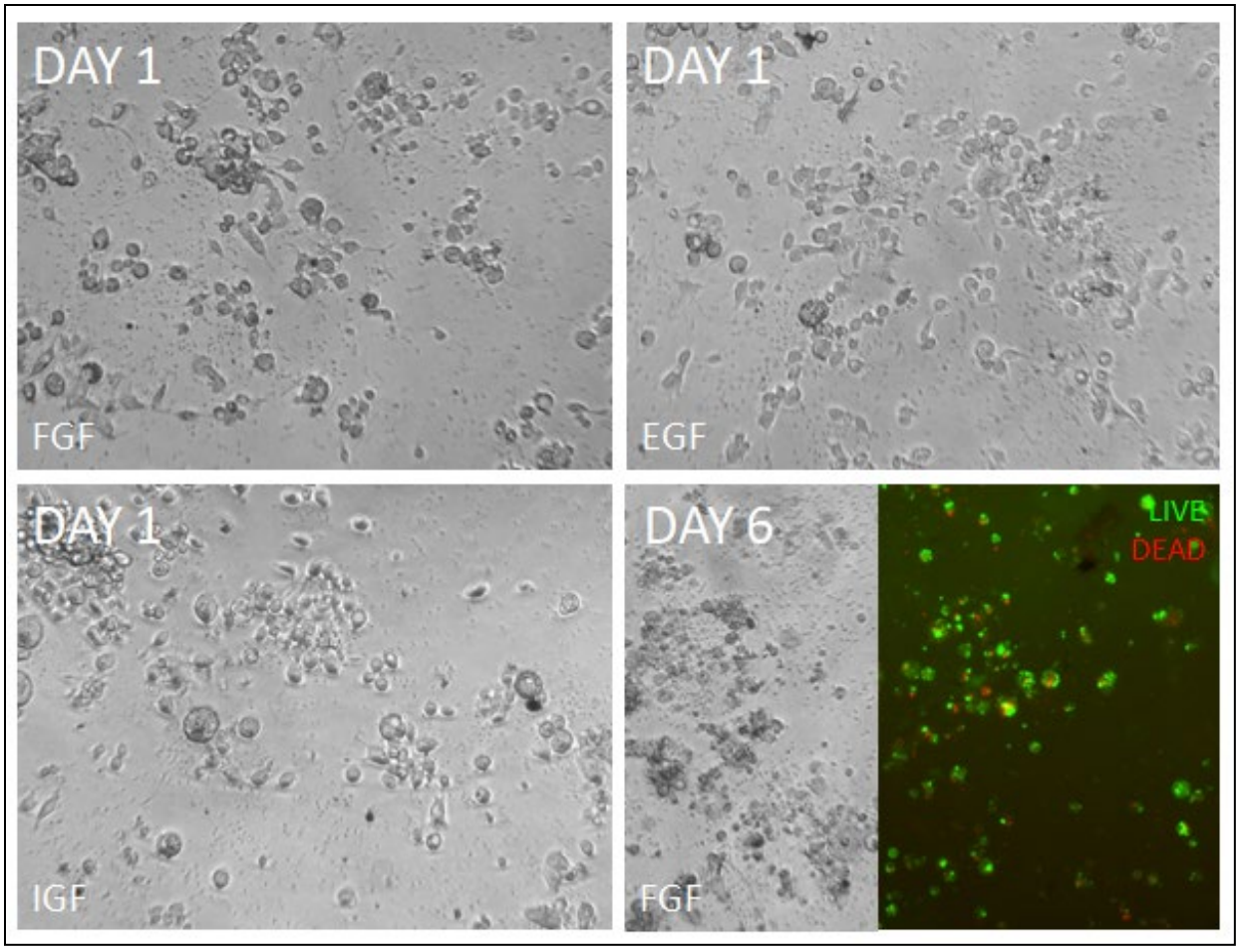


Figure 11 Cultures of quagga mussel embryonic cells. Pronase-dissociated embryonic cells were maintained for 1 to 6 days in Schneider-based culture medium in the presence of the growth factors FGF, EGF or IGF. Calcein AM (Live) / EthD-1 (dead) assay shows the presence of both live and dead cells at day 6 of culture.

3.4 Assessing different transplantation/engraftment methods into live mussels

3.4.1 Transplantation/engraftment methods into live mussels

While developing mussel cell culture protocols, we started assessing the feasibility of directly injecting DN mussel cells into live mussel tissues by attempting in vivo injections of tracker dyes. Examination of various mussel tissues at 24 and 72 hours following injection of VybrantTMDiI showed robust fluorescence staining in a variety of tissues (Figure 12 A-B), which suggests that direct injection may be an efficient strategy to transfer donor cells to live host mussels. To better assess the dispersion of prospective donor cells, we repeated the injection experiments using 2-µm red fluorescent red latex polystyrene beads (Figure 12 C-F). As shown in Figure 12 E and F, we detected the presence of fluorescent beads in most of the tissues examined at 1 and 3 days post-injection. We are continuing to explore injection strategies with various donor cells and improving tissue targeting.

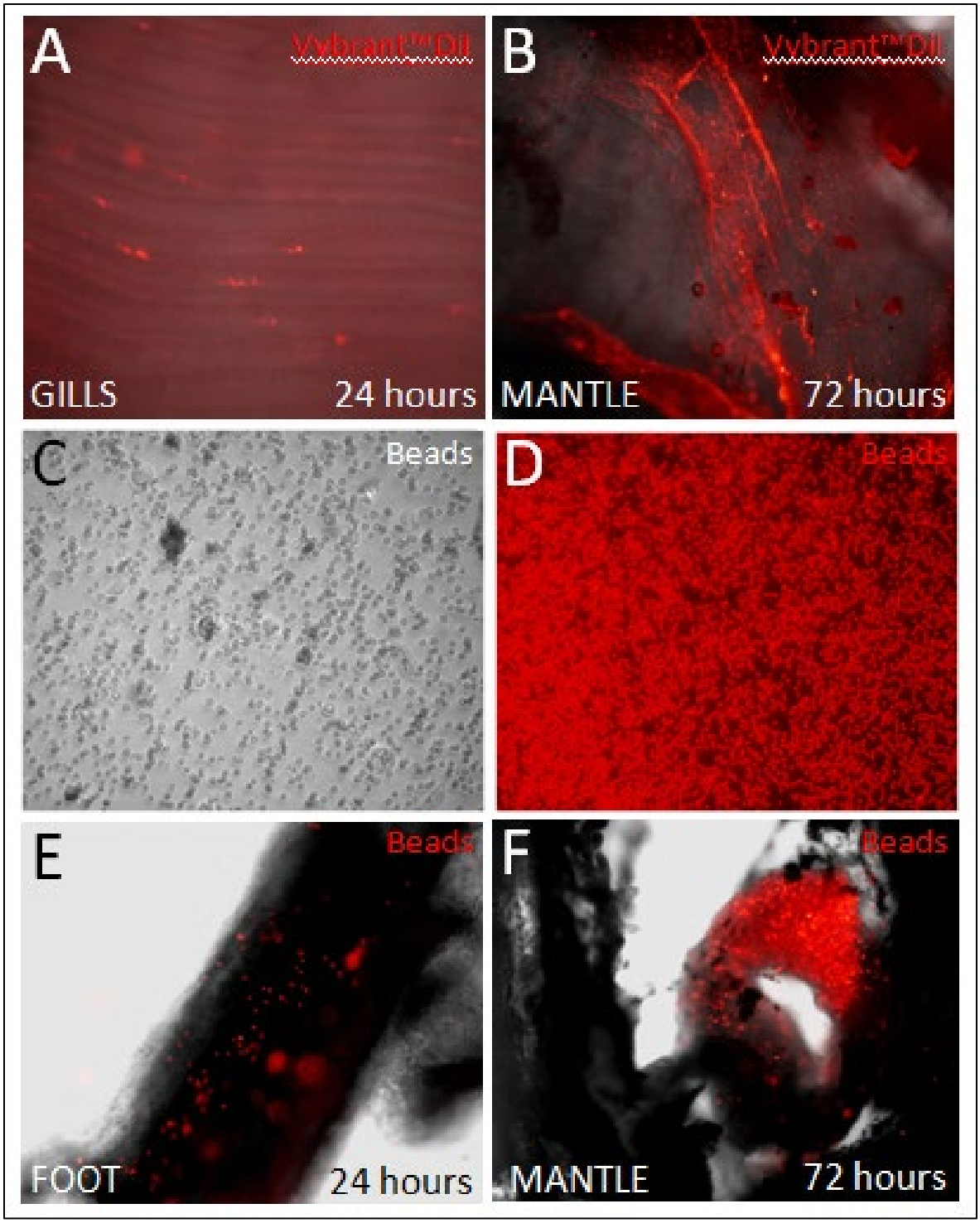


Figure 12 In vivo injections of dye and beads in live host quagga mussels. (A, B) Fluorescence imaging of host gill and mantle tissues 24 and 72 hours after injections of Vybrant™DiI. (C,D) Brightfield (C) and fluorescence (D) imaging of 2um red latex polystyrene beads. (E,F) Fluorescence imaging of quagga foot and mantle tissues 24 and 72 hours following injection of fluorescent beads into live mussels.

3.4.2 Tracking donor cells in live host mussels

A key prerequisite to evaluating the efficacy of transplantation/engraftment strategies is the ability to reliably identify and track donor cells. We focused on the use of previously described fluorescent markers that had proven successful at labeling cells in other model systems. We first tested VybrantTMDiI, a fluorescent dye that stably incorporates into cell membranes and is easily detectable by fluorescence microscopy. As shown in Figure 13A, staining of quagga gill cells with VybrantTMDiI resulted in the labeling of a reduced number of cells. Whether this low staining efficiency in mussel cells is due to unique membrane properties or altered physiological state non permissive to VybrantTMDiI entry has yet to be determined. We then explored the potential of two additional dyes - FM™1-43FX and CellTracker™Green CMFDA (5-chloromethylfluorescein diacetate) (CMFDA™) -, that are non-toxic, water-soluble, that becomes fluorescent when incorporated in the cell membrane or cytoplasm. Robust staining of quagga gill cells (Figure 13 B and C) was observed and retained for several days, which make FM™1-43FX and CMFDA™ dyes strong candidates for tracking donor cells. Co-staining of stained cells with the dead cell marker Eth-D1 allowed us to verify that positive cells were viable. Our next step will be to inject labeled donor cells in live host mussels.

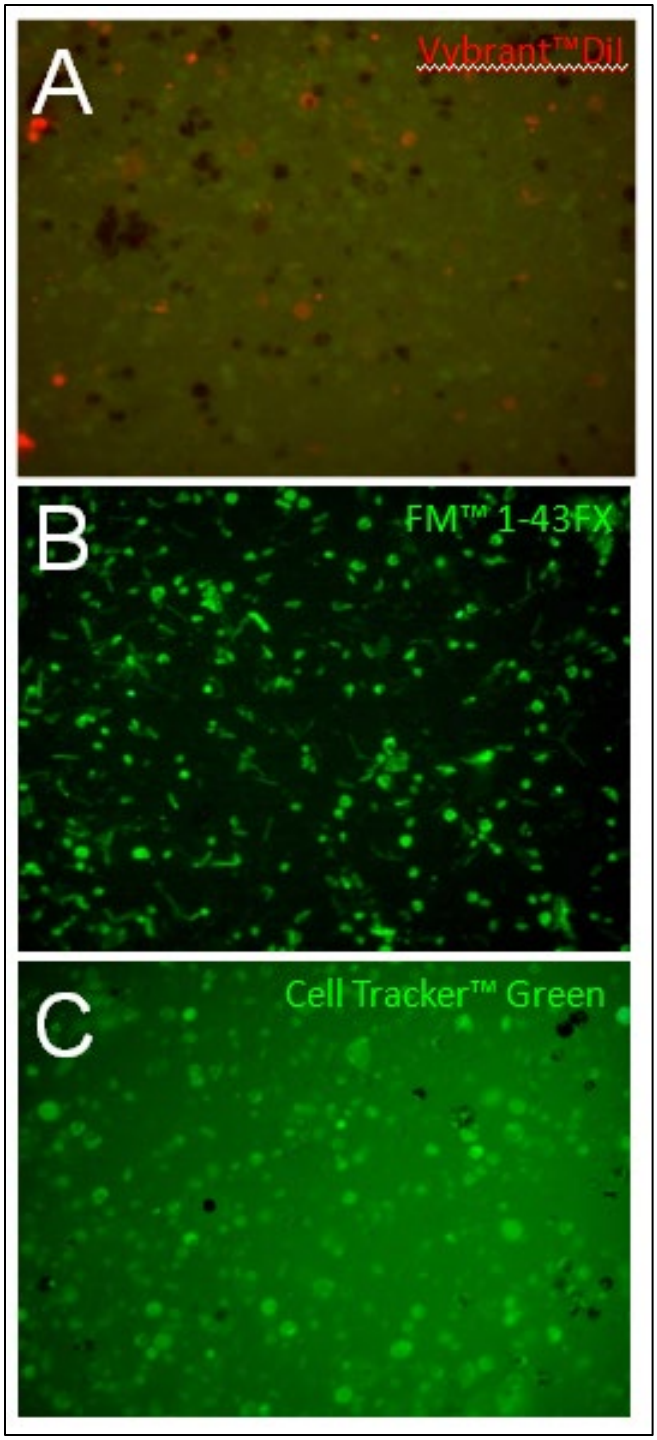


Figure 13 Tracking donor quagga mussel gill-derived cells. Staining of gill cells with Vybrant™DiI (A), FM™1-43FX (B) and Cell Tracker™green CMFDA (C).

3.5 Invasive mussel genes, mRNAs, and expression vectors

Quagga mussel (QM) sequence, gene, and gene structure information provided by Dr. Passamaneck (see Materials and Methods) was used in several ways:

- To provide the nucleic acid sequence of open reading frames (ORFs) encoding proteins of interest to the project such as the cell cycle regulator p53.
- To provide the sequence of gene regions that control expression such as promoters and polyadenylation signals for use in construction of expression vectors that should be functional in mussel cells.
- To provide sequence information for production of primer pairs for analysis of SNPs or qPCR analysis of mRNA expression levels.

Since QM p53 is a primary target for CRISPR/Cas9-mediated mutation, significant effort has been put into understanding this gene and its organization. A schematic of a portion of the characterized QM p53 gene is shown in Figure 14A. The p53 protein has a critical determinant common to the p53 proteins of all species that is central to function. This determinant, referred to as "RCPNH" (reflecting the amino acid composition), is located in the putative Exon 6 of the QM p53 gene (Figure 14A). CRISPR/Cas9-induced mutations that create a frame-shift upstream of the Exon 6 RCPNH determinant are predicted to destroy p53 functionality and foster loss of cell cycle control. The location of the three primary Cas9 targets in Exons 4 and 6 are indicated on the schematic and described in greater detail in a later section of Results.

QM gene control regions, including promoters and polyadenylation elements, have also been identified and used in the construction of dreissenid mussel expression (DME) vectors to drive transgenes (i.e., oncogenes such as SV40 Large T-antigen) in mussel cells. Expression control elements – primarily promoters – sourced from dreissenid mussels are critical to the project because expression control regions are often not interchangeable between phyla. Promoters from human genes may not drive high-level expression in QM cells and QM promoters may not be strongly expressed in human cells. If we test methods of gene transfer into QM cells by assaying expression or a reporter gene with a promoter that in itself is non-functional, transduction efficiency cannot be reliably measured. For this reason, we needed to identify and subclone several promoter regions with high-probability of at least some expression in QM cells or tissues.

The QM elongation factor 1-alpha (EF1a) gene structure and sequence were identified by Dr. Passamaneck, and the putative proximal promoter and polyadenylation signal region were (Figure 14 B and C) synthesized and subcloned into an expression vector. Our analysis of the EF1a gene and proximal promoter suggested that the presence of a short 60 bp Exon 1 immediately followed by a 650 bp intron. The presence of introns within an expression cassette can significantly enhance the expression of transgenes, so as shown in Figure 14B, the final subcloned promoter cassette contains: 1) 1 kB of proximal promoter, 2) a probable 60 bp non-coding exon, 3) a 650 bp intron, 4) a splice acceptor from Exon 2, and 5) a polylinker for transgene insertion. At the 3' end of the expression cassette, an EF1a gene fragment extending from the stop codon approximately 390 bp downstream and encompassing an AATAAA motif was subcloned to act as a mussel-sourced polyadenylation signal (Figure 14C).

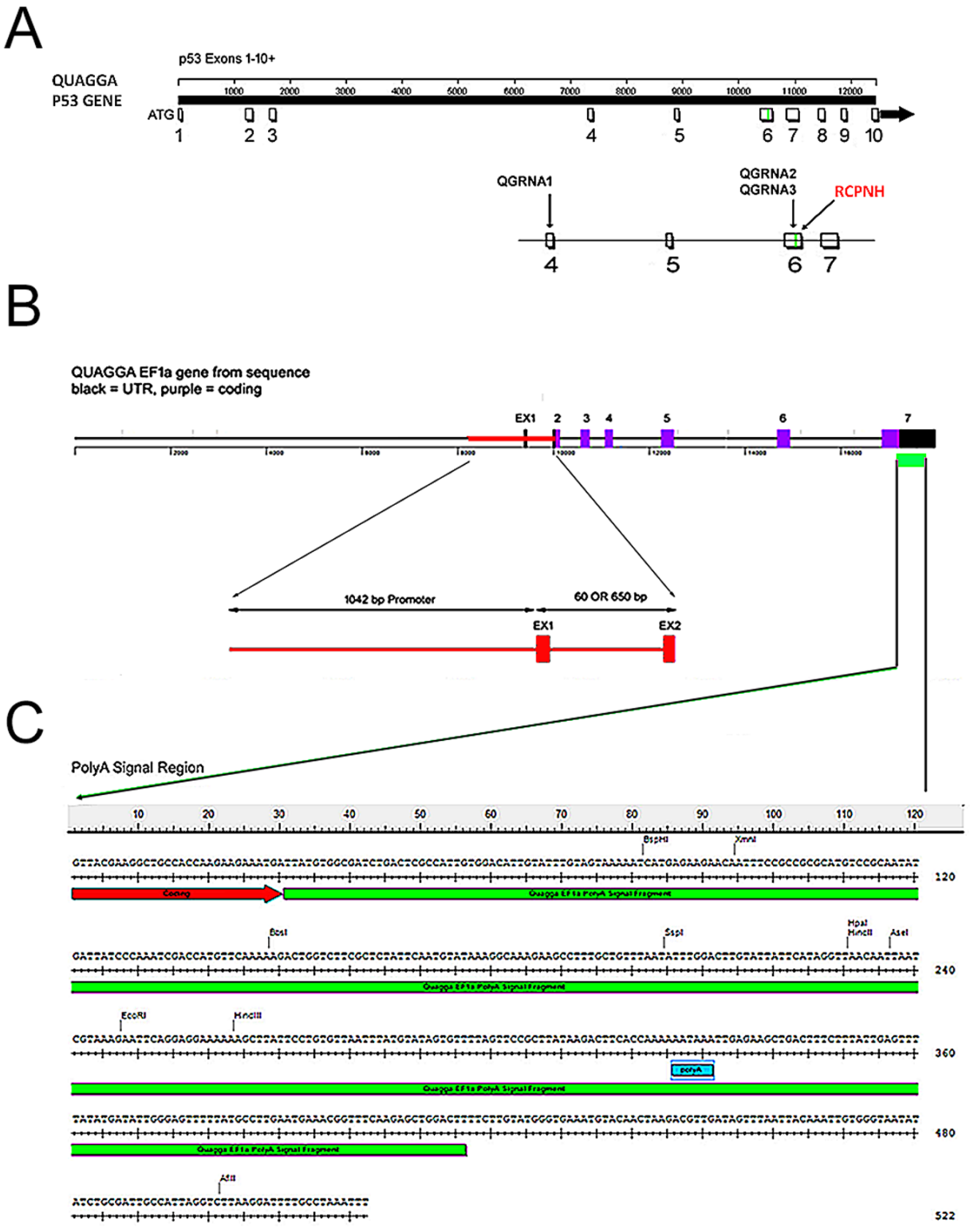


Figure 14 Examples of QM genes characterized and used in construction of expression vectors. A) Map of exons 1-10 of the QM p53 gene and locations of CRISPR/Cas9 recognition sites 1,2, and 3. RCPNH

indicates the location of the critical protein determinant for p53 function. Mutations producing a frame shift upstream of this determinant are predicted to eliminate p53 function. B) Schematic of the QM EF1a gene displaying the promoter region (red) and the 3'UTR and PolyA signal subcloned and used in expression vectors. C) Detail of the QM EF1a gene 3'UTR and AATAAA site.

A variety of reporter genes for fluorescent protein products (eGFP, mCherry, eYFP) or for reporters that are readily detected and quantified (firedrosophila or gaussia luciferase) were inserted between the QM EF1a promoter and QM EF1a polyA signal sequence to create DME expression vectors (Figure 15). Over the course of the project, several additional promoters were subcloned for testing including a 972 bp proximal promoter for QM Ubiquitin-1 (UBI1) gene and a 681 bp promoter for QM Ubiquitin-2 (UBI2). Another promoter fragment used specifically for testing in the insect cell lines is a promoter referred to as mini-AC5 which is a 373 bp basal promoter region of the drosophila actin-5C gene known functional in insect cell types and used as a positive control and for purposes of comparison. All these promoter variants are shown schematically in Figure 15 for the FFLUC DME vector variant used in many experiments.

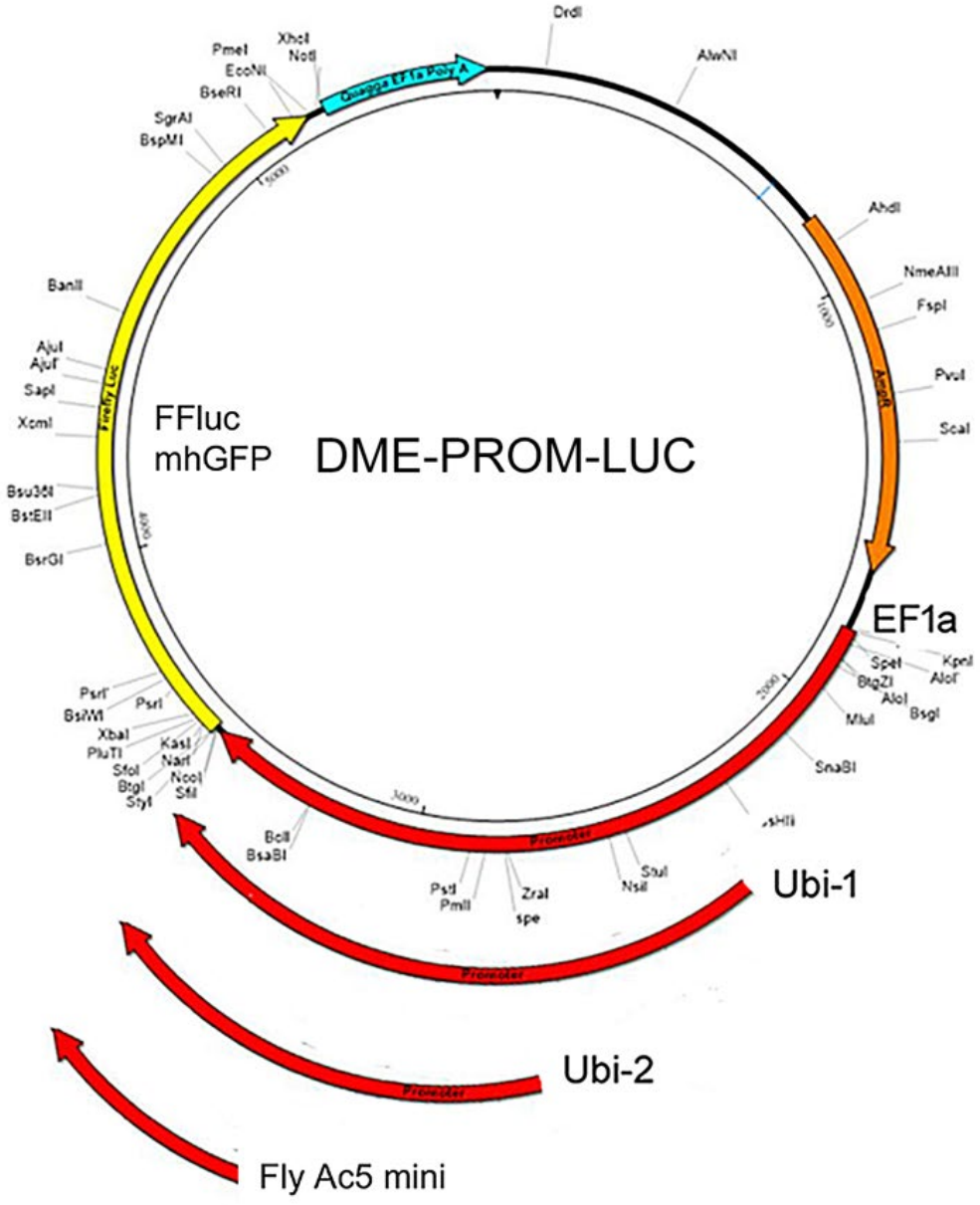


Figure 15 Schematic of dreissenid mussel expression (DME) vectors with promoter variants. The EF1a (1.6kb), Ubiquitin-1 (972bp), Ubiquitin-2 (681bp), and drosophila mini-AC5 (373bp) promoter variants are shown.

3.5.1 Modeling mussel expression vectors in human and insect cell types

Figure 16A shows that human HEK cells transduced with DME vectors containing promoters for QMEF1a, UBI1, or UBI2 with eGFP are clearly fluorescent compared to untransfected controls.

This indicates that all three of the QM promoters isolated are intrinsically functional as promoter elements. The same experiment performed on HEK cells with the FFLUC variant of each vector (Figure 16B) confirmed readily detectable expression of the luciferase transgene from QM promoters. Even though these expression levels are not particularly high compared to strong mammalian promoters like the human cytomegalovirus immediate early promoter (CMV-ie) that are at least 10-fold higher (data not shown), the levels of expression for QM EF1a, UBI1, and UBI2 are comparable to many mid-range mammalian promoters.

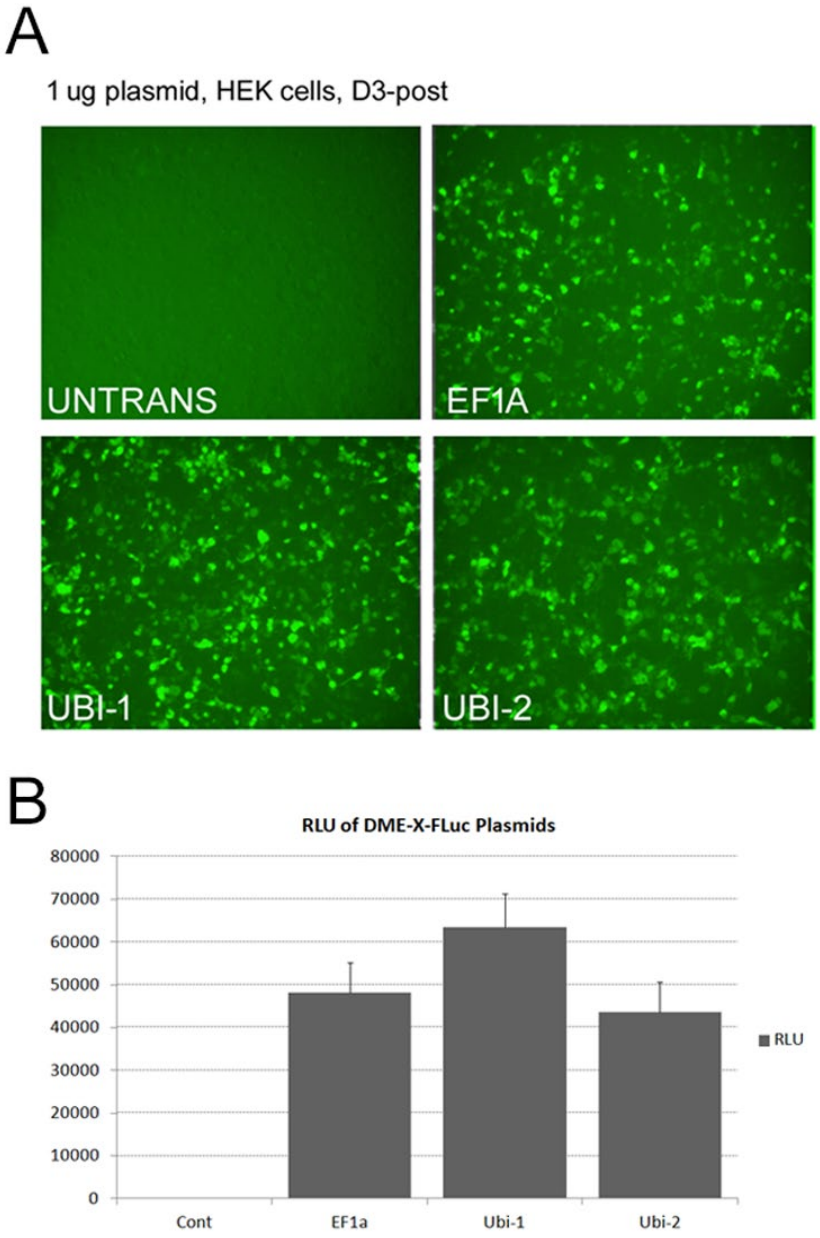


Figure 16 Expression of QM promoters in human HEK cells. A) HEK cells transduced with DME vectors with EF1a, UBI-1, or UBI-2 promoters with an eFGP reporter gene. Green fluorescence is indicative of vector expression. UNTRANS indicates untransfected negative control cells. All cells were imaged for the same time to allow comparison. B) The same experiment using a firedrosophila luciferase (FFLUC)

reporter gene confirming expression of all three DME vectors in transduced human HEK cells. Relative light units are plotted (10s counting of emitted light after luciferin injection).

A comparison of QM promoter vectors and the drosophila mini-AC5 promoter vector revealed that all three QM-derived promoters are also functional in insect cells at levels approximating 20-40% of the strength of a bona fide drosophila promoter (Figure 17A).

In mammalian cells, if the tetracycline transactivator (Gossen and Bujard, 1992) and its highly-specific response element (together known as TTA-TETO) are included between the promoter and the reporter gene, this combination of factors has the potential to increase overall reporter expression even further. A variation of this cassette that includes a G418 resistance gene (Neo), and IRES element in addition to TTA-TETO (together referred to as "NIT") can often boost expression even higher.

When these enhancers were inserted into the UBI1 and UBI2 DME vectors, as expected, overall reporter expression was increased 2-5-fold with TTA-TETO alone and 9-10-fold with NIT compared to the promoters alone (Figure 17B). Together, these results predict that the DME vectors thus far produced and found functional in both human and drosophila cells will almost certainly express if delivered to a QM cell nucleus, providing us with several options for testing of microinjection or other transduction methods.

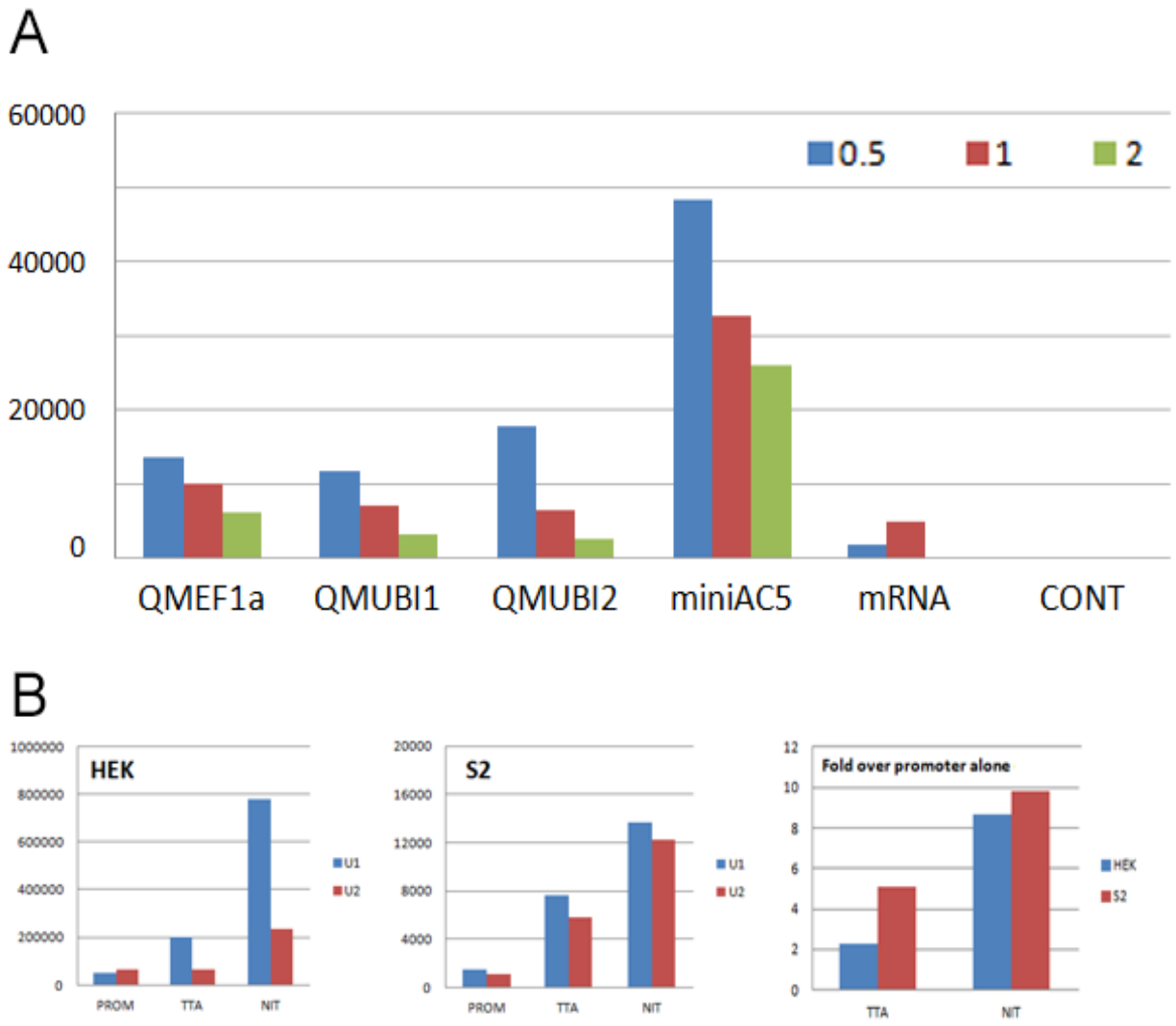


Figure 17 Expression of QM promoters in insect cells and use of expression enhancers in DME vectors. A) Representative experiment showing the relative expression of FFLUC in drosophila S2 cells transduced with different concentrations of DME vectors with EF1a, UBI-1, UBI-2, and mini-AC5 promoters. mRNA indicates wells transduced with FFLUC mRNA and CONT indicates untransduced cells. B) FFLUC expression in HEK (left) or S2 cells (middle) from DME vectors with the UBI1 (U1) or UBI2 (U2) promoters alone or with addition of the TTA-TETO or NIT cassette for expression enhancement, as labeled. The graph on the right shows the fold-change from TTA-TETO or NIT addition relative to promoter only for transduced HEK or S2 cells.

3.5.2 Transduction and viral infection of adult and embryonic QM cells

Although transduction and measurement of reporter expression was readily accomplished for both human and drosophila cells, more than dozen plasmid transduction methods, each with a multitude of subvariants were tested on adult QM cells with no evidence of reporter expression. As shown in

other sections of this report, this is almost certainly due to a lack of nuclear envelope breakdown that accompanies mitosis in all dividing cells. Since QM cells show no evidence of mitosis in culture, transduced plasmids will not transcribe, and reporter protein will not be produced.

Viruses have evolved to transfer genetic material into the cell nucleus and often into genomic DNA in the absence of cell division, so we also tested the recombinant virus types used commonly with mammalian cells to see if expression could be observed in QM cells. Lentiviral vectors constructed by our group using QM promoters to ensure transgene expression were created and packaged into high-titer viral stocks. In addition, we obtained viral stocks for AAV serotypes 1, 2, 5, 6, 8, 9, DJ, DJ8, and DJ9 as well as high-titer stocks of recombinant adenovirus and tested these all for their capacity to infect primary QM adult gill cell cultures. As shown in Figure 18, none of these methods resulted in detectable reporter expression in adult QM cells.

METHOD	NUCLEIC ACID	CONTROL CELLS	TARGET CELLS	OUTCOME	NOTES
CAL-PHOS CO-PRECIPITATION	MCHE/FLUC RNA, DNA	HEK/S2	MUSSEL	NEGATIVE	No precipitate in mussel medium
LIPOFECTAMINE 2000	MCHE/FLUC RNA, DNA	HEK	HEK/DISS MUSS	NEGATIVE	
LIPOFECTAMINE RNAiMAX	MCHE/FLUC RNA, DNA	HEK	HEK/DISS MUSS	NEGATIVE	
LIPOFECTAMINE 3000	MCHE/FLUC RNA, DNA	HEK	HEK/DISS MUSS	NEGATIVE	1X, 2X, 4X, 8X Concentrations
POLYETHYLENEIMINE	MCHE/FLUC RNA, DNA	HEK	HEK/DISS MUSS/WHOLE GILL	NEGATIVE	
ELECTROPORATION (Zap Media I-IV)	MCHE/FLUC RNA, DNA	HEK	DISS MUSS	NEGATIVE	EX1: 100, 200, 300V at 125/250/500/960 Uf
ELECTROPORATION (Zap Media I-IV)	MCHE/FLUC RNA, DNA	ND	DISS MUSS	NEGATIVE	EX2: 600, 1200, 1800V at 25uF
ELECTROPORATION complete mussel medium	MCHE/FLUC RNA, DNA	ND	WHOLE GILL AND GILL CELLS	NEGATIVE	100, 200, 300V at 125/250/500/960 uF600, 1200, 1800V at 25uF
Glass bead-mediated membrane disruption	MCHE/FLUC RNA, DNA	ND	GILL CELLS	NEGATIVE	
Mirus TRANSIT-In	MCHE/FLUC RNA, DNA	HEK/S2	GILL CELLS/4hEm/24hrEm	NEG/POS?/POS?	FLUC MRNA POS
Mirus TRANSIT-2020	MCHE/FLUC RNA, DNA	HEK/S2	24hrEm	NEG	FLUC MRNA POS
Mirus TRANSIT-X2	MCHE/FLUC RNA, DNA	HEK/S2	ND	ND	
Mirus TRANSIT-LT1	MCHE/FLUC RNA, DNA	HEK/S2	ND	ND	
Qiagen Effectene Reagent	MCHE/FLUC RNA, DNA	HEK/S2/Sf9	GILL CELLS/4hEm/24hrEm	POS?	FLUC MRNA POS
AAV Serotypes 1-9	GFP	HEK	GILL CELLS	NEGATIVE	
AdV	GFP	HEK	GILL CELLS	NEGATIVE	
Recombinant Lentivirus (HIV)	YFP/mCHE/PURO/TAG	HEK/S2/Sf9	ND	PENDING	
Recombinant Retrorivus (MMLV)	YFP	HEK	GILL CELLS	NEGATIVE	

Zap Medium I	Leibovitz L15 (15%), HEPES
Zap Medium II	Leibovitz L15 (15%)
Zap Medium III	0.23% saline in sterile mussel water
Zap Medium IV	0.23% saline in laboratory pure water

Figure 18 Table summarizing transduction and infection methods tested on QM cells. Abbreviations: MCHE = mCherry reporter, FLUC = firedrosophila luciferase reporter, PURO = puromycin resistance gene, TAG = SV40 Large-T-antigen, DISS = dissociated, MUSS = quagga mussel, Em = whole embryo or embryonic cells, ND = not done, EX = experiment, NEG = negative result, POS = positive result, Zap medium = medium used with cells for electroporation only.

Although adult-derived QM cells do not divide, cells of 1-2 day QM embryos proliferate and divide (see Figure 10) and experiments are underway to determine if they can be transduced with plasmid DNA. An alternative to DNA transduction is mRNA transduction that requires that nucleic acid only enter the cytoplasm for production of an encoded reporter protein. Although transduction of

mRNA does not result in stable transgenesis, if progress can be made in the introduction of mRNA into QM cells, it follows that these advances may be eventually used with DNA.

Adult cells show no hint of expression of transduced mRNAs, however, mRNAs for the red fluorescent reporter mCherry and for firedrosophila luciferase were observed in transduced embryonic QM cells using insect-based transfection reagents such as Mirus TRANSIT-Insect® or Qiagen Effectene®. The level of transduction is orders-of-magnitude lower than the same mRNAs and reagents used with insect S2 or SF9 cells but are nevertheless the first indication that dreissenid mussel cells are capable of any foreign nucleic acid expression.

These data are shown in Figure 19. Figure 19A shows red fluorescence for mCherry from capped polyadenylated mRNA transduced into drosophila S2 cells as indicated in the legend. Figure 19B shows moth SF9 cells transduced with mRNAs for eGFP and mCherry for 1 or 2 days as indicated. eGFP protein matures rapidly with peak fluorescence between 12-24 hours after transfection, whereas mCherry protein matures slowly and displays peak fluorescence 2-3 days post-transduction. Figure 19C shows rare but detectable mCherry-positive embryonic QM cells visible 3 days post-transduction using Qiagen Effectene® reagent with 0.4 or 2 μg/well of mRNA (Mirus TRANSIT-Insect displayed too much background autofluorescence in QM cells to make a determination).

Similar experiments transducing embryonic QM cells with FFLUC mRNA confirmed the results using fluorescent reporter mRNA, albeit with 50-100-fold lower activity than drosophila S2 cells treated in parallel (Figure 19C, right). It must be noted that QM embryonic cell wells only contained 3-5K cells while S2 wells contained the optimal 50K cells, possibly accounting for some of the difference in overall signal. Using larger numbers of QM embryonic cells and further optimizing ratios of transduction reagents should improve mRNA and DNA transduction of QM embryonic (and adult) cells in future experiments.

These results suggest that existing reagents for the chemical-mediated transduction of QM cells may be of limited effectiveness even on mitotic embryonic cells. Direct delivery methods such as microinjection should be more effective and pilot studies focused on QM embryo microinjection are just beginning and are described below.

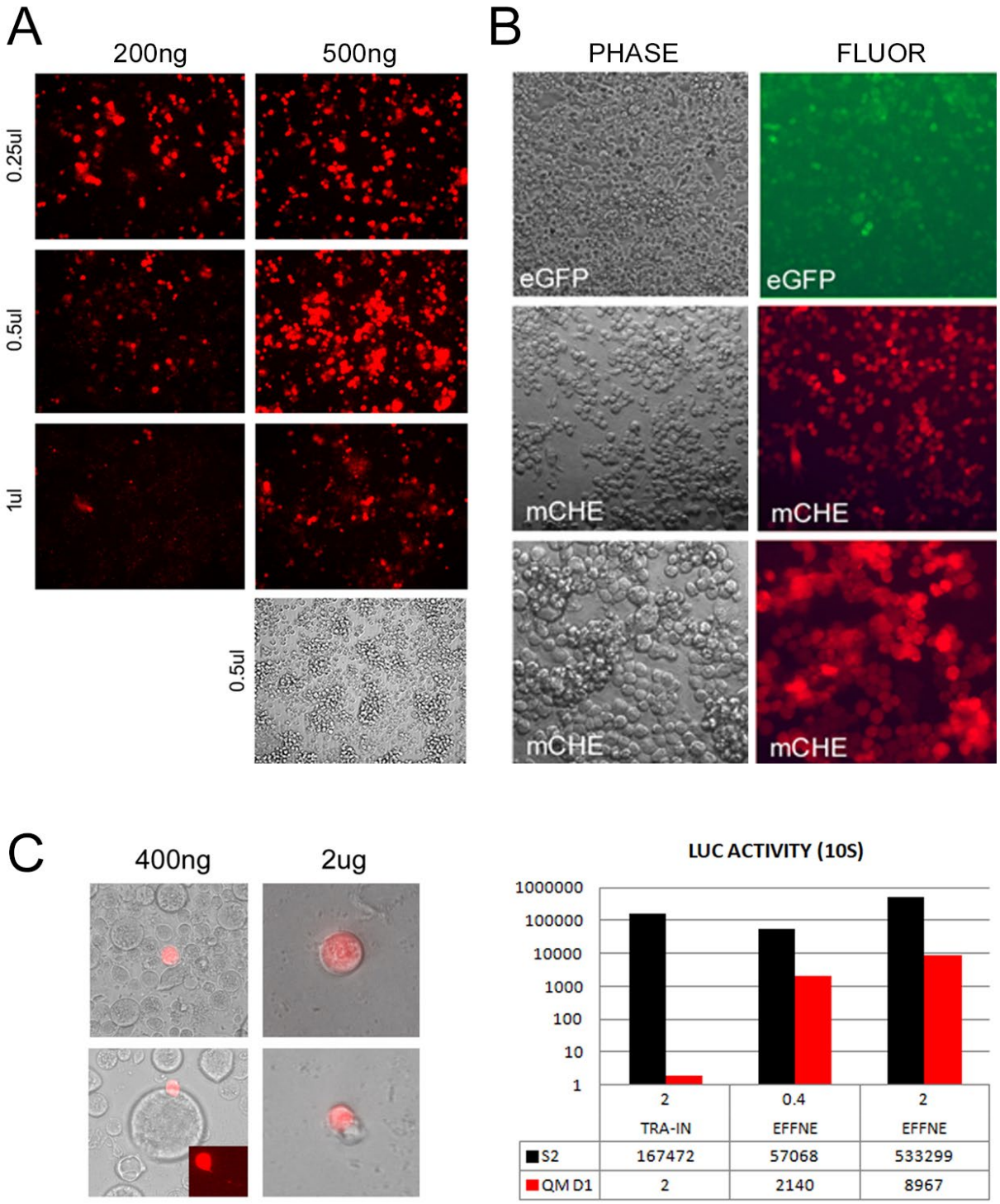


Figure 19 Transduction of insect cells and mussel cells with mRNAs. A) Drosophila S2 cells transduced with 200 or 500 ng of mCherry mRNA using 0.25, 0.5, or 1 µl or TRANSIT-Insect® reagent. Red color indicates fluorescence (400X). The panel at the bottom is a phase-contrast image of cells with 500ng of mRNA and 0.5 µl of TRANSIT showing no change in morphology or indications of toxicity in transduced

cells. B) Moth SF9 cells transduced with 1 µg of eGFP or mCherry mRNA using Effectene® reagent. Images on the left are phase-contrast images. Images on the right are fluorescent images. Green or red color indicates positive signal. The upper GFP and mCherry images were taken on Day 1 post-transduction, and the bottom images were taken on Day 3. eGFP is observed soon after transduction and is not visible much past 24 hours, but mCherry protein matures slowly and gains visibility over several days. C) Transduction of dissociated embryonic mussel cells. Panels on the left show examples of the 8-12 cells found with visible red fluorescence using Effectene®. The graph on the right shows relative light units of luciferase activity in S2 and QM embryonic cells transduced with 0.4 or 2 µg of FFLUC mRNA using Transit® or Effectene®. This is a pilot experiment that requires additional repetitions to confirm findings.

3.5.3 Production and validation of QM p53 CRISPR/Cas9 reagents for knock-out of QM p53

Until microinjection of QM embryos comes online in upcoming experiments, we have pre-validated QM p53 CRISPR Cas9 reagents by creating a mammalian cell model of QM p53. Previous experiments in our laboratory indicated that mammalian fibroblasts lines of most species could be transduced with ribonucleoprotein Cas9-gRNA complexes with nearly 100% efficiency (data not shown), so we reasoned that if we could introduce the region of QM p53 containing our genomic target DNA into mammalian cells, we could at least confirm functionality of our selected gRNAs and examine some parameters that influence efficiency. As described in Materials and Methods, a 450-bp portion of the QM p53 mRNA encompassing Exons 4-6 was stably introduced into mouse 3T3 fibroblasts using a G418-resistant retroviral vector. Cas9-induced cuts that create insertion/deletion mutations known as "indels" (Singh et al., 2015) within any of the three target loci have a chance of disrupting an Msp1 restriction site partially overlapping each of the Cas9 cut sites (Figure 20A). Loss of a functional Msp1 site within PCR fragments produced from genomic DNA of targeted cells will indicate Cas9 activity and the relative efficiency of mutation. The observed digestion pattern will always underestimate the efficiency of targeting for two reasons: 1) Some indels will be too small to disrupt the Msp1 site and 2) Some Cas9 cuts repair with no change in sequence. Nevertheless, this method should still provide an idea of relative targeting efficiency when comparing among different gRNAs and target sites to determine which are potentially strongest and weakest.

As shown in Figure 20B, the appearance of Msp1 uncut bands of the proper size in all gRNA-treated cells and the absence of uncut bands in the DNA of cells with no gRNA treatment (0 nM) reveals that all three gRNA are functional at all concentrations tested. The intensity of uncut bands of gRNA-3 (404 bp) appear more prominent than the 404bp bands of gRNA-2, suggesting that gRNA-3 may be the first choice for injection of QM embryos. gRNA-1 cannot be ruled out, however, because even though the band at 323 bp indicative of mutation appears fainter, the Msp1 site is 4-bp distant from the Msp1 site meaning that only large deletions of 6-8 bp will reach the Msp1 site while smaller (and more common) mutations of 1-3 bp will be missed. Taken together, these data suggest that the gRNAs we have, either singly or in a pool, should be adequate to induce knock-out of QM p53 when introduced into 1-2 cell stage QM embryos by microinjection.

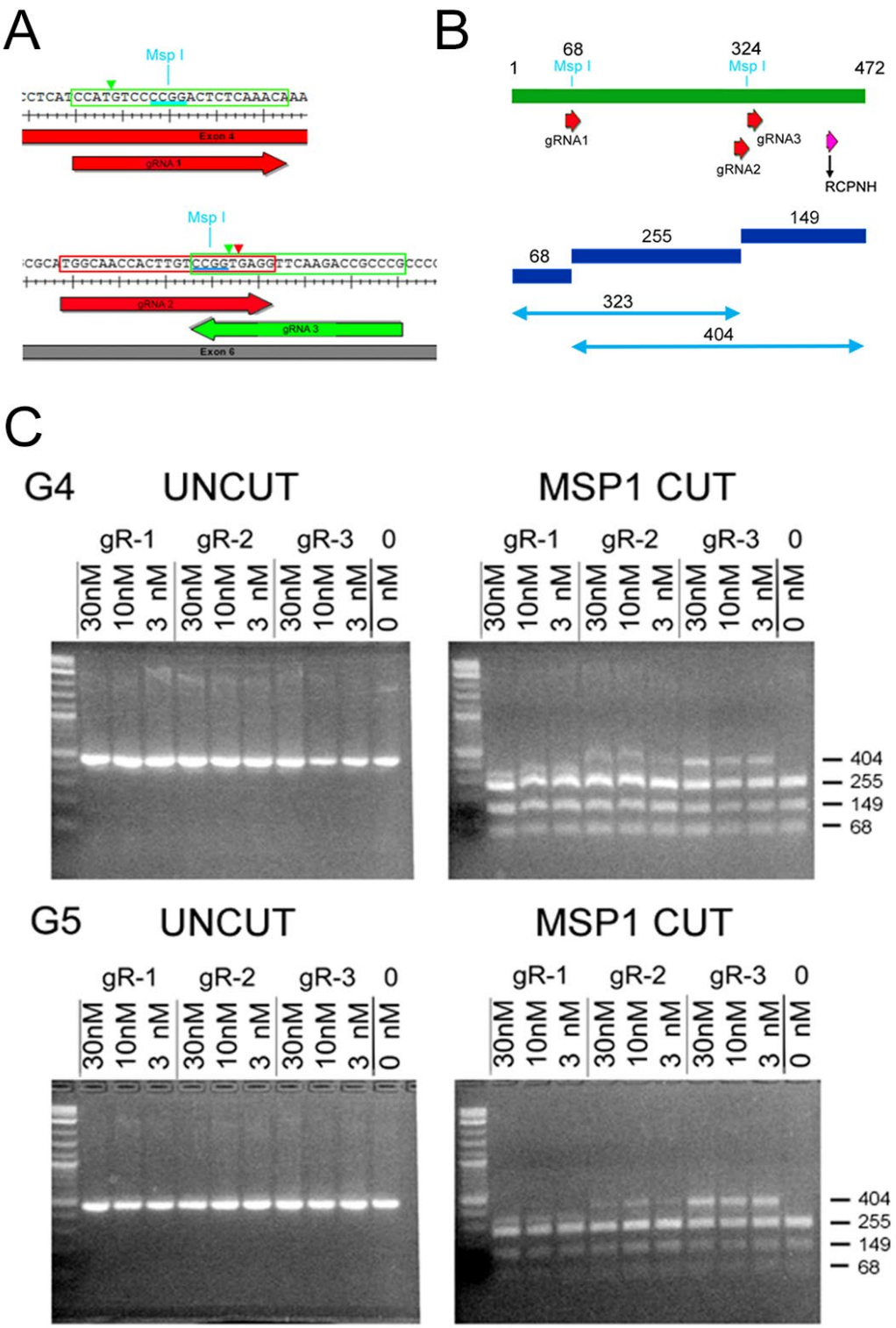


Figure 20 gRNAs in Exons 4 and 6 of QM p53 and test targeting in mouse 3T3 cells genetically modified to carry a fragment of QM p53. A) The recognition site of each gRNA (red or green) in exon 4 (upper) or exon 6 (lower) relative to 5′-CCGG Msp1 sites proximal to the Cas9 cut site (arrow heads). B) The size of the PCR product for QM p53 amplified from genetically modified 3T3 cells and sizes of individual bands

generated after digest with Msp1 (dark blue). If either the Msp1 site in exon 4 or exon 6 is disrupted by mutation, an uncut band of 323bp (exon 4) or 404bp (exon 6) will be observed, indicating mutational targeting. C) 3% Agarose gel analysis of the PCR amplification product produced from genomic DNA harvested from 3T3 lines G4 or G5 transduced with different gRNAs (gR-1,2, or 3) at different concentrations (30, 10, 3, or 0nM) as indicated. Gels on the left are uncut samples (showing the 472bp intact fragment) and gels on the right are cut with Msp1. Results from both independent lines G4 and G5 appeared almost identical, as shown.

3.5.4 Pilot testing of microinjection of fertilized QM embryos

To date, we have had four sessions of pilot microinjections of 1-2 cells stage QM embryos with the assistance of Dr. Jose Cibelli in the Dept. of Animal Science at Michigan State University. For these tests, we fertilized QM oocytes in our laboratory and then quickly drove them to Dr. Cibelli's laboratory on the MSU campus (approx. 20 min from Biomilab) for microinjection (Figure 21 A-C). In these four sessions we learned:

- QM 1-2 cell stage embryos are more fragile than many other embryos worked with previously (cow, zebrafish, drosophila, etc.) but that issues of fragility may be overcome by gentle handling.
- Commercial microneedles with a 2-μm tip opening may be optimal.
- Material appears to enter the zygote cytoplasm from the needle.
- Following injection, embryos appear relatively normal for 30-60 minutes.
- Embryos experience only minimal development post-injection and likely survive only for a few hours.

We are optimistic that microinjection in our own in-house facility currently under construction will overcome issues with QM microinjection revealed in these test sessions.

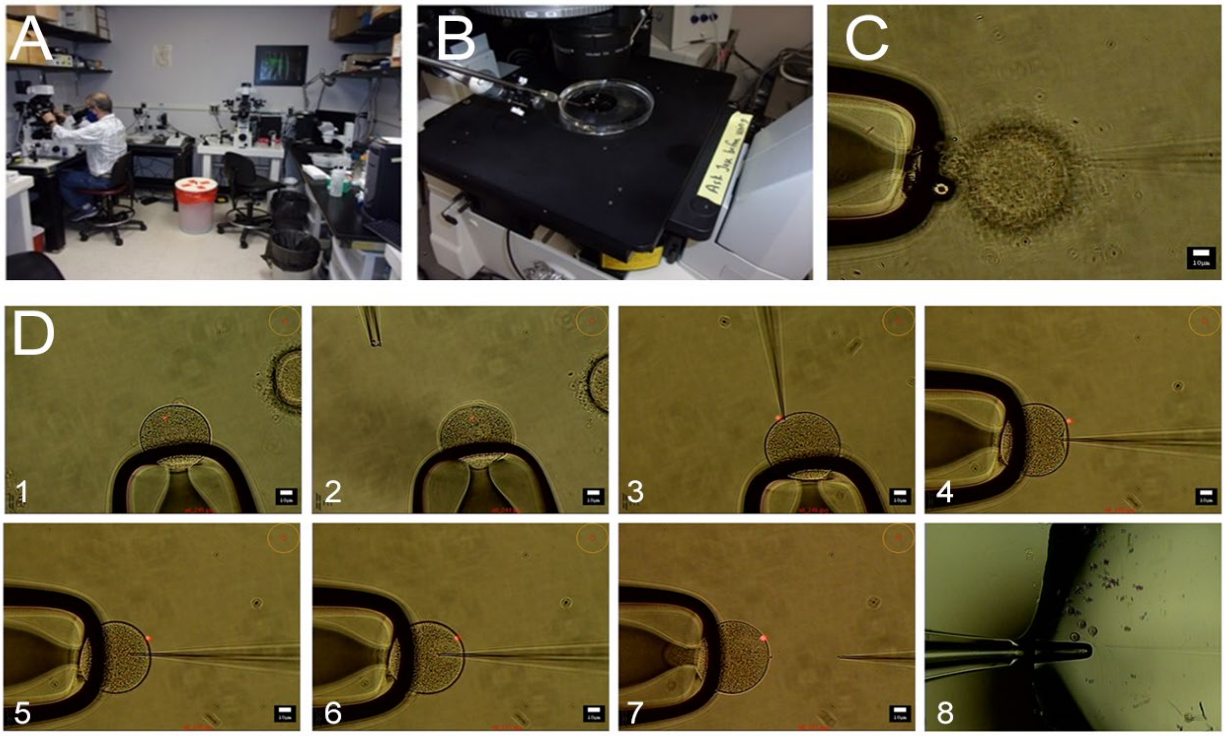


Figure 21 Pilot studies of QM embryo microinjection. A) The microinjection facility in the Cibelli lab at MSU. B) Mussel embryos in medium droplets under oil in preparation for microinjection. C) A QM egg still surrounded with hundreds of sperm cells being drawn toward a holding pipette. The holding pipette could be used to remove sperm and a gelatinous substance associated with the zygote surface. D) Progressive photos of a cleaned zygote being microinjected (the camera was rotated at frame 4 to improve the field of view). In the last panel, the injected embryo is sequestered to the side using the holding pipette to pool it with other injected embryos for later removal to fresh medium for further culture.

4. Discussion

Significant progress has been made toward the proposed invasive mussel eradication agent under development; however, a complete lack of mitosis in explanted and dissociated tissues (and the apparent scarcity of dividing cells in vivo) has slowed efforts to introduce stable transgenes or knock-out endogenous genes to induce transformation. Nevertheless, dramatic progress has been made, many possible avenues of genetic modification have been tested and eliminated, and potential pathways to success identified for implementation in the coming years. Advances within the first three years of the project are summarized as follows:

1. **Established a live quagga and zebra mussel aquaculture station in our laboratory.** Live quagga and zebra mussels are sourced from collaborator Dr. Ashley Elgin at NOAA. Since most mussels come from deep waters, the vast majority are quaggas. Mussels can be maintained healthy for >1 year in this facility.

2. **Established methods for dissociation and long-term (> 6 weeks) culture of quagga mussel (QM) cells in vitro.** Dozens of combinations of culture media, growth factors, temperatures, dissociation methods, and other variables were tested and refined to identify conditions for healthy, long-term cell culture (> 3mo). Cells of the adult gill and 24 hr. embryo are of greatest utility thus far.
3. **Sequencing and analysis of QM genes, gene structures, and mRNAs.** Using QM genome sequencing data provided by our Reclamation collaborator Dr. Yale Passamaneck, we have been able to analyze (i.e., QM p53) and clone multiple QM genes and gene control regions and perform quantitative RNA analysis on multiple genes of interest.
4. **Creation of plasmid and viral expression vectors using mussel-sourced genetic elements predicted to be functional in QM cells and tissues.** We have isolated and tested three QM promoters and constructed at least two dozen vector variations using a variety of reporter genes or oncogenic proteins for eventual use on QM cells.
5. **Establishment of human and insect cell culture as a proxy for QM cell culture.** We hypothesized that human HEK cells and insect cell lines such as drosophila S2 and fall army worm Sf9 cells may have overlapping properties with QM cells and therefore employed these cell lines for preliminary testing of mussel expression vectors. We determined that QM promoters and control regions are functional in both mammalian and insect cell lines, suggesting that they are also likely to be functional in QM cells and tissues.
6. **Established successful protocols for controlled spawning of live QMs and creation of early embryos.** We can now successfully create zygotes and embryos that live for > 5-6 days in vitro.
7. **Pilot microinjection of 1-2 cell stage embryos.** We have had several sessions of microinjection of 1-2 cell stage embryos and have recently begun set-up of a microinjection station in our own lab.
8. **Successful low-efficiency introduction of mRNA into cultured QM cells.** We have identified a transfection agent that can introduce mRNA into QM cells at very low efficiencies. We hope to leverage this low efficiency into more efficient delivery of foreign nucleic acids in the future.
9. **Successful introduction of substances and cell analogs in live mussels.** We have established methods for the introduction of tracker dyes or microbeads into the bodies of live mussels with no significant negative consequences.

A summary of the barriers to progress that we have identified over the same period and that will need to be overcome in the next phase of the project are as follows:

1. **We confirm that QM tissues and cells display essentially no cell division within 24 hours of explant or dissociation.** As suggested in a handful of published reports, QM cells can survive in culture but do not divide. In the absence of cell division, DNA introduced into the cell cytoplasm by a multiplicity of established methods cannot enter the nucleus and incorporate into the genome. At present, transduction of adult QM cells and expression of transgenes does not work.
2. **The absence of mitotic cells in explant culture likely reflects very low levels of true cell division in vivo.** Research we have performed over the last two years indicates that the absence of dividing cells in vitro reflects scarce mitotic cells in vivo, even in gonadal tissue. Furthermore, damage to live mussels (i.e., by needle damage) does not trigger detectable regeneration and mitotic re-entry. We also have preliminary evidence that EdU-labeled cells (Edu labels replicating DNA) in vivo likely arise more from endoreduplication (genome

replication without cell division) or DNA repair, than true mitosis. This is supported by older literature.

3. **Viral infection with established recombinant viral vectors does not work.** We have tested MMLV and lentiviral vectors constructed with QM promoters, multiple serotypes of AAV and adenoviral vectors and detected no gene transfer or reporter expression. Existing viral vectors appear unable to enter end express in QM cells.

The immediate focus of the project going forward is to leverage our success in spawning mussels and producing zygotes by IVF into successful gene transfer using either classic transduction techniques or microinjection. All data with adult cells indicate that classical transduction does not work on dissociated cells; however, it is possible that ongoing experiments with dissociated embryonic cells will prove more amenable to DNA uptake as suggested by the mRNA transduction experiments of Figure 19.

Transduction experiments with embryonic cells will continue, but the use of microinjection to introduce new DNA or CRISPR/Cas9 nucleoprotein complexes likely hold more promise. We believe that completion of our in-house microinjection station will improve survival of injected embryos for several reasons.

First, embryo injection is a race against time because the fertilized egg surpasses the 1-2 cell stage (best for microinjection) within 1-2 hours. If we can perform the IVF within proximity to the microinjection station, we can begin injections of zygotes within minutes of fertilization without the delay of relocating to a separate location. This will result in many more zygotes at the appropriate stage that can be injected during each session.

Second, there is considerable movement (and some temperature fluctuation) that is inherent to vehicle travel both before and after injection at a remote location. We speculate that this movement may result in "bruising" of the embryos, particularly after injection, that may result in high mortality. With injected embryos travelling only a few feet from the microinjection station to the incubator, we believe that damage and temperature fluctuation will be minimal and eliminated as a variable in the process.

Last, and most important, with all aspects of microinjection in one location, we will be able to repeat microinjection sessions with enough frequency to make strong experiment-to-experiment comparisons to refine methods and provide us with optimal injection and survival conditions for treated quagga and zebra mussel embryos.

There are a limited number of laboratories world-wide that are focused on cell and molecular biology of invasive mussels or even mollusks in general. On the road to the eradication agent, this project is making significant contributions to our understanding of molluscan cellular physiology, the establishment of methods for the genetic modification of molluscan species, and factors that could impact mollusk aquaculture. It is our hope that the successful development of the dreissenid eradication agent envisioned, and the knowledge gained in the process, will ultimately help us to suppress invasive mussels, support native bivalves, and return North American aquatic ecosystems to their natural state.

- Data Location -
    - Share Drive folder name and path where data are stored: \\bor\do\TSC\Jobs\DO_NonFeature\Science and Technology\2018-PRG-Biomilab Disseminated Neoplasia 1
    - Point of Contact name, email, and phone: Sherri Pucherelli, spucherelli@usbr.gov, 303-445-2015
    - Short description of the data: Final report (at this time Biomilab will retain all the data in their files)

5. References

Ahuja, D, Sáenz-Robles, MT., Pipas, JM. (2005) SV40 large T antigen targets multiple cellular pathways to elicit cellular transformation. Oncogene, 24, 7729–7745.

Bender, HS., Marshall Graves, JA., Deakin, JE. (2014) Pathogenesis and molecular biology of a transmissible tumor in the Tasmanian devil. Annu. Rev. Anim. Biosci., 2, 165-187.

Carballal, MJ., Barber, BJ., Iglesias, D., Villalba, A. (2015) Neoplastic diseases of marine bivalves. J. Invertebr. Pathol., 131, 83-106.

Canesi L, Miglioli T and Fabbri E (2022) Physiological roles of serotonin in bivalves: possible interference by environmental chemicals resulting in neuroendocrine disruption. Front. Endocrinol. 13:792589.

Cheng TC, La Peyre JF, Buchanan JT, Tiersch TR, Cooper RK (2001) Cryopreservation of heart cells from the eastern oyster. In vitro Cell Dev. Biol. Anim. 37:237-244.

Collodi P., Kamei Y, Ernst T, Miranda C, Buhler DR, Barnes DW (1992) Culture of cells from zebrafish (Brachydanio rerio) embryo and adult tissues. Cell. Biol. Toxicol. 8:43-61. doi: 10.1007/BF00119294. PMID: 1591622.

Díaz, S., Cao, A., Villalba, A., Carballal, MJ. (2010) Expression of mutant protein p53 and Hsp70 and Hsp90 chaperones in cockles Cerastoderma edule affected by neoplasia. Dis. Aquat. Organ., 90, 215-22.

Duffy, MJ., Synnott, NC., Crown, J. (2017) Mutant p53 as a target for cancer treatment. Europ. J. Cancer., 83, 258-265.

Elston, RA., Kent, ML., Drum, AS. (1988) Transmission of hemic neoplasia in the bay mussel, Mytilus edulis, using while cells and cell homogenate. Dev. Comp. Immunol., 12, 719-727.

Gómez-Mendikute A, Elizondo M, Venier P, Cajaraville MP (2005) Characterization of mussel gills in vivo and in vitro. Cell Tissue Res. 321:131-140.

Gossen M, Bujard H. (1992). Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. Proc Natl Acad Sci U S A. Jun 15;89(12):5547-51.

Hansen EL (1976) A cell line from embryos Biomphalaria glabrata (Pulmonata): establishment and characteristics" . Invertebrate Tissue Culture: Research Applications. Ed. K. Maramorosch. USA: Academic Press Inc. 77-97.

Horvath, T. (2008). Economically viable strategy for prevention of invasive species introduction: Case study of Otsego Lake, New York. Aquatic Invasions, 3, Issue 1: 3 – 9.

Labun K, Krause M, Torres Cleuren Y, Valen E. (2021). CRISPR Genome Editing Made Easy Through the CHOPCHOP Website. Curr Protoc. Apr;1(4):e46.

Macpherson, PCD, Suhr ST, Goldman, D (2004). Activity-dependent gene regulation in conditionally-immortalized muscle precursor cell lines. Journal of Cellular Biochemistry 91(4): 821-839.

Mateo, DR., MacCallum, GS., Davidson, J. (2016) Field and laboratory transmission studies of haemic neoplasia in the soft-shell clam, Mya arenaria, from Atlantic Canada. J. Fish Dis., 39, 913–927.

McCartney MA, Auch B, Kono T, Mallez S, Zhang Y, Obille A, Becker A, Abrahante JE, Garbe J, Badalamenti JP, Herman A, Mangelson H, Liachko I, Sullivan S, Sone ED, Koren S, Silverstein KAT, Beckman KB, Gohl DM. (2022). The genome of the zebra mussel, Dreissena polymorpha: a resource for comparative genomics, invasion genetics, and biocontrol. G3 (Bethesda). Feb 4;12(2).

Murchison, EP. (2008) Clonally transmissible cancers in dogs and Tasmanian devils. Oncogene, 27, S19–S30.

Murchison, EP., Wedge, DC., Alexandrov, LB., Fu, B., Martincorena, I., Ning, Z., Tubio, JM., Werner, EI., Allen, J., De Nardi, AB., Donelan, EM., Marino, G., Fassati, A., Campbell, PJ., Yang, F., Burt, A., Weiss, RA., Stratton, MR. (2014) Transmissible [corrected] dog cancer genome reveals the origin and history of an ancient cell lineage. Science, 343, 437–440.

Murgia, C., Pritchard, JK., Kim, SY., Fassati, A., Weiss, RA. (2006) Clonal origin and evolution of a transmissible cancer. Cell, 126, 477–487.

Muttray, AF., O'Toole, TF., Morrill, W., Van Beneden, RJ., Baldwin, SA. (2010) An invertebrate mdm homolog interacts with p53 and is differentially expressed together with p53 and ras in neoplastic Mytilus trossulus haemocytes. Comp. Biochem. Physiol. B. Biochem. Mol. Biol., 156, 298-308.

Neumann D and Kappes H (2003) On the growth of bivalve gills initiated from a lobule-producing budding zone. Biol. Bull. 205:73-82. doi: 10.2307/1543447. PMID: 12917224.

Nogueira LS, Wood CM, Gillis PL and Bianchini A (2013) Isolation and fractionation of gill cells from freshwater (Lasmigona costata) and seawater (Mesodesma mactroides) bivalves for use in

toxicology studies with copper. Cytotechnology 65:773-783. doi: 10.1007/s10616-013-9647-2. PMID: 24081614.

Quinn, B., Costello, MJ., Dorange, G., Wilson, JG., Mothersill, C. (2009) Development of an in vitro culture method for cells and tissues from the zebra mussel (Dreissena polymorpha). Cytotechnology, 59, 121–134.

Ram JL, Crawford GW, Walker JU, Mojares JJ, Patel N, Fong PP, Kyozuka K. (1993) Spawning in the zebra mussel (Dreissena polymorpha): activation by internal and external application of serotonin. J. Exp. Zool. 265: 587-598.

Senut MC, Sen A, Cingolani P, Shaik A, Land SJ, Ruden DM (2014) Lead exposure disrupts global DNA methylation in human embryonic stem cells and alters their neuronal differentiation. Tixicol. Sci. 139:142-161.

Singh, P., Schimenti, JC., Bolcun-Filas, E. (2015) A Mouse Geneticist's Practical Guide to CRISPR Applications. Genetics, 199, 1–15.
Suhr ST, Gage FH. (1999). Gene therapy in the central nervous system: the use of recombinant retroviruses. Arch Neurol. Mar;56(3):287-92.

Tomasovic SP and Mix MC (1974) Cell renewal in the gill of the freshwater mussel, Margaritifera: an autoradiographic study using high specific activity tritiated thymidine. J. Cell Sci. 14:561-569. doi: 10.1242/jcs.14.3.561.

Vassilenko, EI., Muttray, AF., Schulte, PM., Baldwin, SA. (2010) Variations in p53-like cDNA sequence are correlated with mussel haemic neoplasia: A potential molecular-level tool for biomonitoring. Mutat. Res., 701, 145-152.

Walker, CW., Van Beneden, RJ., Muttray, AF., Böttger, SA., Kelley, ML., Tucker, AE., Thomas, WK. (2011) p53 Superfamily proteins in marine bivalve cancer and stress biology. Adv. Mar. Biol., 59, 1-36.

Weiss, RA., Fassati, A. (2015) The clammy grip of parasitic tumors. Cell, 161, 191-192.

Wright DA, Setzler-Hamilton EM, Magee JA, Harvey HR (1996) Laboratory culture of zebra (Dreissena polymorpha) and quagga (D. Bugensis) mussel larvae using estuarine algae. J. Great Lakes Res. 22:46-54.

Xiang L, Kong W, Su J-T,Zhang GY, Xie L-P, Zhang R-Q (2014) Amorphous calcium carbonate precipitation by cellular biomineralization in mantle cell cultures of Pinctada fucata. PLoS One, 9(11):e113150.

Yoshino, TP., Bickham, U., Bayne, CJ. (2013) Molluskan cells in culture: primary cell cultures and cell lines. Can. J. Zool., 91, 1-28.