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Culturing embryonic cells from the parthenogenetic clonal marble crayfish (Marmorkrebs) *Procambarus virginalis* Lyko, 2017 (Decapoda: Astacidea: Cambaridae)

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ABSTRACT

The parthenogenetic marbled crayfish, or Marmorkrebs (*Procambarus virginalis* Lyko 2017), is an emerging model organism. We describe a method to isolate cells from early-stage embryos and culture them *in vitro*. The identity of the cells was confirmed by sequencing the cytochrome c oxidase subunit I (COI) gene. This technique can be applied for use in the manipulation of embryonic parthenogenetic crayfish cells.

Key Words: cell culture, embryos, invasive species, model organisms, ontogeny, techniques

INTRODUCTION

The marbled crayfish or Marmorkrebs (*Procambarus virginalis* Lyko 2017 (Lyko, 2017a), formerly *P. fallax* f. *virginalis* (see Martin *et al.* 2010)), has many biologically significant features. It is the only obligate parthenogenetic decapod crustacean species (Scholtz *et al.*, 2003) as a result of having a triploid genome (Vogt *et al.*, 2015; Martin *et al.*, 2016), which results in all individuals being genetically identical (Martin *et al.*, 2007; Gutekunst *et al.*, 2018). It has no known native habitat but it is a potential or actual invasive species that has been introduced on three continents and established populations in multiple countries (Hossain *et al.*, 2018). Marmorkrebs were rated fourteenth on a list of top invasive species in Europe (Nentwig *et al.*, 2018).

The Marmorkrebs is also an emerging model organism in laboratories (Vogt, 2008b; Jirikowski *et al.*, 2010; Vogt, 2011; Faulkes, 2016; Hossain *et al.*, 2018; Vogt, 2018a) in areas such as ethology (Hossain *et al.*, 2019; Takahashi *et al.*, 2019), developmental biology (Rieger & Harzsch, 2008; Shinji *et al.*, 2019), epigenetics (Gatzmann *et al.*, 2018; Vogt, 2018b), toxicology (Naboka *et al.*, 2018; Velisek *et al.*, 2019), and cancer research (Vogt, 2008a; Lyko, 2017b). Marmorkrebs are the first, and currently only, decapod crustacean species with a known complete genome sequence (Gutekunst *et al.*, 2018). Having a sequenced genome creates possibilities to apply many genetic and molecular techniques that have long been applied in other model organisms, but only rarely with crayfishes. There are many pragmatic reasons to develop Marmorkrebs as a genetic model for crustaceans, notably understanding and managing its introduction and global spread as a non-native crayfish species.

One obstacle to using Marmorkrebs as a model for developmental biology and molecular biology is that the embryos contain large amounts of yolk, making eggs mostly opaque (Alwes & Scholtz, 2006). Imaging embryonic Marmorkrebs cells is difficult compared to model organisms with transparent embryos, such as the amphipod *Parhyale hawaiensis* (Dana, 1853) (Browne *et al.*, 2005), zebrafish (*Danio rerio* (Hamilton, 1822)) (Lawrence, 2007), or tunicates (Miyamoto, 1985). Manipulation of embryonic cells in other models has also led to the development of transgenic animals (Hogan *et al.*, 1994; Offield *et al.*, 2000), which has furthered the utility of several animal models into multiple avenues of research. One possible solution would be to use cell culturing techniques (Toullec, 1999) to separate the developing cells from the yolk. Cells from adult crayfishes have been cultured successfully (Birmelin *et al.*, 1998; Liu *et al.*, 2011; Ding *et al.*, 2012), but not from mature eggs. Embryonic cells have been cultured from shrimps (*Penaeus* spp.) (Toullec *et al.*, 1996; Fan & Wang, 2002; Hu *et al.*, 2008) and prawns (*Macrobrachium rosenbergii* De Man, 1879) (Frerichs, 1996), suggesting that culturing embryonic crayfish cells is feasible. We report a technique for isolating and culturing cells derived from the mature eggs of *P. virginalis*.

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METHODS

Recently laid Marmorkrebs eggs were collected from a female using an established colony at the University of Texas Rio Grande Valley (Jimenez & Faulkes, 2010) and allowed to stabilize at room temperature in minimal light conditions for 7–9 d (from initial collection) in non-treated, previously aged water. Embryo development was observed and grouped based on internal density of the cellular mass. Embryo selection was contingent on approximately a 70–80% internal cellular mass compared to overall embryo mass. Once the selected embryos reached approximately 65% internal mass, the water was then serially diluted with DMEM (Invitrogen, Waltham, MA, USA), supplemented with 10% FBS + DNAse and RNAse every twenty-four hours on the following gradient: 25%, 50%, and 75%. The nucleic acid enzymes DNAse and RNAse were added to reduce the potential for maternal genetic contamination on the external surface of the eggs, as they were removed while still attached to the mother. The resulting 3:1 DMEM stabilized for 24 h in minimal light conditions at room temperature. Cells were then collected from embryos at the early gastrulation stage (Alwes & Scholtz, 2006) and all cells incubated at 28°C + 5% CO_2.

Pre-prepared T25 flasks (2) stabilized in the incubator (28°C + 5% CO_2) for 45 min, containing 3.5 ml DMEM-F (10% FBS + 5X anti-anti (Gibco, Dublin, Ireland); 200 µl polyethylene, and 200 µl nitrofuranozone/potassium dichromate). To perform the initial wash, selected embryos were removed intact using a modified 1.0 ml micropipette tip, then transferred to a 1.5 ml tube containing 0.5 ml DMEM-F, supplemented with 1.0 ml polyethylene (5.4% stock solution) and 1.0 ml nitrofuranozone/potassium dichromate. Embryos were washed so as not to disrupt the membrane, then gently pulsed in a microcentrifuge. The supernatant was discarded and immediately replaced with 1.0 ml of non-enhanced media (DMEM-N). Two additional washes were performed with 1.0 ml of DMEM-N as described above. Embryos were then homogenized and resuspended in 1.0 ml of DMEM-N. A volume of 0.5 ml of homogenized cell suspension was added to each of the pre-prepared flasks mentioned above (day 0, initial seeding) and incubated at 28°C + 5% CO_2. The media was changed on day 5 post seeding (5.0 ml pre-warmed DMEM-F).

Attached cells were split by cell scraping on days 7 (P2), 9 (P3), and 13 (P5) (P denotes passages 2, 3, and 5, respectively), and incorporated antimicrobial and antifungal treatments using the following method. Cell culture media was removed from each flask and stored for subsequent testing, and then immediately replaced with 2.0 ml of DMEM-F (with 50 µl polyethylene, 50 µl nitrofuranozone/potassium dichromate) and allowed to set for 5 min at room temperature. Media was then aspirated and replaced with 3.0 ml of pre-warmed (28°C) DMEM-F. Attached cells were then gently scraped, transferred, and combined to a single 15.0 ml conical tube with an additional 4.0 ml DMEM-F (with 100 µl polyethylene, 100 µl nitrofuranozone/potassium dichromate) and homogenized. Cell suspension was centrifuged at 500 g for 5 min at room temperature, and immediately resuspended in 10.0 ml of DMEM-F. Next, 1.0 ml of cell suspension was transferred to a pre-warmed (28°C + 5% CO_2) T75 flask containing 19.0 ml of DMEM-F (with 50 µl polyethylene, 50 µl nitrofuranozone/potassium dichromate). The remaining cell suspension was then centrifuged at 500 g for 5 min at room temperature. Two 1.0 ml aliquots (post-wash) of supernatant were set aside for sterility testing (targeting fungal and algal contaminants), and the remaining supernatant was archived. Cell pellets were resuspended and cryopreserved with freezing media (5% DMSO + 10% FBS). A 500 µl volume of each aliquot (P3) was then independently transferred to Murishige and Skoog (MS) media agar plates (supplemented with Gamborg's vitamins; Caisson Laboratories, Smithfield, UT, USA) for algal/fungal sterility testing of the microbial treatment regimen used on the cell cultures, and allowed to incubate in moderate light conditions at room temperature for three weeks. All images were visualized with an FV-10i LIV Confocal Scanning Laser Biological Microscope (Olympus, Tokyo, Japan).

The cell suspension derived from the fifth passage (P5) was used for subsequent genetic analysis. No further polyethylene and nitrofuranozone/potassium dichromate were used for expansion of cell cultures after the third passage (P3). Cells preserved from the initial seeding were also thawed and seeded into a new flask to determine viability approximately 7 d after cryopreservation using the methods described previously.

To confirm the identity of the cells, total DNA was extracted (Qiagen, Hilden, Germany) from homogenates generated by scraping a confluent P5 cell monolayer using the manufacturer's protocol. Next, PCR amplicons were generated using crayfish-specific primers for cytochrome c oxidase subunit I (COI) (Martin *et al.*, 2010; Vogt *et al.*, 2015). Amplification was as follows: 94 °C for 2 min followed by 5 cycles for 1 min at 96 °C, 1.5 min at 45 °C, and 1.5 min at 72 °C, with 35 cycles of 93 °C for 1 min, 50 °C for 1.5 min, 72 °C for 1.5 min, with a 5.0 min extension at 72 °C. Previously published sequences for the COI gene (Folmer *et al.*, 1994; Braband *et al.*, 2006) were used to generate PCR primers that produced amplicons for DNA sequencing (LCO1490: 5'-GGTCAACAAATCATAAAGATATTGG-3'; HC02198: 5'-TAAACTTCAGGGTGACCAAAAAATCA-3'). The resulting PCR products were ligated into commercial cloning vectors (Invitrogen), generating TOPO-COI. Each clone was sequenced for DNA analysis (Genewiz, La Jolla, CA, USA), and aligned to the COI gene from the *Procambarus virginalis* crayfish genome (accession #KT074364.1) using LaserGene 12 and the BLAST tool (United States National Library of Medicine, 1988).

RESULTS

Initial attempts to culture cells derived from crayfish eggs were unsuccessful due to contamination of algae and fungi derived from the parental aquatic environment. An aseptic technique, with antifungal and antialgal treatment (50 µl polyethylene, 50 µl nitrofuranozone and potassium dichromate), was thus required to control contamination. Initial isolation trials were discarded due to rapid filamentous growths and algal structures observed within 48 h of seeding, prior to increasing anti-anti concentration up to 5× and incorporating the polyethylene and nitrofuranozone/potassium dichromate treatments. Upon confirmation of cellular isolation and cultivation during the initial seeding, minimal (if any) algal/fungal contamination was evident following the application of the antialgal/antifungal treatment. Pre- and post-wash supernatant aliquots were tested for algae and fungal contaminants on 1× Murishige and Skoog Salts with Gamborg's B5 vitamins with 1.5 % plant tissue culture-grade agar. After a three-week incubation period in moderate light conditions at room temperature, no visible algal or fungal growths were observed when compared to the positive controls. This indicated that the polyethylene and nitrofuranozone/potassium dichromate treatments were successful in reducing the presence of microbial and fungal contaminants, and demonstrated the relative cellular viability given the treatment concentrations.

After initial transfer to the flask (i.e., 0 h), some membranous tissue from the embryos was visible, floating free in media, with some cells visible, which was anticipated, given the likely developmental stage of the cells (Fig. 1A). The cells were not significantly dense and maintained a protein-like base structure, as independently organizing cells were visible within the clusters. The cell cultures appeared to have high rates of metabolism, as the pH of the culture medium became more acidic as the cell cultures progressed over time, indicated by the phenol red present in the culture media changing into an orange-reddish hue. The cell

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cultures, however, appeared healthy and proliferating. Upon observation, the cell population appeared to be comprised of two different morphologies: most cells had a small, circular appearance (Fig.1A), while others had a larger, more irregular, shape. Both types remained attached to the extracellular matrix, suggesting that some type of structural attachment proteins was produced during cell expansion.

Most of the previously free-floating structures (assumed to be cellular debris) had attached to the bottom of the flask after 12 h. By 24–36 h, more clusters had anchored to the flask, but they were slightly orange in color and less translucent. All cells were attached by day 5, and numerous clusters of cells had appeared in relatively large patches within the flask. Microscopic analysis of the predominant cell type showed a morphology consistent with embryonic cells (Fig. 1B).

Following the initial isolation of cells from mature crayfish eggs, we next attempted to passage the putative cells. Cultures derived from the initial isolation were gently scraped, and the media was transferred to a new flask. By the second passage (P2), many cells were still attached, and the density against the background of the flask had increased to an opaque translucent consistency (data not shown). Once these cells had reached ~ 75% confluency, the monolayer was scraped and moved into a new flask, termed P3 (Fig. 2A). We then repeated the entire passaging process two more times, until we had generated a fifth passage (P5), that appeared to be a visible cellular monolayer of very small, apparently undifferentiated cells (Fig. 2B), which suggested that the expansion of the cells in culture were viable. The P5 culture was seeded at approximately 30% confluency andhad reached ~ 90% confluency, within 48 h. Examination of the predominant cell type after P5 was consistent with the previously identified cell type (Fig. 1B), suggesting that the initial cell isolation had produced an embryonic cell capable of expansion (Fig.2C).

To confirm that the cells isolated from the culturing of the cellular material derived from mature crayfish eggs were, in fact, of crayfish origin, PCR amplicons were generated using primers specific for cytochrome c oxidase subunit I (COI). The resulting PCR products were ligated into TOPO-TA vectors, generating TOPO-COI (Fig. 3A). DNA sequencing of the TOPO-COI clones confirmed that the cultured cells were of crayfish origin: sequences

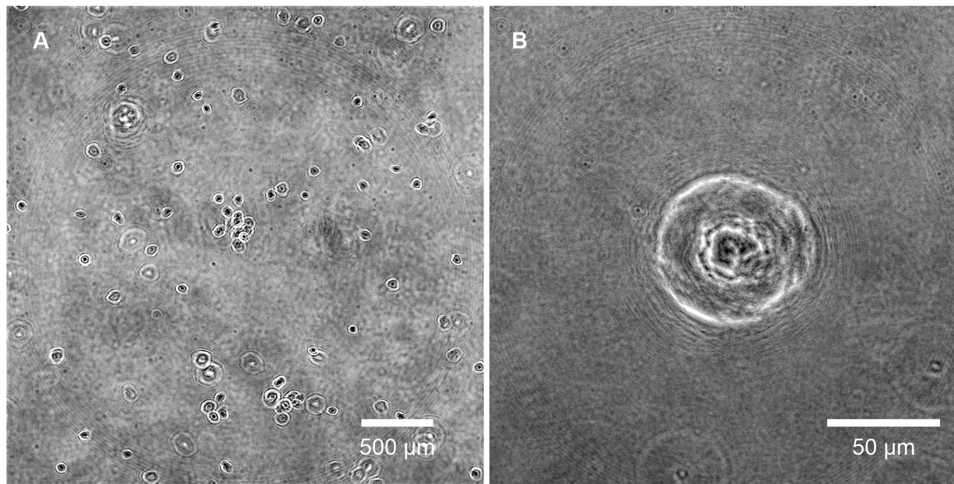

Figure 1. Cells isolated from Marmorkrebs eggs aggregate and attach to solid substrates. Cellular aggregates anchored at the base of the flask and maintained a significant number of cells within them (**A**). Once the cell clusters were removed with media, the scraped cell passages did not have consistently prominent cell aggregates and became absent over by the second and third passages. Examination of single cells within the flask showed a predominate cell type with a morphology resembling that of embryonic cells (**B**). The morphology of these aggregates was significantly different from the fungal containments in the initial isolation attempts.

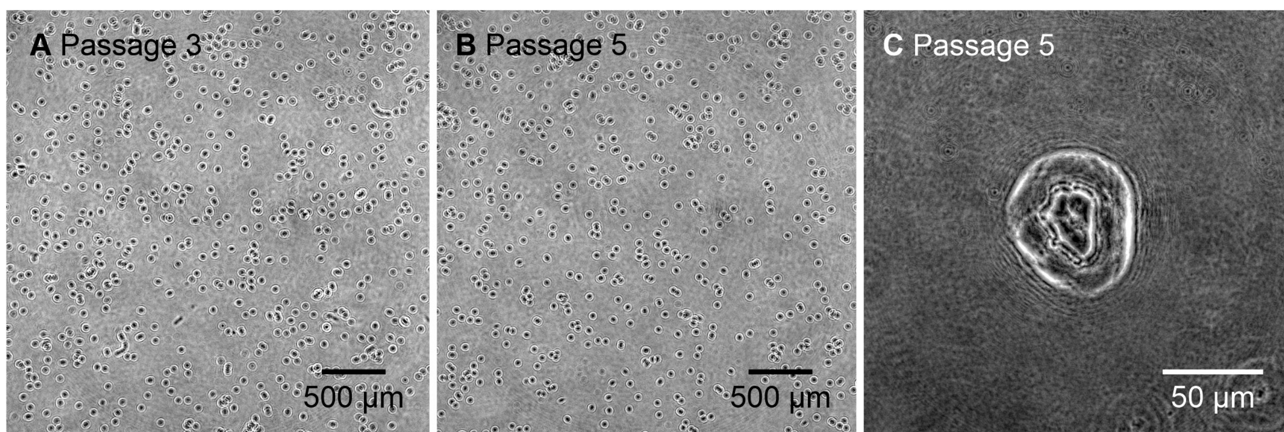

Figure 2. Culturing of cells isolated from Marmorkrebs eggs demonstrates expansion. Cells isolated from crayfish eggs were seeded into T25 flasks and split over the course of three passages, two of which are shown (P3 and P5) (**A**, **B**). Confluency in the flasks increased during each subsequent passage, from ~ 60% in P2, to complete (100%) confluence in P5. The morphology of the cells following P5 was consistent with that observed following the initial isolation of cells (**C**).

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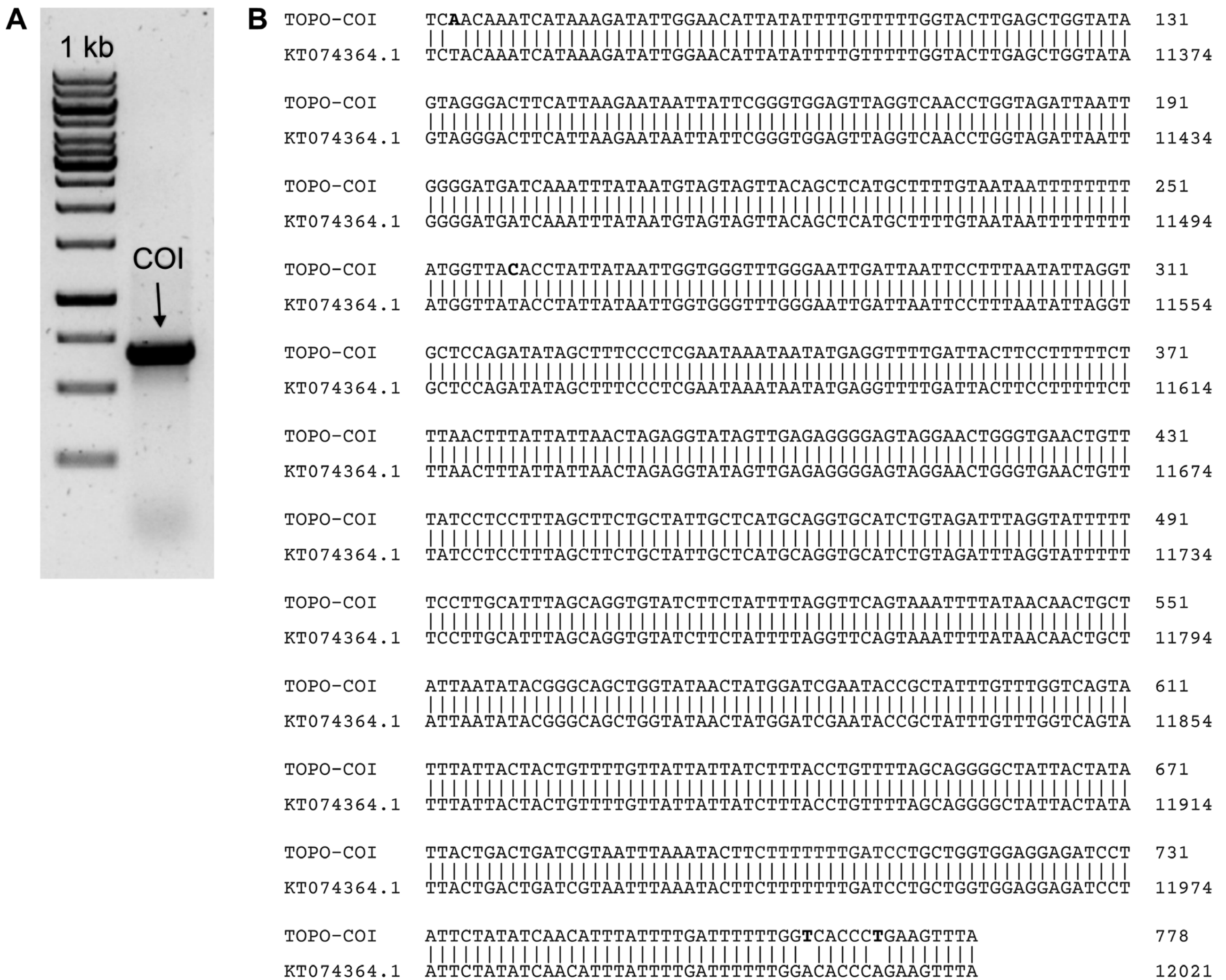

Figure 3. DNA isolated from embryonic cells (P5) was used to PCR-amplify the COI gene. The 29 resulting PCR products were ligated into TOPO-TA cloning vectors for further analysis. DNA 30 isolated from TOPO-COI plasmid (**A**) was sequenced. DNA sequencing confirmed the identity 31 of the PCR product as the COI gene derived from *Procambarus virginalis* (Genbank # 32 KT074364.1), with ~ 99% alignment to consensus sequence (**B**) via Jotun-Hein (nucleotide differences shown in bold).

of COI showed approximately 99% alignment to *Procambarus virginalis* (Fig. 3B).

DISCUSSION

Crayfishes have long been a successful model organism for ecology (Stein, 1977; Lodge *et al.*, 1994; Wilson *et al.*, 2004), behavior (Bovbjerg, 1953), and neurobiology (Wiersma, 1947; Kennedy *et al.*, 1966), but research on decapod crustaceans has been slow to reap the rewards of new techniques in genetics, cell biology, and molecular biology, such as *in situ* hybridization, transgenic approaches (including the CRISPR/CAS system), RNA interference (RNAi), and morpholino-mediated gene knockdown. The ability to isolate and culture cells derived from the mature eggs of Marmorkrebs creates the possibility for using cultured cells as a testbed for many of these techniques before they are applied to the more technically challenging system of the whole embryo, juveniles, or adults.

We cannot definitely state that these cells are embryonic because they did not display the classical characteristics others have reported for embryonic cells derived from aquatic animals (Liu *et al.*, 2011), but they do bear some resemblance to murine embryonic cells (Baillie-Johnson *et al.*, 2015). These cells, however, did appear to undergo some differentiation during culturing. The initial cultures were primarily free-floating cells, but subsequent passaging produced cell cultures that adhered to the plastic substrate in tissue culture flasks. The adherence was strong enough to require gentle scraping to remove attached cells, and the inoculum recovered was able to successfully initiate a new culture post-transfer. We also observed robust expansion, as the cultures displayed ~ 90% confluence at 48 h post-seeding. Traditional isolation of cells derived from mammalian embryos generally produce totipotent cells that can differentiate into many different cell lineages (Kuijk *et al.*, 2010). A mouse embryo at the blastocyst stage consists of three cell lineages: the inner cell mass, the trophectoderm, and the primitive endoderm. The trophectoderm, which is detectable at the morula stage, is the first morphologically distinct cell type of the trophoblast lineage and gives rise to the placental trophoblasts. The yolk-sac endoderm is produced from the primitive endoderm and is visible at the late blastocyst stage as a layer on the surface of the inner cell mass. The inner cell mass, in turn, forms the epiblast, which is the first morphologically distinct cell type of the fetal lineage and, eventually, produces the embryo (Rossant, 2008). It is possible that the cells derived from the mature Marmorkrebs eggs undergo a similar pathway of differentiation, and the initial cells we isolated were

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some type of totipotent or pluripotent stem cell that differentiated into a cellular morphology resembling that of a standard mammalian cell capable of producing structural proteins for attachment to the extracellular substrate present in the culture flasks, as a result of our culturing. This suggests that it may be possible to "push" the outcome of this differentiation into another cell type through alteration of the growth environment, as is commonly done in mammalian bone marrow cells, such as when generating immature dendritic cells from immature monocyte populations (Lutz *et al.*, 1999).

In addition to our observations regarding the growth and morphology of the cells isolated from mature crayfish eggs, we also confirmed the identity of the cells by genetic analysis of the COI gene. Our PCR-amplified COI gene was genetically similar (~ 99%) to the published COI genetic sequences from *Procambarus virginalis*, both in terms of size and nucleotide composition. While the possibility for genetic transfer from the mother (when the eggs were first removed prior to manipulation) could be cited as a source of DNA, and not the cell cultures described herein, we attempted to limit this by using enzymes capable of degrading DNA and RNA in all of our initial isolations, and used the cells in the last-passage (P5) cultures as a source of DNA. It is difficult to imagine that an extracellular amount of DNA could remain as a maternal contaminant, and then persist over a 14-d period in a population of cells that are continually expanding and being transferred to new flasks. Furthermore, we did observe a total of four nucleotide differences between the published COI mitochondrial gene sequence and our PCR amplicon; however, three of the changes are very close to the 5'/3' ends of the PCR amplicon, which may be more of a reflection of the annealing of PCR primers rather than true genetic differences between the DNA sequences.

One of the most attractive possibilities of applying such molecular techniques to Marmorkrebs is in the development of stable strains of transgenic crayfish. Transgenic crayfish have been produced (Sarmasik *et al.*, 2001a; Sarmasik *et al.*, 2001b), but based on examination of the citations of these articles, no other researchers have adopted these techniques for crayfishes. Researchers attempt to minimize genetic variation in many laboratory animals by inbreeding, but inbreeding can cause deleterious phenotypes, and there can be greater phenotypic variation than expected (Anonymous, 2009). It can be very difficult to maintain inbred strains extinct in sexually reproducing organisms (Beck *et al.*, 2000) unless they can be frozen (e.g., *Caenorhabditis elegans*) (Stiernagle, 1999). Because Marmorkrebs are genetically uniform (Martin *et al.*, 2007) and clone themselves naturally (Scholtz *et al.*, 2003), any modification to a single individual (such as a modified gene) can be passed to all her daughters with no extra husbandry. While the generation time of Marmorkrebs is longer than many other model organisms (Seitz *et al.*, 2005), the parthenogenetic reproduction of Marmorkrebs means that new strains should be available indefinitely.

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