Optimization of media and transfection with SV40 large T antigen and c-myc for the establishment of an immortal sea urchin cell line

An undergraduate research project

By Owen Stechishin

Submitted in partial fulfillment of the requirements for the Bachelor of Science degree at Malaspina University-College, Nanaimo, British Columbia

April 13, 2006
ABSTRACT

Immortal cell lines are cell cultures capable of indefinite growth without reaching senescence. Although many vertebrate and some terrestrial invertebrate cell lines have been produced, the establishment of an immortal marine invertebrate cell line remains an elusive goal. In general, marine invertebrate cell culture is greatly hindered by the lack of proliferation and long term viability of cultured cells. As such, much work in this field has focused on identifying media supplements and source cells amenable to long-term culture as well as optimizing immortalization methods. We surveyed seven media supplements (fetal bovine serum, newborn calf serum (NCS), horse serum, chicken serum, two commercial lipid mixtures and coelomic fluid) for their abilities to promote proliferation and long-term viability in culture. We also experimented with embryonic source tissues and developed an oncogene-mediated immortalization technique in hopes of producing transgenic marine invertebrate cells with greater proliferative capacity and decreased fastidiousness. We report that 1% supplementation of Leibowitz’s L-15 media with NCS or chicken serum afforded enhanced viability of coelomocytes as indicated by esterase activity and long term cell-to-substratum attachment compared to unsupplemented L-15 (statistical significance achieved for NCS; p<0.05, Duncan’s multiple range test). We also observed that embryonic tissue was capable of in vitro proliferation, but had significant contamination with protists, leading to rapid demise of cultures within 2 weeks. We observed the formation of colonies in coelomocyte cultures transfected with c-myc and SV40 large T antigen and report stable integration of the c-myc gene sequence in one cell culture five weeks after transfection; however, dramatically increased proliferation was not apparent over a 7 week period.
INTRODUCTION

Immortal cell cultures are invaluable resources in modern biology; their capacity for indefinite growth without reaching senescence makes them veritable biological factories for biotechnology and pharmacology as well as excellent model systems for pathophysiology, cellular, and molecular biology research (Freshney, 1994; Lodish et al., 2004). Although a wide range of vertebrate cell lines are available, there are only about seventy immortal invertebrate cell lines, none of which come from marine invertebrates (Hink, 1979; Odintsova et al., 1994). The establishment of an immortal cell line from sea urchins would be an important initial step towards filling this void.

An immortal sea urchin cell line is particularly attractive because of its many potential applications in a wide range of fields. Sea urchins have long been an important model organism for studies in developmental biology and embryology. Sea urchins have also recently become of particular interest to pharmacology due to the biologically active quinone compounds and pigments produced by several species (Bulgakov et al., 2002). Finally, sea urchins are also emerging as an important aquaculture species, necessitating the establishment of cell lines to study and develop treatments for diseases that could plague aquaculture operations.

Attempts at establishing immortal marine invertebrate cell lines from a variety of source organisms including sponges, molluscs, crustaceans, urochordates, and echinoderms have been made repeatedly over the past four decades (Rinkevich, 1999; Moss et al., 2000). The majority of the primary cultures in these experiments have exhibited short lifespans on the order of 4 to 8 months, although there has been one report of prawn (*Penaeus monodon*) ovary primary cultures remaining alive in excess of 17 months (Fraser and Hall, 1999). However, all marine invertebrate primary cultures have consistently displayed very low proliferative levels with cell
degeneration dominating over proliferation. Additionally, all attempts at subculturing from these primary cultures have proven unsuccessful (Bulgakov et al., 2002).

As such, continuing research in this field has focused on isolating source tissues with the highest potential for immortalization, optimizing the efficiency of the immortalization methods and identifying media supplements capable of supporting long term culture of marine invertebrate cells. Selecting source tissues with a high capacity for proliferation and minimal risk of microbial contamination is the primary starting point for long term marine invertebrate cell culture. In the case of sea urchins, coelomocytes have been widely used because of their abundance, ease of collection, and inherent immune functions that minimize microbial contamination. However, despite the potentially long lifespans of coelomocytes in culture, high levels of coelomocyte growth and proliferation in vitro have yet to be documented (Moss et al., 2000; Massey, 2004; Mason 2005; Mounce 2005).

A variety of other source tissues have also been experimented with in order to select cells that have a higher inherent capacity for cell division with less fastidious requirements and more robustness under sub-optimal conditions. In particular, reproductive tissue has been used in several studies. Ovarian explants from the prawn Penaeus monodon have been reported to remain alive in culture for more than 17 months (Fraser and Hall, 1999). Similarly, embryonic tissue has been demonstrated to be mitotic in culture and has been used in several studies as a cell source for immortalization and culture substrate selection experiments (Odintsova et al., 1994; Bulgakov et al., 2002).

The second main facet of cell immortalization is the actual transformation process that produces the immortal cell line. Early methods for establishing vertebrate and insect cell lines relied on either random mutagenesis or spontaneous genetic changes in senescent primary
cultures to cause mutant, immortal cell lines to emerge. Although spontaneous transformation occurs fairly readily in the cells of some organisms such as rodents, it is a much rarer occurrence in primary cultures of many other vertebrate and invertebrate cells (Lodish et al., 2004). As such, transformation of primary cultures with oncogenes, such as \textit{c-myc} and SV40 large T antigen, has been widely employed to alter cell cycle regulation and induce the emergence of an immortal cell line (Mayne et al., 1986; Khoobyarian and Marczynska, 1993).

The third main area of research in this field is devoted to determining the optimal medium formulation for long term cultivation of marine invertebrate cells. Most work in the field of tissue culture has focused on mammalian cells; as a result, many of the media that are used today for culturing invertebrate cells rely heavily on formulations used for vertebrate cell cultures. In particular, fetal bovine serum (FBS) is commonly added to culture media to supply the diverse array of micronutrients, growth factors and hormones that cells normally obtain from their environment within the host organism.

However, many marine invertebrate cell cultures pose a unique challenge as FBS is unable to support growth of these cells. Although it has been demonstrated that FBS does contain beneficial compounds for invertebrate cell growth, there appear to be significant concentrations of other inhibitory compounds in FBS such that the normal FBS supplement proves inhibitory to cell survival while low concentrations are insufficient to support cell proliferation (Odintsova et al., 1994; Willoughby and Pomponi, 2000). Thus, it seems possible that the continued failure of immortalization procedures for marine invertebrate cells may be due in part to inadequate media.

In light of the inadequacies of FBS, a plethora of media formulations and supplements have been proposed as substitutes for FBS. It has been hypothesized that some of the inhibitory compounds present in FBS may be unique to this particular serum, and as such, other vertebrate
sera may be better able to supplement the growth of marine invertebrate cells (Moss et al., 2000). It has also been proposed that it may be possible to eliminate the need for serum supplementation altogether. In essence, the primary function of serum supplements is to supply essential mitogenic factors that are not constituents of the basic media, but must be present in the external environment of the cell (Freshney, 1994). As such, supplementation of media with tissue or interstitial fluid extracts may be able to supplant the role of serum.

Besides inadequacies in serum supplementation, it has also been proposed that traditional basal media formulations may be inadequate for the needs of marine invertebrate cells in vitro. Most basal media do not contain any lipids in their formulae. However, it has generally been found that most vertebrate and invertebrate cells in culture are not capable of essential fatty acid synthesis and have limited ability to carry out fatty acid elongation and desaturation (Goodwin, 1991). Preliminary experiments by Moss et al. (2000) have suggested that lipid supplements may be able to increase cellular RNA synthesis in primary cultures of the starfish Marthasterias glacialis.

In addition to the incapacity of many cells for de novo lipid synthesis, some cell membrane lipids have also been found to be highly tissue and species specific. Furthermore, the presence or absence of certain lipids or lipoproteins appears to have an important role in cell differentiation and growth (Goodwin, 1991). Therefore, given the potential inability for essential fatty acid synthesis, the dynamic needs for highly specific lipids, and the physiological differences between marine invertebrates and mammals, it seems possible that mammalian sera could fail to provide one or more essential lipids to cultured cells and necessitate lipid supplementation in order to establish proliferative sea urchin cell cultures.

In summary, we sought to enhance in vitro proliferation and establish an immortal sea urchin
cell line by identifying improved media supplementation regimes and source tissues, as well as
developing a transgenic immortalization method. We report that 1% supplementation of
Leibowitz’s L-15 media with NCS or chicken serum afforded enhanced coelomocyte
proliferation as indicated by esterase activity and cell-to-substrate adhesion compared to
unsupplemented L-15. We also observed in vitro proliferation of embryonic tissue, but
encountered significant problems with protozoan contamination. Finally, we transfected
coelemocytes and embryonic cells with c-myc and SV40 large T antigen and report the
production of a stable integrant of c-myc in one coelomocyte culture; however, we did not
observe any significant changes in cell proliferation over a 7 week period.

MATERIALS AND METHODS

Source Cells:

Green sea urchins (Strongylocentrotus droebachiensis) were collected from the Strait of
Georgia off central Vancouver Island. Coelomocytes suspended in coelomic fluid were
extracted directly from the perioral membrane with a syringe prerinsed with 0.1 mL Versene
(Gibco, USA) to prevent clotting. Coelomocyte concentrations were determined by direct count
and diluted in artificial sea water (see Table 1) to 4-6 x 10^6 cells/mL prior to introduction into
culture or subsequent manipulation.

The procedure for collecting embryonic tissue has been previously defined (Johnson, 1995).
Briefly, urchins were induced to spawn in mid-January to early February by injection of 1-2 mL
of 0.5 M KCl through the perioral membrane into the coelomic cavity. Gametes were collected
dry from the gonadopores on the aboral side of the urchin. Eggs were washed once in filtered
sea water (0.2 µm, Millipore, USA) and seeded to cover ⅓ of the bottom surface of formalin-free
fingerbowls filled with 200 to 300 mL of filtered sea water. In later batches of embryos, egg
density was reduced to cover ¼ of the bottom surface in order to reduce microbial growth. Dry
sperm was diluted (1 drop in 50-75 mL of filtered sea water) to obtain a slightly cloudy
suspension; 10 to 15 drops of diluted sperm were then added to a bowl of eggs and gently mixed.
Embryos were incubated at 10°C for 3-4 days, until development of the mid to late prism stage
(see Fig. 1).

Embryos were pelleted (400 RPM, 4 min) and washed four times in autoclaved Ca/Mg free
artificial sea water (0.53 M NaCl, 10.7 mM KCl, 2.4 mM NaHCO₃, 11.3 mM Na₂SO₄, (Johnson,
1995)). A mixture of fully dissociated cells and cell clusters was obtained and resuspended in
artificial sea water at 2-6 x 10⁶ dissociated cells/mL (undissociated cell clusters not included in
count).

Fig. 1: *Strongylocentrotus droebachiensis* embryos in the mid to late prism stage, prior to
dissociation in Ca/Mg free seawater for preparation of embryonic cell suspensions.
Table 1: Basal media formulations used in cell culture experiments.

<table>
<thead>
<tr>
<th>Basal Medium</th>
<th>Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>Artificial Sea Water (referred to as ASW)</td>
<td>0.42 M NaCl, 9.0 mM KCl, 9.3 mM CaCl₂, 18.9 mM MgSO₄, 31.0 mM MgCl₂, 2.1 mM NaHCO₃; filter sterilized prior to use (0.2 µm, Millipore, USA)</td>
</tr>
<tr>
<td>Double Strength Leibowitz’s L-15 (referred to as ds L-15)</td>
<td>double strength L-15 (Sigma Chemical Company), 1000 units/mL penicillin-streptomycin (Gibco, USA); purchased sterile from manufacturer</td>
</tr>
</tbody>
</table>

Media:

Artificial sea water and L-15 were used as the basal media (Table 1). L-15 stock solutions were prepared at twice the recommended concentration to allow additions to be made while maintaining the recommended osmolarity and proportions of media components. Nonetheless, the double strength L-15 stock was sufficiently diluted with ASW or ASW and coelomic fluid to give a net single strength concentration in all coelomocyte and embryonic cell culture experiments involving L-15 basal media (see Table 2). A commercial antibiotic/antimycotic mixture (Gibco, USA) was added at twice the manufacturer’s suggested concentration for embryonic cell culture, in an attempt to combat increased levels of contaminants.

A variety of supplementation regimes with newborn calf, horse, fetal bovine, and chicken sera (Sigma Chemical Company) were employed. Supplements of two defined lipid mixtures (Sigma Chemical Company: Lipid Mixture 1 L-0288, referred to as Lipid 1; Chemically Defined Lipid mixture (1000X) L5146, referred to as Lipid 2) were also used (see Table 3).

Coelomic fluid supplements were also prepared. Coelomic fluid was extracted from urchins, allowed to clot at 10°C overnight, and centrifuged (10 000 RPM, 10 min). The supernatant was then filter sterilized (0.2 µm, Millipore, USA) and stored at 10°C (referred to as cell-free coelomic fluid).
Table 2: **Summary of cell culture conditions.** Tissue culture flasks were used for long term coelomocyte and embryonic cell culture to allow for media exchange and subculturing. Ninety-six well plates were used for short term media supplement assays on coelomocytes and embryonic cells involving fluorometric cell enumeration. Long term culture was performed only with L-15 basal media; media supplement assays were performed with both L-15 and ASW.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Source Tissue</th>
<th>Culture Vessel</th>
<th>Basal Media</th>
</tr>
</thead>
<tbody>
<tr>
<td>Long Term Culture</td>
<td>Coelomocytes</td>
<td>25cm² culture flasks</td>
<td><strong>L-15:</strong> 50% ds L-15, 30 % ASW, 20% coelomocyte suspension in coelomic fluid</td>
</tr>
<tr>
<td>Embryonic Cell</td>
<td>Dissociated Embryos</td>
<td>25cm² culture flasks</td>
<td><strong>L-15:</strong> 50% ds L-15, 30 % ASW, 20% ASW-embryonic cell suspension, ds antibiotic/antimycotic (Gibco, USA)</td>
</tr>
<tr>
<td>Culture</td>
<td></td>
<td>96 well plates</td>
<td><strong>L-15:</strong> 50% ds L-15, 50% ASW-embryonic cell suspension, ds antibiotic/antimycotic (Gibco, USA)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>96 well plates</td>
<td><strong>ASW:</strong> 20% ds L-15, 30% ASW, 50% ASW-embryonic cell suspension, ds antibiotic/antimycotic (Gibco, USA)</td>
</tr>
<tr>
<td>Media Supplement</td>
<td>Coelomocytes</td>
<td>96 well plates</td>
<td><strong>L-15:</strong> 50% ds L-15, 50% ASW-coelomocyte suspension</td>
</tr>
<tr>
<td>Assays</td>
<td></td>
<td>96 well plates</td>
<td><strong>ASW:</strong> 20% ds L-15, 30% ASW, 50% ASW-coelomocyte suspension</td>
</tr>
</tbody>
</table>

Table 3: **Summary of supplementation regimes tested in this experiment.** Control, unsupplemented cultures were prepared for each basal media. nt = not tested.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Basal Media</th>
<th>NCS (%)</th>
<th>FBS (%)</th>
<th>HS (%)</th>
<th>CS (%)</th>
<th>Lipid 1 (%)</th>
<th>Lipid 2 (%)</th>
<th>Coel. Fl. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Media Supplement</td>
<td>L-15</td>
<td>1,2,5,10</td>
<td>1,2,5,10</td>
<td>1,2,5,10</td>
<td>1,2,5,10</td>
<td>0.5,1,2,5</td>
<td>0.5,1,2,5</td>
<td>5,10,15,30</td>
</tr>
<tr>
<td>Assay</td>
<td>ASW</td>
<td>1,2,5,10</td>
<td>1,2,5,10</td>
<td>1,2,5,10</td>
<td>1,2,5,10</td>
<td>0.5,1,2,5</td>
<td>0.5,1,2,5</td>
<td>5,10,15,30</td>
</tr>
<tr>
<td>Long Term Culture</td>
<td>L-15</td>
<td>1,10</td>
<td>1,10</td>
<td>1,10</td>
<td>1,10</td>
<td>1.5</td>
<td>1.5</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>ASW</td>
<td>nt</td>
<td>nt</td>
<td>nt</td>
<td>nt</td>
<td>nt</td>
<td>nt</td>
<td>nt</td>
</tr>
<tr>
<td>Embryonic Cell</td>
<td>L-15</td>
<td>1,2,10</td>
<td>1</td>
<td>1</td>
<td>nt</td>
<td>nt</td>
<td>nt</td>
<td>20</td>
</tr>
<tr>
<td>Culture</td>
<td>ASW</td>
<td>nt</td>
<td>nt</td>
<td>nt</td>
<td>nt</td>
<td>nt</td>
<td>nt</td>
<td>nt</td>
</tr>
</tbody>
</table>
Cell culture:

For long-term cultures, 800 µL of coelomocyte or embryonic cell suspension, containing 5x10⁶ cells, were placed in 25cm² tissue culture flasks (Becton Dickinson, USA). Cells were covered with 3.2 mL of L-15 media with or without supplementation. The seven media supplements were tested in long term cell culture experiments, with two replicate cultures at low supplementation (1% for sera, 0.5x for Lipids 1 and 2, and 20% for coelomic fluid) and single cultures at high supplement concentrations (10% for sera, 5x for Lipids 1 and 2; coelomic fluid not assayed above 20%; see Table 3). Cells were incubated at 10°C and observed daily for changes in cell morphology and density.

Subculturing was attempted on several cultures at 60-70% confluence. Cells were mechanically dislodged with a cell scraper (Becton Dickinson, USA) and resuspended in the culture medium. One half of the resuspended cells were transferred to a new flask and 2.0 mL of fresh media were added to each flask. Alternatively, cells were enzymatically dissociated according to the procedure previously defined by Freshney (1994). Briefly, the culture media was removed and cells were washed twice with ASW. The cells were then washed with 3 mL of 0.25% trypsin (Gibco, USA) and allowed to incubate with the residual trypsin until the cells detached and “rounded up” (10 min). Three mL of fresh media were added and the cells were resuspended; half of the resuspended cell mixture was transferred to a new flask and 2.5 mL of fresh media were added to each flask.

Media Supplement Assays:

Supplements were tested on coelomocyte and embryonic cell cultures in L-15 and ASW (see Table 2). Supplements of newborn calf, horse, fetal bovine, and chicken sera were assayed at concentrations of 1, 2, 5, and 10 % (v/v). Lipids 1 and 2 were tested at concentrations of 0.5, 1,
2, and 5 times the manufacturer’s suggested concentration. Cell-free coelomic fluid supplementation was assayed at concentrations of 5, 10, 15 and 30% (v/v) (see Table 3).

Coelomocytes in coelomic fluid and dissociated embryonic cells were diluted in ASW and 100 µL aliquots were seeded into 96 well plates (Becton Dickinson, USA) to give 2-4 x 10^5 cells/well. Cells were not seeded in the top row of each plate; 100 µL of ASW was added instead to serve as a control for microbial contamination and baseline for fluorometric cell enumeration. One hundred µL of basal media (with or without supplementation) were added to each well. Six 100 µL replicates of each supplement concentration were placed in each column of wells, along with two columns of unsupplemented basal media as controls. The outer perimeter wells on every plate were not used in this assay because the baseline fluorescence values were consistently higher than those measured in the inner wells. Duplicates of each plate were prepared and incubated at 10°C. The entire supplement assay was repeated twice with source cells from randomly selected urchins used to seed one set of duplicate plates.

Cell densities in the replicate plates were assayed after 1 and 2 weeks according to the 5-carboxyfluorescein diacetate acetoxy methyl ester (CFDA-AM) fluorometric assay for esterase activity previously defined (Ganassin et al., 2000). Briefly, 100 µL of CFDA-AM (Invitrogen, USA) diluted to 0.5 µM was added to each well and incubated for 30 min in the dark. Fluorescence (excitation wavelength: 468±20 nm; emission wavelength: 528±20 nm) was measured with a fluorescent plate reader (Bio-Tek Instruments, USA). To account for the esterase activity exhibited by the serum supplements, the baseline fluorescence for each supplement concentration (top control row: no cells) was subtracted from the measured fluorescence intensities in the column. Since our fluorometric assay was optimized for
measuring cell densities between $5 \times 10^4$ to $1 \times 10^6$ cells/mL, minimal cell densities present in some supplements were arbitrarily recorded as 10 000 cells/mL.

Linear regression (Excel, Microsoft, USA) of fluorescence intensities for standard dilutions of coelomocytes generated a calibration curve ($y = 872.63x + 114.5; r^2 = 0.9782$) used to convert fluorescence intensity to cell density. ANOVA (Excel, Microsoft, USA) was performed to test for the presence of differences (statistical significance $p<0.05$) between the cell densities observed in the supplemented and unsupplemented media. Multiple pair-wise comparisons between the cell densities observed in each supplement concentration and those of the control wells on each plate were made with Duncan’s multiple range test (statistical significance $p<0.05$). Survival and growth were calculated by the formulae below.

$$\text{survival} = \left( \frac{\text{average esterase activity in supplemented media}}{\text{average esterase activity in unsupplemented media}} \right) \times 100\%$$

$$\text{growth} = \left[ \frac{\text{average esterase activity at week 2}}{\text{average esterase activity at week 1}} - 1 \right] \times 100\%$$

**Transgenic Cells:**

Plasmids pSVmyc-Mg (c-\textit{myc}) and pRSV-BneoN136 (SV40 large T antigen – neo$^R$) were amplified in \textit{DH5}\textalpha E. coli and extracted with the UltraClean\textsuperscript{TM} Mini Plasmid Prep Kit (MoBio Laboratories, USA) according to the manufacturer’s instructions (see Fig. 2). Plasmid identity and purity were confirmed with restriction digest (EcoR1) and 0.8% agarose gel electrophoresis (see Fig. 3).
Fig. 2: Structure of pSVmyc-Mg and pRSV-BneoN136 plasmids. pSVmyc-Mg contains the human c-myc gene driven by an SV40 promoter; no neomycin resistance gene was present on this plasmid. pRSV-BneoN136 contains the SV40 large T antigen gene driven by RSV and SV40 promoters; a neomycin cassette derived from pMCNeo(poly A) (Stratagene, USA) was also present.
Fig. 3: **Restriction digest to confirm plasmid identity and purity.** Lanes 1 and 8 contain 1 kb ladder marker DNA (New England Biolabs, USA); the numbers along the sides represent the approximate sizes of the fragments in kilobases. Lanes 2 and 5 contain no DNA. Lanes 3 and 4 contain undigested and EcoRI digested pSVmyc-Mg. In lane 4, a band of approximately 11 kb (corresponding to single cut, linearized pSVmyc-Mg) as well as two bands at 8 and 3 kb (corresponding to the predicted fragments for complete digestion) were observed. Lanes 6 and 7 contain undigested and EcoRI digested pRSV-BneoN136. In lane 7, three bands at 3.0, 1.8, and 1.0 kb were observed, which correspond to the predicted sizes of the 3 major fragments from the plasmid map.

The *c-myc* and SV40 large T antigen constructs were electroporated into coelomocytes in coelomic fluid (immediately extracted from the urchin prior to electroporation) and dissociated embryonic cells in ASW. Eight hundred μL of source cell suspension were placed in a 4 mm (800 μL) Multiporator cuvette (Eppendorf, Germany) with 2 μL of plasmid preparation to give a final DNA concentration of 5-20 μg/mL. Cell density was within 2-6x10⁶ cells/mL as recommended by the Multiporator operation manual (Eppendorf, Germany). Electroporation was performed with a pulse width of 25 μs at 250V; two protocols involving a single pulse or two consecutive pulses with a 1.0 min interval were used. Electroporated cells were allowed to
rest for 5 minutes prior to being added to 3.2 mL of culture media and incubated in 25cm² culture flasks (Becton Dickinson, USA). Untransfected control cultures (from the same urchin or batch of embryos) were also prepared. Cells were grown in unsupplemented L-15 as well as L-15 supplemented with 1% NCS, 1% HS, 1% FBS, 1x lipid 1, 1x lipid 2 or 20% coelomic fluid. G-418 (A.G. Scientific, USA) selection at 400 µg/mL (as recommended by the manufacturer) for the neomycin resistance gene on the pRSV-BneoN136 plasmid was performed on several coelomocyte and embryonic cell cultures.

Transfection success was determined by visible inspection of cultures for the development of cobblestone regions characterized by dense, confluent cell growth, which is indicative of clonal expansion. Integration of the plasmids was assayed with PCR using primers specific for regions of the plasmid constructs. Cells from cobblestone regions of transfected cultures at 3 or 5 weeks post-transfection were removed and total cellular DNA was extracted with DNAzol (Invitrogen, USA) according to the manufacturer’s instructions. Total cellular DNA was also extracted from coelomocytes 3 days after transfection and from untransfected cells. Cells were lysed by agitation and DNA was precipitated with anhydrous ethanol, washed in 75% ethanol, and resuspended in distilled water.

PCR was used to detect the presence of the plasmids in the DNA extracts. The primers were 5’-AGGATCTCCTGTCATCTCACC-3’ and 5’-AAGAACTCGTCAAGAAGGCGA-3’ for the pRSV-BneoN136 plasmid (targeting the neomycin cassette - amplicon 492bp). The primers 5’-TCTTCCCCCTACCCGCTCAAC-3’ and 5’-ACCCTGCCACTGTCCAACCTT-3’ were used for the pSVmyc-Mg plasmid (targeting the c-myc gene sequence – amplicon 411bp). PCR reactions contained 2 mM MgSO₄, Taq reaction buffer, 150 nM of upstream and downstream primers, 400 nM dNTPs, 2 U Vent polymerase (New England BioLabs, USA), and 2 µL of DNA extract.
Reactions were run for 39 cycles of 1 min at 94°C, 1 min at 62°C, and 3 min at 72°C, with a final 10 min extension at 72°C. PCR products were analyzed with 1.2% agarose 1X TAE gel electrophoresis.

RESULTS

Media Supplementation Assays:

In order to optimize the culture conditions for sea urchin cells we tested a variety of media supplementation regimes for their abilities to promote cell survival and growth. Fetal bovine serum (FBS), newborn calf serum (NCS), horse serum (HS), chicken serum (CS), coelomic fluid, and lipid mixtures 1 and 2 were tested on coelomocyte cultures in L-15 and ASW media. Cell densities were measured after 1 and 2 weeks with a CFDA-based fluorometric assay for cellular esterase activity.

We found significant differences in the esterase activities of the coelomocyte cultures after 1 and 2 weeks of incubation in the 7 different media supplements. As Figs. 4, 5, and 6 show, no supplementation regime was able to drastically increase coelomocyte survival during the first week in culture above that observed in the unsupplemented media. Modest increases in survival in a dose dependent manner were observed for 1 to 5% chicken serum supplementation of ASW (Fig. 5-H), with 25% greater growth in 5% chicken serum attaining statistical significance (p<0.05; Duncan’s multiple range test). However, the increased survival afforded by chicken serum was short term and quickly abrogated by cellular degeneration during the second week in culture.
Fig. 4: Effects of horse serum (HS), and fetal bovine serum (FBS), supplementation on coelomocytes grown in L-15 and ASW. Cellular esterase activities were measured with a CFDA-AM fluorometric assay after 1 and 2 weeks of incubation. Survival was determined as the ratio of the esterase activities in the treatment wells to the unsupplemented (control) well after 1 week. Growth is the difference in esterase activity for a treatment between the first and second weeks, expressed as a percentage of the first week’s esterase activity. Error bars represent standard deviation; means marked with an asterisk differ significantly from the unsupplemented (control) mean (Duncan’s multiple range test, p<0.05).
Fig. 5: Effects of newborn calf serum (NCS), and chicken serum (CS), supplementation on coelomocytes grown in L-15 and ASW. Cellular esterase activities were measured with a CFDA-AM fluorometric assay after 1 and 2 weeks of incubation. Survival was determined as the ratio of the esterase activities in the treatment wells to the unsupplemented (control) well after 1 week. Growth is the difference in esterase activity for a treatment between the first and second weeks, expressed as a percentage of the first week’s esterase activity. Error bars represent standard deviation; means marked with an asterisk differ significantly from the unsupplemented (control) mean (Duncan’s multiple range test, p<0.05).
Fig. 6: Effects of lipid 1, lipid 2, and cell-free coelomic fluid supplementation on coelomocytes grown in L-15 and ASW. Cellular esterase activities were measured with a CFDA-AM fluorometric assay after 1 and 2 weeks of incubation. Survival was determined as the ratio of the esterase activities in the treatment wells to the unsupplemented (control) well after 1 week. Growth is the difference in esterase activity for a treatment between the first and second weeks, expressed as a percentage of the first week’s esterase activity. Error bars represent standard deviation; means marked with an asterisk differ significantly from the unsupplemented (control) mean (Duncan’s multiple range test, p< 0.05).
Most supplementation regimes proved to have neutral or negative effects on cell survival. Supplementation with FBS and lipid 1 in ASW and L-15 had no statistically significant effect on cell survival over the range of concentrations assayed. Lipid 2 also had no significant effect on cell survival in ASW, but resulted in a dose dependent decrease above the manufacturer’s suggested concentration in L-15. Similar dose dependent patterns of decreased survival were observed for horse and newborn calf sera above 2% supplementation in ASW and L-15. Two supplements, chicken serum in L-15 and cell-free coelomic fluid in both L-15 and ASW, appeared to be significantly toxic to coelomocytes, as they approximately halved cellular esterase activity in a dose-independent manner during the first week of incubation.

In addition to monitoring cell survival, we also measured the change in esterase activity between the first and second weeks of incubation. In many cases, the trends observed in the cell survival data were also present in the observed growth rates. Similar to the 50% reduction in cell survival that was observed after the first week, cell-free coelomic fluid supplementation resulted in an additional 25-50% decrease in cell density over the second week. Lipid 1 appeared to have no significant effect on coelomocyte growth in supplemented L-15 or ASW. Lipid 2 as well as FBS displayed a moderate dose dependent reduction in cell growth that became statistically significant at high concentrations. Dose dependent decreases in growth were also observed with horse serum in both L-15 and ASW, as well as with NCS in ASW. Contrary to the dose dependent increase in cell survival observed with chicken serum in ASW over the first week, a dose dependent decrease in cellular growth was observed over the second week.

Nonetheless, substantial increases in cell growth were observed in three supplement regimes. Although 10% HS supplementation of ASW appears to be quite toxic and causes a 70% reduction in cell density within the first week, it is capable of stimulating significant proliferation
(150% increase) in the surviving cells over the second week (Fig. 4-B). A similar effect was also observed with 10% NCS in L-15 (Fig. 5-E); although minimal cell density (10 000 cells/mL) was present after the first week, a more than 4 fold increase occurred by the second week in culture. Increased growth was also observed for low concentrations (1-2%) of NCS and chicken serum in L-15. While only the 50% increase in growth for 1% NCS was statistically significant, these two supplement regimes displayed a similar pattern in which growth becomes increasingly inhibited as supplement concentration is increased above 1%. Nonetheless, 1% supplementation of L-15 with NCS displayed the greatest enhancement of growth, while having minimal toxicity to the coelomocytes.

In addition to monitoring the effects of the various supplements on coelomocytes over the first couple of weeks in culture, we were also interested in observing any effects that the supplement regimes may have on cellular morphology, cell-to-substratum attachment, long-term viability, and potential for subculturing. As such, we initiated a series of coelomocyte cultures in 25cm² tissue culture flasks. While this did not allow us to use our fluorometric assay for cell enumeration, tissue culture flasks provided a treated substrate to promote cell-to-substratum attachment and allowed for routine media exchange and subculturating necessary for long-term culture.

Although we generally did observe both attached and free floating cells in all media supplement regimes, 1% NCS supplementation in L-15 consistently enhanced cell adhesion. As such, after several days of incubation, coelomocyte cultures in 1% NCS formed partial monolayers and were dominated by the presence of very large, granular, flat, fibroid cells with spreading pseudopodia (Fig. 7A). Significant cellular adhesion was also apparent in media supplemented with FBS and HS, although the initial attachments were more transient and greater
proportions of suspended cells were present after 3 to 4 weeks of incubation. High concentrations (5 to 10%) of FBS, NCS, or horse serum (not shown) favored cell-to-cell adhesion, resulting in large spherical clumps of cells with some individual free-floating or attached cells. In contrast, fewer coelomocytes in unsupplemented media initially attached to the flask and these attachments were more transient. After 2-3 weeks of incubation in unsupplemented L-15, many attached cells dissociated and assumed a large, round, granular morphology (Fig. 7B). The predominance of round, loosely-adherent, petalloid coelomocytes was also observed in media supplemented with coelomic fluid and lipids 1 and 2 (not shown).

Fig. 7: **Morphologies of coelomocytes in culture were affected by media supplement regimes.** Coelomocytes in 1% NCS supplemented L-15 typically were quite large, fibrous, granular, and highly adherent (A). Coelomocytes cultured in unsupplemented L-15 maintained the round, loosely-adherent petalloid morphology (B) observed upon initial extraction from the urchin.

In accordance with the results from the short term media supplement assays, we did not observe drastic increases in cell density in almost all supplementation regimes. Our ability to distinguish growth, especially in supplement formulations that resulted in decreased initial cell survival, was limited by coelomocyte clumping and ambiguity over the viability of free-floating...
cells. Nonetheless, we did observe that only 1% NCS supplementation afforded the production of significant regions of monolayer growth.

As such, two subculturing protocols were attempted on several 1% NCS cultures as they approached 60-70% confluence at 2-3 weeks in culture. Both protocols resulted in significant reductions in cell-to-substratum reattachment in the daughter cultures. Only a small proportion of the cells (¼ to ⅓) reattached, and growth in the daughter cultures remained quite slow, with approximately 25% confluence achieved over the next three to four weeks. However, subcultured flasks, like most other cultures in supplement regimes that did not have significant initial toxicity, displayed lifespans on the order of 8 to 10 weeks, after which significant degeneration lead to rapid demise of the culture.

Embryonic Cell Culture:

In light of the low capacity for growth that coelomocytes have generally demonstrated in culture, we sought to examine other potential source tissues. In particular, we experimented with embryonic tissue in hopes of exploiting its inherently higher capacity for plasticity and growth. Unfortunately, we encountered significant obstacles with microbial contamination during the isolation of dissociated embryonic cells. Significant reductions in microbial contamination were achieved by disinfection of urchin gonadopores with a 70% ethanol wash prior to spawning, dry collection of gametes, and reduction of egg density to cover ¼ of the finger bowl surface prior to fertilization in filter sterilized sea water. Nonetheless, complete sterility was not achieved and inevitable contamination with ciliates and flagellates (penicillin/streptomycin and antibiotic/antimycotic resistant) lead to the demise of embryonic cultures by the second week of incubation.
Fig. 8: **Effects of media supplements on sea urchin embryonic cells in culture.** Cellular esterase activities were measured with a CFDA-AM fluorometric assay after 5 days of incubation and expressed as a percentage of the esterase activity measured in the control (unsupplemented L-15) cultures. All serum and coelomic fluid supplements were added to L-15 media. Error bars represent standard deviation. The 1% FBS cultures were observed to have noticeably greater protozoan contamination than the other treatments.

Nonetheless, we were able to collect some initial data regarding the esterase activities of embryonic cells in several supplement concentrations after 5 days in culture. As Fig. 8 shows, we found enhanced cellular esterase activities were afforded by low concentrations of NCS, FBS and horse serum in comparison to esterase activities observed in unsupplemented L-15 cultures. In agreement with our previous media supplement assays with coelomocytes, we again observed that coelomic fluid supplementation resulted in a reduction of esterase activity. We were also somewhat surprised to observe 3 times greater esterase activity for cells in unsupplemented ASW than in unsupplemented L-15. Visual observations of cell densities in the individual wells generally conformed to the trends present in the esterase activity data, except in the case of FBS supplementation, where cell density was clearly not 5 times greater than in L-15 and significant...
protozoan growth was obvious. However, protozoan contaminants likely had a non-trivial contribution to the all measured esterase activities, so these data can only be regarded as a crude, qualitative comparison between the supplements and not an absolute measure of their true capacity to stimulate embryonic cell growth.

*Cell Immortalization:*

In order to enhance the long term survival and proliferation of sea urchin cells in culture, we sought to produce transgenic coelomocytes and embryonic cells by transfection with the proto-oncogene c-myc or SV40 large T antigen. Transfected cells, along with untransfected controls from the same sample of coelomocytes or batch of embryos, were incubated in a series of media formulations in tissue culture flasks. Unfortunately, we did not observe drastically increased growth rates in any of the transfected cultures. Nonetheless, several coelomocyte cultures did manifest small, isolated colonies of cells, which were relatively homogenous in cell type and had a cobblestone appearance at the base of the colony (see Fig. 9-A,B). We also found that ciliate and flagellate contaminants in embryonic cell cultures appeared to be largely unaffected by electroporation and effectively overran the culture within a couple weeks.
Fig. 9: **Production of coelomocyte colonies observed 3 to 4 weeks after transfection with pSVmyc-Mg (A) and pRSV-BneoN136 (B).**

G-418 selection of coelomocyte and embryonic cell cultures transfected with the SV40 large T antigen plasmid (bearing a neomycin resistance gene) was attempted. We observed that G-418 did noticeably reduce coelomocyte and embryonic cell density by about three quarters. We had also hoped that G-418 might serve as a selective agent against protozoan contaminants in transfected embryonic cell cultures; unfortunately this clearly was not the case as protozoan density and growth appeared to be largely unaffected throughout two weeks of G-418 selection.

Evidence for stable integration of the *c-myc* and SV40 large T antigen plasmids was obtained through PCR. To maximize our chances of finding stable transfectants, we extracted genomic DNA from cobblestone regions in 8 coelomocyte cultures at 3 or 5 weeks after transfection. Additionally, genomic DNA was extracted from 4 coelomocyte cultures 3 days after transfection. DNA from untransfected cultures was also prepared as a control for primer specificity. As the untransfected controls in lanes 15 & 16 of Fig. 10 show, the PCR primers were highly selective for sequences found only on the plasmid constructs and not within the urchin genome. We
observed a single band (lane 4) at 700 bp (which was also amplified in the \textit{c-myc} positive control) that was produced from one \textit{c-myc} transfectant 5 weeks after transfection; no bands were observed in any of the other genomic DNA extracts.

![Image of gel electrophoresis](image)

**Fig. 10:** \textbf{Evidence for stable integration of pSVmyc-Mg was obtained through PCR amplification of a 411 bp region of the \textit{c-myc} gene sequence.} Lanes 1, 11 and 22 contain 1 kb ladder marker DNA (New England Biolabs, USA); the numbers along the sides represent the approximate sizes of the fragments in kilobases. Lanes 2&5, 6&9, and 10&13 contain PCR products amplified using the pRSV-BneoN136 primers with transgenic coelomocyte DNA five weeks, three weeks and three days, respectively, after transfection with pRSV-BneoN136. Lanes 3&4, 7&8, and 12&14 contain PCR products amplified using the pSVmyc-Mg primers with transgenic coelomocyte DNA five weeks, three weeks, and three days, respectively, after transfection with pSVmyc-Mg. Lanes 15 and 16 contain PCR products from untransfected coelomocyte DNA amplified with the pRSV-BneoN136 and pSVmyc-Mg primers, respectively. Lanes 17 and 19 contain PCR products amplified from the pRSV-BneoN136 vector; lane 18 contains the PCR product amplified from the pSVmyc-Mg vector. Lanes 20 and 21 are negative controls run with no DNA and the pRSV-BneoN136 and pSVmyc-Mg primers, respectively.
DISCUSSION

The data presented in this study clearly demonstrate that media supplement regimes can have significant effects on coelomocyte viability and growth in culture. Even though most supplements proved to have a negative rather than positive effect, we did observe a statistically significant ($p<0.05$, Duncan’s multiple range test) 50% enhancement of growth with 1% NCS supplementation of L-15 compared to unsupplemented media. Although not statistically significant, enhanced growth was also observed with chicken serum supplementation of L-15.

However, in both cases, the enhancement afforded by 1% supplementation declined in a dose dependent manner as serum supplementation was increased to 2 and 5%. Therefore, it seems that while NCS and chicken serum contain components that are stimulatory to coelomocyte proliferation, they also contain inhibitory components that negatively impact growth at higher serum concentrations. More severe patterns of dose dependent decreases in cell viability and growth were also seen with FBS and horse serum supplementation. This general pattern of little effect with FBS supplementation at low concentration and dose-dependent toxicity at higher concentrations (>5%) has been shown to occur in a number of previous studies with several different marine invertebrate cell types (Moss et al., 2000; Willoughby and Pomponi, 2000).

From the results of our study, the inhibitory effects of serum supplementation are clearly not unique to FBS, but rather are shared by at least 3 other vertebrate sera. Nonetheless, in light of the enhanced esterase activity that we observed with NCS supplementation, it seems possible that an effective media supplement could be developed by fractionation of NCS to enrich the growth stimulating components, and most importantly deplete the toxic components, allowing supplementation above 1% to be employed without significant cytotoxicity.

Although we had initially hypothesized that lipid supplementation may have an important role
in promoting cell viability and proliferation, our data did not support this hypothesis. Lipids and lipo-protein complexes are known to have critical roles in cell signaling, growth, and differentiation; lipid supplementation is also often critical for long-term survival and proliferation of many fastidious cell types (Darfler, 1990; Goodwin, 1991). However, as cellular lipid requirements are often highly specific, our data do not necessarily discount the importance of lipids in sea urchin cell cultivation, but rather suggest that the two commercial lipid mixtures of vertebrate phospholipids, sphingolipids, and hormones that we selected did not contain any lipids beneficial to sea urchin cells under the culture conditions employed in this experiment (Conforti, et al., 1990; Goodwin, 1991). Nonetheless, the lack of significant cytotoxicity of these lipid mixtures is surprising, as it has been suggested that inappropriate vertebrate sphingolipids present in FBS may be an important inhibitory component to invertebrate cells in FBS-supplemented media (Harpin et al., 1990).

While we had expected most of the vertebrate sera or lipids to likely be ineffective, and even toxic to urchin cells, we were surprised by the drastic, inhibitory effects of cell-free coelomic fluid supplementation. Although one previous study had reported that 10% coelomic fluid had no effect on urchin neuronal cultures, we had hypothesized that coelomic fluid extracts would contain endogenous coelomocyte growth factors and have few inhibitory compounds (Moss et al., 1998). At the very least, we expected coelomic fluid to have a neutral effect on coelomocytes in culture. However, this clearly was not the case, as a consistent 50% reduction in cell survival and growth was observed for 5 to 30% supplementation in L-15. In ASW, the effects were even more striking, with a dose-dependent reduction of 50-70% in cell viability and growth. As such, it seems that the coelomic fluid extracts used in this study not only failed to stimulate proliferation, but also significantly reduced survival of coelomocytes in culture.
In our long term culture experiments, we also observed more rapid degeneration of coelomocytes and greater numbers of what appeared to be apoptotic vesicles during the first five weeks in coelomic fluid supplemented media than in unsupplemented L-15. Thus, it seems that coelomic fluid supplementation may accelerate coelomocyte apoptosis in vitro. While there certainly may be drastic differences between the in vivo coelomic environment and our in vitro system of coelomic fluid-supplemented media, these observations lead us to conjecture that coelomic fluid may contain factors capable of causing cytotoxicity under certain circumstances.

Two such scenarios seemed plausible to us. First of all, urchins may contain humoral factors capable of recognizing allogenic cells. In our experiments, the urchin that the cell-free coelomic fluid was prepared from was a different individual than the urchin that the coelomocytes were extracted from. Since it is known that that many Echinoderms will reject allografts, it is possible that the coelomocytes could be recognized as foreign cells by humoral factors in the coelomic fluid (Gross et al., 1999). However, it is believed that Echinoderm rejection mechanisms are cell-mediated, as humoral factors such as hemolysins, agglutinins, and lectins have only been shown to bind erythrocytes, zymosan particles, and lipopolysaccharide, but not allogenic cells (Canicatti, 1991). As such, a yet undiscovered humoral factor capable of recognizing allogenic cells would clearly be necessary for this hypothesis to be correct. Experiments in which coelomocytes were grown in media supplemented with cell-free coelomic fluid from the same urchin would be useful to confirm or disprove this hypothesis.

Alternatively, coelomic fluid may contain factors that serve to accelerate apoptosis of aging coelomocytes in vivo. The lifespans of many vertebrate immune cells are known to be under strict regulation; as coelomocytes are generally regarded as the Echinoderm homologs of vertebrate innate immune cells, it seems possible that a similar mechanism of coelomocyte
regulation may have evolved within the urchin (Smith et al., 1996; Matranga et al., 1999).

However, significant regeneration of coelomocytes would clearly be necessary in such a negatively selective environment. Since we did not observe growth in coelomic fluid (but our NCS and chicken serum results clearly demonstrate that coelomocyte growth is possible in vitro), coelomic fluid itself must not be sufficient to stimulate coelomocyte proliferation. Therefore, additional growth factors not present in coelomic fluid, or possibly even cell-to-cell contacts present in the in vivo environment, likely play a significant role in inducing proliferation. Thus, the results of this and other studies suggest that while the natural urchin growth factors may seem like a promising approach for developing effective media supplements for marine invertebrate cells, it is clearly not as simple as adding crude tissue extracts to media (Moss et al., 1998; Moss et al., 2000). As such, future tissue extract fractionation studies and identification of individual urchin growth factors will likely be necessary for development of an effective urchin-derived media supplement.

While most previous attempts at media supplement assays have generally focused on increases in DNA, protein, or esterase activity during the first 3 to 7 days in culture as an indicator of success, we were primarily interested in identifying supplements that afforded enhanced long-term maintenance and growth of sea urchin cells in culture. As such, while the presence of essential nutrients and the absence of inhibitory compounds may be critical to determining short term survival, other factors of the cell culture environment can have a profound effect on long term viability in culture. In particular, cell-to-substrate attachments have been demonstrated to be important for promoting the metabolism and long term viability of many vertebrate and marine invertebrate cells (Ben-Ze’ev et al., 1980; Odintsova et al., 1994). Horse serum, FBS, and NCS have been previously shown to enhance marine invertebrate cell adhesion (Benson and
Chuppa, 1990; Odintsova et al., 1994; Moss et al., 2000); in agreement with these findings we observed a noticeable increase in cell-to-substratum adhesion over the first three weeks in cultures supplemented with these three sera as well as with chicken sera. However, we were most encouraged by the noticeably increased long term cell adhesion and formation of monolayers afforded by 1% NCS supplementation of L-15. As such, it seems quite possible that the enhanced adhesion afforded by NCS supplementation may be an important component of the enhanced proliferation measured between the first and second weeks in culture.

Although the availability of a suitable culture media is certainly a critical requirement for cell culture, the physical attributes of the source tissue also have a drastic impact on the success of cell cultures. Coelomocytes have been used as a source tissue in several studies because they offer a readily-available, heterogenous cell population with low risk for microbial contamination; unfortunately, this and other studies have observed that coelomocytes seem to have a fairly low propensity for growth in culture (Massey, 2004; Mason, 2005; Mounce, 2005).

Embryonic source tissue has been previously explored as a cell source in hopes of exploiting its greater proliferative capacity (Odintsova et al., 1994; Bulgakov et al., 2002). While we were able to observe some proliferation of embryonic cells in our study, we encountered significant obstacles with protozoan contaminants. These protists proved to be largely unaffected by antibiotics, antymycotics, electroporation, and selection in G-418; as such, they were able to rapidly overrun embryonic cell cultures within two weeks. Although we made numerous attempts and alterations to our gamete collection and embryo rearing procedures, we were unable to prepare dissociated cell suspensions without the presence of ciliates or flagellates. Similar problems with marine heterotrophic protists in embryonic and other marine invertebrate source tissue preparations have been reported by several other studies (Ilan et al., 1996; Rinkevich and
Protozoan contamination is a serious obstacle in tissue culture as there are few selective agents capable of effectively killing protozoa without harming other eukaryotic cells (Freshney, 1994). However, the situation is even worse in marine invertebrate cell culture as the cells are slow growing and easily out-competed by protists (Rinkevich, 1999). As such, the high potential for protozoan contamination clearly overshadows any greater proliferative capacity offered by embryonic cells and improved methods of sterile embryonic cell collection will have to be developed in order to have any reasonable chance of success in long term embryonic cell culture.

The establishment of an immortal cell line is a difficult goal to achieve; while the selection of highly mitogenic cells and development of media capable of supporting the cells in vitro is essential to any long-term culture attempt, many cell types do not readily undergo spontaneous immortalization. As such, alteration of gene expression by insertion of transgenes is often necessary to deregulate growth control and avoid senescence. While this approach has been widely used to create many immortal vertebrate cell lines, it has achieved much less success with marine invertebrate cells. Currently, there have been only two previous studies that were able to demonstrate increased proliferation and dedifferentiation into tumor-like structures of sea urchin and sand dollar embryonic cells as a result of transfection with the yeast transcriptional activator Gal4 (Bulgakov et al., 2002; Odintsova et al., 2003). While we are not the first to experiment with transfection of oncogenes, we are one of the few to report stable integration of an oncogene into sea urchin cells (Mason, 2005). Since plasmid integration into the host genome is critical to preventing its rapid degradation by cellular nucleases, stable transfection is a significant step toward the eventual establishment of an immortal cell line through transgenesis.

Unfortunately, while we were able to confirm the presence of a portion of the c-myc plasmid in
at least one of our cultured cells, we did not observe significant increases in proliferation or alteration to a transformed state. However, since we determined stable transfection with PCR using primers targeted for a 411 base pair stretch of the c-myc gene sequence, we can only be absolutely confident of the integration of that particular gene segment into the urchin genome. Stable integration is a very rare event that occurs through random recombination between the plasmid construct and the genomic DNA (Lodish et al., 2004). Although homology between the plasmid and the insertion site may favor recombination, the particular portion of the plasmid that is inserted and the insertion site in the genome is still largely determined by chance. As such, it is quite possible that the lack of overt phenotypic effects of c-myc integration could easily be due to insertion of only a portion of the c-myc gene, resulting in an incomplete, non-functional transcript. Similarly, disruption of the promoter region preceding the gene or integration into a region of transcriptionally inactive heterochromatin could easily inhibit any potential expression of c-myc, regardless of whether the actual coding sequence is complete or not.

Furthermore, it has yet to be conclusively determined whether the human c-myc protein is capable of actually inducing deregulation of growth control in urchin cells. Altered c-myc expression is often characteristic of many immortal cancer cells and has been previously used to immortalize primary vertebrate cell cultures (Mayne et al., 1986; Khoobyarian and Marczynska, 1993; Schmidt, 1999). While it has been found that myc genes are conserved throughout higher eukaryote evolution, and there is evidence for a homolog of c-myc in the starfish, it has yet to be established whether human c-myc has any effect on urchin cells (Facchini and Penn, 1998). Small, but critical, differences in amino acid sequences between human and Echinoderm myc homologs could potentially render human c-myc unable to interact with urchin mitotic regulatory pathways. Alternatively, it has also been found that high levels of c-myc expression can be
highly toxic to some vertebrate cells (Pelengaris et al., 2000). Thus, even if human c-myc could induce deregulation of growth control in urchin cells, the high level of expression driven by the constitutive SV40 promoter, could still prove inhibitory to cell growth and survival.

Therefore, further research is clearly necessary to ascertain the potential effects of c-myc and other human oncogenes on marine invertebrate cells. In the case of our stable c-myc integrant, RT-PCR detection of c-myc mRNA would be helpful in determining the status of the c-myc gene construct and confirming that expression is occurring. Furthermore, we were only able to recover a single culture with a stable integrant; production of more stable integrants is clearly necessary to determine c-myc’s potential as a transforming agent for marine invertebrate cells and have any reasonable chance of establishing an immortal cell line.

In summary, this study applied a three-tiered approach to identify improved media supplements, source tissues, and immortalization methods for long term culture and establishment of an immortal sea urchin cell line. Like many attempts before us, we encountered significant obstacles with ineffective or toxic media supplements and microbial contamination. However, we were able to achieve modest success with increased coelomocyte esterase activity and long term cell-to-substratum attachment in 1-2% NCS and chicken serum supplemented media; as such, these data are some of the few positive results in marine invertebrate media development and potentiate future enhancements through fractionation studies of these sera. Additionally, we were able produce a stable integrant for at least a portion of the c-myc gene sequence in coelomocytes and observed the production of cobblestone regions in transgenic cultures. While transformation of coelomocytes with c-myc did not noticeably increase proliferation, stable integration is nonetheless the first step in the process of immortalization by transgenesis. As such, it is hoped that the results of this study will contribute to the field of
marine invertebrate cell culture and the ultimate goal of establishing an immortal cell line.

ACKNOWLEDGEMENTS

I would like to acknowledge the Fisheries and Aquaculture Department at Malaspina for collecting the urchins and caring for them throughout the duration of this project. I would also like to thank all the faculty and technicians of the Biology Department at Malaspina for the assistance and advice they provided me throughout the project. In particular, I must thank Robert Wager for his patience and invaluable support with the technical aspects of my project. Furthermore, a grateful thank you goes to Dr. Allan Gibson for his many ideas and substantial knowledge of urchin biology and embryology, which he generously contributed to this project. I would also like to thank Dr. Phil Dauk for his assistance with the statistical analyses in this study. Finally, I must applaud my supervisor, Dr. Rosemarie Ganassin, for her invaluable support, inspiration and advice, as well as the great deal of latitude she allowed me in defining my project and exploring its many facets.
LITERATURE CITED


Mason, C. 2005. Transfection of purple sea urchin (Strongylocentrotus purpuratus) coelomyctes with plasmids containing SV-40 large T antigen and C-Myc gene sequences. Malaspina University-College Undergraduate Research Project - Biology 491, Malaspina
University-College.


I certify that I have obtained and attached a written permission statement from the owner(s) of each third party copyrighted matter to be included in my research project report, allowing distribution as specified below.

I certify that the version I submitted is the same as that approved by my research advisor.

I hereby grant to Malaspina University-College or its agents the non-exclusive license to archive and make accessible, under the conditions specified below, my research report in its entirety. I retain all other ownership rights to the copyright of the research project. I also retain the right to use in future works (such as, but not exclusively, journal articles) all or part of this research project report.

Signature: ______________________

Student name: Owen Stechishin

Date: May 15, 2006

Project Approved by: ______________________

Research Advisor: Dr. Rosemarie Ganassin, Biology Department, Malaspina University-College.