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Molluscan cells in culture: primary cell cultures and cell lines

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Abstract

In vitro cell culture systems from molluscs have significantly contributed to our basic understanding of complex physiological processes occurring within or between tissue-specific cells, yielding information unattainable using intact animal models. *In vitro* cultures of neuronal cells from gastropods show how simplified cell models can inform our understanding of complex networks in intact organisms. Primary cell cultures from marine and freshwater bivalve and gastropod species are used as biomonitors for environmental contaminants, as models for gene transfer technologies, and for studies of innate immunity and neoplastic disease. Despite efforts to isolate proliferative cell lines from molluscs, the snail *Biomphalaria glabrata* Say, 1818 embryonic (Bge) cell line is the only existing cell line originating from any molluscan species. Taking an organ systems approach, this review summarizes efforts to establish molluscan cell cultures and describes the varied applications of primary cell cultures in research. Because of the unique status of the Bge cell line, an account is presented of the establishment of this cell line, and of how these cells have contributed to our understanding of snail host-parasite interactions. Finally, we detail the difficulties commonly encountered in efforts to establish cell lines from molluscs and discuss how these difficulties might be overcome.

Keywords

Primary cell culture; *in vitro*; cell line; Mollusca; *Biomphalaria glabrata* Say 1818 Bge cell line

Introduction

The ability to isolate and maintain defined cell types in culture provides a valuable tool for analyzing complex molecular interactions at the organ/tissue level when these phenomena are intractable in intact organisms. Such “simplified” *in vitro* systems are amenable to more precisely controlled experimental manipulation. Primary cell cultures may be established by enzymatic dissociation of cells comprising a given tissue and placing these cells into culture, or by allowing cells to migrate from pieces of tissue (explants) that have been placed into culture. Primary culture-derived cells may proliferate, but the number of *in vitro* cell-cycle

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divisions is limited. However, although the vast majority of primary cell cultures yield cell populations with limited proliferative capabilities, on rare occasions, primary cells replicate repeatedly such that cell lines can be isolated. Cultured cells that are capable of proliferating indefinitely under *in vitro* conditions likely derive from single cellular lineages, and are referred to as cell lines. For metazoan invertebrates, the significant impact of the availability of cell lines on research may best be illustrated by the arthropods. The more than 500 insect cell lines currently in existence (Lynn 2007), many from well-established model systems, have engendered rapid advances in a variety of fields, some of which extend well beyond basic or applied entomology. For example, cell lines have played key roles in elucidating complex physiological processes (Fallon and Gerenday 2010; Valanne et al. 2011), in advancing molecular bioprocessing such as the development of eukaryotic gene expression systems (Hitchman et al. 2011; Moraes et al. 2012), in the production and screening of biologics including vaccines or pesticides (Barrett et al. 2010; Cox and Hollister 2009; Smagghe et al. 2009), and in the development of tools and protocols for whole organism transgenesis (Mathur et al. 2010; Isaacs et al. 2011) and functional genomic approaches (Gunsalus and Piano 2005). Perhaps the most useful applications of insect cell lines have been in the cultivation of viruses, many of which have been incorporated into the biotechnological advances mentioned above. Smagghe et al. (2009) provide a comprehensive review of the impact of insect cell cultures on basic and applied research.

In stark contrast to the insects, only a single cell line has been established from molluscs; namely the *Biomphalaria glabrata* Say, 1818 embryonic (Bge) cell line (Hansen 1976). This is despite concerted past efforts to isolate and establish additional lines (Bayne 1998; Rinkevich 2005, 2011). The Bge cell line was derived from a freshwater snail that serves as an important intermediate host for the human blood fluke, *Schistosoma mansoni* Sambon, 1907, the causative agent of schistosomiasis or “snail fever” in the new world and sub-Saharan Africa (Hotez 2008). Bge cells have been extensively studied, and these investigations will be reviewed in detail later in this paper. However, despite this paucity of continuously proliferating cell lines, primary cell cultures from a variety of molluscan species have been used to advance our understanding of complex physiological processes that could not have been investigated within the whole, intact animal. The purpose of this review is not to give a comprehensive literature review of molluscan cell culture studies, but to provide examples of the preparation and culture of cells from a variety of tissues, and an overview of the current status of cell culture as it is being applied to the broad disciplines of molluscan neurobiology, immunobiology, toxicology, and genetics, and as tools in the development of transgenic technologies. We also discuss technical difficulties commonly encountered in attempts to generate primary cell cultures and to derive cell lines, and discuss ways to avoid or minimize such difficulties.

Molluscan Primary Cell Cultures

As a practical matter, molluscan cells can be derived from virtually any tissue and placed into *in vitro* culture. However, their abilities to survive *and* thrive in this artificial environment depend on a myriad of variables including avoidance of damage during tissue isolation and cellular disaggregation, contamination with microorganisms, and the need to ascertain those culture conditions that are both physically and chemically nurturing to isolated cells. Unfortunately, overcoming these constraints is not an easy matter, and has resulted in a focusing of studies on a relatively small subset of tissues and cell-types derived from those tissues. The choice of which cells are targeted for *in vitro* investigation naturally depends on the kinds of questions being addressed, but is also driven by the frequency of success and the ease of establishing primary cultures of cells of interest. The relatively low number of published reports involving molluscan primary cell cultures (Rinkevich 1999, 2005) clearly indicates that there still exist many unknown barriers to successful cell

cultivation in molluscs. How some of these barriers may be overcome when attempting to establish primary cell cultures will be described in detail later in this review. Similarly, the number of mollusc species used as source organisms for cell culture studies remains small, and, perhaps not surprisingly, the ones that are used are those with commercial value (e.g., oysters, clams, mussels, abalone), that are medically important (snail hosts of human disease, e.g., *Biomphalaria*), or that have served as well-studied model organisms in specific disciplines (e.g., the gastropods *Aplysia*, *Lymnaea* and *Helisoma* in neurobiology).

Depending on the molluscan species, tissues of choice, and the conditions under which they are held, isolated cells placed into primary culture have exhibited either short-term (days) non-proliferative characteristics (most common), or the capability of longer-term maintenance in culture (weeks to months) with or without accompanying proliferative activity. Both short- and longer-term cell cultures have been exploited as investigative or analytical tools or as “simplified models” for advancing understanding of complex physiological systems. To summarize these advances we have taken a tissue/cell-oriented approach to review how primary cell cultures derived from various molluscan tissues or embryos have been successfully used to address important basic or applied questions.

Primary cell cultures from embryonic/larval origin

Because of their high proliferative potential and the relatively large proportion of undifferentiated cells (pluripotent “stem” cells), molluscan larval stages or early developing embryos have been considered a prime source for establishing long-term primary cell cultures or identifying cell lines spontaneously arising in such primary cultures. However, relatively few studies have been reported in which successful initiation of proliferating primary cell cultures has been accomplished using intact larval stages or embryos as source material. Because of its medical importance, the freshwater snail *Biomphalaria glabrata* Say, 1818 has been intensely studied. Basch and DiConza (1973) used chemical dissociation of embryo fragments to create primary cell cultures using various complex media. Several cell types were observed including fibroblast-like and epithelioid-type cells early (3–7 days) in culture, later giving way to “polygonal cells” at 2–3 weeks, and finally “muscle” cells dominating cultures at 4–5 weeks in culture. However, cultures failed to thrive beyond this time. In a major breakthrough, Hansen (1976), using cell suspensions of 5-day old *B. glabrata* embryos, isolated the first, and currently the only, molluscan cell line (Bge cell line) derived from primary cultures. How this cell line was derived and its many applications to molluscan cell research are described in more detail in this review.

Primary cell cultures that survived long term also have been reported from larval marine bivalves. Primary cultured cells derived from *in vitro* fertilized trochophore larvae remained viable at 4 months for the scallop *Mizuhopecten (Patinopecten) yessoensis* Jay, 1857 (Odintsova and Khomenko 1991), ~3 months for abalone *Haliotis rufescens* Swainson, 1822 (Naganuma and Degnan 1994), and 2.5 months for mussels *Mytilus trossulus* Gould, 1850 (Odintsova et al. 2010). It is interesting that under similar culture conditions, subsets of cells were observed to differentiate into myocytes (Naganuma and Degnan 1994; Odintsova et al. 2010) and neurons expressing FMRFamide/5-HT (Odintsova et al. 2010) and α B3-like integrin receptors (Odintsova and Maiorova 2012). In a novel application of primary embryonic cell cultures, Boulo et al. (2000) successfully infected cells derived from oyster (*Crassostrea gigas* Thunberg, 1793) larvae with a pseudo-typed pantropic retroviral vector expressing the luciferase (*luc*) transgene. This report, together with earlier demonstrations of transient transfection of Bge cells (Lardans et al. 1996; Yoshino et al. 1998), represent the first attempts to utilize cultured molluscan cells as targets of gene transfer experiments.

Hemocyte primary cultures

Hemocytes, a term that refers to cells freely circulating in the hemolymph (=blood) of molluscs and arthropods, exist as several cell types. Molluscan hemocytes vary morphologically and biochemically (Cheng 1975; Granath and Yoshino 1983; Cavalcanti et al. 2012), express different surface antigens (Yoshino and Granath 1983; Dikkeboom et al. 1988) and differ functionally, such as in regards to phagocytosis, cell signaling pathways, etc (see reviews by Anderson 2001; Canesi et al. 2002; Loker 2010). Virtually all of these studies were performed on cells collected in hemolymph and placed into short-term culture in the presence of an artificial medium (e.g., buffered, salt-balanced solutions, or a complex medium). Perhaps because the hemolymph residing within the hemocoel of these organisms is essentially aseptic, and many of its cells are defensive phagocytes, cultures free of microbial contamination are usually easier to establish than when other tissue types are used. However, because the majority of circulating hemocytes are terminally differentiated, they tend to be short-lived under *in vitro* conditions, surviving for only 2–3 days. For many applications, e.g., morphological or functional testing, such short culture times are usually sufficient to perform useful experiments. For other applications, e.g., testing the effects of low-level environmental toxicants or growth factors on hemocyte viability or function, establishment of longer-term cultures would be required. To date, only abalone *Haliotis* spp. and the freshwater mussel *Dreissena polymorpha* Pallas, 1771 (Parolini et al. 2011) have yielded hemocytes that appear capable of longer-term survival under *in vitro* conditions. Contaminant-free isolation and maintenance of hemocyte primary cultures for 7–10 days have been attained for *Haliotis tuberculata* L., 1758 (Lebel et al. 1996; Farcy et al. 2007; Latire et al. 2012) and *H. midae* L., 1758 (van der Merwe et al. 2010), and 15 days for *D. polymorpha*, providing *in vitro* systems for testing effects of ecotoxin-exposure or other stressors on innate immune function (Galloway and Depledge 2001) or for investigating tissue repair processes (Serpentini et al. 2000). Importantly, these cells can be cryopreserved with post-thaw viabilities of 96% and 71% after 2 and 6 days, respectively (Poncet and Lebel 2003). However, hemocytes were maintained in a frozen state for only 4 days, so the consequences of long-term storage are not known. Contrary to the relatively short-lived cultivation times of “normal” hemocytes, neoplastic or “cancerous” hemocytes spontaneously arising in marine clams (*Cerastoderma edule* L., 1758; Twomey and Mulcahy 1988; *Mya arenaria* L., 1758; Smolowitz et al. 1989) and mussels (*Mytilus edulis* L., 1758; Elston et al. 1988) can survive for months in culture, even after cryopreservation. For example, suspension cultures of *Mya* hemic neoplastic cells can be maintained for > 3 months (both pre- and post-cryopreservation), with doubling times of 1.8–2.4/day before onset of senescence at ~80 hr of continuous culture (Walker et al. 2009). Because of the positive correlation between environmental pollutant levels and incidence of hemic neoplasias (e.g., Reinisch et al. 1984; Delaporte et al. 2008; Pariseau et al. 2009) and the finding of functionally defective p53 tumor suppressor gene mutations in transformed cells (reviewed by Walker et al. 2011), there is now considerable interest in using these spontaneous “cancer”-like neoplasias as models for investigating the ecotoxicological basis of tumorigenesis.

Finally, molluscan hemocyte cultures have been used to investigate the interactions between infectious pathogens and the molluscan host innate cellular immune system. For example, Bayne et al. (1980a, b) developed an *in vitro* cell-mediated cytotoxicity (CMC) assay in which the snail-infective larval stage (sporocyst) of the schistosome blood fluke *S. mansoni* is co-cultured with hemocytes derived from parasite-resistant and -susceptible strains of *B. glabrata* snail hosts. Using this assay, encapsulation reactions by only the resistant-snail hemocytes were effective at killing sporocysts, reflecting the behavior of resistant hemocytes under *in vivo* conditions (Loker et al. 1982). Since its development this *in vitro* cell assay has been used extensively to investigate basic cellular and molecular mechanisms

of immune recognition and hemocyte effector function in this host-parasite system (Bayne 2009; Yoshino and Coustau 2011). *In vitro* hemocyte cultures also have been used to investigate protozoan pathogens of commercially important bivalves. The intracellular parasites *Perkinsus marinus* Mackin, Owen and Collier, 1950 and *Bonamia ostreae* Pichot, 1979 are examples of oyster pathogens that naturally invade hemocytes and profoundly affect their immune capabilities. *In vitro* hemocyte cultures play a central role in investigating the basic mechanisms of parasite invasion and survival within cells (Robledo et al. 2004; Alavi et al. 2009; Morga et al. 2009), as well as host responses leading to resistance to parasite infection (Villamil et al. 2007; Hughes et al. 2010; Morga et al. 2011). Primary hemocyte cultures will continue to be important for addressing a range of basic and applied questions related to molluscan immune function.

Neuronal primary cell cultures

Because of cellular and molecular complexity of the nervous systems of higher metazoans, animals with simpler systems linked to defined behaviors and that can be experimentally manipulated continue to be utilized as research models. Included among these model organisms are several molluscan species of the genus *Aplysia*, *Lymnaea*, *Helisoma* and *Helix*. However, as suggested by Bulloch and Syed (1992), even with their “simple” nervous systems they are still complex organisms with highly integrated neural networks. Therefore, early on, investigators focused on isolation and cultivation of nerve cells located in defined ganglia in order to understand nerve cell growth and regeneration, neuroplasticity, and sensory connectivity to physiological processes and behavior in the intact animal. A review by Schmold and Syed (2012) details the many important contributions to neuroscience made by studying the molluscan nervous system in intact or semi-intact animals, including identification and mapping of neural circuitries controlling rhythmic behaviors such as respiration (Haque et al. 2006; Bell and Syed 2009) or feeding (Yoeman et al. 1995; Horn et al. 2004), or more complex processes like learning and memory (Lee et al. 2008a; Glanzman 2009; Nargeot and Simmers 2011). These types of experimental approaches are critically important as they provide the linkage between behavioral responses and the nerve centers (ganglia) involved in controlling such activities. However, a limitation of using intact or semi-intact organismal preparations is that it is difficult, if not impossible, to identify which neuronal cell(s) are associated with specific behaviors or the molecular mechanisms involved in regulating *in vivo* neuronal cell function. The need to develop simplified *in vitro* culture systems for the maintenance of molluscan nerve cells was recognized early on (Kaczmarek et al. 1979; Wong et al. 1981), thereby setting the stage for further refinement to and detailed descriptions of cultivation methods for model snails such as *Helisoma* (*Planorbella*) *trivolvis* Say, 1817 (Cohan et al. 2003) and *Aplysia californica* James Graham Cooper, 1863 (Lee et al. 2008b; Zhao et al. 2009), as well as other molluscan species. During this time, new investigative approaches also were emerging including electroporation (Lovell et al. 2006), new patch-clamp methods, (Py et al. 2010), development of silicon chip-based neurocircuitries (Birmingham et al. 2004) and live cell imaging (Lee et al. 2008b; Suter 2011) of cultured neurons, thus providing additional means of addressing questions at the single cell and molecular levels.

Over the last 30 years, primary culture of neuronal cells has become the experimental approach-of-choice for addressing fundamental questions regarding their varied functions. Importantly, cells in culture have been validated as accurately reflecting *in vivo* neuronal function by cell ablation-complementation (Syed et al. 1992), and by anti-sense blocking (Milanese et al. 2009). In other studies, *in vitro* vs. *in vivo* cellular responses to specific chemical agents were compared (Fiumara et al. 2005) and found to be similar. Schmold and Syed (2012) have reviewed the extensive literature on *in vitro* neuronal cell research and how these simple culture models have contributed to a fundamental understanding of

complex neural networks in regard to their control of behavior and learning/memory in molluscs. The following are examples of the main types of research conducted using cultured neuronal cells. (1) identification and functional characterization of neurotrophic factors regulating neurite outgrowth and regeneration (Spence et al. 1998; Goldberg 1998; Munno et al. 2000; Dmetrichuk et al. 2008; Milanese et al. 2009; Nejatbakhsh et al. 2011 and others), (2) growth cone motility and development (Spencer et al. 1998; Dmetrichuk et al. 2008; Suter 2011), (3) neurite-soma and soma-soma synaptogenesis (Munno et al. 2000; Hu et al. 2004; Onizuka et al. 2012) and (4) neuronal receptors and cell-to-cell neurotransmitter signaling (White and Laczmarek 1997; Mapara et al. 2008; Giachello et al. 2010; Ye et al. 2010).

Organ-derived primary cell cultures

Primary cultures of cells derived from tissues of solid organs have been developed for both basic and applied research purposes. Organ-specific cells can provide insights into the mechanisms regulating the functioning of that organ or tissue. On the applied side, cells in culture may be used to produce specific cell products of interest or as monitors of environmental pollutants. Regardless of the particular purpose, however, attempts to establish primary cell cultures generally has met with limited success, primarily due to issues related to microbial contamination. As summarized below, the organs most frequently serving as tissue sources for primary cell cultures have been the heart and mantle, mainly from bivalve species.

Heart primary cell cultures—Heart cells have enjoyed greatest success as research subjects in primary culture due to the relative ease of removal from the organism, the fact that the source tissue *in vivo* is not heavily contaminated with microorganisms, the availability of methods for minimizing microbial contamination from adjacent tissue or other sources, and the ease of dissociating and isolating heart cells while retaining high viabilities. Methods for establishing primary cell cultures have been described for snails *B. glabrata* (Bayne et al. 1975), oysters *Crassostrea* spp. Dall, 1909 (Le Deuff et al. 1994; Chen and Wen 1999; Domart-Coulon et al. 1994; 2000; Pennec et al. 2002), marine clams *Mya arenaria* L., 1758 (Kleinschuster et al. 1996); *Meretrix lusoria* Roeding, 1798 (Chen and Wen 1999) and *Ruditapes decussatus* L., 1758 (Hanana et al. 2011), scallop *Pecten* spp. Muller, 1776 (Le Marrec-Croq et al. 1999) and squid *Alloteuthis subulata* Lamarck, 1798 (Odblom et al. 2000). The only studies on gastropods have focused on the freshwater snail *B. glabrata*. Chernin (1963) cultured whole, trypsin-treated hearts and described hemocyte and “polygonal epithelial” cell migration from cultured whole organs. Hemocytes were short-lived but epithelial cells could be maintained for ~6 weeks in culture. Bayne et al. (1975) reported the maintenance of adherent cells emigrating from cultured heart and gonad explants. Although non-mitotic, these cells survived for >1 year in culture, one of the longest times thus far recorded for molluscan primary cell cultures. Cells cultured from bivalve hearts, whether oysters or clams, were initially mostly round cells (=hemocytes), epithelial-like cells and fibroblast-like cells (Chen and Wen 1999; Hanana et al. 2011). Within days in culture, snail, oyster and clam heart cells would commence “beating” either as individual cells or in cell clusters forming contractile networks (Domart-Coulon et al. 2000; Pennec et al. 2002; Hanana et al. 2011), and were identified as cardiomyocytes based on behavior and the presence of intracellular myofibrils by TEM imaging (Le Deuff et al. 1994). Cardiomyocyte cultures from oysters could be maintained for periods of 2–3 months (Domart-Coulon et al. 1994; Pennec et al. 2002), and from clams up to 5 months (Chen and Wen 1999). Several characteristics of primary cardiomyocyte cultures have made these attractive model systems for electrophysiological studies (Odblom et al. 2000; Pennec et al. 2004) and for environmental toxicological testing/monitoring (Domart-Coulon 2000; Pennec et al. 2002); namely their contractility (beating) phenotype, their long-term viability under *in*

vitro conditions, and tolerance to and high viability following cryopreservation (Cheng et al. 2001). Because of the robustness of bivalve heart cells in culture, they also have been used to test various promotor-reporter gene constructs for introducing and expressing transgenes in oyster cells (Boulo et al. 1996; Cadoret et al. 1999).

Mantle primary cell culture—Interest in the cultivation of mantle cells has been driven mainly by their applications to ecotoxicological testing and to understanding the cellular and molecular bases for biomineralization processes in nacre-producing mollusc species. Establishment and maintenance of epithelial cell primary cultures has been achieved in pearl oysters (Perkins and Menzel 1964; Awaji and Suzuki 1998; Sud et al. 2001), mussels *Mytilus galloprovincialis* Lamarck, 1819 (Cornet 2006) and *D. polymorpha* (Quinn et al. 2009), and abalone *Haliotis tuberculata* L., 1758 (Poncet et al. 2000; Sud et al. 2001). The maximum duration of cell maintenance in culture ranged from 2–5 weeks with cell viabilities of 60–75% (Perkins and Menzel 1964; Poncet et al. 2002; Gong et al. 2008a; Quinn et al. 2009), although Suja and Dharmaraj (2005) reported the establishment of cell cultures from explanted *Haliotis varia* L., 1758 mantle tissue surviving a mean of 102 days and a maximum of 1 year. Under their culture conditions, proliferating mantle cells were subcultured at monthly intervals for 3–6 passages before termination. Importantly, mantle cells referred to as “granulocytes” produced crystals with high calcium content, reinforcing the value of this and other primary culture systems for studying bio-mineralization mechanisms involved in shell and pearl formation (Poncet et al. 2000; Sud et al. 2001; Gong et al. 2008a, b). The qualities of long-term *in vitro* maintenance and proliferative capacity associated with cultured mantle cells also offers new approaches for monitoring low-level environmental mutagens/carcinogens (Cornet 2007) and other environmental toxins.

Gill, digestive gland and gonad primary cell cultures—Although not as extensively studied as other tissues, primary cell cultures derived from digestive glands (Robledo and Cajaraville 1997; Mitchellmore et al. 1998; Le Pennec and Le Pennec 2001; Faucet et al. 2003), gills (Faucet et al. 2003; Gomez-Mendikute et al. 2005) and gonads (Bayne et al. 1975) also have been successfully established. Typically, primary cultures were initiated by organs first being cut into small tissue fragments, followed by various primary cell extraction approaches including obtaining cells directly from explant tissues without treatment (Bayne et al. 1976; Faucet et al. 2003), enzymatic treatment alone prior to explant culture (Robledo and Cajaraville 1997; Le Pennec and Le Pennec 2001; Quinn et al. 2009), mechanical disruption alone creating cell suspensions (Faucet et al. 2003) or combining enzymatic and mechanical treatments to obtain cell suspensions (Robledo and Cajaraville 1997; Gomez-Mendikute et al. 2005). Microbial contamination is one of the major barriers to the establishment of primary cell cultures from gill and digestive gland tissues, due most probably to the abundance of microbes in gill mucus and within the digestive system (Robledo and Cajaraville 1997; Gomez-Mendikute et al. 2005). In addition, precise assessment of cell viability is often difficult because primary cell populations represent multiple cell-types (see Gomez-Mendikute et al. 2005 for gills, and Faucet et al. 2003 for digestive gland) that are not uniformly represented, or are present in cell aggregations or clumps as is the case for cultured digestive gland acini (Le Pennec and Le Pennec 2001). Despite these difficulties, high-density cultures with ~80% viability have been reported for zebra mussel *D. polymorpha* Pallas, 1771 digestive gland and gill cells maintained for 8–15 days (Quinn et al. 2009; Parolini et al. 2011). In marine mussels *Mytilus* spp. maintained in short-term cultures (up to 96 hr), viabilities of 50 to >90% for gill cultures (Faucet et al. 2003; Gomez-Mendikute et al. 2005) and 80–85% for primary digestive gland cell cultures (Robledo and Cajaraville 1996; Faucet et al., 2003) have been achieved. Finally, in terms of application, because of natural contact of gill and digestive gland tissues with the outside environment through their respiratory/osmoregulatory and feeding activities, primary cell

cultures from these organs have been recognized as ideal systems for the biomonitoring of a wide range of chemical contaminants found in aquatic systems. Examples of investigations using these cultured cell systems include those assessing the effects of environmental pollutants on cell viability or redox enzyme activities (Le Pennec and Le Pennec 2003; Labieniec and Gabryelak 2007; Parolini et al. 2011) or chemicals exerting genotoxic activities leading to DNA damage (Mitchelmore et al. 1998; Bolognesi et al. 1999; Bolognesi and Fenech 2012).

Molluscan Cell Lines

One of the potential highly-valued outcomes of efforts to establish primary cell cultures is the isolation of embryonic or adult tissue “stem cells” (Rinkevich 2011) and their subsequent *in vitro* propagation as immortalized cell lines. The fact that during the long history of molluscan tissue culture research only one cell line, the *Biomphalaria glabrata* embryonic or Bge cell line, has been established is testament to the difficulty in replicating *in vivo* physiological conditions conducive to supporting stem cells under culture conditions, in particular the specific *in vitro* conditions that support expression of genes responsible for regulating cell replication.

Establishment of the Bge cell line

The neglected tropical disease schistosomiasis continues as one of the most important of global infectious diseases, infecting an estimated 240 million people world wide and with over 700 million at risk of infection (Hotez 2008). The etiological agents for this disease in humans are the blood-dwelling parasitic flatworms of the genus *Schistosoma* - mainly *S. mansoni* Sambon, 1907, *S. haematobium* Bilharz, 1856, *S. japonicum* Katsurada, 1904 and *S. mekongi* Voge, Bruckner and Bruce, 1978. The life cycle of the blood flukes is complex, involving two hosts, a mammalian definitive host and freshwater gastropod intermediate hosts. In the western hemisphere and across sub-Saharan Africa, most of the reported cases of schistosomiasis are caused by *S. mansoni*, which utilizes snails of the genus *Biomphalaria* as its obligatory snail host. Thus, the *Biomphalaria glabrata*-*S. mansoni* system has been, and continues to be, extensively used as an important experimental model for investigating the physiological parameters regulating larval development and host-parasite compatibility (Bayne 2009; Loker 2010; Yoshino and Coustau 2011).

Recognizing the limitations of investigating snail-schistosome interactions under *in vivo* conditions, the U.S. National Institutes of Health awarded four research contracts in 1970 with the singular aim of developing a cell line from *B. glabrata*. Over a 3-year contract period, successful primary cultures were established from larval stages (whole egg masses or separated capsules containing trochophore-stage embryos) of *B. glabrata*. Cells that persisted *in vitro* for six weeks included fibroblast-like cells, contractile muscle cells, and epithelial-like cells (Basch and DiConza 1973). Primary cell cultures of explanted heart and gonad tissues from adult snails also were established, some lasting for >1 year (Bayne et al. 1975). Unfortunately these efforts yielded no cell lines. During this time, Eder L. Hansen (Hansen 1976) also used embryos to initiate cell cultures that exhibited characteristics similar to those described by Basch and Diconza (1973), but also noted new colonies of adherent, fibroblast-like cells that continued to proliferate upon subculture and multiple passages. These continuously propagating cells became known as the Bge cell line. Since the cell line was isolated in a laboratory that also cultured cells from the fruitfly, *Drosophila melanogaster* Meigen, 1830, and Schneider's *Drosophila* medium was used as a base component of the snail medium, further characterization of the cells via serologic, karyotype, behavioral and enzyme electrophoretic assays were conducted to verify the *Biomphalaria* origin of the cells (Bayne et al. 1978). Results of this study, in particular the karyotype analysis, confirmed that the Bge cell line was indeed from the *B. glabrata* snail

(Hansen 1976; Bayne et al. 1978). Thus, the establishment of a cell line from the molluscan intermediate host of *S. mansoni* has provided a critical tool for researchers to address long-standing questions concerning the regulation and maintenance of parasite-host interactions in this model system. The identity of the Bge cell line was recently confirmed using DNA sequence data (Lee et al. 2011).

Application of Bge cells to investigating snail-larval schistosome interactions

Because of its origin from *B. glabrata*, a snail host of *S. mansoni*, the Bge cell line (Fig. 1) has been used in several studies of the *in vitro* interactions between these cells and schistosome larval stages. Co-culture of the schistosome miracidial stage (snail-infective stage) and Bge cells results in the transformation of the miracidia to the first intra-molluscan stage of the parasite, the primary or mother sporocyst, followed by the adherence of cells over the surface of sporocysts forming cellular encapsulations within 48 hr of co-culture (Fig. 2). Encapsulated mother sporocysts continue to develop, eventually forming a second generation of sporocysts (daughter sporocysts) within brood capsules that finally emerge as free daughters (Fig. 2; 2–4 wks), which are capable of producing the final intramolluscan larval stage, the cercaria (Fig. 2; 6 months) (Yoshino and Laursen 1995; Ivanchenko et al. 1999). It is notable that this ability of Bge cells to promote *S. mansoni* larval development is not restricted exclusively to the Bge-*S. mansoni* system, but can also facilitate larval development of other *Schistosoma* spp. and even non-schistosome trematodes (Ataev et al. 1998; Coustau and Yoshino 2000).

The ability of Bge cells to encapsulate co-cultured sporocysts appeared very similar to the interaction of snail hemocytes and sporocysts when miracidia infect innately resistant snails (Loker et al. 1982) or when sporocysts encounter snail hemocytes in *in vitro* co-cultivation cellular-mediated cytotoxicity (CMC) assays (Bayne et al. 1980*a, b*). Based on these observations, Bge cells have been used to gain insight into the mediators of the *in vivo* interactions of *S. mansoni* and the *B. glabrata* hemocytes. For example, it was shown that, in the presence of various carbohydrates (fucoidan, mannose-6-phosphate, dextran-sulfate, and carragenans), the binding of Bge cells to the tegumental surface of the *S. mansoni* sporocyst is inhibited (Castillo and Yoshino 2002). Additionally, surface proteins of Bge cells were shown to bind fucosyl determinants present on the surface of larval sporocysts (Castillo et al. 2007), possibly implicating Bge cell surface lectins (~35–150 kDa) in Bge cell-larval sporocyst interactions. Further evidence that lectin-like proteins on Bge cells may serve as pattern recognition receptors (PRRs) mediating immune reactivity to larval schistosomes comes from the discovery of a highly diverse family of lectin-like proteins, the fibrinogen-related proteins (FREPs), in *B. glabrata* (Adema et al. 1997; Zhang et al. 2004) and the demonstration of their association with innate immune resistance in the *S. mansoni*-*B. glabrata* system (Hanington et al. 2010). Not only have Bge cells been shown to express FREPs (Zhang and Loker 2004), but FREP gene and protein expression could also be silenced by RNA interference, thus providing tools to functionally characterize these proteins in Bge cells (Jiang et al. 2006). Additionally, other lectin-like proteins believed to be acting as PRRs are expressed by Bge cells including one containing a carbohydrate recognition domain homologous to mammalian selectins (Duclermortier et al. 1999) and a tandem-repeat galectin that is also present in hemocytes of *B. glabrata* (Yoshino et al., 2008). Because of the molecular and functional similarities between hemocytes and Bge cells (Yoshino et al. 1999), efforts to identify and characterize other carbohydrate-binding proteins present in Bge cells, as well as their ligands, will contribute to our understanding of innate immune recognition in this system.

During miracidial transformation to primary sporocysts within the snail host (Fig. 2), larvae release a mixture of molecules into the surrounding medium including an array of glycoproteins (Guillou et al. 2007; Wu et al. 2009). Hemocytes, when exposed to these

larval transformation products (or LTPs; Wu et al. 2009), exhibit altered *in vitro* motility, phagocytic activity, and capacity to encapsulate sporocysts (Bayne 2009; Yoshino and Coustau 2011) as well as modulation of specific proteins (e.g., HSP70; Zahoor et al. 2010) that are associated with schistosome resistance in snails (Ittiprasert et al. 2009; Ittiprasert and Knight 2012). To better understand how LTPs interact with snail cells at the molecular level, Bge cells were treated with LTPs of *S. mansoni* and *Echinostoma caproni* Richard, 1964 and transcript expression was evaluated. *In vitro* exposure of Bge cells to LTPs was found to elicit differential expression of various genes including cytochrome c, methyl-binding proteins, glutamine synthetases, and protease inhibitors from the Kunitz family (Coustau et al. 2003). In reciprocal experiments, molecules produced by Bge cells in culture had a profound influence on gene expression in culture-reared *S. mansoni* sporocysts. For example 4 to 6-day old sporocysts exposed *in vitro* to Bge cell-conditioned media displayed numerous changes in gene expression for chaperonins/stress proteins, glutaminyl-tRNA synthetase, thioredoxin reductase, elongation factor 1-alpha, multiple ribosomal proteins and proteins with unknown function (Coppin et al. 2003; Vermeire et al. 2004; Taft et al. 2009). Recent demonstration of non-random spatial repositioning of genes (actin and ferritin) in Bge cells co-cultured with *in vitro* transforming *S. mansoni* miracidia (Knight et al. 2011) suggests novel pathways by which parasites may regulate gene expression in these cells. Clearly, there is considerable molecular cross-talk between larval trematodes and Bge cells that influence metabolic and regulatory pathways in both. The challenge for future investigations is to better understand the functional consequences of this molecular interaction.

Signal transduction pathways and mediators of signaling activity

In order to gain a better understanding of how schistosomes and snail host cells “communicate” following host infection, the *in vitro* Bge cell-*S. mansoni* model has been used to explore the signaling pathways activated in Bge cells by larval contact. Hemocytes of *B. glabrata*, when exposed to schistosome LTPs were differentially modulated in their expression of ERK mitogen-activated protein kinase (MAPK) (Zahoor et al. 2008, 2010), suggesting a direct molecular interaction between LTP binding and cell activation. Similarly carbohydrates commonly associated with LTP have been shown to modulate snail hemocyte ERK and p38 MAPKs and protein kinase C (PKC) pathways (Plows et al. 2005; Humphries and Yoshino 2008). Using Bge cells as a model, reactivities that parallel those of hemocytes also have been demonstrated; e.g., LTP-mediated activation of a Bge cell p38 MAPK that is believed to function in cellular differentiation and survival (Humphries and Yoshino 2006) and PKC involvement in Bge cell spreading (Humphries et al. 2001) that is similar to that exhibited by *Lymnaea stagnalis* L., 1758 hemocytes (Walker et al. 2010). Other immune response mediators of *B. glabrata* snails have been investigated using Bge cells including a macrophage migration inhibitory factor (MIF) family member that is thought to be involved in immune-related gene expression and apoptosis in hemocytes (Baeza Garcia et al. 2010), putative interleukin-1 responsive receptor(s) (Steelman and Connors 2009), an intracellular receptor for activated PKC (RACK) (Lardans et al. 1998), an insulin receptor family homologue (*BglR*) that may regulate Bge cell proliferation (Lardans et al. 2001), and a recently described mannose 6-phosphate receptor-dependent protein pathway of intracellular lysosomal enzyme targeting and sorting (Amancha et al. 2009). Functional involvement of the Man-6-P protein in parasite-host interactions is suggested by previous reports that mannose-conjugated BSA stimulates reactive oxygen species production in snail hemocytes (Hahn et al. 2000) and that Bge cell binding to sporocyst *in vitro* is inhibited by free Man-6-P (Castillo and Yoshino 2002). Although very little is still known about the signaling pathways or their upstream (receptors) and downstream (transcription factors, effector proteins) network connections present in *B. glabrata* and related snail species, the Bge cell

line will continue to serve as an available tool for investigating the signaling systems in this parasite-snail host system.

Changes in the Bge cell line since establishment

After establishment and validation of the Bge cell line (Hansen 1976; Bayne et al. 1978), cells were deposited with the American Type Culture Collection (ATCC) as well as distributed to several laboratories worldwide. Presently, the Bge cell line is no longer available through the ATCC and the last reported cell line obtained from this source failed to yield sustained viable cultures (Odoemelam et al. 2009). To our knowledge, the fate of the cell line is dependent upon a few laboratories (e.g., C. J. Bayne, Oregon State University; E.S. Loker, University of New Mexico and T.P. Yoshino, University of Wisconsin-Madison) and there is concern not only that the original cell line stocks have been lost, but the continuous passaging of the existing cell line for over 30 years in different laboratories may have resulted in genomic changes or contamination by foreign cell lines. Given this latter concern, Odoemelam et al. (2009) conducted a reassessment of chromosomal structure of the Bge cell line obtained from two separate laboratory sources. Karyotype analysis revealed a dramatic change in chromosomal content from the original diploid count of $2n=36$ to cells exhibiting extensive aneuploidy with modal metaphase chromosome complements of 63 and 67 for two separate lab sources. Although the nomenclature of chromosome groups used in the Odoemelam et al. study (2009) was similar to that used by Bayne et al. (1978), the types of chromosomes present within each of the 6 group varied, and included an additional unassigned group consisting of chromosomes exhibiting an unclassifiable morphology. However, despite these chromosomal changes, Bge cells still display morphological and functional characteristics similar to those of hemocytes of *B. glabrata* snails including their fibroblast-like morphology, ability to attach to and encapsulate *S. mansoni* larval stages, and, importantly, their continued ability to express genes and gene products specific to *B. glabrata* (e.g., Yoshino et al. 2008; Garcia et al. 2010; Lee et al. 2011). Although it is clear that this cell line has undergone genomic/chromosomal changes, these cells still represent the only *in vitro* cellular/molecular system related to *B. glabrata*, and remains the only cell line derived from any lophotrochozoan. As such the Bge cell line will continue to serve as an important investigative tool. That being said, however, investigators should be mindful of the genomic changes this cell line has undergone and should, therefore, continue to monitor functional/molecular characteristics of this cell line in future studies.

Difficulties in establishing molluscan cell lines, and possible solutions

Attempts to discover what it will take to enable, provoke, then sustain, proliferation in explanted molluscan cell populations have always been met with a barrage of difficulties. Such difficulties must be anticipated, and alternative means considered for circumventing the challenges that they present. Molluscs - the most species-rich of non-arthropod phyla - are enormously diverse, occupying niches in all oceanic habitats, in brackish and fresh water, and on land. The major clades have been around since the Cambrian. The variety of their body plans and life styles is remarkable, ranging from primitive solenogasters that resemble flatworms to the largest, most intelligent invertebrates - the cephalopods. The single universal feature of molluscs gives the phylum its name - a soft body. While this soft body, covered by its muco-ciliary epithelium, is fully exposed to the environment in slugs, advanced cephalopods and aplousobranchs, it is protected to some degree in most of the bivalves, gastropods, chitons and other molluscs. Unlike their other nearly universal feature (the radula), these exposed soft body parts comprise one of the difficulties that a cell culturist confronts when attempting to establish a cell line. This is due to the microbiota that commonly makes this mucus-rich environment its home.

Difficulty 1: Primary cultures are not axenic; unwanted symbionts or microbial contaminants are present

It is not uncommon to find oneself culturing organisms that were members of the microbial flora associated with the tissues that were used to initiate cell cultures. Unless painstakingly procured in a germ-free state, embryos and larvae bring along both prokaryotic and protistan microbes, and fungi. Juveniles and adults, as we have pointed out, have the muco-ciliary surface epithelium with a resident microbiota. Unfortunately, external surfaces are not the only places where symbionts reside: the molluscan gut is endowed with a wonderfully diverse microbiota! In order to minimize the chances of symbiont contamination, some investigators have used egg masses whose surfaces have been harshly treated to eliminate contaminating organisms. When Hansen (1976) prepared freshly laid egg masses of *B. glabrata*, she first washed them in 0.2% iodine, followed by 0.001% Hyamine-1622, then incubated them in sterile water with antibiotics. After 4–5 days, the embryos were dissected into buffered saline with antibiotics at 10 × the normal strength, and then rinsed in the same. If juveniles or adults (instead of earlier developmental stages) are to be the source of tissue for cell culture, decontamination of the body surface needs to be at least as rigorous. To prepare tunicate *Polyandrocarpa misakiensis* Michaelsen, 1904 tissues for cell culturing, Kawamura and Fujiwara (1995) dipped animals in 99% alcohol then burned their surfaces for a few seconds. Elevated levels of antibiotics can be helpful for microbial control during the preparation and early culture phases, but potential deleterious effects of supra-normal levels of antibiotics are unknown. With the tunicates, fractions of molecules from the donor animals themselves were added initially to media and found to help control microbes. Our own lab protocols call for the use of antibiotics at higher-than-normal levels for the first few days of culture: ten-fold higher has been used during tissue removal and preparation, then the levels are reduced in a step-wise manner during the first several days of culture. While most bacteria can be controlled with antibiotics, fungi and protists are less prone to selective killing: their eukaryotic status means that chemicals that harm them are more likely to harm molluscan cells at the same time. While antifungal agents may be helpful, cultures found to have yeast, fungi or protists are often discarded.

Difficulty 2: Obtaining populations of proliferating cells for use in setting up primary cultures

So as to improve one's chances of finding proliferating cells in early cultures, some investigators have chosen to use embryos, larvae or gonads. Of these, gonads have the advantage of being internal: this allows the investigator to clean and sterilize the surface of the mollusc prior to removal of the tissue from a presumed more sterile microenvironment. However, embryos and larvae are appealing because they harbor growing cell populations and potentially serve as sources of undifferentiated stem cells that could lead to establishment of a cell line. For some species, hatchery-based methods for their preparation have been well established for hundreds of years, as for example in oyster hatcheries. Their appeal is enhanced by the availability of plentiful material, all of the same age and possibly genetically uniform. The hurdle, however, is the difficulty of ensuring that primary cultures are axenic – not a trivial matter. Approaches described above (antibiotics and repeated sterile washes) need to be adapted according to the particular situation. If the tissue from which cell lines are to be derived is difficult to obtain in the quantities that would be needed to initially screen variables (media, protocols, etc), consideration can be given to use of alternate cells for this purpose. Easily isolated and cultured hemocytes or coelomocytes may meet the need as described earlier in this review. Kawamura and Fujiwara (1995) achieved successful cell cultures by obtaining tunicate mesenchymal cell. One need not assume that quantitative measures of cell viability, DNA synthesis, mitotic frequencies or cell proliferation are essential. Visual inspection of primary cultures serves as an excellent means to judge what is working well, is non-destructive, requires no reagents and can be

done inexpensively, with numerous cultures in reasonable time. Changes in cell morphology, loss of substrate adhesiveness and/or cell clumping may be associated with loss of cell viability.

Difficulty 3: Osmolar values of internal fluids and culture media

The osmotic values of molluscan blood (hemolymph) range from ~100 mOsm in freshwater species to ~1000 mOsm in marine species; terrestrial species tend towards intermediate values (Machin 1975). Mammalian blood approximates 300 mOsm, so components of culture media are formulated accordingly; in most cases, their use with molluscan tissue and cell cultures requires adjustment. In the case of freshwater species, optimum levels of classical cell culture media may be as low as ~1/3 the levels used for mammalian cells, whereas marine and terrestrial species will need additional osmolytes, such as inorganic ions, buffers and chelators. In optimizing the conditions for establishing and growing tunicate cell lines, Kawamura and Fujiwara (1995) made the surprising discovery that isosmotic conditions and pH at normal levels were detrimental to cell proliferation! By lowering the mOsm value of the medium from ~1000 to ~800, and holding the pH in the 6.0 – 7.0 range, cell growth was restored after cessation at more ‘physiological’ levels. To achieve success in cell culturing of difficult species, one needs to ‘think outside the box’, even if this means going against consensus views.

Difficulty 4: Not knowing the role of the extracellular matrix (ECM): adhesion preferences

Cells attached to untreated glass or plastic surfaces of culture plates in suitable media often look appealing for long term culturing. But it is not known if such cells would be more likely to divide if allowed access to extracellular matrix material. Some ECM components, such as collagen, are commercially available, but it may be advantageous to test ECMs prepared ‘in house’ from the target species. Such homologous matrices are likely to better capture and display autologous growth factors (Kawamura and Fujiwara 1995). Finally, one also may consider coating of surfaces with chemicals that impart an electro-positive surface charge such as poly-L-lysine, to enhance cell adherence.

Difficulty 5: Not knowing the optimum varieties and concentrations of both inorganic (including H ion) and organic components of media

In their successful efforts to establish cell lines from a budding tunicate, Kawamura and Fujiwara (1995) found that cells benefitted most from Dulbecco’s Modified Eagle Medium (DMEM) when this was present at just 17% [1 part in 6] in the medium! We confirmed (Bayne and Parton 2004, 2005; Barnes et al. 2004) that such low concentrations of nutrient media are well suited to cell cultures of one other marine invertebrate, an echinoderm *Stongylocentrotus* sp. Brandt, 1835. We found that a mixture of DMEM, L-15 and F-12 supported sea urchin cells nicely. The benefit of dilution could be due to one or more components present at inhibitory concentrations. Whether commercially available media or tailor-made media are used to supply nutrients and salts, lower than ‘normal’ amounts of nutrient media deserve to be evaluated for cell cultures from both aquatic and terrestrial species. Referenced here are examples of culture medium compositions for several commonly used freshwater (Basch and DiConza 1973; Hansen 1976; Quinn et al. 2009) and marine (Le Deuff et al. 1994; Chen and Wen 1999; van der Merwe 2010) gastropod and bivalve molluscs.

Difficulty 6: Not knowing the extent of damage done by free radicals or other toxic compounds during explant preparation and culture

Tissue architecture and cell integrity are compromised, at least to some extent, by the process of preparing tissues for primary culturing. Such injuries can release or lead to the

production of deleterious molecules (e.g. lysosomal enzymes) or radicals such as reactive oxygen and nitrogen species. Consequently, tissues need to be handled and processed gently in preparation for *in vitro* culture. Beyond that, washes at lowered temperatures (4–6°C) may slow damaging processes. Also, scavengers or antioxidant agents may be added to lower the levels of damaging compounds; for example, hydrogen peroxide may be scavenged by commercially available catalase. Some improvements in cell health have been ascribed to the addition of mercaptoethanol to media; this reduces disulfide bonds and, by scavenging hydroxyl radicals, serves as an antioxidant. Oxygen levels in media may be lowered by degassing just before use, or be kept low throughout culture by use of an appropriately gassed chamber. When explanted cells are adjusting to their new conditions, there may be benefits in keeping them at reduced temperatures to slow down any damage inflicted by such unknowns. How long to maintain cultures at reduced temperature before allowing cultures to gradually rise to the normal temperature of the donor animal must be determined empirically, but will likely be within the first day or two.

Difficulty 7: Damage done by intentionally introduced proteolytic enzymes (trypsin, collagenase and others), and by chelators (EDTA, citrate): preparation of primary ‘seed’ cultures and passaging

In the process of preparing tissues for primary culturing, a choice is made to explant tissue fragments composed of hundreds or thousands of cells, or to mince and digest fragments until one has a single-cell suspension with which to work, or some intermediate degree of cell liberation. Intact tissue fragments will probably be more often the most successful to follow, but once again empiricism is called for. The reason for mentioning this is that some invertebrate cells, including Bge cells, are easily damaged by exposures to trypsin and/or EDTA concentrations that are well tolerated by vertebrate cells. Reduced times, temperatures and/or concentrations of enzymes and chelators may be profitably evaluated. There is a second reason to pay attention to these matters. When success comes and that rare culture seems to be proliferating, you will be faced with the need to passage the cells to a new culture container, or run the risk of collapse due to overpopulation. The first passages need not include a division of the population to 2 or more cultures (‘split’). Indeed, since there will not yet be any experience with an optimum passaging protocol, one must anticipate losses in the transfer, and the first passage may be best done to simply move a portion of the proliferating culture to a new well/flask. Never throw out the initial flask or culture plate; feed it. Testing cell survival by prior exposure to various enzymes and media to be used for passaging is advised *before* a proliferating culture emerges. These things can be tested with healthy primary cultures.

Difficulty 8: Not knowing the rates of accumulation of deleterious substances in media

Here again, working at lower temperatures and/or lower pO₂ may be beneficial by slowing the rates of production, release and action of undesired components into explanted tissue and surrounding media. Efforts to reduce the risks of damage during the early hours *in vitro* have included the addition of more media, the subsequent replacement of 20–50% of the media once or twice in the early days with increasing amounts of fresh medium added, and change in the frequency of this regime, in accordance with the apparent needs of the cultures. This calls for subjective assessment, based on the apparent health of the cells: Are most of them alive? Attached to substrate (unless being grown in suspension)? Not simply spherical in morphology? Do they appear phase-bright under phase-contrast optics? Are they intact, without blebbing or obvious membrane lysis? If initial primary cultures are set up as replicates, or if a representative set of cells can be recovered from primary cultures, vital dye staining (e.g., trypan blue, propidium iodide, or acridine orange) can be used to quantitatively assess culture viability.

Difficulty 9: Not knowing what are good and what are bad components in fetal calf serum

Just as immunologists, for decades, used adjuvants to enhance the potency of vaccines without knowing how the adjuvants worked, cell culturists, for decades, have had our ‘dirty little secret’ – addition of fetal calf serum to media because of its magical, mystical properties. Because growth factors and other cytokines and their receptors, cell adhesion molecules and carrier proteins are, to variable degrees, conserved evolutionarily, there is a rational basis for inclusion of FCS in media developed for molluscs. But it is naïve to assume that everything in FCS is beneficial or even harmless; there are ‘badies’ in with the ‘goodies’. This is well illustrated by the frequency at which different labs have experienced difficulties in the maintenance of the Bge cell line (*personal observations*). The line currently thrives in several laboratories but, in others, it has been problematical. In our experience, a probable cause in these cases is an unknown property of the FCS used in the Bge culture medium. There is no way to avoid the need to screen FCS lots to identify those which will support healthy cultures over several weeks, and those that will fail. In addition, in most cases it is critically important that FCS be heat-inactivated before use. Again, from our experience with the Bge cell line, serum is typically inactivated at 65°C for 1 hour, making certain that heating is done with frequent, if not constant, mixing of the serum to ensure uniform heating. Finally, although fetal bovine serum is the most common serum component, other sources, e.g., chicken serum (Cornet 2006), may serve as suitable culture additives. Also it is important to determine the optimum percentage of serum, as lower or higher concentrations than the standard 10% FCS recommended for mammalian media may give improved outcomes (Chen and Wen 1999). Caution is advised in choosing to add homologous serum/plasma, since removal from the mollusc can be followed by increasing toxicity of the plasma (Bender et al. 2002).

Difficulty 10: Not knowing the receptors and the physiological levels of their ligands, including growth factors

Access to genomic databases enables investigators to identify genes encoding receptors that are known to influence cell proliferation in other species. The same holds for growth factors and other cytokines. In this way, one can locate orthologous genes and homologous peptides and proteins in the animal from which one seeks to develop cell lines. Such information may be used to procure the *bona fide* peptides, but it can also direct choices of already available reagents (from heterologous animal sources) for addition to growth media. But here is a precautionary note: with evolutionary distance comes sequence divergence in both receptors and ligands, so supra-physiological levels may be appropriate when factors are being tested on heterologous species.

Difficulty 11: Over-population and under-population in primary cultures, and what should be ‘passaged’?

Intuitively, it is obvious that very low and very high populations of cells in primary cultures are not conducive to optimum cell growth in culture. But what is the optimum number of cells to use when initiating a new culture, or attempting to passage a proliferating population? In the early hours and days of culture, damaged cells and unneeded extra-cellular materials may rapidly make the medium toxic. But, removed from their *in vivo* niches, cells *in vitro* may benefit from or even require molecules released from other healthy cells. Ten thousand cells/mm² (or 3×10^5 cells in 100 μ L) has been a number used successfully for primary cultures of tunicate cells (Kawamura and Fujihara 1995). This is similar to Hansen’s use (1976) of 10^6 cells in 300 μ L when she set up primary cultures of *B. glabrata* embryo-derived cells. It should be noted that these numbers are higher than cell densities one might generally use for primary cultures. If sufficient numbers of cells can be obtained during primary cell preparation, establishing multiple cultures with varying ‘seed’ cell numbers may help to set an optimum initial cell density. A decision also will need to be

made as to whether to retain any floating cells. They can be removed during feeding events, but this may be throwing out the materials that would otherwise yield the sought-after proliferating population! It may even be wise to retain some floating material when cultures are passaged.

Difficulty 12 and hope: Cellular quiescence - the 'counterpart to proliferation' (Coller et al, 2006)

Rinkevich (2011) has commented that, within the first days after primary cultures are initiated, cells generally enter a quiescent state. This state is defined as a reversible arrest of population growth and cell proliferation (Coller et al. 2006), and is ascribed to the actions of 'diverse anti-mitogenic signals'. Products from just some of the cells in a culture can decelerate proliferation of an entire population. *The basis for hope*: Transcriptomic studies of such cellular states (Coller et al. 2006) provide a basis for optimism that the genomic basis for such cell behaviors may soon come into better focus, enabling us to identify the genes whose transcripts have deleterious consequences. Considered together with increased understanding of what is responsible for the totipotency or pluripotency of various types of stem cells (Yamanaka 2008), it may not be too much to expect that efforts to establish cell lines from animals that have heretofore been problematic will become increasingly rational, and less reliant on trial and error with excessive numbers of variables. The regenerative capacities of many invertebrate species are taken as strong evidence for the presence of stem cells. It is entirely reasonable to suggest that the molluscan homologs of relevant transcription factors might be used to restore stem cell status to mollusc cells *in vitro*. Retrovirus-mediated transfection experiments have shown that factors such as Oct-3/4, Sox2, KLF4 and c-Myc, which are highly expressed in embryonic stem cells, can be used to transform mouse fibroblasts into pluripotent stem (iPS) cells (Yamanaka 2008). Protocols for genetic manipulations of Bge are already available (Yoshino et al. 1998), and Bge cells have been used in a variety of ways, including as 'feeders' to condition and change media so that the intramolluscan stages of *S. mansoni* survive better *in vitro* (Yoshino and Laursen 1995; Ivanchenko et al. 1999). This illustrates its potential to beneficially 'condition' media for other molluscan cells. The most powerful basis for hope as we continue these endeavors is the existence of the Bge cell line. The species of origin was recently re-confirmed (Lee et al. 2011), so, despite years of frustrating failures, we can be confident that there is no absolute block to the *in vitro* proliferation of molluscan cells.

Conclusions

In vitro cultivated molluscan cells represent invaluable investigative tools that have contributed significantly to many disciplines including neurobiology, immunology, toxicology, environmental science, functional genomics, and others. Cultured neuronal cells from ganglia or other nerve tissues have served as model systems for investigating neurite growth, nerve cone motility, regeneration, synapse formation, and how neural networks are coordinated to achieve higher-level neural functions. Primary cell cultures originating from various tissues including hemolymph (hemocytes), heart, mantle, digestive gland and gill can persist for extended periods *in vitro*, making them suitable systems for electrophysiological research, metabolic or immunological studies, monitoring environmental toxins, and development of transgenic technologies. Attempts to generate continuously proliferating cell lines by establishing primary cell cultures from embryos or larval stages during early development have largely been unsuccessful. To date only one cell line, the *Biomphalaria glabrata* embryonic (Bge) cell line, has been produced. Because this cell line was derived from *B. glabrata*, snail intermediate host of the human blood fluke, *Schistosoma mansoni*, it has become a valuable research tool providing insights into the molecular bases for snail host-parasite immune compatibility, serving as a model for gene

transfer approaches in snails, and enhancing our ability to cultivate schistosome larval stages *in vitro*. These achievements illustrate the importance and usefulness of establishing cell lines from other molluscan species, although many barriers must first be overcome in order to reach this goal. New genomic information gleaned from model organisms may provide the keys for future breakthroughs in molluscan cell culture.

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Figure 1.
Typical morphology of adherent spreading cells of the *Biomphalaria glabrata* Say, 1818 embryonic (Bge) cell line. Nomarski optics. 400x.

Figure 2.

Co-cultivation of primary sporocysts of the human blood fluke *Schistosoma mansoni* Sambon, 1907 with cells of the *Biomphalaria glabrata* Say, 1818 embryonic (Bge) cell line at different times of *in vitro* culture. **48 hr**: Sporocysts are completely encased (encapsulated) with Bge cells that strongly adhere to the larval surface. **2 wk**: Bge-cell-encapsulated primary sporocysts contain brood capsules in which motile secondary or daughter sporocysts develop. **4wk**: Secondary sporocysts eventually emerge from the primary sporocysts as motile free larvae and, in turn, become encapsulated by Bge cells. Multiple generations of secondary sporocysts may be produced under culture conditions. **6 mo**: After prolonged cultivation of Bge cell-sporocyst co-cultures (6–9 months), cercariae, the final intramolluscan stage of development, emerge from sporocysts.