Investigation of the somatic cellular physiology of a marine tunicate.

Masters Thesis Proposal

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Contents

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Proposal

Introduction

- General lack of marine invertebrate cell lines. (Domart-Coulon and Blanchoud 2022)
- Exception is sponge cell line.

Cellular homeostasis acts by maintaining the integrity of the internal environment in the response to environmental fluctuations. If perturbations of the extracellular environment occur at a magnitude or rate beyond the capacity of homeostasis, the cellular stress response is induced. The cellular stress response describes a set of physiological mechanisms associated with rapid generation of phenotypes capable of withstanding extreme stress.

As such, through an evolutionary lense, this can be refered to as stress-induced evolution

Genome reorganization events that occur with macromolecular damage, genome chaos, and DNA repair infidelity are stress-induced adaptive evolutionary strategies that promote cell survival during periods of extreme stress (Kültz 2005, 2020; Rosenberg et al. 2012; Liu et al. 2014).

ROUGH OUTLINE:

- talk about homeostasis

-homeostasis different than the cellular stress response

- the cellular stress response involves physiological mechanisms to circumvent cell death by stress-induced evolution

- stress induced evolution applicable at the cell population level,
- advances cell survival through rapid generation of useful phenotypes
- particularly this can occur through dna methylatio, increasdeed mustation rates chromoanagenesis,

Removal from the *in vivo* context presents somatic cells with severe stress that likely induces several methods of genome transformation which then are directionally selected for their ability to proliferate *in vitro* (Liu et al. 2014).

Utilizing this framework of stress-induced cellular evolution, this project aims towards promoting an immortalized cell line from somatic cells of the marine tunicate, *Botryllus schlosseri*.

B. schlosseri is a cosmopolitan, sessile, colonial species currently found in the shallow, temperate, coastal areas of all continents except Antarctica (Zwahlen et al. 2022). Each mature colony of *B. schlosseri* is composed of genetically identical modules, termed zooids, that are embedded in a gelatinous tunic and share a single vasculature system (Manni et al. 2019). *B. schlosseri* colonies begin life as free-swimming larva that settle and metamorphoses into a single founding oozooid. Through blastogenesis, new blastozooids develop and are organized into a colonial star-shaped system. Over the course of its life, a healthy *B. schlosseri* colony will undergo weekly blastogenic cycles, where parental zooids are synchronously reabsorbed and replaced with a new set of blastozooids (Ricci et al. 2016; Manni et al. 2019). This species of *Botryllus* is of significant interest as an experimental model system in the fields of cell biology, immunology, and developmental biology due to its tightly regulated blastogenic cycle, close phylogenetic relation to vertebrates, and ease of rearing individuals in a lab setting.

Previous work exploring *B. schlosseri* cell line development isolated primary cell cultures from the epithelial and blood tissue of lab-reared colonies (Rinkevich and Rabinowitz 1993, 1997; Rabinowitz and Rinkevich 2004a, 2011; Rabinowitz et al. 2009).

However, all previous attempts of *B. schlosseri* primary cultures hit a state of cellular quiescence where cell division ceases approximately 24 to 72 hours post-isolation; ultimately leading to a loss

of cell viability after 4 weeks *in vitro* (Rinkevich and Rabinowitz 1993; Rabinowitz and Rinkevich 2011; Terzi et al. 2016; Domart-Coulon and Blanchoud 2022).

Given such limited life span and proliferative capabilities, there is a clear need to elucidate the ideal culturing conditions for primary cultures of this species prior to experimentally invoking mechanisms of stress-induced evolution.

Objectives

The overarching hypothesis is that *in vitro* cellular immortalization of *B. schlosseri* is limited by biological constraints that may be overcome through: 1) the optimization of primary culturing methods and 2) stress-induced adaptive evolution of primary cultures.

Methods

The first aim, involving the optimization of primary cell culturing methods for *B. schlosseri*, will be addressed by identifying the ideal combination of complete culture media and substrate that most improves cell viability and proliferation. Blood and epithelial cells of *B. schlosseri* will be isolated using the previously established methods described in Rabinowitz and Rinkevich (2004a), Rabinowitz and Rinkevich (2004b), and Rinkevich and Rabinowitz (1993). Primary cultures will then be maintained at 15 to 20 °C, in a sterile, humidified cell incubator and grown in tunicate culture medium (TCM), pH 8.0. TCM will be formulated in-house and is comprised of 12% supplements and antibiotics, 38% artificial seawater, and 50% single strength commercially available liquid media (Rabinowitz and Rinkevich 2004a, b). We will then assess the proliferative effects of multiple conditions on primary cultures of *B. schlosseri*. Six culture media will be tested as the single strength liquid media component in the TCM: 1) DMEM with 4500 mg/l D-glucose without Gln; 2) HAM F-12 with Gln; 3) Biotarget-1 without Gln; 4) Fischer's medium without Gln; 6) Leibovitz L-15 with Gln; 7) DCCM-1 without Gln. Three substrates will be tested: 1) Collagen, type VII from rat-tail; 2) Methocel-methylcellulose; 3) Fibronectin (bovine). Three supplemental

organic osmolytes will be tested: 1) taurine; 2) betaine; 3) glycine. We will, replace the corresponding amount of NaCl in TCM with up to 50 mM of organic osmolyte supplement. In marine invertebrates, organic osmolytes aid in offsetting ionic stress as well as offer indirect cytoprotective effects such as antioxidation, calcium modulation, and cell membrane stabilization. The listed organic osmolytes were selected as they are most ubiquitously utilized by shallow-water marine invertebrates (Yancey 2005). We will measure cell growth and viability using the high-throughput assay, ViaLight, which quantifies cellular ATP. Furthermore, transcriptome and proteome dynamics will be evaluated over the course of the transition from *in vivo* to *in vitro*. Protein and RNA will be sampled daily from primary cultures beginning at day 0 when tissue is first seeded into culture plates, until cessation of cell proliferation or after 1000 cell passages.

To address aim 2, we will expose primary cultures of *B. schlosseri* to UV-B irradiation and genotoxicants such as benzo[a]pyrene and nickle chloride along with multi-stresssor combinations thereof (Blewett and Leonard 2017; Banni et al. 2017; Guo et al. 2019; Qarri et al. 2020). These will act as directionally selective stressors in the culturing environment. For each stressor, a range-finding experiment will be conducted to identify the median lethal dose (LD50) for epithelial and blood cells of *B. schlosseri*. We will conduct exposures above the LD50 of each stressor at dosages that maximally reduce the initial cell population but yields proliferative cultures after 1-week post-recovery. DNA damage will be measured using the alkaline comet assay (Banni et al. 2017). From cultures that yield the greatest proliferation, protein and RNA will be extracted after the 1-week recovery period once the culture has reached monolayer confluence or proliferation has ceased.

As described above for both aim 1 and 2, RNA will be collected and quantified prior to conduction of RNA-seq at the University of Washington, Seattle Superfund Functional Genomics and Bioinformatics Core Facility. Estimation of transcript abundance and differential expression analysis will be conducted using Cufflinks (Trapnell et al. 2012) or DeSeq (Roberts and Gavery 2018) tools. Transcriptomic data will be stored and managed using Roberts Lab owned computing resources at the University of Washington, Seattle. Data generated from RNA-seq experiments will be utilized in targeted quantitative polymerase chain reaction (qPCR) analyses. Additionally, for both aims, protein will be collected and sent to our collaborators at the University of California, Davis, who will then generate corresponding proteome data. Proteome and transcriptome data will be paired

for functional enrichment analysis with Genesis (Sturn et al. 2002), PANTHER (Mi et al. 2017), KEGG mapper (Kanehisa and Sato 2020), and String (Crosara et al. 2018).

Results

For aim 1, we seek to elucidate the ideal culture media, substrate, and supplement combination that extend the longevity and proliferation of primary cultures of *B. schlosseri* beyond previously documented maximums. Maintaining sterile primary cultures of any cell strain for long periods of time *in vitro* increases the probability of spontaneous immortalization (Gardell et al. 2014). For aim 2, we are aiming to facilitate adaptive stress-induced evolution of *B. schlosseri* primary culture cell populations towards phenotypes more capable of enduring *in vitro* conditions and thus enhances the potential for cell immortalization. Through functional enrichment and network analysis we expect to identify gene and proteome networks that underlie the successful cellular transition to the *in vitro* context which may then inform further synthetic biological approaches that specifically target genes critical to cellular immortalization if cells do not spontaneously immortalize within the context of this project.

Interpretation

Significance

To date, there are no cell lines available from any marine invertebrate species despite decades of extensive research efforts (Rinkevich 2005; Cai and Zhang 2014; Domart-Coulon and Blanchoud 2022). Much of the published work relating to marine invertebrate primary cultures focuses on applied aspects such as ecotoxicology (Yoshino et al. 2013; Ladhar-Chaabouni and Hamza-Chaffai 2016; Rosner et al. 2021). As such, there has not been a sustained effort towards synthesizing an understanding of the *in vitro* requirements of marine invertebrate cells (Rinkevich 2011; Domart-Coulon and Blanchoud 2022). Although marine invertebrates are a highly diverse group, establishment of a clear primary culture methodology for any marine invertebrate would be of great

value across species. This possibility is best exemplified by the breakthrough in *in vitro* cell culturing conditions for insects (Grace 1962), which has since given rise to nearly 1000's of insect cell lines within the span of 50 years (Domart-Coulon and Blanchoud 2022). Additionally, in utilizing -omics analyses in our process of *B. schlosseri* cell line development we are integrating a novel yet critical component that departs from earlier cell line development work conducted on this species.

Marine invertebrates are of interest as potential "bioreactors" (Romano et al. 2022). Therefore, the establishment of cell lines from marine invertebrates would serve as a means to sustainably harvest bioactive compounds which could be of use commercially across several fields such as pharmaceuticals, cosmetics, biomaterials, and more. Furthermore, the continued global worsening of perturbations within coastal ecosystems as a result of anthropogenic activity, necessitates methods of rapid and high-throughput *in vitro* screening. The generation of a *B. schlosseri* cell line would represent a powerful high-throughput tool in identifying molecular responses that predict adverse outcomes to anthropogenic stressors such as those resulting from marine contaminants and climate change.

Timeline

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