Expression of *survivin* and *mortalin* during visceral regeneration in *Parastichopus californicus*

by
Amy L. Tan

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ABSTRACT

Echinoderms are capable of regrowing an astonishing amount of tissue, from arms to internal organs to the majority of the body during asexual reproduction. The molecular control of regeneration has been studied in all classes of echinoderms, but always under conditions of acute stress (irritant injected to induce evisceration or surgical removal of tissue). *Parastichopus californicus* experience atrophy of their viscera (digestive tract, gonads, circulatory system, and respiratory trees) on a yearly cycle. After the cucumber has a month-long torpor period, the viscera regrow. While the regeneration cycle of *P. californicus* is unique, no studies have been done comparing the molecular control of this regeneration with that of echinoderms undergoing regeneration following acute stress evisceration. The development genes *survivin* and *mortalin* are upregulated during regeneration in *Holothuria glaberrima* following injury or evisceration. The purpose of this study is to examine the expression of *survivin* and *mortalin* and their protein products during the annual visceral degeneration and regeneration cycle of *P. californicus*.

INTRODUCTION

Regeneration of tissue occurs in many groups of vertebrates and invertebrates. The ability to regrow lost or damaged tissue is highly advantageous for organisms, allowing them to recover from acute stress such as disease, injury due to predation, and loss of fragile body parts. Other organisms have tissues which are lost or atrophied and regrown on a regular recurring cycle, such as reindeer antlers or the reproductive organs of birds.

Echinoderm Tissue Regrowth

Adult echinoderms have regeneration abilities possibly unsurpassed by any other invertebrates (Candia Carnevali 2006). Crinoids, ophiuroids, and asteroids are able to regenerate arms lost to mutilation or predation. Asteroids take this process a step further with their ability to regenerate an entire body from a single arm, provided the arm has a portion of the central disc attached. Ophiuroids and holothuroids are also capable of a similar asexual propagation with adults splitting into two to three pieces and regrowing the rest of the body (Candia Carnevali 2006, Patruno *et al.* 2001, Uthicke 1997).

Echinoderms also have the ability to regenerate internal structures. Asteroids can regenerate their viscera, specifically the pyloric caeca, during regeneration of an arm and if the pyloric caeca have been removed artificially (Anderson 1962). Crinoids are able to regrow their visceral mass following artificial removal (Kondo and Akasaka 2010). Holothurians can regenerate both radial nerve cords and extensive areas of muscle following acute injury (Mashanov *et al.* 2012, García-Arrarás *et al.* 1999, Murray and García-Arrarás 2004).

Holothurians, especially, are capable of regrowing internal organs following evisceration, including the digestive tract, respiratory trees, and gonads (García-Arrarás and Greenberg 2001, Shukalyuk and Dolmatov 2001, Mashanov *et al.* 2005). Visceral regeneration has been studied extensively in several species of sea cucumbers. These studies have examined acute evisceration of the internal organs, usually triggered by injection of some irritant, and monitored the regrowth pattern of the tissue and the expression of genes during the regrowth period (Mashanov *et al.* 2010, Mashanov *et al.* 2012, Ortiz Pineda *et al.* 2009, Suárez-Castillo and García-Arrarás 2007).

There appear to be two patterns by which visceral regeneration occurs. Gut regrowth in *Apostichopus* begins with thickening of the free edges of the mesenteries which were left behind when the viscera were expelled. This free edge thickens along the length of the body, resembling a blastema in the process, eventually forming the intestinal lumen (García-Arrarás and Greenburg 2001, Murray and García-Arrarás 2004). In *Holothuria*, gut regrowth takes place via two separate rudiments. One rudiment grows anterior to posterior while the second grows posterior to anterior, beginning from the cloaca. The tips of the regenerating portions are covered by coelomic epithelium and cells differentiate while they are in this tip region. The rudiments extend through the body of the animal, eventually fusing together (Mashanov *et al.* 2005, Shukalyuk and Dolmatov 1999). It appears that, in both patterns of regeneration, regrowth of other visceral structures proceeds only after gut regeneration is highly advanced so that tissues associated with the hemal system, respiratory trees, and gonads are the last to form (García-Arrarás and Greenburg 2001).

Gene Expression During Regeneration

During this regrowth phase, a number of genes are expressed above or below levels seen in non-regenerating animals. Developmental pathway genes *Hox 5, 9, 10,* and *12,* along with *Wnt 14*, *TCTP, BMP-1, survivin,* and *mortalin* were all over-expressed during *Holothuria glaberrima* visceral regeneration, while *myotrophin* and *forkhead box K1* were down-regulated (Ortiz-Pineda *et al.* 2009, Mashanov *et al*. 2010). Cytoskeleton protein genes *Actin 1* and *2* and *Tubulin alfa* and *alfa-1* were also over-expressed during regeneration, while *Actin 3, Myosin-11,* and *gelsolin precursor* were under expressed. *Echinonectin*, *Collagen alfa-1*, *Laminin alpha,* and *Tenascin-R*, all extracellular matrix genes, were over-expressed during regeneration as well (Ortiz-Pineda *et*

al. 2009). The ependymin-related gene, *EpenHg,* was also up-regulated during *H. glaberrima* visceral regeneration. In other organisms, ependymin aids in regeneration, formation, and stabilization of axonal pathways (Schmidt *et al.* 1990). Although its precise physiological role in holothurian regeneration is still unknown, this up-regulation is worthy of note as it was the first ependymin sequence reported for an invertebrate (Suárez-Castillo and García-Arrarás 2004).

While many, or homologs, of these genes are useful for organisms that are regenerating, *survivin* and *mortalin* are of further interest as their protein products have been implicated in tumor growth.

Survivin

Expression of *survivin* has been documented in human lung cancers as well as in the stomach and liver during normal function. In humans, the protein survivin is found almost exclusively in tumor cells and developmental cells due to its roles in inhibiting apoptosis and regulating cell division (Deguchi *et al.* 2002). Directly or indirectly, survivin inhibits the activity of caspases, resulting in a disruption of apoptosis. While cells are dividing, survivin is first seen on the centromere, then in the spindle midzone or kinetochore, with any expression being limited to the cleavage plane by the time the cell has reached telophase and cytokinesis (Chiou *et al.* 2003). Survivin knockout mouse embryonic stem cells showed disrupted microtubule formation and polyploidy during development, both resulting in embryonic death (Chiou *et al.* 2003).

Based on studies with mouse livers, survivin appears to play a role in the proliferation and differentiation of cells during regeneration (Deguchi *et al.* 2002). Survivin mRNA and protein have both been observed in normal gastric mucosa of rats and humans. As these epithelial cells are replaced at a rapid rate (turnover rate of 3-5 days), survivin may aid in regulating this

process (Chiou *et al.* 2003). A homolog of *survivin* is also upregulated during visceral regeneration in the echinoderm *Holothuria glaberrima*, with significantly higher levels of expression seen at 21 days post evisceration in the anterior gut rudiment (Mashanov *et al.* 2010).

Mortalin

Mortalin has multiple roles within living cells, ranging from beneficial to cancer promoting. It is an essential member of the Hsp70 family of proteins, found in almost all normal tissues, but is not inducible by heat (Wadhwa *et al.* 2002). Instead, mortalin is expressed in response to glucose-deprivation, calorie restriction, low doses of ionizing radiation, and other mild stressors. In its "housekeeping" and "guardian mode[s]," expression of mortalin is beneficial to the organism. Mortalin is responsible for aiding in the translocation of proteins, both import and export, in the mitochondria (Kaul *et al.* 2007). Most impressively, knocking in extra copies of hsp70F, a mortalin homolog, resulted in extended longevity in *Caenorhabditis elegans*. There is also evidence that similar over-expression extends the life of human fibroblasts *in vitro* (Yokoyama *et al.* 2002). During visceral regeneration of *H. glaberrima*, a mortalin homolog is expressed at higher than normal levels during the first two weeks of regrowth, with especially strong expression 12-14 days post-evisceration (Mashanov *et al.* 2010).

Similar to survivin, mortalin is capable of inhibiting apoptosis. Mortalin binds to p53, preventing it from inducing apoptosis, as well as inhibiting cell division. Because of its regulation of apoptosis and also of oxidative stress, altered regulation of mortalin may contribute to both aging and old age pathologies. In Parkinson's disease patient brains, however, mortalin levels are substantially decreased (Kaul *et al.* 2002).

Regeneration in *Parastichopus californicus*

While many studies have examined regeneration in echinoderms following acute stress events, relatively few studies have examined regeneration which follows a different pattern of tissue loss and regrowth. *Parastichopus californicus*, in addition to regrowing tissue following acute injury or evisceration, loses and regrows its viscera on a yearly basis, seemingly without any underlying acute stress event. Instead, *P. californicus* experiences a diapause beginning every year around September. At this time, the gut, gonads, circulatory system, and respiratory trees atrophy and the cucumber remains in a state of torpor until regrowth begins about one month later (Fankboner and Cameron 1985). In only one other species of sea cucumber has there been any evidence of a similar evisceration/atrophy-regeneration cycle, *Eupentacta quinquesemita*, a species with a range similar to *P. californicus* (Byrne 1985). Although hypotheses exist as to why *P. californicus* undergoes this yearly atrophy and regeneration, the mechanisms, molecular bases, and triggers remain elusive. This yearly cycle may be triggered by changes in temperature, altered photoperiod, changes in salinity, or bacterial load in the gut. As far as is currently known, regrowth of tissues occurs independent of the mechanism of tissue loss--the regrowth will follow the same pattern, adjusting for severity of repair needed, whether the tissues have been lost on an annual cycle or due to injury (Brocke and Kumar 2008). As the genes and proteins responsible for aiding in tissue repair are distinct from those which deal with the initial trauma of tissue loss (immune/stress response vs. regeneration response), the regulation of tissue regrowth may occur in the same manner whether the trigger is acute stress or a chronic cycle (stress or otherwise). While it seems intuitive that the regulation of regrowth would remain the same, this problem has not yet been experimentally examined.

Research Purpose

The purpose of this study is to examine changes in the regulation of *mortalin* and *survivin* and their products during the annual cycle of degeneration and regeneration of the viscera in *Parastichopus californicus*.

Research Objectives

- To examine if *survivin* and *mortalin* are up or down regulated at certain points of the degeneration/regeneration cycle.
- To look for the protein products of *survivin* and *mortalin*.
- To compare regulation patterns of *survivin* and *mortalin* in *P. californicus* to regulation patterns in regeneration due to acute stress in other invertebrates.

METHODS

Timeline

This experiment will be conducted at three times of the year, chosen in order to coincide with expected viscera states of *Parastichopus californicus.* A summary of the methods can be found in Figure 1.

1. Summer: July - August 2014. *P. californicus* have intact viscera and are active both moving and feeding.
2. Fall: October - November 2014. Atrophy of the viscera occurs around September so *P. californicus* should have no viscera and be in torpor by this time.

3. Winter: December 2014 - January 2015. *P. californicus* are regrowing the viscera and beginning to be active.

Experimental Procedures

Parastichopus californicus will be collected by scuba from depths of 10 to 20 meters from waters around the Rosario Beach Marine Laboratory, Anacortes, WA (48°25'22"N 122°40'21"W). Cucumbers will be dissected and the state of the viscera recorded as present, absent, regeneration, or atrophying. During each season, five cucumbers of the representative viscera state for that season will be collected for sampling. Samples will be collected from the digestive tract, respiratory trees, body wall, muscle, and coelomic fluid. Samples will be frozen on dry ice for storage until analysis for *survivin* and *mortalin* expression and protein presence.

Tissue samples will be ground in liquid N_2 with mortar and pestle, and homogenized in TRI Reagent®. Use of TRI Reagent® as a lysis buffer allows extraction of both RNA and protein from a single sample. Homogenization and extraction of RNA and protein layers will be done according to Sigma-Aldrich manufacturer's protocol (Mashanov *et al.* 2010, Tan *et al.* 2011, Unajak *et al*. 2006). Extracted total RNA will be used to synthesize cDNA which will be amplified by PCR (Méndez *et al*. 2000). The primers for PCR amplification of *survivin* and *mortalin* are listed in Appendix A. PCR products will be analyzed using electrophoresis to check for presence and relative quantity of *survivin* and *mortalin* transcripts. Presence and relative quantities of mortalin and survivin proteins will be determined using 2-D gel electrophoresis followed by western blots (Migliore *et al.* 2007, Franco *et al.* 2011).

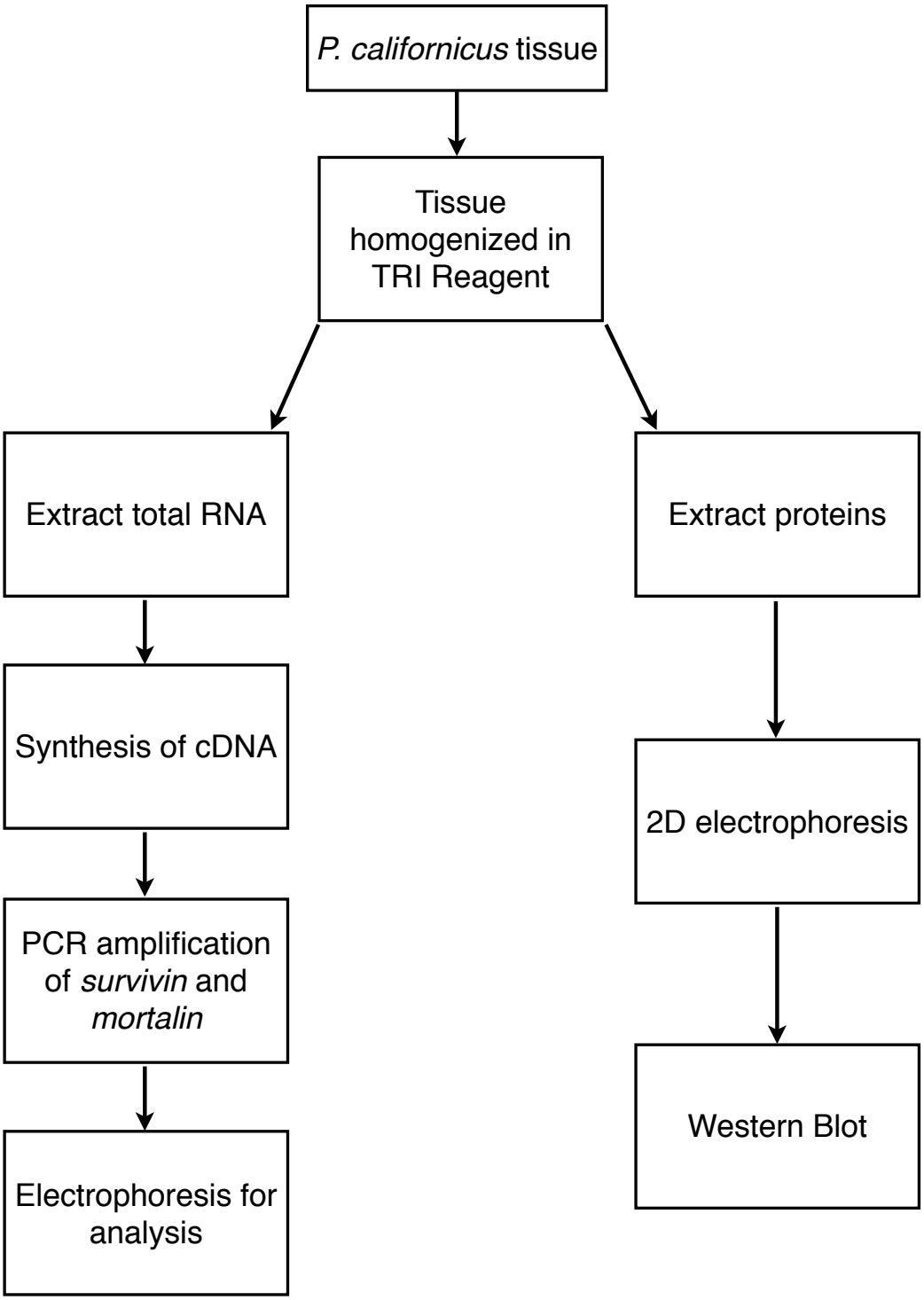

Figure 1. A flow chart of experimental procedures to be conducted on *Parastichopus californicus* tissue samples each season (summer, winter, spring).

RESULTS

I expect that expression of *mortalin* will increase during times of regeneration. Levels of both gene transcripts and proteins are expected to be highest during the winter regeneration period. During the fall, *mortalin* transcripts and proteins may be expressed at moderate levels. While low levels of *mortalin* transcripts and proteins are expected within the digestive tract during the summer, if *mortalin* transcripts are seen elsewhere during this time, it is expected that the protein product will not be translated (Table 1).

Based on the mortalin sequence of *Holothuria glaberrima* from BLAST and analysis with an online protein prediction program, mortalin has a predicted iso-electric point (pI) of 8.44 and molecular weight of 82.96 kD. The predicted pI and molecular weight allow the protein product to be identified using 2-D gel electrophoresis and Western blots from samples taken during different gut states in *P. californicus.*

Expression of *survivin* RNA and protein are expected to increase during the winter gut regeneration period. Low levels of expression of both *survivin* RNA and proteins may be seen in the digestive tract during the summer. If *survivin* is expressed in the muscle, body wall, and/or coelomic fluid during the fall or summer, it is likely that the protein product will not be translated (Table 1). Survivin has an iso-electric point of 4.72 and a weight of 16.66 kD in *Holothuria glaberrima*, based on the BLAST amino acid sequence and an online protein pI and weight prediction software.

Table 1. Expected expression levels of *survivin*, *mortalin*, and their protein products in *Parastichopus californicus* during the annual cycle of visceral regrowth. (-) indicates down-regulation and number reflects relative level of down-regulation. (+) indicates up-regulation and number reflects relative level of up-regulation.

	Coelomic Fluid	Muscle	Body Wall	Digestive Tract	Respiratory Trees
Summer					
survivin	- -	- - -	- - -	+ +	- - -
mortalin	- -	- - -	- - -	+ +	- - -
Survivin	- -	- - -	- - -	+ +	- - -
Mortalin	- -	- - -	- - -	+ +	- - -
Fall					
survivin	- -	- -	- -	n/a	n/a
mortalin	+ +	+	+	n/a	n/a
Survivin	- -	- -	- -	n/a	n/a
Mortalin	++	+	+	n/a	n/a
Winter					
survivin	+ +	+	+ +	+ + +	+ + +
mortalin	+ +	+	+ +	+ + +	+ + +
Survivin	+ +	+	+ +	+ + +	+ + +
Mortalin	+ +	+	+ +	+ + +	+ + +

DISCUSSION

Based on regeneration research done so far, there is no reason to suspect that the mechanism of regrowth will vary in *P. californicus* from any other holothuroid. There are no cases of regeneration currently known in which the regeneration triggered by acute stress has a different molecular control then regeneration triggered by chronic stress or annual cycle (Brocke and Kumar 2008). Thus, because high levels of mortalin and survivin expression have been seen during regeneration in *H. glaberrima*, it is expected they will be expressed in *P. californicus* during regeneration.

Mortalin expression is most probable during times of gut regrowth. Mortalin has been found in the normal tissue digestive tube using *in situ* hybridization in *H. glaberrima* (Mashanov *et al.* 2010). Mortalin expression in the cells of the digestive tract of *H. glaberrima* and *P. californicus* year-round could be explained by the rapid turnover such cells often experience. Aside from this, it is unlikely that *mortalin* expression will occur during intact viscera periods because *mortalin* expression in other systems is triggered by conditions such as glucose-deprivation and calorie restriction, both of which are unlikely with an intact digestive tract and non-torpor states (Kaul *et al.* 2007). For this same reason, moderate levels of *mortalin* transcripts and proteins may be seen during no-gut periods, as the apoptosis-inhibiting role of the protein could aid in survival of the cucumber tissues under stressful conditions.

Raised levels of mortalin expression are seen in tumor cells. Astrocytes in three grades of tumor severity showed progressively increasing numbers of mortalin-positive cells (Takano *et al.* 1997, Kaul *et al.* 2002). Colorectal cancers also consistently over-express mortalin, with high levels of expression being correlated with poor clinical outcomes, independent of other factors

usually indicative of prognosis (Dundas *et al.* 2005). The elevated level of mortalin is likely one of the contributing factors to the longevity of tumor cells as the protein inhibits apoptosis. This same inhibition of apoptosis is probably why *mortalin* is up-regulated and the protein over-expressed during regeneration of tissues.

Expression of survivin is most probable during winter visceral regeneration because it inhibits apoptosis and regulates cell division. For the same reason, however, expression of *survivin* is unlikely outside of the regrowth period as it inhibits apoptosis and is not known to have any beneficial roles outside of development and regeneration. *In situ* hybridization found *survivin* transcripts in the digestive tract of *H. glaberrima* (Mashanov *et al.* 2010). It is likely that *P. californicus* will show a similar expression of *survivin* in the digestive tract during the summer months. As in humans, expression of *survivin* in this area could be linked to the rapid turnover of cells seen in the digestive tract (Chiou *et al.* 2003).

Survivin mRNA and protein have been observed in human tumor cells. Survivin occurs at high levels in the cells of EML4-ALK-Positive (echinoderm microtubule-associated protein-like 4 anaplastic lymphoma kinase) lung cancers (Takezawa *et al.* 2011, Tanizaki *et al.* 2012). High levels of survivin are also seen in cells of colorectal tumors and are correlated with poor prognosis and shorter survival of patients (Kawasaki *et al.* 1998). High levels of the gene transcript and protein are probably advantageous to tumor cells because survivin regulates cell division and apoptosis.

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APPENDIX A

PCR primers used for amplification of cDNA for *mortalin* and *survivin* (Mashanov *et al.* 2010).

	Primer Sequence (5' → 3')
Survivin	
Surv-PCR-F	TTACCACTGCCCAACAGACA
Surv-PCR-R	TCCTCCCATGGATCATCACT
Mortalin	
Mort-PCR-F	GGCATCTTCAGCCCTCCAA
Mort-PCR-R	GCAGCCTGTCTGTTCAGTTCTTG

Proposed Budget

Product	Amount	Justification
Liquid N_2 fills	$180.00	Three tank fills at $60/refill
TRI Reagent	$53.20	Tissue homogenizer, 25 ml
1-bromo-3-chloropropane	$39.70	RNA and protein extraction, 200 ml
Isopropanol	$48.80	RNA and protein extraction, 500 ml
Guanidine hydrochloride	$32.20	RNA and protein extraction, 25 g
Anhydrous-alcohol	$25.33	RNA and protein extraction, 500 ml
GenElute PCR Clean-up Kit	$109.50	cDNA amplification, 70 purifications
Nuclease-free H_2O	$93.00	RNA and protein extraction, 2 x 1L
Isoelectric focusing strips	$651.00	2-DE analysis, 7 packages of 12 strips at $93/pkg
2-DE rehydration buffer	$41.00	2-DE analysis, 10 ml
Mortalin antibodies	$316.00	Western blots, 400 μl
Survivin antibodies	$350.00	Western blots, 100 μl
Primers for *mortalin* and *survivin*	$31.16	PCR analysis
SCUBA air refills	$108.00	Two divers with 3 dives each season to collect organisms at $6/refill
Transportation to Rosario Beach Marine Lab from College Place, WA	$206.40	Two round trips (480 miles) at $0.43/ mile
Total Expenses:	**$2285.29**	