Rapid Wound Repair in the Ctenophore *Mnemiopsis spp.*

by

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Abstract

The ability to quickly and efficiently close a wound is an integral part of any organism's ability to survive. Marine organisms are exposed to numerous environmental pressures that demand the need for a rapid repair response. The delicate marine comb jelly *Mnemiopsis leidyi* faces damage from predation, as well as objects found in its natural surroundings, such as coarse sea grass and sediment. This damage presents the need to quickly and efficiently repair the wound to prevent further injury or infestation by degradative microbes that enter exposed mesoglea and gastrovascular canals. *Mnemiopsis* has evolved a rapid, scar-free wound repair mechanism that quickly seals the mesoglea away from the external environment. Here I describe the morphological changes that occur during repair, the development of a ctenophore wound cell culture system, and provide an initial look at gene expression during the repair response.

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Chapter 1: Wound Repair: A Review

Importance of wound repair:

Wound repair is integral to the survival of all organisms. Without the ability to repair damage, organisms would succumb to the pressures associated with normal everyday existence. Repair mechanisms vary depending on the needs of the organism, but maintain the common goal of restoring damaged structures to their previous undamaged state. The manner and effectiveness in which repair is carried out depends on the complexity of the system in question, and the resources it has available to undergo repair. Wound repair can occur in something as simple as a single cell, and is evident at the tissue and organ level of organization.

Model systems and the conservation of wound repair:

Wound repair has been studied in a number of vertebrate models including mice (Cheon et al., 2006; Safer et al., 2005), rabbits (Kitazawa et al., 1990), pigs (Wang et al., 2009), frogs (Bement et al., 1999), newts (Donaldson et al., 1985) and of course humans (Singer and Clark, 1999). Wound repair studies have also been conducted in a number of invertebrates including planarians (Sanchez Alvarado, 2004), leeches (Tettamanti et al., 2005), cnidarians, including jelly fish (Lin et al., 2000) and *Hydra* (Gierer et al., 1972) , squid (Detrait et al., 2000), and insects (Galko and Krasnow, 2004; Ramet et al., 2002).

Many similarities exist between repair processes. For example, during mammalian cutaneous repair[1] a clot forms at the wound site to stem blood loss as well as provide a matrix for fibroblasts and other infiltrating wound cells to repair the damaged tissue (Clark, 1996). Clot[2] or plug formation is also a key step in epithelial wound repair[3] in *Drosophila melanogaster* larvae. Absence of a clot in *Drosophila* results in improper re-epithelialization, and the wound never properly heals (Galko and Krasnow, 2004). The same holds true for mammalian cutaneous repair, as a lack of clotting results in continuous extravasation[4] into the damaged tissue. Cells that normally migrate into the wound site and use the clot as a scaffold can no longer do so if the clot is removed, thereby decreasing cell differentiation and signaling required for proper healing. This demonstrates the necessity of clot formation in both responses.

Cells that migrate into the wound site typically close the wound. Keratinocytes[5] and similar epithelial cell types migrate into the wound to reestablish epithelial integrity. The Jun-N-terminal kinase pathway and Wnt/ß-catenin pathways are critical to maintaining proper cell migration, proliferation, orientation and polarity at the wound site in both insect and mouse models. Disruption of the Wnt/ß-catenin pathway results in excessive proliferation of fibroblasts in mouse cutaneous wounds (Cheon et al., 2002). Malfunction of the Wnt/ß-catenin pathway in insect epithelial wounds causes a loss of cell polarity (Schlessinger et al., 2007), demonstrating the importance of this pathway not only in mediating proper cell proliferation but also proper cell polarity and therefore appropriate migration.

Inactivation of the Jun-N-terminal kinase (Jnk) pathway in healing *Drosophila* larvae inhibits proper re-epithelialization, resulting in a disorganized array of epithelial cells (Galko and

[1] Repair takes places in the epidermis and underlying dermis
[2] Fibrin-fibrinogen matrix secreted by platelets to stop bleeding
[3] Repair occurs in epithelium and overlying cuticle
[4] Passage or leakage of fluid into tissues, usually blood
[5] Predominant epithelial cell type that secretes keratin

Krasnow, 2004). Mice mutants lacking c-Jun, a downstream target of the Jnk pathway, also displayed improper epidermal healing. In K14:cre:c-*jun*$^{+1/+1}$ mutant mice, keratinocytes could not migrate or flatten out properly, resulting in an inability to migrate underneath the fibrin clot (Li et al., 2003). The loss of proper keratinocyte function resulted in delayed and improper epithelial wound repair. These studies indicate a clear role for the Jnk pathway in maintaining proper re-epithelialization in both the mouse and insect models.

The ability to regenerate also finds common ground across systems. Planarians, when bisected, can regenerate to become whole again thanks to a source of totipotent stem cells distributed throughout the mesenchyme called neoblasts (Agata and Watanabe, 1999). Neoblasts are thought to differentiate to form needed structures in the regenerating planarian. Similarly, the human liver can regenerate up to ~60% of its total mass due to a constant population of adult hepatocytes which rapidly undergo mitotic divisions to replace lost tissue (Fausto, 2000; Michalopoulos and DeFrances, 1997). Another remarkable capacity to regenerate belongs to the salamanders, which can regenerate intact functional limbs when amputated. This process, first described by Ross Harrison and Hans Spemann in 1918 (Harrison, 1918) requires formation of a blastema[6] through dedifferentiation, and proper innervation for regeneration to occur (Kumar et al., 2007). These processes will be reviewed in greater detail below

Repair at the cellular level:

Introduction

Tissues in the human body such as epithelium, skeletal muscle, cardiac muscle, nervous tissue and connective tissue are consistently exposed to stresses associated with everyday existence. Heat/cold shock, oxidative stress, and mechanical stress are a few examples of the pressures facing cells and tissues that constitute the body. For example muscle tissue, such as

[6] Cell mass that grows and differentiates into new tissues, organs or body parts

skeletal and cardiac muscle, is regularly exposed to mechanical stress, which results in the disruption of the plasma membrane of cells residing in those tissues (McNeil and Steinhardt, 2003). The disrupted plasma membrane must be repaired quickly to prevent cytoplasmic leakage and possible death of the cell. This is particularly important in the case of irreplaceable cell types with limited regeneration capabilities such as cardiac myocytes and neurons of the central nervous system (CNS), as well as in developing embryos (McNeil and Kirchhausen, 2005). Here I will review some of the ways in which damaged cells heal themselves.

Self-sealing and the "Patch hypothesis"

There are two proposed mechanisms studied in two very different systems for how plasma membranes repair themselves: 1) self-sealing in erythrocytes and liposomes and 2) patch formation in echinoderm eggs. Self sealing occurs when the plasma membrane of a red blood cell is disrupted, and membrane phospholipids surrounding the disruption site quickly reorient to close the wound (McNeil and Kirchhausen, 2005). To reorient, the hydrophilic head groups orient outward creating a membrane tension that acts to pull the break edges together. This type of repair is reserved for smaller wounds (<.2 μm) and requires low levels of extracellular calcium (<1 mM). In light of the observation that human blood calcium levels are typically .94-1.33 mM/L (Moore, 1970), this has lent doubt as to whether or not this type of repair occurs in normal red blood cells, due to the inability of cells to repair at 1.0 mM Ca^{2+} (McNeil and Steinhardt, 2003). However, studies observing this phenomenon in liposome phospholipid membranes have indicated a role of Ca^{2+} rich vesicles in facilitating self-sealing. In this model exocytotic vesicles release Ca^{2+}, reducing plasma membrane tension. This reduction in

membrane tension allows line tension[7], which naturally promotes self sealing, to properly reorient the lipid membrane (McNeil and Kirchhausen, 2005).

The other proposed model of plasma membrane repair also relies on Ca^{2+}, and involves the formation of a "patch" in wounded sea urchin eggs (Terasaki et al., 1997). In this mode of repair extracellular Ca^{2+} enters through the wound and triggers membrane bound vesicle aggregation and the formation of a patch at the disruption site (McNeil et al., 2000). The patch is a result of homotypic vesicle[8] fusion, linking all of the aggregated vesicle membranes together. The fused vesicles form a temporary lipid bilayer, plugging the open wound and preventing further cytoplasmic leakage, acting as a patch (McNeil and Steinhardt, 2003). This response occurs in the case of larger disruptions in which membrane phospholipid reorientation would be insufficient to seal the wound.

This same process can be seen in invertebrate giant axons. In the squid giant axon and crayfish giant axon, vesicles form after extracellular Ca^{2+} enters the disruption site and triggers vesicle formation (Fishman et al., 1995). Breakage of the plasma membrane results in the loss of transmembrane potential. Vesicle formation in the axon is once again dependent on the entrance of Ca^{2+} into the cytoplasm, and quickly restores membrane resting potential (Detrait et al., 2000). Plasma membrane repair quickly restores cell integrity and proper membrane potential, and is integral in preventing the loss of damaged cells.

Actomyosin ring

Another mode of healing in single cells involves the formation of a contractile ring in the cortical membrane of healing *Xenopus* oocytes. The work of Bement et al. (1999) showed that ring formation involves the rapid recruitment of filamentous actin (F-actin) and myosin II to the

[7] Force created by an increase in free energy where membrane lipids are packed together
[8] Vesicles having the same fundamental structure (i.e. homotypes)

wound site, and that the disruption of that ring inhibited proper repair. The actin and myosin come together at the wound edge to form a contractile actomyosin ring around the wound site. Recruitment occurs within 30 seconds of wounding, resulting in rapid closure of the injured oocyte cortex. Healing occurs quickly and can close a 100 μm diameter wound in the injured actin-rich oocyte cortex in just 15 minutes. Disruption of the actomyosin ring using Cytochalasin D[9], Latrunculin B[10], and Butanedione[11] all resulted in the inhibition of wound repair. The actomyosin ring mechanism is thought to work in conjunction with the vesicle fusion model of membrane repair mentioned above, with the vesicles sealing the cell while the purse-string mechanism reestablishes the cortical cytoplasmic organization.

These mechanisms demonstrate that wound repair occurs at the level of individual cells. Rapidly restoring the integrity of the plasma membrane helps prevent the loss of the cell, which is of particular importance in the case of irreplaceable cell types such as cardiac myocytes, CNS neurons and developing embryos.

Repair at the tissue level:

Epidermis/Dermis (Cutaneous) and Epithelial wound repair

In all phyla cutaneous wound healing follows a similar series of events: 1) clotting or plugging of the wound, 2) cell migration, 3) wound closure, 4) re-epithelialization, and 5) remodeling. This fundamental series of events occurs in wound repair in most vertebrate and invertebrate systems examined to date, including (but not restricted to) rat corneal healing (Lu et al., 2001), porcine skin repair (Lynch et al., 1989; Wang et al., 2000; Wang et al., 2001a), murine epidermal repair (Cheon et al., 2006; Cheon et al., 2002); and epithelial wound repair in *Drosophila melanogaster* (Galko and Krasnow, 2004; Ramet et al., 2002).

[9] Disrupts actin filaments
[10] Sequesters G-actin and prevents F-actin assembly
[11] Myosin ATPase inhibitor

A major outcome of cutaneous repair in most organisms involves the formation of a scar. During mammalian cutaneous repair, fibroblasts lay down type I collagen, and left over collagen forms the scar. The extent of scarring depends on the size and severity of the wound (Singer and Clark, 1999). There are a few notable exceptions to this rule including oral mucosal healing (Szpaderska et al., 2003) and mammalian fetal healing (Adzick and Longaker, 1992; Lorenz and Adzick, 1993; Lorenz et al., 1992). Scar-free healing will be discussed later.

Drosophila epithelial wound repair

Epithelial wound healing has been studied at three major stages of development in the fruit fly, *Drosophila melanogaster*: embryos (Wood et al., 2002), larvae (Galko and Krasnow, 2004), and adults (Ramet et al., 2002). This review will mainly focus on adult and larval repair, which heal in a very similar fashion, and contain a number of processes that can be seen in wound repair in other systems.

The first step after an epithelial wound is inflicted in *Drosophila* is extravasation[12], which initiates the clotting cascade. The second step involves coagulation which involves the release of clotting factors by hemocytes[13] present at the wound, one of which is hemolectin, a clotting protein (Scherfer et al., 2004). Hemolectin bears a von Willebrand factor domain[14] and two discoidin domains[15], both of which are key clotting factors in other systems, and loss of hemolectin results in loss of coagulation (Dushay, 2009). Two enzymes, transglutaminase and prophenoloxidase, also play key roles in clotting and the innate immune response respectively (Bidla et al., 2005; Theopold et al., 2004). Transglutaminase is an important cross linker that links soluble clotting proteins from the plasma together in the clot (Wang et al., 2001b).

[12] Leaking of blood into surrounding tissues following blood vessel injury
[13] Invertebrate immune cells found in the hemolymph
[14] Coagulation factor domain important in ensuring adhesion of platelets to the wound site
[15] Coagulation factor domain that binds phospholipids on the surface of endothelial cells and platelets

Prophenoloxidase is responsible for the hardening of the cuticle during insect development, and has been implicated in the formation of the melanized clot that forms in the ruptured cuticle after wounding (Theopold et al., 2002).

The cuticle is the insect exoskeleton and as such, it provides structural support as well as the first line of defense in insect immunity. The cuticle consists of three layers: 1) the outermost envelope; 2) the epicuticle, and the 3) underlying procuticle. The cuticle is secreted in between masses of protruding microvilli called plaques, which act as an anchor to the epithelium underneath (Moussian et al., 2005). When the cuticle and epidermis are breached, epidermal cells surrounding the wound site synthesize and release a variety of antimicrobial peptides including cecropins[16] and attacins[17] (Boman and Hultmark, 1987). Insect hemocytes act much like mammalian macrophages[18] and natural killer cells[19]. This creates a barrier of cells between invader and host (Nappi and Ottaviani, 2000). The encapsulated cellular material then becomes melanized, forming a protective matrix around the invading microbes (Nappi and Vass, 1993).

Melanin is a common pigment compound found across the entire animal kingdom. In insects it plays a key role in host defense, wound healing and cuticle hardening. The formation of the melanin matrix results in the production of various reactive oxygen species, such as quinones, that are produced by the activity of phenoloxidase during melanization (Sugumaran, 2002). Reactive oxygen species are cytotoxic to both the invading pathogen and the insect host. Therefore, the melanin matrix is important in both trapping and killing invading microbes, as well as preventing the spread of toxic elements throughout the open hemolymph (Carton and Nappi, 1997).

[16] Antimicrobial peptide that lyses bacterial cell membranes
[17] Antimicrobial peptide that binds LPS and inhibits outer membrane protein synthesis
[18] Leukocyte involved in phagocytosis of cell debris and bacteria during inflammation
[19] Inflammatory leukocytes that degrade phagocytosed material through cytolytic activity

Once clotting has occurred, epithelial integrity must be reestablished. Re-epithelialization replaces the damaged epithelium in both mammalian and insect wound repair. A key difference between embryonic wound healing in *Drosophila* and vertebrate systems, is the involvement of an actin purse-string during re-epithelialization. This mechanism consists of an intracellular actin cable that pulls the wound shut in a purse-string like manner (Jacinto et al., 2001), bringing the epithelial edges together similar to that seen during dorsal closure in *Drosophila* morphogenesis (Grose and Martin, 1999). This same phenomenon is also observed during cell division in a number of different systems including HeLa cells (Schroeder, 1970), sea urchin eggs (Schroeder, 1972), and starfish oocytes (Schroeder and Stricker, 1983). During embryonic healing in *Drosophila*, the intracellular actin purse string works in conjunction with actin supported filipodia which protrude from epithelial cells at the wound edge, and attach to and tug upon the underlying substratum to pull the wound shut (Wood et al., 2002).

This is very different from what is observed during larval and adult epithelial wound healing in *Drosophila*, which rely on the action of lamellipodia extended by the migrating epithelial sheets, to seal the wound (Galko and Krasnow, 2004; Ramet et al., 2002). This process is still actin dependent, but the action used to close the wound is very different. Approximately 4 hours after wounding, the disrupted epithelium has shrunk back from the edges of the damaged cuticle, and is made up of enlarged epithelial cells (Fig.1).

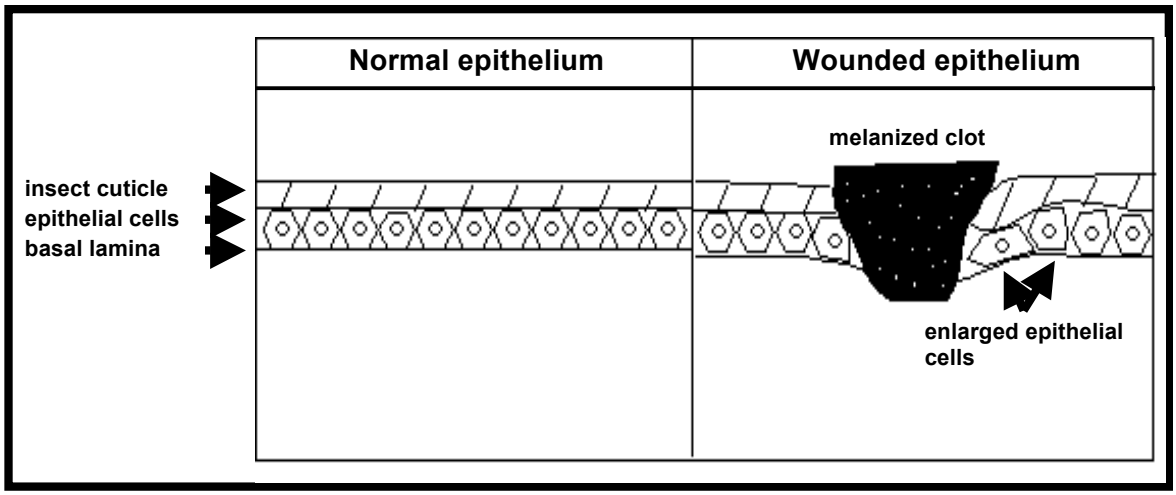

Figure 1: Drawing showing clot formation in *Drosophila*

The larger epithelial cells extend cytoplasmic processes that resemble lamellipodia of migrating mammalian keratinocytes (O'Toole, 2001). By 12 hours post wounding, epithelial cells that make up the two disrupted wound margins begin to migrate underneath the melanin clot and fuse together. By 18-24 hours the epithelium has fully healed, and the enlarged epithelial cells return to their normal size and morphology. Once the epithelium is in place, new cuticle, consisting mainly of chitin, is secreted on top of the new epithelium. The majority of the plug containing the wound debris remains outside of the newly formed layer, and any remaining debris underneath the reestablished epithelium is degraded (Galko and Krasnow, 2004).

Corneal epithelial wound repair

Corneal repair has three distinct, overlapping phases. In the first phase, keratocytes undergo apoptosis (Wilson et al., 2003), hemidesmosomes are lost and epithelial cells flatten out and migrate together as a sheet to cover the exposed wound (Suzuki et al., 2003). There is no mitotic activity in this phase. In the second phase, cells distal to the wound proliferate and differentiate to form the new stratified corneal layers. Finally, in the third phase,

hemidesmosomes re-form and the extracellular matrix is synthesized to create an intact cornea (Agrawal and Tsai, 2003).

These phases are mediated by two growth factors: epidermal growth factor (EGF) and keratinocyte growth factor (KGF) (Imanishi et al., 2000). EGF stimulates cell growth and proliferation of epidermal cells when exogenously applied *in vivo*, increasing the rate of healing in rabbit corneal epithelium, almost doubling the rate of closure from 0.78 ± 0.11 mm^2/hr in control wounds to 1.27 ± 0.17 mm^2/hr in EGF treated wounds (Ho et al., 1974). EGF also promotes growth and proliferation of corneal epithelial cells, keratocytes, and endothelial cells in culture, each of which is involved in corneal healing (Hongo et al., 1992). KGF stimulates keratinocyte differentiation, growth and proliferation. It increases corneal healing rates in rabbits, closing the wound at a rate of 1.85 ± 0.12 mm^2/hr in the presence of KGF as opposed to 1.57 ± 0.16 mm^2/hr in the control (Sotozono et al., 1995). KGF also stimulates keratinocyte proliferation and differentiation in human tissue cultures (Marchese et al., 1990).

Mammalian cutaneous wound repair:

Introduction

Mammalian cutaneous wound repair is a complex process that can be divided into three general, overlapping phases: 1) Inflammation, 2) Re-epithelialization/Granulation, and 3) Remodeling.

Inflammation

A cutaneous wound results in damage to the epidermis and oftentimes, depending on the depth of the wound, the underlying dermis of the skin. Damage to the integument[20] results in the disruption of blood vessels found in those tissues, resulting in extravasation into the tissues

[20] Consists of the epidermis, dermis and hypodermis (innermost skin layer) and their associated structures

surrounding the wound site (Singer and Clark, 1999). Extravasation results in the invasion of platelets into the wound site, which then aggregate by binding to exposed collagen fibrils and release various growth factors that attract inflammatory leukocytes (polymorphonuclear leukocytes, monocytes and macrophages) (Clark, 1996).

Polymorphonuclear leukocytes (PMNs) are components of the innate immune system that include the granulocytic neutrophils (which are the most common), basophils and eosinophils. They are so-called because they bear lobed nuclei. They are usually the first to be recruited to the wound site and are responsible for large-scale removal of debris and bacteria. PMNs make up ~50% of the cells present at the wound site in day 1 after injury (Engelhardt et al., 1998), and are the first step in cleaning the wound site. PMNs release chemotactic agents, oxygen free radicals and proteolytic enzymes which are key to the breakdown of debris and bacteria (Baskurt and Meiselman, 1998). Platelets are also responsible for the release of fibrin, which forms the foundation of the clot. The clot plugs any severed vessels to stop further extravasation, and secretes chemoattractant growth factors, while serving as a provisional matrix for cells migrating into the wound site (Singer and Clark, 1999).

Monocytes[21] and macrophages[22] are also recruited to the wound site at the same time as PMNs, and release numerous cytokines[23] [i.e. PDGF[24] and TGF-ß[25], which are involved in regulating the initial inflammatory response (Riches, 1996)]. Monocytes that migrate into the wound site undergo metamorphosis to become inflammatory macrophages, and by day 2 after injury, represent the majority of the wound response cells (Engelhardt et al., 1998). The increase in macrophage numbers is associated with a decline in PMNs, which are thought be

[21] Inflammatory leukocyte that differentiates into a macrophage
[22] Inflammatory leukocyte whose most basic function is phagocytosis of cell debris and bacteria
[23] Small signaling proteins that elicit many different types of effects on cells
[24] Platelet derived growth factor: stimulates cell growth and proliferation particularly during angiogenesis
[25] Transforming growth factor beta: stimulates cell growth, differentiation and proliferation

phagocytosed by macrophages. As mentioned earlier, the reduction in PMN numbers may have evolved to prevent excessive damage to the wounded tissues (Singer and Clark, 1999).

Macrophages are important components of the inflammation response, and play a key role in initiating the transition from inflammation to repair. At the end of the inflammatory period, inflammatory chemokines and cytokines are gathered and degraded. Fibroblasts are recruited into the wound site from surrounding tissues where they are activated and begin to proliferate, starting the second phase of the wound repair process (Clark, 1996).

Re-epithelialization

Re-epithelialization is an important early step in sealing the wound and protecting its delicate structure from the external environment. It involves the migration, proliferation and differentiation of epithelial keratinocytes to cover the exposed wound site (Raja et al., 2007). Keratinocytes undergo a number of changes in order to migrate into the wound site including: 1) hemidesmosome disassembly, 2) retraction of intracellular tonofilaments and keratin filaments, 3) disbanding of desmosomes, and 4) formation of actin lamellipodia and focal contacts (Santoro and Gaudino, 2005). These steps are mediated by growth factors, matrix metalloproteinases, and integrins.

Both α and ß type integrins expressed on the keratinocyte surface allow the cell to interact with ECM components such as collagen I and fibronectin at the edge of the wound (Singer and Clark, 1999). Matrix metalloproteinases (MMPs) play a key role in degrading and modifying ECM components to facilitate the migration of keratinocytes underneath the fibrin clot (Sternlicht and Werb, 2001). Transforming growth factor- ß (TGF-ß) and macrophage stimulating protein (MSP) are two growth factors important in the regulation of re-epithelialization. Both MSP and TGF- ß stimulate integrin expression, while TGF- ß modulates

a variety of wound responses including: 1) fibroblast proliferation, 2) ECM deposition and 3) myofibroblast differentiation (Santoro and Gaudino, 2005).

Once the keratinocytes have migrated underneath the clot to cover the wound, they proliferate and thereby restore the epithelium to its proper thickness. Proliferation, as with migration, relies on the interaction between integrins, MMPs and growth factors. Epidermal growth factor (EGF), transforming growth factor-α (TGF-α) and keratinocyte growth factor (KGF) all stimulate keratinocyte proliferation (Marikovsky et al., 1993). The extracellular matrix (ECM) has been shown to modulate keratinocyte proliferation, and MMP cleavage of the ECM is thought to release stored growth factors which also mediate proliferation as indicated above (Santoro and Gaudino, 2005). All of these processes are integral to proper re-epithelialization, and could not occur without the interaction of MMPs, integrins and the appropriate growth factors.

Granulation

While re-epithelialization is occurring, the underlying connective tissue is busy reconstructing itself. The newly forming tissue, termed granulation tissue, begins forming 4 days after injury and consists of new blood vessels, loose connective tissue, macrophages and fibroblasts giving it a "granular" appearance (Clark, 1996). Fibroblasts are key players in the formation of new structures within the damaged tissue. Normally, fibroblasts are sparsely scattered throughout the connective tissue, but are recruited into the wound site by the platelet-derived growth factor (PDGF) and TGF-ß working in conjunction with ECM molecules (Singer and Clark, 1999). The fibrin-fibrinogen clot serves as, and is referred to as, a "provisional matrix" for the fibroblasts migrating into the wound, providing structure and a starting point for the rebuilding of lost structure (Greiling and Clark, 1997). After they have migrated into the

wound, fibroblasts begin synthesizing new ECM components such as proteoglycans, elastins and collagen (Singer and Clark, 1999).

A key process in supporting the newly formed granulation tissue is neovascularization, which involves angiogenesis or the formation of new blood vessels. This is necessary for reestablishing blood flow to the damaged tissues. Angiogenesis involves the migration and proliferation of endothelial cells to form new vessels that bud off from the residual vessels (Madri et al., 1996). Several angiogenic factors become up-regulated within the granulation tissue, such as vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), angiogenin, angiopoietin, and thrombospondin, stimulate angiogenesis and so the establishment of blood vessels (Singer and Clark, 1999). The reestablishment of proper blood flow is integral to establishing the normal organization of cutaneous tissue.

Remodeling

This stage of wound repair involves the conversion of the provisional matrix into a collagen-rich scar. The initial fibrin clot is broken down via proteolysis, mainly by plasmin[26], and this is followed by the deposition of a second provisional matrix that is rich in hyaluronic acid and fibronectin (Clark, 1996). The second matrix promotes cell proliferation and migration to help finalize wound repair. As the granulation tissue begins to mature, the secondary matrix is again degraded and a more proteoglycan/collagen rich matrix is formed (Singer and Clark, 1999). The third matrix provides much greater tensile strength, and prepares the wound site for contraction.

Once proper re-epithelialization and maturation of the granulation tissue has occurred, the fibroblasts convert into myofibroblasts characterized by large bundles of actin rich microfilaments (Desmouliere and Gabbiani, 1996). The myofibroblasts act as a single unit,

[26] Proteolytic enzyme that breaks down plasma proteins, namely fibrin

where they contract and cause changes in the surrounding matrix. Contraction stimulated by TGF-ß and PDGF, transmits tension along the myofibroblasts via integrin interactions to collagen fibers interconnected throughout the extracellular matrix (Singer and Clark, 1999). This results in the subsequent contraction of the wound edge boundaries to begin the final stages of wound repair.

During remodeling, collagen is degraded and redeposited. The degradation of ECM collagen is controlled by matrix metalloproteinases (MMPs) which degrade specific types of collagen based upon their isotype (Mignatti et al., 1996). Collagen remodeling, in conjunction with apoptosis of cells that are no longer needed, results in the formation of an acellular scar at adult wound sites (Singer and Clark, 1999). All adult mammalian wounds form a scar; however, fetal wounds exhibit no scar formation up to 24 weeks into gestation (Adzick and Longaker, 1992). This interesting feature has stimulated an increase in the study of fetal wound healing to identify factors that might provide insight into the minimization of scar formation in adult wounds (Bullard et al., 2003; Colwell et al., 2003; Yannas, 2005).

Mammalian Fetal Wound Repair

While scars are seen in adult mammalian cutaneous healing, no visible scar is formed during fetal wound healing. This has been described in human fetuses (Rowlatt, 1979), but also extends to other groups including sheep (Longaker et al., 1990), monkeys (Lorenz et al., 1993), and rodents (Ihara et al., 1990). Scarless fetal healing in humans is dependent on the gestational age, the critical age being less than 24 weeks post conception (Adzick and Longaker, 1992), as well as the wound not exceeding a certain minimum size despite the length of gestation (Cass et al 1997).

Numerous differences between the stages of adult and fetal wound healing have been reported to explain scar formation. During inflammation in adults, polymorphonuclear leukocytes (PMNs) are recruited to the wound where they infiltrate the wound tissue and begin removing debris and bacteria from the wound site (Singer and Clark, 1999)(see above). In fetal inflammation, PMNs do not infiltrate wounded tissues, and macrophages occur in much lower numbers than in their adult counterparts (Adzick et al. 1985). Fetal inflammatory cells respond to chemoattractants and are recruited to the wound site in the presence of irradiated, immobilized bacteria and other nonliving irritants, but lack the ability to phagocytose bacteria like their adult counterparts (Bullard et al., 2003). The lack of mature macrophages and PMNs means less damage through debris removal by PMNs and reduced signaling due to fewer macrophages, which secrete growth factors. These two differences probably play important roles in preventing scar formation at the wound site.

Another key difference between fetal and adult wounds is the composition of the extracellular matrix. Four major differences between fetal and adult ECM are cell adhesion molecules, hyaluronic acid content, collagen composition, and ECM modulators (Colwell et al., 2003). Early in the adult healing response, cell-ECM interactions aid with proper cell invasion into the wound site (Singer and Clark, 1999). Cell adhesion molecules such as fibronectin and the integrins appear earlier in fetal healing, which could facilitate rapid infiltration into wounded tissue (Bullard et al., 2003).

ECM contains a very large amount of glycosaminoglycan (GAG), one of the most common of which is Hyaluronic acid. Hyaluronic acid (HA) is a non-sulfated GAG found abundantly throughout the ECM of most parts of the body, most notably the skin, joints, and the eye (Chen and Abatangelo, 1999). HA is negatively charged, which traps water molecules,

facilitating cell movement through the ECM (Clark, 1996). Fetal fibroblasts continually produce HA, which probably enhances fibroblast migration. HA synthesis decreases as adult fibroblast density increases in the wound tissue (Chen et al. 1988). HA content is also synthesized quickly in fetal tissues, and is found in larger amounts, and lasts longer than in adult wounds (Mast et al., 1991).

Collagen deposition also differs in fetal and adult wounds, with fetal fibroblasts depositing more type III collagen, allowing for the formation of a finer, more reticular network of collagen. This finer network is thought to increase matrix flexibility, and allow for better cell migration compared to the rigid, highly cross-linked collagen matrix seen in adult scar formation (Bullard et al., 2003).

Finally, ECM modulators of repair differ in their function compared to adult wound repair. Growth factor activity differs in adult and fetal wounds. For example, transforming growth factor beta (TGF-ß), a key cytokine that mediates wound healing and which is responsible for collagen and protein deposition in adult wounds, has been shown to play a different role in fetal healing. Firstly, it is present in much lower levels in fetal wounds (Whitby et al., 1991); when added to wounded fetal tissues it induced scar formation (Krummel et al. 1988). It was later discovered that TGF-ß attracts fibroblasts and other inflammatory cell types, as well as stimulates fibroblast production of collagen (Roberts and Sporn, 1996).

Fibroblast growth factor (FGF), a well defined modulator of fibroblast proliferation and activity, is expressed throughout scar formation in adult wounds but disappears by 24 hours in fetal healing (Whitby and Ferguson, 1991). Platelet derived growth factor, another key cytokine to adult repair, induces fibroblast recruitment and collagen deposition in rabbit fetal wounds

(Haynes et al., 1994). The alternative regulatory functions of these cytokines provide at least part of the reason for the profound differences seen in fetal and adult wound repair.

Healing in oral mucosa

Healing of the oral mucosa[27] involves haemostasis, cell proliferation, inflammation, and deposition of a collagen matrix just as we see in cutaneous healing (Walsh et al., 1996). However, oral mucosal wounds heal much more rapidly and do not form a scar. This is very similar to fetal skin healing (Adzick and Longaker, 1992). One possible reason for the rapidity of the oral mucosal response is accredited to the abundance of salivary growth factors, EGF and FGF in particular (Zelles et al., 1995). FGF was found to increase the rate of re-epithelialization in oral mucosal wounds (Kagami et al., 2000), while EGF tends to inhibit terminal differentiation and increases hyaluronic acid synthesis in oral mucosal epithelial cells (Yamada et al., 2004). Like fetal repair, the inflammation stage of oral mucosal repair is reduced, showing fewer neutrophils and macrophages.

Also, IL-6 and IL-8 production are decreased in wounded oral mucosa compared to healing adult skin (Szpaderska et al., 2003). Oral fibroblasts, when placed on an extracellular wound matrix, display increased invasive capabilities much like fetal fibroblasts (al-Khateeb et al., 1997). They also prove resistant to differentiation into myofibroblasts via stimulation by TGF-ß, displaying a scar resistant phenotype (Enoch, 2008). The invasive capabilities of fibroblasts and the reduction of inflammatory cell types and cytokines at the wound site appear to be integral to maintaining scar-free repair in both oral mucosa and fetal skin.

[27] Mucous lined stratified squamous epithelium of the inner mouth

Regeneration and wound repair

Introduction:

Humans are fascinated with the capacity to regenerate, since we are so poor at it ourselves. Even so, some mammalian structures do regenerate: Mammals regenerate various tissues including peripheral nerves, bone, skeletal muscle, liver tissue, and blood vessels (Carlson, 2005); however, they lack the ability to regenerate key structures such as organs (with the notable exception of the liver; see below), and limbs. Adult amphibians, however, do retain a limited capacity to regenerate tissues. Salamanders in particular are notable for their ability to regenerate; indeed the first vertebrate cell culture experiments by Ross Harrison involved salamander tissues, which heal and regenerate readily (Harrison, 1914). Salamanders are capable of regenerating numerous key body parts and organs including limbs, the tail, intestines, the retina, and even portions of the heart (Odelberg, 2005). The ability of a vertebrate animal to regenerate a lost limb is an area of particular interest to researchers trying to unlock the key to regeneration in mammals because regeneration represents a true cure of a disease state (Brockes and Kumar, 2005; Stocum, 2002).

Regeneration studies have also been conducted in a variety of invertebrates. These studies include annelids (Bely, 2005), planarians (Sanchez Alvarado, 2004), cnidarians (Bode, 2003), echinoderms (Carnevali, 2006), ctenophores (Coonfield, 1936; Coonfield, 1938; Henry and Martindale, 2000; Martindale, 1996), and insects (Bosch et al., 2008). Regenerative capabilities in invertebrates range from regenerating individual structures to regenerating entirely new animals. This section will begin with invertebrate regeneration, and move on to regeneration in the mammalian liver and newt limbs.

Invertebrate regeneration

Regeneration in many invertebrate systems, whether it is a lost structure or entire portion of the body, centers on the formation of a blastema. One such invertebrate is echinoderms, which display remarkable regenerative capabilities. Ophiuroid echinoderms (i.e. brittle stars) undergo autotomy, severing their arms as a defense mechanism (Wilkie, 2001). Immediately following autotomy, epidermal cells expand, migrate and spread over the wound and a blastema forms from accumulated coelomocytes[28] (Carnevali, 2006). The coelomocytes proliferate at the distal end of the growing arm, creating the new appendage. Holothuroid echinoderms (i.e. sea cucumbers), following spontaneous evisceration when threatened, form a blastema in the edge of the lamina of the digestive tube (Garcia-Arraras and Greenberg, 2001). The blastema acts as a site for cell recruitment and proliferation as the damaged digestive tract reforms. Asteroid echinoderm larvae can also form entirely new individuals when surgically bisected (Spemann, 1938)

Two other animals that have the ability to regenerate new animals when bisected are the cnidarian *Hydra* and the freshwater planarian *Schmidtea*. Regeneration in *Hydra* was first described by Abraham Trembley (Trembley, 1744), and has been the object of a number of studies (Bode, 2003; Galliot and Schmid, 2002; Gierer et al., 1972). Interestingly, entirely new *Hydra* can form from cell aggregates (Gierer et al., 1972). If *Hydra* are dissociated into suspensions of viable cells and centrifuged, the cells will reorganize into a spherical shell consisting of an endoderm and ectoderm within 24 hours. Next, the heads form and organize the remaining tissue into the body column and foot, acting much like a regeneration blastema, producing new intact *Hydra* (Bode, 2003; Bode and Bode, 1980). New animals can only

[28] Immune cells found in lower coelomates

regenerate from stem cells present in the body column called interstitial cells[29], which constitute the blastema, and can form normal intact animals from as little as 5% of the body column (Bode and Bode, 1980).

The planarian *Schmidtea* can regenerate an intact animal from as little as 1/279th of the original worm (Morgan, 1898). Immediately following injury, rhabdite cells[30] around the wound site secrete their contents, creating a protective mucous coat (Reisinger and Kelbetz, 1964). Within 30 minutes, dorsal and ventral epithelial cells spread over and cover the wound (Sanchez Alvarado et al., 2002). Meanwhile, a blastema forms at the site of injury. In planarians, the regeneration blastema is made up of neoblasts, undifferentiated cells found throughout the body that account for 25-30% of all planarian cells (Reddien and Sanchez Alvarado, 2004). Neoblasts are self-renewing, pluripotent cells that are capable of forming all of the cell types present in adult planarians including epidermal cells, rhabdite cells, muscle cells, and germ cells (Salo et al., 2008; Sanchez Alvarado, 2004). These cells are responsible for forming structures lost as a result of injury, and provide a readily available source of stem cells that can regenerate an intact animal.

Mammalian liver Regeneration

The liver is one of the few tissues that have regenerative properties in mammals. The most commonly studied experimental model of mammalian liver regeneration is the partially hepatectomized rat (Higgins and Anderson 1931). A partial hepatectomy involves the removal of two-thirds, or three of the five lobes, of the liver. The greatly reduced liver is then capable of regenerating the removed portion within 5-7 days (Higgins and Anderson, 1931). Interestingly, it is thought that the liver regenerates through the proliferation of adult hepatocytes present in the

[29] Rapidly proliferating multipotent stem cells found in the body column of *Hydra*
[30] Mucous secreting cells found in some turbellarians, sea stars and mussels

remaining lobes, as opposed to regenerating from the differentiation of liver progenitor, or stem cells (Fausto, 2004). In a normal liver, hepatocytes or oval cells, are typically quiescent and do not undergo cell division; however; during regeneration, these cells undergo 1-2 rounds of cell division to restore the normal number of hepatocytes present in the normal liver (Fausto, 2000). These cells are not stem cells in that they are terminally differentiated adult cells that proliferate to reconstruct the damaged liver.

While adult hepatocytes only undergo 1-2 divisions during normal liver regeneration, their proliferative potential is greater when transplanted into transgenic mice. Mature adult hepatocytes are transplanted into Alb-uPA transgenic mice, which suffer liver damage and subsequent liver failure due to chronic stimulation of cell growth. Once introduced, the transplanted hepatocytes have a high mitotic index, undergoing anywhere from 12-18 rounds of division to replace the defective liver cells (Rhim et al., 1994; Rhim et al., 1995). Furthermore, the liver has been shown to increase or decrease in size when transplanted from one individual to another to meet the new body requirements of the recipient (Francavilla et al., 1988; Francavilla et al., 1994). Clearly, the liver has tremendous regenerative capabilities, and regenerates through the proliferation of terminally differentiated adult hepatocytes that normally constitute the liver. This is a unique model of regeneration in that it does not rely on stem cells to grow lost tissues.

Newt Regeneration

The first step in limb regeneration in the adult newt *Notophthalmus viridescens*, is the formation of the wound epidermis. The work of Repesh and Oberpriller demonstrated the initiation of wound epidermis formation by observing the activity of migrating keratinocytes. Once a newt limb is amputated, the epidermal cells surrounding the amputation site migrate and cover the surface of the wound. These cells remove their desmosomal attachments, and extend

pseudopodia to migrate and recover the wound (Repesh and Obepriller, 1978, 1980). Keratinocyte migration is accomplished through the interaction of integrins on the surface of the keratinocytes and the fibrinogen and fibronectin found in the limb blood clot (Donaldson and Mahan, 1983; Donaldson et al., 1989). Closure of the wound is usually completed by 24 hours, and the newly formed epithelium thickens over the next few days to form the wound epidermis (Mescher, 1996). The wound epidermis can consist of up to 15 layers, compared to the normal 3-4 in normal newt epithelium, and is also called the apical epithelial cap (Campbell and Crews, 2008). The thickened wound epidermis is thought to be integral to the initiation of limb regeneration.

After the formation of the wound epidermis, cells in the underlying tissues, including fibroblasts, muscle cells and cartilage cells, dedifferentiate to form a mass of stem cells (Nye et al., 2003). The term dedifferentiated refers to cells that have reverted to a prior state to generate the new limb. This has been clearly demonstrated both *in vivo* and *in vitro* using skeletal muscle cells. Myotubes and myofibers at the amputation site revert from a differentiated multinucleate form to single nucleus progenitors (Brockes and Kumar, 2002). Therefore, newt regeneration occurs via the action of local progenitor cells as opposed to the pluripotent stem cells[31] that are typically associated with regenerating tissues.

The newly dedifferentiated progenitors accumulate at the distal end of the amputated limb to form a mass of cells termed the regeneration blastema. The regeneration blastema takes on a cone-shaped appearance, as the blastema cells begin to differentiate and form new structures (Whited and Tabin, 2009). The blastema regenerates structures in a proximodistal fashion, always giving rise to structures distal to the location of the blastema (Brockes, 1997). The presence of the blastema itself is not sufficient to completely regenerate severed salamander

[31] Stem cells capable of differentiating into cells present in any of the tissues in the body

limbs, since the removal of the nerves innervating the limb results in the absence of limb regeneration (Kumar et al., 2007). Thus, limb regeneration occurs as a result of a balanced interaction between the wound epidermis, the blastema, and innervation of the amputated limb.

Newt limb regeneration progresses in a manner reminiscent of limb bud development in newt embryos. At the molecular level there are three important developmental genes that are expressed during limb regeneration including *Hox, Msx* and *Shh* (Han et al., 2005). *Hox* and *Msx* are both homeobox genes, and *Shh* is a hedgehog gene. Homeobox genes were first discovered in 1983 in *Drosophila* by two separate labs, one is Switzerland (McGinnis et al., 1984) and one in Indiana (Scott and Weiner, 1984). Their first described function was in determining the anterior-posterior gradient in *Drosophila* eggs, but since that time they have been found to regulate a host of other morphogenetic processes in *Drosophila* such as limb formation (Vachon et al., 1992), and apoptosis during morphogenesis (Lohmann et al., 2002). Hedgehog genes, first discovered in 1978 by Eric Weischauss and Christiane Nusslein-Volhard, are responsible for segmentation in *Drosophila* embryos (Nusslein-Volhard and Wieschaus, 1980). Sonic Hedgehog, one of the three members of this group, has been shown to be involved in muscle (Currie and Ingham, 1996), brain (Herzog et al., 2003) and spinal cord (Lewis and Eisen, 2001) patterning in zebrafish embryos. Both *Hox* and *Shh* are regulators of morphogenesis, essential to proper limb formation in the embryo, and appear to play a role in mediating adult newt limb regeneration.

Some members of the regeneration response have been identified. The work of Kumar et al. (Kumar et al., 2007) has demonstrated a receptor-ligand interaction between nAG the ligand, and Prod1 the receptor that is required in mediating limb regeneration. Prod1, a protein anchored to the blastemal cell surface, is critical for the determination of the proximodistal

identity of cells in the regenerating tissues. This was proven by the conversion of distal cells into more proximal cell types when Prod 1 was introduced into a regenerating limb blastema (Echeverri and Tanaka, 2005). This led to the search for ligands that interact with Prod 1 while the blastema is regenerating the limb.

They found nAG, a protein that interacts with Prod1 to stimulate blastema cell proliferation *in vitro*, and through this interaction, can rescue dennervated limb blastemas. It does so by re-establishing nerve outgrowth into the blastema, reinitiating the proximodistal growth of the amputated limb. Another player, CD 59, a cell surface protein, has also been implicated in determining proper proximodistal identity as the blastema forms the new limb (Echeverri and Tanaka, 2005; Silva et al., 2002).

Introduction to the phylum Ctenophora:

Systematics and phylogeny

There are approximately 150 known species of ctenophores, also known as comb jellies (Mills, 2001). Systematists divide ctenophores into Class Nuda and Class Tentaculata; all members of Class Tentaculata bear tentacles at some point during their life, while genera Beroe and Neis, the only members of Class Nuda, bear none. In contrast to the few members of Nuda, Tentaculata is defined as containing eight Orders: Cydippida, Lobata, Platyctenida, Ganeshida, Cambojiida, Cryptolobiferida, Thalassocalycida, and Cestida (Mills and Haddock, 2007).

Ctenophores are found in both the open ocean as well as coastal marine environments. The best-described coastal ctenophores are all from Class Tentaculata: *Mnemiopsis, Bolinopsis* (Order Lobata), and *Pleurobrachia* (Order Cydippida). The most numerous open ocean ctenophores, the lobates *Leucothea verrugicornis* and *Ocyropsis maculate*, are also from Class Tentaculata (Mills and Haddock, 2007). The molecular phylogenetic relationship between the

major ctenophore orders was first demonstrated by Podar et al in 2001 using 18S rDNA. The 18S rDNA analysis revealed a different phylogeny of the ctenophore orders when compared to placement by morphological analysis.

Introduction to *Mnemiopsis leidyi:*

Ecological significance

The subject of this thesis is the lobate ctenophore *Mnemiopsis leidyi* (see Fig. 2). The native range of *Mnemiopsis* extends along the Atlantic coast of the Americas from as far south as Argentina to as far north as Cape Cod, Massachusetts, including the Caribbean and the Gulf of Mexico (Edmiston, 1979; Harbison et al., 1978). Its role as an invasive species has been well documented, and recent studies have clarified its migration from the New World to the Baltic, Black, Caspian, Mediterranean, and North Seas, probably through ballast water in large tanker vessels (Reusch et al., 2010; Shiganova and Malej, 2009).

Once introduced to their new habitat, *Mnemiopsis* affects local fish populations by out-competing them for food, and eating their eggs and larvae. *Mnemiopsis* has spread to numerous new environments, thrusting *Mnemiopsis* into the spotlight and awakening concern among marine scientists because of its extremely efficient and voracious feeding mechanisms, which allow it to very effectively penetrate novel food webs to divert the normal trophic energy flow. These characteristics have made understanding all aspects of the biology of this organism particularly ecologically relevant to invasion scientists.

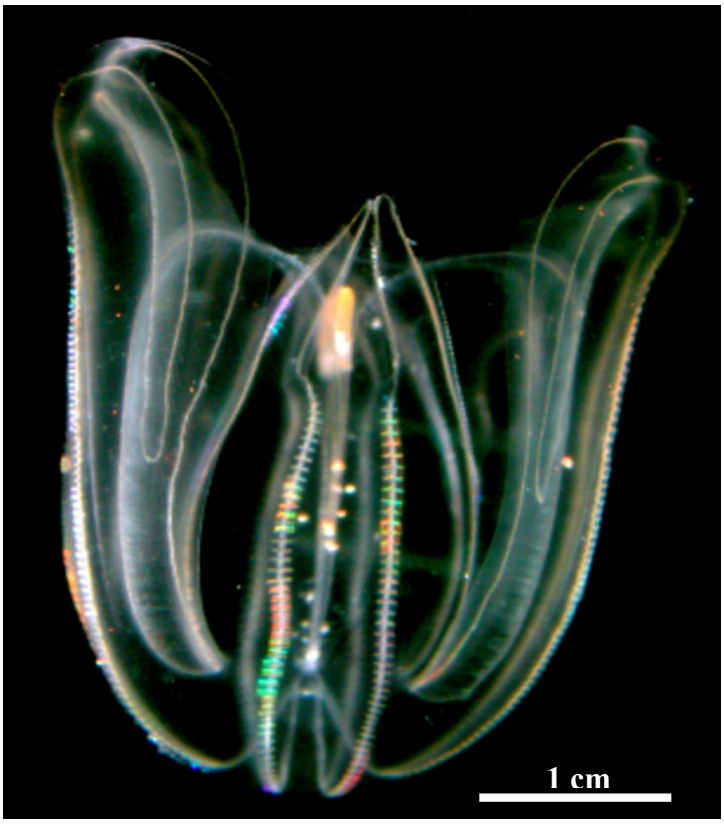

Figure 2: A live *Mnemiopsis leidyi.*

Body plan

The basic ctenophore body plan can be described by examining the structure of Order Cydippida. This is because all ctenophores of Class Tentaculata, including *Mnemiopsis*, develop from cydippid larvae (Tamm 1982). All ctenophores display an oral-aboral axis that runs from the aboral organ (the statocyst) to the oral pole (Fig. 3). The oral-aboral axis occurs at the intersection of the stomodeal plane and the tentacular plane, thereby creating what is often termed biradial symmetry (Fig. 4), but which is perhaps more accurately described as dually bilateral. Ctenophores are considered to be triploblastic (Martindale et al., 2002), consisting of an endoderm, ectoderm, and a gelatinous mesoderm called mesoglea that makes up the bulk of the animal (Fig .5).

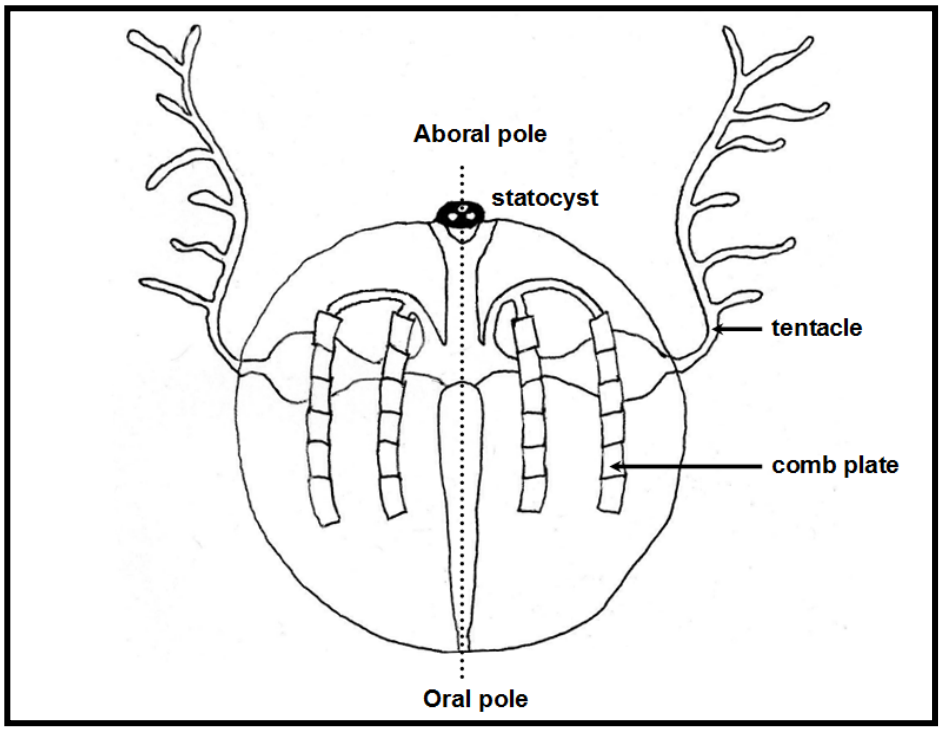

Figure 3: Oral-aboral axis (dotted line) in ctenophores adapted from (Martindale, 2005).

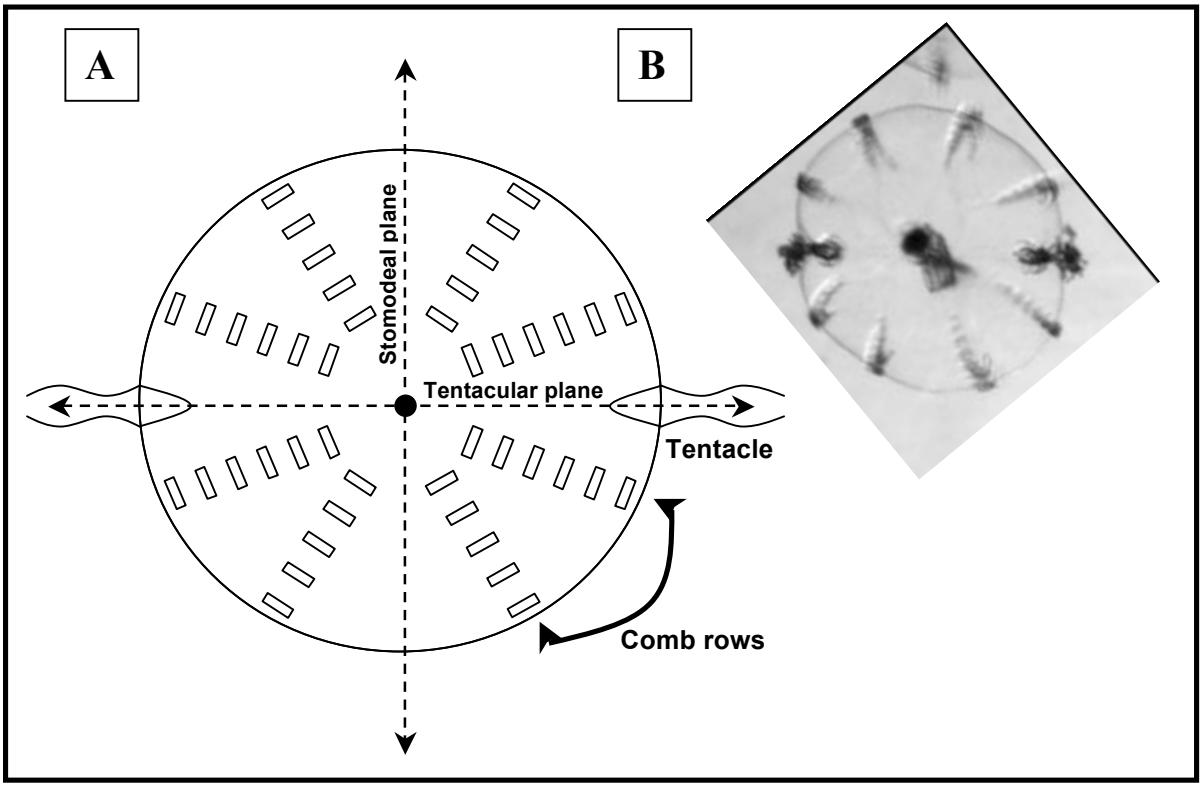

Figure 4: A) Larval body plan of *Mnemiopsis leidyi*. Black represents the statocyst (part of the aboral organ) viewed from above. B) Brightfield image of larval *Mnemiopsis*.

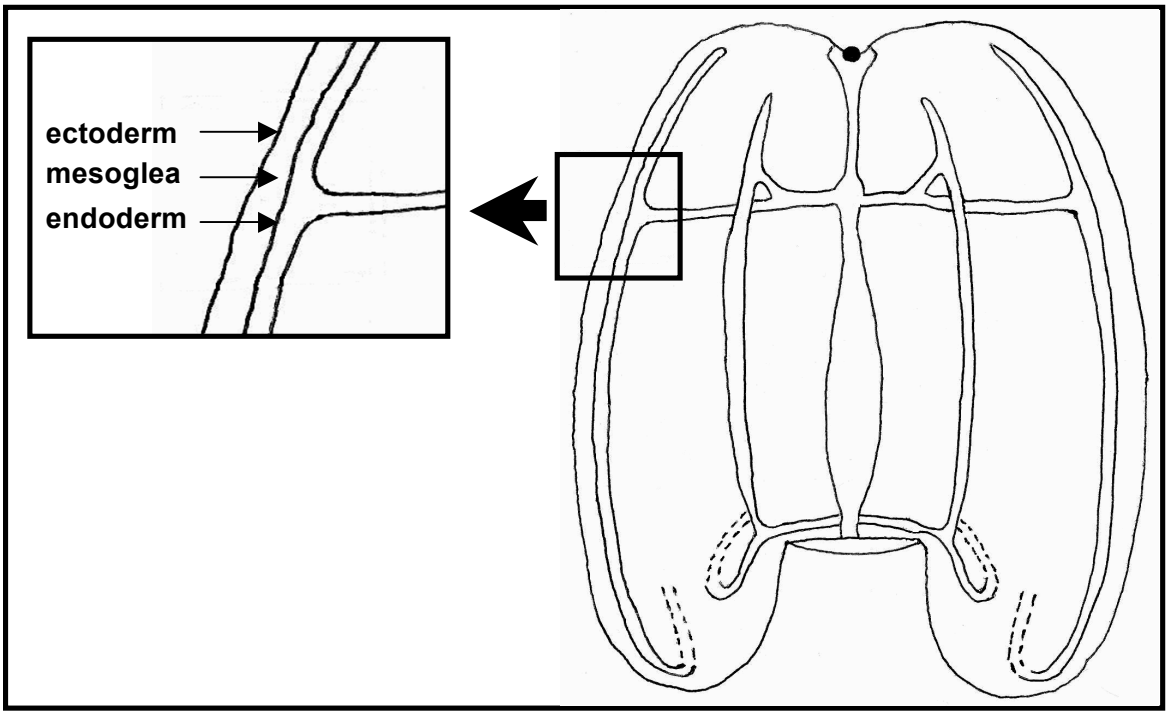

Figure 5: Arrangement of ctenophore tissue layers adapted from Mayer 1912.

Molecular significance

Expressed sequence tag (EST) libraries have been developed to provide snapshots of gene expression in an organism. ESTs are 200-800 bp nucleotide sequences created through the sequencing of the 5' and 3' ends of cDNA molecules that have been reverse transcribed from expressed mRNA. ESTs have been popular since their introduction as a primary resource in the human genome project, mapping new genes and identifying the coding regions of those genes (Adams et al., 1991). Since EST characterization does not involve a full sequence read, the investigator must very carefully construct his libraries via overlap of redundant sequences. Despite the potential pitfalls associated with this "best guess" approach, EST technology has proven to be a useful tool for the study of gene expression in many organisms (Nagaraj et al., 2006). As more EST libraries become available, the researcher has greater and greater ability to construct a more accurate view of animal phylogeny.

The evolutionary significance of ctenophores has recently been revealed more thoroughly by using a broad gene sampling approach. EST-based analyses provided controversial insight into the relative placement of Phylum Ctenophora. Dunn et al., in 2008, looked at 77 taxa including members from key groups such as mollusks, annelids, nemerteans, chaetognaths, echinoderms, hemichordates, chordates, cnidarians, poriferans, as well as ctenophores. They examined 150 different genes that encoded ribosomal proteins, structural proteins, actin and actin-related proteins, and proteins involved in cellular differentiation and proliferation, thereby providing a look at a much broader range of taxa and genes than previously possible.

Maximum likelihood and Bayesian analyses produced phylogenetic trees that showed strong support for the placement of Phylum Ctenophora as a sister group to all other metazoa, indicating ctenophores to be one of the earliest representatives of complex multi-cellular organisms. Dunn et al., used as outgroups[32], six different unicellular eukaryotes, the choanoflagellates *Monosiga ovata* and *Sphaeroforma arctica*, a member of Filastera *Capsaspora owczarzaki*, the amoeba *Amoebidium parasiticum*, and two types of yeast: *Saccharomyces cerevisiae* and *Cryptococcus neoformans*.

While the Dunn et al. results place *Ctenophora* at the base of the Animalia, this work was recently refuted by a German group (Pick et al., 2010). In the Pick et al. study, the same taxa were used, with the addition of 12 sponge taxa, 1 additional ctenophore, 5 cnidarians and 1 placozoan. They also only used choanoflagellates as an outgroup. Their findings place the phylum Porifera as the sister group to all other metazoa, and ascribe the Dunn et al. findings to artifacts from using too distant a set of outgroup organisms (i.e. fungi). The results in the Pick et al. study indicate that the 'deep time' base of the animal evolutionary tree is still a highly controversial topic. However, as molecular genetic tools become more readily available,

[32] Represent organisms that show some sequence or trait similarities to the other groups but are separate

investigators will be able to better understand the role of ctenophores in the evolutionary timeline.

Although its precise phylogenetic placement is unknown, Phylum Ctenophora is clearly one of the most ancient of all metazoan taxa. That means that many of the genes in higher metazoa may be derived from an extremely early metazoan ancestor common to ctenophores. For this reason, understanding the molecular events underlying processes such as ctenophore morphogenesis, regeneration and wound repair should provide a foundation for understanding how these processes work in all animals.

Why study wound repair in Mnemiopsis?

Mnemiopsis leidyi is an ideal system for studying wound repair at both the morphological and the molecular level. As mentioned above, recent phylogenomic analyses in which many genes were examined in a broad number of taxa place *Mnemiopsis* and its fellow ctenophores at the base of the metazoan tree. Conventional wisdom might suggest that such deeply derived and apparently unusual animals are too distant from modern vertebrates to be of value for studies relevant to mammalian biomedical science. However, there is precedence for the recognition of a similarly ancient organism having a full complement of genes found in vertebrates, as was recently shown by the publication of the full genome of the cnidarian *Nematostella* (Putnam et al., 2007). Interestingly, *Nematostella* has a more complete genetic complement than either *Caenorhabditis* or *Drosophila*, which have reduced genomes, making it potentially a superior organism for a complete understanding of the phylogenetic background of cellular or tissue processes.

The benefits of using *Mnemiopsis* as a model for wound repair extend beyond just its evolutionary placement. Fundamentally, it is an easy animal to study. *Mnemiopsis* is readily

obtained along the U.S. Gulf coast and U.S. Eastern Sea Board, can be cultured in the laboratory, and is easy to manipulate in the lab. The transparent epithelium allows for easy visualization of the cellular and structural changes that occur in the epithelium and the underlying mesoglea during repair. Furthermore, the wound response is very rapid compared to other epithelial repair systems, healing an epithelial wound in just minutes. The increased rate of repair does not lead to large amounts of debris or imperfections at the wound site, as might normally be expected. Also, no scar is seen once the wound has healed completely, a characteristic normally reserved for fetal repair (Adzick and Longaker, 1992), or repair in a limited number of organisms such as planarians and salamanders (Kumar et al., 2007; Reddien and Sanchez Alvarado, 2004)(please see the previous discussion of this issue in the Introduction).

The *Mnemiopsis* genome is currently in development. Once complete, it will indicate once and for all the molecular phylogenetic location of *Mnemiopsis* relative to all other animals, and that *Mnemiopsis* studies will be further justified once this information becomes available. A complete genetic map of this animal will go far to establish it as a model system for the analysis of fundamental properties of cellular and molecular events in all animals.

Chapter 2: Basic Light Microscopy Observations

Introduction:

To fully characterize the stages of any wound repair response, there must be a visual record of the cellular morphologies and movements. Analyses of cell morphologies, rates of movement and the timing of repair stages can reveal much about the role of those cells in a dynamic process such as wound repair. There are approximately a half dozen light microscopy techniques available that facilitate this undertaking, such as brightfield, darkfield, phase contrast, differential interference contrast, Hoffman Modulation Contrast©, and fluorescence microscopy.

The microscopic examination of the *Mnemiopsis* wound repair process poses unique challenges. DIC, Hoffman and phase contrast work well with the transparent tissue of *Mnemiopsis*. Immunohistochemistry and immunofluorescence, which reveal the presence of particular molecules in tissue, also work well with *Mnemiopsis*, again because of the clarity of the tissue. The availability of fluorophores that excite at different wavelengths such as rhodamine, fluorescein and Alexa dyes, used in conjunction with nuclear stains such as 4',6-diamidino-2-phenylindole (DAPI) and propidium iodide, allow simultaneous visualization of multiple molecules in a cell.

An early step in a successful wound response is to perform rapid closure of the wound to seal it off from the external environment. While a cut is normally closed over a period of hours to days in humans in the absence of dressings, the ctenophore *Mnemiopsis*

leidyi heals small epithelial wounds in as little as 30 minutes with little or no scarring. *Mnemiopsis* can also seal and regenerate subrow vascular canals in as little two hours. The fetal-like lack of scarring (Rowlatt, 1979; Adzick, 1992) and extremely rapid healing response makes *Mnemiopsis* an ideal model organism for wound healing research. Here I will describe detailed investigations of the cellular structures involved in ctenophore wound healing.

Objectives and Observations:

Since the wound repair process in *Mnemiopsis* has never been previously described, there are many fundamental questions that need to be addressed. Specifically, this research will attempt to answer the following questions: 1) What types of cells are found in *Mnemiopsis*, 2) What is the apparent function of each cell type, 3) Where is each cell type localized, 4) What is the mechanism of cellular motion, 5) What cells involved in the repair processes, 6) Where are the cells involved in the repair process localized prior to and during a wound response, 7) When does cell proliferation occur, 8) How do cells interact with each other and with acellular structures such as the mesoglea, and 9) How quickly does the wound heal. The purpose of this thesis section is to begin to answer these questions.

Objective 1: Describe the overall series of events that occur during ctenophore epithelial wound repair.

Epithelial wound repair in *Mnemiopsis* proceeds in a consistent and reliable series of events. Those events are: 1) Breach of the epithelium; 2) Underlying mesoglea degradation; 3) Initiation of cell migration; 4) Muscle fibers converge on the wound; 5) Cell aggregation; 6) Re-epithelialization; 7) Contraction of muscle fibers and underlying multipolar cells; and 8) Cell dispersal.

Low magnification (4X) time lapse video recordings of repair were taken using an Olympus SZ11 zoom stereo microscope equipped with a 2X magnifying objective.

Objective 2: Determine the rate at which an epithelial wound heals.

Mnemiopsis repairs epithelial wounds at a very rapid rate. The rate of epithelial repair can be defined as:

1) Rate of reduction of the wound area

2) Rate of movement of the cells surrounding the wound

3) Rate of movement of the epithelial edges inward to close the wound

Here the rate of epithelial repair is calculated by measuring the rate of reduction in the area of the wound. This was calculated by averaging the rate of wound closure in mm^2/minute from ten series of wound images. Images were taken every minute using brightfield microscopy in conjunction with time-lapse video recording, same as above. The wound area was then measured using a stage scale and recorded by digital imaging, and the rate of closure calculated at each time point.

Objective 3: Observe what happens to the mesoglea underlying the wound.

Once the epithelium is wounded, a cavity forms as seawater rushes into the wounded area. Observations concerning the wound cavity include:

1) Sea water rushing into the wound results in the degradation of the mesoglea underlying the wounded area.

2) The wound cavity initially appears acellular, but becomes cellular as wound cells migrate in and accumulate.

Images of wound cavity formation were initially observed using time-lapse video recordings. Differential interference contrast (DIC) microscopy using 10X and 20X objectives revealed the acellular nature of the cavity.

Objective 4: Describe the cellular response(s).

One of the first striking observations made concerning the wound repair process was the very 'granular' appearance of the mesoglea and epithelium immediately surrounding the wound site. This led us to ask 1) what were the granular elements and 2) what was the source of the migratory cells? Finally, 3) how many cells were involved in a typical repair response?

To visualize cell migration and wound closure I used brightfield microscopy in conjunction with time-lapse video recordings. This allowed me to determine when cell migration began, and the exact time course of the repair response. Cell morphologies were observed using Differential Interference Contrast (DIC).

Cells at the wound edge were stained using Hoechst 33258 DNA stain, and then counted manually using the ImageJ point selection tool. Ten images of wound edges were counted and wound edge area was calculated using a scale. The total cell number/mm^2 for each image was then averaged.

Objective 5: Establish the importance of contraction in ctenophore wounding.

Contraction plays a role in repairing epithelial wounds in *Mnemiopsis*. Contraction in this system can refer to:

1) Contraction of cells surrounding the wound

2) Localized contraction of smooth muscle fibers running from ectoderm to endoderm.

3) Contractile cells surrounding the wound muscle.

4) Contractile cells using actomyosin based contraction.

During the repair response, the animal contracts its body to bring the damaged edges closer together. This contraction is accomplished by true smooth muscle fibers present throughout the mesoglea of *Mnemiopsis*. This, coupled with the rapid migration of the wound cells towards the wound site, indicates a strong dependence upon actin in the repair response. The Alexa 488 phalloidin, which binds filamentous actin, was used to stain actin at the wound site. Muscle fiber involvement was observed using darkfield microscopy and differential interference contrast (DIC).

Objective 6: Compare inner lobe epithelial repair to outer epithelial repair.

Majority of work in this system was done using the outer epithelium of the animal. However, upon observation of the inner oral lobe, there is a distinctly different repair response. In this response, Migratory cells enter into the wound cavity and accumulate to form a network of cells. Initial observations on the activity of these at the wound site cells are as follows:

1) Cells migrate to the wound site by climbing through the mesoglea.

2) Cells attach to smooth muscle fibers, using them to enter the wound cavity.

3) Cells are recruited to span across the wound site; recruited cells strengthen the bridge and increase contractility (i.e. cells are not just linear but bifurcate....forming a web).

Another cell type involved in the repair response can be found in the epithelium, and plays a role in forming the new epithelium. Some observations on this cell type are:

1) Cells have a vesiculated morphology

2) Cells change shape and migrate through the epithelium to the open wound

3) Cells at the wound edge release their vesicular contents, decreasing in size

Observations at the wound site were observed using differential interference contrast (DIC) microscopy using 10X, 20X and 40X objectives.

Objective 7: Describe what happens once the wound has healed.

One of the most unique aspects of wound repair in *Mnemiopsis* is the absence of scar formation upon completion of the response. Completion of repair involves:

1) Epithelial cells and underlying migratory cells disperse

2) Cells return to their original distribution in the mesoglea

Completion of repair and cell dispersal was observed using differential interference contrast (DIC) and phase contrast microscopy using 10X and 40X objectives.

Materials and Methods:

Animal collection

Coastal *Mnemiopsis leidyi* used for wounding experiments were collected along a range of locations from Dauphin Island, AL to Woods Hole, MA. Collection sites listed from west to east include: Dauphin Island, AL; Pensacola, FL; Panama City, FL; Apalachicola, FL; Dickerson Bay, FL (Gulf Specimen Marine Supply); Charleston, SC; Englehard, NC; Nags Head, NC; Lewes DE; Chesapeake Bay stations 707, 724, 744,758, 804, 818, 834, 845, 858, and 908 (courtesy of MOVE cruises with Dr. Eric Wommack. University of Delaware); and Woods Hole, MA. Animals were collected by dipping from the surface, or via slow tows using a plankton net and kept in large buckets or plankton kreisels. Wound healing experiments were typically carried out within 48 hours of collection on healthy animals.

In vivo wound experiments

Individual *Mnemiopsis* were placed into a finger bowl or small Petri dish to perform all microsurgical procedures. All standardized wound incisions were made while observing the preparation with a stereo microscope (SZ11, Olympus; Center Valley, PA) to ensure proper

wound size and placement. Wounds were made in the epithelium of the animal near the apical end between the subsagittal and subtentacular comb rows near the aboral end (Fig. 1B). Incisions were made with a Gills Welsh Vannas iridectomy scissors (E3388, Stortz Instrument Co.; St. Louis, MO) and measured on average 3 mm long by 3mm deep. Wound size was measured by taking an image of the wound at time zero and using digital image processing software (Image J, NIH, Bethesda, MD) calibrated to the objective and projection lens of the microscope from an image of a scale at the same magnification; the same technique was used to establish scaling for all light micrographs.

The repair process was recorded by time-lapse video. Images were taken with a Sony Hyper HAD monochrome CCD camera (SPT-M124, Sony Security Systems Broadcast and Professional Group, Park Ridge, NJ), and recorded on a Mitsubishi VHS time-lapse videocassette recorder (HS-480U, Mitsubishi Electronics of America Inc., Cypress, CA). Images of the healing wound were also taken at one minute intervals using a digital monochrome CCD camera (Q-ICAM *Fast*, QImaging Corp, Vancouver, BC, Canada) linked to a computer running Image Pro Plus (Media Cybernetics, Bethesda, MD). In each case, the rate of wound closure was analyzed using digital imaging software as described above.

A compound microscope (BHS, Olympus Corporation, Center Valley, PA) equipped for fluorescence, phase contrast and differential interference contrast microscopy was used to photograph cell-cell and cell-mesogeal interactions at the wound site using 20X and 40X objectives. Phase contrast images of the healing wound were also taken shipboard using a Nikon digital camera (Nikon 990 Coolpix, Melville, NY) attached to a Zeiss Axiovert 135 inverted microscope via a custom adaptor (MMCool, Martin Microscope, Easley, SC), and equipped with a strobe for bright, sharp imaging to eliminate shipboard movement.

Actin staining

Sections of wounded oral lobes comprising approximately 1/5, or 6-10 mm of the total length of the animal, were removed from healthy *Mnemiopsis leidyi*. Animals used for wound experiments typically averaged 3-5cm in length from the apical organ to the tip of the oral lobes, resulting in lobe sections ranging from 60-100 mm^2. Removed lobes were then fixed in 4% paraformaldehyde, 0.14 M NaCl, 0.2 M sodium cacodylate buffer, also containing 0.0165 μM Alexa488 phalloidin (Molecular Probes, Invitrogen, Carlsbad, CA) to stain actin, and allowed to sit for 30 minutes at room temperature (adapted from Tamm and Tamm 1989). Preparations were imaged by upright (BHS Olympus) or inverted (Axiovert 135) microscopes, using 488 nm excitation.

Hoechst 33258 staining

Sections of wounded lobe were removed as mentioned above and stained for DNA with a vital, fluorescent stain, Hoechst 33258, which permeates live cells, eliminating the need for fixation. The Hoechst stock solution (10 mg/mL) in ultrapure water was diluted 1:1000 in filtered seawater to give a final working concentration of 1 μg/mL. The dye was allowed to permeate cells for 30 minutes and then viewed on an Olympus BHS compound microscope equipped for fluorescence with a 365 nm excitation Ploem cube (Model 11000, Chroma Technology Corp., Brattleboro, VT).

Results:

Rate of epithelial repair:

Wounds made to the epithelium in *Mnemiopsis* initiate a rapid repair response that can heal as quickly as 30 minutes (Fig.6). The rapidity of the response was measured by taking time-

lapse videos of healing epithelial wounds and then measuring the rate at which the wound closed. Ten series of images were taken, using ten different wounds on ten separate animals, and the wound was measured as a function of time using ImageJ image analysis software. Measurements were averaged to give an estimate of the overall rate of epithelial repair and, the average was calculated to be 0.255 ± .1 mm^2 $minute^{-1}$.

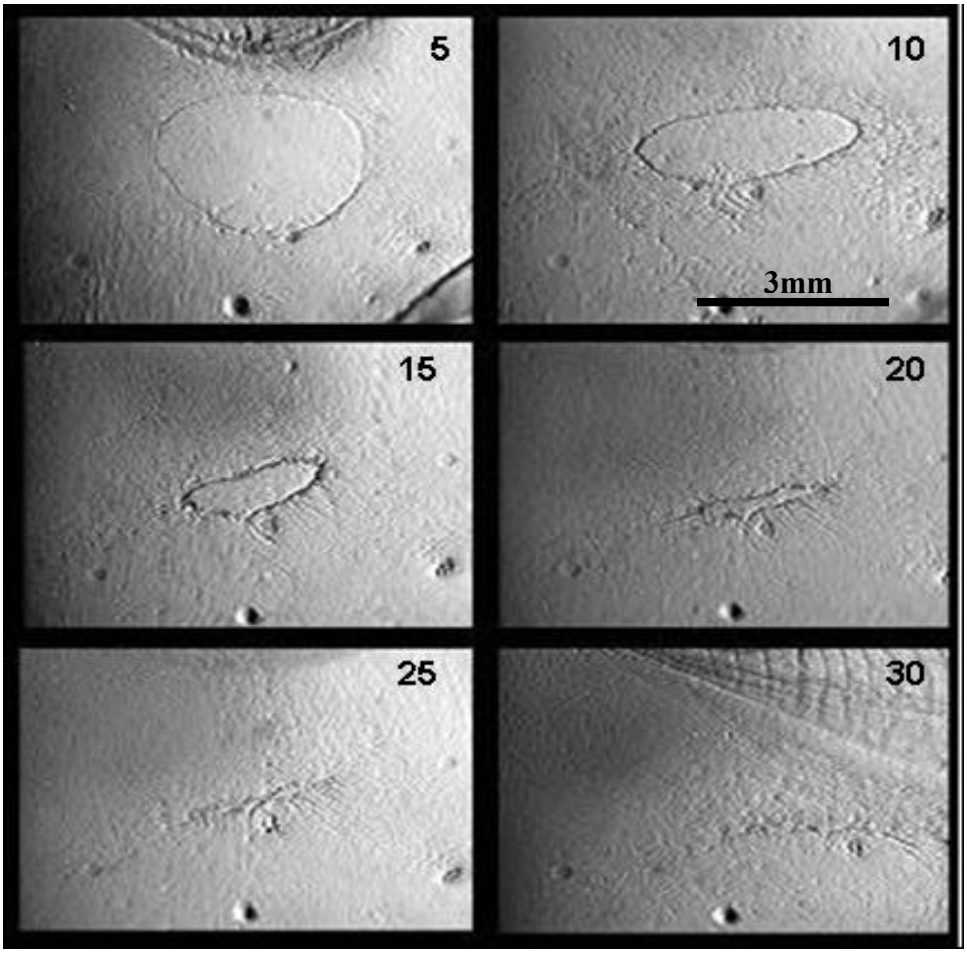

Figure 6: Brightfield microscopy of an epithelial wound healing in 30 minutes

Progression of ctenophore epithelial wound site events post-wounding

Epithelial damage and degradation of underlying mesoglea:

After cuts are made to the epithelium using iridectomy scissors, the wound grows wider as seawater rushes in. The seawater appears to profoundly degrade the underlying mesoglea (Fig.

7), because a cavity immediately appears beneath the breached epithelium. The cavity is evident by the bunched appearance of the epithelium at the wound edges and the acellular appearance of the wound cavity.

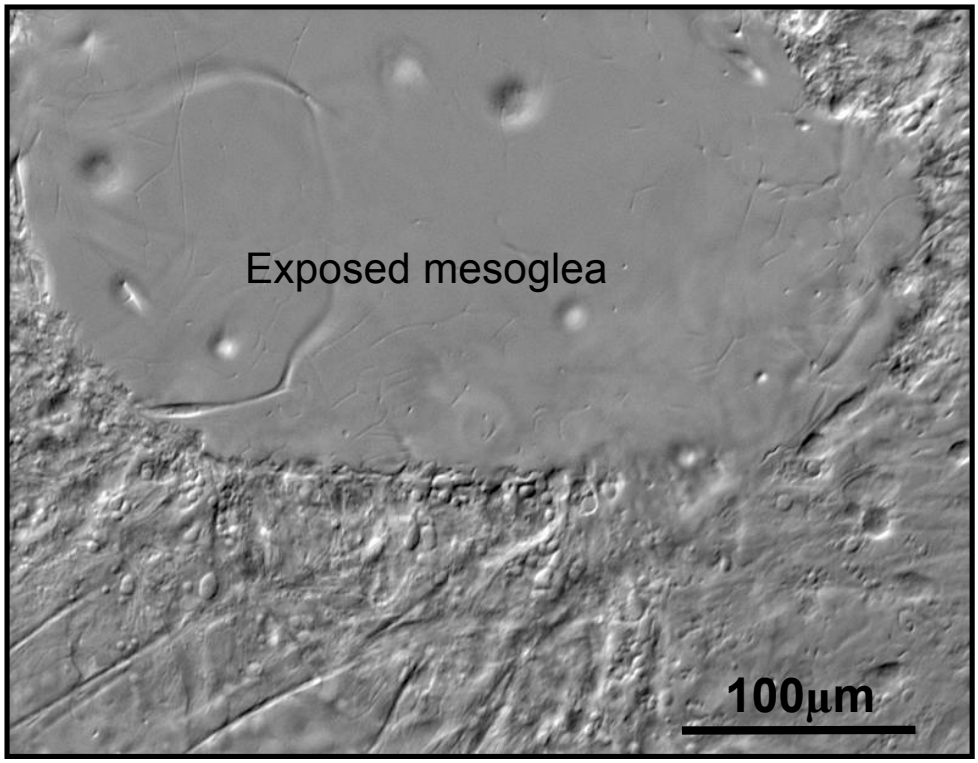

Figure 7: Differential interference contrast microscopy showing cavity formation

Initiation of cell migration:

Breach of the epithelium initiates an ordered series of events that work together in repairing the wound. The exact signal of this initiation is unclear. Exposure to seawater appears to trigger the migration of thousands of migratory cells out of the mesoglea to the wound cavity and epithelial edges. Migration is clearly evident by 5 minutes of wounding. The (at least hundreds) of newly recruited cells give the mesoglea under the wound, and under the epithelium, a distinctly granular appearance. Cells near the sea water interface (i.e. cells of the epithelium and the edge of the wound) can be visualized using the membrane permeant nuclear stain Hoechst 33258, which clearly shows nuclei when excited at 365 nm (Fig. 8).

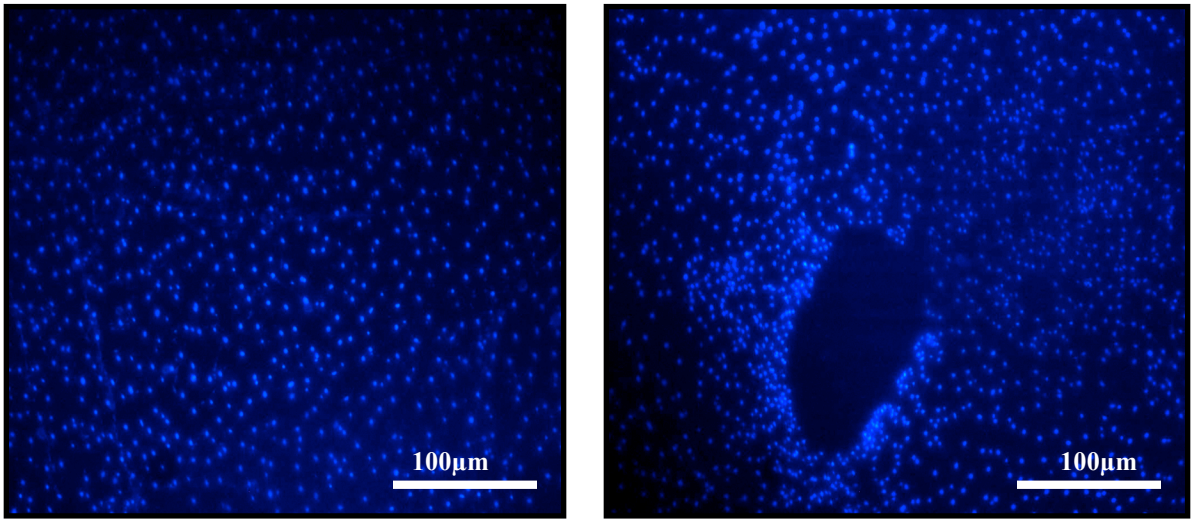

Figure 8: Hoechst staining of normal (Left) versus 5 min. post wounding (Right)

Hoechst staining reveals a noticeable increase in the number of cells around the wound as they migrate towards the newly exposed region. Cells continue to migrate into the wound site as repair proceeds.

Muscle fibers at the wound site:

The mesoglea of *Mnemiopsis* contains giant true muscle fibers (Anderson, 1984; Hernandez-Nicaise et al., 1984). Muscle fibers at the wound site serve three functions, 1) as a scaffold for recruiting new cells, as well as a site for the nucleating new cellular outgrowth, and 2) as stabilizers around the wound edges; later they 3) act to pull the wound edges closed via a purse string mechanism. Cells migrating into the wound cavity can clearly be seen attached to muscle fibers in the wound cavity (Fig. 9). The fibers serve as a scaffold for cells migrating into the wound. The muscle fibers also appear to play a role in stabilizing the wound site. Normally, the muscle fibers of the oral lobe run longitudinally along the oral-aboral axis of the body. However, upon wounding, the muscle fibers closest to the wound become reorganized (Fig. 10).

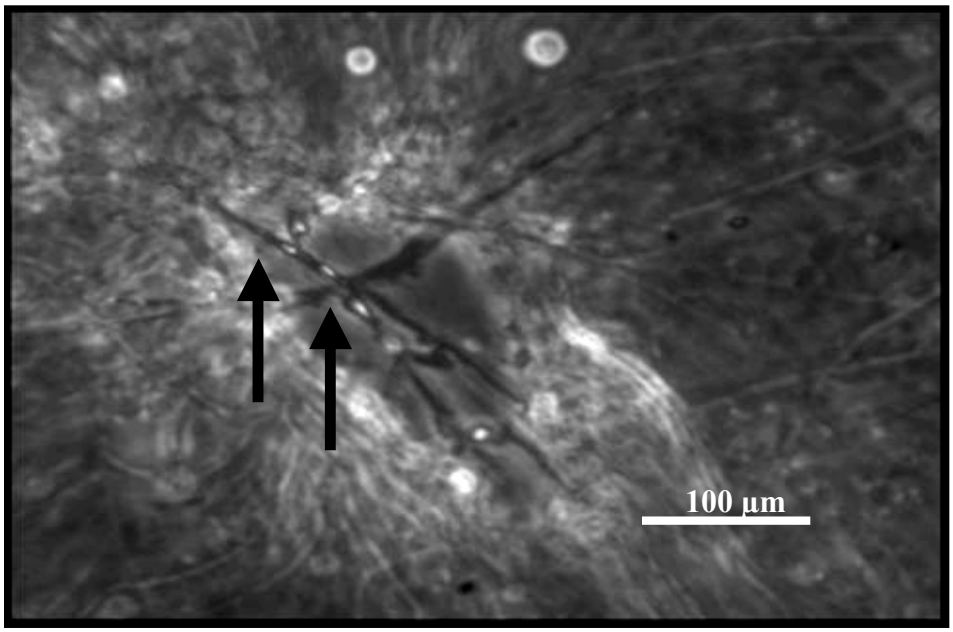

Figure 9: Phase contrast image of muscle cells. Note phase-bright nuclei of mesogleal cells, which have become attached to muscle fibers in the wound cavity

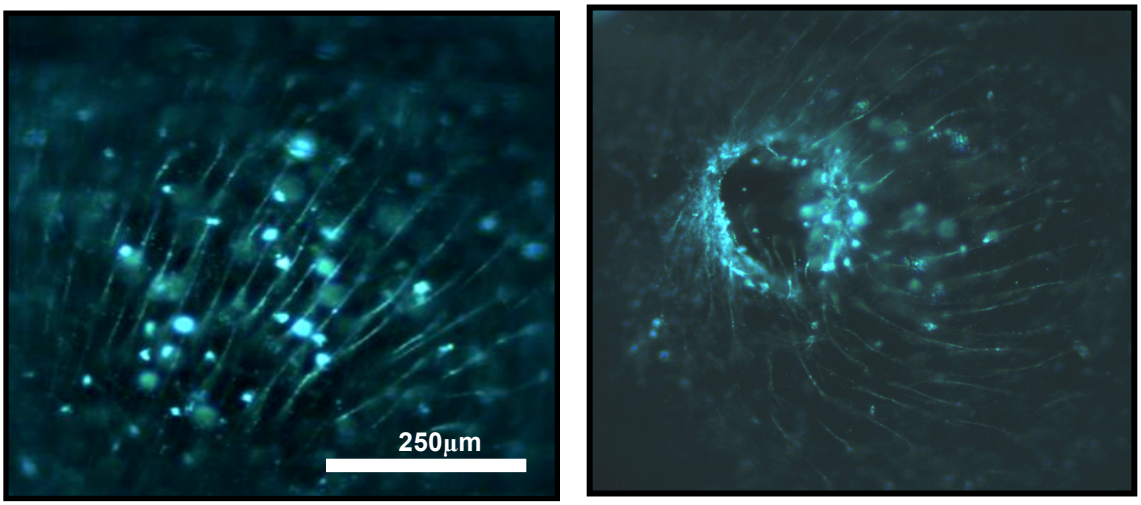

Figure 10: Darkfield image of normal muscle fiber conformation (left) compared to 15min. post-wounding (right). Note how the muscle fibers tend to converge upon the wound site.

Cells surrounding the wound site are rich in F-actin, as shown by using the filamentous actin (F-actin) probe Alexa-488 phalloidin. Staining reveals an abundance of actin at the wound site, not only in the muscle fibers but also in the wound cells migrating into the wound (Fig. 11 A and B).

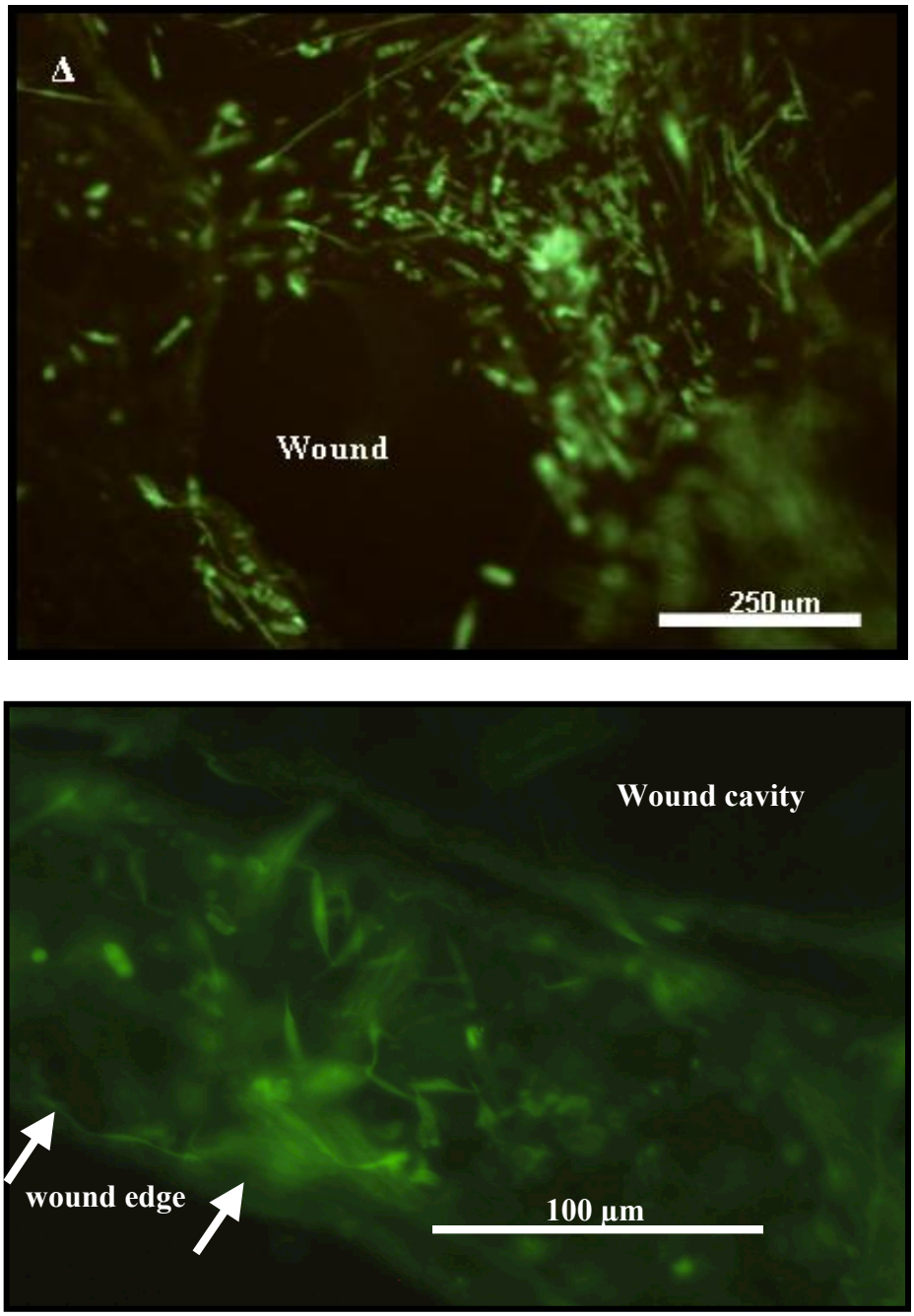

Figure 11: F-actin rich cells revealed by Alexa 488 phalloidin staining.

Cell aggregation:

Here, a simple nuclear stain helps to demonstrate a rough estimate of the magnitude of cells required to quickly heal a small wound in the epithelium of *Mnemiopsis*. Nuclear stains allow the visualization of how many mononucleate cells are present in the tissue. By 10 minutes

into wounding, a large number of cells collect at the wound edge (Fig. 12).

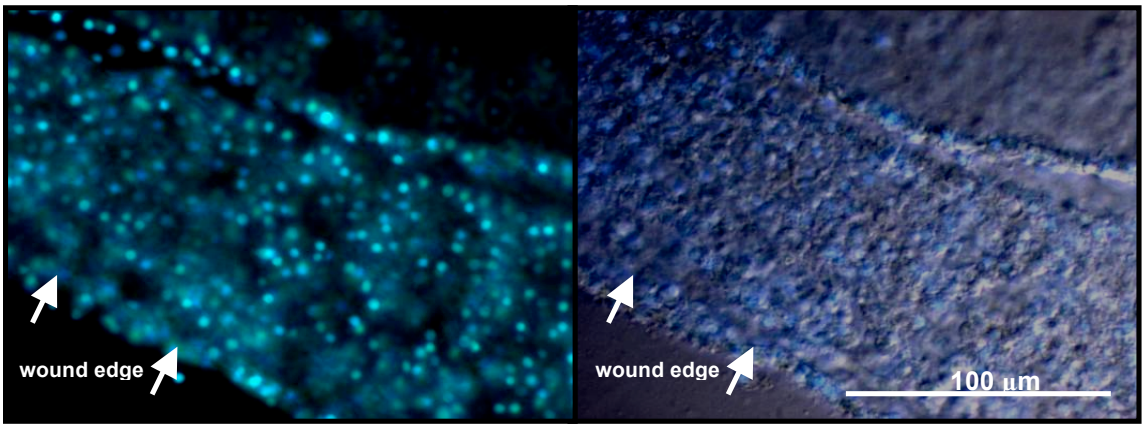

Figure 12: (left) DAPI nuclear stain of a 10 min. wound edge, and (right) the corresponding DIC image.

The area of the wound edge pictured in Figure 12 is 0.02 mm^2, and cell counting revealed ~300 cells in that area. To determine approximate cell number at the wound edge, 9 more wound edges were examined. These numbers were then compared to the same area in ten images of non-perturbed tissue. Cell nuclei were then counted using ImageJ. Cell counts revealed an average of 150 cells in .02 ± .01 mm^2 of wound edge tissue, compared to 65 cells in .02 ± .01 mm^2 of normal tissue. Cell number at the wound edge varied, and did not appear to be dependent on wound size.

Reorganization:

There are two main cell types active at the wound site, epithelial cells with a vesiculated surface and multipolar cells (Fig. 13), and they are involved in two distinct processes that occur simultaneously to heal the wound. The first process, labeled reorganization, occurs in the wound cavity as multipolar migratory cell types aggregate and bind together extending their processes to attach and link to multiple points along the wound edges (Fig. 14A). These cells form a network in the wound cavity and work in conjunction with the contracting muscle fibers to help bring the wound edges together (Fig. 14B).

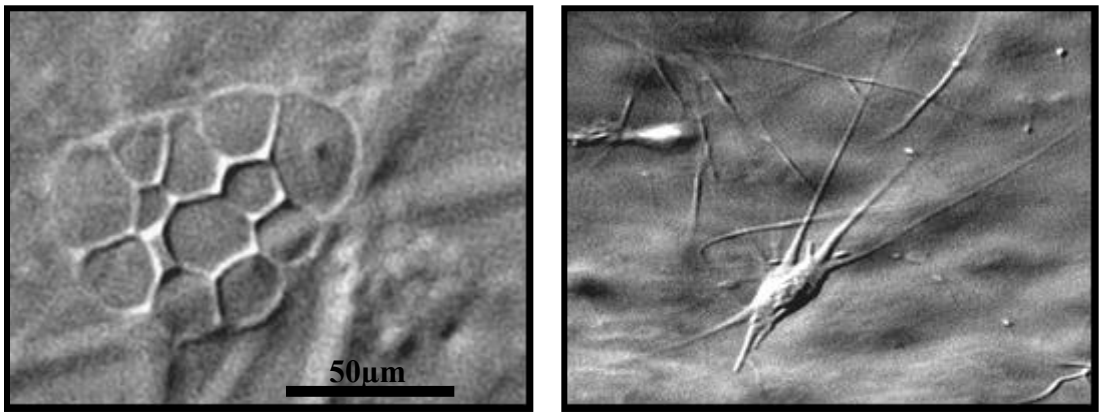

Figure 13: The two main cell morphologies seen at the wound site, vesiculated epithelial cell (left) and multipolar cell (right).

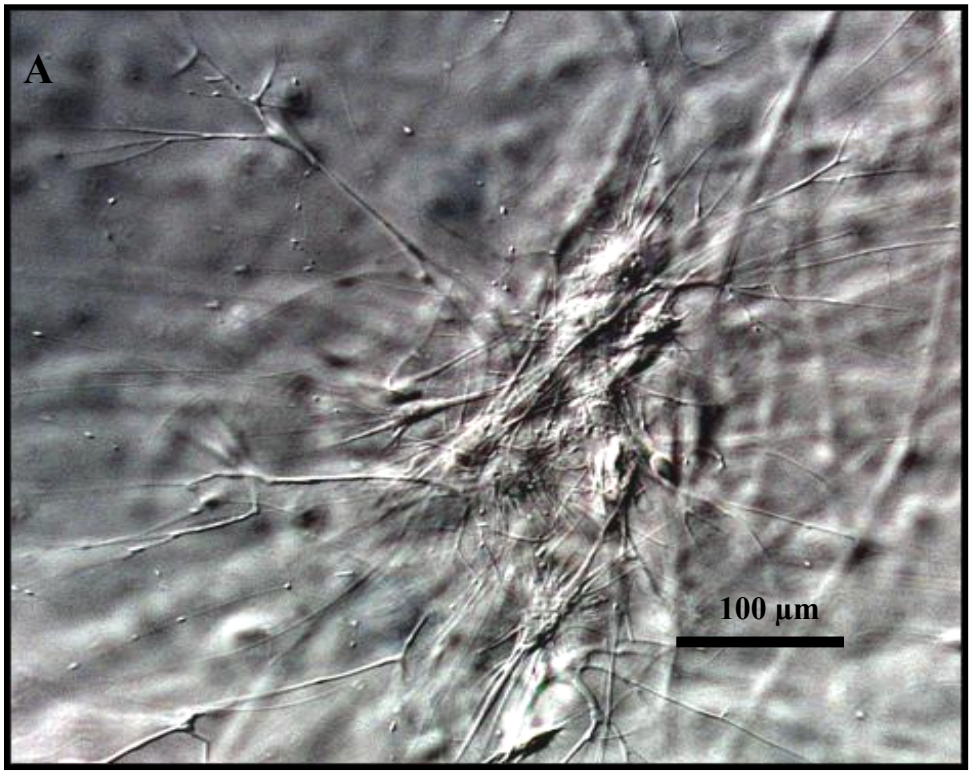

Figure 14A: DIC at ~2 mm deep of cell network within the wound cavity.

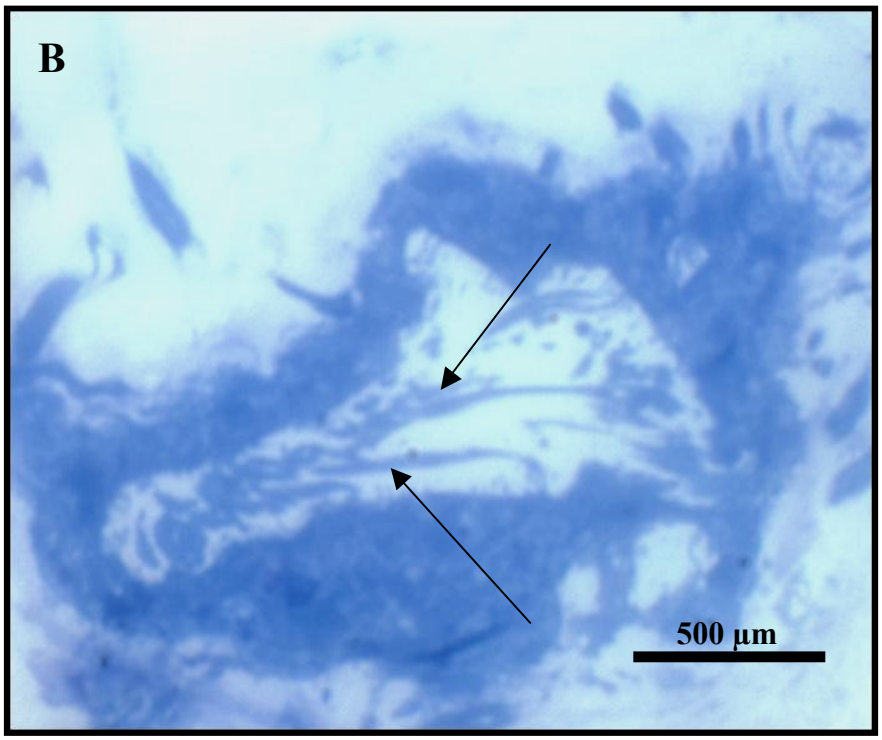

Figure 14B: 0.25µm section stained with Richardson's stain showing multipolar cells stretching across the wound cavity.

7) Re-epithelialization:

While multipolar cells aggregate in the wound cavity, above them (i.e. nearest to the sea water) epithelial cells migrate in a vectorial manner to the open wound. The epithelial cells contain numerous putative secretory vesicles on their surface. The cells, which are flattened and approximately oval (Fig. 15A), become more elongated when migrating to the wound edge (Fig. 15B). Once the epithelial cells have reached the wound, they clump together and shrink in size (Fig. 15C), possibly releasing their vesicular contents. There is no clear and visible nucleus in the epithelial cells, and vesicle number on the cell surface is inconsistent varying from 7-12 vesicles.

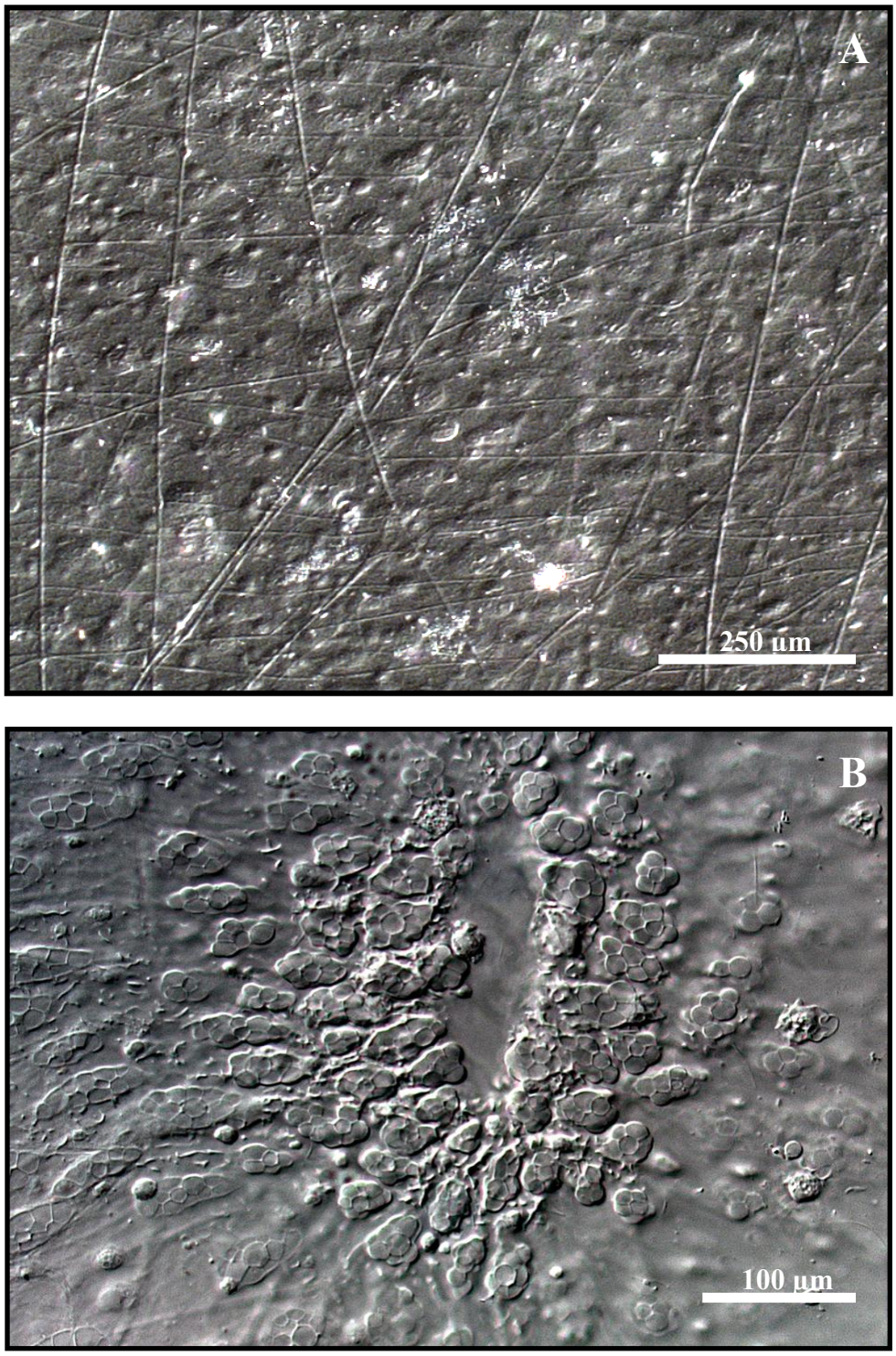

Figure 15: A) Normal circular appearance of epithelial cells
B) Cells become elongated and migrate to wound edge

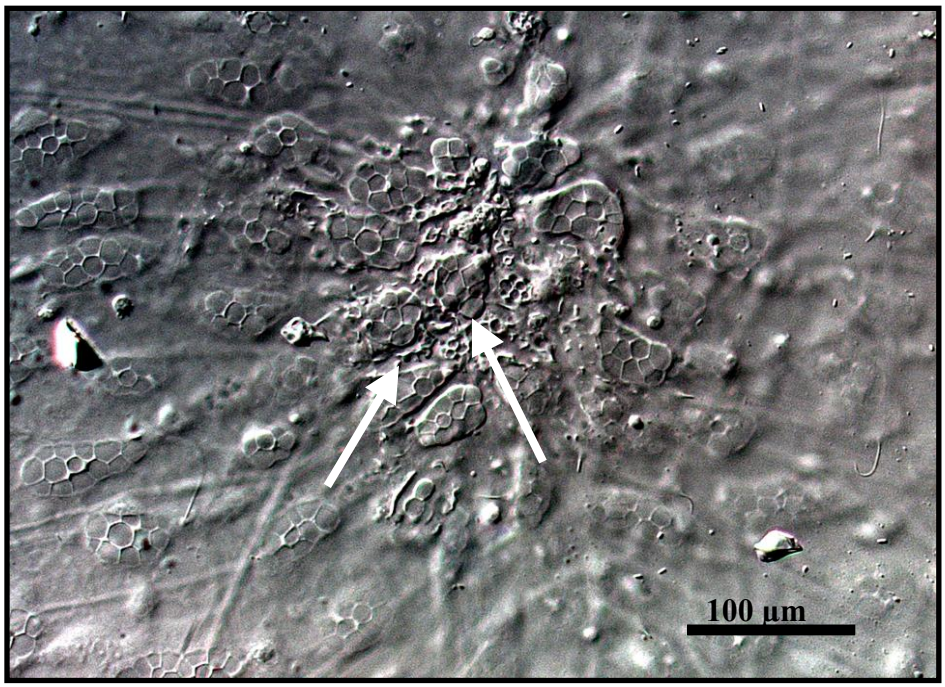

Figure 15: C) DIC showing epithelial cells shrinking in size as the wound closes

8) Cell Dispersal and completion of the healing response:

Once the epithelial wound edges have come together, the epithelial cells return to their normal circular conformation and begin moving away from the wound (see Fig. 16).

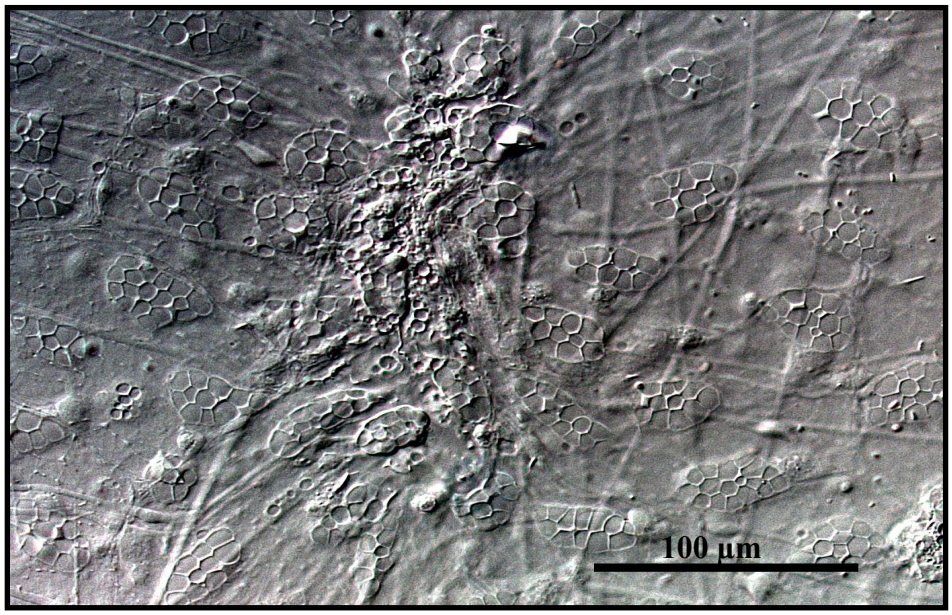

Figure 16: DIC image showing cell dispersal as the wound finishes healing

These cells also appear to return to their normal arrangement, as seen prior to wounding. Examination of the epithelium post healing reveals the absence of any scar or sign that a healing response has occurred.

Discussion:

Epithelial wound repair in *Mnemiopsis* proceeds very rapidly. Repair is 15X faster than adult rabbit cornea, 60X faster than adult mouse epidermis, and 4,000X faster than embryonic *Drosophila* epithelia (Fig. 17).

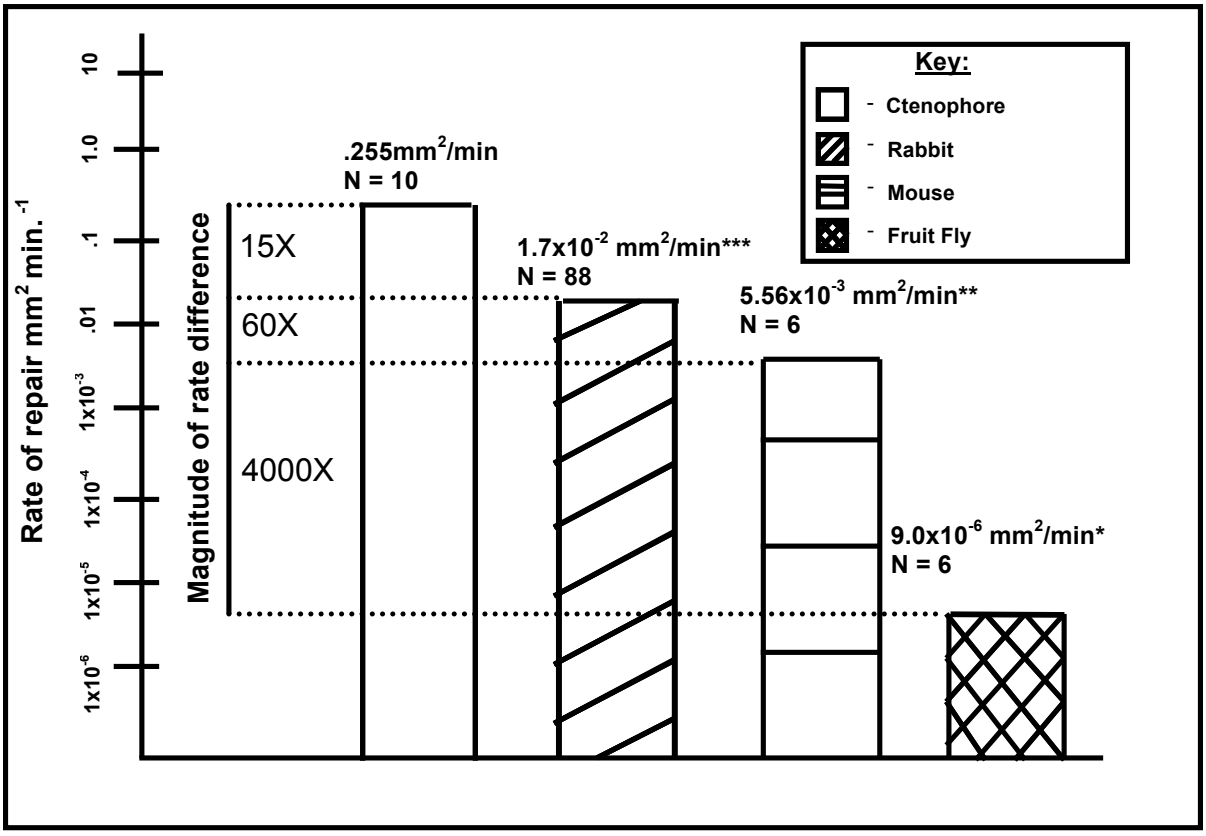

Figure 17: Logarithmic representation of the rate of cutaneous repair in ctenophores compared to rabbit, mouse and fruit fly. *** (Kitazawa et al., 1990);** (Safer et al., 2005); * (Wood et al., 2002).

Epithelial healing cascade:

The wound cascade progresses in a very time specific and ordered manner (Figure 18) and can be broken down into the following stages: 1) Damage to the epithelium, 2) Underlying mesoglea degrades, 3) Initiation of cell migration, 4) Muscle fibers reorientation, 5) Cell aggregation, 6) Re-epithelialization, 7) Contraction of muscle fibers and underlying multipolar cells, and 8) Cell dispersal. These steps overlap to completely and efficiently heal an epithelial wound.

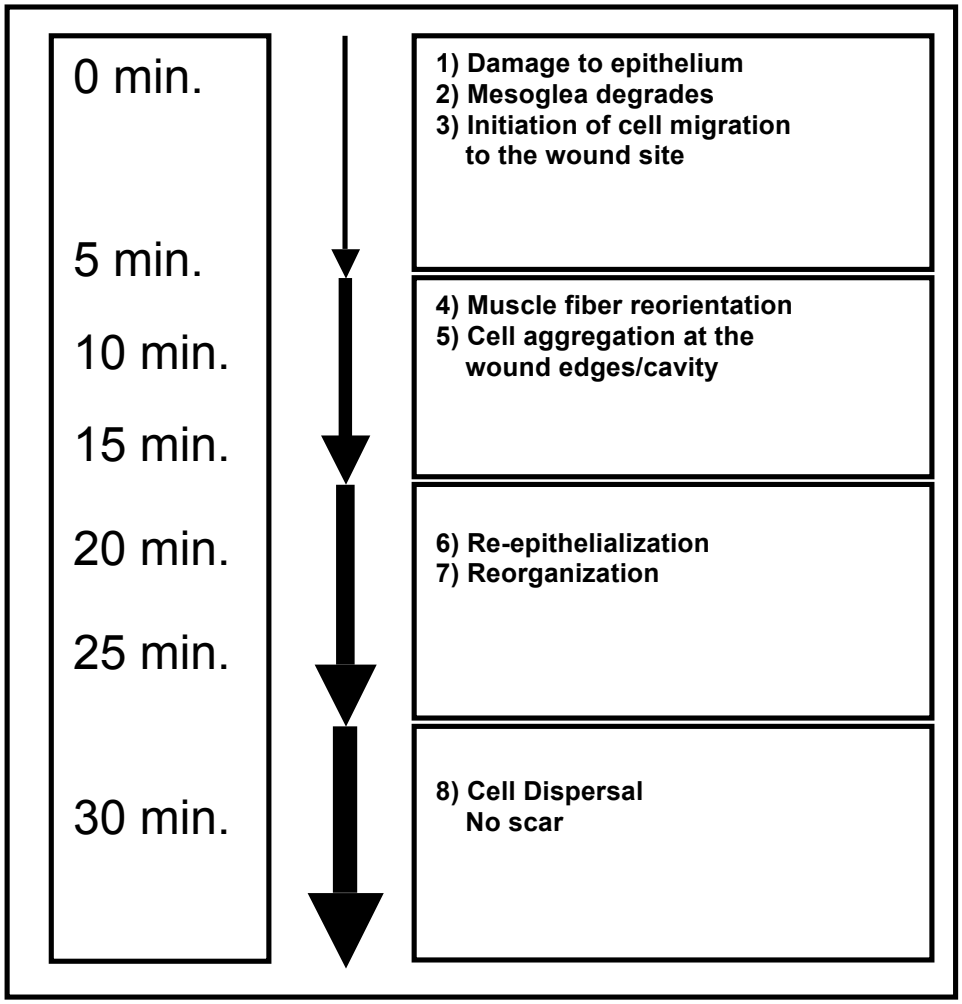

Figure 18: Progression of ctenophore wound repair.

Cell types involved in repair:

There are at least three main cell types responsible for the repair response, epithelial cells, multipolar cells, and muscle fibers. The epithelial cells may or may not have many vesicles covering the surface of the cell. The cells clearly shrink, suggesting that they are releasing contents from the vesicles on the surface. The contents of these vesicles may be a cellular glue responsible for temporarily sealing the fused lobular epithelial edges while the repair response completes. Whatever the case, these cells play an integral role in forming the new epithelium.

The multipolar cells clearly accumulate in the wound cavity, creating a network of cells that link the wound edges together. These cells play a myofibroblast-like role, migrating into the wound cavity and providing added contractile force to the closing process. This is supported by the large amount of F-actin that can be viewed in cells localized around the wound. These cell types also link to muscle fibers present in the wound cavity, using them as a scaffold that may provide even more stability to the wound site.

Muscle fibers surround the closing wound, thereby forming an actomyosin based purse-string such as is seen in dorsal closure in *Drosophila* (Redd et al., 2004). The large number of muscle fibers drives the contraction needed to rapidly close the wound. The muscle fibers, in conjunction with the myofibroblast-like cells may account for how *Mnemiopsis* is able to contract and reseal an epithelial wound as quickly as it does. Muscle fibers at the wound edge helps to maintain structural integrity, as well as allowing rapid cellular migration into the wound to begin repair.

Conclusions:

Although the most dramatic aspect of ctenophore wound repair is its speed, the most important question is how this model relates to other wound repair systems, most importantly

humans. All organisms bear the need to heal as rapidly and effectively as possible, leading to morphological and procedural similarities that can be observed in healing processes across phyla. Two major repair models are mammalian cutaneous wound repair and epithelial wound repair in *Drosophila* larvae (reviewed in Chapter 1). Mammalian wound repair has been thoroughly characterized, as it pertains directly to understanding and improving repair in humans. *Drosophila* has been established as an ideal invertebrate model due to the information now known about it at the genomic level. Comparisons can be drawn between these two systems, and ctenophore wound repair.

For example, both cutaneous wound repair in mammals and *Drosophila* epithelial repair involve re-epithelialization. Re-epithelialization is integral to sealing the wound site from the external environment. This not only serves as a mechanical barrier but also helps to prevent infection. Re-epithelialization is also observed in ctenophores as cells in the epithelium migrate to the wound and form the new epithelium. Another example is when cells migrate out of surrounding tissues to the site of a cutaneous/epithelial wound. In both *Drosophila* and mammalian models, cells migrate out of the underlying tissues to the site of the surface wound, generally using the clot as a matrix through which they migrate. There they can begin their various roles in the wound repair process. This can also be observed in ctenophores: As the cells migrate into the wound site they bind to each other as well as pre-existing muscle fibers. This subsequent aggregation, along with muscle fibers around the wound, forms a contractile network capable of pulling together the wound edges, eventually sealing off the wound.

An understanding of ctenophore wound repair could lead to treatment options that increase the speed of human/domestic animal repair processes in response to acute injury. This system provides an easily accessible and manipulatable system to study scar-free wound repair.

Chapter 3: Cell Culture Observations:

Introduction

Cell cultures allow us to get a better look at cell activity. Cell culture lines such as HeLa cells, embryonic stem cells, cancer cell lines, Chinese Hamster Ovary (CHO) cells, Newt A1 limb cells, *Xenopus* A6 kidney cells, 3T3 mouse fibroblasts, and Madin Darby canine kidney (MDCK) cells have greatly enhanced our understanding of how cells interact with each other and their surroundings. Findings in the cultured cells can then be applied to the *in vivo* models to see if the effect obtained applies to the actual system.

While a great deal has been tested in mammalian cell lines, there has been a recent surge in the study of marine invertebrate cell cultures. This is due to the need for cell culture lines from species important in both the pharmaceutical and aquaculture industries, as well as the stem cell like capabilities documented in a variety of marine invertebrates (Rinkevich, 2005). Primary cell cultures have been developed in the Phylum Porifera (Mueller et al., 1999; Willoughby and Pomponi, 2000), Cnidarian (Anderson, 1984; Przysiezniak and Spencer, 1989), Crustacean (Fraser and Hall, 1999; Owens and Smith, 1999), Mollusk (Barik et al., 2004; Chen and Wang, 1999), Echinodermata (Odintsova et al., 1999), and Urochordata (Rabinowitz and Rinkevich, 2003).

Two cell culture systems that have produced interesting results in the context of this study have been derived from phylums Porifera and Cnidaria. In the Porifera, a number of primary cell culture lines have been developed to test gene expression (Krasko et al., 1999),

synthesis of biological products (Kelve et al., 2003), and cell viability (Sipkema et al., 2004). Cnidarian primary cell cultures have been used to demonstrate the importance of cell-ECM interactions in maintaining cell differentiation and migration (Schmid et al., 1999). Problems exist with all of these cell lines, including the lack of long term (more than one month) cultures; however, these cell lines have shed light on the functions and capabilities of a number of marine invertebrate cell types.

This chapter describes the initial description of a primary marine invertebrate cell culture developed from migratory wound cells removed from healing *Mnemiopsis leidyi*. The wound cell cultures typically lasted 14 days. The cells seek each other out to form tissue-like masses starting at 24 hours post plating. The observations reported here describe how cultured ctenophore cells interact *in vitro*, without changing or adding anything to the culture environment. I developed this culture with the hope of producing a viable continuous ctenophore wound cell line, through which a better understanding of cell-cell interactions and cell functions that may give rise to a scar free wound repair site could be gained. This goal has not yet been achieved; nevertheless, useful observations on the basic cell activity were gained.

Objectives and Hypotheses

Objective 1: Determine the cell morphologies present *in vitro*

Cell morphology can reveal a great deal about the functions of that cell. During the *in vivo* response there appear to be at least 3 major cell morphologies: multipolar cells, bipolar cells, and stellate cells, which act to repair the wound.

Hypothesis 1: Wound cell cultures display the same cell ***morphologies*** observed *in vivo*.

Objective 2: Determine cultured cell functions *in vitro*

Different morphological cell types observed in the *in vivo* response appear to have distinct functions. The epithelial cells migrate together to create the new epithelium, and the migratory cells in the wound cavity form a network underneath the wound.

Hypothesis 2: Cells in the wound cell primary cultures display similar ***functions*** to those observed *in vivo.*

Materials and Methods

Cell Cultures

Oral lobe segments, measuring 5mm x 5mm, were removed from intact *Mnemiopsis* and placed into a sterile 1.5 mL microcentrifuge tube. Lobes were then homogenized for 5 minutes using a sterilized plastic mini homogenizer used to break the tissue up in a mortar and pestle like fashion. Homogenized tissue was centrifuged at 14,000 rpm (16,000g) in an Eppendorf 5415C microcentrifuge (Eppendorf North America, Hauppauge, NY) for 15 minutes to ensure separation of solid and liquid phases.

The solid phase, consisting of cell debris, was spread out into a 35mm plastic Petri dish using ethanol-treated coverslips and allowed to air dry. The liquid phase consisted of a ~ 1:1 ratio of sea water to interstitial fluid, and contains a 1 μg/ml final concentration solution of penicillin-streptomycin which was added to inhibit bacterial growth. Small (~1mm) strips of aggregated wound cells were excised 15 minutes post-wounding and placed onto the dried cellular substrate. 500 μL of the liquid phase was then added to create conditions mimicking the natural wound environment. This volume was used because it was just enough to completely

cover the dried substrate. Cultures were incubated at 4 ºC, as animals used for culturing were collected from cold water and stored in a cold room at 10 ºC, and monitored closely for a week.

Images of cultured cells were collected at 48, 72, 96 and 120 hours using a Zeiss Axiovert 135 inverted microscope set up for Hoffman Modulation Contrast and Phase Contrast Microscopy using a 20X, 0.5 n.a. long working distance objective. Pictures were taken with a digital color CCD camera (Micropublisher 3.3, QImaging Corp, Vancouver, BC, Canada) linked to a Windows XP Dell computer, and processed using digital imaging software (Image Pro Plus 5.1, Media Cybernetics, Bethesda, MD, USA).

Results

Progression of the cell cultures:

All images, graphs, tables and statistical values were obtained from four successful wound cell cultures out of 8 attempts. These cultures displayed similar growth, cell numbers, and cell interactions (described in detail below), and the time progression of the cultures is discussed in this section. At 24 hrs post plating (PP) no cell types could be seen on the culture substrate. The pieces of wound tissue appeared intact, and no cell migration or accumulation could be observed. At 48 PP, multipolar and bipolar cell types began to associate with, but without touching, the spherical cells. By 72 PP, multipolar cells could be seen to be buried between numerous small groups of spherical cells (Fig. 20A). These groups of cells were still small and isolated, forming only three small groups of cells, consisting of 18.75 ± 6.5 cells.

By 96 PP, the cells formed masses of what appeared to be organized tissue, although the masses did not look like native ctenophore tissues. At 96 PP, many cells displayed interconnectivity, having large clusters of spherical cells linked together by long fibrous

extensions (Fig. 20B). This interconnectivity resembles the multipolar cells that migrate to the wound site, aggregate, and span the wound gap during the *in vivo* repair response. Ninety-six hour cultures, when the number of distinguishable morphologies increased, will be discussed in greater detail in the next section. By 120 PP cells began to form large aggregates that resembled organized tissue, consisting of a rudimentary epithelial layer and possible underlying muscle fibers (Fig. 20C). Individual cells within the cell aggregates could no longer be discerned and individual cell morphologies seen earlier in culture were only visible at the edge of the tissue mass.

Wound cell cultures survived ~14 days, but the culture became homeostatic around day 7. Nothing was added to the culture during the two-week span (i.e. more interstitial fluid, growth factors, nutrients), and cell-cell interactions were recorded as they occurred without alteration to the culture environment. To determine the importance of the ctenophore liquid overlay, a wound culture was prepared using filtered seawater (no interstitial fluid) as an overlay. Wound cells introduced into the seawater overlay culture displayed no migration or difference in morphology, and shrank, displaying a spherical appearance within a few hours of plating. No additional change could be observed at 24 hours, and the culture was photographed and terminated at 48 hours post plating (Fig. 19).

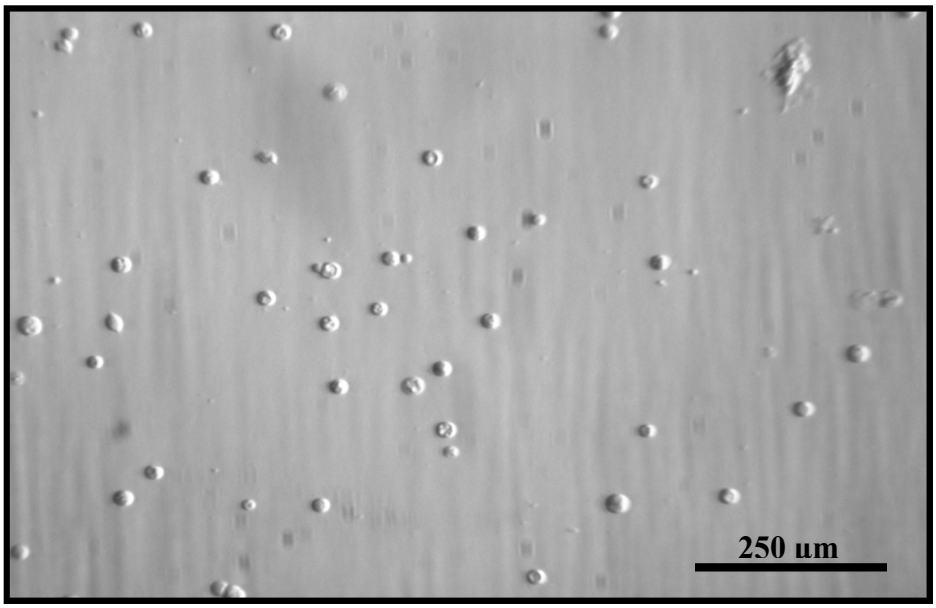

Figure 19: Ctenophore wound culture overlayered with seawater.

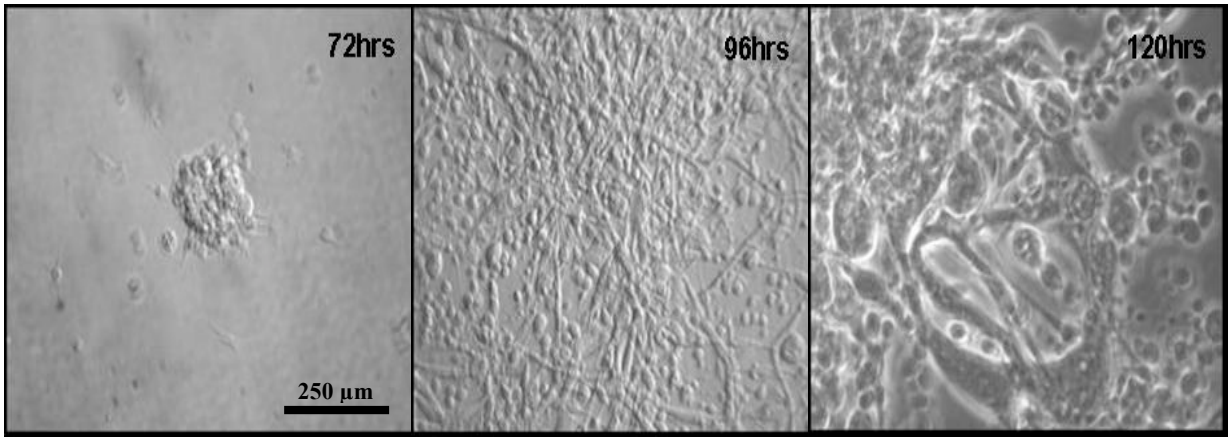

Figure 20: Hoffman Modulation Contrast images of cell cultures at A) 72hrs., B) 96hrs., and Phase contrast at C) 120hrs.

Cell Morphologies and possible functions at 96 hrs:

Several distinct cellular morphologies were observed in culture. The distinct morphologies are most obvious at the 72-96 PP stage, although some can be observed at earlier time points. This appears to be the most active time for the cultured cells, and perhaps the best timeframe to determine their possible *in vitro* functions. Whether these morphologies each

represent different functional cell types, or different stages in what is a highly active and constantly changing population of cells, is unclear. 5 morphological cell types were observed *in vitro*. They are 1) spherical, 2) spindle, 3) bipolar, 4) stellate, and 5) multipolar (Fig. 21).

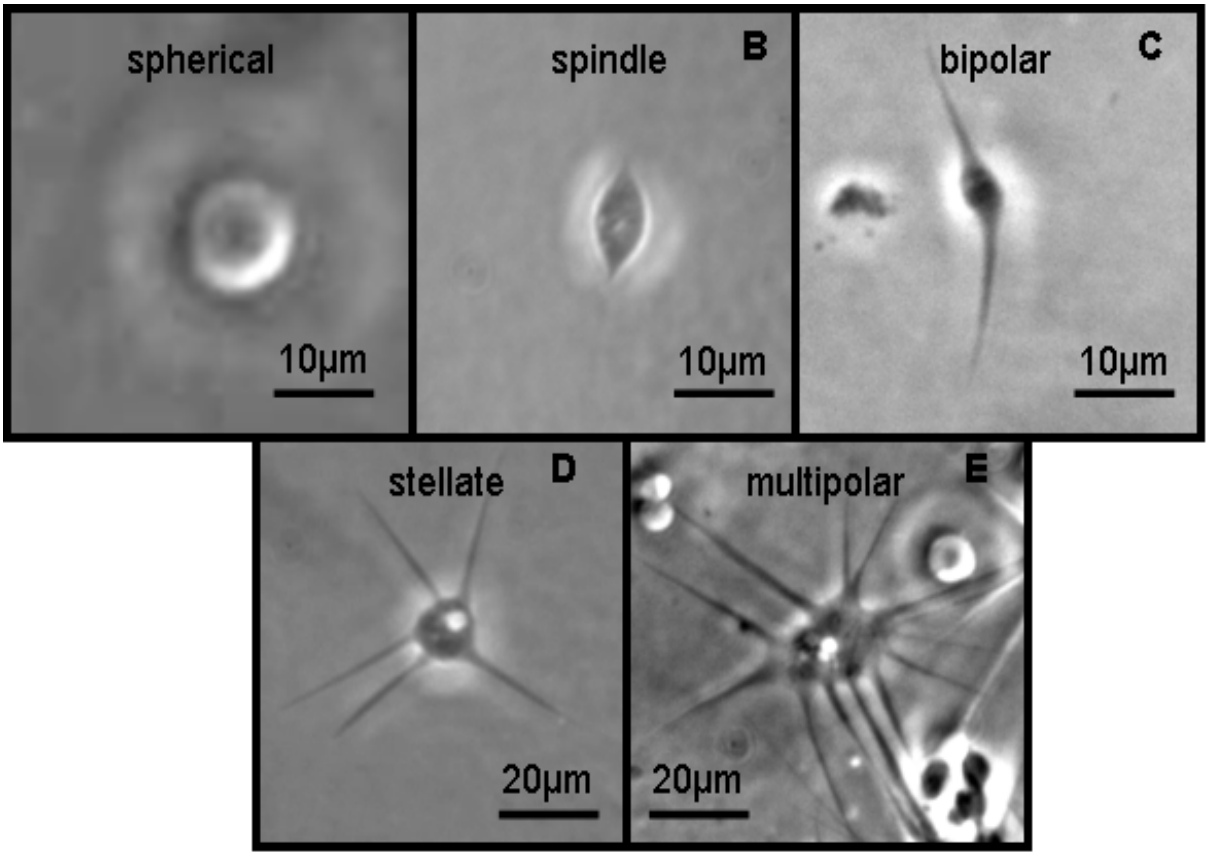

Figure 21: Cell morphologies observed *in vitro*

Four of the *in vitro* morphologies were observed during the *in vivo* wound repair response (Fig. 22). The possible *in vivo* function of these cell types was discussed in detail earlier in this thesis (see Chapter 2).

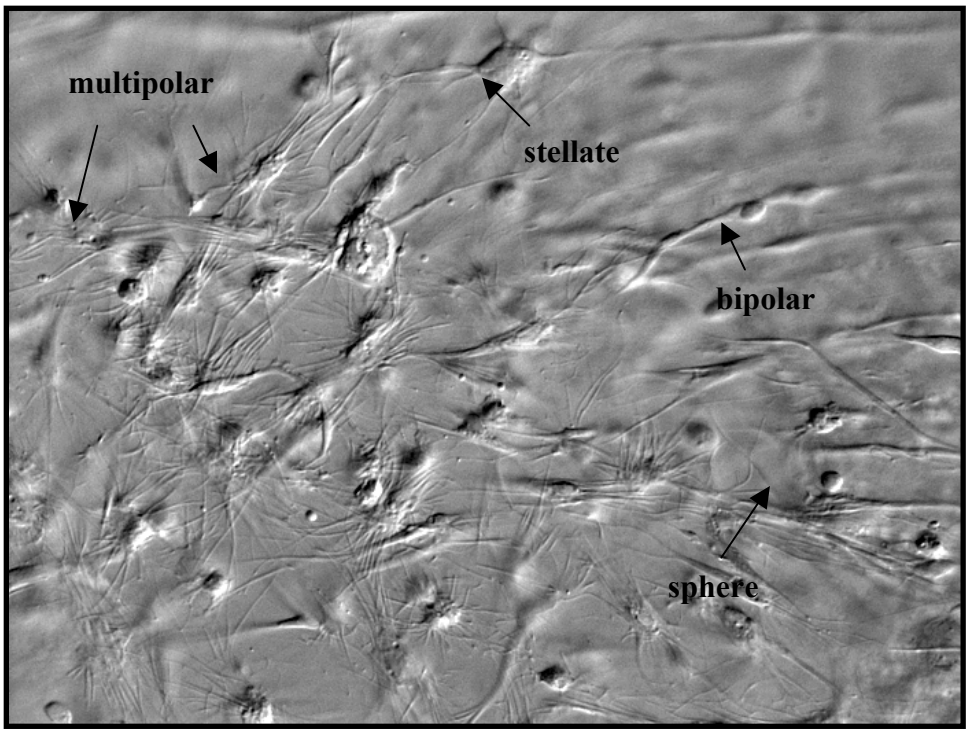

Figure 22: Cell morphologies observed *in vivo*.

The first morphological cell type is the *Spherical Cell* (SC). SCs are the most prevalent cell type *in vitro*, comprising about 54% of the total population at 96 hours. SCs are small, averaging 14.75 ± 4.43 μm (n=100) across the cell body, and have no cellular processes. They are typically found in large groups, and appear to be brought together by the multipolar cells described below.

The second morphological cell type is the *Spindle Cell* (SpC), which display distinctly tapered ends. These cells comprise about 8% of the total population *in vitro* and are slightly larger than the spherical cell type, averaging 30.5 ± 9.02 μm (n=25) across the cell body. Spindle cells are typically found in close proximity but not attached to large masses of spherical cells. Time-lapse imaging reveals that the spindle cells stay anchored in place while other cell types move around them.

The third morphology is the *Bipolar Cell* (BC), which makes up 10% of the total population *in vitro*. BCs average 20.73 ± 4.93 µm (n=25) across the length of the cell body, and 65.45 ± 14.22 µm including the two cellular processes. They move in a directional manner, extending a single lamellipodium, while the opposing tailing process remains anchored. The cells move through the culture at a rate of 12.44 ± 4.07 µm minute^{-1}. The process being extended forward has a more condensed appearance than its anchored counterpart, and pulls the cell forward in an "inch worm" like manner. In the early stages of culturing, between 48 and 72 hours, bipolar cells can be observed moving around the culture environment by themselves (Fig 23A). However, by 96 PP they link together at the edges of large SC aggregates (Fig. 24B).

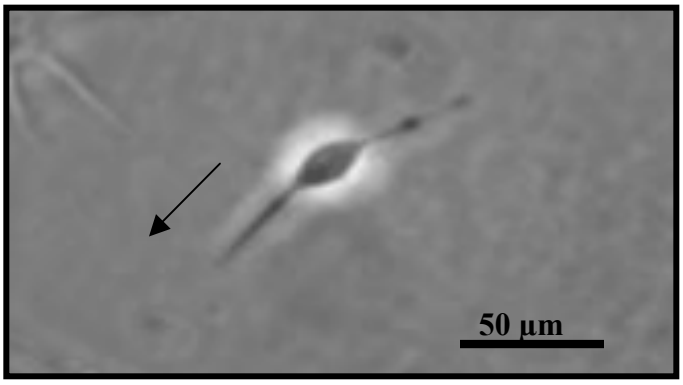

Figure 23A: Individual cultured BC. Arrow indicates direction of cell movement 72 PP.

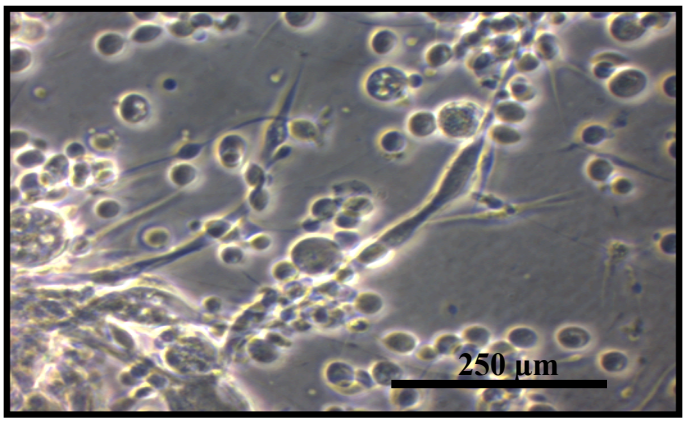

Figure 23B: Linked and extended cultured BPs 96 PP.

The fourth morphological type is the *Stellate cell* (StC). StCs account for 10% of the population. They average 18.25 ± 5.2 µm (n=25) across the cell body and measure 55.75 ± 13.89 µm (n=25) including their 5 distinct pseudopodia. This cell type exhibits a wide range of motion, moving in any direction by extending two processes in the direction of movement while the other three act as anchors (Fig. 24). StCs actively migrate throughout the culture moving at 21.14 ± 4.41 µm minute^{-1} (n=5).

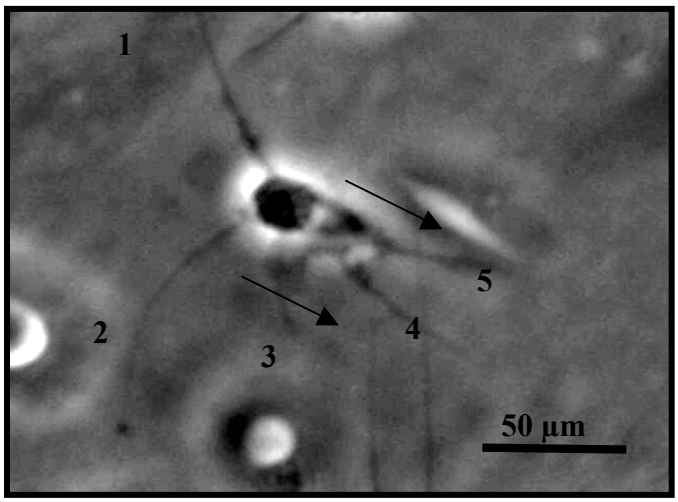

Figure 24: Phase image of a StC moving in culture 96 PP.

The fifth morphological cell type is the *Multipolar cell* (MC). This morphology represents roughly 9% of the total population of cells. It has the largest cell body, averaging 31.88 ± 9.19 µm (n=25) across the cell body, and also has numerous cellular processes that increase the overall cell size to 73.75 ± 15.62 µm (n=25) in total size. These cell types are very similar in morphology to the StC cell type, but differ on a few reliable and distinct morphological attributes. Firstly, they typically have 6-12 cellular pseudopodia, while StCs always have five. Secondly, they are broad and flattened, and closely apposed to the substrate with a much less dense-appearing cytoplasm.

Multipolar cells are very motile, but do not move directionally like SCs and BCs. MCs move in a distinct manner that appears to be tightly coupled to the function of these cells in the culture environment. MCs often aggregate in groups of 5 to 50 cells. MCs stack of top of each other and appear to stick to one another, making exact determination of cell number difficult. Large aggregates of MCs then accumulate SCs (Fig. 25). MC processes are important for SC accumulation, as their extended lamellipodia can act as an anchor to collect SCs. MC movement has no apparent pattern, until SC collection has occurred, where they then move vectorially to other SC cell aggregates.

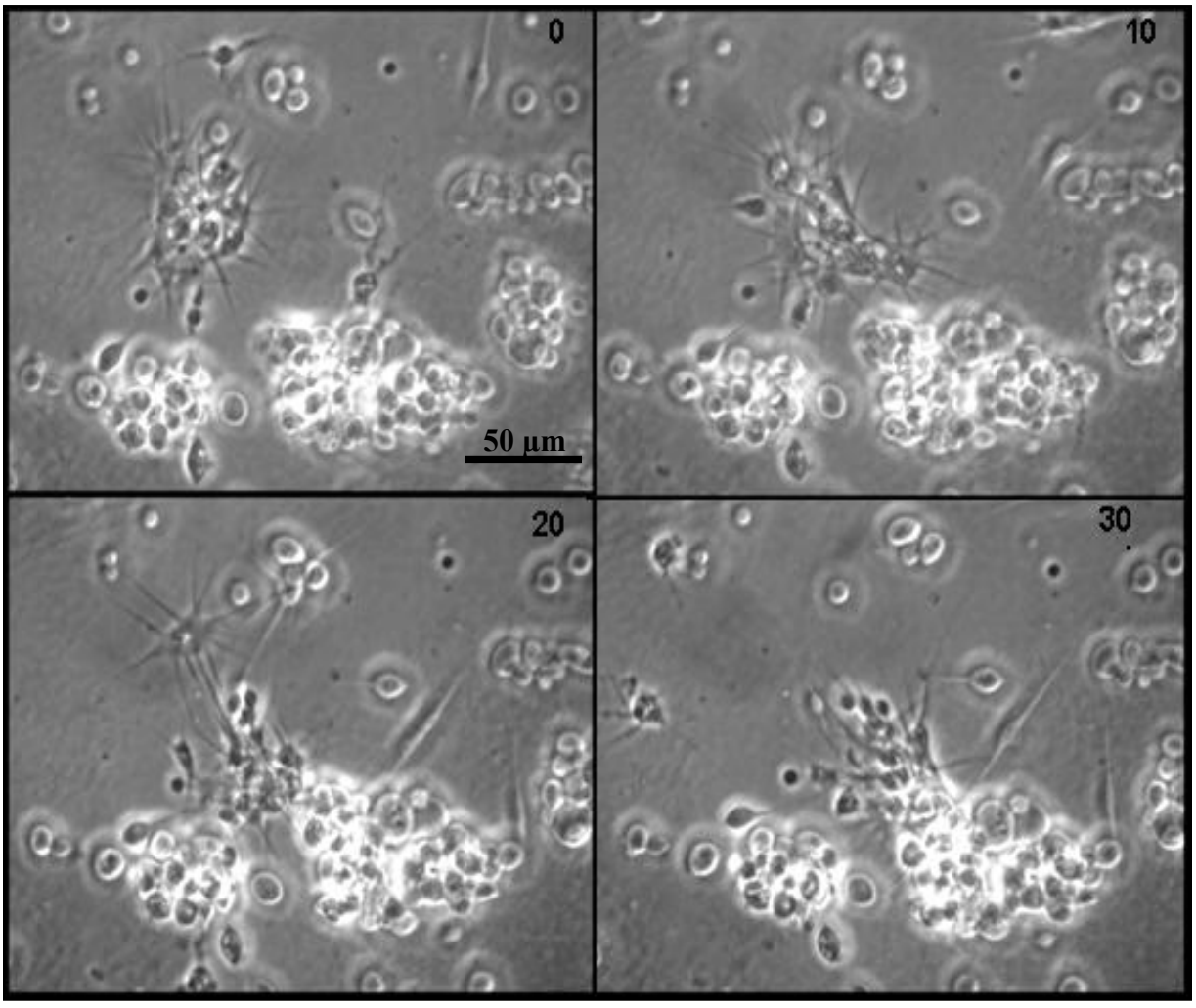

Figure 25: Phase image of MCs collecting SCs *in vitro.*

Three other morphologies occur outside of the previously described morphological cell types. I call this group *Transitional Cell* (TC) types, which I have named unipolar (U), tripolar (Tp) and quadripolar (Qp) morphologies, because they could not be placed in any of the other five morphological categories. They make up a small portion of the total population, at 9% for all three morphologies, and are not consistently associated with any of the other cell types. Number of cells counted (Table 1), percentage of each cell type (Fig. 26), cell size (Table 2) and histograms showing distribution of cell size are all summarized below (Figs. 27 & 28).

Morphology	**Number of cells**	**Percent of total population**
Spherical	153	53%
Spindle	25	9%
Bipolar	28	10%
Transitional*	26	9%
Stellate	28	10%
Multipolar	26	9%

Table 1: Number of cells counted for percentage of each cell type *in vitro*.

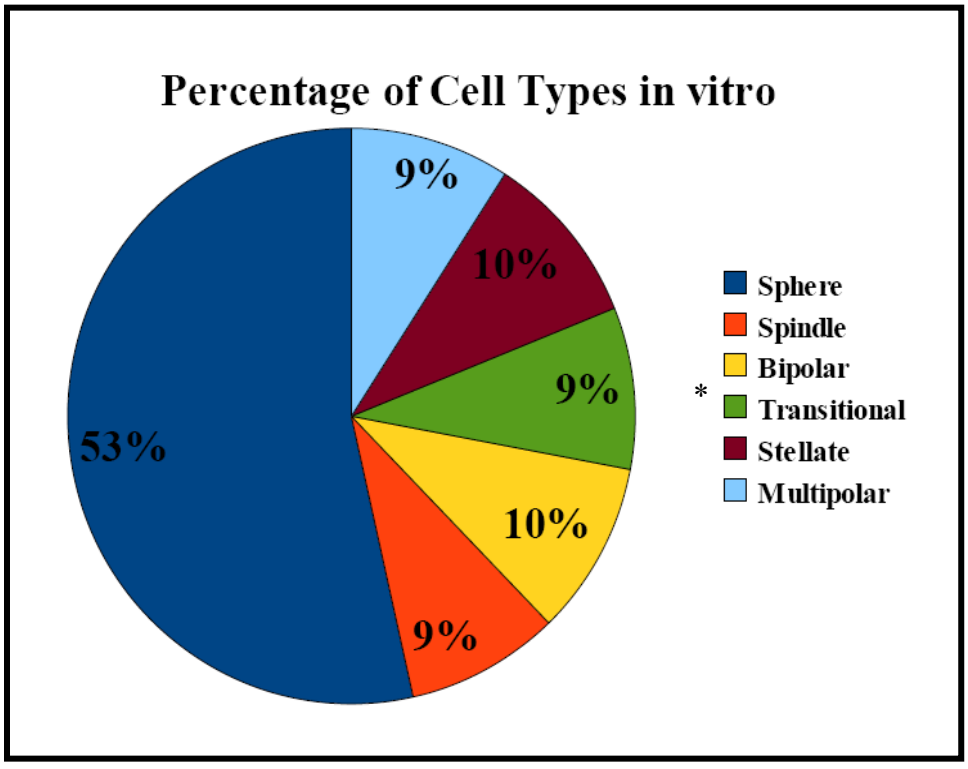

Figure 26: Pie chart showing percentage of cell morphologies present at 96 hr culture stage.
*Transitional morphology includes unipolar, tripolar and quadripolar.

Sample	# of Cells Counted	Cell size (avg.)	Standard Deviation
Sphere	100	14.75	4.43
Multipolar (Body)	25	31.88	9.19
Multipolar (Processes)	25	73.75	15.62
Stellate (Body)	25	18.25	5.2
Stellate (Processes)	25	55.75	13.89
Spindle	25	30.5	9.02
Bipolar (Body)	25	20.73	4.93
Bipolar (Processes)	25	65.45	14.22

Table 2: Cell size across the cell body and the whole cell including processes (when applicable).

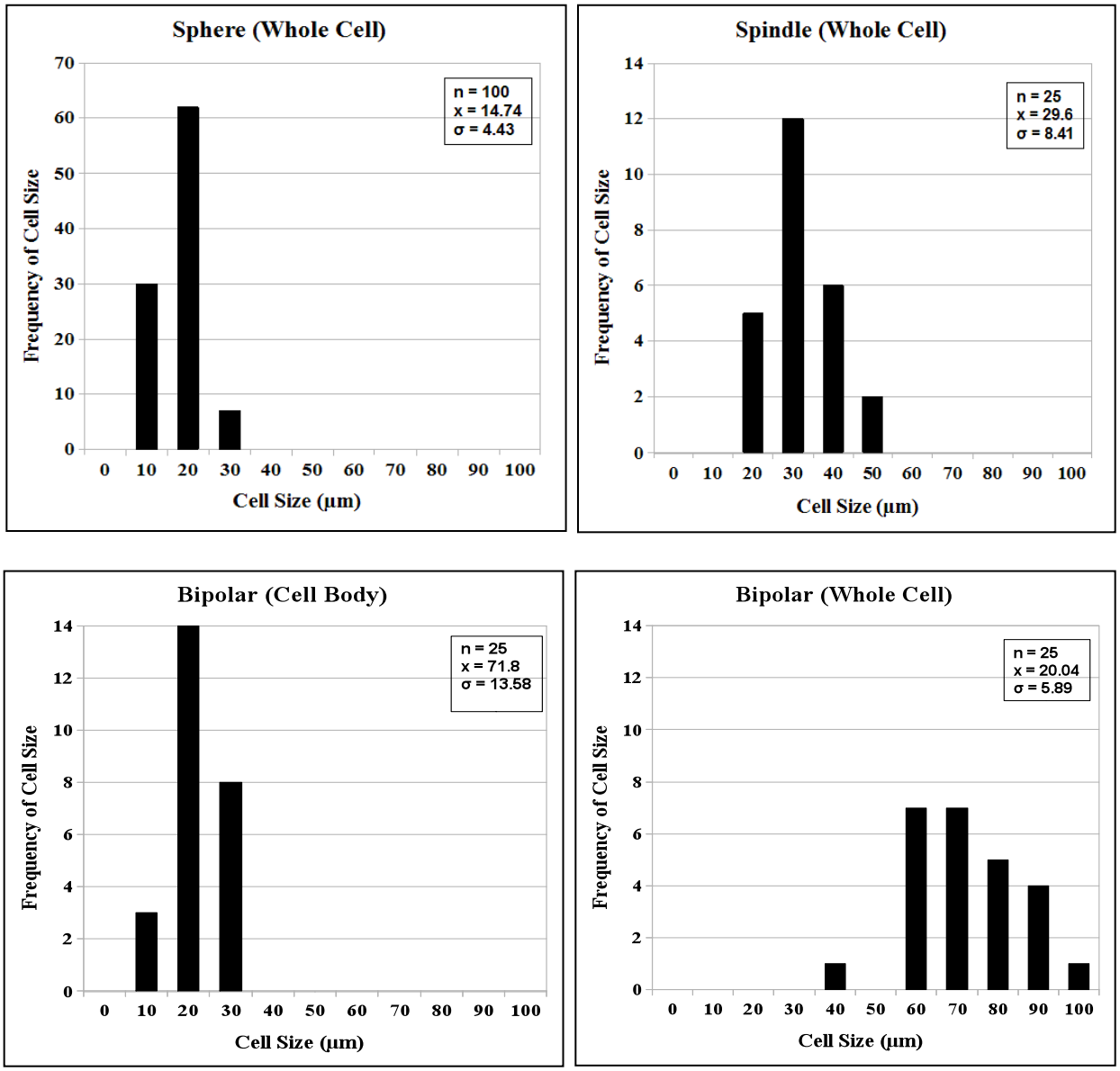

Figure 27: Spherical, Spindle and Bipolar cell sizes and frequency of occurrence.
n = number of cells counted, $\bar{x}$ = sample mean, σ = standard deviation.

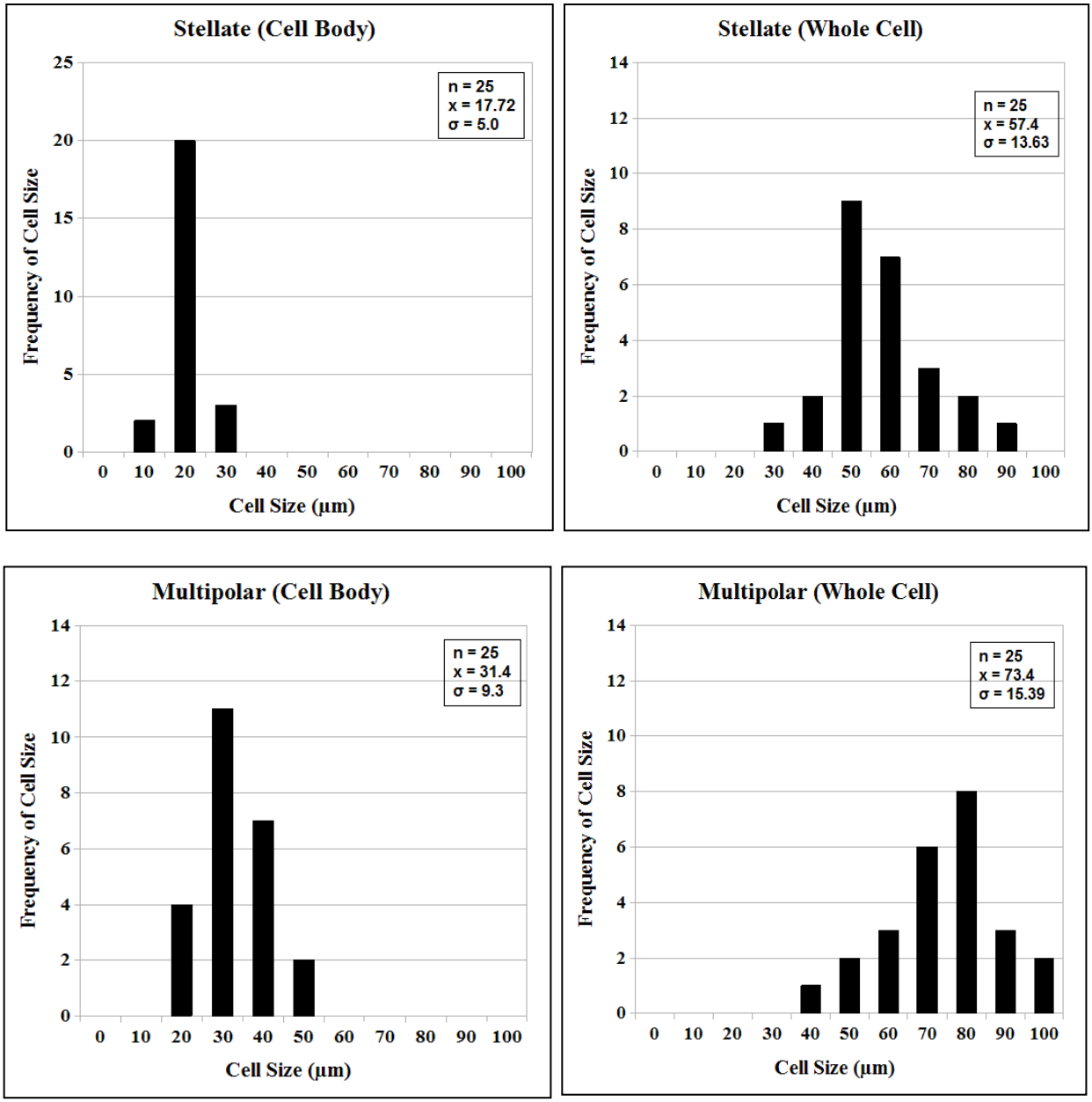

Figure 28: Histograms showing the Stellate and Multipolar cell sizes and how frequently they occur. n = number of cells counted, $\bar{x}$ = sample mean, σ = standard deviation.

Discussion

Wound cells that aggregated at the edge of an epithelial wound in *Mnemiopsis* can be grown in primary cell cultures. The cultures last no more than 2 weeks, with the majority of cellular activity taking place from 72-120 hours post plating (Fig. 29). Cell cultures were

designed using ctenophore tissue to create the culture environment. The dried cellular substrate is made of mesoglea and cell debris, and allows plated wound cells to readily migrate and interact with one another. The liquid overlay, which is made up of interstitial fluid from the cells mixed with residual sea water left over from lobe removal, is integral to cell culture survival. *Wound cell cultures failed to survive when filtered seawater was used as the overlay*.

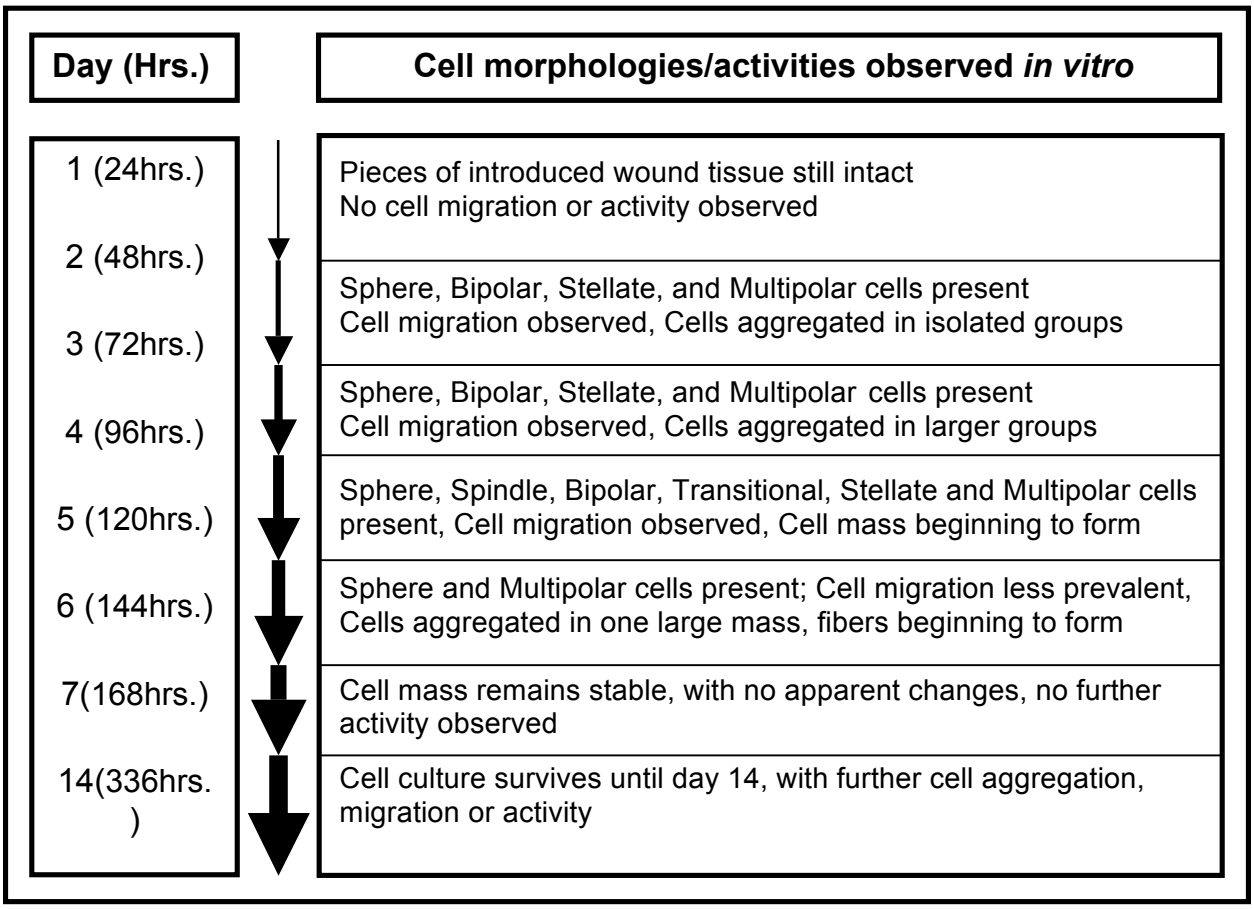

Figure 29: Timeline of cell morphologies and activities observed *in vitro.*

There were five main cell morphologies observed in culture, Sphere (SC), Spindle (SpC), Bipolar (BC), Stellate (StC) and Multipolar (MC). Three other morphologies were present, Unipolar, Tripolar, and Quadripolar, but they were present in low levels as discussed above. I have combined these three morphologies because I believe they represent transitional states between the other cell types, or include cells in which the processes that are unaccounted for are

embedded in the underlying substrate. Out of the five other morphologies, the BC, MC and StC appear the most active, and the SC and SpC appear to be stationary cell types. Here I will discuss what is known about each cell type and elaborate on their possible function.

SC cells lack cellular processes and do not migrate in culture. Instead, this cell type is collected and appears to be a building block for the newly forming cultured tissue. Its *in vitro* function is consistent with the observation that it is the most numerous cell type, consisting of over half of all the cells. It is unclear whether these cells are terminally differentiated, or if they are precursors to BCs, MCs or SpCs, since SCs have not been observed to convert to any other cell type. SpCs bear small cellular processes, and like their morphologically similar counterpart, the SCs, are also stationary. SpCs may be: 1) BCs with their processes embedded and invisible within the underlying substratum or 2) SCs differentiating into BCs. The exact nature and function of the SpC cell type is unclear, but it is a consistently observed morphology.

BC cells have two pseudopods and move in an inchworm-like fashion around in culture. This is one of two cell types that actively migrate *in vitro*, and as described previously resembles a bipolar cell morphology observed *in vivo*. As stated in Chapter 2, BCs clearly link together around 96 hours post plating, forming long fiber-like extensions which closely resemble muscle fibers around a healing epithelial wound. The StC cell type has five distinct pseudopodia, and appears to use two of those processes to pull itself forward while the other processes remain anchored. This is one of the three cell morphologies *in vitro* that can be clearly observed in the *in vivo* response, and is the final migrating cell type found in culture. It often appears to associate with but not touch large groups of MCs. In places where large aggregates are present, StCs appear to remain at the edge of the action, possibly playing an organizational role. StCs may flatten and produce more processes to become the MC cell type, discussed below.

MCs can have up to 12 processes, and are the largest of the culturable cell types. MCs aggregate, forming large masses of cells that move as a group *via* the extension of pseudopodia. Any time masses of MCs were seen, a large group of SCs lay on top. These masses appear to be the basis for the large aggregates of tissue that form 96-120 hours post plating. Accumulation is similar to the behavior seen during an *in vivo* wound response, where the homologous multipolar cell type aggregate underneath the open wound and form a contractile mass of cells that appear to help to bring the wound edges closer together during healing (see Chapter 2).

Conclusions

Ctenophore wound cell cultures allow visualization of cells involved in the *in vivo* wound repair response. The culture system has revealed how some of the mesogloeal cells may normally interact *via* their *in vitro* activities. The observations described here were possible because the culture system allowed the isolation of individual wound cell types from their surrounding tissues. While this allowed a clear view of the activities of the individual cells, generation of a reduced preparation *via* primary cell culture prevented any further signaling from the intact animal. Although this may be interpreted to be a limitation, in fact the investigator can, in a cell culture, more easily examine the effects that pharmacological agents, growth factors, electric currents, or a number of other potential cellular signaling events could have on each of the different cultured cell types, or on the cell masses. The results can be compared to those tested *in vivo*, as well as in invertebrate, mammalian, and other relevant cell lines.

Further development of this culture to produce a sustainable cell line would create a primary cell culture in a marine invertebrate that is both ecologically and evolutionarily relevant (see Chapter 1). The first step needed to improve this culturing system is to increase the length that the culture survives. This wound cell culture is similar to other marine invertebrate cell

cultures in that it does not last longer than 1 month (see Introduction). Increasing the time the culture survives would allow testing of the effects of exogenously applied growth factors on the cultured wound cells. Production of a sustainable wound cell line would allow easy access to another avenue of studying the cell types involved in this rapid repair response.

Chapter 4: Molecular Analyses of Wound Repair

Introduction

Advances in molecular techniques have greatly enhanced our understanding of when genes are expressed or suppressed, and how this regulation of gene expression brings about morphological and functional changes in cellular activity. It can be argued that the reverse transcription polymerase chain reaction (RT-PCR) is one of the leading tools of the ongoing development of cell and molecular biology. Reverse transcriptase, the enzyme upon which RT-PCR is dependent, is an RNA dependent DNA polymerase that was originally discovered to be encoded by retroviruses, which have an RNA genome (Baltimore, 1970; Temin and Mizutani, 1970). This enzyme can be paired with conventional PCR to provide a look at the expression of specific genes. RT-PCR involves a reversal of the direction of flow of genetic information through the reversed transcription of mRNA sequence into DNA. The DNA product of RT-PCR is called complementary, or cDNA, because it is generated as a result of complementary base pairing to the mature, processed mRNA product found in the cytoplasm.

RT-PCR allows the investigator to examine gene expression at set time points in a cell life cycle. Just like the original polymerase chain reaction i.e., PCR (Saiki et al., 1985), target genes are amplified using specific primer sets specific to a unique sequence within the targeted gene. With this technique, researchers can target and amplify single genes or fragments of a gene out of an entire genome. I have used RT-PCR to provide a first look at gene expression during wound repair in *Mnemiopsis leidyi*.

The common marine comb jelly *Mnemiopsis leidyi* can heal small ectodermal epithelial wounds in as little as thirty minutes with no scarring. Several distinct morphological changes are observed in the cells that migrate to the site of repair. There are some common wound repair players involved in this response. Understanding the morphological and molecular expression changes that the cells undergo at the molecular level could provide insight into the rapid, scar-free nature of this healing mechanism.

Description and reasoning behind EST target choice:

ADF and Arp 2/3

It has long been demonstrated that actin and actin binding proteins (ABPs) play an integral role in the motility of eukaryotic cells. One ABP pair, 18.5 kDa actin depolymerizing factor (ADF) protein works together with (the 21 kDa protein) cofilin to regulate the assembly and disassembly of actin filaments. ADF and cofilin bind to G-actin monomers, and mediate the depolymerization of actin filaments through two mechanisms: 1) severing (Pollard and Borisy, 2003) or 2) increasing the rate of dissociation at the pointed (minus) end (Carlier et al., 1997). The interaction between these two proteins is in part responsible for treadmilling in actin filaments, where they tend to rapidly polymerize the actin filament at the barbed end while depolymerizing at the opposite or 'pointed' end (dos Remedios et al., 2003). This ability to rapidly assemble and disassemble actin filaments accounts for the ability of many cell types to actively respond and migrate when signaled during processes such as neuronal outgrowth, phagocytosis, endocytosis, receptor signaling, morphogenesis and of course, wound repair (Van Troys et al., 2008).

The basis behind actin-based movement in many migratory cell types lies in the formation of flat lamellipodia, which are protrusions of the membrane created via actin

polymerization. One model involves the formation of an ABP complex in which cofilin, ADF and Arp 2/3, a 220 kDa complex of 7 proteins that bind and nucleate actin polymerization, drive the formation of actin filaments which underlie lamellipodia in invasive cells (Andrianantoandro and Pollard, 2006). While the exact mechanisms remain unknown, the importance of ADF/cofilin in mediating cell migration is certain.

ADF and cofilin are both highly conserved proteins, and contain 30-40% amino acid homology from vertebrates to protists, for example in swine (*Sus domesticus*), *Xenopus*, *Caenorhabditis*, *Acanthomoeba*, *Dictyostelium*, and *Toxoplasma* (dos Remedios et al., 2003). Multipolar, stellate, and bipolar wound repair cells in *Mnemiopsis* actively migrate toward and into the wound site (see Chapter 3). Actin staining, discussed in Chapter 2, reveals much actin in the wound cells. This evidence supports the possible presence of an actin filament turnover mechanism responsible for actin based migration of wound cells in *Mnemiopsis*. This places both of these proteins as possible important members of the healing cascade in *Mnemiopsis* at both the evolutionary and functional level.

Apoptosis Inhibitor 1

Apoptosis, or programmed cell death, plays an important role in the regulation of numerous cellular processes such as development, the immune response, and wound repair (Greenhalgh, 1998; Sang and Blecha, 2009; Vaux and Korsmeyer, 1999). Apoptosis is mediated by caspases, which are inhibited by proteins found in both vertebrates and invertebrates known as inhibitors of apoptosis (IAPs) (reviewed in (Verhagen et al., 2001). Numerous IAPs have been identified and are known to actively regulate apoptosis in ways that directly affect wound repair (Rai et al., 2005).

During mammalian corneal wound healing, IAPs are key initiators and mediators of the proliferation and migration of active corneal keratocytes (Wilson et al., 2007). PFT-α, an IAP associated with the inhibition of p53 (a tumor suppressor gene), has been shown to accelerate epithelialization and blood vessel formation during mammalian cutaneous wound repair (Vollmar et al., 2002). In invertebrates, IAPs have been identified to mediate apoptosis in a number of organisms (Deveraux and Reed, 1999). These include but are not limited to: 1) regeneration in planarians (Hwang et al., 2004), 2) the immune response in mosquitos (Christophides, 2002), and 3) early development in nematodes and fruit flies (Meier et al., 2000). It is clear that apoptosis, and its inhibitors, play an important role in regulating a host of cellular processes and could be key modulators of wound repair in the ctenophore.

Hsp 60

Heat shock proteins (Hsps) are highly conserved proteins whose induction was first reported in response to elevated temperatures by *Drosophila* (Ritossa 1962). Hsps are found in virtually all organisms, and are known to be synthesized in response to elevated temperatures and a variety of other forms of stress (reviewed in Lindquist 1988). Hsps are classified based on their molecular weight in kilodaltons, and Hsp 27, 60, 70 and 90 in particular have been shown to be expressed in the healing epidermis of wounded mice (Laplante et al., 1998). This research revealed that while Hsps were expressed constitutively in normal unwounded epidermis, they were subsequently modified during the wound repair response. It also showed that the different Hsps were expressed in different epithelial cell types in the wound, showing a level of specificity to the action of these proteins in wounded tissues. Hsp 60 was found in the basal most cell types, the basal and suprabasal cells, Hsp 90 and Hsp 27 were found in the higher suprabasal cells, and Hsp 70 was found throughout the regenerating epidermis.

Other evidence has also indicated an important role for heat shock proteins in wound repair. The addition of exogenous Hsp 70 to epidermal wounds in mice was shown to enhance the rate of wound closure by 60% when compared to control mice (Kovalchin et al., 2006). Hsp 70 also plays an integral role in the proper healing of damaged gastric mucosal cells, as shown by the enhancement of gastric healing in the presence of gastric cells over expressing Hsp70 (Odashima et al., 2002; Odashima et al., 2007; Tsukimi et al., 2001). The smaller heat shock proteins have been implicated in wound healing as well. Hsp 27 has recently been shown to enhance cell migration, focal adhesion, and invasiveness of mouse fibroblasts by mediating ECM degradation via MMP-2 and cell interaction with the actin cytoskeleton (Lee et al., 2008). The involvement of Hsps in a variety of aspects of wound repair made Hsp 60 and Hsp 90 obvious choices to look at changes in expression.

Innexin

One way that cells communicate with one another is through the formation of hexameric protein channels called gap junctions. In vertebrates, the hexameric proteins are termed connexins, and approximately 20 variants have been discovered and described in mammals, fish, amphibians and chickens (Phelan and Starich, 2001). Connexins are not found in invertebrate systems; however, gap junctions are comprised of a functionally and structurally similar, yet genetically distinct set of proteins called innexins. In the popular genetic models *Caenorhabditis elegans* and *Drosophila melanogaster* there are currently 25 and 8 known innexins respectively, with homologs from other invertebrates such as the grasshopper, polychaete, the medicinal leech, and hydra being recently discovered (Alexopoulos et al., 2004; Phelan, 2005). Additional members of the gap junction protein family are referred to as pannexins, which are innexin homologues that are found in vertebrates. Currently three homologs have been identified in

human, mouse, rat and zebrafish models (Phelan, 2005). Connexins and pannexins play a crucial role in regulating a host of vertebrate cellular functions (D'Hondt et al., 2009), but my discussion will focus primarily on the role of innexins in invertebrate cell-cell communications and stabilizing cell-cell interactions.

Intercellular communication via innexins appears to play a key role in a number of developmental processes. For example, innexin 2 is involved in foregut and epithelial morphogenesis, and innexin 4 is involved in germ cell differentiation in *Drosophila* (Bauer et al., 2002; Bauer et al., 2005). Mutations in these genes results in developmental defects in *Drosophila* embryos. In addition, innexins are transcriptionally regulated by the WNT signaling pathway (reviewed in (Bauer et al., 2005).

Both of these interactions indicate a possible role for innexins in the proper orientation and communication of polarized epithelial cells. Innexins also play a role in proper orientation of the blastema in regenerating planarians, mediating gap junction signaling in blastemal cells. This importance was proven by disrupting gap junctions in regenerating planarians *via* heptanol, which resulted in a number of defects (i.e. no tail, two heads) during regeneration (Nogi and Levin, 2005). Based on research indicating innexins may be conserved across the animal kingdom (Panchina et al. 2000), and the role the important role that innexins play in proper cell orientation during development and regeneration may indicate a key role for these invertebrate intercellular communication genes. The expression of an innexin homologue during the wound response in *Mnemiopsis* would suggest a role for these gap junction proteins in maintaining proper polarity of the wound cells forming the new epithelium. Gap junctions would allow the cell-cell communication needed to coordinate the rapid changes that occur at the wound.

Selenoprotein

Selenoproteins are a group of proteins that account for the majority of circulating selenium in the body in the form of selenocystene amino acids. There are 25 selenoproteins identified in the human genome, and they portray a variety of functions, most common of which is the reduction of oxygen free radicals (Moghadaszadeh and Beggs, 2006). Out of the 25 known selenoproteins, selenoprotein P and selenoprotein S have both been shown to play a role in inflammation, and thus have a possible role in ctenophore wound repair.

Selenoprotein P was first discovered in 1977 in rat blood plasma (Herrman, 1977), and accounts for more than 50% of circulating selenium in rat and human plasma (Deagen et al., 1993). This class of proteins is negatively regulated by inflammatory cytokines such as TGF-ß, IFN-γ, TNF-α and IL-1ß (Mostert, 2000). This indicates a possible role for Selenoprotein S in affecting inflammation, which in turn suggests a regulatory role during wound repair.

Selenoprotein S is normally associated with the membrane of the endoplasmic reticulum, and is responsible for mediating proteasome-based degradation of misfolded proteins (Ye et al., 2004). This protein, like selenoprotein P, plays a regulatory role during inflammation. Suppression of selenoprotein S expression via RNAi results in an increase in the expression of proinflammatory cytokines IL-6 and TNF-α (Curran and al, 2005). This suggests a possible role for both selenoprotein P, and selenoprotein S in influencing inflammation during a wound repair response.

TGF-ß

Transforming Growth Factor-ß (TGF- ß) is an important cytokine found in the process of wound repair across the animal kingdom. TGF- ß interaction with its corresponding receptor

results in the phosphorylation of Smad proteins[33], which act as transcriptional regulators of a host of cellular responses (Massague 2000). Smad proteins can also interact with downstream components of other signaling pathways, indicating a diverse role of this signaling pathway (Attisano and Lee-Hoeflich, 2001). TGF-ß is involved in the regulation of keratinocyte migration during re-epithelialization, stimulation of angiogenesis, proliferation of fibroblasts, myofibroblast differentiation, as well as replacing needed components of the extracellular matrix (Werner 2003).

The TGF- ß superfamily is seen throughout Kingdom Animalia, and the conserved structure and sequence homology present among the different subtypes has allowed the identification of members in both vertebrate and invertebrate organisms (Herpin et al., 2004). This includes the discovery that Decapentaplegic (Dpp) in *Drosophila* portrays a high sequence homology to vertebrate TGF- ß subtypes, which suggests that the origin of this superfamily occurred in an early invertebrate system (Herpin et al., 2004; Padgett et al., 1987). Due to recent research indicating the ctenophore as a possible common ancestor to all metazoans (Dunn et al., 2008), discovery of a TGF- ß homolog in the ctenophore wound response could identify one of the earliest examples of this growth factor superfamily.

Objectives and Hypotheses

Objective 1: Determine how gene expression changes during the wound response

Damage to the epithelium initiates a stepwise series of events that work together to repair the wound. Standardized epithelial wounds made in the lab measured ~3mm in diameter and healed in ~30 minutes. In a 30 minute healing response, time 0, 15 minutes, and 30 minutes

[33] Homologs of *Caenorhabditis* SMA proteins, and *Drosophila* 'mothers against decapentaplegic' (MAD) proteins. Transcriptional regulators of TGF-ß activity.

represent major time points. Wound cells removed from the wound site at 15 minutes should represent a midpoint in repair, and 30 minutes a near completion time point. Gene expression at these times can then be compared to the time 0 controls.

Hypothesis 1: Gene expression of chosen targets will differ at time 0, 15 and 30 minutes post-wounding.

Materials and Methods

Collection of wound tissue

Incisions measuring ~3 mm in diameter were made to the epithelium using iridectomy scissors as previously described. Pieces of non-wounded epithelium were excised and pipetted using a 3.0 mL disposable pipette within 15 seconds into a sterile 1.5 mL microcentrifuge tube containing 250 µl of RNA Later for preservation of mRNA (Ambion, Applied Biosystems, CA). Strips of wounded tissue from the wound edge were removed at 15 and 30 minutes and placed into 250 µl of RNA later. Samples were stored at 4 ºC.

Treatment for RNAase contamination

All micropipette tips and microcentrifuge tubes were autoclaved and the 3.0 mL disposable pipettes, and iridectomy scissors were treated with RNase Zap (Ambion, Applied Biosystems, CA) to prevent RNAase contamination of the samples.

RNA extraction

Samples stored in RNA Later were centrifuged at 14,000 rpm (16,000g) for 5 minutes to pellet cellular debris at the bottom of the tube. The supernatant, containing RNA Later solution containing aqueous RNA, was removed by pipetting with RNAase-free pipette tips, and RNA was extracted using the protocol and materials included in the PrepEase RNA Spin Kit (USB

Corporation, OH). Final extracted mRNA was eluted into ultrapure water and stored at -25ºC until processed for Reverse Transcription PCR.

Primer Design

The NCBI database was queried for all available ctenophore sequences. The query yielded 972 hits, consisting of 960 EST sequences and 12 nucleotide sequences. The available sequences were then investigated to identify sequences homologous to gene sequences that might be involved in wound repair in other organisms. The majority of relevant sequences were expressed sequence tags obtained from *Mnemiopsis leidyi* larvae (Kevin Pang and Mark Martindale). Forward and reverse primers were designed for each gene target by entering the sequence information into Integrated DNA Technologies Primer Quest primer design tool, and one pre-existing *wnt* primer set was also used (Table 3).

RT-PCR

Reverse Transcription PCR used the USB One Step RT-PCR kit (USB Corporation, OH). Reactions were set up according to the recommended protocol, except that reaction volumes were halved to produce a 25 µl final reaction volume. Primers were used at a final concentration of 0.4 µM. The volume of extracted RNA added to the RT-PCR reaction was adjusted to provide a final concentration within 1 ng to 1 µg, in the accepted range of the protocol. Amplification conditions were optimized for an ideal annealing temperature for the respective primer sets (provided by IDT Primer Quest), and were as follows: reverse transcriptase production of the first ssDNA product at 45 ºC for 30 min., initial denaturation at 95 ºC for 3 min., then 40 cycles of Taq-based amplification as follows: (denaturation at 95 ºC for 30 s, re-annealing at 60 ºC for 30 s, elongation at 72 ºC for 1 min.), followed by a final elongation step of 72 ºC for 3 min., and dropped to a holding temperature of 6 ºC.

Agarose gel of cDNA amplicons

Amplicons were examined for yield and compared to a wide range molecular weight ladder (All Purpose HiLo Ladder, Bionexus Inc, CA) by electrophoresis through 1% agarose (BP-160, Fisher Scientific) gels run for 30 minutes at 95 V and viewed on a UV transilluminator. Gels were stained by adding 1 µl ethidium bromide incorporated into the 25 mL gel before it solidified. Gels were then imaged with a high performance CCD camera (COHU, CA) and analyzed using GelPro Plus (Media Cybernetics, MD).

Further amplification of cDNA amplicons

Due to low initial yields, RT-PCR amplicons were always further amplified to obtain stronger bands. This eliminated the ability to determine expression, but allowed the detection of products present in low amounts. 25 µl PCR reactions were set up as follows: 12.5 µl GoTaq Green Master Mix (1X final, Promega, Madison, WI), 1.0 µl forward primer (0.4 µM final), 1.0 µl reverse primer (0.4µM final), 0.5 µl $MgCl_2$ (0.5 mM final), 1.0 µl RT-PCR amplicons, 9.0 µl ultrapure water. Products were amplified as follows: 95 ºC for 3 min., then 32 cycles of 95 ºC for 30 s, 60 ºC for 30 s, 72 ºC for 1 min., then 72 ºC for 3 min., and finally held at 6 ºC. Samples were run on an agarose gel as described above.

Primer target (NCBI accession #)	**Primer sequence (5'-3')**
Arp 2/3 complex **(CF931440)**	Forward – AGTTTCTCAGACGAAGTTTTCGTTA Reverse - CTTCTGTAGCTTTACAATGCAGTCA
HSP60 **(CF925354)**	Forward – TTGTTGAGAGAACTATGAGGGAATC Reverse – AAAATCTAGAGCARCACCARWA
Innexin **(CF925368)**	Forward – AGTACATATTCCTCATCCTCTGGTG Reverse – CCAGCTCAAACAAAACCTCTGATA
Selenoprotein S **(CF925396)**	Forward – TCCTGTACTGACTAACAACAACCAG Reverse – ACCCCAGAAAGTAGAGAAGAAGTGT
TGF-ß nuclear protein **(CF931336)**	Forward – AGTTAATGTTTCAGAGTTGGGACTG Reverse – CTTTAAAATGCGACTATTTGATGGT
Wnt*	Forward – GGGGAATTCTGYAARTGYGG Reverse – AAAATCTAGAGCARCACCARWA

Table 3: List of forward and reverse sequences of primers used in RT-PCR amplification.*Degenerate primer set used in human thyroid cells (Helmbrecht 2001).

Results

EST targets were chosen based on their role in wound repair in other organisms as described in the introduction section above. A list of successfully amplified targets and a brief description of the general function of each target is provided in Table 4, below. Five ESTs: Arp 2/3, Hsp 60, Innexin, Selenoprotein S, and TGF- ß worked consistently, and were used to provide an initial look at the molecular regulation of repair.

Target	**Functional Significance**
Actin Depolymerizing Factor	Cytoskeletal component; involved in actin filament turnover
Arp 2/3 complex	Cytoskeletal component; serves as the nucleation site for F-actin
Heat Shock Protein 60	Activated in response to stress, molecular chaperones involved in protein folding, protein trafficking, etc…
Innexin	Gap junction protein
Selenoprotein S	Plasma protein; possibly involved in response to oxidative stress and inflammation (Curran 2005)
TGF-ß nuclear protein	Growth Factor; involved in the regulation of cell proliferation and differentiation
Wnt*	Signaling proteins involved in the regulation of a host of processes including wound repair

Table 4: Targeted ESTs and their possible function in wound repair. *denotes degenerate primers directed against conserved regions of *wnt* (Helmbrecht et al., 2001).

Arp 2/3 (~300bp) was amplified at all time points (0, 15 and 30 minutes) post wounding. This target was subsequently used as a positive control to ensure correct sample amplification when testing other targets.

The first three products that were successfully amplified were Hsp 60, TGF-ß, and Selenoprotein S (Fig. 30). Hsp 60 (~400 bp) is present throughout the wound response, amplifying at all three time points. Interestingly, both TGF-ß and Selenoprotein S only amplified at one of the three time points. TGF-ß (~350 bp) amplified at 15 minutes, with no band showing up at 0 or 30 minutes, while Selenoprotein S (~550 bp) amplified at 30 minutes,

with no bands appearing at 0 or 15 minutes. The final EST target that amplified was Innexin (Fig. 31). Innexin amplified at both 15 and 30 minutes post-wounding, but was absent at time 0.

In conjunction with the EST primer development, four degenerate primers were selected that targeted highly conserved regions of proteins involved in E-cadherin, fetal Hox, Notch-Delta 4 and Wnt signaling (Table 5). Out of these targets only the Wnt degenerate primer set produced a product (~500bp). This product was slightly larger than the 400-450 bp products obtained from previous use of the primers (Gavin et al., 1990; Helmbrecht et al., 2001). Bands were observed at time 0 and 30 minutes, but only a large smear was observed at 15 minutes post wounding, with the highest concentration appearing around 200bp (Fig. 32). In the 0 and 30 minute lanes, no visible primer front was observed, the only case where this occurred.

Target	**Functional Significance (Primer sequence source)**
E-cadherin	Transmembrane protein; found in epithelium, involved in cell-cell adhesion (Matsuyoshi 1997)
Hox (fetal)	DNA sequence: regulation of developmental processes such as morphogenesis (Stelnicki 1997)
Notch-Delta 4	Determination of cell fate, especially during developmental processes (Yoneya 2001)
Pten	Tumor suppressor gene; involved in regulating keratinocyte migration (Bostrom 1998; Zhao 2006)
Wnt*	Signaling proteins involved in the regulation of a host of processes including wound repair (Helmbrecht 2001)

Table 5: List of degenerate primer targets and their significance to wound repair. *denotes working primer set.

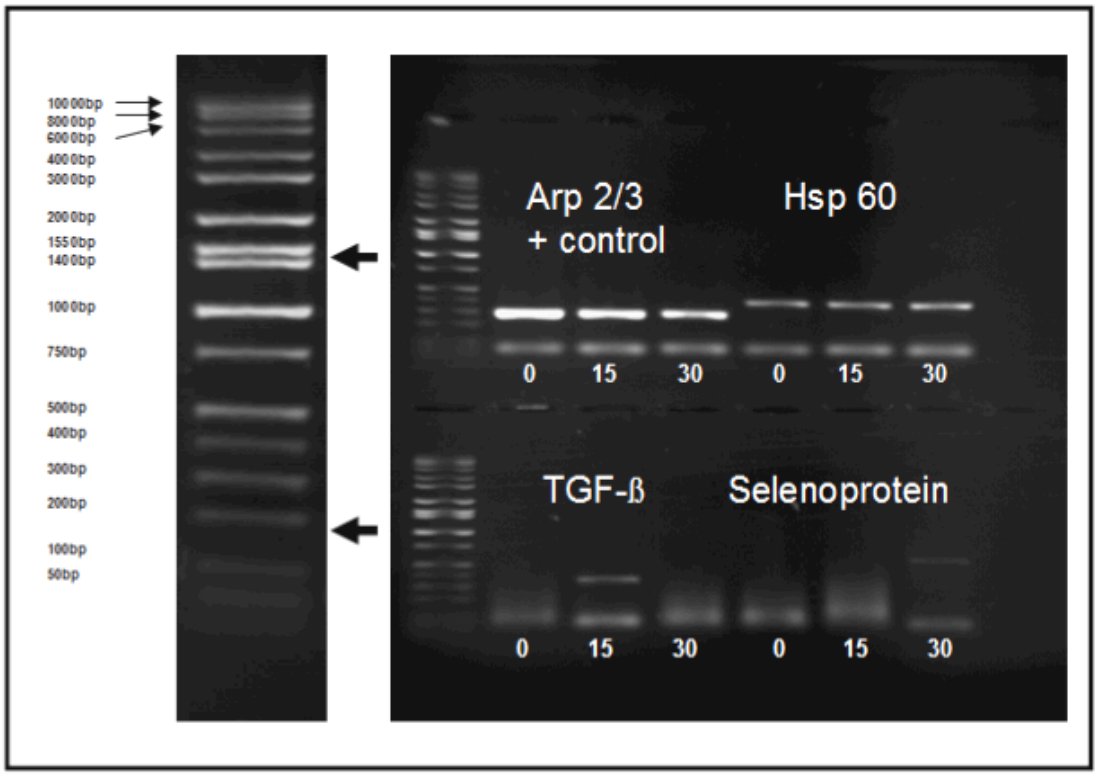

Figure 30: Agarose gel showing changes in Hsp 60, TGF- ß, and Selenoprotein gene expression. Arp 2/3 was used as a positive control for all 0, 15 and 30 minute tissues.

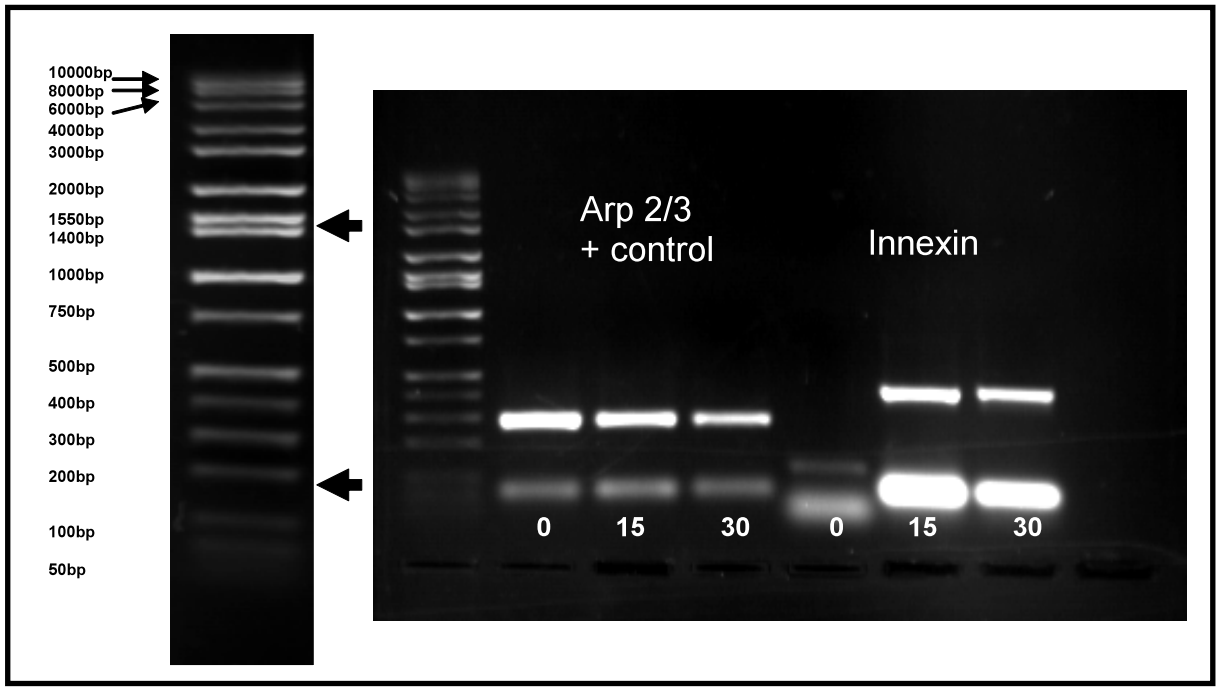

Figure 31: Agarose gel showing changes in Innexin gene expression.

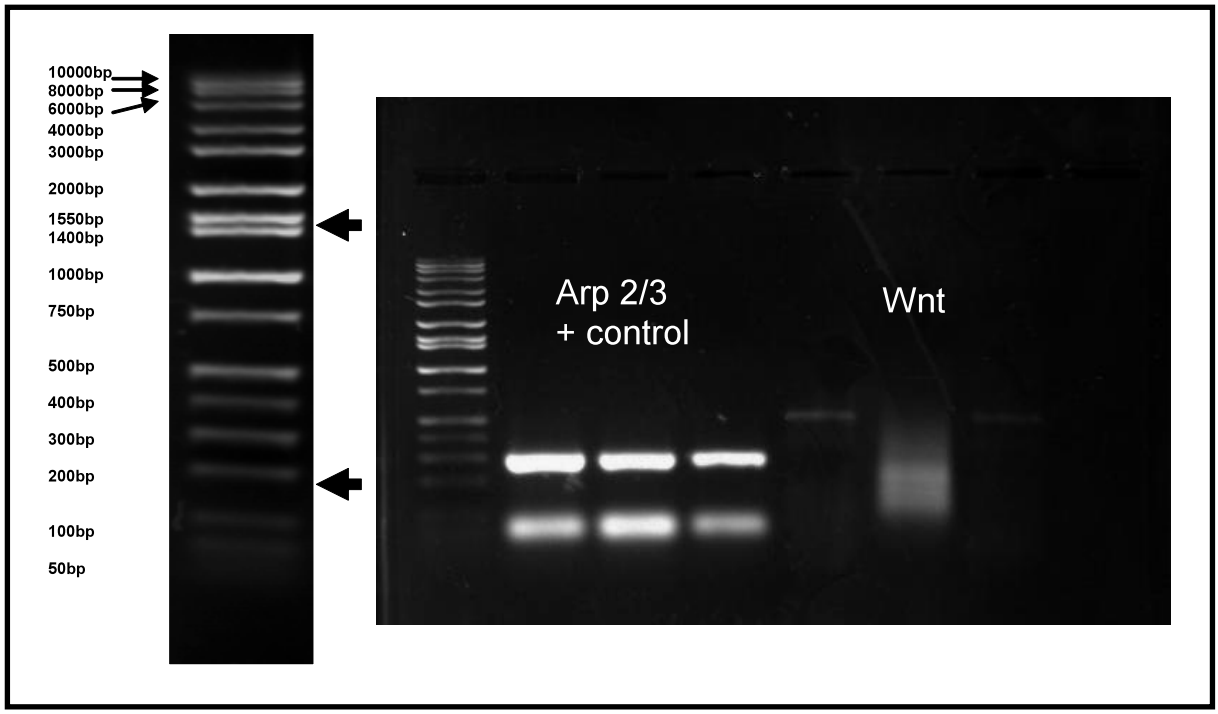

Figure 32**:** Agarose gel showing changes in Wnt gene expression.

Discussion

This work provides a first look at gene expression during wound repair in *Mnemiopsis*. EST analysis shows that different targets amplified at the different time points during repair. Arp 2/3 amplified at a consistent level at time 0, 15 minutes after wounding, and 30 minutes after wounding. Arp 2/3 plays an important role in actin filament nucleation and turnover in migrating cells. That role, coupled with the fact that wound cells in *Mnemiopsis* appear to actively migrate throughout the repair response, and are probably ready to move immediately upon wounding, suggests that Arp 2/3 should be present throughout wound repair. This made Arp 2/3 an excellent internal positive control to ensure the sample was working, and in looking at the expression patterns of other amplifiable EST targets.

Four EST targets were amplified, with Arp 2/3 as a positive control, to look at how their expression differed at 0, 15 and 30 minutes. Hsp 60, a ubiquitous molecular chaperone shown to be involved in wound repair, was, like Arp 2/3, present at 0, 15 and 30 minutes post wounding. Expression appeared to remain constant at all three time points, but bands were fainter than the Arp 2/3 bands. These results suggest that Hsp 60 is expressed throughout the wound response, as well as in time 0 controls. This result is expected because chaperones such as Hsp 60, as discussed above, are found commonly throughout tissues due to demands caused by stress.

The second target used was TGF-ß, a growth factor with a number of functions during wound repair as discussed above. The TGF- ß gene product amplified only at 15 minutes, being absent at time 0 and 30 minutes post wounding. These results are interesting, and suggest that TGF- ß is not present at levels high enough to be detected in time 0 tissues. The situation quickly changes upon wounding, with an amplifiable target being present at 15 minutes into wounding. The effect of TGF- ß does not persist throughout the wound response, indicated by the complete absence of a band at 30 minutes. These results suggest that TGF- ß is present in low or non-existent amounts prior to wounding, becomes strongly expressed midway through the repair response, and then ceases to be present near the end of the response. This provides a first look at the expression of a TGF- ß homolog involved in wound repair in *Mnemiopsis*.

The third target was Selenoprotein S, which was chosen due to its role in protecting cells from oxidative stress (Curran and al, 2005; Mostert, 2000). Selenoprotein S was amplified at 30 minutes, but not at 0 or 15 minutes. This infers that Selenoprotein is present in low or non existent levels in non wounded adult *Mnemiopsis* tissues. Unlike TGF- ß, Selenoprotein expression appears near completion of repair.

The fourth EST used was Innexin, an invertebrate gap junction protein. Innexin did not amplify in the 0 minute control, but appeared at both 15 and 30 minutes into wounding. This indicates that innexin is present in low or non existent levels in normal tissue, but is actively involved in wound repair throughout the response.

The final set of amplicons used degenerate primer sets as described in Table 4. The only gene target that amplified from the degenerate primer set was a *wnt-like gene* , which was originally observed in human thyroid carcinoma cells (Helmbrecht et al., 2001). Degenerate primers were targeted to highly conserved regions of the *wnt* gene (indicate above or below, where exactly you made the choices). Bands were amplified at 0 and 30 minutes, but only a smear was seen at 15 minutes. This implies that *wnt* is found in both control tissues, as well as 30 minutes into the response. The smear at 15 minutes may be a result of non-specific primer binding producing heterogenous products, or mRNA degradation, but was a common occurrence when amplifications were repeated using wound tissues from different animals.

Conclusions

These results were intended to provide an initial look at gene expression during wound repair in *Mnemiopsis*. What the results indicate is that certain targets are present at the different major time points in healing. Further work is needed to gain a better understanding of the exact time frame some of these targets are in fact most active, due to the use of only 15 and 30 minutes into healing. However, this does provide a starting point for more specific gene expression analysis, and proves that wound repair in *Mnemiopsis* is a viable system to study changes in expression during repair.

Each of the EST targets was chosen for its possible involvement in the wound repair cascade in *Mnemiopsis*. Arp 2/3 plays a role in the nucleation of actin filaments during

lamellipodial based cell migration. *Mnemiopsis* wound cells actively migrate both *in vivo*, and *in vitro*, and at least three of the observed morphologies, bipolar, stellate and multipolar cells display movement via cell processes. Presence of Arp 2/3 at 0, 15 and 30 minutes would be expected in an organism 'primed' for immediate wound repair activity, as actin filament turnover in such a case would be an active process in both normal and wounded conditions.

Heat shock proteins are active players in protecting cells from stress, and Hsps 10, 27, 60, 70 and 90 all play a role during wound repair (see Introduction). Presence of Hsp 60 at all three time points during repair in *Mnemiopsis* is not surprising, as Hsps are found in all organisms (Lindquist and Craig, 1988), and thus would be expected to be present not only during wound repair, but also in non wounded tissues. Under non-wounded conditions, Hsps repair misfolded proteins and are induced during a multitude of stresses, including oxidative and mechanical stress and other conditions that require the production of new protein, which would be nearly continuous in strongly stressed animals such as delicate ctenophores.

Selenoproteins respond to oxidative stress, and selenoprotein S, which amplified at 30 minutes into wound repair in *Mnemiopsis*, has been shown to regulate inflammation (see Introduction). This result is interesting, as inflammation is one of the first stages of mammalian wound repair, but selenoprotein activity only comes into play at the end of *Mnemiopsis* wound repair. A possible explanation for this is the involvement of selenoprotein in managing oxidative stress may come into play late in the response to eliminate free radicals produced by damage or the repair response itself.

Innexins are invertebrate gap junction proteins involved in cell-cell communication. Unpublished work from Tamm and Moss using the injectable dye tracer Lucifer Yellow CH, demonstrating dye coupling; as well as multiple electrode implantation studies coupled with field

potential recordings (Moss, 1986 [thesis]), suggests that a gap junction-like organelle must exist in ctenophore tissues. However, innexin has not been directly demonstrated in ctenophores to date. The role of innexin in invertebrate gap junctions made it the perfect choice to try to look at possible cell communication during the wound repair response.

The fact that innexin is present during wound repair, but does not appear in non wounded tissues, indicates that these proteins are produced in response to wounding. A possible explanation for this is the accumulation of wound cells at the wound site. These cells clearly join together in large groups, both at the epithelial layer, and in the underlying mesoglea (see Chapter 2). There is a clear order and precision to how the cells interact with one another, possibly facilitated by the presence of gap junctions in these large cell aggregates, which would stabilize cell-cell interactions.

TGF-ß is a growth factor family commonly involved in mediating wound repair mechanisms across the animal kingdom. TGF-ß, in this case, appears only at 15 minutes into wound repair in *Mnemiopsis*, suggesting a possible role for this growth factor roughly halfway through the response. These results could suggest that TGF-ß is involved in mediating cell signaling responsible for the cell migration, reorganization and possibly differentiation that is associated with cells involved at this time point in repair (see Chapter 2). Due to the diverse role of TGF-ß in mediating cell proliferation and differentiation (see Introduction), careful regulation of when it is produced could prevent hyperproliferation or differentiation events that could lead to cellular irregularities or the introduction of scarring at the wound site. This might account for its expression only at 15 minutes, and expression ceases near the end of repair.

The purpose of this work was to provide a first look at gene expression in *Mnemiopsis* wound repair that can serve as a foundation for future molecular work in this system. These

results mainly included the use of short length (~500bp or less) EST sequences, and represent only three time points of the repair response. The use of longer sequence targets, whose expression changes or remains constant at a larger number of time points, and the use of quantitative PCR, will provide a much better idea of expression changes during this response. Future efforts, building up these results, can hopefully begin to determine any upregulation and/or downregulation as it occurs throughout repair. Efforts in this area will be facilitated by a ctenophore genome project, currently underway, and further strengthen use of *Mnemiopsis* as a model to study rapid scar-free wound repair.

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