Slime Gland Cytology and Mechanisms of Slime Thread Production in the Atlantic hagfish
(Myxine glutinosa)

by

Timothy McEwen Winegard

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ABSTRACT

Slime Gland Cytology and Mechanisms of Slime Thread Production in the Atlantic Hagfish

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Timothy McEwen Winegard
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Advisor: Dr. D.S. Fudge

The multicellular slime glands of hagfish are functionally and structurally unique among cyclostomes. The holocrine release of cells from these glands results in the formation of a fibrous slime that viscously entraps seawater to discourage attacks by gill-breathing predators. Past research into the cytology of the slime gland, and processes of gland thread cell (GTC) and gland mucous cell (GMC) maturation, leads to a number of hypotheses on the mechanism of slime thread assembly in GTCs. Using a number of cell manipulation and imaging techniques, I reveal: (1) a previously undescribed cell in the hagfish slime gland, (2) the intermediate filament and microtubule distribution in hagfish gland cells, and (3) mechanisms of slime thread assembly. The findings test a number of hypotheses and provide the basis for further analysis of cytoskeletal element distribution and interaction in hagfish gland cell types.
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# Table of Contents

List of Tables.............................................................................................................................. v  
List of Figures...................................................................................................................................... vi  
  General Introduction Figures........................................................................................................ vii  
  Chapter 2 Figures................................................................................................................... viii  
  Chapter 3 Figures................................................................................................................... ix  
List of Abbreviations..................................................................................................................... x  
List of Movies..................................................................................................................................... xi  
General Introduction................................................................................................................... 1-12  
Chapter 2........................................................................................................................................ 13-43  
  Introduction................................................................................................................................. 14-17  
  Materials and Methods................................................................................................................ 17-28  
  Results........................................................................................................................................ 28-33  
  Discussion.................................................................................................................................. 33-42  
Chapter 3........................................................................................................................................ 43-78  
  Introduction................................................................................................................................. 43-50  
  Materials and Methods................................................................................................................ 50-63  
  Results........................................................................................................................................ 63-69  
  Discussion.................................................................................................................................. 69-78  
General Discussion....................................................................................................................... 79-84  
References....................................................................................................................................... 85-88  
Appendix.......................................................................................................................................... 89
List of Tables

Table 1: Organic and inorganic osmolytes that were added to commercially available media to raise osmolality to approximately 1000 mOsmol/L

Table 2: Ethanol dehydration series illustrating the dilutions (%) and rinsing times (minutes)

Table 3: Paraffin dewaxing protocol for embedded hagfish glands and tissues.
List of Figures

Chapter 1

Figure 1: Large volume of fibrous slime released by a Pacific hagfish (*Eptatretus stoutii*).

Figure 2: Hagfish thread skeins and mucin vesicles.

Figure 3: Transverse section of a slime gland of *Polistostrema stoutii* by Newby (1946).

Figure 4: Cross-sectional views through hagfish slime threads at different stages of maturity (Downing et al., 1984)

Chapter 2

Figure 1: Differential interference contrast image of isolated hagfish gland cells.

Figure 2: Differential interference contrast images of immature hagfish gland thread cells.

Figure 3: Histologically stained gland thread cells from differentiation through slime thread production.

Figure 4: Differential interference contrast image of an isolated immature hagfish gland thread cell (left) adjacent to a large mature thread skein.

Figure 5: Differential interference contrast imaging of hagfish slime gland cells stained with the vital exclusion dye trypan blue.

Figure 6: Differential interference contrast image of a hagfish gland thread cell of intermediate maturity (left) next to a large mature gland mucous cell (right).

Figure 7: Differential interference contrast image of a gland thread cell of intermediate maturity and gland mucous cell.

Figure 8: Transmission Electron Micrographs of a hagfish gland mucous cell of intermediate maturity.

Figure 9: Paraffin embedded gland mucous cells and surrounding tissue.

Figure 10: Polyhedral cells of *M. glutinosa* slime glands.

Figure 11: Epifluorescent image of immunolabelled hagfish gland cells.
Figure 12: Confocal image of fluorescently labeled hagfish gland cells.

Figure 13: The distribution and ultrastructure of the unidentified cell from hagfish slime glands.

Figure 14: Composite image of 221 individual photos taken at 200x magnification in differential interference contrast.

Figure 15: Hematoxylin and eosin staining of a hagfish slime gland.

Figure 16: A collection of images illustrating the hagfish gland pore and associated cell types.

Figure 17: Epifluorescent images of a paraffin embedded hagfish gland.

**Chapter 3**

Figure 1: Focused Ion Beam (FIB) milled mature gland thread cell imaged with scanning electron microscope (FIB image probe 30 KV:80pA, EHT = 5 kV).

Figure 2: Transmission Electron Micrograph of immature gland thread cell with short helical length of slime thread.

Figure 3: Focused Ion Beam (FIB) milled intermediately mature gland thread cell imaged with scanning electron microscope (FIB image probe 30 KV: 80pA, EHT = 5 kV).

Figure 4: Focused Ion Beam (FIB) milled mature gland thread cell imaged with scanning electron microscope (FIB image probe 30 KV: 80pA, EHT = 5 kV).

Figure 5: Transmission Electron Micrographs of *M. glutinosa* gland thread cells in progressive stages of maturation.

Figure 6: Change in relative slime thread density through five categorical stages of slime thread maturation.

Figure 7: Hagfish slime thread cross-sections at various time points in development.

Figure 8: Transmission electron micrographs of intermediately mature gland thread cell.

Figure 9: Epifluorescent and differential interference contrast images of paraffin embedded hagfish gland thread cells at various time points in development.

Figure 10: Epifluorescent images of paraffin embedded hagfish glands cells.
Figure 11: Immunofluorescent image of mature gland thread cell and undescribed cell type.

Figure 12: Confocal image of fluorescently labeled hagfish gland thread cell.

Figure 13: Transmission electron micrographs of hagfish gland thread cell slime threads and organelles within the cytoplasm.

Figure 14: Transmission electron micrographs of hagfish gland thread cell slime threads.
List of Abbreviations

Cells and cell features

DNA - deoxyribonucleic acid  
EC - epithelial cell  
ESC - epidermal skin cell  
GIC – gland interstitial cell  
GMCs - Gland mucous cells  
GTCs - gland thread cells  
IF - intermediate filament  
MRZ – mitochondrial rich zone  
AF – actin filaments  
MT – microtubule  
PCs - Polyhedral cells  
RNA - ribonucleic acid

Chemicals, media and concentrations

BSA - bovine serum albumin  
CM - culture media  
ddH2O - double de-ionized water  
DMG - dimethylglycine  
EtOH- ethanol  
kDa - kilodalton  
L-15 - Leibovitz 15  
M - molar  
mM - millimolar  
NaCl - sodium chloride  
PBS - phosphate buffered saline  
Sodium citrate - sodium citrate trihydrate  
TMAO - trimethyl aminoxide

Stains and fluorescent dyes

DAPI - 4’,6-diamidino-2-phenylindole, dilactate  
H&E - hematoxylin and eosin

Imaging techniques

AOTF - acousto optical tunable filter  
DIC - differential interference contrast  
FIB/SEM - focused ion beam/scanning electron microscopy  
TEM - transmission electron microscopy/transmission electron micrograph
List of Movies

Chapter 3

Movie 1: Compiled image series of scanning electron micrographs taken during the process of focused ion beam (FIB) milling of a critical point dried gland thread cell (GTC) from *Myxine glutinosa* (FIB image probe 30 KV: 80pA, EHT = 5 kV).

Movie 2: Compiled image series of scanning electron micrographs taken during the process of focused ion beam milling of a plastic embedded gland thread cell (GTC) from *Myxine glutinosa* (FIB image probe 30 KV: 80pA, EHT = 5 kV).
CHAPTER 1: GENERAL INTRODUCTION
Hagfishes

Hagfishes and lampreys belong to an ancient lineage (Superclass: Agnathans, Class: Cyclostomata) that evolved over 350 million years ago and are believed to be the sister group to vertebrates (McCauley and Kuratani, 2008; Near, 2009). The out-group position of hagfishes in the vertebrate phylogeny makes them an important organism for understanding the evolution of vertebrate physiology (McCauley and Kuratani, 2008). Studies on hagfishes date back to Linnaeus (1758) and Darwin (1842) (Fernholm, 1981). Hagfishes are marine bottom-dwellers that are found at depths from 2500 m to 30 m in both the Atlantic and Pacific oceans. There are approximately 67 known species of hagfishes and 40 species of lamprey; however, only a few hagfish species have been used in research: *Eptatretus stoutii*, *Myxine glutinosa*, *Polistotrema stoutii*, *Eptatretus deani*, and *Eptatretus burgeri*. These primarily scavenging organisms play a number of important ecological roles such as substrate turnover (Martini, 1998). Hagfishes are also commonly known to the public for their use in “eel-skin” leather products and culinary dishes.

Hagfish slime glands and slime

In the scientific community, hagfishes are well known for their slime producing ability (Figure 1). This defensive behavior appears to have been conserved among all extant species of hagfishes and may be one of the keys to their survival over the last 300-400 million years (Fernholm, 1981; Lim et al., 2006; Zintzen et al., 2011). When threatened or provoked by gill-breathing predators such as fishes, the fibrous slime released by the hagfish aggregates across the predators gill surface reducing the respiratory flow of water so severely that the aggressor aborts the attack and possibly suffocates (Fernholm, 1981; Lim et al., 2006; Zintzen et al., 2011). No successful predation of hagfish by gill-breathing predators has ever been observed (Zintzen et al.,
The importance of slime to hagfish survival may explain why 3-4% of their wet body mass consists of slime exudate (Fudge et al., 2005). This amount of exudate contains approximately 400 km of intermediate filament (IF) protein fiber (~3000 skeins/µL, x15 cm slime thread/skein = 450 m/µL)

The slime is comprised of two primary constituents that develop within highly specialized cells of the slime gland: (1) large ellipsoidal bundles of thread (150 µm major axis) rich in intermediate filament (IF) proteins, and (2) small disc-shaped mucin vesicles (3 µm in diameter) containing high concentrations of glycoproteins (Figure 2; Newby, 1946; Fernholm, 1981; Downing et al., 1984; Spitzer et al., 1988; Luchtel et al., 1991; Herr et al., 2010; Winegard and Fudge, 2010). The gland thread cells (GTCs) and gland mucous cells (GMCs) that give rise to these constituents have attracted the attention of cytologists for over 150 years (Kolliker, 1860). Contraction of the highly-vascularized smooth musculature surrounding the gland causes the forceful holocrine ejection of both GTCs and GMCs into the surrounding seawater (Newby, 1946; Fudge et al., 2005). Whole slime maturation involves the rupture and elongation of mucin vesicles (released from the GMCs) into strands that adhere to the thread skeins (released from the GTCs) and initiate unraveling via the transmission of hydrodynamic mixing forces—such as those generated by the hagfish as it tries to escape predation (Fudge et al., 2005; Winegard and Fudge, 2010). In its mature state the slime is comprised of a complex network of threads, mucin strands and viscously entrained seawater (Fudge et al., 2005; Winegard and Fudge, 2010).

Hagfishes possess between 75-100 segmentally paired slime glands that line the ventro-lateral sides their eel-like body. Few studies have examined the cellular composition and structure of these glands. Newby (1946) provided the first qualitative description of slime glands from Polistostrema stoutii (the California hagfish). He identified five major components: (1)
undifferentiated cells lining the inside of the gland capsule, (2) connective tissue trabeculae that extend into the gland adjacent to capillaries of the gland lumen, (3) gland thread cells, (4) gland mucous cells, and (5) a reticular connective tissue that extends between the trabeculae and developing GTCs and GMCs (Figure 3; Newby, 1946). Lametschwandtner et al. (1986) presented evidence of a significant difference in the vascularization of slime glands from *Eptatretus stoutii* and *Myxine glutinosa*. Vascular corrosion casts and scanning electron microscopy were used by the researchers to illustrate that capillary loops penetrated into the gland lumen of *E. stoutii* but not *M. glutinosa*. These findings bring to light a number of questions regarding gland cytology and structure reported by Newby (1946). One of the main objectives of Chapter 2 of this thesis is to assess the cytology and structure of slime glands from *M. glutinosa* to determine the basis for Newby’s (1946) observations in *P. stoutii*.

*Holocrine secretions by cyclostomes*

The differentiation of epidermal and epithelial cells into mucous and proteinaceous cells has allowed for the adaptive radiation of these cell types to serve a wide variety of functions among animals (McCallion, 1956; Quay, 1972). Within cyclostomes there are four types of secretory cells arising from a common germinative epidermal cell line, including: (1) small and superficial mucous cells, (2) large gland mucous cells of hagfish, (3) granular cells or epidermal thread producing cells, and (4) thread cells of the hagfish (Quay, 1972). Hagfishes exhibit all four of these secretory cell types while lampreys only have (1) and (3). Within hagfishes the release of epidermal secretory cells occurs via the holocrine mode from both unicellular and multicellular glands (Quay, 1972; Downing, 1981).
Elements of the cytoskeleton

Microtubules (MTs), intermediate filaments (IFs), and actin microfilaments (AF) constitute the cytoskeleton of all eukaryotic cells (Herrmann and Aebi, 2000). Although the study of the cytoskeleton began early in the twentieth century, it was not until the latter half of the century that the cytoskeleton was delineated into three distinct components. AFs were the described by Straub (1942), MTs by De Robertis and Franchi (1953) and IFs by Ishikawa et al. (1968). These pioneering discoveries led to the characterization of cytoskeletal elements and their functions in a wide variety of cell types. The filament systems formed by IFs, MTs, and AFs serve a wide variety of functions, such as: connecting the nucleus and cell membrane, anchoring organelles within the cytoplasm, and maintaining cell shape/integrity (Goldmann et al., 1985; Hermann and Aebi, 2000). Interactions between these cytoskeletal elements are facilitated by a wide array of associated proteins. For example, the IF associated protein plectin is known to mediate IF interactions with MTs (Svitkina et al., 1988). IFs and MTs will be discussed in more detail in a later section.

GTC development

Although the literature on whole-gland structure is limited, a number of studies have attempted to elucidate patterns of slime thread production and coiling within hagfish GTCs. GTCs develop from the periphery of the slime gland to the gland lumen where the ellipsoid-shaped cells grow to approximately 150 µm along their major axis, and about half as wide along the minor axis. Newby (1946) observed a 3-fold increase in the diameter of these cells destined to become GTCs. Increases in cell diameter were followed by a uniaxial lengthening of the cell prior to thread development (Newby, 1946). Newby (1946) described the appearance of granules in association with fine fibers as the differentiating GTCs lengthened to approximately 4 times
their original diameter. He illustrated through hand-drawn figures that the intracellular positioning of these granules formed two elongated spirals located either proximally or distally to the long-axis of the cell. Newby (1946) postulated that the granules (of origin unknown to him) provided a scaffolding on which short segments of fibers (not at that time known to be IFs) longitudinally aligned to form one continuous and highly-coiled thread. He also noted a significant widening of the slime thread although he did not speculate as to how this may occur (Newby, 1946).

Fan (1965) suggested that the formation of the thread is initiated by the production of 12-nm filaments by ribosomes found freely within the cytoplasm of the GTC. This may suggest that the granules observed by Newby (1946) were actually ribosomes or small clusters of cytoplasmic ribosomes (polysomes). Expanding upon the previous conceptual model, Fan (1965) proposed that these filaments joined both longitudinally and laterally to form the mature thread. However, his explanation also lacks sufficient mechanistic insight regarding filament organization into a single tightly-coiled thread.

Terakado et al. (1975) provided the first in-depth analysis of thread development. These researchers suggested that the development of the thread was initiated by the parallel arrangement of filaments 9-12 nm in diameter (D) as well as 35-55-nm (D) tubular structures, which they believed provided the axis for thread development. Terakado et al. (1975) observed changes in the size and frequency, of both the filaments and tubular structures, as a function of thread maturity. The researchers proposed that the 9-12-nm (D) filaments rearranged within the developing thread to form thinner 3-6-nm (D), and 1-3-nm (D) filaments. They also observed that the tubular structures (later discovered to be microtubules) disappeared during the final stages of slime thread maturation (Terakado et al., 1975; Downing et al., 1984).
Downing et al. (1984) presumed that the site of thread formation lay distal and adjacent to the conically-shaped mitochondrial rich zone (MRZ) where the thread skein grows to occupy more than 70% of the cell’s volume. The researchers hypothesized that thread development began with the parallel arrangement of IFs and MTs distal to the MRZ. Using TEM, they illustrated that thread production involved the concurrent arrangement of 10-12-nm IFs and MTs in a highly organized pattern, such that the thread increased in both length and diameter simultaneously. More specifically, Downing and colleagues postulated that the parallel arrangement of IFs and MTs occurred via their circumferential recruitment around the primal thread. Within these filamentous arrangements of IFs, they demonstrated that the presence of MTs was both spatially and temporally variable along the length of the developing thread (Downing et al., 1984; Figure 4). These researchers hypothesized that the MTs may play both structural as well as physiological functions within the developing thread. Structurally, MTs may provide a scaffolding on which IFs can assemble. Physiologically, they may provide a pathway within the thread for the regulation of cytoplasmic composition or abundance and/or the movement/rearrangement of filaments within the developing thread. However, the significance of MTs in IF-MT interactions during the development of this single, tapered, cylindrical thread remains to be determined. In addition to the preceding observations and suggestions, Downing et al. (1984) also provided a qualitative analysis of a shift in thread development characterized by a change in a substance/structure associating with the periphery of the slime thread. They reported using TEM a helically wound fiber around threads of early stages in development. They also demonstrated that a substance of indiscernible ultrastructure replaced the helical wrapping in subsequent stages of maturity.
It is clear from the literature that thread assembly involves interactions between IFs and MTs that result in a large (~15 cm) bi-directionally tapered thread that is precisely coiled within the confines of the GTC (Fan, 1968; Terakado et al., 1975; Downing et al., 1984; Spitzer et al., 1988; Fudge et al., 2005). However, it is not known whether observations of thread development by past researchers (i.e. Newby, 1946; Downing et al., 1984) apply to all species of hagfishes.

Other remaining questions that I will address in this thesis include: (1) What is the mechanism of thread assembly in *M. glutinosa* GTCs? (2) What is the significance of the helical wrapping observed by Downing et al. (1984)? (3) What is the function of MTs in slime thread development and within the GTC? (4) Do increases in thread length and girth happen in concert, or are they ever uncoupled? (5) What is the significance of changes in thread density with maturity? More generally the question of how GTCs manufacture a 15 cm long protein thread in their cytoplasm remains unanswered.

**GMC development**

Hagfish-gland mucous cells (GMCs) represent a highly specialized member of the epidermal/epithelial mucous cell line (Quay, 1963; Leppi, 1968; Luchtel et al., 1991). The divergence of GMCs from their epidermal relatives is supported by differences in their histological staining (Leppi, 1968). Within the slime gland, the GMCs develop into large cells (~100-150 µm in diameter) containing high quantities of ~3-5 µm disc-shaped mucin vesicles bound by a single 9-10-nm membrane (Luchtel et al., 1991). The glycoproteins contained within the mucin vesicles are comprised of 77% protein (rich in proline, threonine and valine), 12-18% carbohydrate and 5% lipid (Lehtonen et al., 1966). The terminal carboxyl groups of the glycoprotein’s oligosaccharide side chains are responsible for many of the histochemical
reactions observed in these cells (Leppi, 1968). For example, the negatively charged groups result in positive reactions with basic dyes such as hematoxylin.

**Intermediate filaments (IFs) and hagfish slime threads**

Intermediate filaments, such as those comprising the hagfish slime thread, are a highly diverse superfamily of non-polar proteinaceous fibers composed of two distinct protein subunits (63.5 and 64.5-65.5 kDa) that combine to form a wide array of cytoskeletal networks (Downing et al., 1981; Aeby et al., 1988; Steinert and Roop, 1988; Koch et al., 1994). Each protein subunit has a tripartite organization that consists of a helical central rod domain as well as non-helical terminal N and C-domains (Koch et al., 1994). IF proteins are identified by the relatively high sequence identity (~30%) of this central rod domain shared between the six known IF protein types (Aebi et al., 1988; Steinert and Roop, 1988). These IF protein types include: (1) the acidic keratins, (2) the basic keratins, (3) desmin, vimentin etc., (4) neurofilament proteins (light, medium, heavy) etc., (5) the nuclear lamins, and (6) phakinin and filensin (Strelkov, 2003; Iwatsuki and Suda, 2010). The homology of the central rod domain shared by IFs can be much higher (70-95%) within a given family (Strelkov et al., 2003).

Hagfish slime thread IFs can be divided into two groups of thread polypeptides (1) alpha (643 residues, 66.6 kDa) and (2) gamma (603 residues, 62.7 kDa) (Spitzer et al., 1988). The gamma polypeptide is also represented by a post-translational beta form that occurs during late stages of GTC development (Spitzer et al., 1984; Spitzer et al., 1988). Respectively, the alpha and gamma thread polypeptides share less than a 30% homology with their most closely related type II and type I IF proteins (Koch et al., 1994). They also exhibit a high threonine content (alpha, 13%; gamma, 10%) when compared to the other IF types (2-5% on average) (Koch et al., 1994). The significance of high numbers of threonine and serine residues in the hagfish IFs has
not been elucidated, however, it is known that threonine and serine represent sites for the modulation of IF assembly and organization through post-translational modifications such as phosphorylation (Iwatsuki and Suda, 2010). The threonine and serine residues of hagfish IF polypeptides may provide sites for post-translational modifications of these proteins such as those observed by Spitzer et al. (1988).

**Microtubules (MTs)**

Microtubules are polar, hollow tubes (25 nm O.D.) comprised of alpha and beta tubulin proteins (Hawkins et al., 2010). MT assembly involves the interaction of alpha and beta tubulin proteins to form heterodimers which assemble end-to-end to form protofilaments. A number of protofilaments bind laterally to form a sheet that is subsequently rolled into the MTs characteristic shape (Hawkins et al., 2010). The dynamic polymerization and depolymerization of MTs occurs primarily from the ‘plus’ end of the MT (Hawkins et al., 2010). Functionally, MTs have been shown to play a number of crucial roles in cell division (dissociation chromosomes) and intracellular transport of materials (Hawkins et al., 2010). In addition, MTs play a number of structural roles such as maintaining cell shape and providing mechanical support for cell products such as cilia (Hawkins et al., 2010).

**IF-MT interactions**

IFs and MTs have been extensively studied as distinct components of the cytoskeleton. However, the functional interactions between IFs and MTs are not as well understood (Bloom et al., 1985; Goldman et al., 1985; Svitkina et al., 1996; Draberova and Draber, 1993). It is known that IF and MT proteins frequently colocalize in cells (Schliwa and van Blerkom, 1981; Bloom et al., 1985; Draberova and Draber, 1993). In some cell types IF networks appear critically dependent on their association with MTs in the cell (Schliwa and van Blerkom, 1981; Bloom et
The application of MT dissociation drugs results in the paranuclear collapse of IFs in a number of cell types (Bloom et al., 1985; Fuchs and Weber, 1994). Bloom et al. (1985) suggested that these findings provided evidence that IF organization is dependent on MTs.

The hagfish GTC provides an opportunity to expand the study of dynamic interaction between IFs and MTs to a uniquely modified system. The interaction of IFs and MTs in the GTC results in the synthesis of a 15-cm thread that represents one of the only extracellular uses of IFs known in the animal world. MTs may be critical in slime thread formation and organization, however, the exact role they play remains unknown (Terakado et al., 1975; Downing et al., 1984).

Thesis Objectives

This thesis is comprised of two data chapters that will be submitted for publication and that have been written in manuscript format. The first data chapter presents a qualitative analysis of cell distribution and patterns of development in the slime glands of M. glutinosa. The questions addressed in this study include: (1) How do GTCs and GMCs develop within the slime gland of M. glutinosa? (2) What are polyhedral cells and what is their function? (3) What is the nature of Newby’s reticular network? (4) Is the nature of the slime gland epithelium continuous or discontinuous? and (5) Where is the site of cell proliferation in slime glands? To address these questions a number of cell manipulation and imaging techniques were employed.

Chapter 3 presents quantitative and qualitative analyses of slime thread synthesis by M. glutinosa GTCs. Thread assembly involves IF-MT interactions that result in a large bi-directionally tapered thread that is precisely coiled within the confines of the GTC. However, it is not known whether observations of thread development by past researchers apply to all species.
of hagfish. As such I asked: (1) What is the mechanism of thread assembly in *M. glutinosa* GTCs? (2) What is the significance of the helical wrapping observed by Downing et al. (1984)? (3) What is the function of MTs in slime thread development and within the GTC? (4) Do increases in thread length and girth happen in concert, or are they ever uncoupled? (5) What is the significance of changes in thread density with maturity and do these changes have anything to do with the post-translational modifications described by Spitzer et al. (1988)? We propose that: (H1) Hagfish thread assembly is initiated within the MRZ with the alignment of 10-12-nm IFs, (H2) Hagfish slime threads grow in length and girth via the helical wrapping of IFs, (H3) The incorporation of MTs into the developing slime thread increases the flexural stiffness of the thread and acts as a scaffolding for the support of newly generated thread loops adjacent to the nucleus, (H4) A phase shift in thread assembly occurs once the slime thread reaches its full length, (H5) The phase shift involves a controlled dissassembly of 10-12-nm IFs via phosphorylation of gamma IF polypeptides and the reassembly of IF proteins/subunits into a ‘super’ fiber prior to maturity, (H6) Following the phase shift subsequent growth involves changes in girth only via the addition of IF subunits, (H7) MTs are removed in fully mature threads and IF subunits fill the spaces they occupied in development.

Ultimately, these data chapters provide a comprehensive look at hagfish slime glands and will serve as a crucial basis for subsequent studies of its nature.
CHAPTER 2: THE DEVELOPMENT AND DISTRIBUTION OF CELLS IN ATLANTIC HAGFISH \textit{(Myxine glutinosa)} SLIME GLANDS
Abstract

A plethora of epidermally derived unicellular and multicellular glands exist for the purpose of generating mucous and/or proteinaceous secretions. The defensive fibrous slime released by hagfishes represents an extreme example of how these glands have been modified to serve a wide variety of functions. Little is known about the hagfish slime gland, the development and organization of its constituent cell types, or its variation among hagfish species. Previous studies have demonstrated significant morphological differences between slime glands of Pacific and Atlantic species of hagfish. The main focus of this study will be to answer the question of whether slime gland cytology is consistent among studied species of hagfish, given the differences in vascularization observed in the literature. This study presents: (1) the first application of cell culturing techniques and immunofluorescence with slime gland cell types from the Atlantic hagfish (*Myxine glutinosa*), (2) a characterization of *M. glutinosa* slime gland cytology with a focus on intermediate filament and microtubule distribution and organization, and (3) the discovery of a new cell type in the hagfish slime gland.

Introduction

The differentiation of epidermal and epithelial cells into basic but dichotomous lines of mucous and proteinaceous cells has allowed for the adaptive radiation of these cell types to serve a wide variety of functions within the animal world (McCallion, 1956; Quay, 1972). Within cyclostomes (hagfishes and lampreys) there are four types of secretory cells arising from a germinative epidermal cell line, including: (1) small and superficial mucous cells, (2) large gland mucous cells of hagfish, (3) granular cells or epidermal thread producing cells, and (4) thread cells of the hagfish (Quay, 1972). Hagfishes exhibit all four of these secretory cell types whereas lampreys exhibit only (1) and (3). Within hagfishes the release of epidermal secretory cells
occurs via the holocrine mode from both unicellular and multicellular glands (Quay, 1972; Downing, 1981). The focus of this chapter will be the multicellular hagfish slime gland.

One-hundred segmentally paired glands line the ventro-lateral sides of a hagfish’s eel-like body. Within these glands the fibrous intermediate filament (IF) and mucin-like slime constituents of the slime are produced by gland thread cells (GTCs) and gland mucous cells (GMCs) respectively (Figure 2; Newby, 1946; Blackstad, 1968; Leppi, 1968; Terakado et al., 1975; Winegard and Fudge, 2010; Herr et al., 2010). Early light microscope investigations into the distribution and development of cells in the slime gland suggested that the gland is comprised of five major components: (1) undifferentiated cells along the inside of the gland capsule, (2) connective tissue trabeculae that extend into the gland, (3) gland thread cells, (4) gland mucous cells, and (5) a reticular connective tissue that extends between the trabeculae and developing GTCs and GMCs (Newby, 1946). The GTCs and GMCs are believed to originate from a common precursor cell such as the cells along the inside of the gland capsule or the polyhedral cells that surround the stalk of the gland pore (Newby, 1946). The entire gland is surrounded by a vascularized connective tissue capsule, a smooth muscle layer and large myotomal bands of striated muscle (Newby, 1946; Lametschwandtner et al., 1986).

Hagfish gland thread cells (GTCs) represent the most specialized proteinaceous cell type of cyclostomes (Quay, 1963). GTCs develop from the periphery of the slime gland to the gland lumen where the ellipsoid-shaped cells grow to approximately 150 µm along their major-axis, and about half as wide along the minor axis. Interactions between IFs and MTs within the GTC result in the assembly of a large (~15 cm) bi-directionally tapered thread that is precisely coiled within the confines of the cell (Fan, 1968; Terakado et al., 1975; Downing et al., 1984; Spitzer et al., 1988; Fudge et al., 2005).
Hagfish gland mucous cells (GMCs) represent a highly specialized member of the epidermal/epithelial mucous cell line (Quay, 1963; Leppi, 1968; Luchtel et al., 1991). The divergence of GMCs, from their epidermal relatives, is supported by differences in their histological staining (Leppi, 1968). Within the slime gland the GMCs develop into large cells (~100-150 µm in diameter) containing high quantities of ~3-5 µm disc-shaped mucin vesicles bound by a single 9-10-nm membrane (Luchtel et al., 1991).

The polyhedral cells (PCs) and ‘small’ cells inside the gland capsule are not mentioned outside of Newby’s (1946) publication on the slime gland. Newby (1946) suggested that the polyhedral cells (found at the base of the gland pore) and ‘small’ cells (found at the periphery of the inside of the slime gland), are similar in morphology and staining characteristics and that these cells do not form a true epithelial lining of the gland. He also posited that the ‘small’ cells represent the undifferentiated cells of the slime gland, from which both the GTCs and GMCs develop, and that the undifferentiated cells may in turn be derived from the polyhedral cells of the gland pore. He further suggested that the polyhedral cells are derived from the epidermis of the skin, which would require them to migrate from the epidermis through the gland pore.

Newby (1946) provided a hand-illustrated account of what he referred to as trabeculae and reticular connective tissues in slime gland of P. stoutii. Although he was unable to provide insight into the identity and possible functions of these structures, he did posit that they might be involved in supporting vasculature within the gland. Lametschwandtner et al. (1986) revealed that capillary loops extended into the glands of E. stoutii but not M. glutinosa suggesting significant differences in gland structure may exist between species of hagfishes. Lametschwandtner et al. (1986) did not corroborate Newby’s (1946) observations of either
trabeculae or connective tissue inside the gland so the natures of these tissues observed by Newby (1946) remains to be determined.

Although a basic framework for gland morphology and composition has been developed for *Polistostrema stoutii* there has been very little synthesis of the literature on what is known about GTCs (Fernholm, 1981; Downing et al., 1984), GMCs (Blackstad, 1963; Leppi, 1968), polyhedral cells (Newby, 1943), and the connective tissue/trabeculae of the gland interior (Newby, 1946) from other species of hagfishes. As such, the goal of this study was to re-examine questions about hagfish slime gland cytology and organization using specimens of Atlantic hagfish (*M. glutinosa*). The questions addressed in this study include: (1) How do GTCs and GMCs develop within the slime gland of *M. glutinosa*? (2) What are polyhedral cells and what is their function? (3) What is the nature of Newby’s reticular network? (4) Is the nature of the slime gland epithelium continuous or discontinuous? and (5) Where is the site of cell proliferation in slime glands? To address these questions a number of cell manipulation and imaging techniques were developed and employed.

**Materials and Methods:**

*Animal collection and care:*

Specimens of Atlantic hagfish (*Myxine glutinosa* Linnaeus) were collected in Passomoquody Bay, New Brunswick, Canada using baited traps deployed along the seafloor. Following collection hagfish were transported to the Hagen Aqualab at the University of Guelph, where they were housed in 2000-L, environmentally controlled, aquatic recirculation units containing artificial seawater (10°C, 34‰). All housing and feeding conditions were in accordance with the University of Guelph Animal Use Protocol 05R154.
Animal anesthesia and slime removal

Hagfish were anesthetized following a clove-oil anesthetic protocol (Winegard and Fudge, 2010). Following anesthesia the slime glands of the hagfish were electrostimulated (60Hz, 18V) using a Grass SD9 electric stimulator (Grass Instruments, Quincy, MA, USA). Electrostimulation was performed 1 or 2 weeks in advance of post-mortem harvesting of the glands to attempt to increase the ratio of immature: mature cells (as shown by Spitzer et al., 1988).

Animal euthanasia and disinfection

Hagfish were euthanized using a large scalpel blade to sever the notochord and dorsal nerve cord directly posterior to the cranium. Following euthanasia the hagfish were disinfected via whole body immersion in 70% EtOH for 5-10 seconds. During disinfection the rostral end of the hagfish was held approximately 2 cm out of the EtOH to prevent EtOH from entering the blood sinus of the hagfish and coming into contact with the slime glands where it could kill the gland cells.

Animal dissection and harvesting of the slime glands

Hagfish were dissected inside a sterilized biological flow cabinet, using a sterilized tray and scissors (autoclaved and sprayed with 70% EtOH). The surgical area of the hagfish was sponged with 70% EtOH and a 6-cm incision down the dorsal midline of the hagfish was made starting from its rostral end. Perpendicular cuts from this dorsal incision to the ventral midline created a rectangular flap of skin on each side. Rat-toothed forceps were used to grasp dorsal muscle tissue firmly while hemostats were used to grasp and pull on each flap in turn. By carefully pulling the flap ventrally and away from the muscle tissue, the slime glands, attached to
the skin at the gland pore, were pulled from between the large myotomal bands of skeletal muscle running dorso-ventrally along the length of the hagfish.

Fine forceps were used to grasp the capsule of each exposed gland, and gently pull it away from the skin to expose the gland pore. Iridectomy scissors were used to cut the slime gland pore where it is attached to the epidermis. Following excision the slime glands were transferred into a sterile culture dish containing 10 mL of 400 mM sodium citrate + 5 mM EGTA (~1020 mOsmol/kg; 11 °C; pH 7.3; sterile 0.22 filtered) on a chiller-re-circulator microscope stage chiller. The glands were rinsed by moving them back and forth in the solution before being transferred into hagfish gland cell culture medium (see Media Preparation below). No anti-bacterial or anti-fungal agents were used in the sodium citrate or culture medium for these steps because the time course in which the tissue is in contact with these solutions was not long enough for them to be effective. This process of harvesting glands was repeated until all glands from a hagfish were removed.

_Gland digestion and cell dissociation_

Once all of the glands were harvested, sterile fine forceps were used to transfer the glands into a 35 mm culture dishes containing 5 mL of rinse solution (sodium citrate). The glands were then torn in half using forceps before being transferred back into culture medium. The cells (mostly mature) in sodium citrate that pelleted out of the gland during were collected using a 5mL serological pipette and centrifuged in 15-mL falcon tubes at 500 g for 2 mins. The supernatant was decanted with special attention to avoid mucin strands and associated threads. The gland cells were resuspended in a small volume of culture medium using a micropipette and a large 1 mL pipette tip that had been cut to increase its bore and thereby reduce the pipetting
velocity. Gland cells were transferred into 35-mm culture dishes containing hagfish CM and maintained in an incubator at 11 °C.

Some of the procedures described below, for example, some electron microscopy preparations, required cells to be manipulated in suspension. For these procedures, the glands were sequentially digested in 5 mL of collagenase digestion solution (1mg/mL in hagfish CM; 20 °C). Gland digestion involved 1 hr sequential rinses of the glands with the collagenase solution (5 mL) in 35 mm culture dishes on a rotator-shaker plate. Following every 1-hour rinse the remaining gland fragments were transferred into fresh collagenase solution and the remaining digestion solution containing dissociated cells was pipetted into a 15-mL falcon tube. The falcon tubes were then stored in the incubator at 11 °C to allow the cells to pellet out of solution. Following 24 hours in the incubator the supernatant digestion solution was aspirated from the cells and the cells were resuspended in fresh culture medium before being transferred via pipette into 35-mm culture dishes and placed back into the incubator.

*Concentrating gland cells and exchanging cell media*

Gland cells were concentrated for fixation and imaging using a number of techniques. These include centrifugation of the cells in media at 500 g for 2 minutes, allowing the cells to pellet out of solution via passive settling, or by pipetting the cells into a cell manipulation chamber that allowed media to flow through and captured cells on a mesh. The method used depended on the final outcome desired as all techniques result in rupturing of some cells and subsequently the formation of mucin strands in the cell culture. Centrifugation resulted in the highest degree of mucin strand formation and these mucin strands were able to unravel skeins and remove the plasma membranes from intact cells.
**Media preparation**

Hagfish are true osmoconformers and as such their tissues, and the cells that comprise them, have osmolalities equal to that of their environment (i.e. seawater). This presents a unique challenge to establishing a primary cell culture when using cell culturing media intended for use with mammalian or teleost fish cells, which typically have osmolalities about one third that of seawater. Previous work by Herr et al. (2010) demonstrates a number of organic osmolytes that occur in the hagfish slime gland at relatively high concentrations, including: Trimethyl amine oxide (TMAO), betaine, and dimethylglycine (DMG). Using these data about the slime gland we have come up with a number of custom media cocktails that all exhibit an osmolality of approximately 1000 mOsmol/kg (see media preparation). With the osmotic contribution of NaCl, TMAO, and DMG held approximately constant among the media the concentration of betaine was varied to account for the inherent differences in osmolality found between the commercially available media types. Leibovitz L-15 was decided upon because of its frequent use in elasmobranch primary cell culture and strong pH buffering capacity under atmospheric carbon dioxide levels. All media were sterile filtered using 0.22 µm Millipore Stericups, pH adjusted to 7.3 using 1 N NaOH (because DMG acidifies the L-15 media), and combined with FBS (10%), Pen/Strep. (10,000 units/mL; 10,000 µg/mL), and Fungizone (2.5 µg/mL) just prior to use.

**Collagenase digestion solution**

The hagfish slime gland is surrounded by a collagenous capsule. Therefore, to expose young cell types found on the inside wall of the gland capsule, excised glands were exposed to collagenase digestion solution that was prepared in hagfish cell media. This solution contained: collagenase (1 mg/mL), fetal bovine serum (10%), penicillin (10,000 units/mL), streptomycin
(10,000 units/mL), and Fungizone (2.5 ug/mL). To maintain the sterility of the culture the digestion solution was pH adjusted to 7.3 and sterile filtered using a 0.22-µm syringe tip filter.

**Cell viability testing with trypan blue**

To determine the viability of recently dissociated gland cells or cells in culture a micropipette with a large bore tip was used to gently re-suspend cells in the 35-mm culture dishes. Following re-suspension a 500-µL sample from the culture dish was taken and placed in a 2-mL microcentrifuge tube. The cells were spun down at 500 g for 2 minutes or placed in the incubator overnight to passively settle out of solution (approximately 12 hours). Following concentrating the cells in the microcentrifuge tube the supernatant media was decanted and replace with 15 µL of media and 15 µL of trypan blue. The cell pellet was gently but thoroughly flushed before a 20-µL sample was taken and placed on a glass slide for observation under a microscope. Trypan blue is a vital exclusion dye, which only penetrates the membrane of dead not living cells. Using a 10x objective lens 3-4 images were taken of each sample. In each of these images the total number of cells, as well as the total number of dead (or blue stained) cells were counted to calculate cell viability within the culture.

**Fixatives**

A number of fixatives were used in preparation for the various tissue embedding and microscopy procedures employed in this study. For transmission and scanning electron microscopy a fixative solution containing 3% paraformaldehyde and 3% glutaraldehyde prepared in 0.1-M sodium cacodylate (~980 mmol/kg) was used. In preparation for paraffin embedding both 10% buffered formalin and 3% paraformaldehyde in PBS were utilized.

All fixatives containing paraformaldehyde were prepared in a fume hood using a heated stir-plate to warm the appropriate salt solutions (for example 0.1-M sodium cacodylate) to 60 °C
before the addition of paraformaldehyde powder. A temperature probe connected to the stir-plate was used to monitor the temperature of the fixative solution, and a pH probe allowed the solutions pH to be brought to 7.3. Paraformaldehyde was dissolved into solution using 1 N NaOH. The pH of the fixative solution was adjusted using 1N HCl. In the electron microscopy fixative an appropriate volume of 50% glutaraldehyde in water was added to a final concentration of 3%.

Ethanol Dehydration

Following fixation a 3 x 10 minute rinse of the cells/glands with the appropriate rinse (sodium cacodylate or PBS) was performed. The final rinsate was removed from the vial and approximately 10 mL of 30% ethanol added onto the manipulation chamber. Precautions were taken to avoid drying of the cells/glands between subsequent dehydration steps of the following ethanol dehydration series. Samples were left in 100% EtOH until being embedded in paraffin or plastic.

Paraffin embedding and staining of hagfish tissues

Hagfish tissues including whole slime glands, whole body cross-sections and whole glands with skin and musculature were fixed using both 10% buffered formalin and 3% paraformaldehyde prepared in PBS. The tissue fixation protocol developed in this study was adhered to for the fixation of all tissues. Tissues were paraffin embedded in the University of Guelph Animal Health Laboratory using standard protocols. Hagfish tissues were sectioned using a microtome to 4 µm sections and placed on glass slides for both histological and immunological staining. Whole glands were stained with: (1) hematoxylin and eosin (HandE), (2) Masson’s Trichrome, and (3) Basic fuschin methylene blue. Gland, skin and musculature sections were stained only with HandE.
De-waxing Paraffin Embedded Hagfish Gland Cells

Prior to immunological labeling of the hagfish glands and associated tissue the paraffin sections were de-waxed using a xylene and EtOH de-waxing series (see appendix). Individual glass slides were treated in 50 mL falcon tubes containing approximately 40 mL of each of the rinsing solutions.

Primary antibodies

Immunological labeling of the intermediate filaments (IFs) in hagfish gland cells was performed using the following dilutions and primary antibodies, respectively: IF antibody (1) 1:400, pan-Cytokeratin of mAbs at 200 ug/mL (Santa Cruz); IF antibody (2) 1:400; hagfish slime thread antibody. Primary antibody labeling of MTs in the gland cells was done using a 1:500 dilution of anti-alpha-tubulin (bovine), mouse IgG (Invitrogen; A11136; 236-10501; 50 ug/mL). Due to the process by which the two IF primary antibodies were made only IF antibody (2) could be used in combination with the MT primary antibody to meet the secondary antibody binding requirements. IF antibody (2) was used in the majority of IF labeling because IF antibody (1) and the MT primary antibody could not be used in concert. Their fairly comparable performance (see Results) further supported the use of IF antibody (2) in the fluorescent labeling experiments.

Fluorescent Conjugated Secondary Antibodies and Stains

The secondary antibody Alexa Flour 488 goat anti-mouse IgG (Invitrogen; A10680; IgM (H+L);2 mg/mL) was used at a dilution of 1:400 for fluorescent labeling of IF primary antibody (1) and the anti-alpha-tubulin antibody. Alexa 568 goat anti-rabbit (Invitrogen; A11011; IgG (H+L); 2 mg/mL) was used at a dilution of 1:400 for the fluorescent labeling of IF primary antibody (2). The nuclei of cells were fluorescently labeled using 4’,6-diamidino-2-phenylindole, dilactate (DAPI; Invitrogen; L21873W) at a dilution of 1:500.
**Immunofluorescence of hagfish gland cells**

Immediately following slide de-waxing, primary antibodies were prepared to their respective dilutions in a single 1.5-mL microcentrifuge tube by diluting the antibodies in an appropriate volume of 1% bovine serum albumin (BSA) prepared in PBS. When more than one antibody was being used, the antibodies were prepared together in a single tube.

De-waxed slides were then removed from ddH$_2$O and excess water gently tapped from the slides. A hydrophobic barrier was then drawn around the de-waxed tissue sections using a Liquid Blocker super PAP pen (Daido Sangyo Co., Ltd., Tokyo, Japan). This step not only contained the stains as they were applied to the sections but also reduced the volume of antibodies needed and maximized exposure of the tissue to antibodies and fluorescent stains. After the hydrophobic barrier dried the slides were transferred to 60-mm tissue culture dishes to prevent debris from collecting on them and to maintain stable incubation conditions. 200 µL of antibody, BSA solution was then applied at the corner of the hydrophobic barrier so as not to disturb the tissues and to minimize bubble formation. Primary antibodies were left to incubate at 4 °C for 12 hours in covered culture dishes.

Following the 12-hour incubation the primary antibody solutions were removed, one at a time, using a micropipette. The tissue sections were then rinsed one time with 200 µL of 1% BSA in PBS for a duration of 5 minutes.

Secondary antibodies and fluorescent stains (i.e. DAPI) were prepared immediately before their use by diluting them in a 1% BSA in PBS solution and storing them in the dark until used. Following the removal of the BSA-PBS rinse solution from a corner of the hydrophobic barrier the secondary antibodies and fluorescent stains were applied and allowed to incubate in the culture dishes for 30 minutes at room temperature (20 °C). Subsequently these were removed
from the tissue sections and the sections rinsed using fresh PBS (no BSA) for 15 minutes in 5 minutes intervals. After the final rinse the PBS was removed and excess PBS gently tapped off of the tissues section. DakoCytomation Fluorescent Mounting Medium was then placed on top of the tissues sections before covering the sections with a cover glass (0.17 mm) and sealing the slide with clear nail polish. The nail polish was allowed to cure for a minimum of 30 minutes before visualization.

**Plastic embedding hagfish glands**

Hagfish glands were plastic embedded using a Polysciences Poly/Bed®812 (Luft formulations) embedding kit. Plastic embedding of the glands was performed for analysis of gland cells using both transmission electron microscopy (TEM) and focused ion beam (FIB) scanning electron microscopy (SEM). The Poly/Bed®812 plastic embedding media was prepared in 100-mL volumes by combining 48 mL of Poly/Bed®812, 31 mL of dodecenyl succinic anhydride, 21 mL of methylnadic anhydride, and 2 mL of 2,4,6-Tris(dimethylaminomethyl)phenol. Ethanol dilutions of this stock Poly/Bed®812 solution were made in 3:1, 2:1, 1:1 and 0:1 ratios (EtOH:Poly/Bed®812).

Freshly collected slime glands were transferred into fixative solution (3% pfa, 3% gta, 0.1-M sodium cacodylate; 11 °C) and fixed for 1 hour on a rotator-shaker plate. The glands were rinsed for 30 minutes in 10-minute intervals in fresh 0.1-M sodium cacodylate rinse solution (pH 7.3). Following the final rinse, glands were post-fixed for 2 hours at room temperature with an osmium tetroxide solution (1% in 0.1-M sodium cacodylate). Additional rinses were performed (3 x 10 minutes) in 0.1-M sodium cacodylate before exposing the glands for 1 hour to a 2% uranyl acetate in 0.1-M sodium cacodylate solution. Following an additional 3 x 10 minute rinsing in sodium cacodylate the glands were dehydrated in ethanol (see dehydration series).
Plastic infiltration of the hagfish glands immediately followed the final dehydration step (i.e. 100% EtOH). In 1 hour intervals the glands were exposed to the following EtOH: Poly/Bed®812 ratios: 3:1, 2:1, 1:1, 0:1. The final infiltration step involved a 12-hour exposure to the Poly/Bed®812 stock. The glands were transferred between infiltration solutions using fine forceps and left on a rotator/shaker plate operating at 50 RPM for each infiltration step. Prior to curing the Poly/Bed®812, the glands were transferred into a silicone mold and covered in fresh Poly/Bed®812. The Poly/Bed®812 was then set to cure in an oven at 60 °C for 24 hours. The cured plastic blocks containing the glands were allowed to cool for at least one hour before being removed from the silicone mold.

*Epifluorescent and brightfield Imaging*

Live cells, as well as fluorescently labeled and histologically stained hagfish tissue slides were analyzed using a Nikon Eclipse 90i Epi-fluorescent microscope. Brightfield differential interference contrast (DIC) images and fluorescent images were taken using a cooled monochrome digital camera (Q-Imaging Retiga 1300; Surrey, BC, CAN) driven by NIS Elements AR software. Fluorescent monochrome images were taken using DAPI, FITC and TRITC filter cubes to image the DAPI, AlexaFluor 488, and AlexaFluor 568, respectively. The monochrome images were then assigned color channels in Adobe Photoshop CS5 based on the emission wavelengths of the secondary antibodies and fluorescent stains used, when applicable. Double and triple immunofluorescent images were then assembled into multichannel images using Adobe Photoshop CS5. Brightfield color images were taken using a Q-imaging EXi 12-bit color camera (Q-Imaging; Surrey, BC, CAN) driven by NIS Elements AR software.
Confocal imaging

Confocal imaging was performed using an Leica DM6000B microscope in conjunction with Leica TCS SP5 system and Leica DFC350FX monochrome camera. A Radius laser (405 nm; 20% Acousto Optical Tunable Filter (AOTF)) was used for near UV excitation of the DAPI stain, an argon laser (50 mW; 20% AOTF; 488 nm) was used for excitation of the AlexaFluor488, and an HeNe (543 nm; 50% AOTF) for excitation of AlexaFluor568. The Leica SP5 system allowed for the attenuation of the laser lines with its AOTF. The SP5 spectral detection system was used to reduce overlap in the emission spectra of the different fluorophores thereby creating custom detection filter settings and eliminating cross-talk in the fluorescent emissions from the different lasers used.

Transmission Electron Microscopy (TEM)

TEM was performed at the University of Guelph’s Electron Microscopy Unit using a Philips CM10 (tungsten filament 80 kV) outfitted with a top mount SIS/Olympus Morada 11 MP CCD camera. Sections were cut between 20 and 60 nm using a Porter-Blum MT-1 manually driven ultramicrotome and stained with uranyl acetate (2%) and lead citrate (1%) following sectioning.

Results

Gland thread cell (GTC) development

Slime gland harvesting and dissociation illustrated the morphology of live hagfish GTCs at various stages of maturity (Figure 1). Differential interference contrast images and histological staining procedures revealed that the thread skeins of GTCs remain closely proportionate to the cell in size (in both major and minor axes) throughout development (Figure 2, 3). The appearance of a prominent nucleolus in undifferentiated cells may precede cell division (Figure
3.1) as only cells with prominent nucleoli have been observed dividing (Figure 3.1, 3.2). The nuclei of dividing cells (Figure 3.2) and immature GTCs (Figure 2.2) appear similar. Following division the nucleus moved towards one pole of the GTC (Figure 2, 3). Nuclear repositioning was followed by the appearance of a short helical length of slime thread adjacent and apical to the nucleus (Figures 2.2, 2.3, 3.3, 3.4). In both live and paraffin-embedded samples the increase in slime thread length was evident from morphological comparison between GTCs of early and intermediate stages of maturity (Figures 2, 3). However, it was difficult to tell whether slime thread lengthening proceeds for the entire time-course of GTC development because of the complexity of thread coiling (Figure 4). The width of the slime thread increased throughout GTC maturation (Figure 4).

During early stages of thread development the GTC nucleus was round to ovoid in shape and occupied the entire basal side of the maturing GTC, which forced thread development to occur apical to the nucleus (Figures 3.3, 3.4). As thread development proceeded, the nucleus appeared increasingly conical or spindle shaped and coils of slime thread began to extend lateral and basal to the nucleus (Figures 3.5, 3.6). It appears that the nucleus of the GTC constrained how coils of slime thread were laid down and organized into the developing skein.

Staining with hematoxylin and eosin (HandE) revealed the slime thread is eosinophilic. This staining property was exhibited by the bright pink coloration to the thread in DIC (Figure 3.3,3.6). The cytoplasm was eosinophilic in immature GTCs and was markedly less intense than that of the slime thread (Figure 3.3). In GTCs of intermediate to late maturity, the thread skein occupied the majority of the cytoplasmic space and as a result the cytoplasm was difficult to distinguish (Figures 3.3,3.6). The nucleolus was black in appearance relative to the purple of the nucleus suggesting a stronger basophilic reaction (Figure 3.6). The application of a basic fuschin
methylene blue stain highlighted the basophilia of the prominent GTC nucleolus in a deeply-
staining blue/black coloration (Figure 3.2,3.4,inset 3.4). The basophilic nucleus also appeared
purple in coloration (Figure 3.2,3.4,inset 3.4). The thread stained metachromatically and
appeared light pink or purple in comparison to the purple and black of the nucleus and nucleolus
respectively (Figure 3.4 inset). Application of Masson’s Trichrome revealed a dark brown
staining nucleolus and red/brown nucleus.

Live cell culture also revealed that hagfish gland cells are non-adherent to a wide variety
of attachment substrates including: collagen, fibronectin, poly-l-lysine, alcian blue. For this
reason the cells were maintained in suspension.

The viability of gland cells in culture was determined using the vital exclusion dye trypan
blue. Viability testing demonstrated that the protocols used for gland harvesting and cell
dissociation resulted in high numbers of live gland cells (Figure 5.1-4).

*Gland mucous cell (GMC) development*

The hagfish GMC was spherical throughout development with one off-center nucleus and
a single spherical nucleolus of less prominence than that of the GTCs (Figure 6,7). Hagfish
GMCs contained large numbers of disc-shaped mucin vesicles up to 5 µm in diameter (Figures
6,7). TEM revealed that GMCs contain a vast Golgi complex that extended from the centrally
located nucleus to the periphery of the cell (Figure 8.1). The Golgi contained vesicles at various
stages of maturity with the smaller mucin vesicles adjacent to the nucleus and largest towards the
periphery of the cell (Figures 8.1,8.2). Mature (~ 3-5 um diameter) mucin vesicles were visible
in immature GMCs (Figure 8.3).

Immunolabeling for IF and tubulin protein revealed that GMCs exhibit low fluorescent
signals for these proteins. A faint signal for IF protein did appear within the GMCs but it
appeared to be associated with the glycoproteins of the mucin vesicles (Figure 9.1). The development of a GMC in early stages of development resulted in the separation of surrounding epithelial cells from the periphery of the slime gland (Figure 9.1,9.2). Histological preparations of GMCs illustrated that the glycoproteins of the GMC are basophilic (Figure 9.3). A eosinophilic network (light pink) was also visible within figure 9.3 that appeared to extend between the developing vesicles. The eosinophilic cells of the proposed epithelial lining distinguished them from the adjacent gland capsule.

Repeated viability tests using trypan blue revealed that the GMCs remained viable in culture for extended periods of time (close to three months in some cases), although no evidence of gland cell proliferation in culture was observed. This is not entirely surprising as GTCs and GMCs do not divide once differentiated.

*Polyhedral cells of the hagfish slime gland*

The application of histological and immunofluorescent techniques to paraffin embedded hagfish gland sections revealed the polyhedral cells (PCs) mentioned by Newby (1946). PCs can be distinguished from neighboring cell types because: (1) they are rounder and many sided in comparison to the squamous cells (ECs), which line the periphery of the slime gland, and to the squamous epidermal skin cells (ESCs) of the gland pore and skin surface (Figure 10), and (2) they occur in a thick cell layer surrounding the stalk (bottom) of the slime gland pore in comparison to the thin one or two cell layers of ECs and ESCs (Figures 10.2, 10.4).

Epifluorescence and confocal microscopy revealed that PCs express both IF and tubulin proteins (Figure 10.2, 10.3). IFs appeared to form a peripheral network around the cell (Figure 10.2, 10.3). A cytoplasmic MT network was also apparent in the PCs with the greatest intensity of fluorescence in the paranuclear region of the cell (Figure 10.3). PCs stained with HandE
exhibited an eosinophilic cytoplasm and basophilic nucleus with no distinct nucleolus (Figure 10.1).

*New cell in the hagfish slime gland*

Fluorescent staining of paraffin embedded hagfish gland sections revealed the presence of a new cell type that has never been described before within hagfish slime gland (Figure 11). These cells are spindle-shaped with a large centrally located nucleus, and they exhibit strong evidence for extensive cytoplasmic IF and MT networks (Figure 11, 12.1, 12.2). This cell type also exhibited long bifurcating processes that formed connections with other similar cells, the gland periphery and developing GTCs and GMCs throughout the gland (Figure 11, 12.1, 12.2). These cells were abundant and found in close proximity to the developing GTCs and GMCs at all stages of maturity (Figure 12.3). The cells were found within the interstitial spaces of the slime gland between developing GTCs and GMCs (11, 12.3), which gives them an extremely high aspect ratio (Figure 12.2). Each GTC and GMC appeared to associate with one to three of these undescribed cells suggesting they are at least equally as abundant within the gland as GTCs and GMCs.

Epifluorescence also revealed that a monolayer of epithelial-like cells lines the slime gland (Figure 11). The appearance of this monolayer in fluorescence illustrates a distinct transition from the gland interior to the gland capsule. The continuous nature of these cells is indicative of a continuous gland epithelium.

Transmission electron microscopy supported the observation that the newly discovered cell type closely juxtaposes GTCs and GMCs (Figure 13). The cells contained a Golgi apparatus that extended throughout the cytoplasm. Golgi is observed both adjacent to the nucleus (Figure 13.6) as well as within the interstitial space occupied by the cells between developing GTCs and GMCs.
GMCs (Figure 13.4). The cells also exhibit large numbers of vesicles and mitochondria, the former of which appear to fuse between this cell type and adjacent GTC and GMCs (Figure 13.3, 13.6). Cellular processes extending between developing GTCs and GMCs also contain Golgi and mitochondria. In some instances the fusion of vesicles between the cell and GTCs and GMCs was observed (Figure 13.6, 13.7).

**Cell distribution in the hagfish slime gland**

Whole gland histology revealed several distinct regions at the periphery of the slime gland. These regions include: (1) the gland pore and polyhedral cells of the gland stalk/base (Figure 10, 15.1, 16, 17), (2) the epithelial lining of the slime gland (15.2) and (3) the region of major cell proliferation (Figure 15.3ab). The hagfish slime gland represents a clear invagination of the epidermis (Figure 14). The transition between the observed regions can be followed by tracing the epidermis in through the gland pore of Figure 14. The observed cell regions become more distinct in hagfish glands that were slimed 2 weeks before the glands were harvested (Figure 15).

**Discussion**

This study presents a number of novel insights into the morphological and ultrastructural characteristics of hagfish gland cell types, including: (1) the first images of live hagfish gland cells in culture, (2) the first images of polyhedral cells from the hagfish slime gland, (3) the discovery of a previously undescribed cell type within the slime gland, and (4) ultrastructural and immunological analysis of hagfish GMCs. The application of a variety of cell preparation and imaging techniques provided here represent the most comprehensive investigation of hagfish gland cell types to date.
*Gland thread cell development*

The study of GTC development within the slime gland is restricted to a small number of previous studies (Newby, 1946; Blackstad, 1968; Downing et al., 1984; Spitzer et al., 1988). Furthermore, what is known about GTC development has rarely been corroborated by other studies, and it is also unclear how much of this information can be generalized to other hagfish species. The investigation of GTC development presented here is qualitative, but several of its observations will feed into the more detailed analysis of thread production in GTCs in Chapter 3.

The characteristically large nucleus and nucleolus of the GTC (following differentiation) is indicative of cells that require large numbers of ribosomes for protein synthesis. This cellular feature has been used by several researchers to differentiate GTCs from other cell types in the gland (Newby, 1946; Blackstad, 1968; Terakado et al., 1975; Downing et al., 1984). Large numbers of unbound cytoplasmic ribosomes and fewer numbers of ribosomes on the rough endoplasmic reticulum have been observed in hagfish GTCs (Terakado et al., 1975; this study). This suggests protein synthesis is primarily accomplished by cytoplasmic ribosomes which is consistent with observations by Fan (1968) and Terakado et al. (1975).

Changes in nucleus position and morphology were observed from early to intermediate stages of GTC development. The polar repositioning of the GTC nucleus in cells from *M. glutinosa* is consistent with observations by Newby (1946), Terakado et al. (1975) and Downing et al. (1984), which showed similar nuclear repositioning in GTCs from *Polistotrema stoutii, Paramyxine atami, Eptatretus burgeri,* and *Eptatretus stoutii.* The formation of recognizable slime thread following nucleus migration is consistent with observations by past researchers (Newby, 1946; Terakado et al., 1975; Downing et al., 1984). As a result of the consistency
between these observations the migration of the nucleus from the center of the cell to a pole appears influential in shaping the developmental axis (i.e. polarity) of the cell.

The morphological changes in the GTC nucleus, from early (round or ovoid) to intermediate (conical or spindle shaped) stages of thread development, suggest that the shape of the nucleus may be influential in guiding thread organization within the cell. The idea that the conical shape of the nucleus imparts a conical shape on the arrangements of thread loops is supported by Fernholm (1981) who observed the thread uncoils in successive arrangements of loops organized in a conical way. He dubbed these ‘conical loop’ arrangements. It can be hypothesized that cell growth proceeds each successive formation of a conical loop arrangement such that each arrangement is nested within the last. This hypothesis is supported by Fernholm (1981) who observed then when thread from the apical side of the skein is drawn upon with forceps the thread unravels from each conical grouping successively. We propose that the position and shape of the nucleus - as well as increases in cell size that accommodate skein growth - guide thread organization by both restricting the space for thread elongation and the areas in the cell in which the thread may coil.

The eosinophilia of the hagfish slime thread is consistent with IF and keratin staining in the literature (Requena et al., 1990). The strong basophilic reaction of the nucleus and nucleolus with all three of the applied stains is consistent with the large concentrations of nucleic acids (DNA and RNA) found in the nucleus. The entirely RNA composition of the nucleolus in part accounts for its darker coloration as a result of the basic stains. This difference in nucleus and nucleolus staining may also be related to the presence of a high euchromatin to heterochromatin ratio in the GTC nucleus as a result of active transcription of DNA.
The non-adherence of the GTCs to a wide variety of attachment substrates may be consistent with the requirement that the GTCs ‘flow’ from the lumen of the slime gland to the gland pore prior to rupture of their plasma membrane. Significant sites of attachment to neighboring GMCs may result in the premature rupture of GTC and GMC cell membranes. This would result in the release of mucin vesicles or thread within the gland, which could conceivably clog the gland pore. However, as a number of studies have shown that epidermal and epithelial cell derivatives grow most vigorously in co-culture with nurse cells (i.e. fibroblasts; El-Ghalzbzouri et al., 2002) this is also a possibility for improved GTC culture and adhesion.

The formation of mucus from ruptured mucin vesicles in culture is also an obstacle to be overcome. The resultant strands of mucous following vesicle rupture stripped the plasma membranes from cells during centrifugation causing high amounts of cell death. The application of disulfide cleaving compounds such as dithiothreitol and β-mercaptoethanol, although effective at reducing mucin strand density, were ineffective in totally eliminating mucin attachments in the culture and resulted in accelerated cell death.

**GMC development**

Hagfish GMCs represent a unique derivative of the mucous producing epidermal cell lines known in cyclostomes (Quay, 1979). This study presents the first analysis of GMCs using TEM and immunofluorescence techniques. Apart from Leppi’s (1968) insightful account of the histological staining characteristics of GMCs from hagfish, there has been no focus on the development of these cells in the literature. This study finds that GMCs develop from within the proposed epithelial lining of the hagfish gland where their growth results in the separation of a number of adjacent cells from the epithelial lining. These cells appear to remain with the GMC through development and may represent the undescribed cell type from the gland lumen. TEM
revealed that mucin vesicles are produced from an elaborate Golgi apparatus and this process appears to begin early in cell development. Developmentally the hagfish GMC is different from its epidermal relatives because: (1) It develops as a part of a multicellular holocrine gland, whereas the mucous cells and epidermal thread cells of the epidermis are considered unicellular holocrine glands, and (2) hagfish GMCs are stored within a gland prior to their release, whereas epidermal mucous cells (both large and small) release upon reaching the surface of the epidermis.

Immunofluorescent staining of GMCs illustrates relatively little emission of fluorescence for the IF and alpha-tubulin proteins probed. Although it is likely that both IFs and MTs are present within the GMC signal detection may have been limited because of the low cytoplasmic surface area that is exposed between the mucin vesicles for antibody interactions.

The basophilic staining of the hagfish mucins is consistent with findings from Leppi (1968). In general the basophilic properties of mucins such as glycoproteins is consistent with the interaction of hematoxylin with the carboxyl end groups of the oligosaccharide side chains of glycoproteins. The eosinophilic network visible in the GMCs likely corresponds to the cell cytoplasm that exists between closely juxtaposed mucin vesicles. The reticular appearance is thus a result of the narrow cytoplasmic gaps between mucin vesicles and the Golgi of the cell. These observations suggest that the low fluorescent signal for IFs and MTs observed in GMCs is consistent with the observation that little cytoplasmic surface area exists between the vesicles for exposure of these cytoskeletal elements to the antibodies.

*Polyhedral cells of the hagfish slime gland: a forgotten cell type*

The cell types comprising the periphery of the slime gland interior have received very little attention in the literature. In fact, Newby (1946), the first to discuss these cells, suggested
that the slime gland does not have a true epithelial lining. This study finds that an epidermal to epithelial transition occurs at the pore of the slime gland. The lining of the gland consists of polyhedral cells clustering at the base of the gland pore and epithelial cells lining the rest of the inner surface. This study corroborates observations of a morphologically distinct cell type at the gland pores stalk (Newby, 1946). These cells which Newby (1946) dubbed ‘polyhedral cells’ received little attention in his analysis of the gland. The findings of this study suggest an eosinophilic nature to the cytoplasm of both the polyhedral cells and those lining the gland periphery. Immunofluorescence revealed the presence of IF proteins and alpha tubulins in both of these cells. The peripheral IF network, visible in both epifluorescence and confocal microscopy, is characteristic of cells types that experience sequential changes in cell shape brought on by processes of proliferation, movement or invasion of tissues (Iwatsuko and Suda, 2010). A peripheral IF network exists in both polyhedral cells and the epithelial-like cells. Similar IF networks are observed in the epithelial cells of mammalian bladders where they help maintain cell integrity during sequential filling and emptying of the bladder (Hicks, 1965; Iwatsuko and Suda, 2010). The process of gland filling and emptying is somewhat analogous to the bladder, albeit over a longer timescale. This may suggest similar mechanical demands of the cells lining the periphery of the gland.

The large accumulations of PCs exhibiting peripheral IF networks may also suggest structural or mechanical roles of these cells. The large cell layer that the PCs represent may narrow the gland pore to facilitate the holocrine release of the GTCs and GMCs. Studies by Lim et al. (2006) illustrate that the gland expels its contents quite forcefully. Personal observations also suggests that a type of mechanical plug may exist within the gland pore of slime glands that have not recently been ‘slimed’. In these glands, electrostimulation results in the forceful
ejection of slime exudate, suggesting the gland is under high pressure before it releases. The accumulation of PCs near the gland pore may therefore act as a mechanical plug to facilitate the ejection of the slime exudate away from the hagfish. Although further investigation into ‘slimed’ vs. ‘unslimed’ glands is needed to support this assertion.

**The Gland Interstitial Cell (GIC): a new cell in the lumen of the hagfish slime gland**

Until now only GTCs and GMCs were believed to be found in the lumen of the hagfish slime gland. However, this study presents evidence for a third cell type found within the interstitial space of developing GTCs and GMCs. Previous studies likely missed the presence of these cells because they are so narrow, but use of the nuclear stain DAPI highlighted their presence (Figure 11). Subsequent imaging of this cell using confocal, and transmission electron microscopy reveal a number of interesting morphological characteristics of these cells that point toward a number of possible functions. These cells form tight associations with immature and mature GTCs and GMCs in the gland, and they appear to completely occupy the spaces between them. This positioning of these cells between the much larger GMCs and GTCs gives them a spindle like appearance, although their morphology in three dimensions is likely far more complicated, especially considering that their cellular processes occasionally bifurcate (Figure 12.2). Ultrastructural analysis reveals the presence of a large Golgi apparatus with many associated vesicles. Elements of the Golgi appear to extend from the paranuclear space well into the cytoplasmic processes that extend between developing GTCs and GMCs. The fusion of vesicles between the membrane of the GIC and GTCs/GMCs was also observed in TEM, suggesting that these cells may play a feeder role to the GTCs and GMCs. Immunofluorescence supports the observation of IF networks in these cells and provides evidence for a substantial cytoplasmic MT network as well.
These morphological observations suggest that the GICs may play a number of roles within the slime gland. These roles may include but are not limited to: (1) Providing structural support within the gland to developing GTCs and GMCs, (2) Acting as ‘feeder’ or ‘nurse’ cells to the adjacent GTCs and GMCs, and (3) Providing a network within the gland for the retention of immature GTCs and GMCs during a sliming event.

The results of this study suggest that the reticular network observed by Newby (1946) was not connective tissue but in fact a network of GICs extending between developing GTCs and GMCs. This study finds no evidence of trabeculae or capillary loops within the gland of *M. glutinosa* which corroborates vascular corrosion casts of *M. glutinosa* glands done by Lametschwandtner et al. (1986). This study finds no evidence that the GICs are fibroblasts or that they produce an extracellular connective tissue in the gland. The positive reaction in the GICs for the pan-Cytokeratin antibody, which binds type I and type II IF proteins, suggests these cells are not fibroblasts. Fibroblasts utilize type III IFs such as vimentin and as such do not express the keratin proteins probed for with the dye. The pan-cytokeratin dye utilized probes only for type I and type II IFs found in epithelial cell derivatives. Ultrastructural analysis of the GICs shows no evidence of an extracellular matrix secreted by these cells. As such, the GIC is a epithelial cell line derivative that may provide a support network around the GTCs and GMCs of the gland and function as a feeder cell for the developing GTCs and GMCs.

The vesicle fusion between GICs and GTCs/GMCs observed in this study is consistent with the role of nurse cells such as sertoli cells and some fibroblasts. For example, the dependence of certain epithelial and epidermal cell types requires nurse cell support both *in vivo* and *in vitro* (Rheinwald and Green, 1975). It has been shown in keratinizing epitheliums that the
interaction between these keratinizing cell types and fibroblasts is critical in the growth of these epidermal cells (Rheinwald and Green, 1975).

The reticular network that the GICs form within the gland may function as a mechanical means for retaining immature GTCs and GMCs following contraction of the gland (i.e. sliming). This hypothesis is supported by the large proportion of mature thread skeins to immature skeins expressed upon electrostimulation of full slime glands (Spitzer et al., 1988). Although the size of the skeins decreases with successive electrostimulation of the gland, successive contractions may not represent a realistic scenario given the large numbers of glands possessed by hagfish from which slime can be released in the event of an attack. The hypothesis that the GICs are involved in cell retention is also supported by a personal observation that slime glands cannot be completely depleted of cells. A proportion of small, likely immature GTCs and GMCs, are always retained by the gland even after sequential stimulation (Spitzer et al., 1988). This property of the slime gland to retain immature cells is consistent with findings from the parotoid glands of amphibians investigated by McCallion (1956). Furthermore, the lack of noticeable GICs in cell culture suggests that the GICs are likely retained in the gland during a sliming event or damaged beyond recognition following holocrine release of the GTCs and GMCs. This may suggest that intercellular connections between GICs and GTCs/GMCs are weakened or reduced at maturity.

*A revised perspective of cell distribution in the slime gland of *M. glutinosa*

This study finds that the trabeculae observed by Newby (1946) in the glands of *Polistostrema stoutii* are not present in the glands of *Myxine glutinosa*. The reticular network observed by Newby (1946) is explained by the discovery of a network of undescribed cells (the GICs) within the gland. The results of this study suggest that the gland is divided into 4 general
regions: (1) the gland pore and polyhedral cells of the gland stalk/base, (2) the epithelial lining of the slime gland, (3) the region of major cell proliferation, and (4) the gland lumen comprised of GTCs, GMCs and GICs. The presence of large numbers of immature GTCs, GMCs and GICs in the basal portion of slime glands (opposite the gland pore) is indicative of a highly active area of cell proliferation in the gland. If GTCs and GMCs developed in the apical region of the gland (side with the gland pore) the presence of immature cells in this region would result in their release from the slime gland during a sliming event. Present results agree with those of Spitzer et al. (1988), that these immature cells are typically not observed in the exudate released from hagfish slime glands.

**Future Studies**

Numerous questions arise from this work, including: (1) What are the functions of the GICs and PCs in the slime gland? (2) Would the isolation of GICs along with GTCs/GMCs from the gland facilitate long-term growth, differentiation and development of cells in culture? and (3) What role do actin microfilaments play in the slime gland? An expansion of cell culturing techniques such as cell isolation based on type and size, would allow for a wide range of future studies, for example, exploring differential gene expression as a function of cell maturity. This information would be immensely important in determining the proteins that are being produced by GTCs, GMCs and GICs.
CHAPTER 3: SLIME THREAD PRODUCTION BY THE ATLANTIC HAGFISH (M. glutinosa) GLAND THREAD CELL
Abstract

The slime produced by hagfishes contains a unique combination of proteinaceous threads and mucins that viscously entrap seawater to discourage attacks by gill-breathing predators. The intermediate filament (IF) threads that traverse the mass of slime reach up to 15 cm in length and are synthesized for holocrine release by 150-µm gland thread cells (GTCs). Exactly how a 150-µm cell synthesizes a continuous 15-cm IF thread is not well understood. What is known is that microtubules (MTs) are present within bundles of intermediate filament (IF) during the early and intermediate stages of slime thread maturation but subsequently disappear as the GTCs fully mature. This study presents an analysis of: (1) slime thread ultrastructure using focused ion bean scanning electron microscopy and transmission electron microscopy, and (2) the distribution and organization of IFs and MTs using immunofluorescent techniques. Using these techniques we test a number of hypotheses regarding the origin and mechanisms of slime thread assembly. This study reveals that thread assembly involves predictable changes in thread composition, organization, and patterns of IF and MT recruitment over five stages of maturation.

Introduction

Hagfishes, and lampreys, belong to an ancient lineage (Superclass: Agnathans, Class: Cyclostomata) that evolved over 350 million years ago and are believed to be the sister group to vertebrates (McCauley and Kuratani, 2008; Near, 2009). Hagfishes are well known for their slime producing ability. This defensive behavior has been conserved among all extant species of hagfishes studied and may be one of the keys to their survival over the last 300-400 million years (Fernholm, 1981; Lim et al., 2006; Zintzen et al., 2011). The slime is comprised of two primary constituents that develop within highly specialized cells of the slime gland: (1) large ellipsoidal bundles of thread (150 µm major axis) rich in intermediate filament (IF) proteins, and (2) small
disc-shaped mucin vesicles (3 µm in diameter) containing high concentrations of glycoprotiens (Figure 2; Newby, 1946; Fernholm, 1981; Downing et al., 1984; Spitzer et al., 1988; Luchtel et al., 1991; Herr et al., 2010; Winegard and Fudge, 2010). Contraction of the highly-vascularized smooth musculature surrounding the gland causes the forceful holocrine ejection of both GTCs and GMCs into the surrounding seawater (Newby, 1946; Fudge et al., 2005).

Intermediate filaments, such as those comprising the hagfish slime thread, are composed of two distinct protein subunits that combine to form a wide array of cytoskeletal networks (Downing et al., 1981; Aebi et al., 1988; Steinert and Roop, 1988; Koch et al., 1994). Each protein subunit has a tripartite organization that consists of a helical central rod domain as well as non-helical terminal N and C-domains (Koch et al., 1994). Hagfish slime thread IFs can be divided into two groups of thread polypeptides (1) alpha (643 residues, 66.6 kDa) and (2) gamma (603 residues, 62.7 kDa) (Koch et al., 1994; Koch et al., 1995). The gamma polypeptide is also represented by a post-translational beta form that occurs during the late stages of slime thread development (Spitzer et al., 1984; Spitzer et al., 1988). The alpha and gamma thread polypeptides share less than a 30% homology with other IF types and exhibit a relatively high threonine content (alpha, 13%; gamma, 10%) when compared to the other IF types (2-5% on average) (Koch et al., 1994). Threonine and serine represent sites for the modulation of IF assembly and organization through post-translational modifications such as phosphorylation and dephosphorylation (Iwatsuki and Suda, 2010). This may suggest that hagfish IF polypeptides exhibit a high degree of competence for modifications in their assembly and organization based on their high threonine and serine content.

Hagfish slime develops within segmentally paired epidermal involutions, or slime glands, that line the ventro-lateral sides of the animal (Blomfield, 1882; Newby, 1946; Downing et al.,
Within the slime gland the thread producing gland thread cells (GTCs) are distinguished by their large round nucleus (~15 µm diameter) and nucleolus (~4 µm diameter) (Terakado et al., 1975). Distal to the nucleus lies a conically-shaped region rich in mitochondria (the mitochondrial-rich-zone or MRZ) as well as elements of a Golgi apparatus and rough endoplasmic reticulum (Downing et al., 1984). The characteristic slime thread skein grows to occupy the remainder of the cell (Blomfield, 1882; Newby, 1946; Fan, 1965; Terakado et al., 1975; Downing et al., 1984).

Although the literature on whole-gland structure is limited, a number of studies have attempted to elucidate patterns of slime thread production and coiling within hagfish GTCs. GTCs develop from the periphery of the slime gland to the gland lumen where the ellipsoid-shaped cells grow to approximately 150 µm along their major axis, and about half as wide along the minor axis (Newby, 1946). Within maturing GTCs the slime thread develops via the parallel arrangement of 10-12-nm (D) IFs as well as 35-55-nm (D) MTs distal to the conically-shaped mitochondrial rich zone of the cell (Terakado et al., 1975; Downing et al., 1984). Past researchers have observed changes in the size and frequency, of both the IF and MTs, as a function of thread maturity. Based on TEM images, Downing and colleagues postulated that the parallel arrangement of IFs and MTs occurred via their circumferential recruitment around the primal thread. Within these filamentous arrangements of IFs, they demonstrated that the presence of MTs was both spatially and temporally variable along the length of the developing thread (Downing et al., 1984). These researchers hypothesized that MTs may play both structural as well as physiological functions within the developing thread. Structurally, MTs may provide a scaffolding on which IFs can assemble (Downing et al., 1984). This hypothesis is consistent with Terakado and colleagues (1975) who posited that the tubular structures in the thread provide the

46
axis for thread development. Physiologically, MTs may provide a pathway within the thread for
the regulation of cytoplasmic composition or abundance and/or the movement/rearrangement of
filaments within the developing thread (Downing et al., 1984). However, the significance of MTs
in IF-MT interactions during the development of this single, tapered, cylindrical thread remains
to be determined. Downing et al. (1984) also provided a qualitative analysis of a shift in thread
development characterized by a change in a substance/structure associating with the periphery of
the slime thread. They reported using TEM a helically orienting fiber around threads of early
stages in development (Downing et al., 1984). They also demonstrated that a substance of
indiscernible ultrastructure replaced the helical wrapping in subsequent stages of maturity
(Downing et al., 1984).

The functional interactions between IFs and MTs have only more recently been examined
(Bloom et al., 1985; Goldman et al., 1985; Svitkina et al., 1996; Draberova and Draber, 1993).
Immunofluorescence has revealed that IF and MT proteins often co-localize within the
cytoplasm of cells, and that IFs may even be moved by MT polymerization (Bloom et al., 1985;
Dablerova and Draber, 1993). The most compelling evidence for critical IF-MT interactions has
come from examining the effect of MT dissociating drugs such as colchicine on IF structure
within the cell (Bloom et al., 1985; Draberova and Draber, 1993). It has been shown that the
disruption of MTs within the cell causes the paranuclear collapse of IFs (Bloom et al., 1985).
Bloom et al. (1985) suggest that this may provide evidence that IFs are supported by a MT
scaffolding within the cell.

These previous studies provide a number of interesting observations and insights into
GTC maturation and slime thread development: (1) Hagfish GTCs make a 15-cm long protein
thread, (2) The thread is coiled in a highly organized manner, except for at the apical tip, (3) The
thread grows in both length and girth as the GTC develops, (4) The developing thread contains both IFs and MTs however the MTs eventually disappear, (5) A helical wrapping around the slime thread is present at some stages of thread development, but not all, (6) The relative thread density appears to increase as a function of maturity, and (7) Post-translation modification of slime thread IF proteins has been reported in late stages of GTC development and are known to induce changes in IF assembly and organization.

It is clear from the literature that thread assembly involves interactions between IFs and MTs that result in a large (~15 cm) bi-directionally tapered thread, that is precisely coiled within the confines of the cell (Fan, 1968; Terakado et al., 1975; Downing et al., 1984; Spitzer et al., 1988; Fudge et al., 2005). However, it is not known whether observations of thread development by past researchers (i.e. Newby, 1946; Downing et al., 1984) apply to all species of hagfish. As such we ask: (1) How does the mechanism of thread assembly in *M. glutinosa* GTCs compare to *P. stoutii* and *E. stoutii*? (2) What is the significance of the helical wrapping observed by Downing et al. (1984)? (3) What is the function of MTs in slime thread development and within the GTC? (4) Do increases in thread length and girth happen in concert, or are they ever uncoupled? And (5) What is the significance of changes in thread density with maturity and do these changes have anything to do with the post-translational modifications described by Spitzer et al. (1988)?

Based on the observations of previous workers, and our own (Chapter 2), we propose the following hypotheses, and predictions which allow them to be tested:

(H1) Hagfish thread assembly is initiated within the MRZ with the bundling of 10-12-nm IFs. H1 predicts that: (P1a) similar patterns of slime thread assembly should be observed
between *M. glutinosa* and *P. stoutii*. (P1b) the smallest fibers in the GTC should be observed in the MRZ, (P1c) the smallest slime threads should be comprised of IFs only.

(H2) Hagfish slime threads grow in length and girth via the helical wrapping of IFs. H2 predicts that: (P2a) the helical wrapping should have the same dimensions as other IFs in the thread, (P2b) the angle of the helical wrapping should not be constant, (P2c) IFs in the thread should show evidence of helicity.

(H3) The incorporation of MTs into the developing slime thread increases the flexural stiffness of the thread and acts as a scaffolding for the support of newly generated thread loops adjacent to the nucleus. H3 predicts that thread produced in the absence of MTs should be unorganized.

(H4) A phase shift in thread assembly occurs once the slime thread reaches its full length. H4 predicts that the length of the thread is constant after the phase shift and that only girth increases.

(H5) The phase shift involves a controlled disassembly of 10-12-nm IFs via post-translational modification of the gamma IF polypeptides and the reassembly of IF proteins/subunits into a ‘super’ fiber prior to maturity. H5 predicts that: (H5a) following the phase shift 10-12-nm IFs should not be resolved in the slime thread, (H5b) following the phase shift distinguishable structure within the slime thread should not be visible following maturity, (H5c) relative slime thread density should increase as a function of maturity.

(H6) Following the phase shift subsequent growth involves changes in girth only via the addition of IF subunits. H6 predicts that slime thread taper should decrease with maturity.
(H7) MTs are removed in fully mature threads and IF subunits fill the spaces they occupied in development. H7 predicts that MTs should not be visible in the thread following maturity.

To test these predictions, we used a number of cell preparation and imaging techniques such as immunofluorescence and focused ion beam milling. A number of previously employed techniques such as transmission electron microscopy have also been employed to compare to previous researchers and corroborate findings in this study.

**Materials and Methods**

*Animal collection and care*

Specimens of Atlantic hagfish (*Myxine glutinosa* Linnaeus) were collected in Passomoquody Bay, New Brunswick, Canada using baited traps deployed along the seafloor. Following collection hagfish were transported to the Hagen Aqualab at the University of Guelph, where they were housed in 2000-L, environmentally controlled, aquatic recirculation units containing artificial seawater (10°C, 34‰). All housing and feeding conditions were in accordance with the University of Guelph Animal Use Protocol 05R154.

*Animal anesthesia and slime removal*

Hagfish were anesthetized following a clove-oil anesthetic protocol (Winegard and Fudge, 2010). Following anesthesia the slime glands of the hagfish were electrostimulated (60Hz, 18V) using a Grass SD9 electric stimulator (Grass Instruments, Quincy, MA, USA). Electrostimulation was performed 1 or 2 weeks in advance of post-mortem harvesting of the glands to attempt to increase the ratio of immature: mature cells (as shown by Spitzer et al., 1988).
**Animal euthanasia and disinfection**

Hagfish were euthanized using a large scalpel blade to sever the notochord and dorsal nerve cord directly posterior to the cranium. Following euthanasia the hagfish were disinfected via whole body immersion in 70% EtOH for 5-10 seconds. During disinfection the rostral end of the hagfish was held approximately 2 cm out of the EtOH to prevent EtOH from entering the blood sinus of the hagfish and coming into contact with the slime glands where it could kill the gland cells.

**Animal dissection and harvesting of the slime glands**

Hagfish were dissected inside a sterilized biological flow cabinet, using a sterilized tray and scissors (autoclaved and sprayed with 70% EtOH). The surgical area of the hagfish was sponged with 70% EtOH and a 6-cm incision down the dorsal midline of the hagfish was made starting from its rostral end. Perpendicular cuts from this dorsal incision to the ventral midline created a rectangular flap of skin on each side. Rat-toothed forceps were used to grasp dorsal muscle tissue firmly while hemostats were used to grasp and pull on each flap in turn. By carefully pulling the flap ventrally and away from the muscle tissue, the slime glands, attached to the skin at the gland pore, were pulled from between the large myotomal bands of skeletal muscle running dorso-ventrally along the length of the hagfish.

Fine forceps were used to grasp the capsule of each exposed gland, and gently pull it away from the skin to expose the gland pore. Iridectomy scissors were used to cut the slime gland pore where it is attached to the epidermis. Following excision the slime glands were transferred into a sterile culture dish containing 10 mL of 400 mM sodium citrate + 5 mM EGTA (~1020 mOsmol/kg; 11 °C; pH 7.3; sterile 0.22 filtered) on a chiller-re-circulator microscope stage chiller. The glands were rinsed by moving them back and forth in the solution before being
transferred into hagfish gland cell culture medium (see Media Preparation below). No anti-
bacterial or anti-fungal agents were used in the sodium citrate or culture medium for these steps
because the time course in which the tissue is in contact with these solutions was not long
enough for them to be effective. This process of harvesting glands was repeated until all glands
from a hagfish were removed.

Gland digestion and cell dissociation

Once all of the glands were harvested, sterile fine forceps were used to transfer the glands
into a 35-mm culture dishes containing 5 mL of rinse solution (sodium citrate). The glands were
then torn in half using forceps before being transferred back into culture medium. The cells
(mostly mature) in sodium citrate that pelleted out of the gland were collected using a 5mL
serological pipette and centrifuged in 15-mL falcon tubes at 500 g for 2 minutes. The supernatant
was decanted with special attention to avoid mucin strands and associated threads. The gland
cells were resuspended in a small volume of culture medium using a micropipette and a large 1-
\text{-}mL pipette tip that had been cut to increase its bore and thereby reduce the pipetting velocity.
Gland cells were transferred into 35-mm culture dishes containing hagfish CM and maintained in
an incubator at 11 °C.

Some of the procedures described below, for example, some electron microscopy
preparations, required cells to be manipulated in suspension. For these procedures, the glands
were sequentially digested in 5 mL of collagenase digestion solution (1mg/mL in hagfish CM; 20
°C). Gland digestion involved 1-hour sequential rinses of the glands with the collagenase
solution (5 mL) in 35-mm culture dishes on a rotator-shaker plate. Following every 1-hour rinse
the remaining gland fragments were transferred into fresh collagenase solution and the remaining
digestion solution containing dissociated cells was pipetted into a 15-mL falcon tube. The falcon
tubes were then stored in the incubator at 11 °C to allow the cells to pellet out of solution. Following 24 hours in the incubator the supernatant digestion solution was aspirated from the cells and the cells were resuspended in fresh culture medium before being transferred via pipette into 35-mm culture dishes and placed back into the incubator.

Concentrating gland cells and exchanging cell media

Gland cells were concentrated for fixation and imaging using a number of techniques. These include centrifugation of the cells in media at 500 g for 2 minutes, allowing the cells to pellet out of solution via passive settling, or by pipetting the cells into a cell manipulation chamber that allowed media to flow through and captured cells on a mesh. The method used depended on the final outcome desired as all techniques result in rupturing of some cells and subsequently the formation of mucin strands in the cell culture. Centrifugation resulted in the highest degree of mucin strand formation and these mucin strands were able to unravel skeins and remove the plasma membranes from intact cells.

Media preparation

Hagfish are true osmoconformers and as such their tissues, and the cells that comprise them, have osmolalities equal to that of their environment (i.e. seawater). This presents a unique challenge to establishing a primary cell culture when using cell culturing media intended for use with mammalian or teleost fish cells, which typically have osmolalities about one third that of seawater. Previous work by Herr et al. (2010) demonstrates a number of organic osmolytes that occur in the hagfish slime gland at relatively high concentrations, including: Trimethylamineoxide (TMAO), betaine, and dimethylglycine (DMG). Using these data about the slime gland we have come up with a number of custom media cocktails that all exhibit an osmolality of approximately 1000 mOsmol/kg (see Media Preparation). With the osmotic contribution of
NaCl, TMAO, and DMG held approximately constant among the media the concentration of betaine was varied to account for the inherent differences in osmolality found between the commercially available media types. Leibovitz L-15 was decided upon because of its frequent use in elasmobranch primary cell culture and strong pH buffering capacity under atmospheric carbon dioxide levels. All media were sterile filtered using 0.22 µm Millipore Stericups, pH adjusted to 7.3 using 1 N NaOH (because DMG acidifies the L-15 media), and combined with FBS (10%), Pen/Strep (10,000 units/mL; 10,000 µg/mL), and Fungizone (2.5 µg /mL) just prior to use.

Collagenase digestion solution

The hagfish slime gland is surrounded by a collagenous capsule. Therefore, to expose young cell types found on the inside wall of the gland capsule, excised glands were exposed to collagenase digestion solution that was prepared in hagfish cell media. This solution contained: collagenase (1 mg/mL), fetal bovine serum (10%), penicillin (10,000 units/mL), streptomycin (10,000 units/mL), and Fungizone (2.5 µg/mL). To maintain the sterility of the culture the digestion solution was pH adjusted to 7.3 and sterile filtered using a 0.22-µm syringe tip filter.

Cell viability testing with trypan blue

To determine the viability of recently dissociated gland cells or cells in culture a micropipette with a large bore tip was used to gently re-suspend cells in the 35-mm culture dishes. Following re-suspension a 500-µL sample from the culture dish was taken and placed in a 2-mL microcentrifuge tube. The cells were spun down at 500 g for 2 minutes or placed in the incubator overnight to passively settle out of solution (approximately 12 hours). Following concentrating the cells in the microcentrifuge tube the supernatant media was decanted and replace with 15 µL of media and 15 µL of trypan blue. The cell pellet was gently but thoroughly
flushed before a 20-μL sample was taken and placed on a glass slide for observation under a microscope. Trypan blue is a vital exclusion dye, which only penetrates the membrane of dead not living cells. Using a 10x objective lens 3-4 images were taken of each sample. In each of these images the total number of cells, as well as the total number of dead (or blue stained) cells were counted to calculate cell viability within the culture.

Fixatives

A number of fixatives were used in preparation for the various tissue embedding and microscopy procedures employed in this study. For transmission and scanning electron microscopy a fixative solution containing 3% paraformaldehyde and 3% glutaraldehyde prepared in 0.1-M sodium cacodylate (~980 mmol/kg) was used. In preparation for paraffin embedding both 10% buffered formalin and 3% paraformaldehyde in PBS were utilized.

All fixatives containing paraformaldehyde were prepared in a fume hood using a heated stir-plate to warm the appropriate salt solutions (for example 0.1-M sodium cacodylate) to 60 °C before the addition of paraformaldehyde powder. A temperature probe connected to the stir-plate was used to monitor the temperature of the fixative solution, and a pH probe allowed the solutions pH to be brought to 7.3. Paraformaldehyde was dissolved into solution using 1 N NaOH. The pH of the fixative solution was adjusted using 1N HCl. In the electron microscopy fixative an appropriate volume of 50% glutaraldehyde in water was added to a final concentration of 3%.

Cell suspension preparation for scanning electron microscopy

Following the hagfish dissection and cell dissociation protocols established in this study viable cultures of hagfish gland cells were concentrated via centrifugation (500 g; 2 minutes). The cell pellet was then resuspended in 500 μL of EM fixative and transferred via micropipette
into a custom-made cell manipulation chamber with the bottom mesh in place. The chamber was then situated in a sealable flat-bottom glass vial, before the upper mesh of the manipulation chamber was put in place and an additional 10 mL of fixative solution was added to the glass vial. Fixation was performed for a total of 1 hour with pipette (glass Pasteur) driven flushing of the fixative solution every 15 minutes to ensure adequate mixing inside the manipulation chamber. Following fixation, the fixative solution was removed from the glass vial and drained from the manipulation chamber. 10 mL of 0.1-M sodium cacodylate rinse (pH 7.3) was then added to the glass vial and 3, 15-minute rinses were performed with fresh rinsing solution each time to remove any excess fixative.

**Ethanol dehydration**

Following fixation a 3 x 10 minute rinse of the cells/glands with the appropriate rinse (sodium cacodylate or PBS) was performed. The final rinsate was removed from the vial and approximately 10 mL of 30% ethanol added onto the manipulation chamber. Precautions were taken to avoid drying of the cells/glands between subsequent dehydration steps of the following ethanol dehydration series. Samples were left in 100% EtOH until being critical point dried, or embedded in paraffin or plastic.

**Critical point drying**

The critical point dryer (CPD) was cooled by running cold tap-water through the water jacket for 15-20 minutes. Following this initial cooling period the chamber was filled partially with 100% EtOH. Once all the manipulation chambers were loaded (top up) into the CPD the chamber, it was then filled with 100% EtOH to prevent air-drying of the samples. Making sure that all valves were closed in the CPD system the CO₂ tank valve was opened first, and then each downstream valve to the CPD. The pressure within the chamber at this point reached ~800 psi.
After allowing 2 minutes to completely fill the chamber the CO₂ rinsing was started by alternating 2 minute fills with 2 minute degassing of the chamber. Degassing was performed by gently opening the outflow valve and tapping the outflow tubing to prevent any build-up of solid CO₂. Filling and degassing was performed approximately 4-5 times or until the CO₂ being released took on a feathery appearance. Following the final degassing, the CPD was filled for the final time with CO₂. All the valves were then closed starting from the CPD and moving upstream to the CO₂ tank valve. The cold water running through the CPD water jacket was then turned off and warm water turned on. The warm water increases the pressure in the chamber. Without allowing pressure chamber to rise above 1350 psi a thermometer was inserted into the CPD and the chamber temperature was allowed to rise to 40 °C. The combination of CPD chamber pressure of 1200-1350 psi and a temperature of 40 °C was maintained for 5 minutes. The CPD-bomb was then degassed at a rate of 50 psi/minute to 0 psi and the manipulation chambers were removed for storage in a desiccator.

*Mounting and sputter coating critical point dried samples*

Hagfish gland cells were mounted for scanning electron microscopy on aluminum pin stubs (12.7 mm diameter) using two-sided carbon tape. The gland cells tended to cluster on one of the two mesh sides of the cell manipulation chamber. Once the side with the majority of cells was determined the plastic collar keeping the opposite mesh in place was removed and the manipulation chamber inverted over the two-sided carbon tape. The mesh with cells attached was then gently tapped to release cells from the mesh and onto the two-sided carbon tape. Following the collection of a sufficient number of gland cells on the carbon tape the samples were sputter coated with a 15 nm gold-platinum coating (Model K550; Emitech; Ashford, Kent, England).
**Paraffin embedding and staining of hagfish tissues**

Hagfish tissues including whole slime glands, whole body cross-sections and whole glands with skin and musculature were fixed using both 10% buffered formalin and 3% paraformaldehyde prepared in PBS. The tissue fixation protocol developed in this study was adhered to for the fixation of all tissues. Tissues were paraffin embedded in the University of Guelph Animal Health Laboratory using standard protocols. Hagfish tissues were sectioned using a microtome to 4 µm sections and placed on glass slides for both histological and immunological staining. Whole glands were stained with: (1) hematoxylin and eosin (HandE), (2) Masson’s Trichrome, and (3) Basic fuschin methylene blue. Gland, skin and musculature sections were stained only with HandE.

**De-waxing paraffin embedded hagfish gland cells**

Prior to immunological labeling of the hagfish glands and associated tissue the paraffin sections were de-waxed using a xylene and EtOH de-waxing series (see appendix). Individual glass slides were treated in 50-mL falcon tubes containing approximately 40 mL of each of the rinsing solutions.

**Primary antibodies**

Immunological labeling of the intermediate filaments (IFs) in hagfish gland cells was performed using the following dilutions and primary antibodies, respectively: IF antibody (1) 1:400, pan-Cytokeratin of mAbs at 200 µg/mL (Santa Cruz); IF antibody (2) 1:400; hagfish slime thread antibody. Primary antibody labeling of MTs in the gland cells was done using a 1:500 dilution of anti-alpha-tubulin (bovine), mouse IgG (Invitrogen; A11136; 236-10501; 50 µg/mL). Due to the process by which the two IF primary antibodies were made only IF antibody (2) could be used in combination with the MT primary antibody to meet the secondary antibody requirements.
binding requirements. IF antibody (2) was used in the majority of IF labeling because IF antibody (1) and the MT primary antibody could not be used in concert. Their fairly comparable performance (see Results) further supported the use of IF antibody (2) in the fluorescent labeling experiments.

**Fluorescent conjugated secondary antibodies and stains**

The secondary antibody Alexa Flour 488 goat anti-mouse IgG (Invitrogen; A10680; IgM (H+L); 2 mg/mL) was used at a dilution of 1:400 for fluorescent labeling of IF primary antibody (1) and the anti-alpha-tubulin antibody. Alexa 568 goat anti-rabbit (Invitrogen; A11011; IgG (H+L); 2 mg/mL) was used at a dilution of 1:400 for the fluorescent labeling of IF primary antibody (2). The nuclei of cells were fluorescently labelled using 4’,6-diamidino-2-phenylindole, dilactate (DAPI; Invitrogen; L21873W) at a dilution of 1:500.

**Immunofluorescence of hagfish gland cells**

Immediately following slide de-waxing, primary antibodies were prepared to their respective dilutions in a single 1.5-mL microcentrifuge tube by diluting the antibodies in an appropriate volume of 1% bovine serum albumin (BSA) prepared in PBS. When more than one antibody was being used, the antibodies were prepared together in a single tube.

De-waxed slides were then removed from ddH₂O and excess water gently tapped from the slides. A hydrophobic barrier was then drawn around the de-waxed tissue sections using a Liquid Blocker super PAP pen (Daido Sangyo Co., Ltd., Tokyo, Japan). This step not only contained the stains as they were applied to the sections but also reduced the volume of antibodies needed and maximized exposure of the tissue to antibodies and fluorescent stains. After the hydrophobic barrier dried the slides were transferred to 60-mm tissue culture dishes to prevent debris from collecting on them and to maintain stable incubation conditions. 200 µL of
antibody, BSA solution was then applied at the corner of the hydrophobic barrier so as not to
disturb the tissues and to minimize bubble formation. Primary antibodies were left to incubate at
4 °C for 12 hours in covered culture dishes.

Following the 12-hour incubation the primary antibody solutions were removed, one at a
time, using a micropipette. The tissue sections were then rinsed one time with 200 µL of 1%
BSA in PBS for a duration of 5 minutes.

Secondary antibodies and fluorescent stains (i.e. DAPI) were prepared immediately
before their use by diluting them in a 1% BSA in PBS solution and storing them in the dark until
used. Following the removal of the BSA-PBS rinse solution from a corner of the hydrophobic
barrier the secondary antibodies and fluorescent stains were applied and allowed to incubate in
the culture dishes for 30 minutes at room temperature (23 °C). Subsequently these were removed
from the tissue sections and the sections rinsed using fresh PBS (no BSA) for 15 minutes in 5-
minutes intervals. After the final rinse the PBS was removed and excess PBS gently tapped off
of the tissues section. DakoCytomation Fluorescent Mounting Medium was then placed on top of
the tissues sections before covering the sections with a cover glass (0.17 mm) and sealing the
slide with clear nail polish. The nail polish was allowed to cure for a minimum of 30 minutes
before visualization.

*Plastic embedding hagfish glands*

Hagfish glands were plastic embedded using a Polysciences Poly/Bed®812 (Luft
formulations) embedding kit. Plastic embedding of the glands was performed for analysis of
gland cells using both transmission electron microscopy (TEM) and focused ion beam (FIB)
scanning electron microscopy (SEM). The Poly/Bed®812 plastic embedding media was prepared
in 100-mL volumes by combining 48 mL of Poly/Bed®812, 31 mL of dodecenylic acid,
anhydride, 21 mL of methylnadic anhydride, and 2 mL of 2,4,6-
Tris(dimethylaminomethyl)phenol. Ethanol dilutions of this stock Poly/Bed®812 solution were 
made in 3:1, 2:1, 1:1 and 0:1 ratios (EtOH:Poly/Bed®812).

Freshly collected slime glands were transferred into fixative solution (3%
paraformaldehyde, 3% glutaraldehyde, 0.1-M sodium cacodylate; 11 °C) and fixed for 1 hour on 
a rotator-shaker plate. The glands were rinsed for 30 minutes in 10-minute intervals in fresh 0.1-
M sodium cacodylate rinse solution (pH 7.3). Following the final rinse, glands were post-fixed 
for 2 hours at room temperature with an osmium tetroxide solution (1% in 0.1-M sodium 
cacodylate). Additional rinses were performed (3 x 10 minutes) in 0.1-M sodium cacodylate 
before exposing the glands for 1 hour to a 2% uranyl acetate in 0.1-M sodium cacodylate 
solution. Following an additional 3 x 10-minute rinsing in sodium cacodylate the glands were 
derhydrated in ethanol (see dehydration series)

Plastic infiltration of the hagfish glands immediately followed the final dehydration step 
(i.e. 100% EtOH). In 1 hour intervals the glands were exposed to the following EtOH: 
Poly/Bed®812 ratios: 3:1, 2:1, 1:1, 0:1. The final infiltration step involved a 12-hour exposure to 
the Poly/Bed®812 stock. The glands were transferred between infiltration solutions using fine 
forceps and left on a rotator/shaker plate operating at 50 RPM for each infiltration step. Prior to 
curing the Poly/Bed®812, the glands were transferred into a silicone mold and covered in fresh 
Poly/Bed®812. The Poly/Bed®812 was then set to cure in an oven at 60 °C for 24 hours. The 
cured plastic blocks containing the glands were allowed to cool for at least one hour before being 
removed from the silicone mold.

Epifluorescent and brightfield Imaging
Live cells, as well as fluorescently labeled and histologically stained hagfish tissue slides were analyzed using a Nikon Eclipse 90i Epi-fluorescent microscope. Brightfield differential interference contrast (DIC) images and fluorescent images were taken using a cooled monochrome digital camera (Q-Imaging Retiga 1300; Surrey, BC, CAN) driven by NIS Elements AR software. Fluorescent monochrome images were taken using DAPI, FITC and TRITC filter cubes to image the DAPI, AlexaFluor 488, and AlexaFluor 568, respectively. The monochrome images were then assigned color channels in Adobe Photoshop CS5 based on the emission wavelengths of the secondary antibodies and fluorescent stains used, when applicable. Double and triple immunofluorescent images were then assembled into multichannel images using Adobe Photoshop CS5. Brightfield color images were taken using a Q-imaging EXi 12-bit color camera (Q-Imaging; Surrey, BC, CAN) driven by NIS Elements AR software.

Confocal imaging

Confocal imaging was performed using a Leica DM6000B microscope in conjunction with Leica TCS SP5 system and Leica DFC350FX monochrome camera. A Radius laser (405 nm; 20% Acousto Optical Tunable Filter (AOTF)) was used for near UV excitation of the DAPI stain, an argon laser (50 mW; 20% AOTF; 488 nm) was used for excitation of the AlexaFluor488, and an HeNe (543 nm; 50% AOTF) for excitation of AlexaFluor568. The Leica SP5 system allowed for the attenuation of the laser lines with its AOTF. The SP5 spectral detection system was used to reduce overlap in the emission spectra of the different fluorophores thereby creating custom detection filter settings and eliminating cross-talk in the fluorescent emissions from the different lasers used.
Focused ion beam/scanning electron microscopy (FIB/SEM)

FIB/SEM was performed at McMaster University’s Canadian Center for Electron Microscopy (CCEM) using a Zeiss N Vision40 microscope outfitted with both a field emission Gemini column and SIINT Zeta Ga\(^+\) ion FIB column capable of image resolution down to 1.1 nm and 4 nm, respectively.

A number of sample preparations were made for FIB/SEM. Critical-point dried cells were fixed to aluminum stubs using two-sided carbon tape and sputter coated with a 2-nm gold layer using an Edwards S150B sputter coater. Plastic embedded glands were fixed to aluminum stubs using silver paint and dried at 40 °C. Plastic samples were also sputter coated with a 2-nm gold layer.

Transmission electron microscopy (TEM)

TEM was performed at the University of Guelph’s Electron Microscopy Unit using a Philips CM10 (tungsten filament 80 kV) outfitted with a top mount SIS/Olympus Morada 11 MP CCD camera. Sections were cut between 20 and 60 nm using a Porter-Blum MT-1 manually driven ultramicrotome and stained with uranyl acetate (2%) and lead citrate (1%) following sectioning.

Results

Slime thread assembly begins apical to the nucleus in the MRZ with the formation of 40 nm IF assemblies

Focused ion beam scanning electron microscopy (FIB/SEM) and transmission electron microscopy (TEM) were used to explore the paranuclear region of GTCs, which is believed to be the site of thread synthesis (H1). The side of the cell closest to the nucleus will be referred to as the basal side of the cell. At the apical tip of the nucleus, within the mitochondrial rich zone (MRZ) of the cell, we observed a fine network of threads as small as 40 nm in diameter in both
FIB/SEM and TEM (Figure 1,2). At high magnification we observed that these ~40 nm threads corresponded to assemblies of IFs in the absence of intrathread MTs (Figure 2,3). Structures of similar size and appearance to ribosomes or polyribosomes appeared to cluster on these narrow threads (Figure 3). The findings support the hypothesis that thread synthesis occurs with the bundling of 12-nm IFs in this paranuclear region (H1).

Transmission electron micrographs (TEM) of GTCs through various stages of cell and skein development supported the hypothesis that thread development is initiated adjacent to the nucleus from within the MRZ (H1; Figures 2). Electron micrographs at the scale of the whole GTC revealed that thread development is initiated adjacent to the nucleus (Figure 5.2) following the post-division growth of the GTC nucleus (Figure 5.1, 5.2). This short length of thread developed towards the apical end of the cell prior to the nucleus fully completing its polar migration (Figure 6). Changes in GTC ultrastructure from division (Figure 5.1) through maturity (Figure 5.7) are presented in Fig. 5. The skein and GTC are closely matched in size (length and width) throughout development (Figure 5). The interthread space visible in 5.3 and 5.4 was gradually reduced as the thread increased in diameter.

Relative slime thread density significantly increases near maturity

Relative slime thread density significantly increased following the initiation of intrathread MT removal (Figure 6,7: One-Way ANOVA p<0.01). This finding supports hypothesis 5 that changes in IF and MT organization within the developing thread, prior to maturity, result in an increasingly dense thread that loses distinguishable substructure. Figure 6 represents an analysis of 130 thread cross sections at 5 stages of maturity, such as those in figure 7, from a total of 6 hagfish. The stages of thread development in figure 6 were determined by the IF and MT composition of the thread observed in TEM. For example, the first stage in slime
thread development involved the formation of thread from distinct 12 nm IFs (H3; Figures 7.1, 7.2). Stage 2 in thread maturity involved the integration of MTs into the thread (Figure 7.3). A distinct electron lucent space was observed around the IFs (~6-8 nm) and MTs (~12-14 nm) comprising the early slime thread (Figure 7.1, 7.2, 7.3). MT integration had no significant effect on relative thread density (t-test p>0.05). The third stage in slime thread development was characterized by a reduction in the interfilament spacing of IFs as well as the initiation of MT removal from the thread (i.e. gaps exist where MTs have recently been removed; Figure 7.4). The dissociation of IFs and removal of MTs significantly increased the relative density of the thread (t-test p<0.01). At this stage in thread development large gaps exist where MTs have been removed from the thread (figure 7.4). Stage 4 in thread maturity (figure 7.5) depicts a further reduction in MT frequency, despite an increase in slime thread diameter, and a total compaction of IFs comprising the thread. Stage 5 represents fully mature slime threads that exhibited no intrathread MTs and a totally electron dense IF organization (Figure 7.6). This finding supports hypothesis 7.

The unorganized apical threads of the skein develop in the absence of MTs

No intrathread MTs were found in the characteristically unorganized thread at the apical tip of the skein (Figure 8.1-4) despite the integration of MTs more basally in the thread. In conjunction with the findings that slime thread production began with IF associations adjacent to the nucleus, it is likely that the apical thread of the skein corresponds to the earliest slime thread produced by the GTC. These findings may also suggest that when MTs begin to be incorporated into the thread the apical length of thread has already coiled within the GTC. As such, these findings are consistent with prediction 3 and provide support for the involvement of MTs in controlled thread coiling (H3).
A dense MT network exists within the interthread space of the developing slime thread

Epifluorescent imaging of GTCs at various points in development illustrated a tight association between MTs and IFs in the developing slime thread (Figure 9). In immature GTCs the MT network extended throughout the cytoplasm and zone of thread development (Figure 9.1). A IF network appeared at the periphery of the cell as well as in the zone of thread formation (Figure 9.1). As the GTC matured the primary MT network appeared to recess from the apical pole and periphery of the cell and intensified medially along the long-axis of the cell and in the paranuclear region (Figure 9.2). IF fluorescence appeared more homogeneously distributed through the cell and corresponded to an increase in slime thread presence in the cell (Figure 9.2). In mature GTCs the MT network was most intense in the paranuclear region of the cell with aspects of the network extending both apical and basal of the nucleus along the long axis of the cell (Figure 9.3). IF fluorescence appeared to extend throughout the cytoplasm of the cell (Figure 9.3). A differential interference contrast image illustrates the highly condensed thread bundle (skein) of a mature hagfish GTC (Figure 9.4).

Colocalization analyses of figure 10 and 11 revealed a decreasing colocalization of IF and tubulin labelled proteins as the cells mature, from approximately 20 to 10% colocalization between the IF and MT color channels (Image J Colocalization Highlighter Plugin). A colocalization analysis of a confocal image of the GTC from figure 10 also illustrated a comparative colocalization between the epifluorescent and confocal images of the same GTC suggesting a relatively low cross-talk between the emittance of the IF and MT secondary antibodies (Figure 12). This suggests that the MTs observed in fluorescence were primarily MTs within the interthread space of the developing slime thread.
These findings illustrated a dynamic distribution of tubulin protein in the GTC at various time points in development. The close association observed in these images between the fluorescently labelled tubulin and IF proteins support to the hypothesis that MTs may act as a structural scaffolding for the organization of the slime thread (H3) and represent the first application of immunofluorescent staining in hagfish GTCs.

Changes in slime thread assembly patterns

Comparing stages 1 and 2 to stages 3 and 4 in figure 7, we observed that a flocculent material, of slightly different density to the compacted IF portion of the thread, was visible at the periphery of the thread cross section. This material was not present in stages 1 and 2 and appeared to disappear by full thread maturity at stage 5 (Figure 7). In longitudinal and tangential sections of slime thread in stages 1 and 2 of maturity, a fiber was seen helically wrapping the developing thread (Figures 13.1,13.2,13.3). The mean diameter of this helical fiber measured 12.66+/−0.67 nm and did not significantly differ from the diameter of the known IF portion of the thread (12.31+/−0.4 nm; MATLAB t-test p>0.05). These findings support H2 that the helical wrapping represents new IF being added to the slime thread. The observation of IFs traveling obliquely and perpendicular to the main axis of the thread in stages 1 and 2 of development (Figure 7.1; 7.2; 7.3; 13.1 inset,13.3), provided further support for H2 because evidence for helicity exists within thread cross-sections. The disappearance of the helical wrapping, appearance of the flocculent material, condensing of the IFs, and removal of the MTs may suggest a shift in how the thread is being assembled following stages 1 and 2. This structural shift could represent a change from utilizing 12-nm IFs to build a full-length skein template, to a focus on increasing thread width with smaller IF subunits and is consistent with H5. Such a shift is also be supported by the finding that thread taper decreased with increasing maturity. The
smallest threads (~40 nm) of the paranuclear region shown in figures 1,3 and movie 1 measured a full 27 times smaller than the thread found at the periphery (~1071 nm) of the thread skein within the same image plane. The intermediate diameter of the threads (fully mature thread diameter ~3 µm) suggested that the GTC in figure 1, movie 1 is of an intermediate stage of maturity. The smallest threads of the GTC in figure 4 and movie 2 measured ~210 nm whereas the largest fibers, in the same image plane, measured ~1480 µm. This represented a 17-fold change in thread diameter from the paranuclear thread loops to those at the cell periphery. The larger diameter thread of the GTC in figure 4, as well as the lateral and basal position of thread loops relative to the nucleus seen in movie 2, suggested figure 4 represents a later stage of maturity than figure 1. These findings may suggest that the thread taper decreases with increasing skein maturity, which provides support for H6.

Cell size and nuclear position: possible implications in directing cell coiling

During early to intermediate stages of development the nucleus of the GTC protruded from the basal side of the cell (Figure 1, Movie 1). As thread development proceeded thread loops were deposited both lateral and basal of the nucleus (Figure 4, Movie 2). The position of the nucleus within developing thread bundle (as opposed to basal of the skein throughout development) implicates the nucleus in at least the passive guidance of newly generated thread coils around its position (Figures 5.4,5.6). Figure 5.4 provides a good illustration of the spindle-like role the nucleus may play in thread coiling/organization.

Presence of vesicles within the developing slime thread

TEM illustrated that vesicles occurred within the MT gaps of the developing thread (Figure 14). The gaps, which coursed for lengths of up to 500 µm, were observed to contain large numbers of MTs and their associated cargo (Figures 14.1,14.2). MTs within the larger gaps
were not oriented entirely parallel to each other or the IFs of the slime thread (Figure 14.3). Instead they appeared to bend as they travelled through the slime thread (Figure 14.2). TEM also illustrated the movement of MTs from the interthread space to the intrathread space (Figure 14.4).

Discussion

Slime thread assembly and ultrastructural changes through GTC maturation

This study presents the first application of FIB/SEM for investigating the ultrastructure of the hagfish GTC. Using FIB/SEM and TEM imaging we provide support for the hypothesis that slime thread assembly begins apical and adjacent to the nucleus, within the MRZ of the cell, with the formation of small ~40 nm IF assemblies in the absence of intrathread MTs. Furthermore, this study provides both qualitative and quantitative analysis of ultrastructural changes that within the slime thread at various points in development. These findings support the hypothesis that a phase shift in thread assembly involves the dissociation of 12-nm IFs (found in early stages of development) and reassembly of the IF protein subunits into a ‘super’ fiber.

The observation that the paranuclear region of the GTC is host to the narrowest slime thread sections of the skein (throughout development) corroborates similar findings by a number of past researchers (Newby, 1946; Fan, 1968; Terakado et al., 1975; Fernholm, 1981; Downing et al., 1984; Spitzer et al., 1988; Fudge et al., 2005). A common assumption among past researchers relates the cell area host to the smallest diameter threads to not only the origin of the thread but also the area of active thread length generation. Utilizing FIB/SEM this study investigated the proposed area of thread development apical of the nucleus and revealed a network of fibers on the order of tens of nanometers. As a result, FIB/SEM not only provided a glimpse into the organization of threads in this area but also a look at the smallest recognizable
threads in hagfish GTCs presented to date. TEM ultrastructural analysis of developing GTCs, and their thread composition, reveals that the 40 nm threads observed using FIB/SEM likely correspond to assemblies of IFs (~8) in the absence of MTs. These findings support the hypothesis that thread length is added to the skein via the assembly of IF slime thread, in the absence of MTs, within the MRZ of the cell. As such, MTs are later incorporated into the developing thread where they may play a number of roles in thread organization and assembly (discussed later).

Qualitative and quantitative analysis of slime thread ultrastructure reveal a dynamic yet predictable change in the organization of IFs and MTs throughout slime thread development. This study presents five stages in thread maturation based on changes in the ultrastructural composition and appearance of the slime thread. Stage 1 of thread maturation is characterized by the appearance of circular assemblages of 12-nm IFs separated by a distinct interfilament spacing in cross-section. Stage 2 is characterized the incorporation of MTs into the IF slime thread. Stage 3 slime threads exhibit a type of coalescence between the previously distinct IFs in which the interfilament spacing becomes reduced. Stage 3 also marks the initiation of MT removal from the thread (i.e. MT gaps with no MTs present). Stage 4 illustrates a complete compaction or coalescence in the IF portion of the slime thread resulting in a total reduction in the interfilament space. The number of MTs within the thread cross sections is also reduced from stage 3. Stage 5 illustrates a complete removal of MTs from within the slime thread resulting in an entirely IF thread of indistinguishable substructure. The developmental change in the hagfish slime thread composition has been a subject of discussion by many past researchers. However, it was not known whether slime thread assembly begins with IFs or MTs. This study provides support for the hypothesis that slime thread synthesis begins with IFs and that MTs are only later
incorporated into the thread. The observation that MT frequency increases following the 
beginning of slime thread production and then decreases to zero is consistent with the 
observations of past researchers (Terakado et al., 1975; Downing et al., 1984). This change in 
MT frequency may be explained by roles MTs play in thread coiling and organization during 
thread development and will be discussed later in the chapter. The change in relative thread 
density qualitatively reported by Fan (1965), Terakado et al. (1979) and Downing et al. (1984) 
has not previously been quantitatively investigated. The analysis of thread cross sections at each 
of the 5 stages of thread development reveal a significant increase in relative thread density as a 
result of changes in IF and MT organization.

The presence of large numbers of cytoplasmic ribosomal clusters in TEM as well as 
polysomal clusters decorating the thread surface observed using FIB/SEM are consistent with 
findings from Terakado et al. (1979) who observed polysomal clusters producing fibrous 
products within the cytoplasm of the cell and suggested that these clusters are involved in 
depositing IF proteins onto the thread. Terakado et al., (1979) suggested that cytoplasmic clusters 
of ribosomes are influential in the production of IF protein for thread synthesis. The importance 
of cytoplasmic ribosomes in protein synthesis as opposed to ribosomes bound by the rough 
endoplasmic reticulum (RER) is supported by the relatively little RER, and high numbers of 
cytoplasmic ribosomes observed in TEM. It is easily conceived that the high concentrations of 
ribosomes and mitochondria found in the MRZ provide the necessary combination of energy and 
protein synthesis capacity for the generation of new length to the slime thread skein. Although IF 
protein synthesis and integration into the thread certainly occurs elsewhere in the cytoplasm the 
findings of this study support the view that the MRZ is host to the origin of slime thread 
development.
Slime thread coiling and organization: implications of cell morphology, size, and MT interactions

The morphological analysis of GTCs from division/differentiation through maturity revealed that: (1) the thread skeins closely approximate the size of their respective GTC in both minor and major axes, and (2) that thread coils are deposited apical of the nucleus in immature GTCs but that the coils extend lateral and basal of the nucleus in mature GTCs. These findings raise a number of interesting questions regarding the influence of cell morphology and nucleus position on the organization and coiling pattern of the thread skein. These observations may suggest a passive involvement of cell morphology and nucleus position in guiding cell coiling and organization in the cell. For example, by limiting the space in which the thread can lengthen between the nucleus and the plasma membrane of the cell (i.e. influence of cell size) you force either a coiling or a buckling of the torsionally biased lengthening fiber. Furthermore, by obstructing where the resultant coils/folds or buckles can deposit (i.e. influence of nucleus position) you further regulate the organization of the thread coils.

The characteristic conical group arrangements of the thread skein, as illustrated by Fernholm (1981), are consistent with a thread coiling hypothesis in which the production of each conical grouping of coils is proceeded by a increase in cell size that allows for the production of only one additional conical group arrangement within the last (i.e. between the previous conical group arrangement and the nucleus). Observations by Downing et al. (1984) suggest that the MRZ of the GTC is conical in shape. This study corroborates these previous findings and illustrates that the nucleus and MRZ takes on a spindle-shaped appearance when GTCs of intermediate stages of maturity are observed in longitudinal section (Figure 5.4). In combination with the findings of this study that suggest length is added to the slime thread from within the MRZ it can be conceived that the shape of the nucleus and MRZ give shape to the successive
arrangements of conical groupings that comprise the skein. It appears that by obstructing where the newly formed coils of thread can deposit, the shape of the cell and position of the nucleus may be contributing to the coiling pattern of the thread.

The unorganized apical tip of the thread skein has often been discussed in the hagfish literature as the initiation site of thread skein unraveling (Newby, 1946; Fernholm, 1981; Spitzer et al., 1988; Winegard and Fudge, 2010). However, its characteristic lack of unorganization relative to the remainder of the precisely coiled skein has also intrigued researchers. The findings of this study suggest that the unorganized thread of the skeins apical region correspond to the first lengths of thread produced by a GTC and are likely produced before the large scale integration of MTs into the developing slime thread. These findings support the hypothesis that MTs play a role in thread coiling and organization. The interconnection of MTs and IFs has been shown to have critical implications on the structure of some IF networks (Draberova and Draber, 1993; Carmo-Fonseca and David-Ferreira, 1990). Although the exact structural changes to the thread are not known. It is known that MTs exhibit vastly stiffer mechanical properties than IFs (Hawkins et al., 2010). As a result, their presence within the IF thread would inherently have a stiffening effect on the thread assuming physical linkages between the intrathread MTs and IFs. Although IF-MT interactions are not well understood in hagfish slime threads, MTs and IFs are known to form connections through IF associated proteins such as fillagrins or plectins in many other cell types (Draberova and Draber, 1993; Svitkina et al., 1998; Iwatsuki and Suda, 2010). As such, if one assumes such linkages between the IFs and MTs of the slime thread, then the MTs could conceivably be acting as stiffening elements within the thread to modulate its mechanical properties (i.e. persistence length). This would influence the bending properties of the developing slime thread and may implicate MTs in thread coiling. The findings that the
unorganized threads of the apical region of the thread skein are produced in the absence of MTs, but that MTs are later incorporated in thread development, further implicates the MTs in influencing thread coiling.

This study presents the first application of immunofluorescence to investigate the distribution and association of IFs and MTs in GTCs at various time points in development. These techniques revealed a shift if MT distribution in the developing GTC from a large cytoplasmic network in immature cells, to a more medial and paranuclear network as maturation proceeded. The MT network appears closely associated with both the paranuclear region and the medial long axis of the cell. Although the MT organizing center (MTOC) of a GTC has never been documented, the paranuclear network of MTs in the GTC is indicative of the high levels of MT polymerization occurring from the MTOC. In a plethora of cells the MTOC have been illustrated to occur in the paranuclear region of the cell. The intensity of the tubulin signal along the medial long-axis of the developing GTC may suggest the presence of a MT scaffolding for newly generated slime thread loops. Colocalization analysis of IF-MT distribution in intermediately mature and fully mature GTCs revealed a decrease in colocalization between the two stages of maturity. This is consistent with the overall reduction of intrathread MTs observed both in this study and elsewhere in the literature (Terakado et al., 1975; Downing et al., 1984). The colocalization of IFs and MTs is consistent with interactions observed in other cell types (Carmo-Fonseca and David-Ferreira, 1990; Goldman et al., 2008). However, the overall low colocalization between the IFs and MTs of the GTC, at both intermediate and later stages of maturity, is indicative of the tubulin signal arising primarily from interthread or cytoplasmic MTs. This study as well as Downing et al. (1984) observe that intrathread MTs do not travel the full length of the thread. As such, there are points at which they must enter and exist the
developing slime thread (Figure 14.4). As a result, it may be difficult to distinguish between a interthread and intrathread MT network as it is likely that they both represent extensions of each other. This belief is supported by the observation of vesicles within the MT gaps of the slime thread (Figure 14.1; 14.2). These findings suggest MTs transport vesicles from the Golgi apparatus to vesicles throughout the cell and plasma membrane via both interthread and intrathread pathways. This may implicate the MTs in the transport of the kinases and enzymes necessary for the organizational shift in IFs and MTs seen in the developing slime thread (i.e. post-translational modification of the slime thread). However, further investigation is needed into the nature of the vesicle contents. What we can see about the presence of MTs and their associated gaps in the thread is that the filaments themselves must not be very stiff to allow for the fluid movement of vesicles between them. This may further support the importance of MTs as a stiffening element within the thread.

Changes in slime thread assembly patterns: the helical wrapping and flocculent material

The observation that a helical wrapping surrounds the developing slime thread in early to intermediate stages of slime thread assembly has been made by Terakado et al. (1979), Downing et al. (1984) and this study. The replacement of the helical wrapping during mid-to-late stages of development with indiscernible flocculent material on the periphery of the slime thread has also been observed a number of times via ultrastructural analysis of the slime thread (Terakado et al., 1975; Downing et al., 1984; this study). This study supports these observations and provides a quantitative comparison between the cross-sectional diameter of the helical wrapping substance and the cross-sectional diameter of known IFs within the slime thread. This analysis reveals no significant difference between the cross-sectional diameters of these two filaments and as such supports the hypothesis that the helical wrapping is new IF being added to the thread. This
hypothesis is further supported by the observation that tangentially and longitudinally sectioned IFs appear within cross sections of slime threads in stages 1 and 2 of development. Under this hypothesis the addition of subsequently longer IF helical wraps (much in the way that the IFs themselves are comprised of alpha-helical polypeptides) results in a slime thread of increasing length and width simultaneously. This hypothesis is supported by the observations of this study and those of Downing et al. (1984) that the slime thread increases in both length and width apparently simultaneously. The loss of the helical wrapping and change in thread density following stage 3 of slime thread development corresponds to the appearance of the flocculent material around the thread. Terakado et al. (1979) suggest that this material might represent the deposition of new IF proteins onto the slime thread. This study supports the hypothesis that a phase-change in thread assembly occurs at stage 3 in development. This phase-change appears to involve the disassembly of 12-nm IFs into smaller IF protein subunits. A notion that is supported by ultrastructural analysis of slime thread cross sections by both Fan (1965) and Terakado et al. (1979). Furthermore, we postulate that the phase-change represents a shift from increasing slime thread length to increasing slime thread width. This hypothesis is supported by FIB/SEM analysis of thread skeins at different stages of maturity. Ultrastructural analysis with FIB/SEM suggests that the degree of thread taper decreases as the thread matures because of significant widening of the slime thread adjacent to the nucleus relative to earlier stages of development. This decrease in thread taper may correspond to change in IF organization and assembly observed in this study. This phase-change hypothesis is also supported by ultrastructural analysis with TEM of slime thread composition at different stages of maturity. TEM illustrates a marked condensation of the IF portion of the slime thread resulting in the inability to resolve individual IFs.
The presence of a shift in IF organization and assembly is further supported by Spitzer et al. (1988) who observed a post-translational modification of the gamma-IF polypeptide comprising the hagfish slime thread IFs. The phosphorylation of gamma results in its transition to the beta form of the polypeptide. This shift corresponded to later time points in development according to Spitzer et al. (1988). It has been demonstrated on numerous occasions that post-translational modifications of IFs can play critical roles in IF network assembly and organization (Herrmann and Aebi, 2000; Hyder et al., 2008). This study postulates that the post-translational modification of the gamma-IF polypeptide observed by Spitzer et al. (1988) is consistent with the phase shift from stages 1 and 2 of slime thread development to stages 3, 4 and 5 observed in this study. Ultrastructurally this is observed as a shift from distinct 12-nm IF associations to less distinct associations of IF subunits, presumably comprised of greater concentrations of the alpha-beta IF. Post-translational modifications (PTMs) such as those regulating the shift from gamma IF polypeptide to the beta IF polypeptide have been implicated in driving the functionally diverse roles of IFs in the cell i.e. cell signaling roles and cell structural integrity (Hyder et al., 2008). It has been demonstrated that phosphorylation-mediated PTMs are the most consequential PTM in IF assembly and organization, although others exist. An example of a phosphorylation-mediated PTM is the reorganization of IFs during mitosis (Hyder et al., 2008).

*New hypothesis for slime thread synthesis and organization with the GTC*

Slime thread assembly begins adjacent to the nucleus within the MRZ of the cell where cytoplasmic ribosomal clusters actively synthesize IFs. Newly synthesized IFs begin to helically wrap together in circular organizations of 6-8 IFs to form the first recognizable loops of the slime thread. The thread is produced from within the MRZ to the apical pole of the cell. The integration of MTs into the developing thread stiffens the thread and facilitates its proper coiling
within the confines of the cell. Thread coiling is further influenced by the torsional bias generated by the helical wrapping and integration of newly forming IFs into the thread. As slime thread length increases the conical shape of the nucleus and MRZ obstruct the deposition of newly formed coils. This forces the thread loops to fall around the nucleus and MRZ where they form conical groups. Cell growth allows for each conical loop arrangement to form within the last. Such that the youngest and narrowest thread loops occur closest to the nucleus throughout development. MTs both within and between the developing slime thread create a scaffolding for the separation of the thread loops. A full-length skein template is generated by intermediate stages of development. Following the generation of a template, the post-translational modification of the gamma-IF polypeptide in the thread, induces the disassembly and reorganization of IFs in the thread. This shift in thread assembly involves a reduction in the number of intrathread MTs and a shift from helically integrating IFs (helical wrapping) to the peripheral recruitment of IF protein subunits onto the surface of the thread (flocculent material). The role of MTs in maintaining the interthread space between adjacent loops is gradually reduced as the slime thread thickens and becomes more consolidated via IF subunit recruitment until maturity. The fully mature thread is totally electron dense and comprised of only IF proteins.
CHAPTER 4: GENERAL DISCUSSION
In the following section, I will discuss the main contributions this thesis makes to the literature on slime gland cytology, and I will compare and contrast GTCs with another, possibly related cell type, and I will sketch out some directions for future work that could be based on questions that arose from my research.

Major findings

Chapter 2 provides a qualitative analysis of cell distribution and patterns of development in the slime glands of *M. glutinosa*. The contributions of this chapter to the hagfish literature, include: (1) images of live hagfish GTCs and GMCs at various stages of development, (2) images of ‘polyhedral cells’ from the stalk of the slime gland, (3) the discovery and partial characterization of a new cell type within the hagfish slime gland, (4) the application of immunofluorescence and confocal microscopy for the investigation of IF and MT distribution in hagfish gland cell types, and (5) ultrastructural images of hagfish GMCs. The highlight of this chapter is certainly the discovery of a new cell type within the gland—the Gland Interstitial Cell. These cells exhibit evidence of an epithelial origin and explain the enigmatic “reticular network” within the gland described and named by Newby (1946). The discovery of this new cell type is also exciting because it is equally or more numerous than the GTCs and GMCs. As such, the continued investigation of cell organization and development in the slime gland of hagfish is certainly warranted. Further studies may include: (1) a detailed characterization of the newly discovered cell type to determine the roles of this cell, (2) additional cell-culturing trials in which techniques for cell attachment and isolation are explored, (3) a detailed analysis of the possible functions polyhedral cells play within the slime gland, and (4) additional immunofluorescent investigations into IF-MT interactions and actin microfilament distribution in the slime gland.
Chapter 3 provides both qualitative and quantitative analysis of slime thread development and whole GTC maturation in the slime glands of *M. glutinosa*. The findings of chapter 3 demonstrate: (1) that patterns of GTC thread formation appear to be conserved among hagfishes, (2) thread formation in the hagfish GTC is initiated within the MRZ of the cell with the formation of IF bundles, (3) relative thread density increases as a function of slime thread maturity, and (4) slime thread maturation involves a number of developmental shifts that consist of changes in patterns of slime thread assembly and organization. As a result of these findings, and additional observations from the literature, a new perspective for slime thread development is presented in chapter 3. The findings of this chapter provide the basis for a large number of subsequent studies into slime thread development. Future studies may consider immunolabelling of actin microfilaments to determine their distribution within the GTC and possible associations with the developing slime thread. Fluorescent labeling of ribosomes would yield insight into their distribution within the cell to corroborate ultrastructural observations of their association with the thread. Immunolabeling for keratin 19 would yield insight into the location of differentiating epithelial cell types within the gland. Further investigation into the protein composition of the peripherally associating cell components (i.e. the helical wrapping and flocculent material) is certainly warranted. Further ultrastructural analyses of slime thread coiling and composition using FIB/SEM and plastic embedded slime glands is also warranted to aid in the creation of three-dimensional renderings of GTCs at various stages of maturity. Additional FIB/SEM experiments should replace the sputter coated gold layer of the plastic sections with a tungsten layer to reduce the ‘streaking’ effect of the gold. The resultant data sets should exhibit the necessary consistency in contrast between sections for a comprehensive segmentation of the GTC.
Statement of significance

The findings of this study, and future works into understanding hagfish GTCs, will lead to a better understanding of protein assembly dynamics in general. The GTC provides a unique model for studying the interaction of cytoskeletal elements during the synthesis and assembly of proteins and might elucidate a means of generating a renewable and reliable source of fibrous biopolymers (Downing et al., 1981; Fudge et al., 2010). Currently synthetics account for two-thirds of the world textile market (Fudge et al., 2010). As such, there is an immense importance for developing new fiber sources that are both mechanically consistent in their structural properties and economically viable to produce (Fudge et al., 2010). The mechanical and physical consistency with which hagfish slime threads are produced within the GTC makes this thread source an ideal candidate for large-scale bio-fiber production. As such, developing an understanding of how the slime thread is produced within the GTC will provide the starting point for future industrial textile collaborations. The ultimate goal of these collaborations would be the production of a renewable and reliable fiber source that does not rely on petrochemicals and whose production involves cell media.

Epidermal threads cells (‘Club cells’) and hagfish GTCs

The most commonly used descriptors of hagfish GTCs in the literature are certainly ‘novel’ and ‘unique’. No cells exist that compare to the GTC in their ability to synthesize large quantities of proteins and organize them into an elaborately-coiled thread. As such, relatively few studies have compared the GTC to other thread producing cell types. Nevertheless, it has been suggested that an evolutionary relationship exists between the the ‘club cells’ (Downing and Novales, 1971) or ‘epidermal thread cells’ (Quay, 1972) of cyclostomes (lamprey and hagfish) and the highly specialized GTCs which are restricted to the hagfishes. Club cells are
found in the epidermis of both lamprey and hagfishes. In lamprey the club cells produce a helical thread that extends in clockwise rotations to the apex of the cell (Downing and Novales, 1971). Downing and Novales, (1971) illustrate that the cytoplasm contains large number of ribosomes and mitochondria, a poorly developed rough endoplasmic reticulum (RER) and microtubules that align in parallel with the helical filament of the cell. This description of the club cell closely resembles the ultrastructural findings of this study. The hagfish GTC also contains large numbers of cytoplasmic ribosomes and mitochondria, shows little evidence of an extensive RER, and has MTs that align with IFs within the thread. The immunofluorescent and ultrastructural findings of this study further support the possibility that hagfish GTCs share a number of similarities with the epidermal club cells shared by hagfishes and lamprey. The clockwise coiling pattern of the GTC previously described in the literature (Fudge et al., 2005) further strengthens the commonalities between these cell types. Although it is not clear at this time, the helical wrapping of the thread skein may impart the torsional bias necessary to induce a helical twisting of the slime thread causing a predictable direction of coiling that is consistent with those observed in the epidermal club cells and GTC skeins in hagfish. Downing and Novales (1971) provide little insight into how the thread product of club cells is helically coiled. Further investigation into the relatedness of these two cell types is certainly warranted.

Epidermal thread cells (ETCs) are found in both lamprey and hagfishes. This suggests the formation of polymer threads from the interaction of IFs and MTs evolved before the divergence of cyclostomes. The specialized GTCs found only in hagfish, suggest that the evolutionary changes to IF-MT interactions necessary to modify ETC-like cells into GTC-like cells followed the divergence of lamprey and hagfishes. For example, the inclusion of additional sites for IF post-translational modification (i.e. threonine and serine) may have allowed for this transition in
polymer assembly. Changes in IF-MT organization and assembly may have arisen as a result of
differential selection pressures on the two lineages of cyclostomes (one an anadromous parasite
the other a marine scavenger and predator). The topic of evolutionary changes in IF-MT
interactions has not received significant attention in the literature. However, it has been
suggested that cytoskeletal elements have been highly conserved across eukaryotic taxa because
of their ability to acquire new and elaborate mechanisms of assembly and interaction (Erickson,
2007). Epidermal thread cells and hagfish GTCs certainly make interesting models for further
study on this topic.

Ultimately, the study of hagfish GTCs (and their 15 cm fibers) in this thesis has brought
together a large number of scientific disciplines; such as, cytology, biomechanics, biomimetics,
and ethology. The images and results presented in this thesis represent a significant step forward
in our understanding of the hagfish slime gland and its constituent cell types.
References


Straub, F.B. (1943) in Studies from the Institute of Medical Chemistry *University Szeged* (ed. Szent- Gyorgyi, A.) S. Karger AG, Basel; 3; 23-37


Appendix

Table 1: Organic and inorganic osmolytes added to commercially available media to raise osmolality to approximately 1000 mOsmol/L

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<th>Osmolytes</th>
<th>EMEM (g/100 mL)</th>
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<th>DMEM (g/100 mL)</th>
<th>PBS (g/100 mL)</th>
<th>L-15 (g/100 mL)</th>
<th>HBSS (g/100 mL)</th>
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Table 2: Ethanol dehydration series illustrating the dilutions (%) and rinsing times (minutes)

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<td>15</td>
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Table 3: Paraffin dewaxing protocol for embedded hagfish glands and tissues.

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<tr>
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<td>5</td>
</tr>
<tr>
<td>100% EtOH 1</td>
<td>3</td>
</tr>
<tr>
<td>100% EtOH 2</td>
<td>3</td>
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<tr>
<td>95% EtOH</td>
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<tr>
<td>70% EtOH</td>
<td>1.5</td>
</tr>
<tr>
<td>ddH2O</td>
<td>until ready for IHC staining</td>
</tr>
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CHAPTER 1: INTRODUCTION FIGURES
Figure 1: Large volume of fibrous slime released by a Pacific hagfish (*Eptatretus stoutii*).
Figure 2: Hagfish thread skeins (arrow) and mucin vesicles (arrowhead). A thread skein has been partially unraveled to reveal the tightly coiled nature of the skein.
Figure 3: A transverse section through a slime gland of the tail region of Polistotrema. In the body region the gland would be more flattened and would lie between the myomere and the skin.

Figure 3: Transverse section of a slime gland of Polistotrema stoutii illustrated by Newby (1946). Illustrating his segmentation of the gland.
Figure 4. Cross-sections through slime threads at different stages of maturation illustrating the
tempo-spatial changes in the parallel arrangement of IFs (black dots) and MTs (tubular structures)
scale bar 0.5 um (Downing et al., 1984).
CHAPTER 2 FIGURES
Figure 1: Differential interference contrast image of isolated hagfish gland cells illustrating a variety of maturity stages of both gland thread cells (arrow) and gland mucous cells (arrowhead)
Figure 2: Differential interference contrast images of immature hagfish gland thread cells with increasing lengths of slime thread growing adjacent and distal to the nucleus. Key: asterix, nucleolus; arrowhead, nucleus; arrow, slime thread.
Figure 3: Histologically stained gland thread cells (GTCs) from differentiation through slime thread production. The gland capsule (CAP), epithelial lining (EL) and interior (INT) are denoted. (1) Masson’s Trichrome stained undifferentiated cell, (2) basic fuchsin methylene blue stained dividing cells of the epithelial lining, (3) hematoxylin and eosin (HandE) stained immature GTC with short length of slime thread, (4) basic fuchsin methylene blue stained immature GTC with inset of a slightly later stage of maturity, (5) Masson’s Trichrome stained GTC at intermediate stage of maturity, (6) HandE stained GTC at intermediate stage of maturity. Dashed arrow denotes an endothelial cell forming a capillary within the gland capsule. A large eosinophilic erythrocyte is visible within the capillary. Scale bars are 10 µm.
Figure 4: Differential interference contrast image of an isolated immature hagfish gland thread cell (left) adjacent to a large mature thread skein. The characteristically unorganized apical tip of the mature thread can be seen separating from the rest of the thread bundle. This image also illustrates the drastic change that occurs in thread thickness and length as GTC maturity proceeds.
Figure 5: Differential interference contrast imaging of hagfish slime gland cells stained with the vital exclusion dye trypan blue. (1) Low magnification image of slime gland cells of various stages of development illustrating a large number of live gland thread cells (GTCs) and gland mucous cells. (2) Live GTCs and GMCs, (3) live hagfish GTC, (4) dead hagfish GTC.
Figure 6: Differential interference contrast image of a hagfish gland thread cell of intermediate maturity (left) next to a large mature gland mucous cell (right).
Figure 7: Differential interference contrast image of a gland thread cell (GTC) of intermediate maturity and gland mucous cell (GMC). The polar position of the GTC nucleus is evident in comparison to the position of the GMC nucleus.
Figure 8: Transmission Electron Micrographs of a hagfish gland mucous cell of intermediate maturity illustrating the formation of mucin vesicles from within the large Golgi-complex. (1) GMC, (2) high magnification image of GMC nucleus and surrounding vesicle filled Golgi apparatus, (3) single mucin vesicle within the Golgi of the GMC illustrating the surrounding folds in the Golgi apparatus. Key: asterix, nucleolus; arrow, nucleus; arrowhead, Golgi apparatus
Figure 9: Paraffin embedded gland mucous cells and surrounding tissue, showing the capsule (CAP), epithelial lining (EL) and gland interior (INT). (1) Epifluorescent image showing immunofluorescently labelled intermediate filament protein (red), alpha tubulin (green), and nucleic acids (blue). (2) Differential interference contrast image corresponds to epifluorescent image 9.1. (3) Hematoxylin and eosin staining of an immature hagfish GMC. Key: arrow, gland mucous cell; arrowhead, unidentified cells surrounding the GMC.
Figure 10: Polyhedral cells of *M. glutinosa* slime glands. The gland capsule (CAP), epithelial lining (EL) and interior (INT) are denoted. (1) Hematoxyline and eosin stained hagfish slime gland illustrating the gland pore and associated tissues. The layer of cells denoted by the asterix correspond to the polyhedral cells described by Newby (1946). (2) Epifluorescent image of polyhedral cells of the gland immuno-labelled for intermediate filament (IF:red) and tubulin (MT:green) proteins. (3) Confocal microscopy image of a polyhedral cell demonstrating IF and MT distribution in the cell. (4) Differential interference contrast image of same polyhedral cells in Figure 10.2.
Figure 11: Epifluorescent image of hagfish gland cells immuno-labelled for intermediate filaments (IFs: green) and nuclei (DNA: blue). The capsule (CAP), epithelial lining (EL) and gland interior (INT) are denoted. Key: arrow, unidentified cells connecting the epithelial layer and cells in the gland interior; dashed-arrow, connections of unidentified cells with epithelial cells.
Figure 12: Confocal image of hagfish gland cells fluorescently labelled for intermediate filaments (IFs: red), microtubules (MTs: green) and nuclei (DNA: blue). The capsule (CAP), epithelial lining (EL) and gland interior (INT) are denoted. (1) Arrows denote a number of unidentified cells, arrowhead denotes gland mucous cells (2) Bifurcation of the unidentified cell (dashed arrow), (3) mature gland thread cell with adjacent unidentified cell (arrow).
Figure 13: The distribution and ultrastructure of the unidentified cell from hagfish slime glands. (1) Arrow denotes unidentified cell nucleus between two developing gland thread cells (GTCs). (2) Higher power image of 13.1 showing the unidentified cell nucleus and cellular processes extending between the GTCs. (3) High power image of paranuclear region of the unidentified cell. Dashed arrow denotes a mitochondrion, arrowhead a cluster of intermediate filaments (IFs). (4) Cell process from the unidentified cell between adjacent GTCs with Golgi and IFs visible. (5) Large GTC with closely juxtaposed unidentified cell type. (6) Unidentified cell showing large amounts of Golgi (simple arrow), and vesicle fusion with adjacent gland mucous cell (double lined arrow). (7) Unidentified cell adjacent to developing GTC showing vesicle fusion between the membranes, Golgi and IFs.
Figure 14: Composite image of 221 individual photos taken at 200 x magnification in differential interference contrast. This image illustrates the general layout of the hagfish slime gland among surrounding tissues types. The high resolution nature of this image allows for high magnification inspection of the gland by readers. The asterix denotes the slime gland pore.
Figure 15: Hematoxylin and eosin staining of a hagfish slime gland which had been ‘slimed’ 2 weeks prior to harvesting. The three proposed regions of cell organization along the inside wall of the slime gland. Magnified insets include: (1) the gland pore and polyhedral cell region, (2) the lateral walls of the slime gland composed of epithelial cells. Vascularization of the gland capsule is seen on the left of the figure. Blood cells occur within one of the vessels. (3a,b) developing gland thread cells (GTCs) and gland mucous cells (GMCs) in the proliferative zone of GTC and GMC differentiation.
Figure 16: A collection of images illustrating the hagfish gland pore and associated cell types. The gland capsule (CAP), and gland interior (INT) are labelled as such. (1) Epifluorescent image with intermediate filaments (IFs), microtubules (MTs), and nuclei shown in red, green and blue respectively. (2) Differential interference contrast image (DIC). (3) Confocal image of the gland pore (IFs: red, MTs: green, nuclei: blue). (4) hematoxylin and eosin stained paraffin section of a hagfish gland.
Figure 17: Epifluorescent images of a paraffin embedded hagfish gland focused on the gland pore and constituent cell types. Image illustrates the hagfish gland capsule (CAP), polyhedral cell cluster (PCs) and gland interior (INT). Intermediate filaments (IFs) are visible in red, microtubules (MTs) in green, and cell nuclei in blue. The asterix denotes the gland pore. (1) nuclei of cells stained with DAPI, (2) differential interference contrast image of gland pore region, (3) MTs within gland pore associated cells, (4) IFs within gland pore associated cells, (5) composite image showing nuclei, IFs and MTs.
CHAPTER 3 FIGURES
Figure 1: Focused Ion Beam (FIB) milled mature gland thread cell imaged with scanning electron microscope (FIB image probe 30 KV:80pA, EHT = 5 kV). Asterix (*) denotes nucleolus within the surrounding conically shaped nucleus. Arrow denotes fine paranuclear thread network with fibers as small as 40 nm visible. Outer thread diameters are approximately 1.21 µm. Scale bar = 10 µm.
Figure 2: Transmission Electron Micrograph of immature gland thread cell (GTC) with short helical length of slime thread. Figure insets illustrate two additional levels of magnification with a focus on the mitochondrial rich zone of the GTC and the paranuclear region. Inset (3) illustrates the close juxtaposition between the formation of the primal thread and the nucleus and mitochondria of the cell. Arrow denotes slime thread cross-section.
Figure 3: Focused Ion Beam (FIB) milled intermediately mature gland thread cell imaged with scanning electron microscope (FIB image probe 30 KV:80pA, EHT = 5 kV). Image was taken immediately distal to the cell nucleus in the proposed area of thread development. Arrows denote probable clusters of ribosomes (polysomes) on the thread surface.
Figure 4: Focused Ion Beam (FIB) milled mature gland thread cell imaged with scanning electron microscope (FIB image probe 30 KV:80pA, EHT = 5 kV). Images 1-8 taken from the blunt basal side of the GTC to the apical side. Images seen in cross-section of the long axis of the cell. A Sobel convolution filter was applied. Asterix (*) denotes nucleolus within the surrounding nucleus. Arrow denotes fine paranuclear thread network with fibers as small as 100 nm visible. Arrowhead denotes mitochondria at the apical tip of the nucleus. Scale bar = 10 µm.
Figure 5: Transmission Electron Micrographs of M. glutinosa gland thread cells (GTCs) in progressive stages of maturation. Image illustrates a change in thread electron density and nuclear position as skein development proceeds. (1) Dividing gland stem cell and differentiating daughter cell. (2) Recently differentiated GTC with primal slime thread. (3) Young GTC with slime thread coiling and skein formation beginning. (4) GTC of intermediate maturity with well formed skein. (5) Intermediately mature GTC with large thread skein. (6) Late stage of GTC maturity. (7) Fully mature GTC illustrating electron dense thread and no intra-thread MTs.
Figure 6: Change in relative slime thread density through five categorical stages of slime thread maturation. Stages of slime thread maturity determined by the ultrastructural composition and appearance of thread cross-sections observed in transmission electron microscopy and analyzed via thresholding the protein content of the thread against the background of the image. Stages are defined as the following: (1) Slime thread composed of 12 nm intermediate filaments (IFs), (2) Slime threads composed of 12 nm IFs and 25 nm microtubules (MTs), (3) Intermediately mature slime thread comprised of large numbers of loosely arranged 12 nm IFs as well as 25 nm microtubules (MTs), (4) Intermediately mature slime thread with IFs beginning to condense and large multi-MT associations, (5) Late maturity slime thread with fully condensed IFs and reduced MT numbers, (6) Fully mature slime thread with full IF condensation and no MTs.
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Figure 7: Hagfish slime thread cross-sections at various time points in development. Scale bar is 500 µm. (1) 12 nm intermediate filaments (IFs) associating during early slime thread formation, (2) Slime threads composed of 12 nm IFs and 25 nm microtubules (MTs). (3) Intermediately mature slime thread comprised of large numbers of loosely arranged 12 nm IFs as well as 25 nm microtubules (MTs). (4) Intermediately mature slime thread with IFs beginning to condense and large multi-MT associations. (5) Late maturity slime thread with fully condensed IFs and reduced MT numbers. (6) Fully mature slime thread with full IF condensation and no MTs.
Figure 8: Transmission electron micrographs of gland thread cell at an intermediate stage of maturity illustrating thread cross-sections from the apical region of the cell corresponding to the unorganized threads of the skein. (1) Intermediately mature GTC imaged at the scale of the whole cell. (2) Higher power image of the cell in (8.1) illustrating the apical most region of the cell. (3,4) High power images of thread cross-sections from the apical region of the cell in (8.1) illustrating large assemblies of 12 nm intermediate filaments and no intrathread microtubules.
Figure 9: Epifluorescent (EF) and differential interference contrast (DIC) images of paraffin embedded hagfish gland thread cells (GTCs) at various time points in development. Immunofluorescence is for hagfish IF protein (red), alpha-tubulin (green) and nuclei (blue). All three color channels have been overlayed in 9.1-3. (1) Immature GTC. (2) Intermediately mature GTC. (3) Mature GTC. (4) DIC of mature GTC.
Figure 10: Epifluorescent images of paraffin embedded hagfish glands cells. Image illustrates the hagfish gland capsule (CAP), epithelial lining (EL) and gland interior (INT). Intermediate filaments are visible in red, microtubules in green, and cell nuclei in blue. The arrow, arrow-head and asterix respectively denote the gland thread cell (GTC), gland mucous cell (GMC) and a third previously undescribed cell within the gland (Winegard Cell: WC). (1) Composite image of overlaid red, green and blue color channels. (2) Green color channel. (3) Differential interference contrast (DIC). (4) Blue color channel with the nuclei of GTC, GMC and WC denoted. (5) Composite DIC and red, green, blue overlay. (6) Red color channel.
Figure 11: Immunofluorescent image (IFL) of mature gland thread cell (GTC) and undescribed cell type: (a) Tubulin staining, (b) Hagfish intermediate filament (IF) staining, (c) DAPI staining, (d) overlay of IFL images, (e) overlay of IFL images superimposed on differential interference contrast (DIC) image, (f) DIC image of mature GTC. Arrow denotes the GTC, arrowhead denotes the location of the undescribed cell type.
Figure 12: Confocal image of hagfish gland thread cell (GTC) fluorescently labelled for intermediate filaments (red), microtubules (green) and nuclei (blue). The arrow, arrowhead and simple arrow denote the GTC, gland mucous cell, and a previously undescribed cell type in the gland.
Figure 13: Transmission electron micrographs (TEMs) illustrating the ultrastructure of hagfish gland thread cell slime threads and organelles within the cytoplasm. (1) Main image illustrates a tangentially sectioned slime thread illustrating the helical wrapping found in slime threads of early to intermediate stages of development prior to IF protein compaction within the thread. Inset: cross section through a slime thread of equivalent maturity. (2) TEM illustrates a longitudinally sectioned slime thread with cross-sections through the helical wrapping at the sides of the thread. (3) TEM of mitochondrial rich zone at the apical tip of the GTC nucleus. Numerous mitochondria and IF slime thread assemblies are visible in both tangential and cross-section. (4) TEM of slime thread sections exhibiting a indiscernible flocculent material at the thread periphery. Key: simple arrow head; microtubules, arrowhead; helical wrapping, arrow; mitochondria
Figure 14: Transmission electron micrographs (TEMs) of hagfish gland thread cell slime threads illustrating the compaction of the IF portion of the thread into an electron dense bundle in which numerous microtubules (MTs; arrowhead) can be observed. (1) Vesicles (arrow) of varying shape and size are observed within the longitudinally sectioned slime thread. (2) A large MT gap in the slime thread containing a variety vesicles. (3) A longitudinally sectioned slime thread illustrating a number of MTs within a MT-gap in the slime thread. The bending of the MTs is illustrated in this image. (4) The image illustrates a cross-section through a slime thread illustrating the condensed IF portion of the thread as well as a number of MTs dispersed throughout the thread both singularly and in small groups. A MT-gap appears perpendicular to the longitudinal axis of the thread illustrating a MT that appears to be moving between the cytoplasm and intrathread space of the slime thread.