Coiling and maturation of a high-performance fibre in hagfish slime gland thread cells

Timothy Winegard1, Julia Herr1, Carlos Mena2,3, Betty Lee2,3, Ivo Dinov2,3,4, Deborah Bird5, Mark Bernards Jr1, Sam Hobel2,3, Blaire Van Valkenburgh5, Arthur Toga2,3 & Douglas Fudge1

The defensive slime of hagfishes contains thousands of intermediate filament protein threads that are manufactured within specialized gland thread cells. The material properties of these threads rival those of spider dragline silks, which makes them an ideal model for biomimetic efforts to produce sustainable protein materials, yet how the thread is produced and organized within the cell is not well understood. Here we show how changes in nuclear morphology, size and position can explain the three-dimensional pattern of thread coiling in gland thread cells, and how the ultrastructure of the thread changes as very young thread cells develop into large cells with fully mature coiled threads. Our model provides an explanation for the complex process of thread assembly and organization that has fascinated and perplexed biologists for over a century, and provides valuable insights for the quest to manufacture high-performance biomimetic protein materials.
The hagfish gland thread cell (GTC) is remarkable because of the strength of the thread it produces, but also because of the thread’s impressive length (~150 mm), its exquisite packaging within the cytoplasm, and its ability to deploy rapidly in seawater without tangling. Although the exact site of thread assembly is unknown, it is most likely the region just apical to the nucleus where the thinnest regions of the thread are found. As the GTC matures, loops of thread are generated and organized by an unknown mechanism into what have been referred to as conical loop arrangements. The continuous coiling of the thread into these conical loop arrangements results in a ‘skein’ that is comprised of about 15–20 conical layers of thread loops. In mature cells, these highly organized loops fill most of the cell volume. While others have attempted to describe how the thread is organized within thread cells, these studies were limited by their reliance on surface imaging (scanning electron microscopy (SEM) and light microscopy) or snapshots of the skein interior from cross-sections (transmission electron microscopy (TEM)). Our goal was to understand the three-dimensional (3D) morphology of the coiled slime thread in both mature and immature GTCs in order to gain insight into possible mechanisms of thread production and coiling.

The mature slime thread consists mainly of two intermediate filament (IF) proteins, α and γ. Immature slime threads contain both 12-nm diameter IF and microtubules (MT), but it is unclear which of these elements appears first in nascent portions of the thread. Early in its development, the thread also appears to be wrapped with a distinctive ~12-nm diameter filament of unknown function. The most dramatic change in thread ultrastructure corresponds with the compaction of 12-nm diameter IFs into a dense superstructure in which discrete IFs are no longer visible and MT become surrounded by an electron lucent space presumably freed up by IF compaction. This shift in thread ultrastructure likely corresponds with the post-translational modification of IF proteins within mature GTCs; it also coincides with the loss of the wrapping filament and the appearance of a fluffy rind around the thread periphery. In fully mature threads, MTs are absent, the spaces they occupy are completely filled in, the fluffy rind is gone, and adjacent threads conform to each other leaving only a narrow space of about 40 nm between them.

In this study, we use light and electron microscopy as well as 3D imaging and modelling to describe the pattern of slime thread coiling within developing gland thread cells. Our results lead us to a new understanding of slime thread coiling and maturation, and confirm the observations made by other researchers in a different genus of hagfishes, suggesting that GTC and slime thread maturation may be highly conserved among the hagfishes.

Results

Morphology of developing GTCs. We confirmed using light microscopy that GTCs originate in the slime gland epithelium and move towards the gland lumen as they mature. SEM of isolated skeins that had cracked open revealed the morphology of conical loop arrangements at various places in the skein. To gain deeper insight into thread organization, morphology and maturation, we used TEM to observe GTCs at various stages in development. While changes to the length, diameter and morphology of the thread are the most obvious changes in the ultrastructure of maturing GTCs, changes in nuclear size and shape are also dramatic. The nucleus of an immature (that is, recently differentiated) GTC, identifiable because of its large prominent nucleolus, is round and occupies the majority of the cell volume. As the number of mitochondria and ribosomes in the GTC increase and thread synthesis begins, the nucleus becomes longer and more conical, eventually adopting a spindle shape with a flared base. In fully mature cells, the nuclear spindle recedes, leaving only a hemispherical nuclear cap outside the coiled skein.

3D structure of the coiled slime thread. To understand the precise patterning of staggered loops, we used focused ion beam scanning electron microscopy (FIB-SEM) to generate a 3D model of thread coiling (Fig. 2a) (Supplementary Movie 1). By tracing the 3D structure of a dozen continuous loops using Mimics (v. 15.01) software, we were able to elucidate for the first time the morphology of thread loops within an immature GTC; this enabled us to define the precise spatial relationship between adjacent loops (Fig. 2b) (Supplementary Movie 2). With these data we constructed a 3D model using Maya 2013 software of thread coiling that is built around a repeating loop structure (Fig. 2c) (Supplementary Movie 3). This model reproduces many of the most salient features of natural thread skeins, specifically, the spiralling nature of the conical loop structures, the nesting of these structures within each other, as well as the cabled appearance of the skein where the thread runs circumferentially along the skein surface.
Changes in thread ultrastructure with cell maturation. Our TEM studies largely substantiate observations of thread development described in other species of hagfishes\(^6,7,9\), but they also add to our understanding of the ultrastructural changes that take place during thread maturation (Fig. 3a,b). Our results clearly establish that thread production starts with a small bundle of IFs in a paranuclear region rich in mitochondria and ribosomes. The thread increases in both length and width via the addition of IFs and eventually via the incorporation of MTs. The youngest threads are only 2–3 IFs in diameter (about 30 nm) and more mature ones are about 30 IFs in diameter (about 400 nm). The wrapping filament is clearly visible in immature threads, but it is not clear whether the filament spirals around the thread at a steep angle\(^6\) or exists as separate rings (Fig. 3c). The condensation of IFs corresponds not only with the loss of the wrapping filaments, but also with the appearance of the fluffy rind that has been described by others\(^6,7\). High power TEM revealed the presence of MTs within the post-IF condensation thread (Fig. 3a,d), as described by others\(^6,7\), as well as evidence that the MTs penetrate the thread from the side (Fig. 3d).

Discussion

The changes in nuclear shape observed using TEM correspond with changes in the morphology of the conical loop arrangements observed using FIB-SEM, and suggest that the nucleus acts as a template on which the staggered loops form. Specifically, the loops making up the cones are short near the apical tip of the cell when the cell nucleus is round, and considerably longer towards the basal end as the nucleus becomes more spindle shaped. In this way, the nucleus likely provides an obstruction that limits where the thread can be deposited as it elongates from its site of synthesis. Our data and model suggest that the ascending and descending portions of the conical loops are directly shaped by the nucleus, whereas the circumferential runs, which form the base of each conical loop, are laid down in the basal groove where the nucleus and plasma membrane converge (Fig. 1c). In nearly mature GTCs, the nucleus is reduced to only a fraction of its former size, and its full retreat in a basal direction leads to very short circumferential runs and loops consisting of mostly back and forth axial runs, called ‘inner core loops’ by Fernholm\(^4\) (Fig. 4). While our model explains many aspects of slime thread morphology, it does not explain how the precise staggering of loops arises. The fact that each successive loop is translocated in a clockwise direction relative to its younger neighbouring loop is suggestive of a wheel-like mechanism at work. Although wheels are extremely rare in biology\(^10\), thread coiling may involve just such a mechanism.

Interactions between the IFs known as nuclear lamins and the inner nuclear membrane are well described\(^11,12\), but less is known about how the outer nuclear membrane interacts with cytoplasmic elements\(^13,14\). The patterning of slime thread coiling by the GTC nucleus may provide a new model for exploring these interactions in detail. Our data also raise questions about how such dramatic changes in nuclear size and shape are regulated.

Our observations are consistent with the hypothesis that elongation occurs at a single site near the apical tip of the nucleus\(^6\), but the mechanism of elongation remains elusive. The role of intra-thread MTs is also unclear, although they may be involved in the delivery of IFs and/or IF subunits\(^15,16\) to the interior of the growing thread. Indeed, TEM and FIB-SEM images both demonstrate MTs within the thread and penetrating the thread from the side (Fig. 3d). The 12-nm filament that wraps around the thread (Fig. 3b,c) before the IFs condense is also puzzling. Downing et al.\(^6\) propose that the filament is involved in the addition of IF to the growing thread, but how exactly this would work is difficult to imagine. We suggest that the wrapping filaments may prevent the merging of adjacent loops, which would have obvious negative effects on thread deployment and ultimately on the function of the slime\(^1\). Two other cell types, the hagfish epidermal thread cell and the lamprey skin cell, notably also produce thick helical IF bundles\(^17,18\), in contrast to the branching networks of IFs that are far more common in metazoan cells\(^19\). In hagfish GTCs, this ability to produce a single, unbranched IF bundle has been taken to the extreme and may be one of the key adaptations that allowed hagfishes to evolve their remarkable fibrous defensive slime.

Our results confirm that the condensation of discrete 12-nm IFs is an important step in slime thread maturation and occurs in all the species of hagfish examined thus far\(^6,7\). Previous work suggests that IF condensation may be regulated by phosphorylation of IFs\(^8,9\), which may also mark a transition to thread growth mediated by the direct addition of IF proteins to the condensed thread rather than the addition of discrete IFs to a growing bundle. Recent efforts to make fibres from solubilized hagfish slime thread proteins and the IF protein vimentin yielded threads with promising material properties, but they were clearly inferior to native slime threads\(^20,21\). The changes in thread ultrastructure documented here suggest that the outstanding mechanics of native slime threads likely arise via careful control of protein structure from IF subunits to mature IFs to dense IF superstructure. Unravelling the biochemical and biophysical mechanisms underlying these transitions may lead us to new methodologies for the production of sustainable high-performance protein materials.
Methods
Animal collection, care and procedures. Specimens of Atlantic hagfish (Myxine glutinosa) (used for TEM and FIB-SEM) were collected in Passamaquoddy Bay, New Brunswick, Canada. Pacific hagfish (Eptatretus stoutii) (used for gland histology and whole skin SEM) were obtained from the Bamfield Marine Station, Bamfield, BC, Canada. Hagfishes were housed at the Hagen Aqualab at the University of Guelph. Animals were fed a diet of fish and squid and were maintained at a constant temperature (10°C) in artificial seawater (10× concentrated artificial seawater) with continuously aerated water. Hagfish were anaesthetized with a clove-oil anaesthetic protocol. Following anaesthesia the slime glands of the hagfish were electrostimulated (60 Hz, 18 V) using a Grass SD9 electric stimulator (Grass Instruments, Quincy, MA, USA). For harvested glands, electrostimulation was performed 1 or 2 weeks before tissue collection to increase the ratio of mature glands. Hagfish were killed by severing the notochord and dorsal nerve cord directly posterior to the cranium with a large scalpel. All housing and feeding conditions were in accordance with the University of Guelph Animal Use Protocol 09R128.

SEM of thread skeins from slime exudates. Slime exudate was collected from anaesthetized hagfish as described above and stabilized in an aqueous stabilization buffer (0.9 M sodium citrate, 0.1 M PIPES, pH 6.7). Thread skeins were separated from much smaller mucin vesicles by filtering through 53 μm Nylon mesh. Skeins were fixed in 3% paraformaldehyde in phosphate-buffered saline (PBS) and transferred into 30% ethanol and dehydrated using the following ethanol series for 15 min each: 30, 50, 70, 95 and 100% (3 × 3). The same dehydration series was used for tissues prepared for TEM and histology (see below). Dehydrated skeins were critical point dried and then sputter coated using an Emitech K550 Sputter Coater (Emitech Ltd, Ashford, Kent, England) to produce an ~12-nm Au/Pd coating (20 mA for 2 min). The prepared skein samples were then observed using a Hitachi S-570—Scanning Electron Microscope (Hitachi High Technologies, Tokyo, Japan) and images were captured using the Quartz PCI—Image Management System, v. 8.5.

Slime gland fixation and embedding. Killed hagfish were dissected by first making a 6-cm incision down the dorsal midline starting from the rostral end. Perpendicular cuts from this incision to the ventral midline created a rectangular flap of skin on each side. 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 to gently pull it away from the skin to expose the gland pore. Iridectomy scissors were used to cut the slime gland pore where it attached to the epidermis. Following excision, the slime glands were transferred into a culture dish containing buffered fixative. For TEM and SEM, a fixative solution containing 3% parafomaldehyde and 3% glutaraldehyde in 0.1 M sodium cacodylate was used, followed by 3–10 min rinses with cacodylate buffer. Following fixation, glands were post-fixed with osmium tetroxide (1% in 0.1 M sodium cacodylate) and en bloc stained in a 2% uranyl acetate in 0.1 M sodium cacodylate solution. Fixed, osmicated, stained and dehydrated glands were plastic embedded for TEM and FIB-SEM using a Polysciences Poly/Bed812 (Luft formulations) embedding kit (Polysciences Inc. Warrington, PA). For histology, glands were fixed in 3% parafomaldehyde in PBS and rinsed three times for ten minutes in PBS and dehydrated using the ethanol series described above. Fixed and dehydrated slime glands were paraffin embedded at the University of Guelph Animal Health Laboratory using standard protocols. Hagfish tissues were sectioned using a microtome to 4 μm sections and mounted on glass slides for staining with hematoxylin and eosin (H&E).

FIB-SEM. Before mounting, plastic-embedded glands were sectioned to expose the cells at the periphery and in the lumen of the gland. This was performed using a Porter-Blum MT-1 manually-driven ultramicrotome (Ivan Sorvall, Inc.). Samples were spatter coated with a 5-nm gold coating (Model S150B; Edwards; England) and mounted for FIB-SEM on aluminium pin stubs (12.7 mm diameter) using silver paint. Embedded GTCs were milled away in 90 nm sections using a gallium-focused ion beam (FIB image probe 30 kV; 80 pA, EHT = 5 kV) and SEM images were collected after each layer was ablated.

3D model of continuous thread loops from FIB-SEM data. Grayscale image stacks obtained from FIB-SEM experiments were imported into a 3D visualization software package (Mimics v. 15.01, Materialise NV, Leuven, Belgium) and a length of thread was segmented by manually masking its cross-section over the course of about a dozen staggered loops. Nucleus, nucleus and mitochondria were individually segmented and rendered as separate 3D objects. 3D objects were exported from Mimics into Maya 3D animation software (v. 2013, Autodesk Inc. San Rafael, CA, USA) for further editing and the production of a high-resolution colour image and movie.
TEM imaging. E. Nikon Eclipse 90i microscope. Brightfield colour images were taken using a 3D modelling of skein coiling and organization. uranyl acetate (2%) and lead citrate (1%) following sectioning using a Porter-Blum MT-1 manually-driven ultramicrotome and stained with.


Acknowledgements
We thank Oualid Haddad, Aatsuko Negishi and Nicole Pinto, as well as Dianne Moyle from the University of Guelph Electron Microscopy Unit, Glynis de Silveira and Julia Huang from McMaster University’s Canadian Center for Electron Microscopy, Amy Rowat and Dan Toso from UCL, Susan Lopes from the University of Guelph’s Animal Health Laboratories, as well as Bob Frank, Matt Cornish and Mike Davies from the University of Guelph’s Hagen Aqualab. Funding was provided by NSERC Discovery and Accelerator grants to D.F., an NSERC CGS-M scholarship to T.W., an NSF grant (DUE 0716055, 1023115) to I.D., an NSF GRFP grant (DGE-1144087) to D.B., an NSF grant (BSY-1119768) to B.V.V., and an NIH P41 grant, 5P41EB015922-16, for the Laboratory of Neuro Imaging Resource (LONIR).

Author contributions
T.W. designed and carried out experiments and wrote the manuscript. J.H. prepared transparencies and permissions accompanies this paper at http://www.nature.com/naturecommunications

Competing financial interests: The authors declare no competing financial interests.

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