An investigation of the molecular and structural properties of the mutable collagenous tissue in a European sea cucumber

Némo LEMAIRE[1], Nathalie SINGH[2], Amy LIN[2], Rudy WATTIEZ[3], Jérôme DELROISSE[1], Matthew J. HARRINGTON[2], Patrick FLAMMANG[1]

[1] Biology of Marine Organisms and Biomimetics Unit, University of Mons (UMONS), Mons, Belgium
[2] Department of Chemistry, McGill University, Montreal, Canada
[3] Proteomics and Microbiology Department, University of Mons (UMONS), Mons, Belgium

A connective tissue with unconventional properties

In most connective tissues, e.g. tendons and bones, properties are set in time and changes can only occur irreversibly under a handful of conditions. On the contrary, the **mutable collagenous tissue (MCT)** found in echinoderms is a unique connective tissue that can rapidly alter its **mechanical properties** in response to certain stimuli. The tissue can alternate between **three different stiffness states**. Thanks to this, echinoderms can minimise energy expenditure and are able to express various behaviours associated with their defence, reproduction, or locomotion.

The body wall of **sea cucumbers** (Holothuroidea) is a typical MCT capable of reversible modulation and irreversible softening. In this model, mechanical properties are modified by the secretion of molecular factors in the extracellular matrix, resulting in the formation or rupture of **transient cross-bridges** between collagen fibrils. However, this specific model is still lacking a lot of information regarding the actors of those changes and the way they interact with the collagenous scaffold.

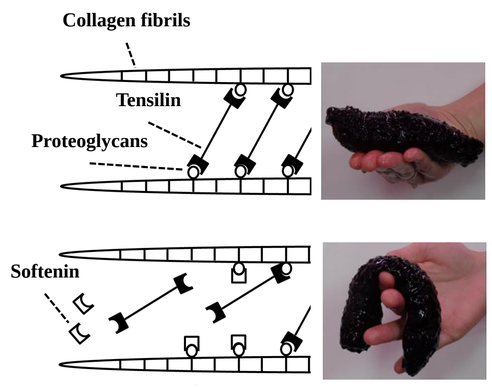


Figure 1: In the standard state (upper picture), collagen fibrils are connected by local cross-bridges made of dimers of a stiffening molecule called **tensilin (stiffener)**. Tensilin is released by specific neurosecretory-like cells found exclusively in MCT. On the contrary the shift to the soft state (lower picture) is assumed to work through a process involving an antagonist molecule named **softenin (softener)**. Both molecules would bind to collagen fibrils by interacting with **surface proteoglycans**. The stiff state (not represented here) is, at the time of writing, poorly understood but could require the stiffening of the microfibrillar meshwork surrounding the collagen in combination to water exudation. Diagram adapted from Takehana *et al.*, (2014)

Investigation of the MCT

- Extracellular matrix interactome
- Nature of the collagenous scaffold
- Identification of molecular actors through their affinity for collagen

Strategy for protein extraction

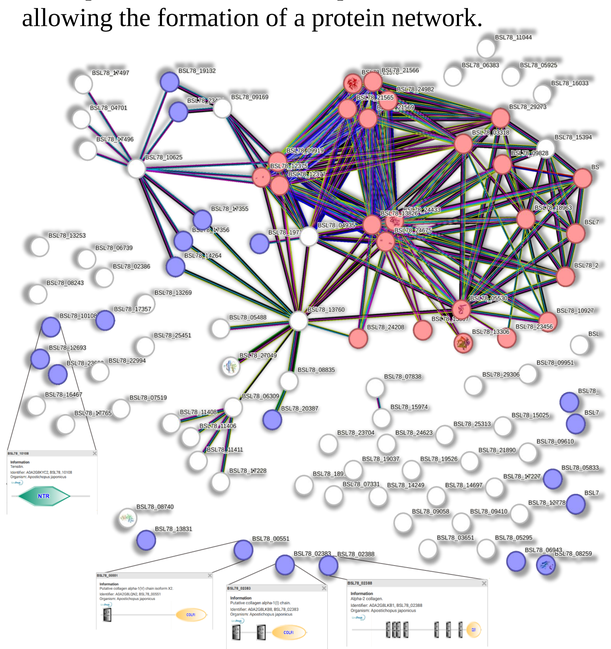
Body wall samples were retrieved from the sea cucumber *Holothuria forskali*, a species inhabiting the European coast. After removal of the pigmented epidermis and muscle layers, the main collagenous part was rapidly subjected to a series of freeze-thaw cycles and centrifugation following Tamori *et al.* (2006). This protocol led to the separation of collagen fibrils and extracellular matrix proteins.

The presence of molecular factors in the protein fraction was assessed based on the knowledge about the activities of stiffener (i.e. tensilin) and softener (i.e. softenin) in sea cucumber's MCT. Extracted proteins were combined with purified collagen fibrils under increasingly destabilising conditions before being co-precipitated.

Isolated fibrils, extracted proteins, and collagen-bound proteins were analysed by nanoscale liquid chromatography coupled to tandem mass spectrometry (nanoLC-MS/MS). Recovered peptides were matched to a previously obtained body wall transcriptome of the species before being further investigated bioinformatically.

MCT interactome

Solubilisation of the whole protein fraction present in the extracellular matrix resulted in the identification of more than 300 sequences by the software Protein pilot. Among those, half were represented by at least two different peptides and were therefore considered to be more than background noises. When computed on the prediction software STRING nearly all sequences showed correspondence to annotated proteins from the better-known sea cucumber *Apostichopus japonicus* allowing the formation of a protein network.



The network is composed of an equal proportion of cellular and extracellular sequences. The former of which is most noticeable by its cytoskeletal proteins (*actin*, *myosin*, and *actin-binding proteins*). Concerning the latter, many sequences are identified by short fragments containing for most nodes a single domain. The last half of the nodes are represented by putative proteins with very little information available.

- Cluster of cytoskeletal proteins
- Next to no interaction within extracellular matrix proteins
- No interaction related to collagen trimers or tensilin

Figure 2. Protein network of ortholog proteins of *Apostichopus japonicus.* The system is composed of 99 nodes (protein) selected by proximity to *H. forskali* sequences. Interaction between proteins are visualised by coloured lines connecting nodes. Nodes composing the cytoskeleton are filled in red, while nodes found in the extracellular region are marked in blue (gene ontology). Non-coloured nodes identify proteins with no associated location. Collagen trimers and tensilin are presented with their predicted domains in supplementary insets.

Isolated collagen fibrils

Among all tested samples, purified fibrils were characterised by the lowest number of associated sequences and detected peptides. Nonetheless, five proteins were consistently found between all our replicates and were, in the case of two transcripts (4466 and 5489), represented by a considerable number of peptides. Sequence analysis using Blast-based approaches and SMART tool revealed that three of these proteins were collagenous in nature and belonged to two different clades of fibrillar collagen. The other two sequences, partially complete, were related to *cadherin-like* and *cubulin-like protein*.

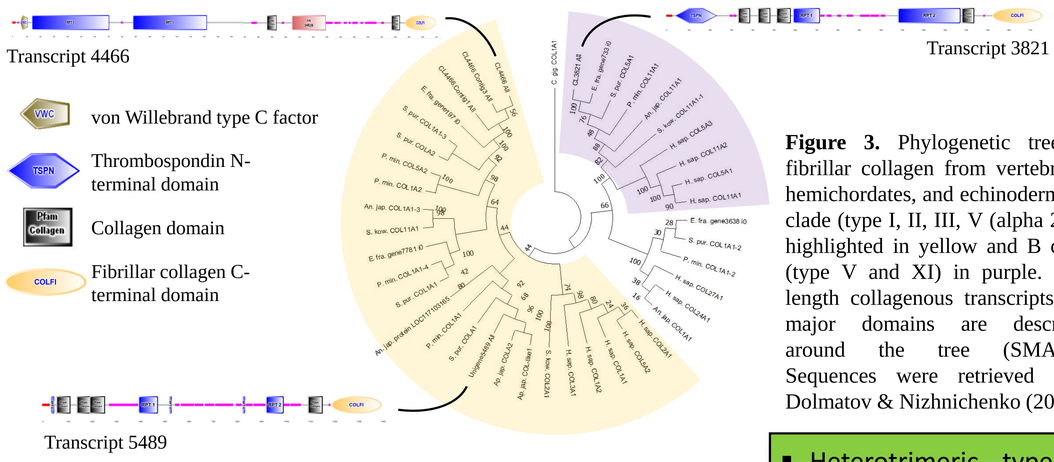


Figure 3. Phylogenetic tree of fibrillar collagen from vertebrates, hemichordates, and echinoderms. A clade (type I, II, III, V (alpha 2)) is highlighted in yellow and B clade (type V and XI) in purple. Full-length collagenous transcripts and major domains are described around the tree (SMART). Sequences were retrieved from Dolmatov & Nizhnichenko (2023)

The identified sequences are reminiscent of alpha 1 and 2 chain from type I (transcript 4466 and 5489, respectively), and type V/XI (transcript 3821) vertebrate *collagens*. The later discovery is unexpected as only few mentions of it have been made in echinoderms. *Type V collagen* differs from usual collagen due to the conservation of its N-propeptide at maturity. This conserved region extends outwards through the gap zone of the fibrils and serves as a binding site for multiple proteins.

- Heterotrimeric type I chain (alpha 1 & 2)
- Heterotypic fibrils (type I/V)
- Involvement of type V collagen during tissue state shifting ?

Collagen-bound proteins

Previous work on the activity of the stiffener tensilin has defined a range of affinity for collagen fibrils. When exposed to 2.5 M of NaCl, collagen aggregates maintained by tensilin lose their structure and regain their isolated form. Since the accepted hypothesis for tissue softening involves competitive mechanisms, a general lookout of collagen-protein interaction could reveal novel actors of those changes.

Figure 4. Heat map and Venn diagram of the detected sequences found within samples. Purified fibrils and extracted proteins were mixed under physiological (0.5 M NaCl) or destabilising conditions (2.5 M NaCl) and collagen-bound proteins were recovered with collagen precipitated by centrifugation (26 000 g, 10 min). The number of unique peptides found by transcript in each condition is written in their corresponding box. Conserved domains were identified with SMART.

A quick look at collagen associated-proteins show that a number of sequences are found in of both conditions. *Tensilin* itself (transcript 6864) is still detected at similar level for 0.5 and 2.5 M. Interestingly, nearly of third of potential candidates are *collagen-like proteins*. Other relevant sequences included *major yolk proteins*, *cubulin-like protein*, *cadherin like protein*, *and cysteine-rich secreted protein*.

- Missing or unrecognizable softening factor → new activity
- Potential relevance of small collagen-like proteins in fibrils structures and/or interactions
- Fragmented sequences

Conclusion

This study, while still requiring further investigations, highlights the detection of a **novel collagen (type V/XI) in sea cucumber connective tissue**, an occurrence only recently suggested in *A. japonicus*. In the MCT, this collagen could serve, directly or thanks to sulphated glycosaminoglycans, as the **binding site for tensilin**. However further understanding of the tissue is impeded by the discrepancy between molecular and physiological data available. Mechanical effectors, such as softenin or NSF, could already be known in public database under a different name, but the lack of precise information makes their identification extremely speculative.

Understanding the ins and outs of the MCT would prove crucial for the fabrication of tunable bio-scaffold with controlled properties. Such materials are already extensively looked after in medical domains for tissue regeneration, drug delivery, xenograft, and cell development.

References: **Tamori, M., Yamada, A., Nishida, N., Motobayashi, Y., Oiwa, K., & Motokawa, T.** (2006). Tensilin-like stiffening protein from *Holothuria leucospilota* does not induce the stiffest state of catch connective tissue. *Journal of Experimental Biology*, *209*(9), 1594-1602. https://doi.org/10.1242/jeb.02178. **Takehana, Y., Yamada, A., Tamori, M., & Motokawa, T.** (2014). Softenin, a novel protein that softens the connective tissue of sea cucumbers through inhibiting interaction between collagen fibrils. *PloS one*, *9*(1), e85644. **Dolmatov, I. Y., & Nizhnichenko, V. A.** (2023). Extracellular Matrix of Echinoderms. *Marine Drugs*, *21*(7), 417.