

# Ring-Mesh Model of Proteoglycan Glycosaminoglycan Chains in Tendon based on Three-dimensional Reconstruction by Focused Ion Beam Scanning Electron Microscopy<sup>\*[5]</sup>

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Tendons are composed of collagen fibrils and proteoglycan predominantly consisting of decorin. Decorin is located on the d-band of collagen fibrils, and its glycosaminoglycan (GAG) chains have been observed between collagen fibrils with transmission electron microscopy. GAG chains have been proposed to interact with each other or with collagen fibrils, but its three-dimensional organization remains unclear. In this report, we used focused ion beam scanning electron microscopy to examine the three-dimensional organization of the GAG chain in the Achilles tendon of mature rats embedded in epoxy resin after staining with Cupromeronic blue, which specifically stains GAG chains. We used 250 serial back-scattered electron images of longitudinal sections with a 10-nm interval for reconstruction. Three-dimensional images revealed that GAG chains form a ring mesh-like structure with each ring surrounding a collagen fibril at the d-band and fusing with adjacent rings to form the planar network. This ring mesh model of GAG chains suggests that more than two GAG chains may interact with each other around collagen fibrils, which could provide new insights into the roles of GAG chains.

Tendon is primarily composed of collagen fibrils and the non-collagen matrix (1). Canty *et al.* (2) suggested that plasma membrane protrusions called fibripositors contribute to the parallel arrangement of collagen fibrils in tendon, especially in the embryonic stage. Decorin and biglycan belong to the small leucine-rich proteoglycan family and consist of a core protein containing leucine repeats with a covalently attached chondroitin/dermatan sulfate glycosaminoglycan (GAG)<sup>2</sup> chain. The majority (>90%) of the tendon proteoglycan is decorin, while tendon also contains small amounts of biglycan (3). The

decorin core protein binds to the surface of collagen fibrils by non-covalent binding and the attached decorin GAG chain extends from the core protein to associate closely with another decorin GAG chain on an adjacent fibril surface (4–6).

Some studies have suggested that the GAG chain forms bridges between collagen fibrils to guarantee mechanical integrity (4, 7–9). In contrast, other studies have suggested that the GAG chain is irrelevant to mechanical integrity (3, 10–12), because results of mechanical tests using decorin-knock-out mice vary depending on the type and region of the tendon (13). Some researchers also reported that decorin might restore the alignment of the collagen fibrils deformed under load, rather than contribute to the transmission of forces (14, 15). However, precise roles of decorin are not well understood, partly because its three-dimensional structure is unresolved.

The structure of decorin GAG chains among collagen fibrils was observed by ultrathin sections of a sample stained with Cupromeronic blue and the critical electrolyte concentration technique, which was used to reveal the sulfated GAGs under a transmission electron microscope (TEM) (5, 16). Scott *et al.* (17) proposed the “shape module model” in which the decorin GAG chain was located perpendicular to the collagen fibril surface and constituted antiparallel duplexes to form inter-fibrillar bridges between adjacent collagen fibrils. Nomura *et al.* (18) proposed the “binding model” of decorin GAG chains in which a GAG chain adheres to the surface of a collagen fibril. On the other hand, radial arrangement of decorin GAG chains between collagen fibrils was suggested in tendon (11, 14). Thus, the three-dimensional structure of GAG chains is still unclear.

In this study, we used a focused ion beam scanning electron microscope (FIB-SEM) to observe the three-dimensional structure of proteoglycan GAG chains in the Achilles tendon of rats. Observation of the surface of resin embedded biological samples using backscattered electron imaging of SEM provides an image that is similar to TEM results (19, 20). Recently, as an alternative for obtaining TEM serial sectioning images, two serial sectioning techniques, using a microtome or focused ion beam (FIB) integrated inside a SEM, known as serial block-face SEM (SBF-SEM) (20) or FIB-SEM (21), respectively, have been developed. These techniques allow reconstruction of a three-

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[5] This article contains supplemental movie and data.

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<sup>2</sup> The abbreviations used are: GAG, glycosaminoglycan; FIB-SEM, focused ion beam scanning electron microscope; TEM, transmission electron microscope.

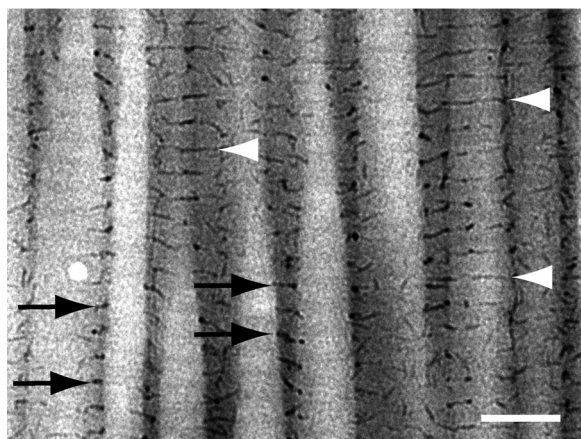


FIGURE 1. **Conventional two-dimensional TEM images of a longitudinal section of collagen fibrils and GAG chains in the rat Achilles tendon.** The GAG chains were stained with Cupromeronic blue, but d-bands of collagen fibrils were not stained. GAG chains were arranged orthogonally to the surface of collagen fibrils (*arrowheads*) and seemed to connect the surfaces of collagen fibrils (*arrows*). Bar, 200 nm.

dimensional image using software from the hundreds of SEM images obtained by repeated sectioning and observation, and have proved to be successful for biological studies, such as following axons and synaptic connections (21), and clarifying mitochondrial fusion and fission (22).

In a study on tendon, SBF-SEM revealed new extensive fine structural information of tendon quantifying tenocyte shape and cell-cell interactions as well as estimating the number and organization of individual collagen fibrils (23). We employed the FIB-SEM technique herein because it is more suitable than SBF-SEM for thin milling to observe fine GAG chains. The GAG chains were found to form a ring mesh-like, planar network structure at the d-band of collagen fibrils.

## Results

**Two-dimensional Image of GAG Chains**—The d-band structure of collagen fibrils was not identified in TEM images because samples were not subjected to electron staining in this study. However, GAG chains were observed in the region presumed to be the d-band of the collagen fibrils separated by an interval of approximately 67 nm (Fig. 1). Most GAG chains were arranged orthogonally between adjacent collagen fibrils. The block-face images from FIB-SEM were identical to TEM images of GAG chains and collagen fibrils. However, the d-band of the collagen fibrils could be observed in block-face images with the back-scattered electron detector. A series of block-face images showed that the GAG chains on the d-band were arranged to surround collagen fibrils (Fig. 2, [supplemental Movie S1](#)).

**Three-dimensional Reconstruction of GAG Chains**—Three-dimensional images of the GAG chains were reconstructed from 250 serial block-face images obtained by FIB-SEM. The reconstructed image showed that the GAG chains formed a planar network and surrounded collagen fibrils ([supplemental Movie S2](#)). This planar network developed at every d-band region. The GAG chains were not distributed like a bridge/chuck-form that connects adjacent collagen fibrils, but they formed a ring mesh-like structure among collagen fibrils (Fig.

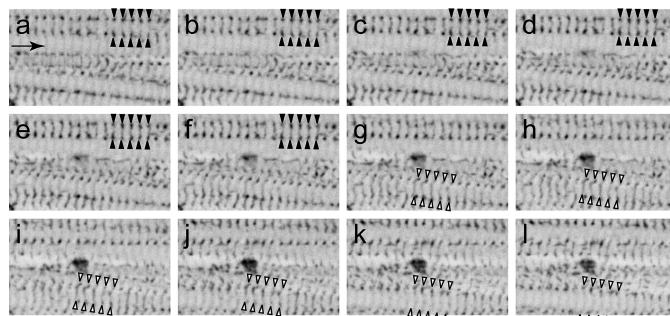


FIGURE 2. **Serial block-face images taken using FIB-SEM.** Serial block-face images were taken with 10-nm intervals (*a-l*). The GAG chains appeared to attach at the d-band of a collagen fibril (*arrow* in *a*). These serial images were used to reconstruct the three-dimensional structure of the GAG chains. *Filled arrowheads* from *a* to *f* show that the GAG chains become longer and finally both ends of the GAG chains are connected to each other. *Open arrowheads* from *g* to *l* show the reverse of *a* to *f*.

3). This network had overlapping thick areas that appeared green-red, probably consisting of multiple GAG chains that were presumed to be the binding site of adjacent collagen fibrils.

## Discussion

This is the first report of the three-dimensional structure of GAG chains reconstructed from FIB-SEM images. The GAG chains were found to form a ring mesh-like structure (Fig. 4*a*) and surrounded collagen fibrils at the d-band of collagen fibrils in the tendon (Fig. 4*b*).

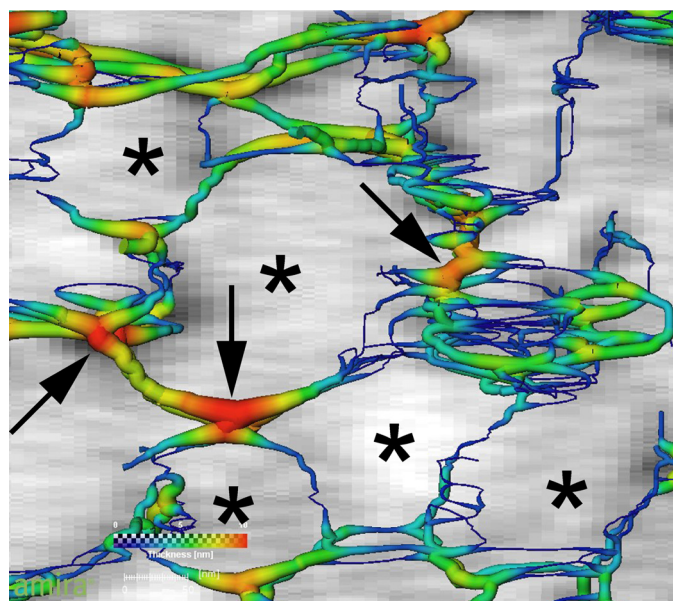
The majority (>90%) of tendon proteoglycan has been reported to be decorin, whereas tendon also contains small amounts of another chondroitin/dermatan sulfate proteoglycan, biglycan (3). Although proteoglycans with keratan sulfate such as fibromodulin and lumican are also expressed in tendon, their distribution differs from that of decorin/biglycan (3, 24, 31), and the amount of keratan sulfate proteoglycans is very small when assessed by enzymatic GAG depletion using chondroitinase ABC (3). Thus, the majority of GAG chains observed in this study can be considered to be decorin GAG chains; although, small amounts of biglycan GAG chains may also be involved.

In a longitudinal thin section of Cupromeronic blue-stained collagen fibrils, GAG chains will give different images according to a particular model. In the “shape module model” (3, 11, 14, 16, 17) in which two GAG chains of decorin form the antiparallel duplex, GAG chains will be observed between collagen fibrils (Fig. 5*a*). In the “binding model” (18, 25) where GAG chains adhere to the surface of collagen fibrils, GAG chains will appear on both sides or on the center of a collagen fibril (Fig. 5*b*). If decorin GAG chains are arranged radially between adjacent collagen fibrils, GAG chains will be observed between collagen fibrils in some longitudinal sections (Fig. 5*c*), but not in others (Fig. 5*d*) depending on the orientation of the tissue section.

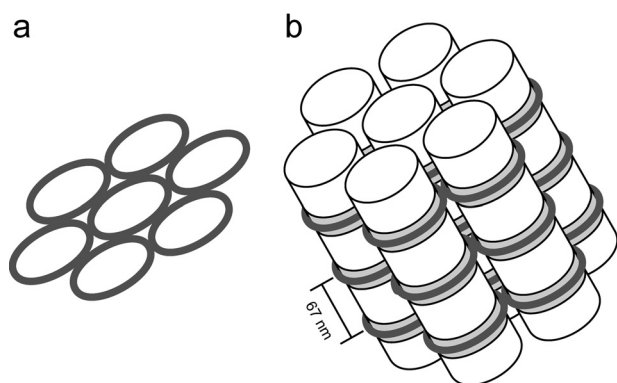
In contrast, in the ring mesh-like model, GAG chains will be observed between or on collagen fibrils in every section, as shown in Fig. 1 (Fig. 5, *e* and *f*). Thus, the “ring mesh model” is consistent with previously observed two-dimensional TEM images of decorin GAG chains (1, 17) and provides an explana-



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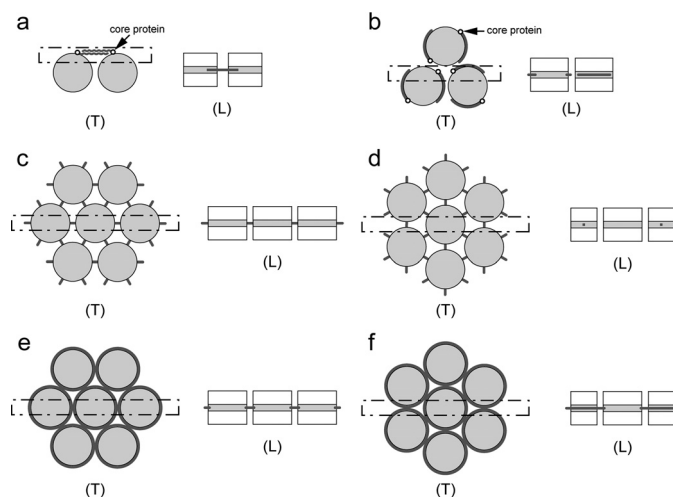
**FIGURE 3. Three-dimensional structure of GAG chains.** Thin layer of animation in supplemental data 2 is shown. GAG chains are depicted in color; blue lines indicate thin GAG chains, while green-red lines (arrows) indicate thicker ones and are possibly binding sites between adjacent ring-formed GAG chains. Unstained collagen fibrils appear in gray in this image and are indicated with asterisks.



**FIGURE 4. Schematic illustration of the ring-mesh model of GAG chains.** *a*, image of the ring mesh-like GAG chains. *b*, three-dimensional scheme of collagen fibrils and GAG chains. Ring mesh-like planar GAG chains (dark gray) are located at the d-band (light gray) of collagen fibrils with an interval of 67 nm.

tion for the inconsistencies when applying the previously hypothesized models described above, or in other words, the previously proposed models of decorin GAG chains (16–18) could be combined to account for three-dimensional structure of the GAG chains in the ring mesh model. The results presented also suggest that more than two GAG chains may bind together because a thick binding area was observed where collagen fibrils are in close contact. How multiple GAG chains interact represents a new study that will be addressed.

In the dermis of the skin, decorin GAG chains were suggested to surround collagen fibrils (18, 25), though the ring mesh-like structure has not been explicitly proposed. In contrast, in the tendon, decorin GAG chains are assumed to have a radial arrangement between collagen fibrils (11, 14), as illustrated in Fig. 5, *c* and *d*. However, the present study indicates that decorin GAG chains in the tendon are not radially arranged but surround collagen fibrils. This discrepancy about



**FIGURE 5. Comparison of TEM images of collagen fibrils and GAG chains in four different models.** “Shape module model” by Scott *et al.* (17) and “Binding model” by Nomura (18) are shown in *a* and *b*, respectively. *T* represents a transverse image, and *L* represents a longitudinal image of a thin section shown by a rectangle in *T*. The collagen fibril is indicated with a large circle and a gray line represents a GAG chain. A core protein of decorin is depicted with a small circle in *T* of *a* and *b*. The diameter of a collagen fibril and thickness of an ultrathin section was set to 200 and 70 nm, respectively. In the “radial arrangement model” shown in *c* and *d*, GAG chains can be observed as *L* of *c* or *d*, depending on the orientation of a thin section. In contrast, in the “ring-mesh model” (*e* and *f*), GAG chains are observed as shown in *L* of *e* or *f*; *L* of *e* is consistent with arrows in Fig. 1, filled arrowheads in Fig. 2*a* and open arrowheads in Fig. 2*l*, whereas *L* of *f* is consistent with arrowheads in Fig. 1, filled arrowheads in Fig. 2*f* and open arrowheads in Fig. 2*g*.

the structure of decorin GAG chains between the dermis and the tendon may be attributable to the thickness of collagen fibrils. The general thickness of ultrathin sections for TEM observation is ~70 nm. The diameter of collagen fibrils in the dermis is approximately 60 nm (25), whereas the tendon contains a large number of collagen fibrils > 100 nm in diameter, which form regular waves called crimps (23, 26, 27). Thus, it is possible that decorin GAG chains in the tendon were not always observed to be straight but were assumed to be helical and wavy, making it difficult to observe GAG chains surrounding collagen fibrils.

Previous studies suggested that decorin is involved in lateral binding of collagen fibrils. Zhang *et al.* (24) reported that the collagen fibrils in the decorin-deficient flexor digitorum longus tendon are comparable to the wild-type control during the early developing stages between days 1 and 10 after birth; however, irregularly contoured fibrils were observed at day 90. This study also reported that biglycan expression increased substantially in decorin-deficient tendons for potential functional compensation. These data indicate that the absence of decorin is associated with dysfunctional regulation of fibril growth resulting in abnormal lateral fusion of collagen fibrils (24). The aberrant collagen fibrils due to uncontrolled lateral fusion were also observed for the tail tendon in decorin-deficient mice (28). An *In vitro* study suggested that decorin GAG chains without the core protein may simply bind to the collagen fibril surface and regulate ties between two or more collagen fibrils (29). Thus, lateral binding of collagen fibrils is a key function of decorin GAG chains. The ring mesh model of GAG chains suggests that lateral binding is stronger than other models because multiple

GAG chains are involved in binding between collagen fibrils in every direction.

It was suggested that the majority of collagen fibrils in mature tendons are short and discontinuous, and that GAG chains transmit tensile strength through a matrix of these fibrils (4). However, other study suggested that most collagen fibrils in the tendon are long and that tensile strength is transferred directly through the fibrils (12). With regard to a role of GAG chains in mechanical properties of tendon, Provenzano and Vanderby (12) suggested that GAG chains in the tendon aid structural integrity by helping to guide collagen fibril maturation, maintaining hydration, and facilitating sliding between adjacent and intertwined fibrils during loading. Fessel and Snedeker (11) enzymatically removed 80% of GAG chains from the tendon and found that its mechanical properties did not change compared with intact specimens. They concluded that the GAG content did not seem to be necessary for elastic tensile mechanics, and tendon GAG chains seem not to act as mechanical crosslinks but rather act to promote collagen fibril sliding under tension (30). The ring mesh model of GAG chains is compatible with these ideas if interactions between GAG chains are not rigid and provide a flexible structure between collagen fibrils.

Recent studies (11, 14) using computational methods suggested that GAG chains in the tendon do not mediate dynamic elastic behavior or regulate the dynamic viscoelastic properties. However, these schematic models of GAG chains assumed the radial arrangement of GAG chains around a collagen fibril. Because we found that the decorin GAG chain makes the ring mesh-like planar structure at every d-band of collagen fibrils, further computational analysis of the ring mesh model of GAG chains may reveal their novel functions.

The present study clarified that GAG chains form a ring mesh-like planar structure in the normal adult tendon. However, organization of collagen fibrils and decorin changes under different physiological conditions of tissue. Further studies on the decorin GAG chain in normal and diseased tissues using FIB-SEM may reveal novel aspects of the dynamic functions of the decorin GAG chain.

## Experimental Procedures

**Specimen Preparation**—The left or right Achilles tendon of four male Sprague-Dawley rats (about 300 g, 12 w; Hokudo Co. Ltd., Sapporo, Japan) were used. All experiments were performed in accordance with the Ethical Guideline for Sapporo Medical University (No. 192, Jan. 17, 2008). The rats were euthanized by overdose of pentobarbital sodium (120 mg/kg, i.p.; Somnopentyl, Kyoritsu Seiyaku, Japan). Tissue samples were obtained from the central one-third of the middle of the Achilles tendon. These samples were sliced into small cubes of  $\sim 1 \text{ mm}^3$  under a dissecting microscope, and fixed and stained with 0.05% Cupromeronic blue (*w/v*) (Seikagaku Corp., Tokyo, Japan) in 0.025 M sodium acetate buffer (pH 5.8) containing 3% glutaraldehyde (*v/v*; TAAB, Berks, UK) and 0.1 M  $\text{MgCl}_2$  for 5 days at 4 °C. Residual stain was removed by rinsing three times in 0.025 M sodium acetate buffer containing 0.1 M  $\text{MgCl}_2$ , and the samples were further stained with 0.034 M  $\text{Na}_2\text{WO}_4$  for 1 h at room temperature. After washing twice in 50% ethanol containing 0.034 M  $\text{Na}_2\text{WO}_4$ , samples were dehydrated through a

graded series of ethanol and transferred to QY-1 (Nisshin EM, Tokyo, Japan) according to standard procedures. Samples were embedded in epoxy resin (Quetol 812, Nisshin EM) and sliced longitudinally. For TEM analysis, ultrathin sections were prepared using an ultramicrotome (Super nova; Reichert-Jung, Vienna, Austria) and mounted on copper grids. The remaining resin block was placed on a holder and used for FIB-SEM observation.

**Transmission Electron Microscopy**—Ultrathin sections were examined under a TEM (JEM 1400; JEOL, Tokyo, Japan) at an acceleration voltage of 80 kV without electron staining. We observed longitudinal sections of collagen fibrils because GAG chains on the d-band can be observed clearly in this orientation.

**Focused Ion Beam Scanning Electron Microscopy**—The observation position of the sample block was identified under FIB-SEM (Helios Nanolab 650, FEI, Eindhoven, Netherlands) at a high voltage of 20 kV. A new surface for serial block-face imaging was generated by FIB-milling at an 83 pA beam current, where gallium ions were accelerated at a voltage of 30 kV. Serial block-face images were obtained with a back-scattered electron detector at an acceleration voltage of 2 kV and working distance of 4 mm; 250 block-face images were obtained (field of view:  $5.08 \times 5.08 \mu\text{m}$ , 10-nm interval). These images were transferred to the 8-bit gray scale tiff image ( $3072 \times 2048$  pixels) for a 3.0  $\mu\text{s}$  dwell time. The voxel size was 1.65 nm/pixel wide, 3.14 nm/pixel high and a depth of 10 nm/pixel. The contrast of the images was inversed. Three-dimensional reconstruction and segmentation of GAG chains were performed by 3D imaging software (AMIRA 5.6, Maxnet Co. Ltd., Tokyo, Japan). The Amira software was used to perform three-dimensional reconstruction from two-dimensional FIB-SEM data sets, which allows reconstructed image evaluation from various angles.

**Author Contributions**—T. W. and K. K. conducted the focused ion beam scanning electron microscopy, data analyses, and interpretation. Y. K. conducted the critical revision of the manuscript for important intellectual content and suggested the model of GAG chains. T. W. and D. S. wrote the manuscript and D. S. provided tissue samples. Y. I. and K. T. provided technical assistance and contributed to the preparation of the figures. K. H. helped with transmission electron microscope analysis. All authors reviewed the results and approved the final version of the manuscript.

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