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Anatomy

Ultrastructural study of the three-dimensional tenocyte network in newly hatched chick Achilles tendons using serial block face-scanning electron microscopy

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ABSTRACT. The lateral cytoplasmic processes of tenocytes extend to form three-dimensional network surrounding collagen fibers. It is unknown whether connections between two cytoplasmic processes involve overlapping of the processes or merely surface contact. In this study, the two-dimensional and three-dimensional structure of tenocytes in the Achilles tendons of the newly hatched chicks were studied using transmission electron microscopy and serial block face-scanning electron microscopy. Observation of the two-dimensional structures revealed various forms of cellular connections, including connections between the cytoplasmic processes of adjacent tenocytes and between the cytoplasmic process of tenocytes and fibroblasts. Three-dimensional observation showed spike-like cytoplasmic processes extending from one tenocyte that interlocked with cytoplasmic processes from other tenocytes. Cytoplasmic processes from each tenocyte within the chick tendons interlocked to ensure a tight cell-to-cell connection around growing collagen fibers. A cellular network formed by these cytoplasmic processes surrounds each collagen fiber. Cell-cell junctions, which were suggested to be gap junctions, observed at sites of cytoplasmic process overlap most likely represent the major route for communication between tenocytes associated with fibroblasts, enabling vital signals important for maintaining the cell and tendon integrity to be transmitted.

KEY WORDS: Achilles tendon, chick, serial block face-scanning electron microscope, tenocyte, three-dimensional

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Tendons are strong cords composed of dense fibrous connective tissue with a complex hierarchical structure in which bundles of collagen fibers are arranged in parallel. Each collagen fiber comprises multiple collagen fibrils and is enmeshed in a network of cytoplasmic processes extending from the surrounding tenocytes. Groups of collagen fibers are surrounded by the endotendineum, a sheath of connective tissue produced by fibroblasts (Fig. 1) [11]. Tenocytes are also called "wing cells" because of their extending cytoplasmic processes, which create cellular networks that stabilize the collagenous products they secrete and facilitate intercellular communication via gap and adherens junctions [5, 6, 9]. Tenocyte processes become thinner as the thickness of the corresponding collagen fiber or collagen fiber bundle increases [11].

Two-dimensional morphological analysis of tenocytes has suggested that their cytoplasmic processes contact those of adjacent cells at points where they overlap [4, 11]. However, recent improvements in serial block face-scanning electron microscope (SBF-SEM) technology, which can acquire continuous images while cutting a tissue sample at the thickness of 100 nm or less, make it possible to carry out three-dimensional analysis at the ultrastructural level to confirm this hypothesis [3]. A study of mouse tail tendons using this technique showed that the cytoplasmic processes of tenocytes arranged in longitudinal arrays along collagen

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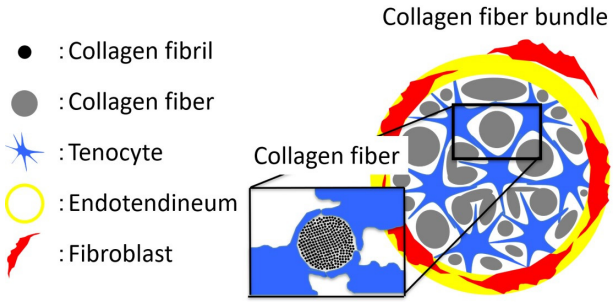

Fig. 1. Diagram showing the organization of a collagen fiber bundle. Tenocyte cytoplasmic processes extend around a group of collagen fibrils forming a collagen fiber. A collagen fiber bundle comprises a group of collagen fibers surrounded by the endotendineum, which is a sheath of connective tissue embedded with fibroblasts.

fibrils form a sort of curtain with a straight edge that overlaps the edge of the corresponding 'curtain' formed by the cytoplasmic processes of adjacent tenocytes [4]. Each collagen fiber is demarcated by a network of these cytoplasmic processes. As growth tendon development, the interval between the tenocytes is expanded. Still, the adjacent tendon cells maintain the connection through the cell-cell junction of the cytoplasmic processes and extend the length of the processes in mouse tail tendons [4]. In addition, a study of metatarsal tendons in chick embryos presented a well-defined image of the overlapping edges of tenocyte cytoplasmic processes arranged in tandem along collagen fibers [7]. However, it is unknown whether tenocyte cytoplasmic processes align and overlap in a similar way in the weight-bearing Achilles tendon in chicks after hatching. Therefore, the aim of this study was to elucidate the three-dimensional structure of the tenocyte network associated with both cellular and non-cellular components of collagen fiber bundles in Achilles tendons in newly hatched broiler chicks.

MATERIALS AND METHODS

Experimental animals

The animal experiments performed as part of this study were carried out in strict accordance with the Rakuno Gakuen University Animal Experiment Guideline (approval number VH18A7) established by the Rakuno Gakuen University Animal Experiment Committee of Rakuno Gakuen University, Ebetsu, Hokkaido, Japan. Four broilers (ROSS 308) obtained within 24 hr after hatching from the university farm were euthanized by intraperitoneal injection with 80 mg/kg sodium pentobarbital (Somnopentyl; Kyoritsu Pharmaceutical Co., Tokyo, Japan) and exsanguinated.

Tissue sample preparation

The Achilles tendon just above the calcaneal tuber was dissected out and immediately fixed by immersing in a half-Karnovsky solution (2% paraformaldehyde + 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4) at 4°C overnight. The contrast of the tissues membrane structures was enhanced for electron microscopy by heavy metal block staining, as described previously [12]. Briefly, the fixed tendons were washed with a solution containing 0.1 M cacodylate buffer (pH 7.4) and 2% OsO_4 (TAAB Laboratories Equipment Ltd., Berks, UK) in 0.15% $K_4(CN)_6$ (Nacalai Tesque Inc., Kyoto, Japan) four times (4 min each) at 4°C, soaked in the same solution for 1 hr, washed with distilled water four times (4 min each), immersed in 0.1% thiocarbohydrazide (Sigma Aldrich, Tokyo, Japan) for 20 min at room temperature, washed with distilled water four times (4 min each), immersed in 2% OsO_4 for 30 min at room temperature, washed with distilled water four times (4 min each), immersed in 1% uranyl acetate at 4°C overnight, washed with distilled water four times (4 min each), and immersed in Walton's lead aspartate solution at 60°C for 30 min. Finally, the tissues were dehydrated with an ethanol series, transferred to QY-1, and finally embedded in epoxy resin (Quetol 812; Nissin EM, Tokyo, Japan).

Transmission electron microscopy

The tissue block was sliced into 80-nm-thick sections perpendicular to the long axis of the tendon using an ultramicrotome (JUM-7; JEOL, Tokyo, Japan). The sections were collected on a 100-mesh copper grid, and the two-dimensional structures were observed under a transmission electron microscope (HT-7700; Hitachi High Technology, Tokyo, Japan) at an acceleration voltage of 80 kV. A sample block containing a large number of tenocytes was selected for three-dimensional structural observation.

Serial block face-scanning electron microscopy

The selected block was attached to an aluminum stub using a conductive adhesive (CW-2400; Chemtronics, Kennesaw, GA, USA). Two series of SBF-SEM images (Apreo VS Serial Block Imaging, Thermo Fisher Scientific, Waltham, MA, USA) were obtained: 1,254 serial images were obtained at low magnification (field of view: 122 μm^2 at 20 nm/pixel, 50-nm interval) and 317 serial images were obtained at high magnification (field of view: 20 μm^2 at 5.0 nm/pixel, 50-nm interval).

Three-dimensional imaging

Three-dimensional images were produced from the serial images using image analysis software (Image Pro 3D module version 10.0.4; Nippon Roper, Tokyo, Japan). The low-magnification images were processed using the software's automatic extraction function so that the cell components were shown in white and the collagen fibers in gray/black. Subsequently, a three-dimensional network of the tenocytes constituting the collagen bundle was constructed. The tenocyte membranes were manually selected from the high-magnification images and used to construct a three-dimensional image of the structure of individual tenocytes and the network they formed.

RESULTS

Two-dimensional structural analysis

Cytoplasmic processes extended from tenocytes perpendicular to the longitudinal array of collagen fibers to form a network within the collagen fiber bundle (Fig. 2A). Two-dimensional observation of the tendon showed that each collagen fiber was surrounded by network of cytoplasmic processes from two to three tenocytes. In addition to tenocytes, fibroblasts, which are responsible for producing the endotendineum (a connective tissue sheath that surrounds each collagen bundle), were also observed to have cytoplasmic processes (Fig. 2B). Tenocyte cytoplasmic processes were found to form two different types of connections: 1) overlapping connections with the cytoplasmic processes of adjacent tenocytes, and 2) extensions toward fibroblasts in the endotendineum (Fig. 2B). Electron density at overlapping processes was higher than it was at non-overlapping processes (Fig. 2C, 2D).

Three-dimensional structural analysis

Low-magnification images: Observation of the serial images acquired at low magnification showed that tenocytes within the collagen fiber bundle generally connected with adjacent tenocytes via their cytoplasmic processes. This could indicate that these tenocytes were building of three-dimensional network to surround each collagen fiber in the bundle (Fig. 3A–C). Fibroblasts were found embedded in the endotendineum sheathing the collagen bundle. Some tenocyte cytoplasmic processes also extended into the endotendineum to contact fibroblast cell membranes (Fig. 3D).

High-magnification images: A three-dimensional image of a group of tenocytes was constructed from highly magnified cross-sectional images obtained at different points along the sectioned tendon. Each tenocyte was assigned a different color so that the connections between the cytoplasmic processes of each tenocyte and those of nearby tenocytes could be clearly visualized (Fig.

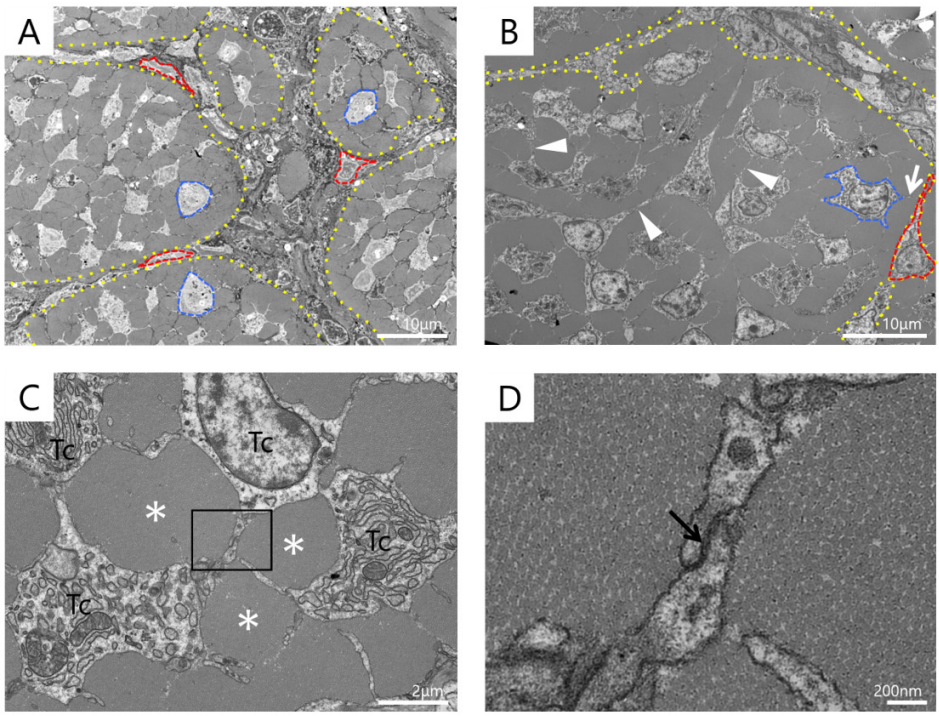

Fig. 2. Transmission electron microscope images of the tendon. A: Tenocytes (blue) are present within each collagen fiber bundle, which is surrounded by the endotendineum (yellow dotted line), but fibroblasts (red) remain on the outside of the bundle. B: The cytoplasmic processes of most tenocytes stretch to form networks surrounding collagen fibers (white arrowheads). Some tenocyte cytoplasmic processes extend to connect with fibroblasts (white arrow). C: Most collagen fibers (*) are surrounded by a network of cytoplasmic processes from three tenocytes (Tc). D: Magnified image of the box in C. Electron density is intense at sites of overlapping processes (black arrow).

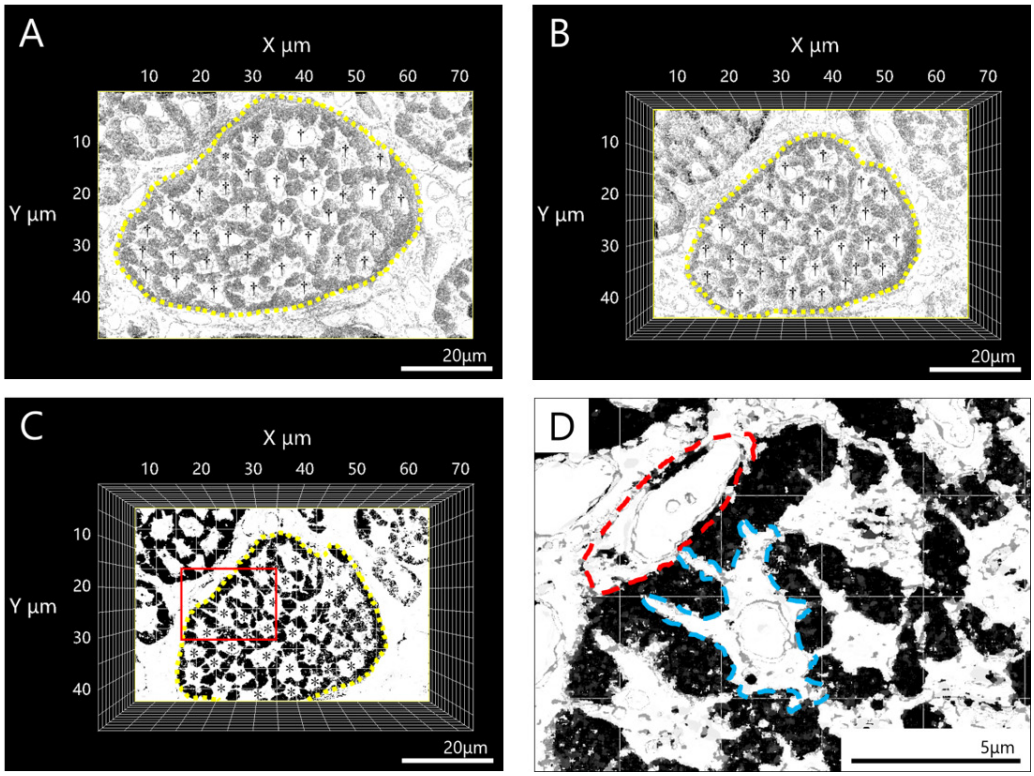

Fig. 3. Topographical images of collagen fiber bundles at the beginning (A), middle (B) and end (C) of the cut tendon. A group of collagen fibers is gathered together to form a collagen fiber bundle, which is enclosed within the endotendineum (yellow dotted line). The cytoplasmic processes of the tenocytes (†) arrayed longitudinally along the collagen fibers extend to form a three-dimensional network around the collagen fibers. Fibroblasts located outside the collagen fiber bundle are also arrayed along the length of the collagen fiber bundle. D: A connection between the cytoplasmic process of tenocyte (blue dotted line) and a fibroblast (red dotted line) can be seen in the magnified box in C.

4A). The three-dimensional image showed that a network of these connecting processes formed around each collagen fiber. When the image was observed obliquely, a tandem array of tenocytes with non-continuous and spike-like cytoplasmic processes was found to run along the length of each collagen fiber (Fig. 4B). However, these spike-like processes were not observed at the ends of the cells (Fig. 4C). Close observation revealed gaps between the ends of two adjacent tenocytes within the tandem array (Fig. 4D). Each collagen fiber was surrounded by a network composed of cytoplasmic processes from multiple tenocytes (Fig. 4A, 4B). The spike-like cytoplasmic processes of some tenocytes appeared to simply interdigitate with the spike-like processes of adjacent tenocytes (Fig. 4E). However, most of the cytoplasmic processes that we observed were not so carefully arranged; indeed, the majority varied widely in terms of shape, size, length, and direction. Primary processes, which extended directly from the cell body, usually gave rise to one or two secondary processes which extended to fill the recesses left by the cytoplasmic processes of the adjacent tenocyte. As the size of the recesses varied, the tips of the cytoplasmic processes that extended into these recesses appeared to enlarge to fit the available space (Fig. 4F). Therefore, tenocyte cytoplasmic processes appear to form a three-dimensional interlocking network.

DISCUSSION

This study used SBF-SEM to analyze the three-dimensional morphology of tenocytes and their connections with collagenous components, neighboring tenocytes, and fibroblasts within collagen fiber bundles in Achilles tendons from newly hatched chick. Analysis of the low-magnification images revealed a three-dimensional network of tenocytes within the collagen fiber bundle, providing a more detailed understanding of these structures than previous reports based on conventional scanning electron microscope (SEM) which were only able to observe the surface layer of the tissues and were thus unable to detect detailed cellular connections [5, 11]. Using SBF-SEM enabled us to clearly visualize process-to-process and process-to-cell connections. Analyzing serial images taken of sections along the length of the tendons allowed us to construct three-dimensional images of both the cellular and non-cellular components of the tendon. Though three-dimensional ultrastructural studies have been used to embryonic, newborn and adult mouse tail tendons, and chick metatarsal tendons [4, 7], these studies only investigated tenocytes within the collagen fiber bundle. To the best our knowledge, this is the first study that has used SBF-SEM to analyze not only inter-tenocyte connections, but also the connections between tenocytes and fibroblasts that form the complete cellular network within collagen fiber bundles.

Our analysis showed that tenocytes formed cellular networks surrounding each collagen fiber by extending their cytoplasmic

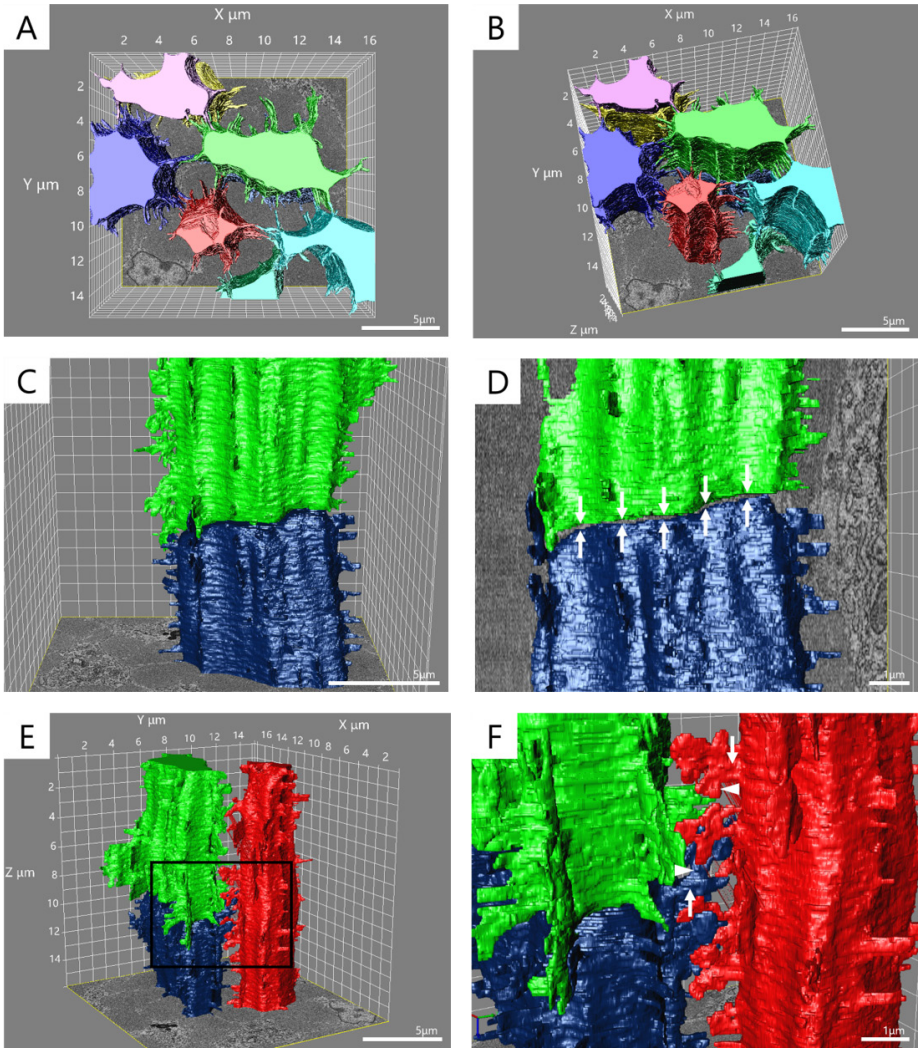

Fig. 4. Three-dimensional reconstruction of tenocytes delineated with different colors. Networks formed by the cytoplasmic processes of all tenocytes surrounding each collagen fiber were clearly observed on a horizontal plane (A). Tenocytes with lateral non-continuous spike-like cytoplasmic processes were arranged in tandem along the length of collagen fibers (B, C). The vertical slice section of C shows gaps between the ends of tenocytes (arrows, D). Differently sized and shaped processes extended to fill the recesses left by the processes extending from the adjacent tenocyte, to form an interlocking structure (E). Primary processes (arrows) and secondary processes (arrowheads) can be seen in the magnified box in E (F). However, these processes were not observed at the terminal ends of these cells, where instead intercellular gaps were clearly seen (D).

processes to join with those of adjacent tenocytes. This was previously reported for rat Achilles tendons using conventional SEM [5]. It is generally accepted that tenocytes produce extracellular matrix and regulate collagen assembly. Cellular communication is required to accomplish these functions. The intense electron density observed where the cytoplasmic processes of two adjacent tenocytes overlapped suggested the presence of a gap junction, which would represent a major communication route between tenocytes in chick Achilles tendons [9]. Gap junctions would allow tenocytes to exchange vital information regarding the fate of collagen fibers [2]. Tenocyte cytoplasmic process elongation may occur following stimulation by transforming growth factor beta 1, which may be released from muscles in response to mechanical forces, as has been described in zebrafish [10]. Therefore, we propose that gap junctions facilitate communication between tenocytes in chick Achilles tendons from muscle junction to bone junction and enable them to form networks surrounding individual collagen fiber bundles.

In addition to various forms of connections between tenocytes, we also observed connections between tenocytes and the surrounding fibroblasts. Tenocytes locating at the periphery of each collagen fiber bundle either extended their cytoplasmic processes into or penetrated the endotendineum to establish direct contact with the fibroblasts embedded within this connective tissue. A SEM study of porcine superficial digital flexor tendons reported that tenocyte cytoplasmic processes simply attach to the endotendineum, but did not report any direct connection between these tenocytes and fibroblasts within the endotendineum [11]. We suspect that direct connections between tenocytes and fibroblasts do exist in this porcine tendon, but that the conventional SEM approach used in the study was not powerful enough to detect these connections. Using SBF-SEM to reconstruct the three-

dimensional tenocyte and fibroblast network could help to elucidate these connections in porcine and the other animal tendons.

Although gap junctions provide a readily available means of communication between tenocytes, the mechanism of communication between tenocytes and fibroblasts was not investigated by the present study. Fibroblasts develop and differentiate in response to transforming growth factor beta 1 [15]. The connections between the tenocytes and fibroblasts observed in this study, whether they are gap junctions or not, may serve as a mode of communication to transmit crucial message from the tenocytes to fibroblasts, and vice versa. As fibroblasts produce fibrous tissue and undergo phenotypic transformation in response to the signal transduction and mechanostimulation [1, 13–15], communication between fibroblasts and tenocytes in the collagen fiber bundle may help maintain the endotendineum and the integrity of the tendinous components of the collagen fiber bundle.

Analysis of our high-magnification images identified tandem arrays of tenocytes arranged along the length of the collagen fibers. The lateral appearance of tenocytes with cytoplasmic processes is dramatically different from the proximal ends of tenocytes arrayed in tandem: at the ends of these cells, cytoplasmic processes are absent, and cell-to-cell connections are two-dimensional in nature. This suggests that tenocytes on a flat surface form a tandem arrangement in which the cytoplasmic processes interdigitate smoothly without vertical interruption (like the teeth of a zipper). At birth, the cytoplasmic processes of tenocytes within the Achilles tendons of the newly hatched chick extend horizontally to interdigitate with those of the adjacent tenocytes. Further three-dimensional elongation of these processes to form an interlocking complex occurs as the collagen fibers thicken, thereby firmly attaching the widening tenocytes to the enlarging collagen fibers [11]. The size and direction of the interlocking processes are dependent upon the recesses formed by their interlocking partners. These interlocking complexes typically appear as spike-like interdigitation. Overlap of cytoplasmic processes between adjacent tenocytes is maintained by the proximal spike-like projections of the two cells. These spike-like interdigitating tenocyte processes in chick Achilles tendons differ from the overlapping processes observed in the mouse tail tendon. In mouse tendons these cytoplasmic processes are not zipper-like, but rather form vertical extensions like curtains or wings (which is why tenocytes are sometimes referred to as wing cells), and adjacent tenocytes connect at the edges of these curtain-like cytoplasmic processes [4]. We think that there are two possible reasons for this apparent difference: either there truly is a difference in tenocyte morphology between mouse tail tendons and chick Achilles tendons, or the observations were made under different condition and at different resolutions.

The three-dimensional images of mouse tail tendon tenocytes reported previously were constructed from continuous images with a pixel size of 10 nm of 41 μm × 41 μm regions of 100-nm-thick sections using SBF-SEM. However, the three-dimensional images presented in the current study of chick Achilles tendons were constructed using continuous images with a pixel size of 5 nm of 20 μm × 20 μm regions of 50-nm-thick sections. This protocol resulted in higher-resolution images; thus, the structures within the chick tendon could be studied in greater detail. It is possible that the spike-like morphology was composed of non-continuous cytoplasmic processes along the parallel collagen fiber array.

This hypothesis could be investigated in future studies by observing the morphology of tenocytes and their cytoplasmic processes during maturation. Besides, present results would provide critical information when analyzing the tenocyte network in the matured Achilles tendon in the future studies. Moreover, analysis of the three-dimensional tenocyte network and the expression of cell adhesion-related proteins (e.g., scleraxis or connexin-43) over time could provide important information for developing regenerative medicine approaches to tendon repair [8, 15].

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