Morphological variety of capillary ends invading the epiphyseal plate in rat femora using scanning electron microscopy with osmium maceration

Tsuneyuki Yamamoto[a], Shigeru Takahashi[a], Tomoka Hasegawa[b], Hiromi Hongo[b], Norio Amizuka[b]

[a] Department of Oral Functional Anatomy, Graduate School of Dental Medicine, Hokkaido University, Kita13 Nishi7, Kita-ku, Sapporo 060-8586, Japan

[b] Department of Developmental Biology of Hard Tissue, Graduate School of Dental Medicine, Hokkaido University, Kita13 Nishi7, Kita-ku, Sapporo 060-8586, Japan

Corresponding author

T. Yamamoto

Department of Oral Functional Anatomy, Graduate School of Dental Medicine, Hokkaido University, Kita13 Nishi7, Kita-ku, Sapporo 060-8586, Japan

yamatsu@den.hokudai.ac.jp

ABSTRACT

Objectives: The function of capillary ends at the epiphyseal plate has been actively investigated. However, their morphology is still poorly understood. This study was designed to examine the capillary ends invading the epiphyseal plate three-dimensionally by scanning electron microscopy and discuss the relationship between their morphology and function.

Methods: Distal halves of the femora of eight-week-old male Wistar rats were used. The specimens were divided into two groups for transmission and scanning electron microscopy. For transmission electron microscopy, sagittal ultrathin sections were routinely prepared after the demineralization of the specimens, and the chondro-osseous junction was examined at the epiphyseal plate. For scanning electron microscopy, the specimens were sagittally freeze-cracked, osmium-macerated, and routinely processed.

Results: Endothelial cells of capillary ends had fine fenestrations, and hence they were distinguishable from perivascular cells (also known as septoclasts). Based on the outline and the presence or absence of pores, the capillary ends were divided into four types: closed dome, closed spire, porous dome, and porous spire. The two dome types generally occupied more than half of a lacuna, whereas the two spire types generally occupied only a small part of a lacuna. The porous types engulfed cellular remnants, indicative of degraded chondrocytes, via their pores. Some of the spire types penetrated the transverse septum.

Conclusions: The morphological variety of capillary ends reflected their functional variety. Observations suggest that the capillary ends change their morphology dynamically in

response to various functions, including the removal of degraded chondrocytes and perforation of transverse septa.

247 words

Keywords: Capillaries, Endothelial cells, Epiphyseal plate, Osmium maceration, Scanning electron microscopy

1. Introduction

During normal growth of long bones, osteoblasts and osteoclasts continuously migrate to the chondro-osseous junction at the epiphyseal plate, also known as the growth plate. Capillary-constituting cells, i.e., endothelial cells and septoclasts (also known as perivascular cells), play a leading role in this migration. Prior to their migration, endothelial cells and septoclasts invade the hypertrophic zone by penetrating the incompletely calcified transverse septum. Capillary invasion is conducted by the vascular endothelial growth factor secreted by hypertrophic chondrocytes. Thereafter, osteoblasts and osteoclasts migrate into opened lacunae through the preformed invading route, located on calcified intercolumnar or longitudinal septa, and form primary trabeculae and then secondary trabeculae [1-4] (Fig.1).

The morphological features and behavior of septoclasts during the preceding invasion have been investigated in detail [1,5-11]. Briefly, septoclasts are mono-nuclear spindle-shaped cells located adjacent to growing capillaries, and hence they are also known as perivascular cells. They contain well-developed organelles: the rough endoplasmic reticulum, the Golgi apparatus, and many lysosomes, including proteinases that digest collagenous matrices, such as cathepsin B and matrix metalloproteinase (MMP)-13. Septoclasts have slender finger-like processes extending to the transverse septum. According to the above findings, septoclasts synthesize cartilage-degrading enzymes and break through the transverse septum, allowing capillaries to invade the hypertrophic zone [4, 6, 8, 9].

The morphological features and behavior of capillary endothelial cells have been investigated. According to Tsuchiya et al. [11], endothelial cells at the chondro-osseous junction show immunoreactivity for MMP-9, which is involved in the degradation of collagens. Similar to septoclasts, endothelial cells have slender cytoplasmic processes extending into the transverse septum. From these findings, Tsuchiya et al. [11] suggested that endothelial cells interact with septoclasts for invasion into the epiphyseal plate. Nevertheless, the behavior of capillary ends during the invasion is still unclear. We postulated that the capillary ends exhibit morphological variety, depending on various functions, e.g., invasion, expansion, and removal of particular structures. This study was designed to observe the capillary ends invading the epiphyseal plate in rat femora and to elucidate whether the endothelial cells exhibit morphological variety. For this purpose, we used scanning electron microscopy with osmium maceration [12] to examine the features of intracellular organelles. In particular, with this method, we were able to observe the capillary morphology three-dimensionally and to identify several kinds of cells, i.e., endothelial cells, chondrocytes, osteoblasts, osteoclasts, and septoclasts.

2. Materials and Methods

2.1 Animals

Eight-week-old male Wistar rats were used. The animals were treated in accordance with the principles of animal care and research use set by Hokkaido University (Approval No. 10-

0081 and 15-0041). The animals were divided into two groups for transmission and scanning electron microscopy.

2.2 Transmission electron microscopy

The animals were anesthetized using an intraperitoneal injection of sodium pentobarbital and perfused with 2.5% glutaraldehyde in 0.06 M cacodylate buffer (pH 7.4). The distal half of the femora was dissected out, immersed in the same fixative overnight, and decalcified in 5% EDTA (pH 7.4) for 3 weeks. Then, the specimens were sagittally cut with a blade into slices of 200–300 μm in thickness, under a stereoscopic microscope. All slices were postfixed using 1% OsO_4 and 1.5% potassium ferrocyanide in 0.06 M cacodylate buffer (pH 7.4) for 1 h. After dehydration in a graded series of acetone solutions, the specimens were embedded in EPON 812. Ultrathin sections were prepared and stained with uranyl acetate and lead citrate for examination using a JEM-1400 transmission electron microscope.

2.3 Scanning electron microscopy

Osmium maceration was conducted following the methods of Tanaka and Mitsushima [12]. After anesthesia, the animals were perfused with a mixture of 0.5% glutaraldehyde and 0.5% paraformaldehyde in 0.06 M cacodylate buffer (pH 7.4). The distal half of the femora was dissected out and immersed in the same fixative overnight. Then, the specimens were treated with 25% and 50% dimethyl sulfoxide and sagittally freeze-cracked on a metal plate in liquid nitrogen. After postfixation with 1% OsO_4 in 0.06 M cacodylate buffer, they were immersed in 0.1% OsO_4 in 0.06 M cacodylate buffer (pH 7.4) for 10–12 days at 20 °C. They

were then treated with 1% tannic acid and 1% OsO_4 for conductive staining [13], dehydrated in a graded series of ethanol solutions, critical-point dried, and coated with platinum-palladium prior to examination using a Hitachi S-4800 scanning electron microscope.

3. Results

3.1 Validity of osmium maceration

Figure 2 shows the chondro-osseous junction between the hypertrophic zone and primary trabeculae. Capillaries, degraded chondrocytes, osteoclasts, osteoblasts were identifiable on the basis of shape, size, and in particular, features of intracellular organelles, which were revealed through osmium maceration.

3.2 Morphological variety of capillary ends

3.2.1 Closed type

The closed type had no or only a few pores in the endothelial cells, and the inner and outer capillary walls were relatively smooth (Fig.3). This type was further divided into two subtypes: closed dome and closed spire. The closed dome type showed a dome- or bulb-like configuration and generally occupied more than half of a lacuna (Fig. 3a, b). In contrast, the closed spire type showed a peaked configuration and generally occupied only a small part of a lacuna (Fig. 3c, d). The two subtypes had structural features in common, i.e., fine fenestrations and slender cytoplasmic processes, some of which penetrated the transverse

septum. The fenestrations assembled into patches (Fig.3b, d) and were accompanied with the diaphragm by transmission electron microscopy (Fig. 3e, f),

3.2.2 Porous type

The porous type had many pores with diameters of 0.1–5.0 μm in the cytoplasm and intercellular spaces of endothelial cells, and it was also divided into two subtypes: porous dome and porous spire (Fig. 4a–c). The morphological differences between the dome and spire types were similar to those between the two closed types. The pores, especially in the dome type, assembled to form a mesh-like structure (Fig. 4a, b). In addition, many cytoplasmic spherules or vesicles were attached to the endothelial cell surface (Fig. 4b). Hence, the capillary walls were more irregular in the porous types than in the closed types. The two porous types had slender cytoplasmic processes and fine fenestrations (Fig. 4a–c). Cellular remnants, indicative of degraded chondrocytes, were engulfed via the pores (Fig. 4d). The engulfment was confirmed under a transmission electron microscope (Fig. 4e). Occasionally, the porous dome type coexisted with the closed dome type (Fig. 5).

3.3 Septoclasts

Septoclasts were found between the endothelial cells and lacuna wall in all types (Fig. 6). They were flattened cells with multi-processes and were distinguished from endothelial cells by the absence of fenestrations. Under a transmission electron microscope, the septoclasts possessed well-developed organelles, such as the rough endoplasmic reticulum,

phagosomes, and lysosomes (Fig. 4e).

4. Discussion

4.1 Morphological variety of capillary ends

This study is the first to report the morphological variety of capillary ends invading the growth plate. The capillary ends were divided into two types, i.e., closed and porous types, and each type was further divided into either the dome or spire type. Consequently, four types, i.e., closed dome, closed spire, porous dome, and porous spire types were distinguished. This morphological variety was postulated to reflect functional variety. Although static micrographs capture only one stage of cell behavior, different functions can still be assigned to particular types.

4.2. Porous dome and porous spire types for removal of chondrocytes

The two porous types, observed under transmission and scanning electron microscopes, may serve to remove degraded chondrocytes through capillaries. Nakamura and Ozawa [6] found that perivascular cells have cytoplasmic projections extending toward degenerated chondrocytes as well as the transverse septum, and hence they suggested that septoclasts absorb degenerated chondrocytes in addition to the uncalcified cartilage. We do not rule out their postulate, but removal of cellular remnants through capillaries may be more effective and efficient. Nevertheless, it is unclear whether the capillary ends become porous only for engulfment of particular structures. The porous types were found to have complicated

structures accompanied with many cytoplasmic vesicles in addition to pores. To date, we do not exclude the possibility that the pores are generated for primary function(s), such as release of particular structure(s) and/or molecule(s), and that engulfment is a secondary function. This postulate is currently under investigation in our laboratory.

Because porous dome and closed dome types were found to coexist, the two types may transform into each other. The closed dome type does not perform the chondrocyte-removing function. Although it is assumed that the closed dome type is in the stable or resting phase, the role of the closed dome type in the development and ossification of the epiphyseal plate remains unclear.

4.3 Closed spire and porous spire types for penetration

It is readily assumed that the capillary ends form a thin and sharp configuration during penetration into the transverse septum, and hence the penetrating function may be assigned to the two spire types. The two spire types, as leading tips, may gradually erode the transverse septum by producing collagen-degrading enzymes, such as MMP-9 [11].

Here, the endothelial tip cells should be discussed. The endothelial tip cells appear at a growing sprout in angiogenesis [14-16]. They are motile and invasive and, furthermore, guide newly generated sprouts. They have extended filopodial processes and are accompanied with abundant, plasma membrane-derived microvesicles. These morphological features indicate that the endothelial tip cells are related to the porous spire type, although it is unclear whether the epithelial tip cells have cytoplasmic pores. Details of

the fine structure, i.e., transmission and/or scanning electron microscopic data of the endothelial tip cells, should be further accumulated to elucidate whether the endothelial tip cells are identical to or different from the porous spire type.

4.4 Septoclasts

Septoclasts were found with all types of capillary ends in this study. They intervened between the capillary ends and lacuna wall. We could not elucidate which of septoclasts and endothelial cells precede or play a central role in the penetration into transverse septa. On the basis of previous immunohistochemical studies [11] and this study, it appears that septoclasts and endothelial cells may cooperate with each other to break through the transverse septum.

5.Conclusion

For the first time, this study revealed the three-dimensional morphological variety of capillary ends invading the epiphyseal plate. The capillary ends were divided into closed dome, closed spire, porous dome, and porous spire types. Findings obtained in this study suggested that the capillary ends changed their morphology dynamically in response to varying functions, including removal of degraded chondrocytes and perforation of transverse septa.

Author contribution

Tsuneyuki Yamamoto: Main researcher involved in all processes of this work, especially osmium maceration and electron microscopic observations.

Shigeru Takahashi, Tomoka Hasegawa, and Hiromi Hongo: Performed sample preparations, including fixation, embedding, and sectioning for transmission electron microscopy.

Norio Amizuka: Participated in the discussion and preparation of the manuscript.

All the above authors have read and agreed to the submission of the manuscript.

Ethical statement

This study followed the principles of animal care and research use set by Hokkaido University (Approval No. 10-0081 and 15-0041).

Conflict of interest

We declare no conflicts of interest.

Acknowledgments

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Figure legends

Fig.1. Schematic diagram depicting the chondro-osseous junction. Prior to cell death hypertrophic chondrocytes (CC) induce calcification (arrows) in the longitudinal septa (LS). Capillaries (CP) and septoclasts (SC) break through the incompletely calcified transverse septa (TS) and invade the hypertrophic zone. Following their invasion, osteoclasts (OC) and osteoblasts (OB) form the primary trabeculae (PT) by matrix absorption and deposition.

Fig. 2. Low magnification of chondro-osseous junction in a rat femur using scanning electron microscopy with osmium maceration. Capillary ends (asterisks), osteoblasts (OB), osteoclasts (OC), and degraded chondrocytes (CC) are seen. Bar = 10 μm.

Fig. 3. Scanning (a–d) and transmission (e, f) electron micrographs of the closed types. **a:** The closed dome type fills a lacuna and has only a few pores. The front side capillary wall has been cut off. Arrow indicates a slender process penetrating the transverse septum. Bar = 2 μm. **b:** Magnification of a square in **a**. Arrows indicate fenestrations. Bar = 0.5 μm. **c:** The closed spire type clinging to a lacuna wall. The front side capillary wall has been partially cut off. The endothelial cell has extended slender processes. Bar = 2 μm. **d:** Magnification of a square in **c**. Arrows indicate fenestrations. Bar = 1 μm. **e:** The closed dome type filling a lacuna. Bar = 2 μm. **f:** Magnification of the capillary wall indicated by arrowhead in **e**. Arrows indicate fenestrations with diaphragm. Bar = 250 nm.

Fig. 4. Scanning (a–d) and transmission (e) electron micrographs of the porous types. **a:** The porous dome type. The front side capillary wall has been cut off. The endothelial cells

have extended slender processes (arrows). Bar = 2 μm. **b:** Magnification of a square in **a**. Many vesicles are attached on the endothelial cells. White arrow and black arrow indicate fenestrations and overlapping cells, respectively. Bar = 1 μm. **c:** The porous spire type penetrating the transverse septum. Arrow indicates slender processes extending from the tip. Bar = 2 μm**. d:** The porous dome type (asterisk) engulfs cellular remnants indicative of degraded chondrocytes (CC). Bar = 3 μm**. e:** Cellular remnants engulfed in the capillary end (asterisk) via the pores. Septoclasts (SC) are in close contact with endothelial cells (EC) and contain well-developed organelles, such as phagosomes, lysosomes, and the rough endoplasmic reticulum. Bar = 5 μm**.**

Fig. 5. Coexistence of closed dome (CD) and porous dome (OD) types. Bar = 2 μm.

Fig. 6. Scanning electron micrographs of septoclasts (SC) and endothelial cells (EC). **a:** A septoclast (SC) intervenes between a lacuna wall and endothelial cells (EC). The front side capillary wall has been cut off. Arrow indicates slender processes of the septoclast. Bar = 2 μm. **b:** Magnification of a square in **a**. The endothelial cell (EC) has fine fenestrations (arrows) and the septoclast (SC) do not. Bar = 1 μm. **c:** A septoclast (SC) and endothelial cells (EC). Bar = 2 μm. **d:** Magnification of a square in **c**. The septoclast contains many vesicular organelles (asterisk) and has no fenestrations. Arrow indicates slender processes sticking to a lacuna wall. Bar = 1 μm.

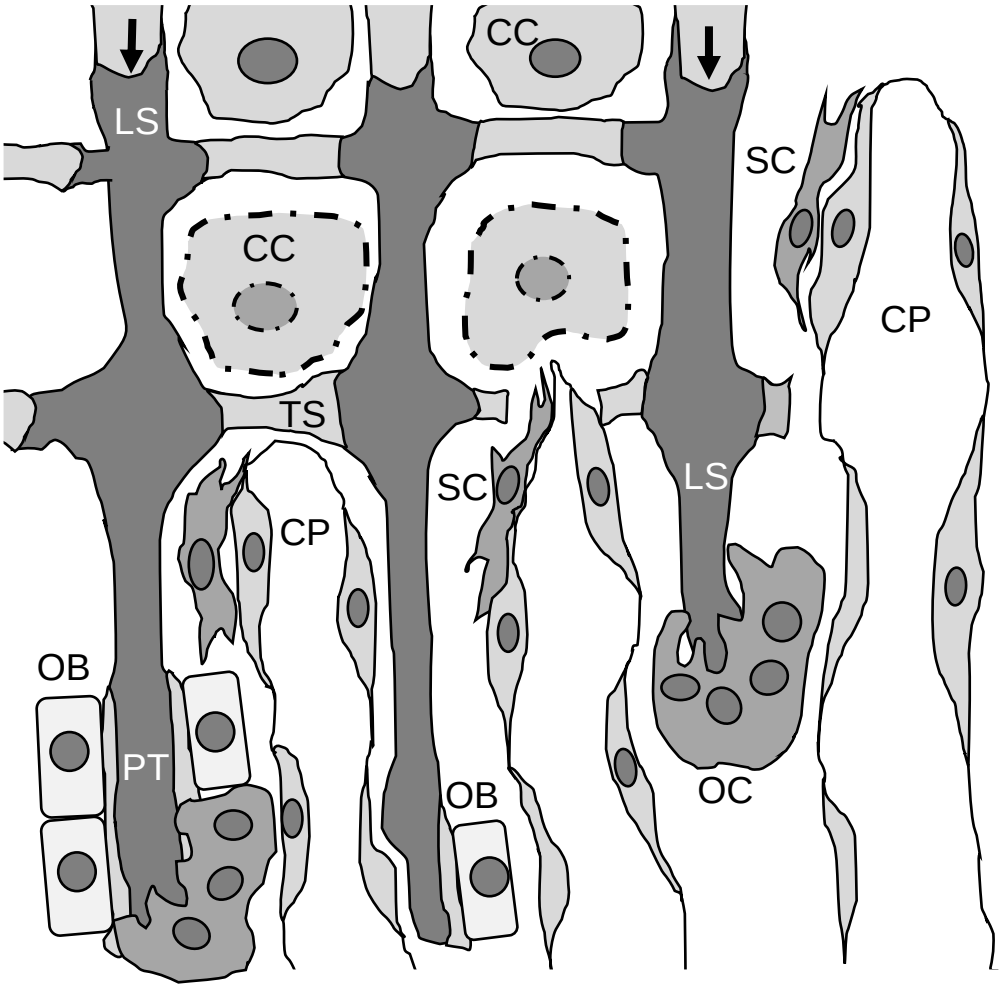
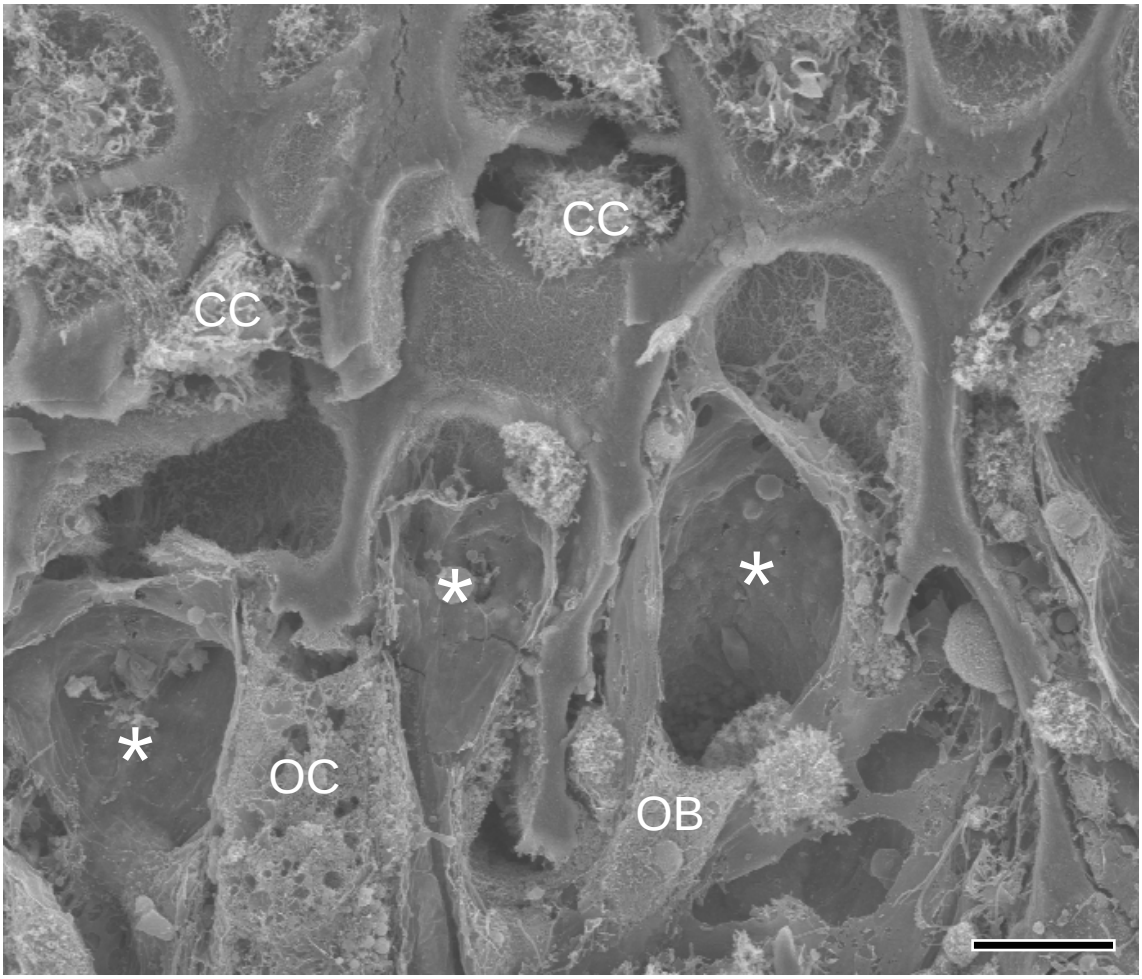


Fig.1



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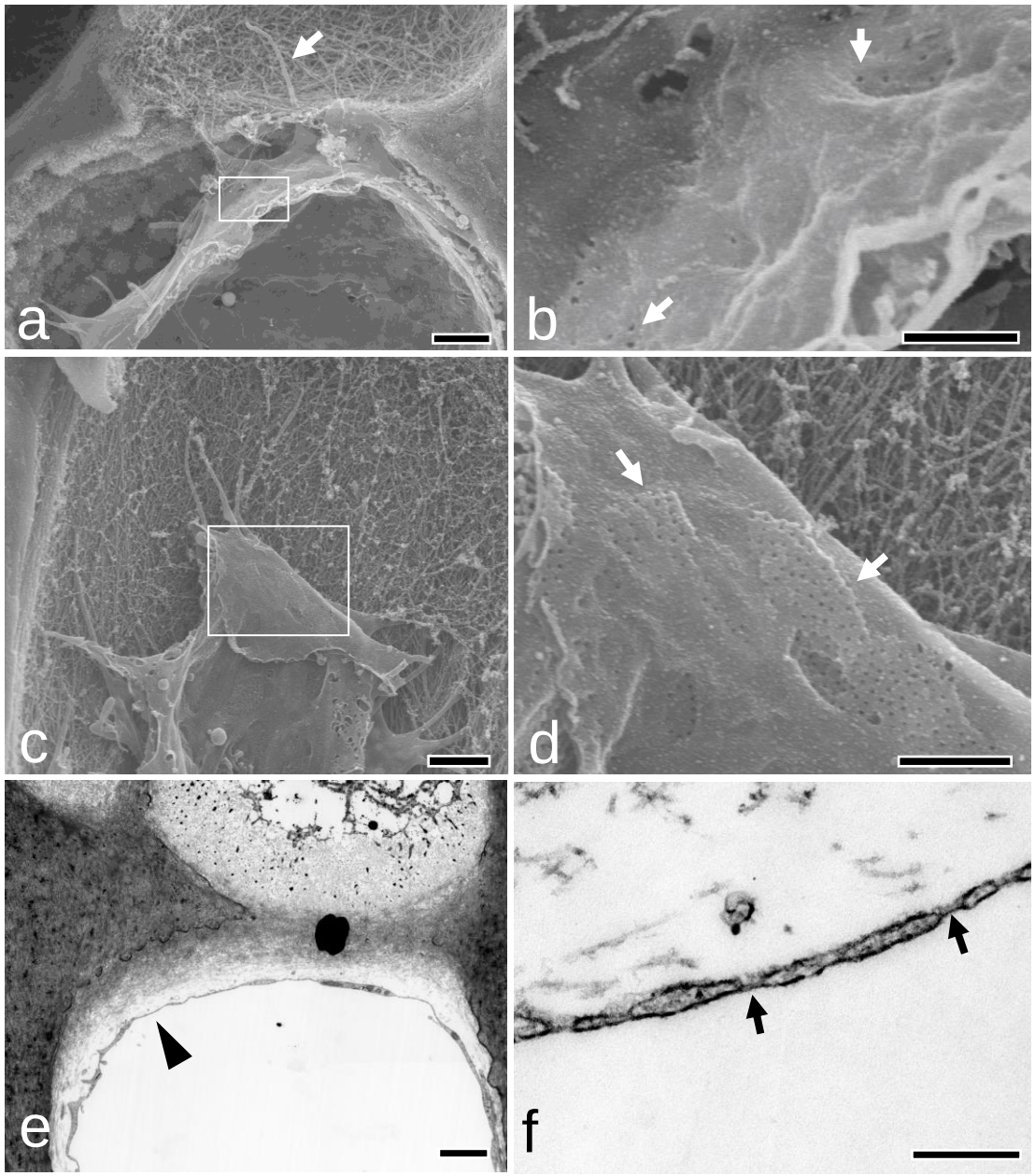

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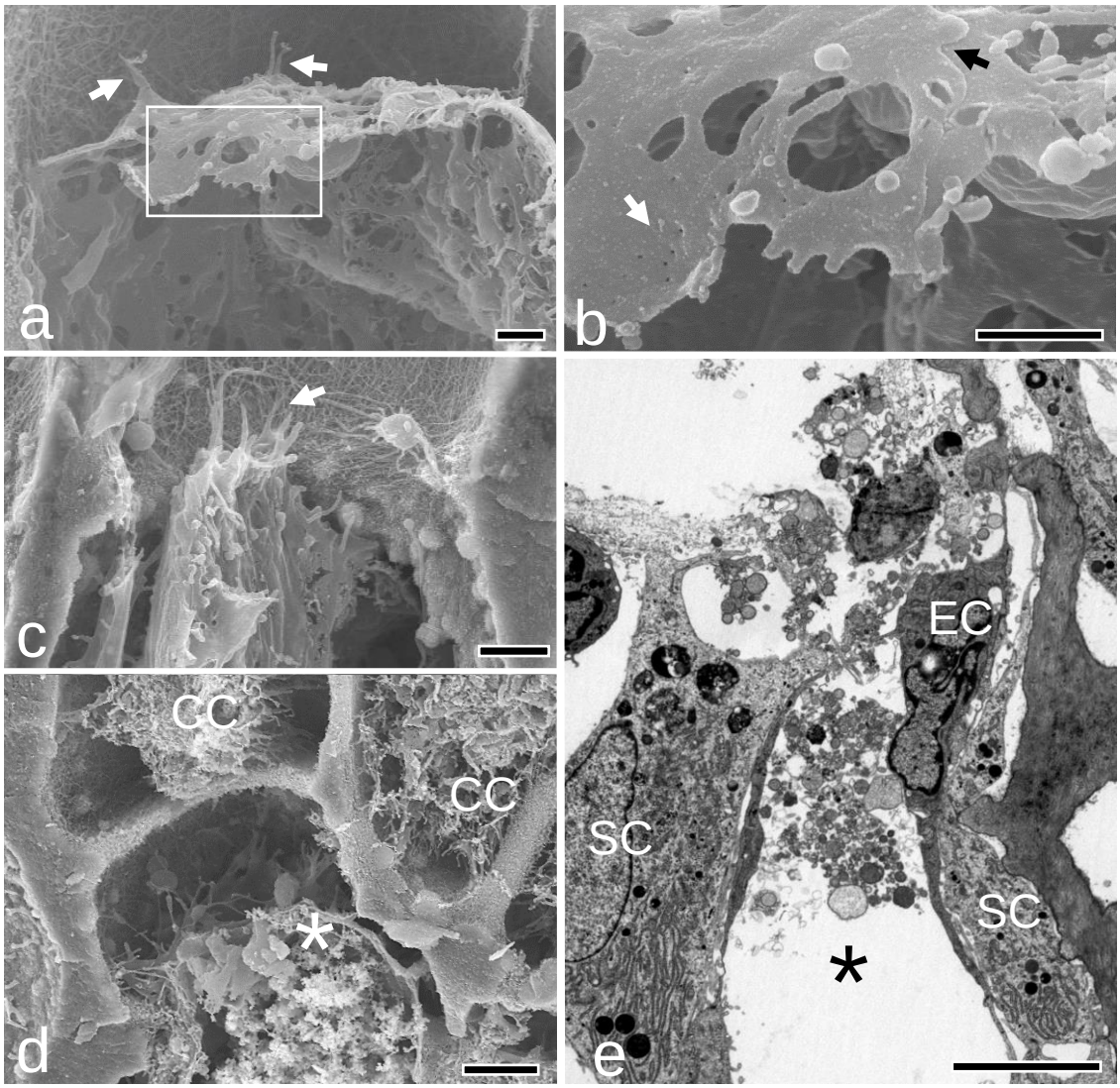

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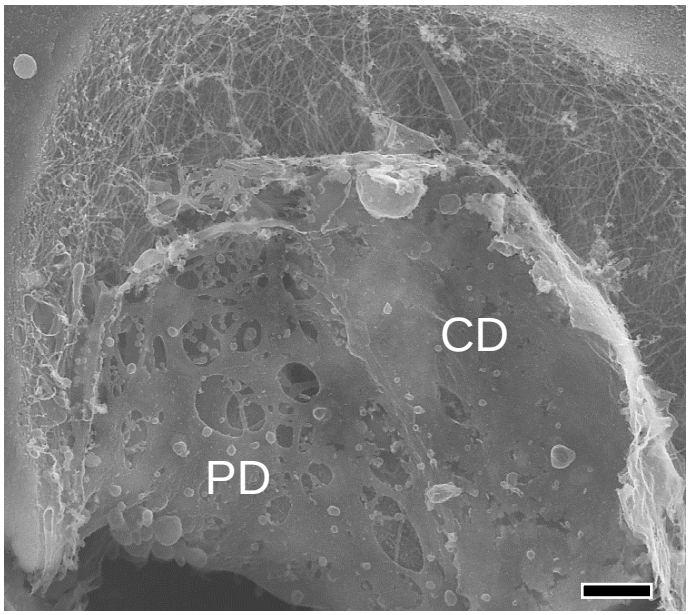

Fig.5

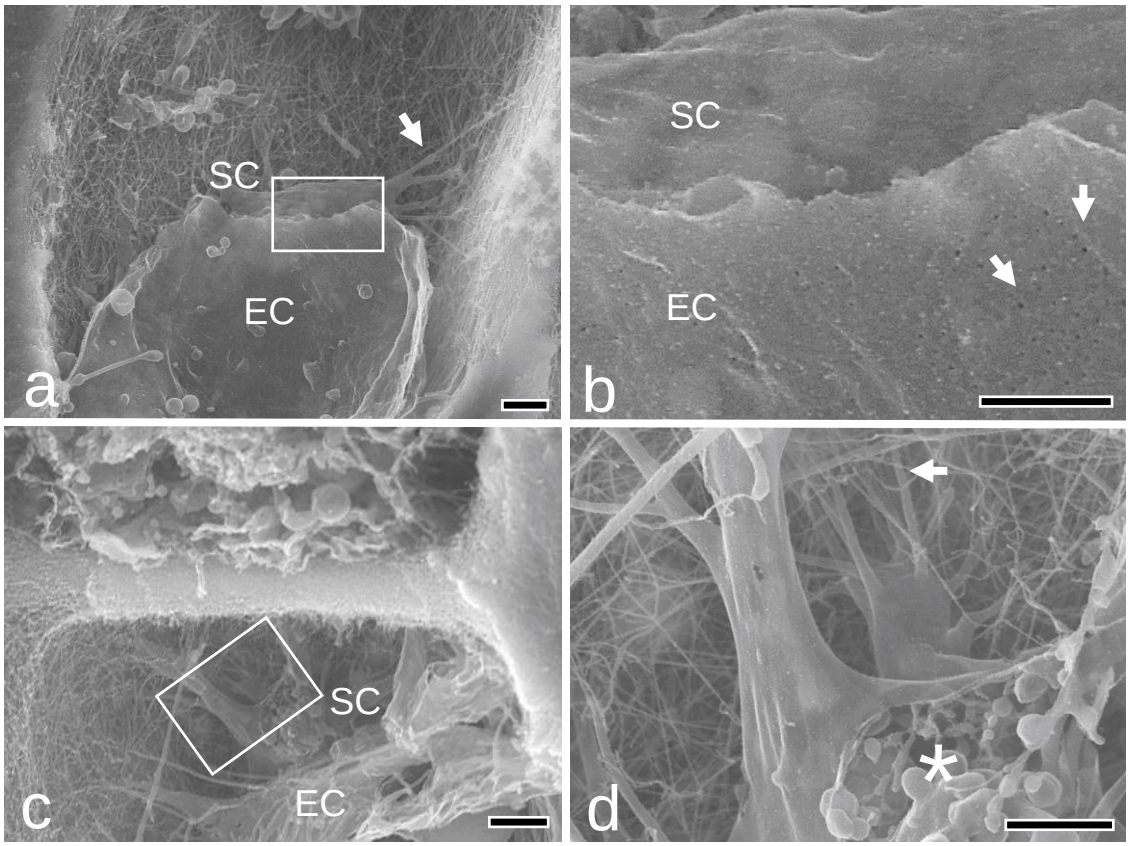

Fig.6