博士論文

Morphological assessment of bone mineralization in tibial metaphyses of ascorbic acid-deficient ODS rats.

（アスコルビン酸合成能欠如(ODS)ラットの骨組織石灰化における組織学的解析）

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Morphological assessment of bone mineralization in tibial metaphyses of ascorbic acid-deficient ODS rats

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ABSTRACT

Osteogenic disorder shionogi (ODS) rats carry a hereditary defect in ascorbic acid synthesis, mimicking human scurvy when fed with an ascorbic acid-deficient (*aa-def*) diet. As *aa-def* ODS rats were shown to feature disordered bone formation, we have examined the bone mineralization in this rat model. A fibrous tissue layer surrounding the trabeculae of tibial metaphyses was found in *aa-def* ODS rats, and this layer showed intense alkaline phosphatase activity and proliferating cell nuclear antigen-immunopositivity. Many osteoblasts detached from the bone surfaces and were characterized by round-shaped rough endoplasmic reticulum (rER), suggesting accumulation of malformed collagen inside the rER. Accordingly, fine, fragile fibrillar collagenous structures without evident striation were found in *aa-def* bones, which may result from misassembling of the triple helices of collagenous α-chains. Despite a marked reduction in bone formation, ascorbic acid deprivation seemed to have no effect on mineralization: while reduced in number, normal matrix vesicles and mineralized nodules could be seen in *aa-def* bones. Fine needle-like mineral crystals extended from these mineralized nodules, and were apparently bound to collagenous fibrillar structures. In summary, collagen mineralization seems unaffected by ascorbic acid deficiency in spite of the fine, fragile collagenous fibrils identified in the bones of our animal model.

200 words

INTRODUCTION

Ascorbic acid, *i.e.*, vitamin C, is essential for the hydroxylation of proline and lysine in the α-chains that occurs in the rough endoplasmic reticulum (rER) during the process of collagen synthesis. Shortage of ascorbic acid may disrupt the process of cross-linking through which collagenous α-chains are bundled so as to form the stable helical structure known as superhelix (9, 28). Unlike humans, wild-type rats can synthesize ascorbic acid; osteogenic disorder shionogi (ODS) rats, however, carry a hereditary defect in ascorbic acid synthesis caused by the absence of *l*-gulonolactone oxidase, which catalyzes the conversion of *l*-gulono-lactone into ascorbic acid (18, 19). ODS rats, when fed with an ascorbic acid-free diet, show low body weight, small craniofacial size, low concentrations of plasma alkaline phosphatase (ALPase) and defective bone formation (23, 35, 30, 36). Accordingly, the hydroxyproline level in collagen synthesis (20) was also decreased (18). Since type I collagen accounts for approximately 90% of the organic fraction of bone (15), the deficiency of ascorbic acid in ODS rats results in reduced bone formation accompanied by deleterious effects in bone mechanical properties (14, 30, 36).

In bone, there have been reports that ascorbic acid not only affects the hydroxylation of collagenous proline and lysine, but also influences osteoblastic differentiation. Ascorbic acid supplementation fostered the synthesis of type I collagen, as well as the expression of ALPase and osteocalcin in osteoblastic cell culture (11, 12). On the other hand, addition of 3,4-dehydroproline or cis-4-hydroxyproline, known inhibitors of collagen hydroxylation, negatively affected the synthesis of type I collagen, and also blocked the overexpression of ALPase and osteocalcin (11, 12). In addition, it was shown that bone matrix collagen plays an important role in regulating osteoblastic adhesion and subsequent differentiation by means of the α2β1 integrin expressed in osteoblasts (33). Ascorbic acid, in summary, appears to affect hydroxylation of collagenous proline and lysine, collagen synthesis, ALPase and osteocalcin expression, and osteoblastic adhesion to bone matrix.

It is therefore feasible that ascorbic acid deficiency could compromise bone mineralization. Osteoblast-induced mineralization is known to start with the secretion of small extracellular vesicles, referred to as “matrix vesicles” (3-5). Matrix vesicles contain needle-like mineral crystals (3), which grow out of the matrix vesicle and form globular

structures branded as mineralized nodules or calcifying globules (5). Mineral crystals extending from mineralized nodules then reach the surrounding collagen fibrils, and mineralization runs along the collagen fibrils to establish a thoroughly mineralized matrix (27). The first step of collagen mineralization, *i.e.*, nucleation of calcium phosphates, has been reported to occur at the "hole zone" between neighboring collagen triple α-helices (17). Many researchers raised the possibility that small crystals nucleate in these hole zones, which form continuous channels opening to the collagen surface (13, 16, 21, 37). In the absence of ascorbic acid, however, the cross-linking collagenous triple α-chains is either completely or partially disrupted, making it impossible for calcium phosphates to nucleate in the hole zones of collagen fibrils. Therefore, the normally-formed cross-linking among collagenous α-chains seems of importance for intact mineralization.

We reported previously that the ascorbic acid deficiency-related reduction in bone mechanical properties is associated with collagen abnormalities in bone of the ascorbic acid-deficient ODS rats (14), and the others demonstrated morphological change of osteoblasts, *e.g.*, round profiles of rER and few osteoid beneath them in the ascorbic acid-deficient ODS rats (30). However, both investigations lacked for preciously clarifying whether the defective mineralization could be seen in this rat model. In this study, therefore, we have examined the ultrastructural alterations of bone mineralization in the metaphysis of ascorbic acid-deficient ODS rats.

MATERIALS AND METHODS

Animals and experimental design

All procedures were performed in compliance with Hokkaido University's guidelines for care and handling of experimental animals and with the approval of Eisai's Ethics Committee. Eight-week-old male ODS rats (CLEA Japan, Tokyo, Japan) were housed (3–4 animals per cage) and acclimated until reaching 13 weeks of age. Water containing 200 mg/dL ascorbic acid and a purified diet containing 0.5% calcium (CLEA Japan) were provided *ad libitum*. Fourteen 13-week-old ODS rats were then divided into two groups: ascorbic acid group (*aa+*, n=7) and ascorbic acid-deficient group (*aa-def*, n=7). Ascorbic acid solution at concentrations of 200 mg/dL or 0.3mg/dL was given as drinking water to *aa+* rats or to *aa-def* rats, respectively, as previously reported (14). Calcein for bone labeling (10 mg/kg, sc, Kanto Chemical, Tokyo, Japan) was administered 12 and 5 days before sacrifice.

Anesthesia was induced with diethyl ether followed by pentobarbital (Nembutal, Dinabot, Osaka, Japan), and the rats were perfused with 4% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.4) through the left cardiac ventricle. Tibiae were dissected free of soft tissue and immersed in the same fixative for additional 12 h at 4°C. After decalcification with a 5% EDTA-2Na solution for 4 weeks at 4°C, some specimens were dehydrated through a graded series of ethanol prior to paraffin embedding. Decalcified and non-decalcified specimens destined for transmission electron microscope (TEM) observations were post-fixed with 1% OsO_4 in 0.1 M cacodylate buffer (pH 7.4) for 4 h at 4°C, dehydrated with ascending concentrations of acetone and embedded in epoxy resin (Taab, Berkshire, UK). Ultra-thin sections from these blocks were obtained with a microtome (Sorvall MT-5000, Du Pont, DD) and stained with tannic acid, uranyl acetate, and lead citrate for TEM examination (Hitachi H-7000 Hitachi Co. Ltd, Tokyo, Japan) at 80 kV. Non-decalcified samples were embedded into methyl methacrylate for calcein labeling observation under fluorescent microscopy (Eclipse E800, Nikon Instruments Inc. Tokyo, Japan).

Histochemistry for ALPase, tartrate resistant acid phosphates (TRAPase), proliferating cell nuclear antigen (PCNA), osteocalcin and osteopontin

Dewaxed paraffin sections were examined for ALPase, TRAPase, osteopontin and osteocalcin

reactivity as previously reported (1, 2). In short, sections were immersed into 0.3% H_2O_2 in methanol for 30 min to block endogenous peroxidase. In order to reduce non-specific binding, 1% bovine serum albumin (Serologicals Proteins Inc. Kankakee, IL) in phosphate buffered saline (1%BSA-PBS) was applied to the sections for 20 min. Slides were then incubated with rabbit antiserum against rat tissue nonspecific ALPase (24) at a dilution of 1: 200, or rabbit anti-osteopontin (LSL Co., Ltd, Tokyo, Japan) at 1:5000, respectively. After being rinsed with PBS, the sections were incubated with horseradish (HRP) peroxidase-conjugated anti-rabbit IgG (Chemicon International Inc., Temecula, CA) for l h. For osteocalcin immunohistochemistry, 1% BSA-PBS treated sections were incubated with goat anti-rat osteocalcin (Biomedical Technologies Inc. St. Stoughton, MA) at 1: 500 in 1% BSA-PBS at room temperature for 1 h, and then reacted with HRP-conjugated anti-goat IgG (Jackson Immunoresearch Laboratories Inc., West Grove, PA). For PCNA immunostaining, histological sections were incubated with antibody against PCNA (Oncogene Research Products, San Diego, CA) at 1: 100, and then, reacted with HRP-conjugated second antibody (Dako Cytomation, Glostrup, Denmark). For visualizing all these immunoreactions, diaminobenzidine tetrahydrochloride was employed as a substrate. For TRAPase detection, sections were incubated in a mixture of 8 mg of naphthol AS-BI phosphate (Sigma, St. Louis, MO, USA), 70 mg of red violet LB salt (Sigma) and 50 mM L(+) tartaric acid (0.76 g, Nacalai Tesque, Kyoto, Japan) diluted in 60 mL of a 0.1M sodium acetate buffer (pH 5.0) for 20 min at 37^{o}C. All sections were counterstained with methyl green, and observed under a light microscope (Eclipse E800, Nikon Instruments Inc. Tokyo, Japan). Images were acquired at various magnifications using a digital camera (Nikon DXM1200C, Nikon).

Von Kossa's staining

Undecalcified epoxy resin sections were incubated with a 5% silver nitrate aqueous solution (Wako Pure Chemical Industries, Tokyo, Japan) at room temperature and under sunlight until turning dark brown (29).

RESULTS

Histological alterations in ascorbic acid-deficient tibial metaphyses

Ascorbic acid-deficient (*aa-def*) ODS rats revealed shortened, irregularly distributed trabeculae beneath the growth plate cartilage of tibiae (**Fig. 1B**), compared with those of *aa+* rats (**Fig. 1A**). These trabeculae were fragmented and surrounded by thick fibrous tissue. This fibrous tissue stained intensely for ALPase, a hallmark of osteoblastic cells, and contained many TRAPase-positive osteoclasts (**Fig. 1E**). In contrast, the ascorbic acid-sufficient, *aa+*, bones revealed ALPase-positive osteoblasts and TRAPase-reactive osteoclasts on the surfaces of metaphyseal trabeculae (**Fig. 1C**). Higher numbers of cells immunopositive for PCNA were seen in the fibrous tissue layer in *aa-def* bones when compared to similar areas in *aa+* metaphyses (**Compare Figs. 1D and F**).

Highly magnified microscopy showed that osteoblasts in *aa-def* bones were not attached to the bone surfaces (**Figs. 2A, B**). Only few osteoblasts were lying on the surfaces of the metaphyseal trabeculae at the chondro-osseous junction; instead, fibroblast-like cells surrounded the fragmented trabeculae in this area (**Fig. 2A**). Trabecular surfaces stained intensely for toluidine-blue (**Fig. 2A**), osteopontin, and osteocalcin (**Figs. 2C, D**). Under TEM, an electron-dense linear structure could be found in a similar location (**Fig. 2B**). The fibroblast-like cells included round and enlarged rER which contained evenly electron-dense material (**Fig. 2E**). Amorphous organic material including fine fibrillar structures were associated with these fibroblast-like cells (**Fig. 2F**). Thus, in the metaphysis of *aa-def* tibia, osteoblasts tended to detach from the bone matrix, and many osteoblasts and fibroblast-like cells in the fibrous tissue layer showed enlarged rER, indicating the accumulation of organic materials inside the rER.

Matrix mineralization in the ascorbic acid-deficient bone

Bone mineralization in the primary trabeculae close to the chondro-osseous junction was examined by calcein labeling, and *aa-def* bones showed a small amount of bone deposition onto mineralized cartilage (**Compare insets of Figs. 3A, B**). Bone matrix could be seen surrounding the cartilage cores of primary trabeculae in *aa+* rats (**Fig. 3A**), whereas *aa-def* cartilage was often seen without a surrounding bone matrix (**Fig. 3B**). Under TEM, in

contrast with normal striated collagen fibrils of *aa+* animals (**Fig. 3C**), amorphous, feather-like, or fibrillar structures were seen associated with osteoblasts with round-shaped rER in *aa-def* specimens (**Fig. 3D**). Thus, the ultrastructure of collagen fibrils was shown to be different between *aa-def* and *aa+* groups.

Next, mineralization in the metaphyseal trabeculae was assessed. In a state of ascorbic acid sufficiency, von Kossa staining revealed numerous granular structures adjacent to mature osteoblasts in the primary trabeculae (**Figs. 4A, B**). Accordingly, TEM observations portrayed numerous matrix vesicles and mineralized nodules, some of which were associated with collagen fibrils (**Fig. 4C**). At a higher magnification, mineral crystals could be seen growing towards the collagen fibrils from the mineralized nodules (**Fig. 4D**). Crystal deposition unrelated to mineralized nodules was not seen in normal collagen fibrils. In *aa-def* metaphyses, von Kossa staining verified poor mineralization and a markedly reduced trabecular number (**Figs. 5A, B**). Fragmented trabeculae neighboring the growth plate cartilage showed granular structures stained with von Kossa's solution. TEM imaging from corresponding areas demonstrated numerous matrix vesicles and mineralized nodules (**Figs. 5C, D**). Therefore, bone mineralization still seemed to take place even in the circumstance of ascorbic acid deficiency. At higher magnification, the fine mineral crystals from the mineralized nodule extended along fine fibrillar structures that were not the mature form of collagen fibrils (**Figs. 5E, F**).

DISCUSSION

To our knowledge, this is the first report on the ultrastructural properties of collagen mineralization in ascorbic acid-deficient bones. Despite a marked reduction in bone formation, ascorbic acid deprivation seemed to have no deleterious effect on mineralization: while reduced in number, normal matrix vesicles and mineralized nodules could be seen in *aa-def* bones. Theoretically, the "hole zone" in collagen fibrils should be occupied by small proteoglycans such as decorin and biglycan (10, 13), and the release of these proteoglycans may trigger crystal nucleation in collagen. In *aa-def* bones, the presence of very fine fibrillar and amorphous materials indicated alterations of the ultrastructural geometry of collagen fibrils, implying the absence or disruption of the hole zones. Nevertheless, as shown here, fine needle-like mineral crystals extending from the mineralized nodules were associated with fine collagenous fibrillar materials. This finding suggests that these fine collagenous fibrillar structures may serve as a scaffold for collagen mineralization.

As reported previously (14, 30), *aa-def* rats had abnormal bone histology: the presences of a fibrous tissue layer in tibial metaphyses, osteoblastic cells detaching from bone surfaces, and osteoblasts that possessed rounded rER locating close to the chondro-osseous junction are all rather peculiar findings. These histological observations appear to be consisting to those by Sakamoto *et al*. (30), and probably indicate reduced osteoblastic function. Ascorbic acid is required for prolyl hydroxylase activity, a rER enzyme that hydroxylates proline residues in the nascent procollagen chains and allows folding of the chains into a stable triple helix (7, 25). In our study, osteoblasts with round rER indicate that ascorbic acid deficiency might inhibit prolyl hydroxylation of proline in collagen, and consequently, accumulation of abnormal collagenous α-helices may result in the enlarged rER as seen in Fig. 2E. Also, the amorphous and/or fibrillar materials associated with the fibroblastic cells and osteoblasts seen in Figs. 2F and 3D, respectively, may be the same structure and suggest that these fibroblastic and osteoblastic cells may be secreting incomplete collagenous triple helices. Yet, the thin calcein labeling implicated a small amount of bone deposition close to the chondro-osseous junction in the *aa-def* group. In this study, as with the previous report (30), the synthesis of collagen fibrils seemed to be negatively affected by ascorbic acid deficiency.

Ascorbic acid has been shown to increase ALPase activity and stimulate expression of osteoblastic hallmarks and production of both type I collagen and parathyroid hormone (PTH)/PTH-related peptide receptor (11-12, 22, 32, 33, 38). In addition, collagen molecules may be involved in interaction with the $\alpha 2\beta 1$ integrins borne by osteoblasts, which transduce signals related to focal adhesion kinase (34). Also, osteocalcin promoter activity appears to be, at least in part, regulated by ascorbic acid (38). In our study, many fibroblast-like cells in the metaphyses stained intensely for ALPase and PCNA, and therefore one can assume that such cells are part of the osteoblastic lineage and are proliferating. Although intense iimunoreactivity for osteocalcin and osteopontin was seen on the bone surfaces, osteoblastic cells were shown to detach from them (Fig. 2). Therefore, osteocalcin- and osteopontin-mediated cell adhesion might be secondary, while anchoring via the collagen secreted by osteoblasts may be vital for osteoblastic adhesion to bone surfaces, as well as to their subsequent differentiation. Thus, our findings seem consistent with the idea that ascorbic acid regulates cell adhesion and the subsequent biological functionality of osteoblasts.

An interesting finding was that, despite marked decreases in bone formation, mineralization seemed unaltered in *aa-def* bones as shown in Fig. 5. The process appeared absolutely normal, as it was mediated by matrix vesicles and mineralized nodules (Compare Figs. 4D and 5E), even though the mineralizing area seemed reduced when compared to that of the *aa+* group (Figs. 4A, B and 5A, B). Under TEM, in both groups it was evident that many mineralized nodules contact with collagen fibrils, and mineral crystals grow along with these collagen fibrils, as previously reported (26). Thus, the association between collagen fibrils and mineralized nodules appears to be the very event that initiates collagen mineralization. However, Hodge demonstrated the presence of a "hole zone" between neighboring tropocollagens, *i.e.*, superhelices, and suggested that mineralization could initiate exactly at this space (17). Many researchers investigated whether very small crystals nucleate in these hole zones (13, 16, 21, 37). There exists an assumption that the "hole zone" is normally occupied by small proteoglycans such as decorin and biglycan (10, 13), which may inhibit the nucleation of mineral crystals in a normal state. It has been shown that decorin-deficient tendons develop abnormal, irregularly contoured collagen fibrils, indicating that small leucine-rich proteoglycans such as biglycan, decorin, fibromodulin, and lumican, as

well as glycoproteins are involved in collagen fibril strucutral maturation and tissue function (8, 39). Sanches *et al*. suggested that the absence of decorin disturbs the lateral assembling of thin fibrils, resulting in thicker, irregular collagen fibrils (31). Biglycan/decorin double-knockout mice are markedly osteopenic, with a complete loss of the basic fibril geometry characterized by a "serrated fibril" morphology (6). As shown in Fig. 3D, *aa-def* bone matrices were composed by fibrillar and amorphous collagenous materials and, at least theoretically, the normal hole zones should be either absent or disrupted. Finding alterations of the ultrastructural geometry of collagen fibrils and normal bone mineralization in the circumstance of ascorbic acid deficiency was rather unexpected. As shown in Fig. 5F, fine needle-like mineral crystals extending from mineralized nodules neighbored fine collagenous fibrillar materials. Therefore, we hypothesize that mineralization in collagen may not occur at the hole zones in well assembled collagen fibrils, but may occur instead on the fine collagenous fibrillar structures that serve as a scaffold for crystal growth.

In summary, our findings provide the basis for further research on the elucidation of the ultrastructural mechanisms of collagen mineralization, since collagen fibrils would serve as a scaffold for mineralization, at least in the model here proposed.

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Figure legends

Figure 1

Bone histology of ascorbic acid-sufficient (*aa+*, A, C, D) and ascorbic acid-deficient (*aa-def*, B, E, F) tibiae

The *aa+* tibia had many metaphyseal trabecules, showing ALPase-positive osteoblasts and TRAPase-reactive osteoclasts (A, C), while the *aa-def* metaphysis showed a few numbers of trabecules (B). The fibrous tissue of *aa-def* tibia (asterisks, insets in B, E and F) included many ALPase-positive osteoblasts, TRAP-positive osteoclasts and PCNA-immunoreactive cells (E, F). Note many PCNA-positive cells in the *aa-def* fibrous tissue compared with *aa+* tibial metaphysic (D, F). epi: epiphysis, GP: growth plate, meta: metaphysis, tb: trabecular bone

Bar: A, B; 1mm, C, E; 100μm, D,F; 50μm

Figure 2

Cell morphology (A, B, E, F) and immunohistochemistry for osteocalcin (C) and osteopontin (D) in the tibial metaphysis of *aa-def* mice

Osteoblasts in *aa-def* bones often detached from the bone surfaces (B), while fibrous tissue, corresponding to Figs.1B-F, surrounded the trabeculae (an asterisk in A). Trabecular surfaces were intensely stained for toluidine blue (arrows, A), osteocalcin (arrows, C) and osteopontin (arrows, D). Under TEM, an electron-dense linear structure could be found on bone surfaces (white arrows, B). The fibroblast-like cells showed enlarged rough endoplasmic reticulum (rER) which contained evenly electron-dense material (white arrows, E). Amorphous organic material including fine fibrillar structures (black arrows, F) were associated with these fibroblast-like cells (F). bm: bone matrix, ob: osteoblast, tb: trabecular bone

Bar: A; 20μm, B; 4μm, C, D; 50μm, E; 3μm, F; 6μm

Figure 3

Bone deposition and osteoblasts in the metaphyseal trabecules close to the chondro-osseous junction of the *aa+* (A, C) and *aa-def* (B, D) tibiae

Toluidine blue staining showed metaphyseal trabeculae composed of surrounding bone matrix and cartilage cores in the *aa+* tibia (A), whereas the *aa-def* trabeculae merely showed cartilage matrix without obvious bone deposition (B). Consistently, two lines of calcein labeling with certain intervals can be seen in the *aa+* mice, while the *aa-def* mice exhibited overlapped two lines of calcein labeling (See insets of panels A and B). TEM observations demonstrated stout collagen fibrils with evident striations (a white arrow, C), while fragile fibrillar collagenous structures without apparent striation were seen in the bone matrix beneath the osteoblasts (a white arrow, D). bm: bone matrix, cc: cartilage core, ob: osteoblast

Bar: A,B; 10μm, C,D; 5μm

Figure 4

Ultrastructural assessment for mineralization in the metaphyseal trabeculae of the *aa+* tibia

Von Kossa staining revealed well-mineralized metaphyseal trabeculae (A) and numerous granular structures in the primary trabeculae (white arrows, B). Under TEM observations, there were numerous matrix vesicles and mineralized nodules. Note some mineralized nodules associated with collagen fibrils (white arrows, an inset, C). At a higher magnification, mineral crystals extending from the mineralized nodules ran along the collagen fibrils (4D). Notice no crystal deposition related to mineralized nodules in normal collagen fibrils. meta: metaphysis, mn: mineralized nodule, mv: matrix vesicle, ob: osteoblast, tb: trabecular bone

Bar: A; 20μm, B; 5μm, C; 1μm, D; 0.1μm

Figure 5

Ultrastructural assessment for mineralization in the metaphyseal trabeculae of the *aa-def* tibia

By von Kossa staining, the mineralized metaphyseal trabeculae were shown to be markedly reduced in number (A), but a few, granular mineralized matrices were seen close to the chondro-osseous junction (white arrows in B). TEM observations verified the presence of mineralized nodules and matrix vesicles in the corresponding trabecules (C, D). At a higher magnification, the fine mineral crystals from the mineralized nodule extended along fine fibrillar structures of immature collagen fibrils (E, F). bm: bone matrix, mn: mineralized nodule, mv: matrix vesicle, tb: trabecular bone

Bar: A; 30μm, B; 10μm, C; 4μm, D; 1μm, E,F; 0.1μm

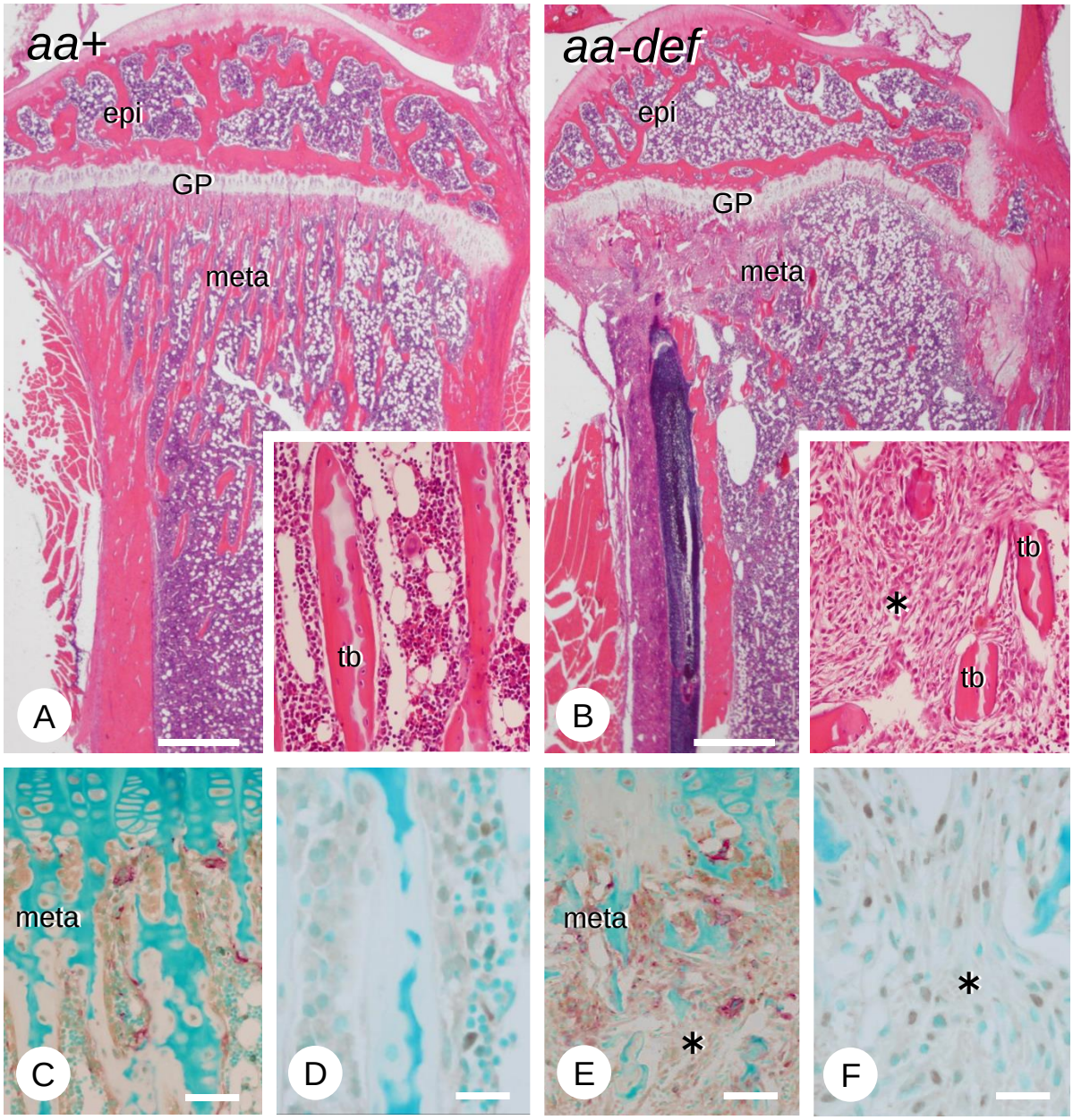

Fig. 1

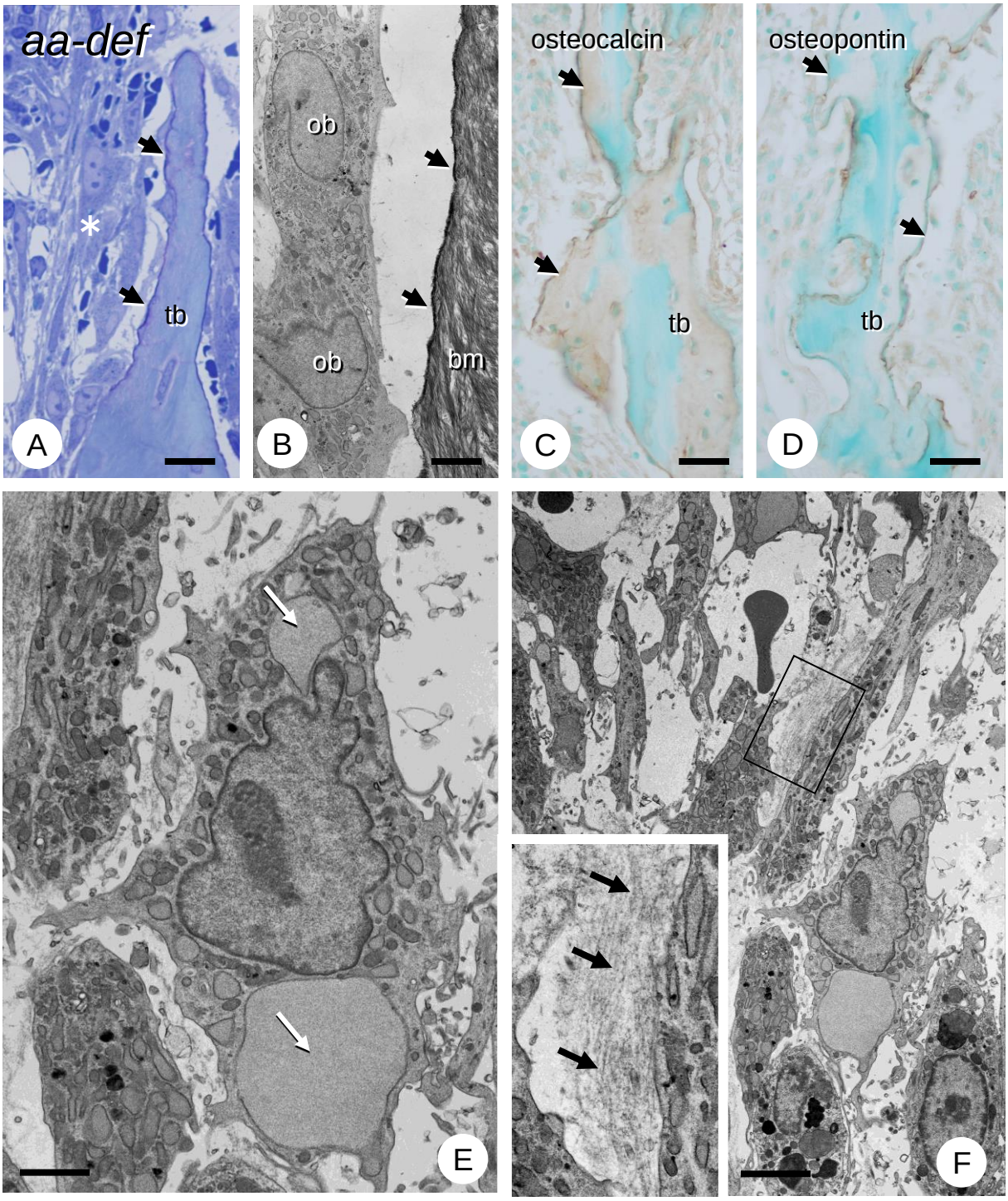

Fig. 2

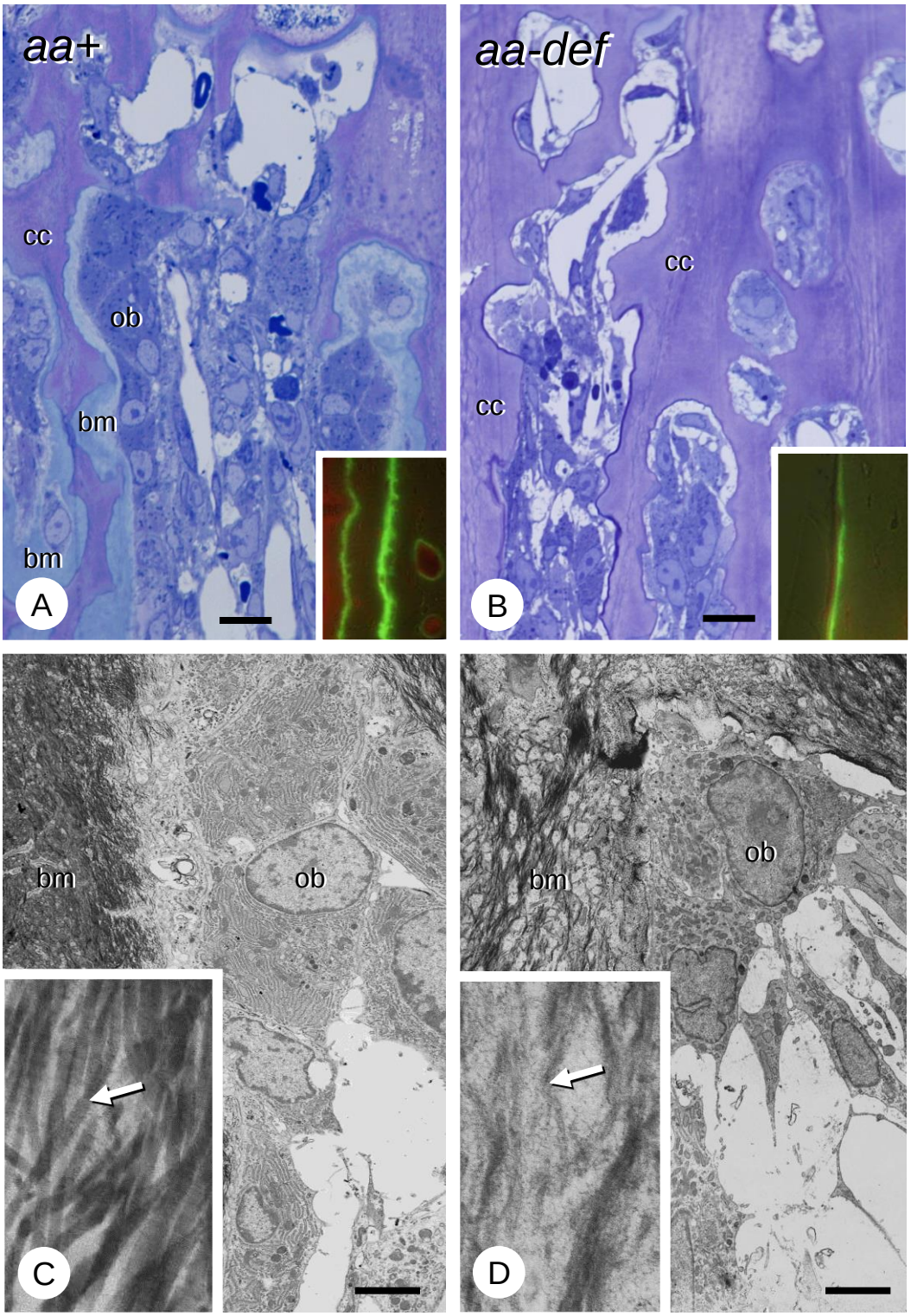

Fig. 3

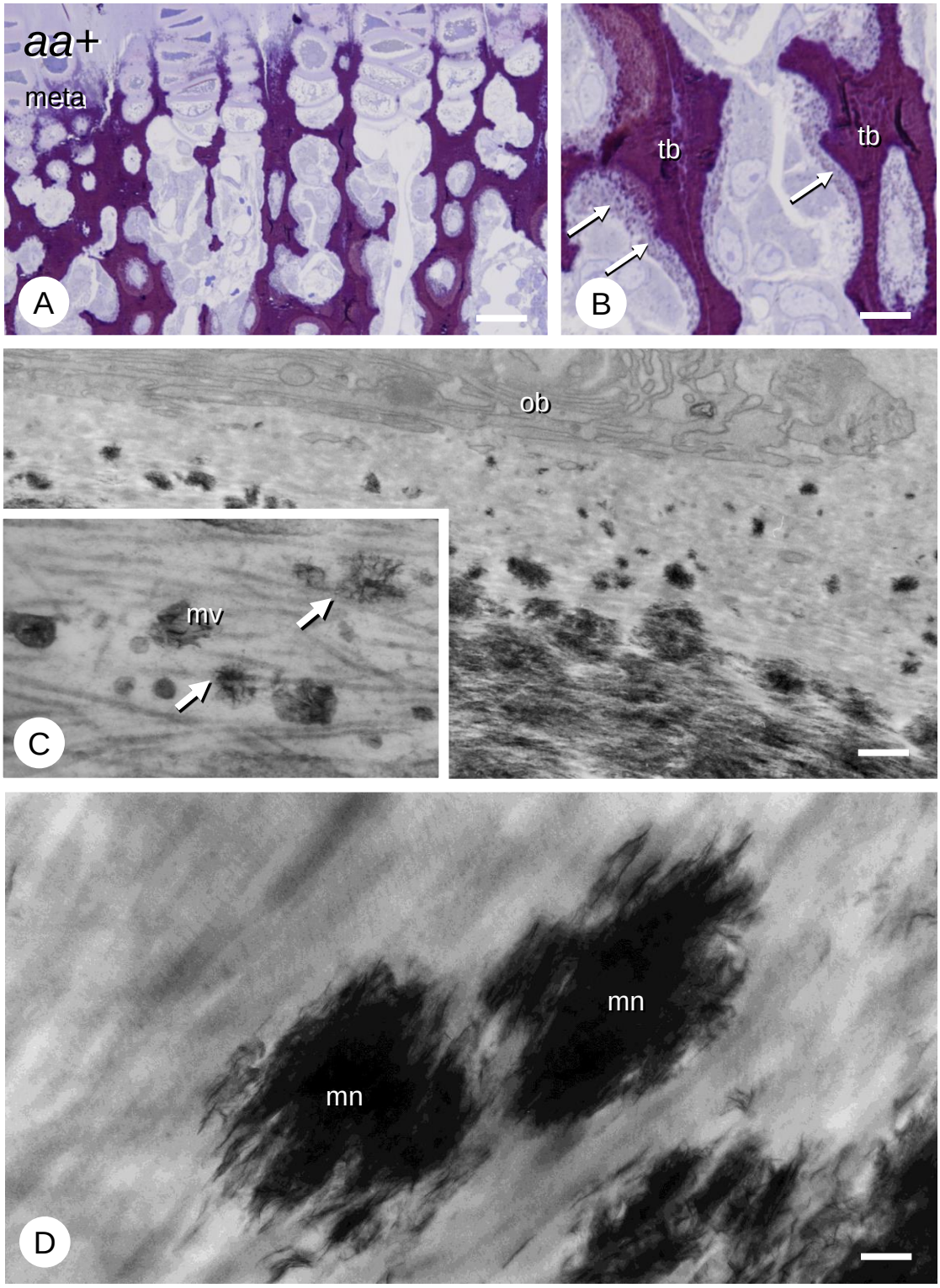

Fig. 4

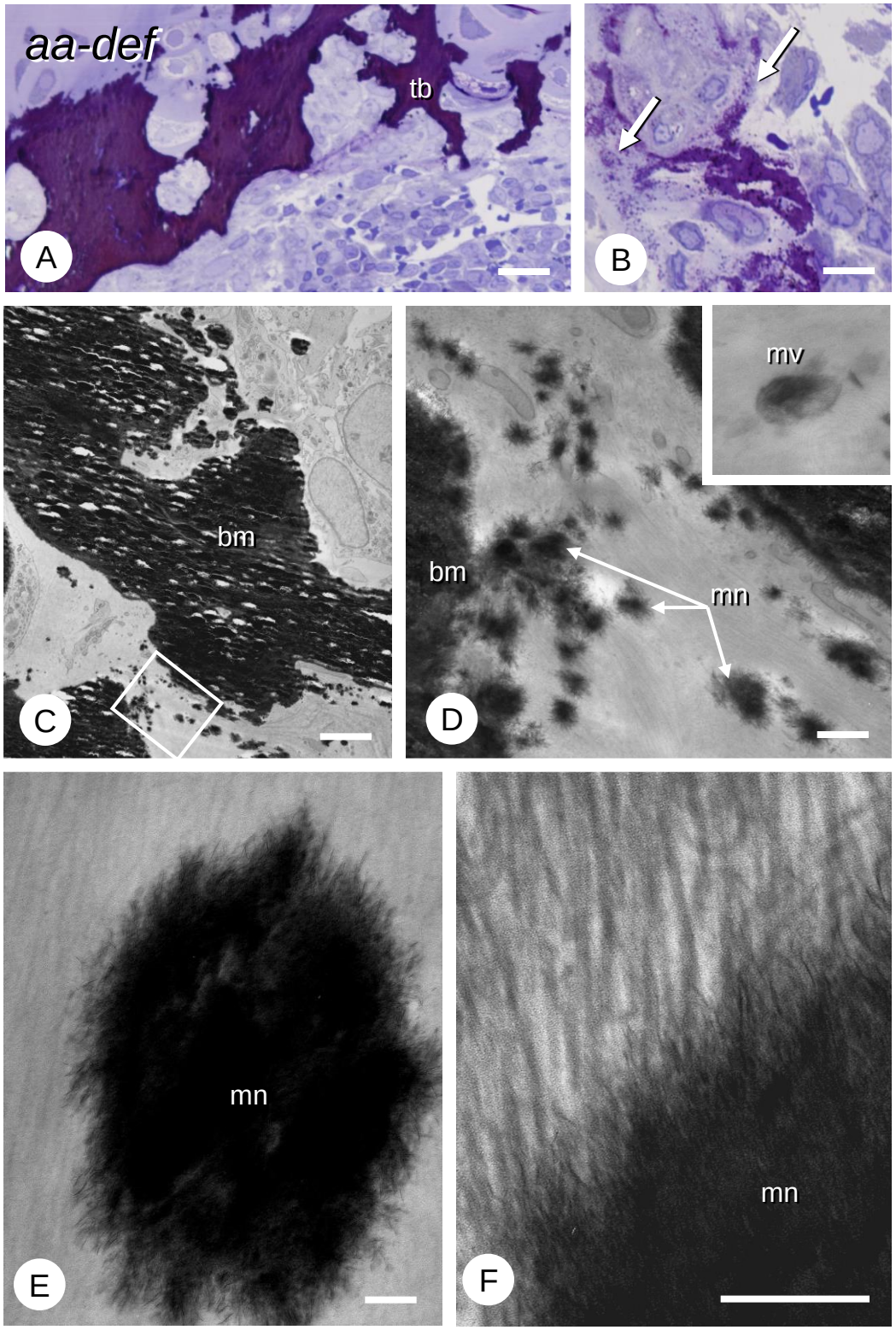

Fig. 5