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Review

# Degradation of extracellular matrices propagates calcification during development and healing in bones and teeth

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ABSTRACT

*Background:* Bone, dentin, and enamel are tissues formed through calcification, a process involving deposition of calcium phosphate minerals on extracellular organic matrices. Calcification, the underlying mechanism of which is unknown, is initiated with mineral deposition followed by advancing of the deposit and subsequent maturation of the mineral crystal.
*Highlight:* We have reviewed the current knowledge of how calcification proceeds during bone development, bone healing, and enamel and dentin development, based on reported studies. Previous studies reported by us and by other authors have suggested that degradation of some extracellular matrix (ECM) proteins is involved in calcification during bone and dentin development and bone healing in a manner similar to that previously reported for enamel development.
*Conclusion:* The ECM proteins may inhibit mineral deposition and calcification, similar to the role of amelogenin during enamel development. The candidates for the amelogenin equivalents in bone and dentin have not been identified. Further studies are required to elucidate the regulatory mechanisms of bone and dentin calcification in light of specific ECM proteins that prevent calcification and enzymes that degrade these ECM proteins.


**Contents**



## 1. Introduction

Bone, dentin, and enamel are specific tissues formed by calcification, a process involving deposition of calcium phosphate minerals on extracellular organic matrices such as collagens, non-

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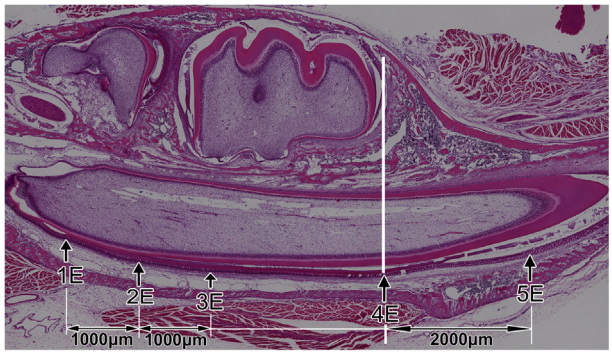


**Fig. 1.** A developing mandibular incisor of a 2-week-old rat, stained with hematoxylin-eosin. Five points were selected in developing enamel (1E, 2E, 3E, 4E, and 5E) for analysis with SEM-EDX. SEM-EDX: scanning electron microscopy with energy dispersive X-ray spectroscopy. * Adapted from Maruyama et al. (2016) [5]. With permission from Elsevier B.V.

collagenous proteins, amelogenin, and non-amelogenin proteins. Following the initial mineral deposition, calcification proceeds, and the mineral crystals mature. Recent studies conducted by us and the other authors suggest that degradation of particular extracellular matrices (ECMs) is a common aspect of calcification in bones and teeth during both development and healing, as described in this review.

## 2. Enamel development

Calcification in enamel has been extensively investigated and a gamut of information thus obtained has helped us better understand the calcification process in bone and dentin. The enamel matrix on the outer layer of dentin is formed by ameloblasts. Enamel calcification begins with the deposition of characteristic, noncrystalline, mineral ribbons that are tightly attached to dentin through a connection to its organic matrix. Moreover, they are in close association with the calcification front or the

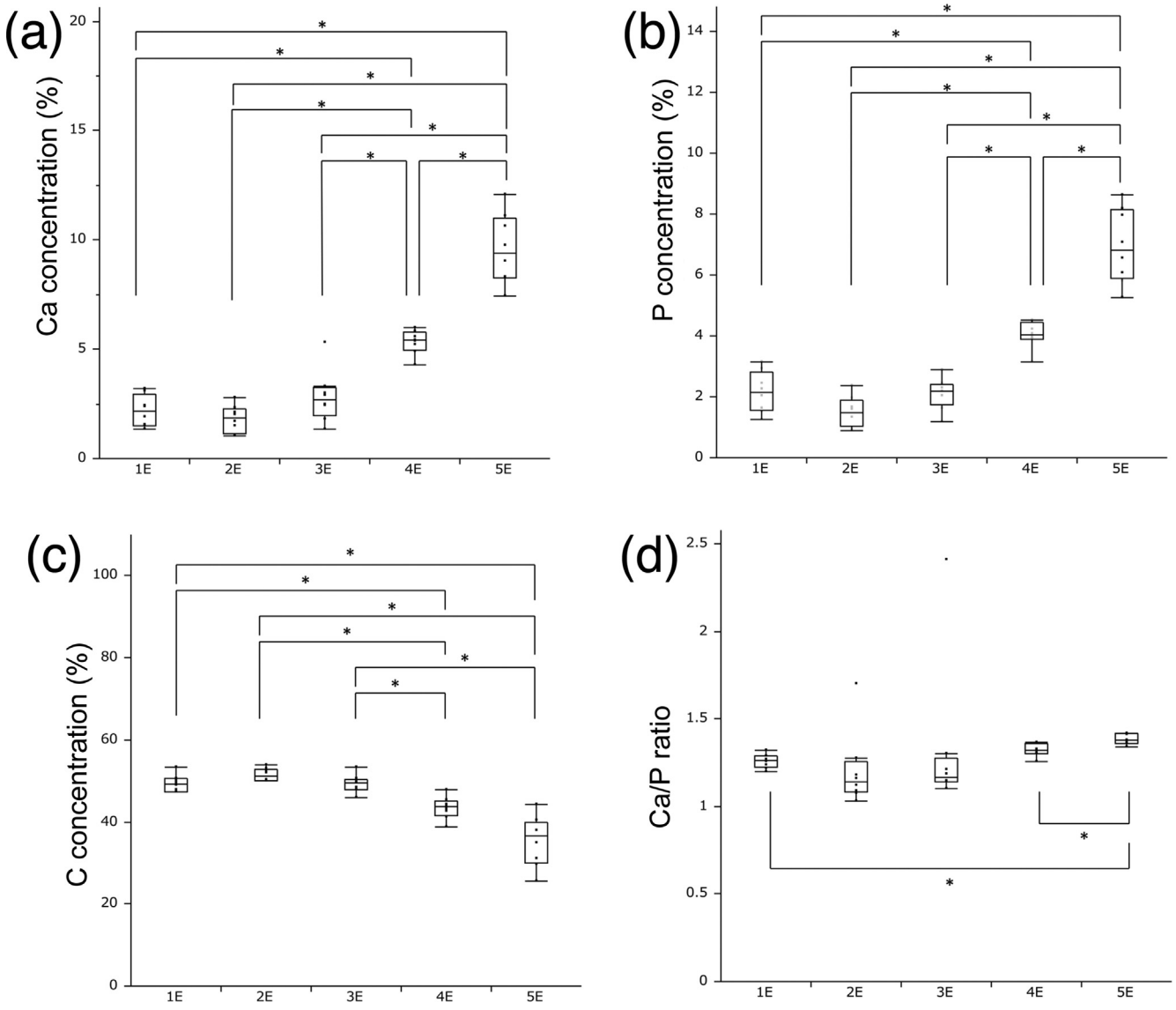


**Fig. 2.** Concentrations of Ca (a), P (b), and C (c), and C/P ratios (d) in developing enamel. Ca: calcium, P: phosphorus, C: carbon * Adapted from Maruyama et al. (2016) [5]. With permission from Elsevier B.V.

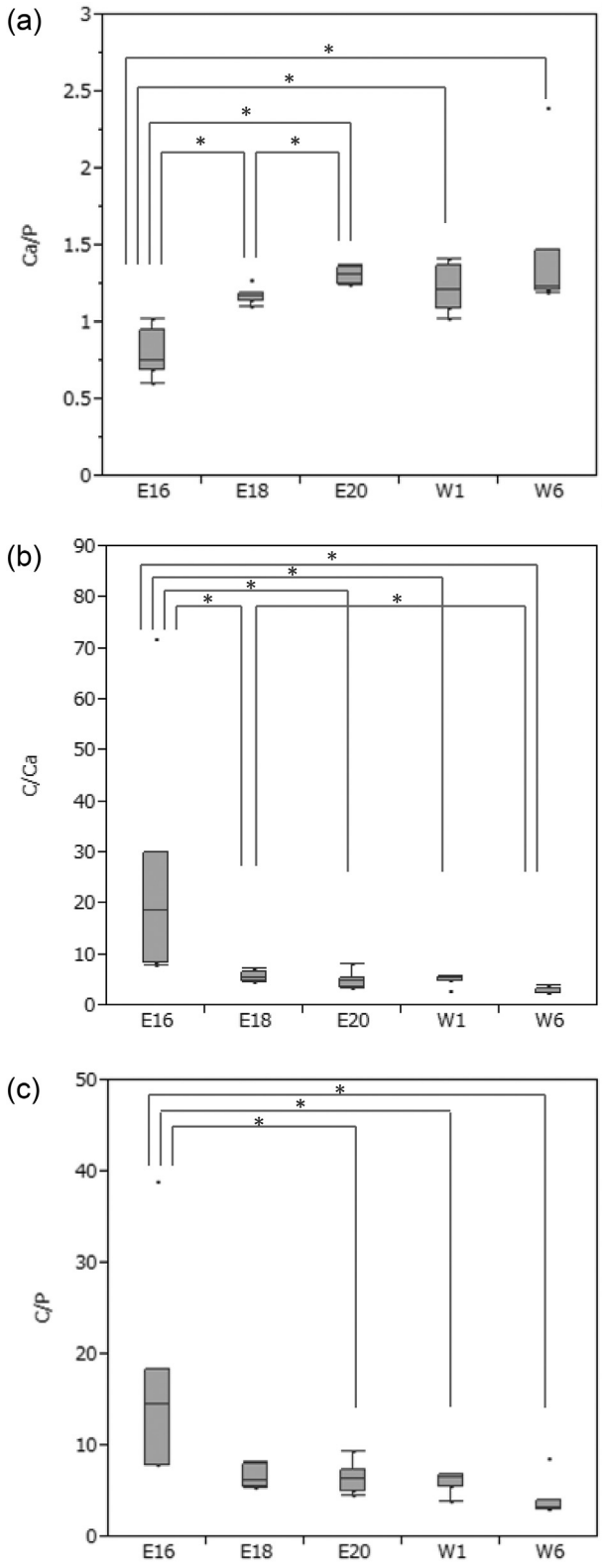


**Fig. 3.** Ca/P (a), C/Ca (b), and C/P (c) molar ratios obtained from SEM-EDX analysis of developing rat calvarial bone. The Ca/P molar ratio increases significantly from embryonic day 16 (E16) to embryonic day 20 (E20). The high C/Ca molar ratio at E16 significantly decreases after embryonic day 18 (E18). The high C/P molar ratio at E16 significantly decreases after E20. W1: week 1, W6: week 6, $^{*}P < 0.05$, SEM-EDX: scanning electron microscopy with energy dispersive X-ray spectroscopy, Ca: calcium, P: phosphorus, C: carbon. * Adapted from Henmi et al. (2016) [6]. With permission from the Japanese Society for Bone and Mineral Research and Springer Japan.

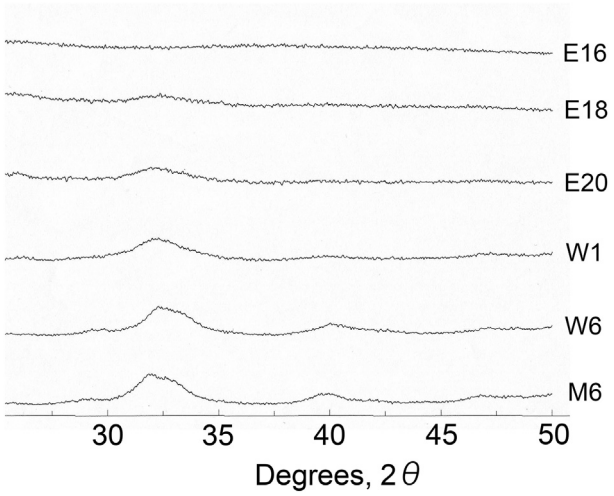


**Fig. 4.** X-ray diffraction patterns of calvarial bone at embryonic day 16 (E16), embryonic day 18 (E18), embryonic day 20 (E20), week 1 (W1), week 6 (W6), and month 6 (M6). The broad 2θ peak around 30°–35° is detected at E18 and becomes gradually higher from E18 to M6, consistent with the advancement of crystallization toward hydroxyapatite from the amorphous state. * Adapted from Henmi et al. (2016) [6]. With permission from the Japanese Society for Bone and Mineral Research and Springer Japan.

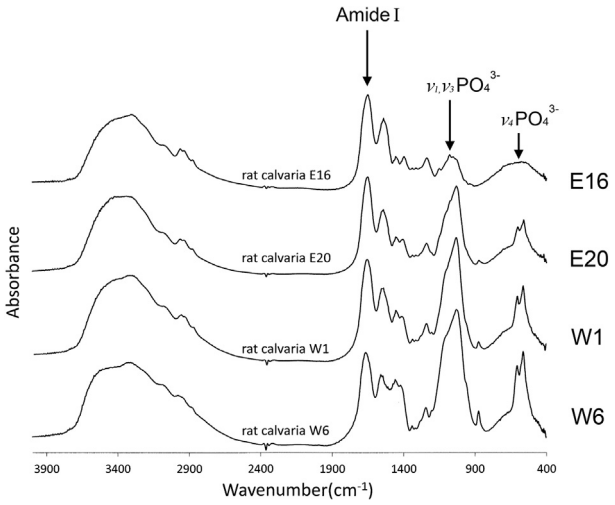


**Fig. 5.** Fourier transform infrared spectra of the calvarial bone at embryonic day 16 (E16), embryonic day 20 (E20), week 1 (W1), and week 6 (W6). With time, the phosphate ν1, ν3 region (900–1200 $cm^{-1}$) changes from a broad band characteristic of amorphous calcium phosphate to a narrow band with a high-frequency shoulder that is typical of poorly crystalline hydroxyapatite. The ν4 region (560–600 $cm^{-1}$) becomes resolved into two distinct peaks characteristic of crystalline calcium phosphate. The peak of the mineral phosphate ν1, ν3 absorbance in relation to that of the protein amide I absorbance around 1655 $cm^{-1}$ becomes gradually higher. * Adapted from Henmi et al. (2016) [6]. With permission from the Japanese Society for Bone and Mineral Research and Springer Japan.

secretory surface of the ameloblast cell membrane [1,2]. Following the initial deposition, the enamel calcification proceeds through advancing of the deposit and the maturation of mineral crystals.

Previous studies, using the respective gene knockout mouse, have shown that enzymes such as matrix metalloproteinase (MMP) 20 and kallikrein (KLK) 4 are essential for the calcification to proceed in the enamel. MMP 20 slowly degrades the enamel proteins to grow crystallites, resulting in a net replacement of proteins by minerals, while KLK4, a serine protease, degrades the accumulated

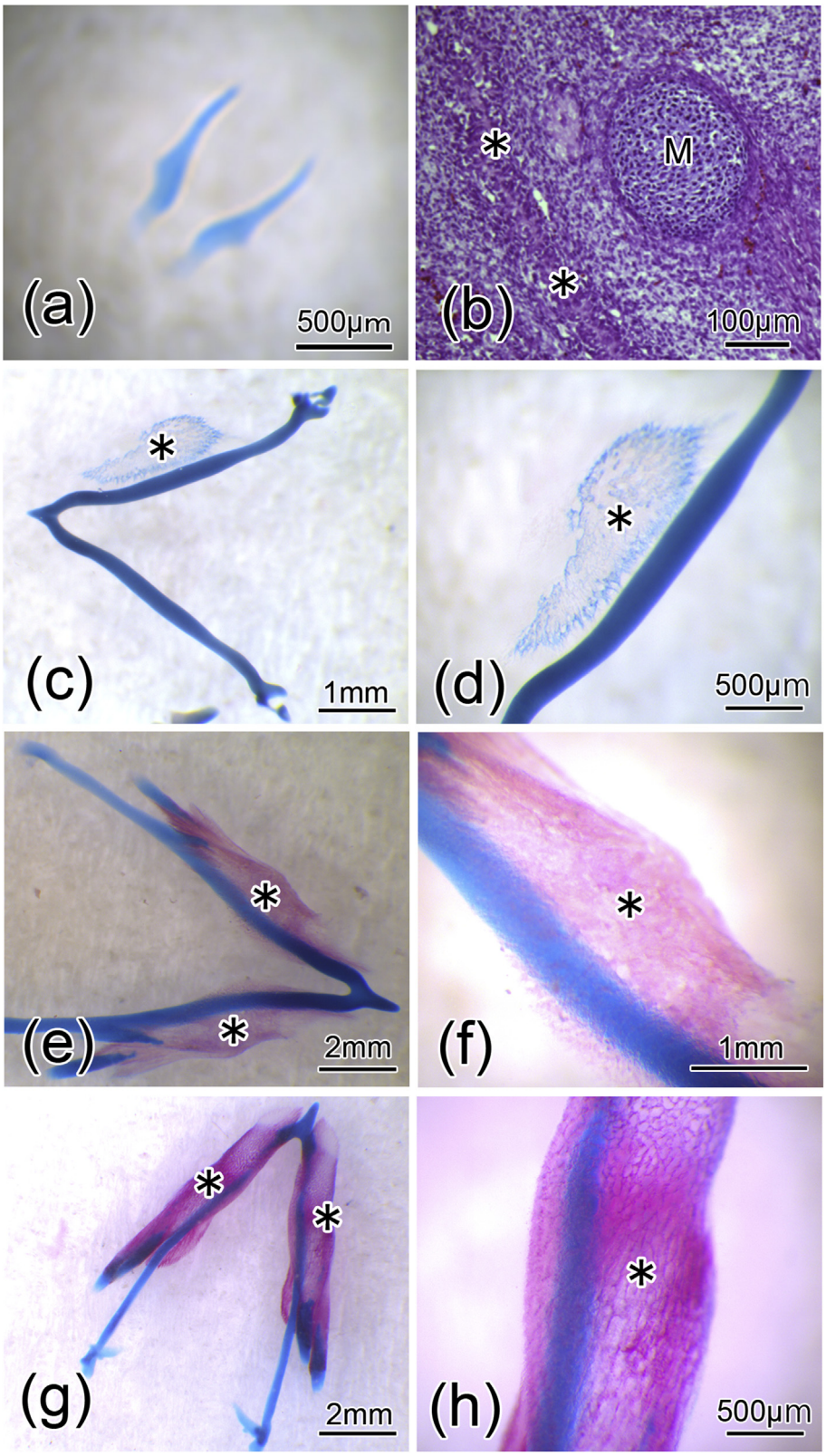


**Fig. 6.** Whole-mount specimens of rat mandibles at embryonic day 15 (E15) (a), Embryonic day 16 (E16) (c, d), Embryonic day 18 (E18) (e, f), and embryonic day 20 (E20) (g, h), and a specimen stained with hematoxylin and eosin of an embryonic day 15 (E15) rat mandible (b). Osteoid (*) is seen around Meckel's cartilage (M) on histology at E15 (b) but not seen in the whole mount specimen (a). Mandibular bone (*), stained with alizarin red, expands around Meckel's cartilage, stained with alcian blue, during embryonic development (c–h).
* Adapted from Henmi et al. (2017) [7]. With permission from Biomedical Research (Tokyo).

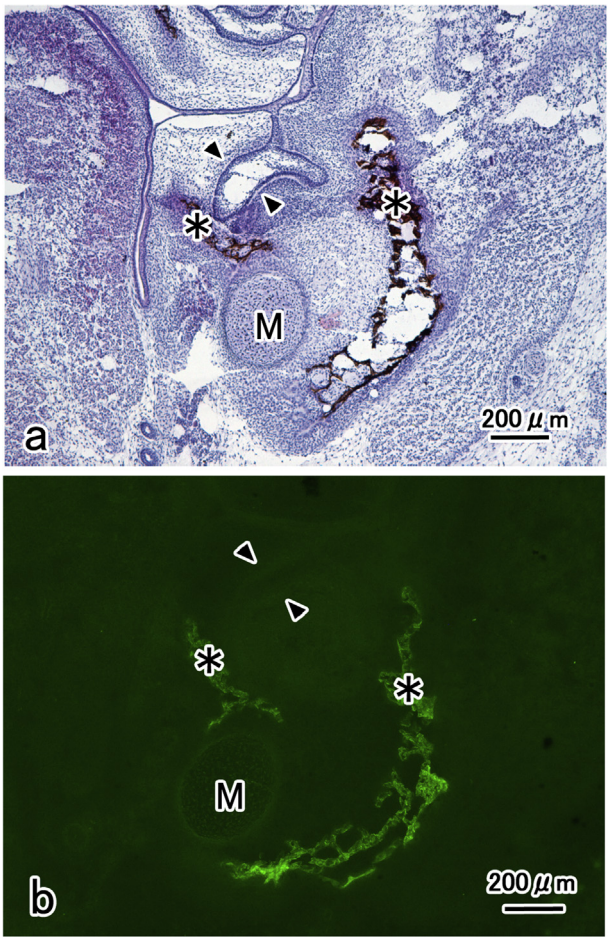


**Fig. 7.** In situ zymography for enzymatic activity of MMPs (gelatinases) in rat mandibles at embryonic day 18 (E18) (a) with corresponding histology of an adjacent section processed with von Kossa and hematoxylin and eosin staining (b) The gelatinolytic activity is localized in forming bone (*). M: Meckel's cartilage, arrowheads: tooth germ. Scale bar = 200 μm.

enamel proteins to allow the crystals to grow until adjacent crystals are in contact, which occurs during the advanced stages of enamel formation [3]. It has been suggested that the maturation of enamel crystals is regulated in part by proteolysis of amelogenin, a major constituent of enamel proteins [4]. Amelogenin may regulate or prevent the mineral deposition process and maturation of the mineral.

In a previous study, we had suggested that the maturation of mineral crystals in the enamel involves degradation of organic components such as proteins [5]. Enamel development in rat mandibular incisors was analyzed by scanning electron microscopy with energy dispersive X-ray spectroscopy (SEM-EDX). Distributions and concentrations of calcium (Ca) and phosphorus (P), which represent the calcium phosphate minerals, and carbon (C), which represents the organic components such as proteins, were examined [6,7]. The concentrations of Ca and P increased, while that of C decreased during the early maturation stage, with these effects being more pronounced in the late maturation stage. The Ca/P ratio increased in the late maturation stage that seemed to correlate with the decrease in C (or the organic components). Enzymes such as MMP20 and KLK4 may be involved in the increase of the Ca/P ratio, thereby modifying the chemical composition and facilitating maturation of the calcium phosphate mineral (Figs. 1 and 2).

## 3. Bone development

Information on the mechanisms that drive the calcification process in bones is limited compared to those involving the enamel.In bone, the ECM molecules accumulate during the embryonic development before mineral crystals are deposited [7–13]. Calcification begins with the deposition of calcium phosphate and proceeds through the maturation and increase in the mineral crystals in the ECM. It has been proposed that the matrix vesicles budded from the plasma membrane of the osteoblasts accumulate calcium and phosphate ions extracellularly and deposit calcium phosphate minerals on the collagen fibrils. Calcification proceeds toward maturation by transformation of the calcium phosphate deposits into the more crystalline apatite and by propagation of the apatite deposition [14–22]. However, the mechanisms governing the propagation of mineral deposition during bone development after the initial mineral deposition by the matrix vesicles is not fully understood [6].

It has been postulated that some bone ECMs or proteins are degraded during bone development and the degradation may contribute to the maturation of the calcium phosphate mineral [23–27], as described in the case of enamel development. We investigated the calcification process of rat calvarial bone during development by analyzing the atomic concentrations and distributions of Ca, P, and C using SEM-EDX. The changes in the crystal structure were examined using X-ray diffraction (XRD) and Fourier transform infrared spectroscopy (FTIR) [6].

Stoichiometric hydroxyapatite (HA) ($Ca_{10}(PO_4)_6(OH)_2$) occurs as a hexagonally packed crystal [28]. In contrast, the biological apatite crystals in bones and teeth consist of poorly crystalline HA with a low Ca/P molar ratio, and contain foreign ions, such as carbonate and fluoride [29–31]. Rat calvarial bone formation starts around embryonic day 16 (E16). XRD and FTIR analysis showed that the amorphous structure of the minerals at E16 gradually transformed into poorly crystalline HA, while the proportion of protein to mineral decreased until postnatal week 6 (Figs. 3–5) [6]. This study suggested that the chemical composition and crystal structure mature while the proportion of organic components such as proteins in the bone matrix decreases during calvarial bone development.

In another study, we reported the comparable process of calcification in rat mandibular bone development using micro-computed tomography (micro-CT) and SEM-EDX [7]. It was suggested that the rat mandibular bone formation is initiated around Meckel's cartilage at embryonic day 15 (E15), and deposition and maturation of the calcium phosphate minerals as well as a decrease in organic components occurs during the development of rat mandibles (Fig. 6).

MMPs break down the ECM, which is required for embryonic development, morphogenesis, and tissue remodeling [32,33]. Gelatinases, a type of MMPs, degrade proteins, such as proteoglycans, that inhibit calcification [24,26,34]. The event of protein degradation has been presumed to facilitate calcification [35] during bone development similar to that of enamel development [27,36–38].

MMP 13 expression in osteoblasts has been reported [39–42]. We have previously reported the gene expression profiles of MMPs 2, 8, and 13 in osteoblasts and osteocytes during bone development in rat mandibles and hind limbs [36,43]. In situ zymography analysis demonstrated the enzymatic activity of MMPs (gelatinases and collagenases) in osteogenic regions of developing mandibular bone (Fig. 7) [38]. Bones of MMP2-deficient mice have been reported to display progressive loss of mineral density or decreased calcification [44,45]. Osteoblasts and osteocytes that secrete MMPs may degrade specific ECM proteins that they themselves produce to advance the calcification process. These ECM proteins may play an

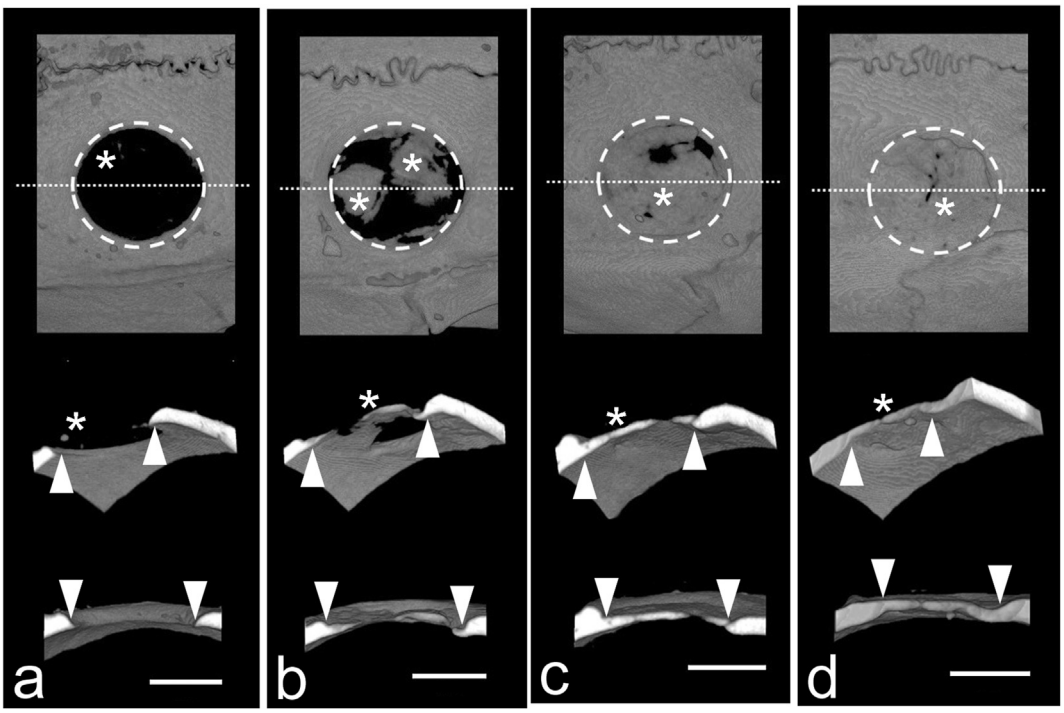


**Fig. 8.** Micro-computed tomography images of bone healing in weeks 1 (a), 2 (b), 4 (c), and 8 (d). The upper images are elevated views of the defect area (dashed circle) from the skin side. The images of the defect area were sectioned along the midline (dashed line), as shown in the middle and lower images. The middle images are views from the dura mater side, and the lower images are views of the mid-plane of the defect area. The periphery of. the defect is indicated with arrowheads. Scale bars = 2000 μm * Adapted from Okata et al. (2015) [52]. With permission from John Wiley & Sons Ltd.

important role in initial bone development. However, they may also prevent calcification from proceeding and, therefore, may be eliminated during calcification [27].

Matrix vesicles have been reported to contain MMPs 2 and 9 [46,47]. These MMPs may degrade ECM proteins such as proteoglycans and may initiate calcification [24,26,34]. Osteoblasts and osteocytes may further degrade the ECM proteins using MMPs and other enzymes to propagate calcification after the initial deposition of calcium phosphate mineral initiated by the matrix vesicles [27].

We have earlier reported the expression of another family of extracellular proteases known as disintegrins, and metalloproteinases with thrombospondin type 1 motifs (ADAMTSs) in developing bones [37,48]. ADAMTSs 1, 4, and 5 were identified in some osteoblasts. These enzymes may degrade proteoglycans such as versican [49] produced by the osteoblasts and accumulated in the extracellular matrix during bone development. It has been suggested that ADAMTSs cooperate with MMPs to remodel extracellular matrices [50,51]. This interaction may drive calcification during bone development.

## 4. Bone healing

Calcification during bone development shows characteristic extracellular events such as the increase and maturation of the mineral crystals and degradation of the extracellular organic matrices. We examined whether bone healing involves comparable events [52].

We studied an experimental model of standard defects on rat parietal bone [27,53], and calcification in the defect during healing was examined with micro-CT and SEM-EDX. The mineral density of the healing bone increased with time. The Ca/P ratio increased, while the ratios of C/Ca and C/P decreased in the healing bone matrix. The results suggested that, as bone heals, the mineral content increases in density and matures in quality, while the organic components decrease (Figs. 8 and 9) [52].

Another of our studies using the same experimental model investigated the gene expression profiles of MMPs 2, 8, and 13 in osteoblasts and osteocytes of the healing bone in addition to type I collagen and osteocalcin [27,54]. The findings suggested that during healing, calcification proceeds when osteoblasts and osteocytes using MMPs, degrade autogenic extracellular matrices and propagate mineral deposition in the bone matrix [52]. This process is similar to that of bone development.

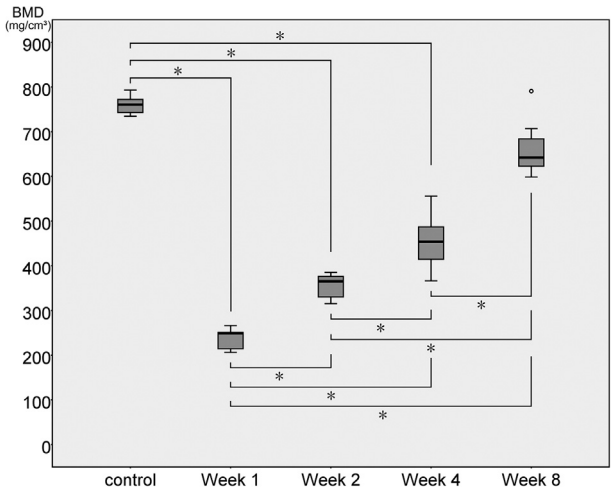


**Fig. 9.** Bone mineral density of healing bone in the defect in weeks 1, 2, 4, and 8 and in the controls. *I < 0.05 *Adapted from Okata et al. (2015) [52]. With permission from John Wiley & Sons Ltd.

## 5. Dentin development

Calcification in dentin has been shown to involve matrix vesicles derived from odontoblasts. The matrix vesicles are thought to provide nucleation sites for calcification of the initial dentin to form the outer layer, known as mantle dentin. In contrast, calcification of the inner layer of dentin, known as circumpulpal dentin, proceeds through the spread of mineral deposition from the pre-existing calcified outer dentinal matrix [55,56]. We examined the process by which the dentin calcification proceeds from the initial dentin [5].

The Ca/P ratio was significantly lower in the initial than in the advanced dentin. In contrast, the ratio was not significantly different among the more developed forms of dentin such as predentin, circumpulpal dentin, and mantle dentin. The initial dentinal matrix, when compared to the other parts of dentin and predentin, may possess distinctive mineral characteristics. The low Ca/P ratio may reflect primary calcification occurring in the initial dentinal matrix, similar to the calcification in initial bone matrix [6]. The amorphous structure of calcium phosphate minerals may be involved in the initial dentinal matrix, whereas poorly crystalline HA may predominate in the predentin, circumpulpal dentin, and mantle dentin.

Enzymes such as MMPs expressed and secreted by odontoblasts [27,43,57] may degrade ECMs that inhibit calcification in the initial dentinal matrix to allow for the growth and concentration of mineral crystals, thus resulting in higher concentrations of Ca and P with a lower C concentration in the dentinal matrix [58,59].

## 6. Conclusions

Similar to that of enamel development, the degradation of some ECM proteins are involved in the calcification process during bone and dentin development and bone healing, as suggested by several authors including us.These ECM proteins may inhibit mineral deposition and calcification that is similar to the role of amelogenin during enamel development. Some candidates for the amelogenin equivalents in bones and dentin could be proteoglycans [58,59], but a conclusive evidence has not been found yet. Further studies are required to elucidate the regulatory mechanisms of bone and dentin calcification in light of specific ECM proteins that prevent calcification and enzymes that degrade these ECM proteins.

## Ethical statement

All the animal experiments in our studies were approved by the Animal Research Committee of Tohoku University.

## Conflict of interest

The authors have no conflict of interest to declare.

## CRediT authorship contribution statement

**Yasuyuki Sasano:** Writing - original draft. **Megumi Nakamura:** Writing - review & editing. **Akiko Henmi:** Writing - review & editing. **Hiroshi Okata:** Writing - review & editing. **Osamu Suzuki:** Writing - review & editing. **Atsuko Kayaba:** Writing - review & editing. **Miyuki Mayanagi:** Writing - review & editing.

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