Biomedical Research **34** (3) 119-128, 2013

Histochemical aspects of the vascular invasion at the erosion zone of the epiphyseal cartilage in MMP-9-deficient mice

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(Received 4 December 2012; and accepted 14 March 2013)

ABSTRACT

We have histologically examined vascular invasion and calcification of the hypertrophic zone during endochondral ossification in matrix metalloproteinase (MMP)-9 deficient (MMP-9$^{-/-}$) mice and in their littermates at 3 days, 3 weeks and 6 weeks after birth. Capillaries and osteoclasts at the chondro-osseous junction showed an intense MMP-9 immunopositivity, suggesting that they recognize chemical properties of cartilaginous matrices, and then release MMP-9 for cartilage degradation. CD31-positive capillaries and tartrate-resistant acid phosphatase-reactive osteoclasts could be found in the close proximity in the region of chondro-osseous junction in MMP-9$^{-/-}$ mice, while in wild-type mice, vascular invasion preceded osteoclastic migration into the epiphyseal cartilage. Although MMP-9$^{-/-}$ mice revealed larger hypertrophic zones, the index of calcified area was significantly smaller in MMP-9$^{-/-}$ mice. Interestingly, the lower layer of the MMP-9$^{-/-}$ hypertrophic zone showed intense MMP-13 staining, which could not be observed in wild-type mice. This indicates that MMP-13 may compensate for MMP-9 deficiency at that specific region, but not to a point at which the deficiency could be completely rescued. In conclusion, it seems that MMP-9 is the optimal enzyme for cartilage degradation during endochondral ossification by controlling vascular invasion and subsequent osteoclastic migration.

Endochondral ossification enables the longitudinal growth of long bones, keeping the balance between chondrocyte proliferation and differentiation (*i.e.,* appositional and interstitial growth) and vascular invasion into cartilage prior to bone deposition (12, 27). The length of the hypertrophic zone appears to be maintained by two mechanisms: 1) the rate at which chondrocytes enter the hypertrophic phenotype and 2) the pace at which vascular endothelial cells invade the hypertrophic zone of the cartilage. After the formation of calcified cartilage matrices, the process of endochondral ossification involves a well-defined series of events in which osteogenic and osteoclastic cells replace calcified cartilage for bone (2). At an erosion zone, vascular endothelial cells invade and collapse incompletely-calcified transverse partitions of cartilage matrix, making a way for osteoclastic and osteoblastic migration. On the other hand, the longitudinal intercolumnar septae are

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structures that calcify after matrix vesicle secretion by hypertrophic chondrocytes (2, 4), serving as scaffolds for the primary trabecular spiculae. Osteoclasts, also referred to as chondroclasts when found at the chondro-osseous junction, resorb the excessive calcified cartilage matrix from longitudinal intercolumnar septae (2, 15, 20), thereby modeling the calcified cartilage parallel to the longitudinal axis of long bones. Therefore, hypertrophic chondrocytes and vascular endothelial cells are crucial for normal endochondral ossification, synthesizing matrix vesicles for cartilage calcification and promoting vascular invasion into the chondro-osseous junction, respectively (2, 4, 15, 20, 21, 27).

Endochondral ossification requires proteinase-driven proteolytic degradation. In that context, matrix metalloproteinases (MMPs) are of paramount importance due to their ability to cleave collagens and aggrecan, the main components of cartilaginous matrices (5, 9). MMP-9, a gelatinase that breaks down components of the extracellular matrix and is highly specific for degraded collagens, is expressed at sites of active tissue remodeling and neovascularization. MMP-9 plays a key role in endochondral ossification by enabling capillary invasion into the hypertrophic cartilage (26). At the erosion zone of the cartilage anlage, MMP-9 was shown to be present in preosteoclasts and in chondroclastic cells. MMP-9 deficient (MMP-$9^{-/-}$) mice have a distinct phenotype, with a substantially larger hypertrophic zone in the epiphysis, impaired endochondral ossification and delayed formation of the bone marrow cavity (26). MMP-$9^{-/-}$ mice showed decreases in trabecular bone formation, chondrocyte apoptosis, vascular invasion and osteoclastic recruitment during development (6, 26).

These findings support the idea that MMP-9 plays a key role in normal endochondral ossification. However, some issues are yet to be addressed: 1) Which cells are the most important for triggering the process of cartilage degradation during endochondral ossification, osteoclasts (chondroclasts) or vascular endothelial cells? 2) Is osteoclastic migration and vascular invasion into hypertrophic chondrocytes altered in an MMP-9-deficient environment? And 3) Does the absence of MMP-9 affect calcification in the epiphyseal cartilage, especially at the hypertrophic zone?

In order to address these questions, we conducted histological examinations on the endochondral ossification in MMP-$9^{-/-}$ mice, aiming specifically at the cellular events taking place at the erosion zone, so that the biological function of vascular endothelial cells in endochondral ossification could be clarified.

MATERIALS AND METHODS

Tissue preparation. All animal procedures were performed in accordance with the guidelines for animal experimentation set by Keio University and Hokkaido University. Three day-, 3 week- and 6 week-old MMP-$9^{-/-}$ mice and their wild-type littermates (n = 6 for each, Jackson Laboratory, Bar Harbor, ME), were anesthetized with an intraperitoneal injection of chloral hydrate and perfused through the cardiac left ventricle with 4% paraformaldehyde diluted in 0.1 M cacodylate buffer (pH 7.4). Tibiae were dissected free of soft tissues and immersed in the same fixative for additional 24 h at 4°C. After decalcification with 5% EDTA-2Na solution for 3 weeks at 4°C, the specimens were dehydrated through a graded series of ethanol prior to being embedded in paraffin. The stained sections were observed under a Nikon Eclipse E800 microscope (Nikon Instruments Inc. Tokyo, Japan), and light microscopic images were acquired with a digital camera (Nikon DXM1200C, Nikon).

Histochemistry for MMP-9, cathepsin K, CD31 and tartrate-resistant acid phosphatase (TRAP). For detection of TRAP activity, as previously reported (3), histological sections were incubated with a mixture of 2.5 mg of naphthol AS-BI phosphate (Sigma, St. Louis, MO), 18 mg of red violet LB salt (Sigma) and 100 mM L (+) tartaric acid (0.76 g; Nacalai Tesque, Kyoto, Japan) diluted in 30 mL of a 0.1 M sodium acetate buffer (pH 5.0) for 15 min at 37°C.

For immunolocalization of MMP-9, -13 and cathepsin K, deparaffinized sections were treated with 0.1% hydrogen peroxide for 15 min to inhibit endogenous peroxidase, and pre-incubated with 1% bovine serum albumin in phosphate buffered saline (BSA-PBS) for 30 min at room temperature. Goat antibody against mouse MMP-9 (R&D Systems, Inc., Minneapolis, MN; dilution 1 : 100), goat antibody to MMP-13 (Chemicon International Inc., Temecula, CA; dilution of 1 : 100), or mouse anti-human cathepsin K (Daiichi Fine Chemical Co., Ltd., Takaoka, Japan; dilution 1 : 200) were applied to the sections overnight at 4°C. The sections were then incubated with horseradish peroxidase (HRP)-conjugated anti-goat IgG (American Qualex Antibodies, San Clemente, CA; dilution 1 : 100) or HRP-conjugated anti-mouse IgG (Chemicon; dilution 1 : 100) at room temperature, respectively. For CD31 detection, histological sections were pretreated

with proteinase K at room temperature for 15 min, and incubated with rat anti-CD31 antibody (BD Bioscience, Qume Drive, CA). After rinsing with PBS, sections were immersed in HRP-conjugated anti-rat IgG (Kirkegaard & Perry Laboratories, Gaithersburg, MD) for 1 h. For negative control, sections were incubated with HRP-conjugated secondary antibodies only. Immune complexes were visualized using 3,3'-diaminobenzidine tetrahydrochloride (Dojindo Laboratories, Kumamoto, Japan). For double staining of CD31/TRAP, MMP-9/TRAP and MMP-13/TRAP, we performed immunohistochemistry for CD31, or MMP-9 and -13, and then, detected TRAP activity as described.

Von Kossa staining. Longitudinal sections of tibial epiphyseal cartilage embedded in epoxy resin were incubated with an aqueous solution of nitric silver until calcified matrix is visible in dark brown color, as reported elsewhere (22).

Statistical analyses for calcified areas in the hypertrophic zone, and the distance between vascular endothelial cells and osteoclasts at the chondro-osseous junction. The hypertrophic zone was identified according to chondrocyte morphology, as hypertrophic chondrocytes feature enlarged, translucent cell bodies. Proliferative and hypertrophic zones of wild-type and MMP-9$^{-/-}$ mice (3 day, 3 and 6 weeks after birth, n = 6 each) were measured with ImagePro Plus 6.2 (Media Cybernetics, Silver Spring, MD), as previously reported (10, 11). The areas of calcified cartilage matrix were also measured after von Kossa staining with the aid of ImagePro Plus (3 weeks after birth, n = 6). The longitudinal distance between invading CD31-positive endothelial cells and neighboring TRAP-positive osteoclasts at the erosion zone (3 weeks after birth, n = 6) was calculated with images taken at 40x magnification. Statistical analysis was performed using Microsoft Excel 2003 (Microsoft Corporation, Redmond, WA). All values are presented as means ± standard deviation. Differences among groups were assessed by unpaired Student's *t*-test, and considered statistically significant when $P < 0.05$.

RESULTS

Larger hypertrophic zone in MMP-9$^{-/-}$ mice and distribution of TRAP, cathepsin K and MMP-9 in wild-type and MMP-9$^{-/-}$ metaphyses

MMP-9$^{-/-}$ mice showed larger growth plate cartilage, which became more evident 3 weeks postnatally (Compare growth plates in Figs. 1B and E). Although the extent of the proliferative zone was similar between the wild-type and MMP-9$^{-/-}$ mice, the hypertrophic zone was enlarged after birth and seemed to protrude towards the center of the bone after 3 weeks (See an arrow in Fig. 1E). Statistical analysis on the areas of the hypertrophic (Fig. 1G) and proliferative (Fig. 1H) zones revealed that only the MMP-9$^{-/-}$ hypertrophic zone was higher up than those of wild-type mice at 3 days and 3 weeks of age. Interestingly, MMP-9$^{-/-}$ mice possessed more metaphyseal trabeculae, at least during the experimental period (Compare Figs. 1A–C and 1D–F). Since 3-week-old MMP-9$^{-/-}$ mice featured the most obvious phenotype, we decided to use this time point to examine the distribution of TRAP, cathepsin K and MMP-9. Both TRAP and cathepsin K activities were found in osteoclasts located at the chondro-osseous junction, with similar intensity in both wild-type and MMP-9$^{-/-}$ specimens (Compare Figs. 2 A, B, D, E). Osteoclasts and vascular endothelial cells were positive for MMP-9 in the erosion zone of wild-type metaphyses, but MMP-9 staining was absent in the hypertrophic zone of wild-type mice (Fig. 2C). In contrast, MMP-9$^{-/-}$ specimens showed hardly any immunoreactivity for MMP-9 (Fig. 2F). In wild-type specimens, osteoclasts at the chondro-osseous junction showed intense MMP-9 staining, but those at the termini of the metaphyseal trabecules revealed strong TRAP activity and weak staining of MMP-9 (Figs. 2G–I).

Intense MMP-13 immunoreactivity in the lower region of the MMP-9$^{-/-}$ hypertrophic zone

Unlike cathepsin K and TRAP, which were similarly present in wild-type and in MMP-9$^{-/-}$ specimens at the third postnatal week, an intense MMP-13 immunoreactivity was seen in the bottom layer of the MMP-9$^{-/-}$ hypertrophic zone (Compare Figs. 3A and B). At higher magnification, it was the cartilage matrix, rather than osteoclasts at the erosion zone, that appeared to have a strong MMP-13 immunoreactivity in the MMP-9$^{-/-}$ hypertrophic zone (Figs. 3C, D). Many granular cartilaginous matrices were also immunopositive for MMP-13. Both wild-type and MMP-9$^{-/-}$ mice showed a slight MMP-13 positivity on the bone surface facing osteoclasts in the terminal regions of metaphyseal trabeculae (Figs. 3E, F). Thus, MMP-13 may be over-produced at the chondro-osseous junction in a state of MMP-9 deficiency.

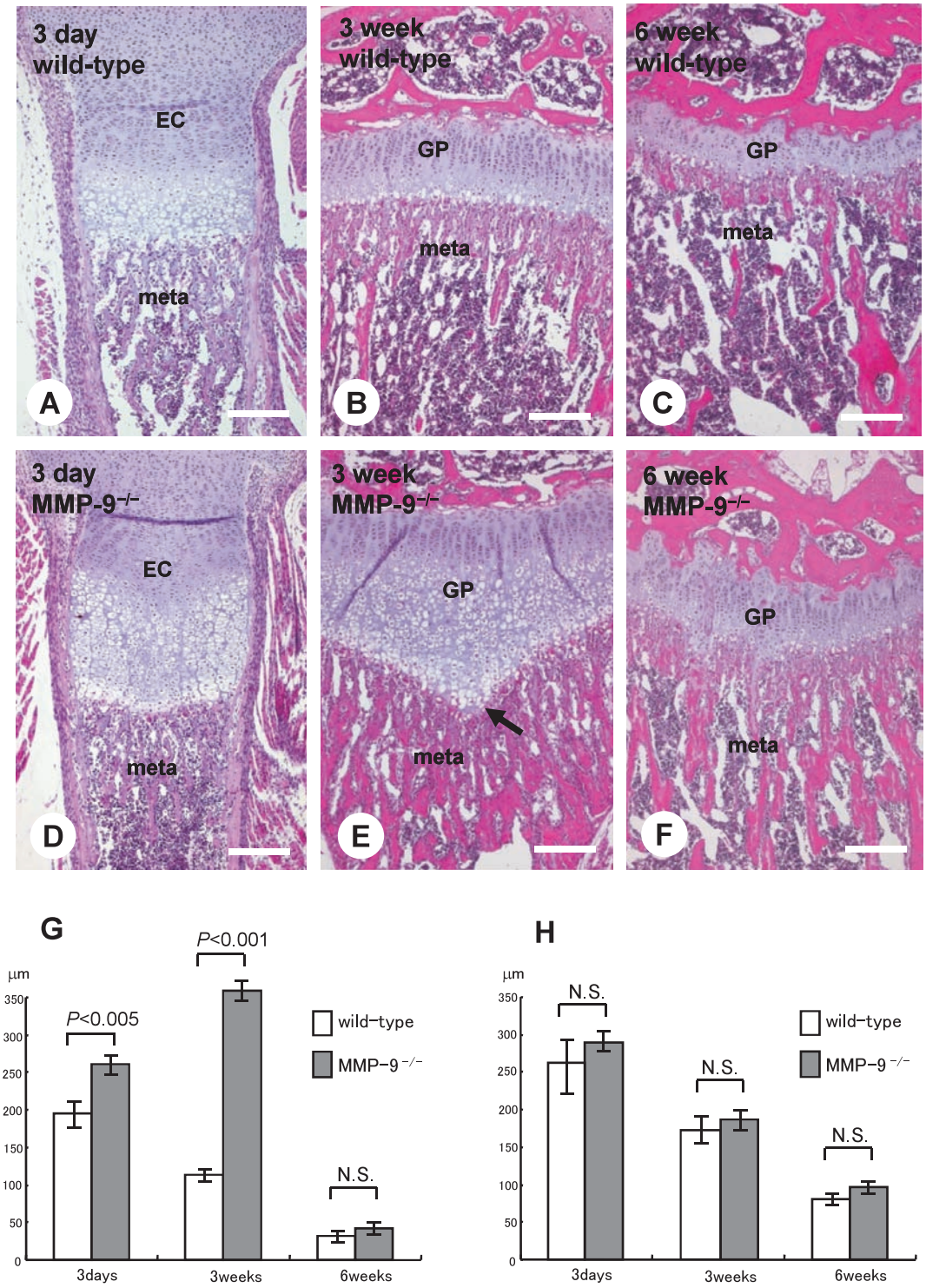

Fig. 1 Histological analysis of the epiphyseal cartilage in wild-type and MMP-9$^{-/-}$ mice. The epiphyseal cartilage (EC) of MMP-9$^{-/-}$ tibiae at 3 days (**D**) and 3 weeks (**E**) after birth show a relatively enlarged hypertrophic zone compared with that seen in their wild-type counterparts (**A**, **B**). The hypertrophic zone in the MMP-9$^{-/-}$ growth plate (GP) at 3 weeks postnatally is enlarged and protruded towards the metaphysis (an arrow, meta) (**E**). However, the expanded MMP-9$^{-/-}$ growth plate (GP) seems to be normalized, or shortened, at 6 weeks postnatally (Compare **C** and **F**). Notice that there are more metaphyseal trabeculae in MMP-9$^{-/-}$ specimens at 3 and 6 weeks. Panels **G** and **H** represent the statistical analysis of the indices for the areas of hypertrophic (**G**) and proliferative zones (**H**). There is a significant increase in the index for the hypertrophic zone in MMP-9$^{-/-}$ tibiae at 3 days and 3 weeks after birth. Bars, 250 μm

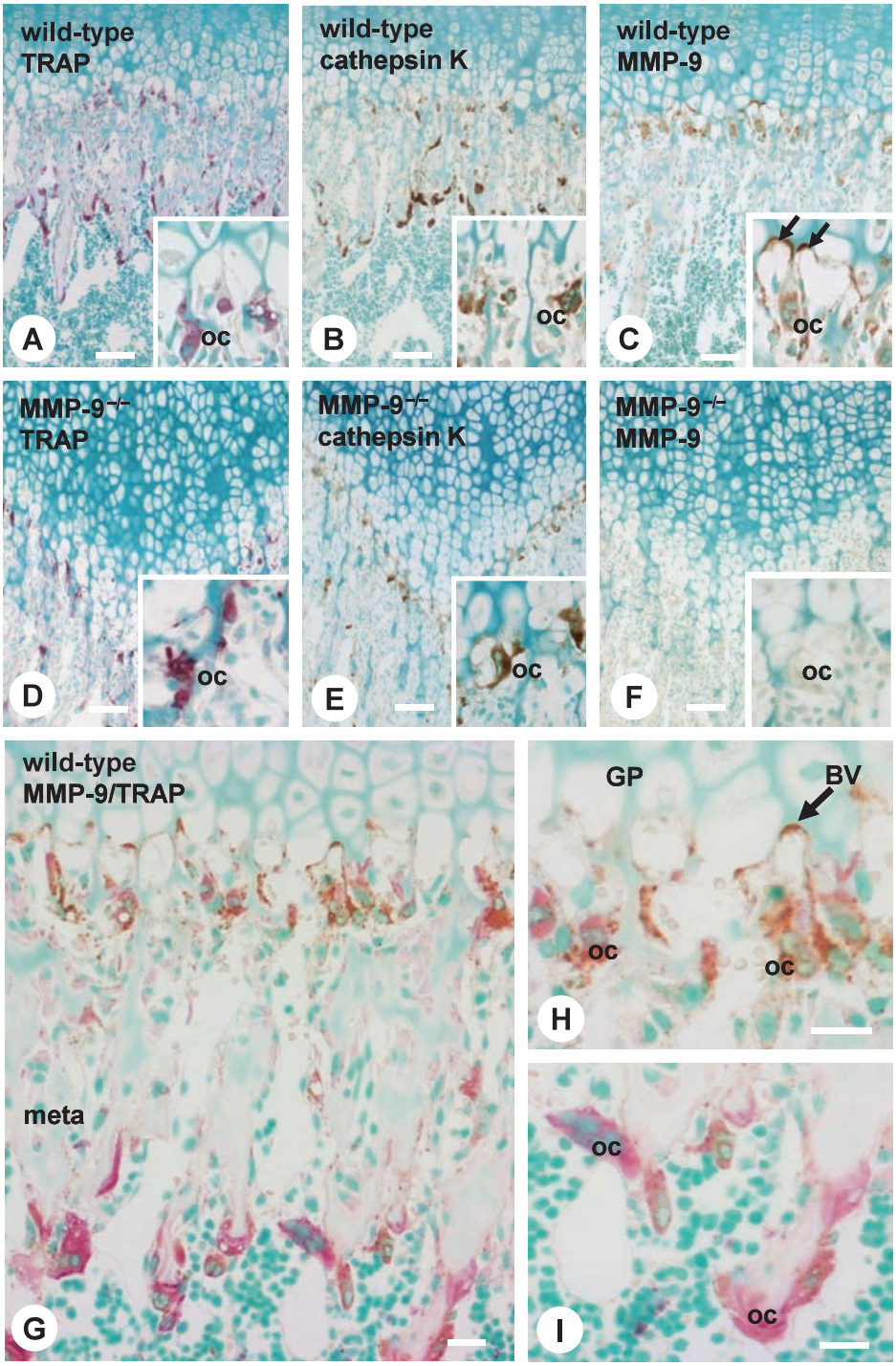

Fig. 2 Distribution of TRAP, cathepsin K and MMP-9 at the chondro-osseous junction. Our immunohistochemistry demonstrates the similar staining of TRAP (**A**, **D**) and cathepsin K (**B**, **E**) in osteoclasts (oc), also referred to as chondroclasts, at the chondro-osseous junction between the wild-type (**A**, **B**) and MMP-9$^{-/-}$ (**D**, **E**) specimens. MMP-9-positive osteoclasts (oc) and blood vessels (arrows) are seen mainly at the chondro-osseous junction of wild-type mice (**C**), whereas no MMP-9 positivity is observed in MMP-9$^{-/-}$ specimens (**F**). Insets indicate highly magnified images of osteoclasts showing TRAP (**A**, **D**), cathepsin K (**B**, **E**) and MMP-9 (**C**, **D**). Double staining for TRAP and MMP-9 in the wild-type specimens reveals that osteoclasts at the chondro-osseous junction are stained more intensely for MMP-9 (brown) rather than for TRAP (red) (**G**). Note osteoclasts (oc) and blood vessels (BV, an arrow) are intensely positive for MMP-9 (brown color) at the chondro-osseous junction (**H**), while osteoclasts (oc) located on the termini of metaphyseal trabeculae show strong TRAP positivity (red, **I**). Bars, A–F: 50 μm, G–I: 30 μm

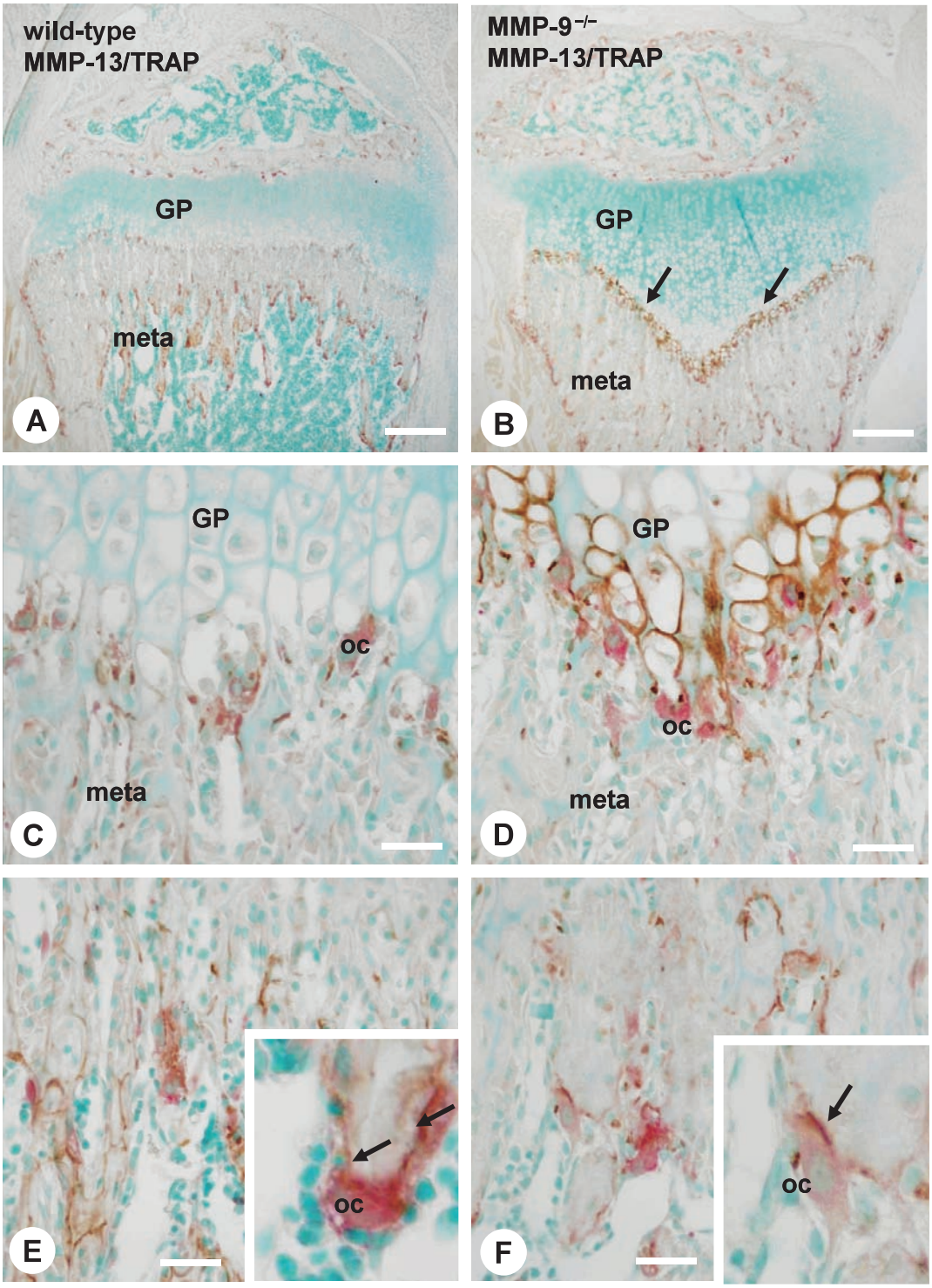

Fig. 3 Immunolocalization of MMP-13 in the wild-type and MMP-9$^{-/-}$ tibiae. Epiphyseal growth plate (GP) and metaphyses (meta) of wild-type (**A**) and MMP-9$^{-/-}$ (**B**) tibiae, showing MMP-13 positivity (brown color) and TRAP-reactive cells (red color). See the MMP-13 positive band (arrows) in the most inferior of the MMP-9$^{-/-}$ hypertrophic zone (**B**). At higher magnification, the cartilage matrix exhibits an intense immunoreactivity of MMP-13 (brown color) at the chondro-osseous junction (**D**), while no obvious positivity for MMP-13 is seen in wild-type counterpart (**C**). Note many granular cartilaginous matrices immunopositive for MMP-13 (Dots intensely stained in dark brown in **C** and **D**). In the trabecular termini, wild-type (**E**) and MMP-9$^{-/-}$ (**F**) tibiae show bone surfaces (arrows) slightly stained for MMP-13, which are close to osteoclasts (oc). Bars, A, B: 150 μm, C–F: 30 μm

Unevenly-gathered osteoclasts and vascular endothelial cells at the chondro-osseous junction of MMP-9$^{-/-}$ specimens

At the third postnatal week, double staining for CD31 and TRAP in wild-type specimens revealed endothelial cells piercing deep into the epiphyseal cartilage, preceding osteoclastic migration (Fig. 4A). In contrast, vascular endothelial cells and osteoclasts were disorderly gathered in MMP-9$^{-/-}$ mice (Fig. 4B). Observing semithin sections of epoxy resin-embedded specimens, wild-type vascular endothelial cells invaded into chondrocytic lacunae prior to osteoclastic migration (Fig. 4C): Wild-type vascular endothelial cells were invading ahead 20.0 ± 6.2 μm away from osteoclasts at the chondro-osseous junction. However, endothelial cells were adjacent to osteoclasts in MMP-9$^{-/-}$ chondro-osseous junction (Fig. 4D), with a markedly-smaller gap between these cell-types (1.04 ± 1.13 μm, $P < 0.005$) compared with that in the wild-type counterparts. Thus, the endothelial cells' invasion prior to osteoclastic migration in normal chondro-osseous junction was disturbed in MMP-9$^{-/-}$ specimens.

Cartilage calcification in the MMP-9$^{-/-}$ hypertrophic zone

Cartilage calcification was assessed by means of von Kossa staining, which showed that although most of the wild-type hypertrophic zone was calcified, only the inferior portion of the hypertrophic zone in MMP-9$^{-/-}$ specimens was calcified (Figs. 5A, B). At higher magnification, randomly running blood vessels and irregularly distributed calcified cartilage cores could be observed in MMP-9$^{-/-}$ tibiae, while blood vessels in wild-type specimens ran parallel to the longitudinal axis of the tibiae (Figs. 5C, D). Statistical analysis showed that the index of calcified areas in the hypertrophic zone was 79.2 ± 7.01% in

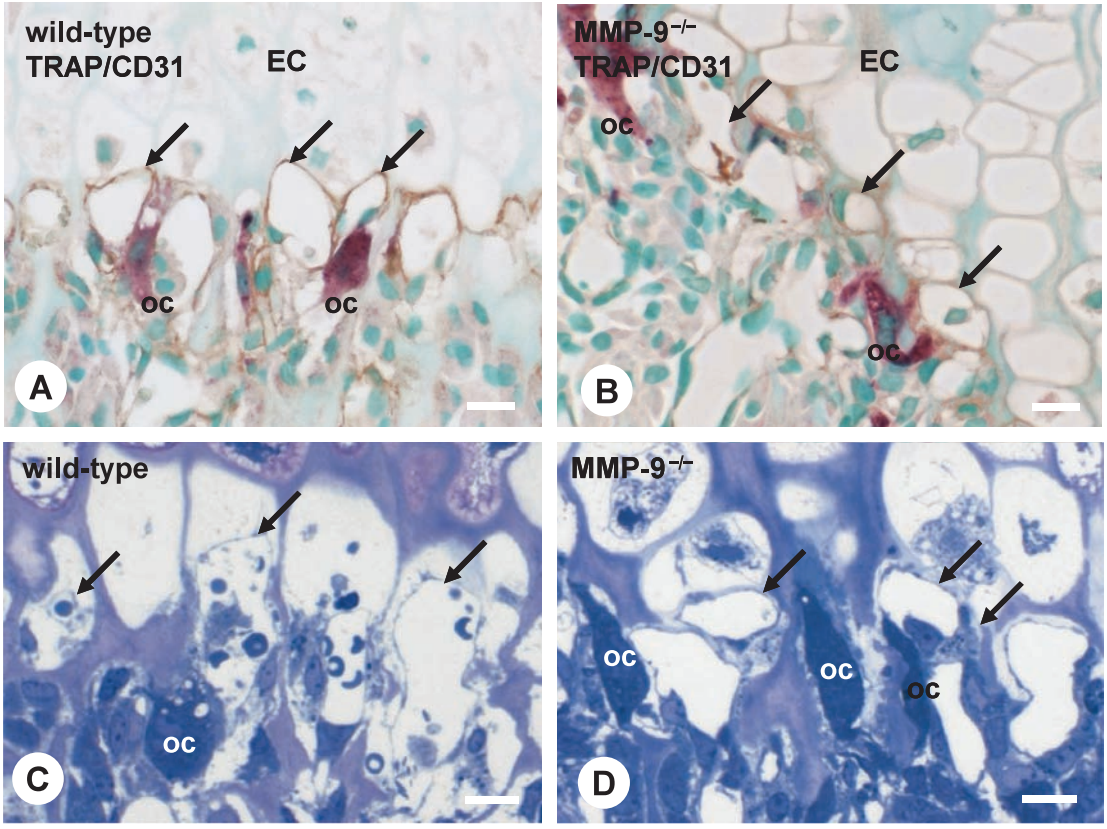

Fig. 4 Double staining TRAP and CD, and high-resolution observations at the chondro-osseous junction of wild-type and MMP-9$^{-/-}$ tibiae. CD31-positive endothelial cells (brown color, arrows), rather than osteoclasts (red, oc), are closer to the epiphyseal cartilage (EC) in the wild-type specimens (**A**), whereas vascular endothelial cells (black arrows) and osteoclasts (oc) are very close (**B**) in MMP-9$^{-/-}$ tibiae. Consistently, semithin sections demonstrate that wild-type vascular endothelial cells (black arrows) invade the chondrocytic lacunae further and prior to osteoclastic migration (oc) (**C**). However, MMP-9$^{-/-}$ osteoclasts (oc) and vascular endothelial cells (black arrows), on the other hand, are located in close proximity (**D**). Bars, A, B: 15 μm, C, D: 10 μm

the wild-type growth plate cartilage, and 27.1 ± 5.06% in the MMP-9$^{-/-}$ specimens (P<0.001) (Fig. 5E).

DISCUSSION

This study contributed to the previous report on the histology of MMP-9$^{-/-}$ mice (26) in the following ways: first and foremost, a large amount of MMP-13 was synthesized in the most inferior region of the MMP-9$^{-/-}$ hypertrophic zone, probably compensating for the lack of MMP-9; second, the distance between invading vascular endothelial cells and os-

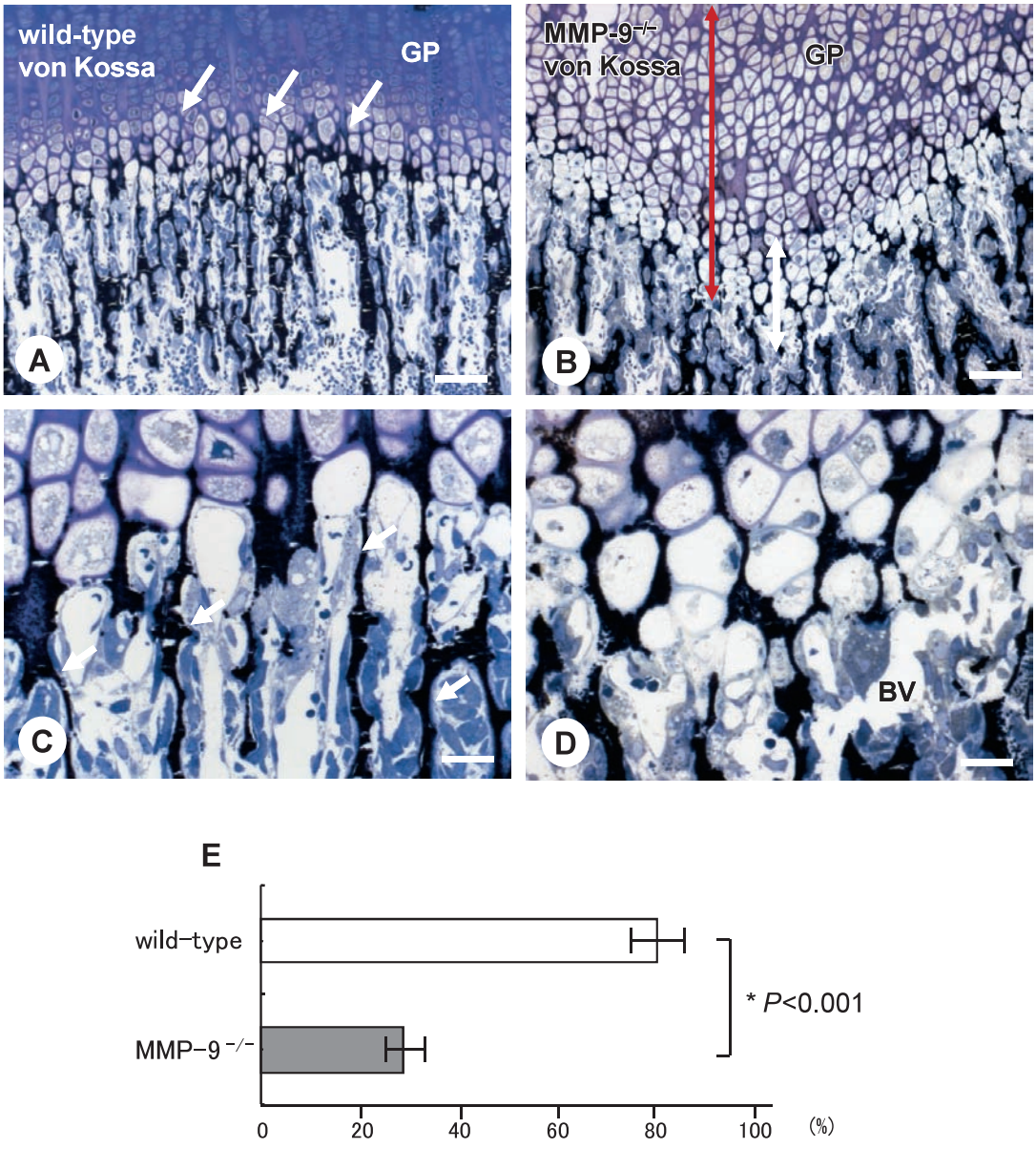

Fig. 5 Von Kossa staining on calcified cartilage matrix of the wild-type and MMP-9$^{-/-}$ hypertrophic zones. White arrows indicate the sites for initial calcification (von Kossa staining begins to be discerned) in wild-type mice (**A**). MMP-9$^{-/-}$ mice had large hypertrophic zone (a red arrow), but only the inferior portion (a white arrow) of the hypertrophic zone (**B**). Note that only the lower region of the MMP-9$^{-/-}$ hypertrophic zone is calcified, whereas the wild-type hypertrophic zone is mostly calcified. Panels C and D are highly magnified images of panels A and B, respectively. Longitudinal calcified primary trabeculae are seen (white arrows, **C**), while calcified cartilage cores (black) show a haphazard pattern in MMP-9$^{-/-}$ specimens (**D**). Notice the tangential arrangement of blood vessels (BV) in this region. Panel **E** represents the statistical analysis of the percentage of calcified areas in the hypertrophic zone. There is a significant difference between wild-type epiphyseal cartilage and MMP-9$^{-/-}$ epiphysis (P < 0.001). Bars, A, B: 50 μm, C, D: 20 μm

teoclasts was remarkably shortened in MMP-9$^{-/-}$ mice; and third, the areas of the calcified hypertrophic zone did not differ between wild-type and MMP-9$^{-/-}$ mice, but the amount of calcified cartilage was markedly reduced in MMP-9$^{-/-}$ mice.

Many reports have suggested osteoclasts and hypertrophic chondrocytes express MMP-9 (19, 24, 26), but some investigators demonstrated MMP-9 also in vascular endothelial cells in fetal stages (25). Consistent with the latter report, our histochemical examinations verified an intense MMP-9 staining in vascular endothelial cells and osteoclasts at the chondro-osseous junction. It seems reasonable that vascular endothelial cells penetrating uncalcified cartilaginous partition would secrete MMP-9 for digesting type II collagen and proteoglycans (5, 9). Osteoclasts following the endothelial cells in the chondro-osseous junction also secreted MMP-9, whereas osteoclasts located on the trabecular termini revealed intense TRAP staining, but showed weak MMP-9 immunoreactivity. It seems likely that endothelial cells and osteoclasts recognize the biochemical properties of the cartilage matrix and then secrete specific proteolytic enzymes at the chondro-osseous junction. Without MMP-9, invasion of vascular endothelial cells may be slowed down, allowing osteoclasts to "catch up" with those cells at the chondro-osseous junction, and therefore, the endothelial cells and osteoclasts were seen in the close proximity in the region of chondro-osseous junction.

Proteolysis by MMP-13 was shown to be required for chondrocyte differentiation in the process of matrix calcification (28). Some reports demonstrated that MMP-13 is expressed in the hypertrophic zone, and it is highly effective for cleaving type II collagen (8, 13, 16). Others have showed MMP-13 synthesis also by osteoclasts (18). In this study, MMP-13 was overexpressed in the most inferior region of the MMP-9$^{-/-}$ hypertrophic zone at the third postnatal day, which strongly indicates that it partially compensates for MMP-9 deficiency. Indeed, Nagai and Aoki reported on the inhibition of growth plate angiogenesis and endochondral ossification with diminished expression of MMP-13 in hypertrophic chondrocytes (17). Such compensation, however, may not be enough to effectively degrade the cartilage, at least up to the third postnatal week. Our observations on 6 week-old MMP-9$^{-/-}$ mice showed a relatively normal height of the epiphyseal growth plate. This may be due to: 1) the rate of chondrocytic proliferation in the proliferative zone, and subsequent transition into the hypertrophic phenotype, which might be slower as mice grow older, and 2) the substitutive function of other proteolytic enzymes such as MMP-13, which also carry on cartilage degradation.

One may wonder why cartilage calcification occurred only in the bottom layer of the MMP-9$^{-/-}$ hypertrophic zone, despite the fact that cells in the upper layer of the hypertrophic zone might secrete matrix vesicles that can form calcified nodules in the intercolumnar septa (2, 20, 21). One possible explanation for this phenomenon is that invading vascular endothelial cells may modulate cartilage calcification carried out by hypertrophic chondrocytes. In the embryo, the center of the cartilage rudiment is composed of hypertrophic chondrocytes. Then, a vascular network forms outside the perichondrium, where calcification occurs (23). Alternatively, some reports suggest that hypertrophic chondrocytes secrete vascular endothelial growth factor (VEGF) targeted to invading endothelial cells, thereby guiding their invasion into the cartilage (7, 12, 14). Thus, cellular interactions between hypertrophic chondrocytes and invading vascular endothelial cells might enable normal endochondral ossification. A recent report indicates that there is an interesting connection among MMP-9, VEGF and osteoclast function during endochondral ossification, even though the functions of VEGF and MMP-9 in endochondral ossification do not completely overlap (19).

While there were reports on the lessened trabecular bone formation during the development of MMP-9$^{-/-}$ mice (6, 26), in this study (as shown in Fig. 1) we found more metaphyseal trabeculae in MMP-9$^{-/-}$ mice than in their wild-type counterparts. We could not clarify why we and others had such discrepancy, but at least, it is likely that the reduced matrix degradation at the chondro-osseous junction may leave many cartilaginous cores behind, which may serve as templates for additional trabecular formation in the metaphyses. In the normal hypertrophic zone, cartilage columns are distributed parallel to the longitudinal axis of the long bone (1, 2, 21); however, MMP-9$^{-/-}$ hypertrophic chondrocytes were not longitudinally arranged (See Figs. 5A and 5D). In a normal state, the longitudinal intercolumnar septae are well calcified and serve as scaffolds for primary trabecular spiculae, which are formed parallel to the bone's longitudinal axis. Differently, calcified cartilage was not longitudinally displayed in the MMP-9$^{-/-}$ hypertrophic zone and, therefore, oblique or haphazardly oriented blood vessels were seen in this area. Furthermore, the unevenly-gathered vascular endothelial cells and osteoclasts may make the

spatial distribution of primary trabecules more disorganized.

In summary, our findings suggest that MMP-9 deficiency may distort the arrangement of calcified cartilage remnants and, consequently, the patterns of vascular invasion.

Acknowledgements

This study was partially supported by a grant from Japan Society for the Promotion of Science (Amizuka N, Maeda T, Saito C, and Kojima T).

REFERENCES

1. Amizuka N, Davidson D, Liu H, Valverde-Franco G, Chai S, Maeda T, Ozawa H, Hammond V, Ornitz DM, Goltzman D and Henderson JE (2004) Signalling by fibroblast growth factor receptor 3 and parathyroid hormone-related peptide coordinate cartilage and bone development. *Bone* **34**, 13–25.
2. Amizuka N, Hasegawa T, Oda K, Freitas PHL, Hoshi K, Li M and Ozawa H (2012) Histology of epiphyseal cartilage calcification and endochondral ossification. *Front Biosci* **4**, 2085–2100.
3. Amizuka N, Kwan MY, Goltzman D, Ozawa H and White JH (1999) Vitamin D3 differentially regulates parathyroid hormone/parathyroid hormone-related peptide receptor expression in bone and cartilage. *J Clin Invest* **103**, 373–381.
4. Anderson HC (1969) Vesicles associated with calcification in the matrix of epiphyseal cartilage. *J Cell Biol* **41**, 59–72.
5. Bergers G, Brekken R, McMahon G, Vu TH, Itoh T, Tamaki K, Tanzawa K, Thorpe P, Itohara S, Werb Z and Hanahan D (2000) Matrix metalloproteinase-9 triggers the angiogenic switch during carcinogenesis. *Nat Cell Biol* **2**, 737–744.
6. Brinckerhoff CE (2002) Matrix metalloproteinases: a tail of a frog that became a prince. *Nat Rev Mol Cell Biol* **3**, 207–214.
7. Carlevaro MF, Cermelli S, Cancedda R and Descalzi Cancedda F (2000) Vascular endothelial growth factor (VEGF) in cartilage neovascularization and chondrocyte differentiation: auto-paracrine role during endochondral bone formation. *J Cell Sci* **113**, 59–69.
8. D'Angelo M, Yan Z, Nooreyazdan M, Pacifici M, Sarment DS, Billings PC and Leboy PS (2000) MMP-13 is induced during chondrocyte hypertrophy. *J Cell Biochem* **77**, 678–693.
9. Egeblad M (2002) New roles for the matrix metalloproteinases in the progression of cancer. *Nat Rev Cancer* **2**, 163–176.
10. Freitas PHL, Hasegawa T, Takeda S, Sasaki M, Tabata C, Oda K, Li M, Saito H and Amizuka N (2011) Eldecalcitol, a second-generation vitamin D analog, drives bone minimodeling and reduces osteoclastic number in trabecular bone of ovariectomized rats. *Bone* **49**, 335–342.
11. Freitas PHL, Li M, Ninomiya T, Nakamura M, Ubaidus S, Oda K, Udagawa N, Maeda T, Takagi R and Amizuka N (2009) Intermittent PTH administration stimulates pre-osteoblastic proliferation without leading to enhanced bone formation in osteoclast-less c-fos(-/-) mice. *J Bone Miner Res* **24**, 1586–1597.
12. Gerber HP, Vu TH, Ryan AM, Kowalski J, Werb Z and Ferrara N (1999) VEGF couples hypertrophic cartilage remodeling, ossification and angiogenesis during endochondral bone formation. *Nat Med* **5**, 623–628.
13. Lin R, Amizuka N, Sasaki T, Aarts MM, Ozawa H, Goltzman D, Henderson JE and White JH (2002) 1alpha, 25-dihydroxyvitamin D3 promotes vascularization of the chondro-osseous junction by stimulating expression of vascular endothelial growth factor and matrix metalloproteinase 9. *J Bone Miner Res* **17**, 1604–1612.
14. Knäuper V, López-Otin C, Smith B, Knight G and Murphy G. (1996) Biochemical characterization of human collagenase-3. *J Biol Chem* **271**, 1544–1550.
15. Marks Jr. SC and Odgren PR (2002) Structure and development of the skeleton. In *Principles of Bone Biology*, Vol.1 (Bilezikian JP, Raisz LG and Rodan GA, eds), pp3–15, Academic press, San Diego.
16. Mitchell PG, Magna HA, Reeves LM, Lopresti-Morrow LL, Yocum SA, Rosner PJ, Geoghegan KF and Hambor JE (1996) Cloning, expression, and type II collagenolytic activity of matrix metalloproteinase-13 from human osteoarthritic cartilage. *J Clin Invest* **97**, 761–768.
17. Nagai H and Aoki M (2002) Inhibition of growth plate angiogenesis and endochondral ossification with diminished expression of MMP-13 in hypertrophic chondrocytes in FGF-2-treated rats. *J Bone Miner Metab* **20**, 142–147.
18. Nakamura H, Sato G, Hirata A and Yamamoto T (2004) Immunolocalization of matrix metalloproteinase-13 on bone surface under osteoclasts in rat tibia. *Bone* **34**, 48–56.
19. Ortega N, Wang K, Ferrara N, Werb Z and Vu TH (2010) Complementary interplay between matrix metalloproteinase-9, vascular endothelial growth factor and osteoclast function drives endochondral bone formation. *Dis Model Mech* **3**, 224–235.
20. Ozawa H, Hoshi K and Amizuka N (2008) Current concepts of bone mineralization. *J Oral Biosci* **50**, 1–14.
21. Poole R (1991) The growth plate: cellular physiology, cartilage assembly and mineralization. In *Cartilage: Molecular Aspects* (Hall BK and Newman SA, eds), pp179–211, CRC Press, Boca Raton.
22. Sakagami N, Amizuka N, Li M, Takeuchi K, Hoshino M, Nozawa-Inoue K and Maeda T (2005) Reduced osteoblastic population and the defective mineralization in osteopetrotic (op/op) mice. *Micron* **36**, 688–695.
23. Sasaki T, Amizuka N, Irie K, Ejiri S and Ozawa H (2000) Localization of alkaline phosphatase and osteopontin during matrix mineralization in the developing cartilage of coccygeal vertebrae. *Arch Histol Cytol* **63**, 271–284.
24. Simsa S, Genina O and Ornan EM (2007) Matrix metalloproteinase expression and localization in turkey (Meleagris gallopavo) during the endochondral ossification process. *J Anim Sci* **85**, 1393–1401.
25. Takahara M, Naruse T, Takagi M, Orui H and Ogino T (2004) Matrix metalloproteinase-9 expression, tartrate-resistant acid phosphatase activity, and DNA fragmentation in vascular and cellular invasion into cartilage preceding primary endochondral ossification in long bones. *J Orthop Res* **22**, 1050–1057.
26. Vu TH, Shipley JM, Bergers G, Berger JE, Helms JA, Hanahan D, Shapiro SD, Senior RM and Werb Z (1998) MMP-9/gelatinase B is a key regulator of growth plate angiogenesis and apoptosis of hypertrophic chondrocytes. *Cell* **93**, 411–422.
27. Wallis GA (1996) Bone growth: coordinating chondrocyte differentiation. *Curr Biol* **6**, 1577–1580.
28. Wu CW, Tchetina EV, Mwale F, Hasty K, Pidoux I, Reiner A, Chen J, Van Wart HE and Poole AR (2002) Proteolysis involving matrix metalloproteinase 13 (collagenase-3) is required for chondrocyte differentiation that is associated with matrix mineralization. *J Bone Miner Res* **17**, 639–651.