Expression of tartrate-resistant acid phosphatase and cathepsin K during osteoclast differentiation in developing mouse mandibles

Megumi Nakamura¹, Naoki Aoyama¹, Satoshi Yamaguchi², and Yasuyuki Sasano¹
¹Division of Craniofacial Development and Tissue Biology, Tohoku University Graduate School of Dentistry and ²Division of Aging and Geriatric Dentistry, Tohoku University Graduate School of Dentistry

(Received 29 July 2020; and accepted 28 October 2020)

ABSTRACT
The present study was designed to test the hypothesis that osteoclasts appear after or at the same time as the initiation of bone mineralization in developing intramembranous bones. We examined mineral deposition via Von Kossa staining to determine when bone mineralization begins, tartrate-resistant acid phosphatase (TRAP) activity and cathepsin K immunoreactivity to identify the presence of osteoclasts, and their mRNA expression levels to assess osteoclastic differentiation in the embryonic mouse mandible. Cathepsin K-immunopositive cells were detected around the same time as the onset of bone mineralization, whereas TRAP-positive cells appeared prior to bone mineralization. Cathepsin K protein was expressed only in multinucleated osteoclasts, whereas TRAP activity was identified in both mono- and multinucleated cells. During bone development, TRAP-positive cells altered their morphology, which was related to the number of their nuclei. The elevated mRNA levels of TRAP and cathepsin K were consistent with the increased percentage of multinucleated osteoclasts and the progression of bone development. Our study revealed that TRAP-positive cells appear prior to bone mineralization, and TRAP- and cathepsin K-positive multinucleated osteoclasts appear at the same time as the initiation of bone mineralization in embryonic mouse mandibles, suggesting that osteoclasts contribute to bone matrix maturation during intramembranous ossification.

INTRODUCTION
During embryonic development, osteogenesis begins with mesenchymal cell condensation at the presumptive bone site (Sasano et al., 2000; Zhu et al., 2001). Most bones, including long bones, develop through endochondral ossification, in which a cartilage template of the future bone is initially formed by chondrocytes that differentiated from mesenchymal cells and eventually replaced by newly formed bone. Contrarily, most craniofacial bones, such as maxillary and mandibular bones, develop through intramembranous ossification. Intramembranous bones are formed by osteoblasts that differentiated directly via mesenchymal condensation without a cartilage template (Erlebacher et al., 1995; Berendsen and Olsen, 2015).

The process of endochondral ossification in long bones has been well studied. Longitudinal growth of the diaphysis occurs at the growth plate, also known as the epiphyseal plate, which is a layer of cartilage located between the epiphysis and diaphysis. At the growth plate, osteoblastic bone formation and osteoclastic bone resorption are highly coordinated. Additionally, bone resorption by osteoclasts on the endocortical surface to expand the marrow cavity and new bone formation by osteoblasts in the periosteum results in the radial growth of the diaphysis (Allen and Burr, 2014; Kenkre and Bassett, 2018).
From the perspective that osteoclast activity is essential for endochondral bone formation, previous studies have identified the presence of osteoclasts in developing long bones using osteoclast-specific markers, such as tartrate-resistant acid phosphatase (TRAP) and cathepsin K (Roach, 1997; Soderstrom et al., 1999; Blumer et al., 2012). TRAP, a glycoprotein with two iron atoms in its active site, is predominantly expressed in osteoclasts and is widely used as a specific molecular marker for osteoclasts (Burstone, 1958; Minkin, 1982; Oddie et al., 2000). This lysosomal enzyme can dephosphorylate bone matrix proteins, including osteopontin and bone sialoprotein (Ek-Rylander et al., 1994). Cathepsin K, a lysosomal cysteine protease, is also exclusively expressed in osteoclasts (Tezuka et al., 1994; Inaoka et al., 1995; Drake et al., 1996). Activated cathepsin K has been shown to degrade the major organic component of bone matrix, type I collagen (Bossard et al., 1995; Dodds et al., 1996; Bromme et al., 1996; Dodds et al., 1998; Kafienah et al., 1998; Li et al., 2002).

Osteoclasts are multinucleated cells resulting from the fusion of mononuclear osteoclast precursors derived from the monocyte/macrophage lineage (Boyle et al., 2003). Prominent morphological characteristics of activated osteoclasts include a ruffled border, multiple plasma membrane folds resembling microvilli, and the sealing zone, a circular adhesive structure at their interface with bone (Pierce et al., 1991; Vaananen et al., 2000). Osteoclasts have the ability to both dissolve minerals and degrade organic components by secreting hydrochloric acid and proteolytic enzymes, including TRAP and cathepsin K, through the ruffled border within the sealing zone. However, they can only resorb mineralized bone and do not resorb unmineralized or demineralized bone as bone minerals are required for their sealing zone formation (Chambers et al., 1984; Saltel et al., 2004).

In intramembranous ossification, few studies have focused on the role of osteoclasts. As a result, little is known about the expression profiles of TRAP and cathepsin K throughout intramembranous bone development. In this study, we hypothesized that osteoclasts appear after or at the same time as the initiation of bone mineralization in developing intramembranous bones. To test this hypothesis, we investigated mineral deposition via Von Kossa staining to determine when bone mineralization begins during mandibular bone development, examined TRAP activity and cathepsin K immunoreactivity to identify the presence of osteoclasts during bone development, and quantified the mRNA expression levels of TRAP and cathepsin K to assess osteoclastic resorption in the developing mouse mandible.

**MATERIALS AND METHODS**

**Experimental animals.** Pregnant C57BL/6 female mice were obtained from the SLC Corporation (Japan SLC, Inc., Hamamatsu, Japan). Embryos 13, 14, 15, and 16 days post coitum (E13d, E14d, E15d, and E16d) were isolated from pregnant mice following the inhalation of an overdose of isoflurane (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan). At each time point, three embryos were analyzed via von Kossa staining, immunohistochemistry, and TRAP staining. E13d, E14d, and E15d (eight embryos each) were subjected to quantitative real-time polymerase chain reaction (qPCR) analysis. All experimental procedures followed the Regulations of Animal Experimentation at Tohoku University, were reviewed by the Institutional Laboratory Animal Care and Use Committee of Tohoku University, and finally approved by the President of the University.

**Von Kossa staining.** Heads were resected from the embryos and immediately fixed in 4% paraformaldehyde (PFA) in 0.01 M phosphate-buffered saline (PBS) (pH 7.4) at 4°C overnight. The fixed, undecalcified specimens were embedded in paraffin after dehydration through a graded ethanol series and cut into 5-μm-thick serial sections. To detect calcium deposits via von Kossa staining, some sections were deparaffinized and exposed to a 5% aqueous silver nitrate solution (Nacalai Tesque, Inc., Kyoto, Japan) for 60 min at room temperature under light illumination. The sections were incubated with a 5% aqueous sodium thiosulfate solution (FUJIFILM Wako Pure Chemical Corporation) for 5 min and then counterstained with hematoxylin and eosin.

**Immunohistochemistry.** The heads of E13d, E14d, and E15d were fixed in 4% PFA in 0.01 M PBS (pH 7.4) at 4°C overnight, decalciﬁed in 10% ethylenediaminetetraacetate (EDTA) in 0.01 M PBS, dehydrated in a series of graded ethanol, embedded in paraffin, and cut into 5-μm-thick serial sections. The sections were deparaffinized and incubated with 0.1% trypsin (Merck KGaA, Darmstadt, Germany) in PBS at 37°C for 15 min. Endogenous peroxidase activity was blocked by incubation with 3% hydrogen peroxide in methanol for 30 min, and nonspecific primary antibody binding was blocked by incubation with 5% normal goat serum in PBS at room tem-
heterogeneity for 30 min. Subsequently, the sections were incubated with a rabbit polyclonal primary antibody against cathepsin K (ab19027; Abcam plc, Cambridge, UK) diluted 1:400 in 5% normal goat serum in PBS at room temperature for 2 h. For the secondary antibody reaction, Histofine Simple Stain Mouse MAX-PO (R) (Nichirei Co., Tokyo, Japan) was applied on the section for 45 min at room temperature. After washing the sections in PBS, the immunoreaction was developed using 3,3′-diaminobenzidine as the substrate. The sections were then counterstained with 1% methyl green. Negative control sections were processed with 5% normal goat serum as a substitute for the primary antibody.

**TRAP staining.** The sections adjacent to those used for von Kossa staining or immunohistochemistry were processed for TRAP staining. The sections from E15d and E16d specimens were decalcified in 10% EDTA in 0.01 M PBS overnight, whereas those from E13d and E14d specimens were not decalcified after deparaffinization. The sections were incubated in a mixture of 0.4 mM naphthol AS-BI phosphate (Nacalai Tesque, Inc.) and 75 mM L(+)-tartaric acid (FUJIFILM Wako Pure Chemical Corporation) in 0.1 M sodium acetate buffer (pH 5.0) for 30 min at 37°C. Next, the sections were immersed in the same buffer containing 0.1% pararosaniline chloride (FUJIFILM Wako Pure Chemical Corporation) until a red color appeared, rinsed with distilled water to terminate the reaction, and counterstained with 1% methyl green.

Sections that included the mandibular left first molar germ with the widest contour were selected from three embryos at each time point, except for E13d. TRAP-positive cells in the left mandible were counted under a microscope and sorted by the number of nuclei. The average number of cells for each embryonic day was calculated and expressed as a percentage of the total cells.

**qPCR analysis.** E13d, E14d, and E15d (six embryos each) were examined. The mandible was resected from the embryos under the microscope, immediately immersed in QIAzol Lysis Reagent (QIAGEN, Hilden, Germany), mechanically homogenized with an ultrasonic homogenizer, and stored at −80°C until RNA extraction. After thawing, total RNA was extracted using an RNaseasy Lipid Tissue Mini Kit (QIAGEN) with DNase treatment and reverse-transcribed into cDNA using random primers (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and SuperScript III Reverse Transcriptase (Thermo Fisher Scientific). Specific primers for TRAP, cathepsin K, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA were designed. The expression level of target mRNAs was normalized to GAPDH as a reference gene. The primer sequences were as follows: TRAP forward, 5′-GGC TAC TTG CGG TTT CAC TAT G-3′, and reverse, 5′-GGG AGG CTG GTC TTA AAG AGT G-3′; cathepsin K forward, 5′-AGC AGT ATA ACA GCA AGG TGG AT-3′, and reverse, 5′-GGG TCA AAC TCG AAC TGT GAT-3′; and GAPDH forward, 5′-AGG TCA ATG AAG GGG TCG T-3′. qPCR reactions were run on a LightCycler Nano System (Roche Diagnostics, Mannheim, Germany) with FastStart Essential DNA Green Master (Roche Diagnostics) under the following conditions: an initial enzyme activation step at 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 10 s, annealing at 60°C for 10 s, and elongation at 72°C for 15 s. Melting curve analysis was conducted by heating from 60°C to 95°C at a temperature transition rate of 0.1°C per second.

**Statistical analysis.** Statistical analysis was conducted using SPSS 22.0 (IBM Japan, Ltd., Tokyo, Japan) to compare the qPCR data. Variations in the mRNA expression levels were analyzed via a one-factor analysis of variance (ANOVA) for each embryonic day. Significant differences in mean mRNA expression levels among the three embryonic days were examined using Bonferroni post hoc tests. P-values < 0.05 were considered statistically significant.

**RESULTS**

**TRAP-positive cells during bone mineralization**

Mineralization and TRAP enzymatic activity were analyzed histochemically during mandibular bone development, focusing on the area close to the first molar tooth germ. Mesenchymal cell condensation with osteoid formation was observed in the putative osteogenic region, but no calcium deposits or TRAP-positive cells were found at E13d (Fig. 1A, B). Although bone matrix mineralization had not yet initiated in E14d mandibles, the osteoid region was extended, and some small TRAP-positive cells were identified on the surface of the osteoid (Fig. 1C, D). At E15d, calcium deposition was detected via von Kossa staining (Fig. 1E). In addition, flat cells expressing TRAP were observed on the surface of the bone matrix (Fig. 1F). At E16d, the mineralized bone area was further expanded, and numerous large
Fig. 1 Frontal sections of the left mandible stained with Von Kossa are presented in A, C, E, and G, and their adjacent sections stained with TRAP are presented in B, D, F, and H. At E13d, no mineral deposition is observed in the presumptive osteogenic region where osteoid formation with mesenchymal cell condensation is evident (A), and no TRAP activity is detected (B). At E14d, mineral deposition is not yet observed (C), but small TRAP-positive cells are observed on the osteoid surface (D). At E15d, calcium deposits stained in brown are detected (E), and TRAP-positive cells are observed on the surface of the bone matrix (F). At E16d, the mineral deposition area is expanded (G), and numerous large TRAP-positive cells are detected (H). Asterisks, the putative osteogenic region or bone matrix; arrowheads, TRAP-positive cells; M1, the lower first molar tooth germ; MC, Meckel’s cartilage. Scale bars = 200 μm (A, C, E, and G); 100 μm (B, D, F, and H).
multinucleated TRAP-positive cells were found in contact with the bone matrix (Fig. 1G, H).

**Morphological changes in TRAP-positive cells related to the number of nuclei**

TRAP-positive cell morphology of E14d, E15d, and E16d was compared at the same and high magnification, and TRAP staining showed that their cell shape was different at each time point (Fig. 2A–C). Almost all TRAP-positive cells were small, round, and mononucleated at E14d, whereas the number of flat TRAP-positive cells with two or three nuclei increased at E15d (Fig. 2A, B). At E16d, large multinucleated cells shaped like jellyfishes were prominent (Fig. 2C). In these cells, the intracellular positive reaction for TRAP activity was strong on the side facing the bone matrix, and multiple nuclei were localized on the opposite side of the bone matrix (Fig. 2D).

Based on morphological observations, we counted the number of nuclei in TRAP-positive cells at each embryonic day and revealed the percentage of cells categorized by the number of nuclei (Table 1). At E14d, mononucleated cells accounted for over 90% of the total number of TRAP-positive cells, and no TRAP-positive cell with three or more nuclei was found. At E15d, the percentage of mononucleated cells decreased to 78.57%, and the proportion of cells with two nuclei increased to 17.86%. In addition, 3.57% of cells contained three nuclei, whereas no TRAP-positive cell with four or more nuclei was found. At E16d, the percentage of mononucleated cells decreased to less than 50%, whereas that of cells with two and three nuclei increased to 22.73% and 12.99%, respectively. Furthermore, large TRAP-positive cells with four or more nuclei accounted for 18.18%.

**Cathepsin K-immunoreactive cells during bone development**

Cathepsin K immunoreactivity was examined immunohistochemically during mandibular bone development to confirm the presence of osteoclasts, and the multinucleated TRAP-positive cells were found in contact with the bone matrix (Fig. 1G, H).

**Fig. 2** High-magnification light microscopic images of TRAP-positive cells. Several small mononucleated TRAP-positive cells (arrowheads) are observed at E14d (A). Flat TRAP-positive cells with two or three nuclei (arrows) are observed at E15d (B). Numerous multinucleated cells shaped like jellyfishes (double arrowheads) are observed at E16d (C). The intracellular TRAP-positive reaction is strong on the side facing the bone matrix, and multiple nuclei are localized on the opposite side of the bone matrix in the jellyfish-like shaped cell (D). Asterisks, the bone matrix; MC, Meckel’s cartilage. Scale bars = 50 μm (A–C); 20 μm (D).
results were compared with the TRAP histochemistry data. No TRAP activity or cathepsin K immunoreactivity was observed in and around the putative osteogenic region where the mesenchymal cells assembled at E13d (Fig. 3A, B). At E14d, small TRAP-positive cells were clearly observed on the surface of the bone matrix, whereas no distinct

| Number of Nuclei | E14d   | E15d   | E16d   |
|------------------|--------|--------|--------|
| One              | 92.21% | 78.57% | 46.10% |
| Two              | 7.79%  | 17.86% | 22.73% |
| Three            | 0%     | 3.57%  | 12.99% |
| Four or more     | 0%     | 0%     | 18.18% |

Table 1  The percentage of TRAP-positive cells according to the number of nuclei

Fig. 3  Frontal sections of the left (A–D) or right (E, F) mandible histochemically stained for TRAP are presented in A, C, and E, and their adjacent sections immunohistochemically stained for cathepsin K are presented in B, D, and F. No TRAP activity or cathepsin K immunoreaction is detected in and around the putative osteogenic region at E13d (A, B). At E14d, several small TRAP-positive cells are observed on and around the bone matrix (C), whereas distinct cathepsin K immunoreactivity is not detected (D). At E15d, both TRAP-positive cells and cathepsin K-immunopositive cells are identified on the bone matrix (E, F). Asterisks, the putative osteogenic region or bone matrix; arrowheads, TRAP-positive cells; arrows, cathepsin K-immunopositive cells. Scale bars = 50 μm (A–D); 25 μm (E, F).
cathepsin K immunoreactivity was observed in the mandible on the adjacent sections stained with TRAP (Fig. 3C, D). At E15d, the relatively large cells on the surface of the bone matrix exhibited both intense cathepsin K immunoreactivity and TRAP activity (Fig. 3E, F).

TRAP and cathepsin K mRNA expression in the mandible

TRAP and cathepsin K mRNA expression levels in mandibles were quantified at E13d, E14d, and E15d and statistically compared among three embryonic days (Fig. 4). The lowest TRAP mRNA level occurred at E13d, and significant differences were observed between E13d and E14d \( (P = 0.004) \) and E13d and E15d \( (P < 0.001) \). Cathepsin K also exhibited the lowest level of mRNA expression at E13d, and significant differences were observed between E13d and E14d \( (P = 0.001) \) and E13d and E15d \( (P < 0.001) \). The expression levels of both TRAP and cathepsin K were the highest at E15d and significantly higher than E14d \( (P < 0.001) \). As indicated above, TRAP enzyme activity and cathepsin K immunoreactivity were detected from E14d and E15d, respectively. In contrast, both TRAP and cathepsin K mRNA expressions were detected at E13d. The mRNA expression levels of TRAP and cathepsin K were increased along with the progress of bone development.

**DISCUSSION**

We showed that cathepsin K-immunopositive cells are identified around the same time as the onset of mouse mandibular bone mineralization, whereas TRAP-positive cells appear prior to bone mineralization. We originally hypothesized that osteoclasts appear after or at the same time as the initiation of bone mineralization as they degrade only mineralized tissues (Chambers et al., 1984; Saltel et al., 2004). The results indicated that cathepsin K expression is consistent with our hypothesis, whereas the detected levels of TRAP activity did not support our hypothesis. Almost all TRAP-positive cells detected before mandibular bone mineralization were mononucleated. In previous studies, double staining for TRAP and nonspecific esterase (NSE), a selective marker for cells derived from the macrophage/monocyte lineage, was employed to distinguish between osteoclast precursors and mature osteoclasts. Baron et al. (1986) revealed that mononucleated osteoclast precursors are derived from the macrophage/monocyte lineage using this double staining technique. Their study revealed that the number of mononucleated TRAP-positive cells increased prior to the formation of multinucleated osteoclasts in a rat mandibular bone resorption model system. In a different study, Dodds et al. (1998) defined mononucleated cells positive for both TRAP and NSE as preosteoclasts and mono- and multinucleated cells positive...
for TRAP and negative for NSE as mature osteoclasts. Based on the above information, the mononucleated TRAP-positive cells found in this study may be preosteoclasts or mature mononucleated osteoclasts just before their fusion to form multinucleated cells. Our results indicate that TRAP is activated in mononucleated osteoclasts or preosteoclasts and multinucleated osteoclasts, whereas cathepsin K protein is expressed only in multinucleated osteoclasts capable of resorbing the mineralized bone matrix. Currently, the function of mononucleated TRAP-positive cells during bone development is unclear. Thus, further investigation is needed to elucidate whether these mononucleated cells are just one step in the process of osteoclast differentiation or play a particular role in osteogenesis.

Previous studies reported that mice lacking genes essential for osteoclast differentiation, such as c-fos, TNF receptor-associated factor (TRAF) 6, receptor activator of NF-κB (RANK), and RANK ligand, exhibit osteopetrosis due to impaired osteoclastic bone resorption, suggesting that bone is formed even in the absence of osteoclasts, although this is not normal (Wang et al., 1992; Dougall et al., 1999; Lomaga et al., 1999; Kim et al., 2000). Therefore, we presume that osteoclasts are involved in bone matrix maturation during osteogenesis; however, in most studies, embryonic bone development has not been investigated in mice deficient in osteoclast differentiation factors. Our study revealed that TRAP-positive cells remarkably altered their morphology during embryonic bone development, which was related to the number of their nuclei. Specifically, mononucleated cells were small and round, cells with two or three nuclei were flat, and most cells with four or more nuclei were shaped like jellyfishes. The jellyfish-like shape of the cells we detected at E16d is considered to reflect the activated state of osteoclasts with a ruffled border (Pierce et al., 1991; Vaananen et al., 2000), suggesting that osteoclasts have already started to resorb bone in E16d mandibles. There may be a difference in the bone quality (i.e., the composition ratio of minerals to organic components) between mice lacking osteoclasts and wild-type mice already at the late embryonic stage.

Both TRAP enzyme activity and cathepsin K immunoreactivity were negative at E13d. However, we detected the mRNA expression levels of both molecules. There are three possibilities for this discrepancy. First is that osteoclast precursor cells express TRAP and cathepsin K at the gene level, but not at the protein level in E13d mandibles. Second is the detection limit of TRAP staining and immunohistochemistry. qPCR can detect mRNA with low expression levels with the use of an amplification technique. The sensitivity for mRNA detection via qPCR may be higher than that for enzyme activity and immunoreactivity using histological methods. Third is the difference in the mandibular bone regions used as samples between qPCR and histological analyses. We used sections containing a limited region of the mandible that included the first molar tooth germ for the detection of TRAP activity and cathepsin K immunoreactivity, whereas the entire mandible was used for mRNA quantification in this study. Therefore, more advanced stages of mandibular bone may have been included in the samples for mRNA quantification since the stage of bone development differs, depending on the location of the mandible (Bhaskar et al., 1953; Slavkin et al., 1989).

Our study revealed that TRAP-positive cells appear prior to bone mineralization, and active multinucleated osteoclasts expressing both TRAP and cathepsin K appear at the same time as the initiation of bone mineralization in embryonic mouse mandibles, suggesting that osteoclasts contribute to bone matrix maturation during intramembranous ossification.

Acknowledgments
This work was supported by JSPS KAKENHI Grant Numbers JP18K09517 and JP18K09760. We thank Mr. Yasuto Mikami, Division of Craniofacial Development and Tissue Biology, Tohoku University Graduate School of Dentistry, for his assistance.

CONFLICT OF INTERESTS
There are no conflicts of interest to declare.

REFERENCES
Allen MR and Burr DB (2014) Chapter 4 - Bone modeling and remodeling. in Basic and Applied Bone Biology (Burr DB, Allen MR, eds), pp 75–90, Academic Press, San Diego.
Baron R, Neff L, Tran Van P, Nefussi JR and Vignery A (1986) Kinetic and cytochemical identification of osteoclast precursors and their differentiation into multinucleated osteoclasts. Am J Pathol 122, 363–378.
Berendsen AD and Olsen BR (2015) Bone development. Bone 80, 14–18.
Bhaskar SN, Weinmann JP and Schour I (1953) Role of Meckel’s cartilage in the development and growth of the rat mandible. J Dent Res 32, 398–410.
Blumer MJ, Hausott B, Schwarzer C, Hayman AR, Stempel J, et al. (2012) Role of tartrate-resistant acid phosphatase (TRAP) in long bone development. Mech Dev 129, 162–176.
Bossard MJ, Tomaszek TA, Thompson SK, Amegadzie BY,
Osteoclasts in developing bone

Boyle WJ, Simonet WS and Lacey DL (2003) Osteoclast differentiation and activation. *J Biol Chem* 271, 12517–12524.

Bromme D, Okamoto K, Wang BB and Biroc S (1996) Human cathepsin O2, a matrix protein-degrading cysteine protease expressed in osteoclasts. Functional expression of human cathepsin O2 in Spodoptera frugiperda and characterization of the enzyme. *J Biol Chem* 271, 2126–2132.

Burstone MS (1958) Histochemical demonstration of acid phosphatases with naphthol AS-phosphates. *J Natl Cancer Inst* 21, 523–539.

Chambers TJ, Thomson BM and Fuller K (1984) Effect of substrate composition on bone resorption by rabbit osteoclasts. *J Cell Sci* 67, 61–71.

Chambers TJ, Thomson BM and Fuller K (1984) Effect of substrate composition on bone resorption by rabbit osteoclasts. *J Cell Sci* 67, 61–71.

Dodds RA, Connor JR, Drake F, Feild J and Gowen M (1998) Osteoclasts: structure and function. *Electron Microsc Rev* 4, 1–45.

Roach HJ (1997) New aspects of endochondral ossification in the chick: chondrocyte apoptosis, bone formation by former chondrocytes, and acid phosphatase activity in the endochondral bone matrix. *J Bone Miner Res* 12, 795–805.

Saltel F, Destaing O, Bard F, Eichert D and Jurdic P (2004) Apoptosis-mediated actin dynamics in resorbing osteoclasts. *Mol Biol Cell* 15, 5231–5241.

Sasano Y, Li HC, Zhu JX, Imanaka-Yoshida K, Mizoguchi I, et al. (2000) Immunohistochemical localization of type I collagen, fibronectin and tenasin C during embryonic osteogenesis in the dentary of mandibles and tibias in rats. *Histochem J* 32, 591–598.

Slavkin HC, Bringas P, Jr., Sasano Y and Mayo M (1989) Early embryonic mouse mandibular morphogenesis and cytodifferentiation in serumless, chemically defined medium: a model for studies of autocrine and/or paracrine regulatory factors. *J Craniofac Genet Dev Biol* 55, 308–327.

Wang QZ, Ovitt C, Grigoriadis AE, Mohle-Steinlein U, Ruther U, et al. (1992) Bone and haematopoietic defects in mice lacking c-fos. *Nature* 360, 741–745.

Zhu JX, Sasano Y, Takahashi I, Mizoguchi I and Kagayama M (2001) Temporal and spatial gene expression of major bone extracellular matrix molecules during embryonic mandibular osteogenesis in rats. *Histochem J* 33, 25–35.