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# Osteopetrosis, osteopetrorickets and hypophosphatemic rickets differentially affect dentin and enamel mineralization

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ABSTRACT

Osteopetrosis (OP) is an inherited disorder of defective bone resorption, which can be accompanied by impaired skeletal mineralization, a phenotype termed osteopetrorickets (OPR). Since individuals with dysfunctional osteoclasts often develop osteomyelitis of the jaw, we have analyzed, if dentin and enamel mineralization are differentially affected in OP and OPR. Therefore, we have applied non-decalcified histology and quantitative backscattered electron imaging (qBEI) to compare the dental phenotypes of $Src^{-/-}$, *oc/oc* and $Hyp^{-/0}$ mice, which serve as models for OP, OPR and hypophosphatemic rickets, respectively. While both, $Src^{-/-}$ and *oc/oc* mice, were characterized by defects of molar root formation, only *oc/oc* mice displayed a severe defect of dentin mineralization, similar to $Hyp^{-/0}$ mice. Most importantly, while enamel thickness was not affected in either mouse model, the calcium content within the enamel phase was significantly reduced in *oc/oc*, but not in $Src^{-/-}$ or $Hyp^{-/0}$ mice. Taken together, these data demonstrate that dentin and enamel mineralization are differentially affected in $Src^{-/-}$ and *oc/oc* mice. Moreover, since defects of dental mineralization may trigger premature tooth decay and thereby osteomyelitis of the jaw, they further underscore the importance of discriminating between OP and OPR in the respective individuals.



## Introduction

Osteopetrosis (OP) is a high bone mass disorder caused by mutational inactivation of genes affecting either differentiation or function of bone-resorbing osteoclasts [1]. We and others have previously reported that OP can be accompanied by severe defects of skeletal mineralization, a phenotype termed osteopetrorickets (OPR) [2–5]. Using mouse models for both diseases we were further able to demonstrate that OPR is the consequence of a combined acidification defect in bone-resorbing osteoclasts and gastric parietal cells [2]. In fact, *oc/oc* mice, which carry an inactivating mutation of the *Tcirg1* gene, encoding a subunit of the vacuolar proton pump, do not only display high bone mass and osteoid enrichment, but also hypochlorhydria and severe hypocalcemia. In contrast, mice lacking the Src tyrosine kinase specifically display high bone mass without hypocalcemia and defective matrix mineralization. The relevance of a discrimination of both phenotypes was further underscored by non-decalcified histology of 21 bone biopsies from non-genotyped individuals diagnosed with OP, where we found an additional enrichment of osteoid in 10 of the cases [2]. These data demonstrated that OP and OPR are distinct disorders with comparable prevalence, which is further supported by the fact that TCIRG1-mutations are found in approximately 50% of the cases with autosomal recessive osteopetrosis [6]. Based on these findings there are at least two clinically relevant questions remain for future research. First, is OPR specifically found in individuals with TCIRG1-mutations? And second, how does OPR, in contrast to OP, affect mineralization of dentin and enamel?

While addressing the first question requires histomorphometric analysis of non-decalcified bone biopsies form a large number of individuals with defined mutations causing osteoclast dysfunction, it appeared reasonable to address the second question by studying the dental phenotypes of $Src^{-/-}$ and *oc/oc* mice. In fact, although it is well documented that both mouse models display a failure of tooth eruption [7,8], there is yet no knowledge regarding specific differences of matrix mineralization within the dentin and enamel phase. In our opinion this is particularly surprising, since it is known that individuals with osteoclast dysfunction often develop osteomyelitis of the jaw, as a consequence of caries and periodontitis [9–14]. Although these dental diseases are generally caused by poor oral hygiene, it is certainly possible that dentin or enamel hypomineralization may contribute to these pathologies. Based on these arguments it was important to address the possibility, that OPR does not only affect bone, but also dental mineralization. Therefore we performed a thorough comparative analysis of the dental status of $Src^{-/-}$ and *oc/oc* mice using non-decalcified histology, μCT-imaging and quantitative backscattered electron imaging (qBEI). For the assessment of matrix mineralization we further included a mouse model of hypophosphatemic rickets ($Hyp^{-/0}$), which is known

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to display defect of dentin mineralization, at least at younger age [15–17].

## Materials and methods

### Mice

The colonies of *Src*$^{-/-}$, *oc/oc* and *Hyp*$^{-/0}$ mice used for this study have been described previously [2,18]. All mouse models were obtained from the Jackson Laboratory and fed a standard rodent diet, with the exception of *Src*$^{-/-}$ mice that were maintained on a liquid diet with equivalent nutrition content (Altromin, C0199) after weaning. All comparisons were carried out on 5 mice per genotype at 2 weeks of age. Given the early lethality of *oc/oc* mice the comparisons at other ages were only performed with wildtype, *Src*$^{-/-}$, and *Hyp*$^{-/0}$ mice (3 mice per genotype). Experiments were approved by the animal facility of the University Medical Center Hamburg-Eppendorf and by the Amt für Gesundheit und Verbraucherschutz (Org139).

### Skeletal and dental analysis

Skulls were fixed in 3.7% PBS-buffered formaldehyde for 24 h. After a 24-h incubation in 70% ethanol the skulls were cut in the midsagittal plane and analysed by contact radiography using a Faxitron X-ray cabinet (Faxitron X-ray Corp., Wheeling, IL, USA) followed by μCT scanning with a voxel size of 6 μm (μCT 40, Scanco Medical, Bassersdorf, Switzerland). After three-dimensional reconstruction teeth were highlighted and pseudo-colored according to the μCT-gray values using Photoshop (Photoshop Cs 4, Adobe Systems Inc., USA). For undecalcified histology skulls were dehydrated in ascending alcohol concentrations and embedded in methylmethacrylate as described before [19]. Sections of 5 μm were cut in the sagittal plane on a Microtec rotation microtome (Techno-Med, Munich, Germany). For ground sections skulls were dehydrated, embedded in methylmethacryltaebased resin (Technovit 7200 VLZ, Germany) and ground to 50 μm as described previously [20]. TRAP activity staining was performed on dehydrated decalcified sections as previously described [21]. Histomorphometric quantification of bone, coronal dentin, enamel and cell numbers was performed using the Osteo-Measure histomorphometry system (Osteometrics, Atlanta, GA, USA).

### Mineral density distribution measurements by quantitative backscattered electron imaging (qBEI)

This method is based on the fact that the intensity of the backscattered electrons shows a high correlation with the mineral content within the anorganic matrix [22–24]. The methylmethacrylate-embedded biopsies were grinded coplanar, surface-polished and carbon-coated as previously reported [25,26]. The scanning electron microscope (LEO 435 VP, Leo Electron Microscopy Ltd., England) was operated at 20 kV at a constant working distance of 20 mm (BSE Detector, Type 202, K.E. Developments Ltd., England). The electron beam was kept constant at 580 pA using a Farraday cup (MAC Consultants Ltd., England). The signal amplification (brightness and contrast) was calibrated during the entire procedure by keeping measurements of carbon and aluminium standards (MAC Consultants Ltd., England) at gray level of $5 \pm 0.5$ and $222.4 \pm 0.5$, respectively. Nine digital BE images were obtained per specimen at a nominal magnification of 100×, corresponding to a pixel resolution of 0.8 μm/pixel. The gray level histograms of bone, dentin and enamel were standardized using a threshold routine (Image J 1.42, National Institute of Health, USA). These values were transformed into calcium weight using a standardization line determined by the gray levels of osteoid (0 wt.% Ca) and hydroxylapatite (39.86 wt.% Ca). Therefore, the gray value of osteoid was obtained by measurements of areas with histologically proven osteoid. The gray value of hydroxylapatite ($Z = 14.06$) was determined theoretically using the calibration line given by the carbon ($Z = 6$, GL = 5) and aluminium ($Z = 13$, GL = 224.4) standards as described previously [23]. The accuracy of this calibration was ensured by EDX measurements (EDAX DX-4, EDAX Inc., USA) of bone and teeth biopsies previously measured by qBEI. For statistical analysis the mean calcium content (Ca mean) and the heterogeneity of the calcium distribution (Ca width) were introduced referring to the expected value and the standard deviation of the calcium distribution. For visualization areas of interest were highlighted and pseudo-colored according to the backscattered graylevels using Photoshop (Photoshop Cs 4, Adobe Systems Inc., USA).

### Statistical analysis

Results are presented as bar graphs, indicating mean ± SD. Statistical analysis was performed using an unpaired, two-tailed Student's *t*-test. For multiple testing *p* values were corrected with Bonferroni adjustment. $p$ Values $< 0.05$ were considered statistically significant.

## Results

### Tooth development and eruption in *Src*$^{-/-}$, *oc/oc* and *Hyp*$^{-/0}$ mice

Given the early lethality of the *oc/oc* mice we first analyzed 2 weeks old mice and evaluated tooth eruption by contact radiography. Here we did not only observe the expected length reduction of the incisors in *Src*$^{-/-}$ and *oc/oc* mice, but also a severe impairment of molar root formation (Fig. 1A). Since it has been hypothesized that the major importance of functional osteoclasts in tooth eruption is the removal of overlying bone to generate an eruption channel [27], we have further used μCT scanning to analyze the incisors of the three mouse models. Unexpectedly, we found that eruption channels were present in *Src*$^{-/-}$ and *oc/oc* mice (Fig. 1B). Since the continuously erupting rodent incisor is not physiological for the situation in humans, we have thereafter focused on molar teeth. Here we found that their growth in *Src*$^{-/-}$ and *oc/oc* mice was not limited by non-resorbed bone matrix overlying the crowns, but rather by the lack of alveolar bone removal underneath this region. Likewise, while the crown length was not reduced in both mutant mouse models, root formation was severely impaired (Figs. 1C and D). To determine whether osteoclastogenesis is associated with root formation, we also performed TRAP activity staining on decalcified molar sections (Suppl. Fig. 1A). Here we observed the presence of osteoclasts on the surface of the alveolar crypt below the developing molars in either mouse model. Histomorphometric quantification further revealed increased numbers of osteoclasts in *Src*$^{-/-}$ and *oc/oc* mice, which is readily explained by the fact that both mutations only cause defects of osteoclast function, but not of osteoclast differentiation (Suppl. Fig. 1B). Since *Src*$^{-/-}$ mice, unlike *oc/oc* mice, do not display increased lethality when they are fed a liquid diet, we next analyzed their molar teeth phenotype at 6 and 24 weeks of age in comparison to wildtype and *Hyp*$^{-/0}$ mice (Fig. 1D and Suppl. Fig. 2). Although we observed eruption of the 2nd molar into the oral cavity at 6 and 24 weeks, all molar teeth of *Src*$^{-/-}$ mice were totally enclosed within alveolar bone at 52 weeks of age. Most importantly, we still observed a specific reduction of the root length in *Src*$^{-/-}$ mice at all ages analyzed. Moreover, all roots were fused to alveolar bone due to loss of periodontal fibers at 24 and 52 weeks of age, a condition called ankylosis [28]. In summary, these data demonstrate that functional osteoclasts are required for removal of alveolar bone to allow proper root formation, and that the impaired tooth development caused by osteoclast dysfunction is similar in OP and OPR.

### Analysis of bone mineralization in *Src*$^{-/-}$, *oc/oc* and *Hyp*$^{-/0}$ mice

Since our previous analysis of these three mouse models was limited to spine and tibia sections [2,18], we first analyzed matrix