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Dental and Cranial Pathologies in Mice Lacking the Cl-/H+-Exchanger CIC-7

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Abstract

CIC-7 is a 2Cl⁻/1H⁺-exchanger expressed at late endosomes and lysosomes, as well as the ruffled border of osteoclasts. CIC-7 deficiencies in mice and humans lead to impaired osteoclast function and therefore osteopetrosis. Failure of tooth eruption is also apparent in CIC-7 mutant animals, and this has been attributed to the osteoclast dysfunction and the subsequent defect in alveolar bone resorptive activity surrounding tooth roots. Ameloblasts also express CIC-7, and this study aims to determine the significance of CIC-7 in enamel formation by examining the dentitions of CIC-7 mutant mice. Micro-CT analysis revealed that the molar teeth of 3-week old CIC-7 mutant mice had no roots, and the incisors were smaller than their age-matched controls. Despite these notable developmental differences, the enamel and dentin densities of the mutant mice were comparable to those of the wild type littermates. Scanning electron microscopy (SEM) showed normal enamel crystallite and prismatic organization in the CIC-7 mutant mice, although the enamel was thinner (hypoplastic) than in controls. These results suggested that CIC-7 was not critical to enamel and dentin formation, and the observed tooth defects may be related more to a resulting alveolar bone phenotype. Micro-CT analysis also revealed abnormal features in the calvarial bones of the mutant mice. The cranial sutures in CIC-7 mutant mice remained open compared to the closed sutures seen in the control mice at 3 weeks. These data demonstrate that CIC-7 deficiency impacts the development of the dentition and calvaria, but does not significantly disrupt amelogenesis.

Keywords

ameloblast; amelogenesis; biomineralization; craniofacial development; pH regulation; enamel; chloride channels

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INTRODUCTION

CIC-7, encoded by *Clcn7* gene, is a voltage-gated $2\text{Cl}^-/1\text{H}^+$ -exchanger (Leisle et al., 2011), and appears to be ubiquitously expressed in all cells examined (Brandt and Jentsch, 1995). The subcellular localization of CIC-7 is at late endosomes and lysosomes, as well as the ruffled border of osteoclasts (Kornak et al., 2001; Kasper et al., 2005). Osteoclasts degrade and resorb bone matrix using a specialized cell compartment, the resorptive lacuna, in which an acidic pH is established by V-type ATPase (V-ATPase) present in the ruffled membrane (Marshansky et al., 2014). The H^+ transport by V-ATPase is electrogenic and requires an anion channel in the same membrane to balance the charges of ion across the membrane (Jentsch, 2007; Stauber and Jentsch, 2013). Loss of *Clcn7* in mice (*Clcn7*^{-/-}) causes lysosomal storage disease (Kasper et al., 2005; Wartosch et al., 2009) and osteopetrosis as osteoclasts fail to form a ruffled border and properly acidify the extracellular space between the osteoclast and the bone (Kornak et al., 2001; Neutzsky-Wulff et al., 2010). Similarly, mutations in the *CLCN7* gene in humans led to autosomal recessive osteopetrosis (ARO), also known as malignant infantile osteopetrosis (Cleiren et al., 2001; Kornak et al., 2001). Certain missense mutations in one allele of *CLCN7* gene lead to autosomal dominant osteopetrosis type 2 (ADO2, Albers-Schonberg disease), a milder and the most common form of osteopetrosis with symptoms not evident until later in life (Cleiren et al., 2001; Del Fattore et al., 2008). Not surprisingly, lack of tooth eruption has been reported in both *Clcn7*^{-/-} mice (Kornak et al., 2001) and in patients with *CLCN7* gene mutations (Xue et al., 2012; Duan, 2014); this is because tooth eruption is critically dependent on osteoclast activity to reabsorb surrounding alveolar bone, which enables root development and the crown of the tooth to erupt into the oral cavity.

In this study we examined whether CIC-7 is also directly involved in amelogenesis, based on the fact that ameloblasts, like osteoclasts, express V-ATPase (Lin et al., 1994; Josephsen et al., 2010; Damkier et al., 2014) and CIC-7 (Lacruz et al., 2013). In addition, the acidic pH of the enamel matrix bordering ruffle-ended ameloblasts (RA) has long been observed (Smith, 1998), although it remains unclear whether the acidic pH is the result of active acid secretion by ameloblasts, or is the by-product of hydroxyapatite crystal synthesis (Damkier et al., 2014). Although a clear correlation exists between osteopetrosis phenotype and the delay or absence of tooth eruption, the structure of the tooth and surrounding bone in osteopetrotic animal models has never been examined in detail. In this study, micro-CT (μCT) analysis and scanning electron microscopy (SEM) were used to examine mineral densities of enamel and dentin, as well as the architecture of the enamel in age-matched *Clcn7*^{+/+} (wild type; WT) and *Clcn7*^{-/-} (mutant) littermates. The μCT whole head scans also allowed us to comprehensively examine calvariae and suture formation in CIC-7 deficient mice.

MATERIALS AND METHODS

Animal study approval

All methodologies and animal manipulations related to this study were approved by the University of Southern California's (USC) Institutional Animal Care and Use Committee (IACUC).

Samples

Six heads from three age-matched male pairs of *Clcn7^{+/+}* and *Clcn7^{-/-}* littermates at ages post-natal (PN) day 19, day 20, and day 21 (one WT and one mutant from each age), were obtained from Dr. Thomas Jentsch (Kornak et al., 2001) having been initially fixed in 10% formalin for 1–2 weeks and then kept in 70% ethanol. Despite the small sample size (n=3 mutants and n=3 controls) there were no sample outliers, and we were able to generate statistically significant data in all parameters examined and reported on.

MicroCT (μ CT) analysis

All six mouse heads were scanned, and reconstructed on a SCANCO μ CT 50 at the University of Southern California's Molecular Imaging Center with acquisition settings of: 70kVp, 85uA, 6W, filter AL 0.5mm, and 10 micron isotropic resolution. Depending on the sample size, acquisitions proceeded for ~ 2 hours. The images were exported in dicom format from the SCANCO workstation, volume/surface rendered, enamel and dentin segmented, and their mean intensity calculated in all sections with AMIRA5.5.0. Data collected from the same genotype, *Clcn7^{+/+}* or *Clcn7^{-/-}*, were considered as a group. Student's t-test was used for statistical analyses.

Scanning Electron Microscopy (SEM)

First, mandibular incisors were dissected and air-dried overnight. About 50 ml of hard EPON embedding material was thoroughly mixed with 25.7ml of resin, 9.3 ml of DDSA, 16.5 ml of NMA, and 0.8 ml of DMP-30 in a 50-ml conical tube within a vented fume hood. The EPON components came from a PELCO Eponate 12™ kit with DMP-30 (Catalogue # 18010, Redding, CA). Next, the EPON embedding mixture was diluted with propylene oxide by 30%, 60%, and 100% and mixed with the incisors for 1 hour sequentially to facilitate infiltration. The embedding EPON was deposited into a vacuum desiccator to eliminate bubbles and then poured into a rubber embedding mold holder topfull. The incisors were added and cured at 60–65°C for 36–48 hours.

Embedding blocks were first ground and polished with course sandpaper to expose the prismatic structure in a sagittal section of the incisors, the surface point of interest, and then continually milled with progressively finer grades of sandpaper down to 2.5-micron silicon carbide (P4000). (Lightly scraping the incisors with a toothbrush was necessary to remove debris during this process.) After cleaning with running water and a toothbrush, the block was then air-dried and acid etched with 1% nitric acid for 45 seconds, followed by a second water rinsing and air-drying. The air-dried sample was then mounted on a SEM stub with double sticky tape. Conductive paint (Colloidal silver liquid, Pelco TED PELLA Inc.) was applied to the whole stub which was then sputter coated with gold/platinum. JEOL JSM 6390LV was used for observation.

Sampling

All 3 wild type animals (ages 19–21) had a similar phenotype as observed by both μ CT and SEM. All 3 mutant animals had a similar phenotype as observed by both μ CT and SEM. Images presented are from the 21 day old mice (PN21).

RESULTS

Dentition of the mutant mice

Three-dimensional μ CT images showed teeth in *Clcn7*^{-/-} animals that were mal-aligned (occlusal plan was disrupted) (Figure 1, panel B compared to Figure 1, panel A), which is likely explained by the fact that all molars remained unerupted at 3 weeks. The incisor teeth of the mutant mice were significantly smaller and distorted in shape compared to those of their wild type littermates (Figure 1, panel B compared to Figure 1, panel A; and panel G). The molar teeth of the mutant mice were absent of any root development as illustrated in Figure 1F when compared to the wild type (Figure 1E). Cross sectional slices (Figure 1, panels C–F) showed that the enamel and dentin densities of the mutant mice were comparable to those of the wild type mice. This result was further confirmed by quantitatively comparing the relative mineral density of all slices (indicated by mean intensity in Figure 1H). The density of enamel and dentin from post-natal (PN) day 19 to PN21 mice showed little change. The data collected from mice of the same genotype at three different time points (PN19, PN20, and PN21) were considered as a group. Neither enamel nor dentin showed any statistically significant difference in density between *Clcn7*^{+/+} and *Clcn7*^{-/-} mice.

The architecture of the enamel was also examined using SEM. The mature portion of the enamel was noticeably thinner (hypoplastic) in *Clcn7*^{-/-} incisors when compared to wild type littermate (~ half the thickness; Figure 2, panel B when compared to panel A). However, when the mature ends of the incisors were observed at higher magnification (Figure 2, panels C and D), the typical enamel rod-interrod organization seen in *Clcn7*^{+/+} mice was also clearly apparent in the *Clcn7*^{-/-} incisors. The enamel crystallites also appeared to be of normal morphology and size in *Clcn7*^{-/-} incisors (Figure 2, panels E and F). Together with μ CT data, these results suggest that the morphology and tooth growth were impacted by the lack of CIC-7 (most notably the lack of molar root formation and the size of the incisor teeth); however, the enamel prismatic structure (rod/interrod architecture) was not seemingly disrupted.

Calvaria of the mutant mice

The whole head was scanned using μ CT with the intent to document any dental abnormalities, however we were struck by some of the abnormal features apparent in the calvarial bones of the mutant mice. We observed that the cranial sutures were wide open (Figure 3, panel D compared to Figure 3, panel C). The bone density of area (4mm-square) surrounding bregma and lambda was quantified by measuring mean intensity for all six samples (indicated by mean intensity in Figure 3I). *Clcn7*^{-/-} mice showed statistically significantly ($p < 0.001$) decreased bone density at bregma and lambda compared to their littermates. (The open sutures in *Clcn7*^{-/-} mice have not been previously reported.) While an impaired ability of osteoclasts to reabsorb bone in *Clcn7*^{-/-} mice may account for an osteopetrosis phenotype, failure of cranial suture closure clearly indicated that there was decreased bone growth at these sites, suggesting the bone formation by osteoblasts was compromised as a result of impaired osteoclast function.

The surface of calvarial bones in *Clcn7*^{-/-} mice was rough and porous (Figure 3 panel D compared to panel C). This was also apparent in the sagittal section of mandible (Figure 1, panel D compared to panel C), which showed that instead of being covered by more dense and compact bone, the surface of alveolar bone had the characteristics of trabecular bone. This observation was consistent with previous studies on the long bones that showed *CIC-7* deficient mice lacked compact cortical bone development (Neutzsky-Wulff et al., 2010). In addition, μ CT slices in transverse and sagittal section showed an increase in density of the cranial bones with the almost complete obliteration of bone marrow space (Figure 3, panels F and H when compared to panels E and G). Increased bone density in the mutant mice was especially noted at the skull base (Figure 3, panel H), where the bone marrow cavity shown in wild type mice (Figure 3, panel G) was almost completely occupied by irregular bone speculae in the mutant mice (Figure 3, panel G).

DISCUSSION

Mice lacking *CIC-7* or its obligate β subunit, *Ostm1*, show severe osteopetrosis as well as lysosomal storage disease (Kornak et al., 2001; Kasper et al., 2005; Lange et al., 2006; Weinert et al., 2014). Without *CIC-7*/*Ostm1* to operate alongside the V-ATPase to dissipate the transmembrane electrical potential difference, osteoclasts fail to acidify the extracellular resorption lacuna (Kornak et al., 2001). Lysosomal pH, on the other hand, is normal in cells lacking *CIC-7*/*Ostm1* due to shunting provided by cation counterflux (Kasper et al., 2005; Steinberg et al., 2010). Knock-in mice, *Clcn7*^{unc/unc}, in which *CIC-7* Cl^-/H^+ exchange is converted to chloride channel, also display osteopetrosis (albeit milder) and lysosomal pathology, indicating that Cl^-/H^+ exchanging activity mediated by *CIC-7*, rather than Cl^- conductance alone, is critical for osteoclast and lysosomal function (Weinert et al., 2010). In addition to *Clcn7*, most of the genes involved in osteopetrosis are associated with the pH regulation in osteoclasts including: carbonic anhydrase II (CAII), which generates a proton and a bicarbonate ion from carbon dioxide and water; anion exchanger AE2, which removes bicarbonate from the cytoplasm by exchange with chloride; and the V-ATPase subunit ATPV0A3 (TCIRG1), which transports protons (Del Fattore et al., 2008; Sobacchi et al., 2013). In many cases of osteopetrotic animals and human patients, the absence of teeth, or severe delay in tooth eruption, is a characteristic feature and often the first sign of osteopetrosis (Van Wesenbeeck and Van Hul, 2005). Osteopetrotic cattle with a *Clcn7* mutation display gross gingival hamartomas, also likely linked to non-erupting teeth (Sartelet et al., 2014). Abnormal tooth eruption has been attributed to the deficiency of osteoclasts and subsequent defect in alveolar bone resorption during tooth development (Van Wesenbeeck and Van Hul, 2005).

Ameloblasts, like osteoclasts, express V-ATPase (Lin et al., 1994; Josephsen et al., 2010; Damkier et al., 2014). The pH at the mature end of enamel fluctuates between acidic and neutral and the pH regulation is important in enamel formation (Smith, 1998). Therefore we intended to examine whether *CIC-7* plays a role in amelogenesis using the *Clcn7* knockout mouse model. In this investigation, we found that at 19–21 days of age the molar and incisor teeth of *Clcn7*^{-/-} mice had failed to erupt as previously reported (Kornak et al., 2001) and that the molar teeth of *Clcn7*^{-/-} mice had no evidence of root formation. In the mutant mice the shape and size of continuously erupting incisors were even more severely affected than

molars, and the enamel thickness of the incisors was approximately 50% less than the wild type. Despite these abnormalities, when examining the enamel and dentin, we found no marked difference in either mineral density content or the prismatic architecture and crystallite structural organization in the mutant mice when compared to wild type mice. These data suggest that although CIC-7 is ubiquitously expressed in most cells, including ameloblasts (Lacruz et al., 2013), the lack of CIC-7 expression in ameloblasts does not grossly impact the function of ameloblasts in directing enamel formation. The chloride channel (CLC) gene family encodes four chloride channels and five $2\text{Cl}^-/\text{H}^+$ exchangers in mammals (Jentsch, 2008; Stauber et al., 2012); and it is feasible that the lacking of CIC-7 conductance in ameloblasts might be carried out (or substituted) by other CLC family members or other channels as observed in lysosomes where a cation conductance suffices for providing electric shunt for acidification by V-ATPase (Steinberg et al., 2010; Weinert et al., 2010). Therefore, observed defects in tooth development of CIC-7 deficient mice are most likely due to abnormal tooth eruption. Consistent with this idea, the incisors, with their continuously erupting nature, are affected much more than molars. A similar dental phenotype has been shown in another osteopetrosis animal model; the Ae2 knockout ($\text{Ae2}^{-/-}$) mice (Josephsen et al., 2009). In the $\text{Ae2}^{-/-}$ mice the development of both the upper and lower incisors are impaired, however the authors noted that in these $\text{Ae2}^{-/-}$ mice the molar teeth had a radio-density comparable to that of wild-type (Josephsen et al., 2009).

With μCT , we noted that cranial sutures remained open at age 21 days, in contrast to the closed sutures seen in the wild type littermates. It seemed paradoxical that osteopetrosis, which is the result of bone overgrowth, and patent sutures, which are indicative of less than normal bone growth, were occurring in the same organism. These contradictory features could be due to differences in various skeletal sites with respect to osteoclastic bone digestion (Everts et al., 2009). This phenomenon is not unique for the $\text{Clcn7}^{-/-}$ mice. Pycnodysostosis, a rare autosomal recessive disease caused by the absence of active cathepsin K, an enzyme involving degrading the organic matrix of bones, shows both osteopetrosis in long bones and patent cranial sutures (Chen et al., 2007). $\text{Ae2}_{a,b}$ -deficient mice, deficient in the main isoforms of anion exchanger 2, which is responsible to keep near-neutral intracellular pH during proton pumping in osteoclasts, exhibit osteopetrosis of long bones but no alterations of calvaria (Jansen et al., 2009). Our observation in calvariae of the $\text{Clcn7}^{-/-}$ mice provided yet another example of bone-site-specific differences in bone cells leading to local differences in bone remodeling (Everts et al., 2009). Deficiency in osteoclast produces unbalanced bone remodeling affecting bone growth in sutures and long bones differently.

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Abbreviations

SEM	scanning electron microscopy
RA	ruffle-ended ameloblasts
WT	wild type
DDSA	dodecenyl succinic anhydride
NMA	nadic methyl anhydride
DMP-30	dimethyl aminomethyl phenol

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Figure 1.

Clcn7^{+/+} (panels A, C, and E) and *Clcn7*^{-/-} (panels B, D, and F) teeth at age PN21. Panels A–F: μCT three-dimensional images (panels A and B) and cross-sectional slices of incisors (panel C and D) and molars (panel E and F) of two typical examples of teeth from *Clcn7*^{+/+} and *Clcn7*^{-/-} littermates. AMIRA software was used to analyze the μCT data. Scale bars (1mm) are included in each panel. Arrowheads show molars that are encased in alveolar bones (panel F). Panel G: the length of mandibular and maxillary incisors. The average and standard deviations (error bars) were plotted using six incisors from three mice of the same genotype as a group. One-tailed paired Student's t tests were run and the length of both mandibular and maxillary incisors in *Clcn7*^{-/-} mice was significantly smaller ($p < 0.001$) than that in *Clcn7*^{+/+} mice. Panel H: the density of mature enamel and dentin, indicated by mean intensity value from μCT scanning, were compared between *Clcn7*^{+/+} and *Clcn7*^{-/-} mice using all three age matched pairs of mice. The averages and standard deviations (error bars) were plotted. Two-tailed paired Student's t tests were run and no statistically significant difference in either enamel or dentin density was identified between *Clcn7*^{+/+} and *Clcn7*^{-/-} mice.

Figure 2.

Scanning electron microscope (SEM) images of tooth enamel. Teeth were embedded in EPON and ground and polished to expose the prismatic structure in a sagittal section of the incisors. Panels A, C and E are images from an incisor of *Clcn7*^{+/+} at age PN21 with 1000 $\times$, 3000 $\times$, and 10000 $\times$ magnification, respectively, whereas Panels B, D and F are from the PN21 *Clcn7*^{-/-} littermate using the same magnification scales. All panels show the mature end of the enamel structure.

Figure 3.

Micro-CT images of *Clcn7*^{+/+} (panels A, C, E and G) and *Clcn7*^{-/-} (panels B, D, F, and G) skulls at age PN21. Images of front view (panels A and B) and top view (panels C and D), and slices of transverse sections (panels E and F) and sagittal sections (panels G and H) are illustrated. AMIRA software was used to analyze μ CT data. Scale bars (1mm) are included in each panel. Arrowheads in panels G and H show lack of bone marrow cavity at skull base in the mutant mouse compared to its wild type littermate. Bone density of areas surrounding bregma (2) and lambda (1) shown as 2mm squares in panel C were quantified. The corresponding areas in panel D (not shown) were similarly quantified. The mean intensity were plotted in panel I.