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The Role of GPR155 in Trans-ameloblast Calcium Transport

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The Role of GPR155 in Trans-ameloblast Calcium Transport

Ida Gorshteyn

Abstract

Purpose: The roles of G protein-coupled receptor 155 (GPR155) have not been previously explored in the context of amelogenesis. This study aims to determine the role of GPR155 in enamel maturation, and specifically its involvement in trans-ameloblast calcium transport. Methods: We collected enamel organ cells from murine first molars, providing ameloblasts at the secretory, transition, and maturation stages of development in both *Nckx4*$^{+/+}$ and *Nckx4*$^{-/-}$ mouse models. RNA was extracted and GPR155 expression in both models were analyzed by qPCR and immunostaining. We used RT-qPCR to characterize the expression of *Orai2* and *Trpm7*, which are known contributors to trans-ameloblast calcium transport. The correlation between the expression of GPR155 and enamel matrix calcification was also analyzed in the lactating mouse. Results: *Gpr155* expression increased in the transition stage, peaked in mid-maturation stage, and slightly reduced in late-maturation stage. There was a significant increase ($P<.05$) of *Gpr155* expression in *Nckx4*$^{-/-}$ maturation stage ameloblasts. Calcium transport associated genes *Trpm7* and *Orai2,* which are co-upregulated with *Gpr155* in the wild type maturation ameloblasts, were also upregulated with *Gpr155* in the *Nckx4*$^{-/-}$ maturation ameloblasts. Finally, immunostaining showed increased signals of GPR155 protein in maturation ameloblasts of lactating mice. Conclusion: Results suggest that GPR155 participates in calcium transport within maturation stage ameloblasts. This less-characterized gene has shown great promise in the realm of calcium signaling and control during the later stage of amelogenesis, contributing to our understanding of how interactions between the enamel matrix and ameloblasts direct the biomineralization of the hardest tissue in our bodies.

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BACKGROUND

Amelogenesis is an ameloblast-directed process of production and deposition of enamel matrix, followed by mineralization of this matrix. This process occurs in three main stages, called pre-secretory stage, secretory stage, and maturation stage. Prior to the pre-secretory stage, the inner enamel epithelium releases growth factors and signaling molecules to promote the differentiation of dental papilla cells into odontoblasts[1, 2]. In turn, the differentiation of pre-secretory ameloblasts begins to take place. In the secretory stage, these ameloblasts build up an organic matrix by secreting the enamel matrix proteins from their distal extensions, called Tomes' processes. The composition of this protein-rich enamel matrix consists of majority amelogenin (90%) and a mixture of tuftelin, enamelin, and ameloblastin et al[1]. In addition to the enamel matrix proteins, secretory ameloblasts also generate proteinases, primarily MMP20, to process matrix proteins and trigger their functional domains to assemble into structural scaffolds. This scaffolding accounts for the directional growth of calcium phosphate-based hydroxyapatite in the enamel matrix[2]. In the maturation stage, enamel matrix proteins are further degraded, endocytosed, and replaced with fluid and minerals[2]. At this point, active transport of mineral ions by ameloblasts modulating between ruffle-ended and smooth-ended morphologies begins. These varying ameloblast morphologies reflect cyclical changes in ameloblast function related to pH regulation and ion transport until the enamel becomes fully crystalized[3]. Thus, the transport of large quantities of calcium is inherently an important biological process of normal amelogenesis or enamel development[2]. Previously characterized molecules that participate in the trans-ameloblast calcium transport include transient receptor potential cation channel, subfamily M, member 7 (TRPM7)[3], store-operated calcium entry pathway members ORAI1/2, STIM1/2[4], apical membrane calcium exchangers NCX,NCKX4, and calcium pump PMCA[2, 5, 6]. In the store-

operated calcium entry pathway, STIM1 acts as a calcium sensor in the endoplasmic reticulum (ER) and communicates with the ORAI1/2 proteins at the ameloblast's basal surface to regulate calcium entry into ameloblasts from the papillary layer into the ER, where it is then stored[2]. Calcium is released from the ER storage when inositol triphosphate receptor (IP_3R) binds to the secondary messenger IP3 and effluxes into the enamel space through NCXs, PMCA, and NCKX4[4]. It is then not surprising that *Nckx4* knockout mice displayed defective enamel as NCKX4 has been linked to the transfer of the calcium ion from ameloblasts to the forming enamel matrix dependent on the transmembrane ion gradient. This role is further bolstered by the finding of NCKX4 immunolocalization at the apical poles of maturation stage ameloblasts[5]. Furthermore, the enamel defects in *Nckx4* null mice resemble the defective enamel of individuals with amelogenesis imperfecta who carry a mutation on the human *NCKX4* gene.

Amelogenesis imperfecta (AI) is a genetic disorder of tooth development which affects about 1 in 14,000 individuals in America[7]. AI may be inherited by autosomal dominant, autosomal recessive, or X-linked transmission[8]. Clinically, AI is characterized by fragile, pitted, or discolored enamel. Furthermore, affected teeth may be overly sensitive due to the variable enamel state and possible dentin exposure, and can present with loss of proximal contacts which leads to excessive trapped food. On the whole, this presentation often causes patients low self-esteem, trouble eating, and pain[7,9]. Because there is relatively little clinical data surrounding AI, clinical treatment planning decisions can often be difficult to make.

Amelogenesis imperfecta has been shown to occur as a result of gene mutations and variants in a clinical setting. Observed clinical cases have been sent for genetic testing and several genetic variants have been identified in humans. Clinically, the types of AI can be subdivided into a few major categories. These are referred to as hypoplastic (Type I),

hypomaturation (Type II), or hypocalcified (Type III). Type I AI is typically defined as failure during the secretory stage[7,8]. The enamel is thin, but mineralized. Type II AI is defined as failure in the maturation stage. The enamel's presentation is full-thickness, but weak and brittle due to the incomplete removal of protein from the enamel matrix. Type III AI is often due to insufficient transport of calcium ions following complete removal of the protein matrix, which can lead to full thickness but extremely soft enamel[1]. These types can be divided further into subtypes distinguished by specific genetic cause and pattern of inheritance. Many of these AI causal genes have been found to be significant in the processes of enamel matrix protein synthesis and secretion, enamel matrix pH sensing, extracellular matrix-ameloblast adhesion, and the removal of the protein-rich matrix and its subsequent replacement with calcified tissue. In short, amelogenesis imperfecta can be described as a genetic fault which disrupts the natural process of amelogenesis.

The University of Leeds Amelogenesis Imperfecta Leiden Open Variation Database (LOVD) details 192 different, published AI gene variants identified in 270 families with AI[9]. Of all these published gene variants, four genes account for the cause of AI in 60.4% of the families included in the database. The most common variants identified were in *FAM83H* (19.3% of cases), *FAM20A* (15.2% of cases), *ENAM* (14.2% of cases), and *AMELX* (11.5% of cases). Among these AI families, 48.9% have autosomal dominant AI, while 40.4% have autosomal recessive, and 11.5% have X-linked inheritance. Of course, the LOVD resource focuses on certain populations for ease of study and the results may thus be biased[9]. *NCKX4* mutations are seen to be the causal factor of AI in 2.2% of families in the database. Of those families, there seems to be no ethnic preference, but they are all autosomal recessive variants[9]. This database is just the beginning of an important collection of information. In addition, more studies are needed

to bolster the existing literature on the genetics of amelogenesis imperfecta[8]. Due to the lack of accessibility to the developmental stages of human enamel organ specimens, enamel researchers rely on mouse models to investigate the genetic causes and molecular mechanisms responsible for amelogenesis imperfecta. Further, the mouse incisor serves as an ideal tool to study enamel development as it has a full representation of ameloblast differentiation and enamel development from cervical loop to incisal tip[4].

As shown by the aforementioned studies, calcium handling is critical for the biomineralization of enamel matrix. The significance of NCKX4 in facilitating calcium movement from the ameloblast into the developing enamel matrix has been evident in the *Nckx4* function deletion mouse model. Maturation ameloblasts from *Nckx4*$^{-/-}$ mice had significantly upregulated *Gpr155* expression as compared to *Nckx4*$^{+/+}$ maturation ameloblasts. GPR155, a novel molecule in term of amelogenesis, has come to our attention recently. In line with our microarray analysis showing a significant upregulation of *Gpr155* in wild type mouse maturation ameloblasts as compared to secretory ameloblasts, Simmer's group also shows a 9.9-fold increase of *Gpr155* in the maturation stage as compared to the secretory stage through a microarray analysis[11]. Moreover, a t-SNE dimensional reduction which visualized the expression profiles of dental epithelial cells demonstrated that *Nckx4* and *Gpr155* were all significantly upregulated in ameloblast maturation[12]. These data led us to investigate herein how GPR155 participates in NCKX4-mediated calcium trans-ameloblast transport.

The existing literature on GPR155 identifies it as a G protein coupled receptor, but much of its potential function and background is unknown. Recent neuroscience research indicates that *Gpr155* and *Nckx4* have similar co-expression patterns in the central nervous system as they do in ameloblasts[13]. *Gpr155* mRNA is expressed in neural tissue during development, with the

highest upregulation in the lateral striatum and hippocampus. Moderate upregulation is also evident in the olfactory bulb, where coincidentally, some of the first *Nckx4* studies were conducted[11,12].

Due to its significant upregulation in the maturation stage ameloblasts in the wildtype as well as in the *Nckx4* knockout mouse maturation ameloblasts, we hypothesize that GPR155 may be involved in regulating the activity of calcium efflux into the enamel matrix. In wild type and *Nckx4*$^{-/-}$ mouse maturation ameloblasts, we also detected significant upregulation of *Trpm7* and *Orai2*, both of which are associated with bringing calcium into ameloblasts from the papillary layer. Therefore, we concluded that *Gpr155* upregulation is associated with increased calcium demand in the enamel matrix and subsequent increased calcium entry into the ameloblast cell and then ultimately into the enamel matrix. We further confirmed the correlation between *Gpr155* expression and ameloblast calcium transport using the lactating mouse model, in which the calcium supply in circulation may be compromised[4,17,20].

Our results indicate that the increased expression of GPR155 in late stages of amelogenesis has an important function in regulating calcium transport during enamel maturation. Furthermore, the significantly increased expression of GPR155 in maturation stage amelogenesis of *Nckx4* knockout mice suggests a negative feedback loop function in which the decreased levels of calcium in the enamel matrix cause an overexpression of the proteins which seek to advance the calcium transport in the absence of sufficient concentrations of calcium. Ultimately, the results and conclusions herein aim to determine the role of GPR155 in enamel maturation, and specifically its involvement in trans-ameloblast calcium transport.

MATERIALS AND METHODS

Animal genotyping:

NCKX4 loss-of-function mouse colony (*Nckx4*$^{-/-}$) was a gift from Dr. Jonathan Lytton, whose team deleted exon 6 and 7 to generate a NCKX4 function deficient mouse model[15]. All animals were maintained in the UCSF animal care facility, which is a barrier facility, accredited by Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). All experimental procedures associated with this established mouse model were approved by the Institutional Animal Care and Use Committee (IACUC) under the protocol AN183449-01. Mouse tails were snipped from the *Nckx4* heterozygous mating pairs. Then the genome DNA was extracted for PCR. Agarose gel electrophoresis was conducted to visualize the PCR products, which were then used to determine the genotyping of each corresponding mouse.

RT-qPCR analysis:

First molars from wild type or *Nckx4*$^{-/-}$ mice were dissected on days P5, P9, P11, P12 and P13 from mandibles, where ameloblasts are progressively at the secretory, transition, early maturation, and late maturation stages of development. First molars from five mice were pooled together as an experimental group. Total RNA was purified using Quick-RNA MiniPrep Kit (Genesee Scientific). Complementary DNA was generated by using reverse transcriptase III (Invitrogen), and gene-specific primers were synthesized for RT-qPCR. SYBR Green Master Mix (Bio-Rad Laboratories) was used in semi-quantitative PCR assays to proportionally indicate the abundance of amplicons according to the manufacturer instructions. The significant difference in the expression levels of *Gpr155* in the variant developmental stages of ameloblasts was determined by one way ANOVA analysis using SPSS Statistics package 19 followed by Tukey Post Hoc Tests. Two-tail t-test statistical analysis was applied to assess the difference in

the expression levels of target genes between *Nckx4*$^{+/+}$ and *Nckx4*$^{-/-}$ maturation stage ameloblasts.

Immunohistochemistry:

Wild type mice (*Nckx4*$^{+/+}$) and *Nckx4*$^{-/-}$ mice at postnatal seven weeks old (P7W) were collected following standard IACUC protocols. Eight week old female littermates were divided into two groups. One group was bred with male wild types and another group was housed pair-wisely to serve as virgin controls. On day 21 of lactation, the pups were weaned, and the lactating mice were euthanized with carbon dioxide asphyxiation followed by cervical dislocation. To characterize the histological phenotype of ameloblast/enamel matrix, mouse incisors were examined because they possess all developmental stages of ameloblasts and enamel matrix. Briefly, the hemimandibles were dissected and fixed with 4% PFA for 24 hours at 4°C, then decalcified in 8% EDTA at 4°C for 3 weeks, with EDTA being changed every other day. The hemimandibles were then processed, embedded and sectioned at the sagittal plane. Sections were stained with hematoxylin & eosin (H&E) for ameloblast and enamel matrix morphological examination. Tissue sections were dewaxed and rehydrated as is routine. For immunostaining, the sections were incubated with 0.15 mg/mL pepsin in 0.2N HCl for 15 minutes at 37ºC to retrieve antigens. Next, the sections were incubated with Universal Protein Blocking Reagent (GeneTex) for one hour, followed by incubation with rabbit anti-GPR155 antibody (Abcam) overnight at 4°C. Sections were then incubated with HRP-conjugated goat-anti-rabbit IgG for 1 hour at RT, followed by incubation with the Vectastain AEC peroxidase substrate kit (Vector Laboratories) as described in the protocol provided by the manufacturer. Sections were counter-stained with Mayer's hematoxylin, dehydrated through ethanol series, dewaxed and mounted. The slides were imaged using a Leica microscope.

RESULTS

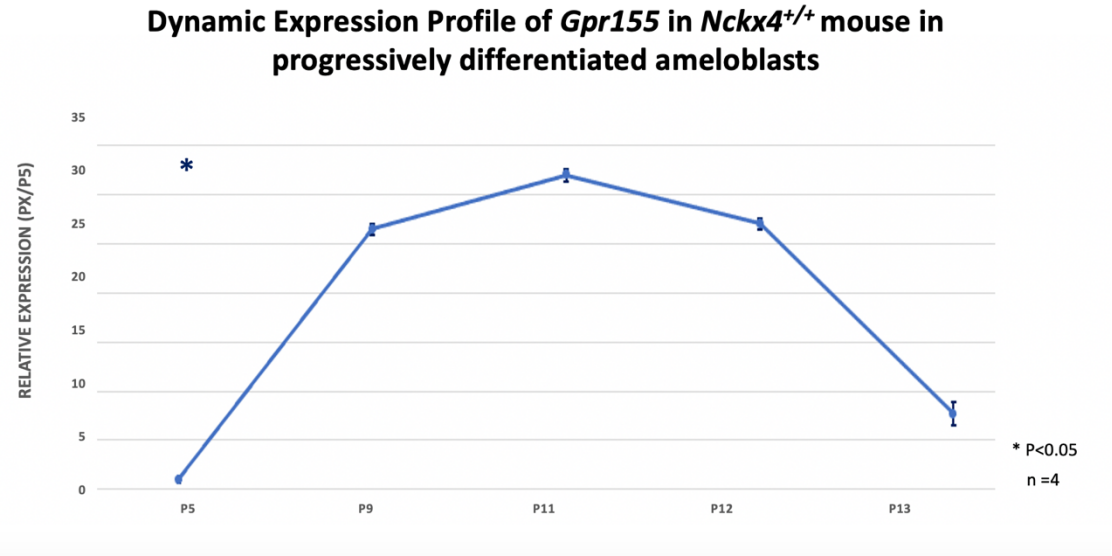

Fig1: Temporal expression of *Gpr155* in ameloblasts at P5, P9, P11, P12, and P13. Compared to ameloblasts at the secretory stage (P5), *Gpr155* expression was significantly increased (P<.05) at P9, P11, P12, and P13.

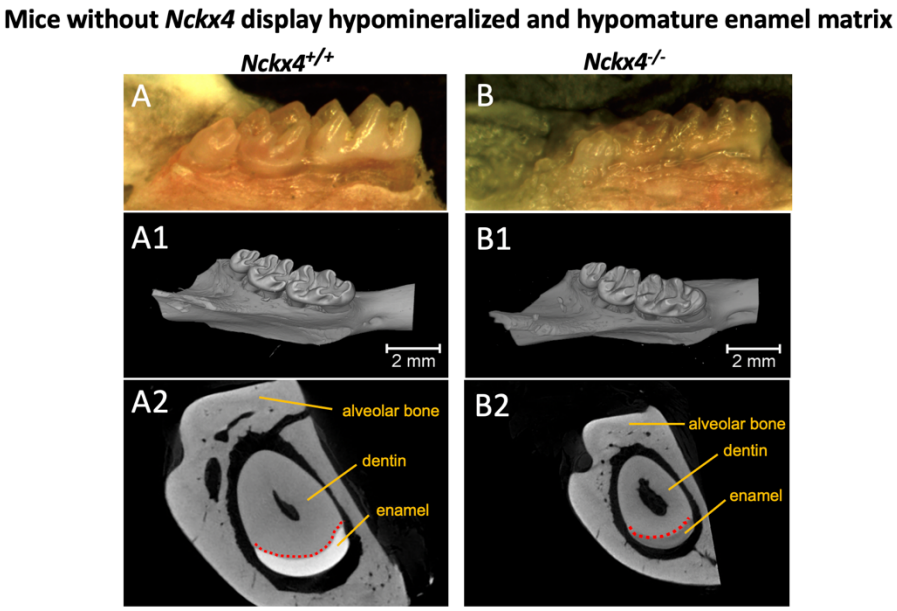

Fig2: Stereo microscopic analysis shows the *Nckx4*$^{+/+}$ mouse incisor had smooth and whitish enamel, and its molar cusps were sharp and well-developed (FigA). *Nckx4*$^{-/-}$ mouse incisor enamel was rough with brown pigmentation, and its molar cusps were worn down and stained (FigB).

Reconstituted microCT image of the mouse hemimandible (Fig1A) shows an intense radiopaque layer covering incisor and molars of *Nckx4*$^{+/+}$ mice, where the enamel layer was clearly contrasted to the underlaying dentin and surrounding alveolar bone (FigA2). Meanwhile, microCT of the *Nckx4*$^{-/-}$ hemimandible showed minimal mineralization of the enamel layer of incisor and molars, not clearly contrasted to the underlaying dentin and alveolar bone (FigB1, B2).

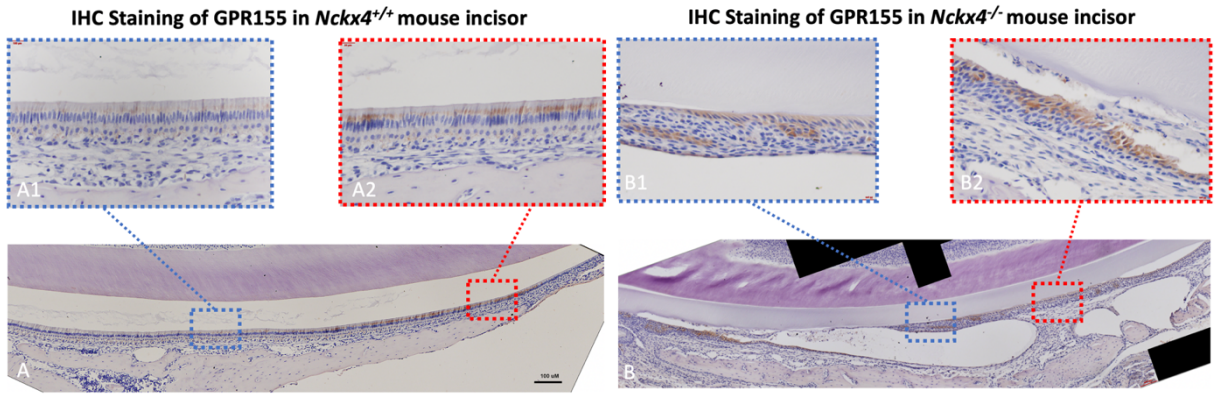

Fig3: Immunostaining of the mouse incisor sagittal section indicates that GPR155 signal was increased in the deformed ameloblasts of *Nckx4*$^{-/-}$ mice. Fig A shows a 10x view of the wild type mouse incisor, with GPR155 upregulation in the single-cell layer of maturation stage ameloblasts (FigA2) as compared with secretory stage ameloblasts (FigA1). Fig B shows a 10x view of the *Nckx4* knockout mouse incisor, where there is upregulation of GPR155 in the maturation stage ameloblasts (FigB2) as compared to the secretory stage ameloblasts (FigB1). However, due to loss of function of *Nckx4,* the ameloblast development has altered and divided into several layers, amongst which the GPR155 is widely distributed (FigB1, B2).

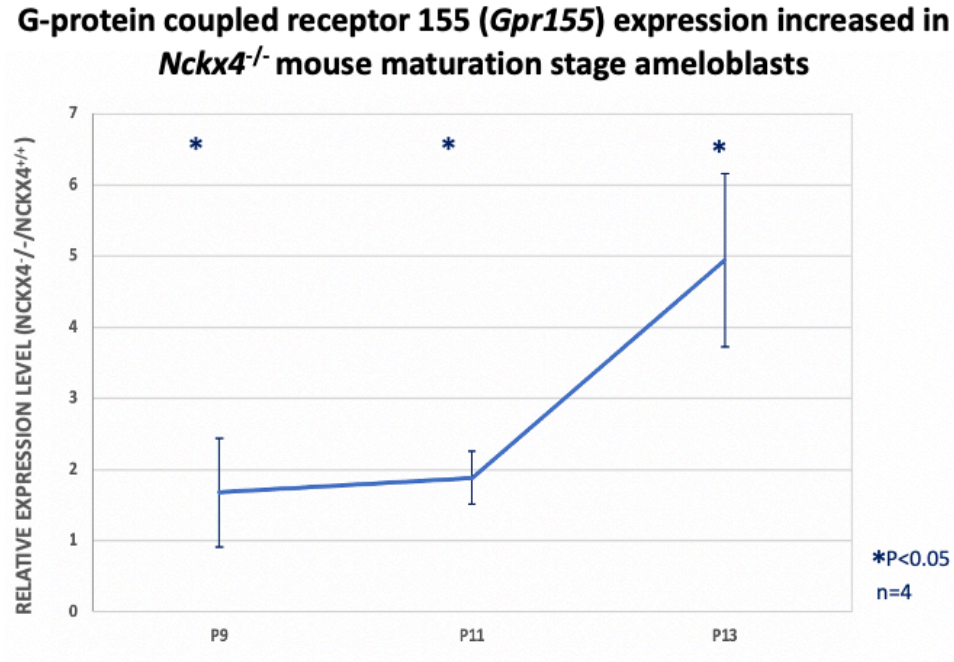

Fig4: *Gpr155* is significantly (P<.05) upregulated in *Nckx4*$^{-/-}$ maturation ameloblasts as compared to *Nckx4*$^{+/+}$ maturation ameloblasts for the timepoints P9, P11, and P13 we detected.

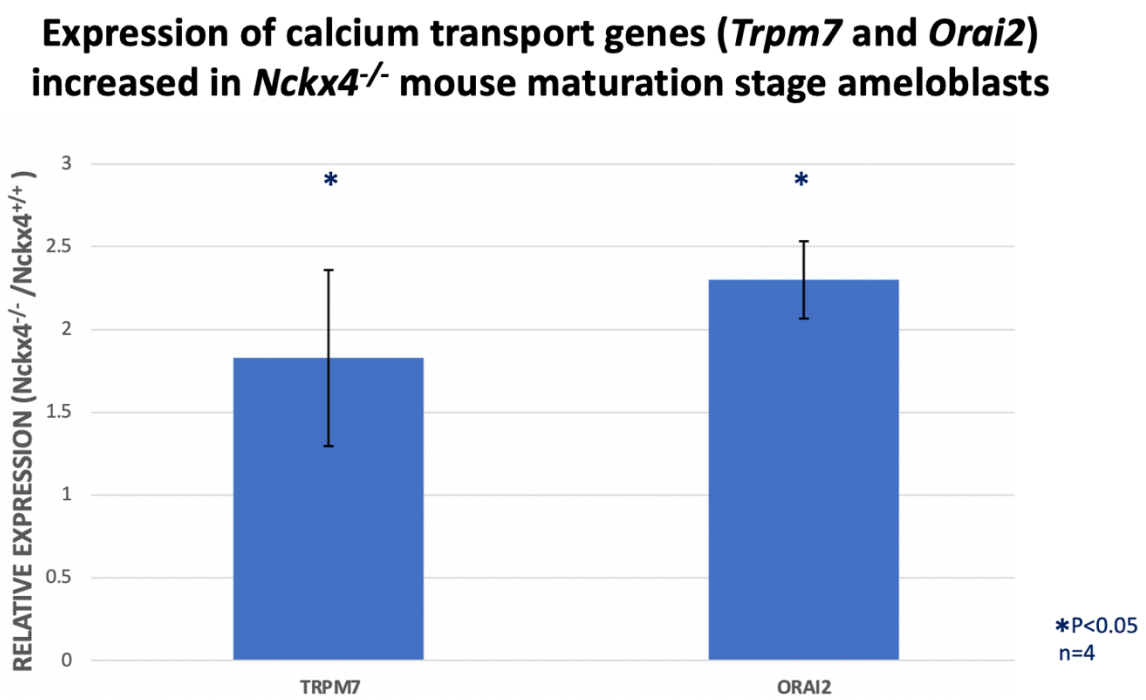

Fig5: Known genes involved in calcium transport, *Orai2* and *Trpm7*, are significantly upregulated (P<.05) in *Nckx4*$^{-/-}$ maturation stage ameloblasts

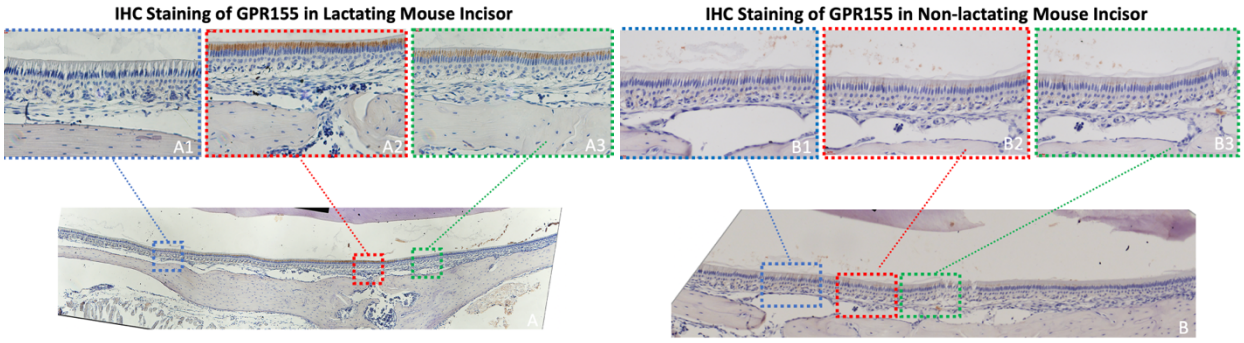

Fig6: Immunostaining of lactating vs non-lactating mouse incisors shows increased protein signal of GPR155 in maturation stage ameloblasts in the lactating model (FigA) as compared to the nonlactating model (FigB). Figs A1-3 illustrate 40x views of secretory, mid-maturation, and late-maturation ameloblasts in a lactating mouse, respectively. Mid maturation ameloblasts (A2) show the most GPR155 expression. Figs B1-3 illustrate 40x views of secretory, mid-maturation, and late-maturation ameloblasts in an age-matched non-lactating control mouse, respectively.

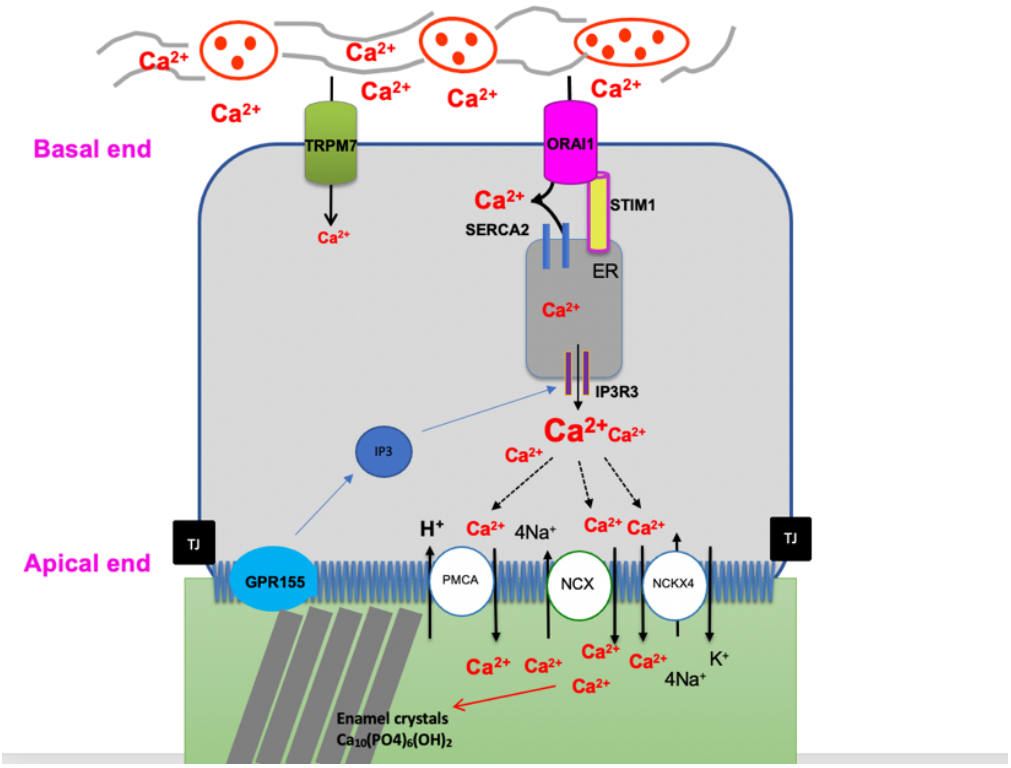

Fig7: Graphic representation of suggested GPR155 mechanism within maturation stage ameloblast cells, in conjunction with other store-operated calcium entry genes and functions. Efficient transport of ion to enamel matrix is critical for enamel maturation.

DISCUSSION

Enamel is the hardest tissue in vertebrates mostly due to its highly mineralized composition. Minerals make up more than 96% of enamel weight[6]. About 86% of the minerals that are required for enamel biomineralization are transported by maturation ameloblasts[7]. Therefore, it would be intuitive to believe that maturation ameloblasts are largely engaged in the calcium delivery process. G protein-coupled receptor 155 (GPR155) has been shown to be a gene significantly upregulated in maturation ameloblasts as compared to secretory ameloblasts through multiple whole transcriptome sequencing analyses[10,11,12]. However, the functions of GPR155 in amelogenesis has not yet been studied. By analyzing the expression levels of *Gpr155* in the progressively advancing ameloblasts, we observed its trajectory expression pattern: the upregulation begins at the transition stage, continuously climbs in the maturation stage ameloblasts, then declines at the end of maturation stage. Utilizing a mouse model with the deletion of NCKX4, a calcium transporter responsible for extruding calcium from ameloblasts into enamel space, we delineate, for the first time, GPR155's engagement in calcium transport during enamel biomineralization.

NCKX4, a calcium transporter, has been determined to be a causal gene for amelogenesis imperfecta[1,23]. NCKX4 has been localized at the apical surface of maturation ameloblasts along with PMCA and NCXs[18,19], which indicates that the function of NCKX4 is to extrude calcium from ameloblasts into the enamel matrix. Previous research studies have demonstrated that store-operated calcium entry pathway (SOCE) elements, including calcium channels ORAI1 and ORAI2, and cation channel TRPM7 are responsible for bringing calcium into maturation ameloblasts. Therefore, we assessed the expression of *Orai2* (the major ORAI molecule in maturation ameloblasts based on our microarray analysis) and *Trpm7* in the *Nckx4*$^{-/-}$ mouse

maturation ameloblasts, and surprisingly found that both of them were upregulated, as was *Gpr155*. This result implied that by sensing the reduced calcium content in enamel extracellular matrix resulting from loss-of-function of NCKX4, maturation ameloblasts responded to import more calcium intending to rescue this deficit. Thus, upregulation of GPR155 is part of the ameloblast response to the limited or deficient calcium supply.

In order to further investigate the correlation between enamel matrix calcium demand and GPR155 expression levels, we used lactating mice, whose circulating calcium may be reduced in order to provide the minerals necessary for the rapid growth of their pups. Secondary to the low supply of calcium in the circulation, we assumed that there is less calcium being provided to the biomineralization process of the continuously growing incisor enamel during this time. Not surprisingly, we detected the increased GPR155 in the maturation ameloblasts of lactating mice as compared to their virgin littermates. This is further evidence indicating that GPR155 might engage calcium demand in the enamel matrix.

We also observed that GPR155 expression reached its peak in the middle of maturation stage of enamel formation both by RT-qPCR and immunostaining. Smith CE has demonstrated that the most calcium influx into the matrix occurs during the middle of enamel maturation stage. These minerals form highly organized hydroxyapatite crystals that account for the great hardness of enamel tissue, which is about 4 times the hardness of bone and dentin.[24] This result also implies the aforementioned role of GPR155 in the calcium transport and enamel biomineralization.

GPR155 is a cell surface protein, which has 7 transmembrane domains. The N-terminus of GPR155 localizes outside of cells and has three consensus N-glycosylation sites[13]. These N-glycosylation sites are proposed to have a “mucin-like stalk” which could help facilitate the

GPCR's interaction with extracellular ligands that cannot directly pass through the cell membrane[25,26]. This interaction then allows for the GPCR to undergo a conformational change into its biologically active form and activates the coupled G-protein to further regulate the function of target proteins and messengers[27,28]. In this case, the activation of this pathway ultimately allows secondary messenger IP3 to interact with the IP3 receptor of the endoplasmic reticulum to release calcium and thereby increase cytosolic calcium ion concentration[29,30]. It is possible that the extracellular ligand responsible for beginning this cascade is calcium, phosphate, pH, hydrolyzed enamel matrix proteins, or molecular remnants of apoptotic ameloblasts.

Amelogenesis imperfecta is a well-known, clinically found tooth disorder in both children and adults alike. However, it is a complex disorder involving many potential gene mutations and much of its underlying genetics is left unknown. The more data and knowledge that we can accumulate about AI, the more clinically relevant decisions and techniques we can develop to treat this population of patients. Understanding GPR155 and its engagement with calcium transport and biomineralization may aid in enamel bioengineering and cure amelogenesis imperfecta.

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