Investigating the Etiology of Primary Failure of Eruption (PFE): A Comprehensive Phenotypic and Genetic Analysis

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ABSTRACT

Heather Morgan Hendricks: Investigating the Etiology of Primary Failure of Eruption (PFE): A Comprehensive Phenotypic and Genetic Analysis (Under the direction of Sylvia A Frazier-Bowers)

The genetic basis of PFE (OMIM ID: 125350), a diagnosis that conveys a poor prognosis in the eruption/ function of teeth, was interrogated. Treatment with a continuous archwire worsens the condition. Two aims tested the hypothesis that PTH1R mutations result in loss of function and that multiple genes cause PFE: to determine 1) the fate of a functional PTH1R mutation and 2) PFE contribution by BMP2 and TNFSF-11. Methods: We used IFA and transfected COS7 cells with either the WT or 1092delG PTH1R mutation sequence to compare the fate of the expressed protein and performed mutational analysis of BMP2 and TNFSF-11 with PCR and sequencing. Results: Sequencing revealed 3 intronic SNPs in TNFSF-11; in silico /functional studies showed expression alterations and structural changes in mutant vs WT PTH1R protein. Conclusion: PTH1R mutations in PFE likely result from diminished function; causative mutations in genes.
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INVESTIGATING THE ETIOLOGY OF PRIMARY FAILURE OF ERUPTION (PFE): A COMPREHENSIVE PHENOTYPIC AND GENETIC ANALYSIS

Introduction

Background and Significance

The process of normal tooth eruption is an integral part of normal tooth development and a thorough understanding of the biologic underpinnings is critical for any dental practitioner. Every member of the dental team needs to have a comprehensive and functional understanding of the patterning and sequencing of eruption in order to provide optimal care. The ability to develop a prioritized diagnosis and treatment plan as a result of this knowledge is especially important for the practicing clinician and therefore makes expanding our understanding of this biologic process (i.e. eruption) more critical. This knowledge also includes the ability to understand when to refer a patient to a specialist, who may be better trained to treat certain diagnoses. The literature has identified and described many different etiologies of eruption disturbances that are not uncommon occurrences within the dental practice, however, the critical step in diagnosing eruption disorders lies in first identifying its etiology out of the wide range of possibilities. It is understood that eruption disorders may stem from local and non-syndromic causes (cysts, ankylosis, lateral tongue pressure, supernumerary teeth, thumb habit,) or may manifest as part of a genetic syndrome (Cleidocranial Dysplasia, Hunter’s Disease and Osteopetrosis)(Figure 1).
**Eruption Disorders and Diagnostic Approaches**

Three categories of eruption disorders exist within the broad spectrum of local/non-syndromic causes.\(^2\) These are Mechanical Failure of Eruption (MFE), ankylosis, and the more perplexing- Primary Failure of Eruption (PFE) (OMIM ID: 125350). MFE is defined an eruption failure due to a mechanical obstruction of a tooth’s eruption pathway- which can often be treated successfully with the removal of the mechanical blockage due to a correct diagnosis.\(^8,13\) Ankylosis is defined as the occurrence when the cementum on the root of the tooth fuses directly to the bone, thereby eliminating the periodontal ligament space (PDL) and halting spontaneous eruption potential.\(^3,4\) However the primary diagnosis is based on clinical appearance; this often includes infraocclusion relative to the adjacent teeth. An important adjunctive diagnostic approach is the diagnosis of ankylosis through radiographic examination; this is based largely on identifying locations of an absence of a visible periodontal ligament space. However, the diagnosis of ankylosis is frequently more subjectively based on the lack of clinically-appreciable mobility, and a sharp sound noted upon tooth percussion with a metal object.\(^5\) It is important to realize the limitations of these diagnostic techniques, in addition to their applications. Currently, the only two ways to definitively diagnose an ankylosed tooth is to apply orthodontic force and wait for the tooth to move, or to luxate the tooth and achieve visual mobility. The ability to implement advanced imaging is a promising tool, however, when evaluating a two dimensional radiograph, the appearance of PDL fusing to bone can either be overstated or completely undetected; this is in part due to the ability, or inability, to radiographically image mineralized tissue that falls within a specific range of density and size.\(^6,13\) To date, there have been no investigations evaluating the ability to diagnose ankylosis using
a Cone Beam CT scan of any size or resolution. Even though the large field of view CBCT scans have an average resolution of 0.3-0.4 voxels, this is still an inadequate resolution to evaluate whether a fusion of cementum and bone exists. The important goal in distinguishing ankylosis from other eruption problems is that unlike PFE, the ankylosed tooth can be extracted and the remaining teeth will likely be responsive to orthodontic treatment. The challenge is however that ankylosis can often be confused with the more enigmatic Primary Failure of Eruption (Figure 2).

Primary Failure of Eruption is poorly understood but clearly falls within the spectrum of Local/Non-syndromic eruption disruptions. First described at UNC by Proffit and Vig (1981), PFE is clinically marked by failure of permanent (adult) tooth eruption in the absence of mechanical obstruction, and affects only the posterior dentition. The use of the term, primary, denotes that the defect is located in the eruption mechanism of the tooth itself. PFE diagnosis has been characterized by distinction of two different patterns of clinical presentation: Type 1 PFE and Type 2 PFE. Type 1 PFE presentation consists of a progressive, anterior-posterior lateral open bite that worsens distal to the most mesially positioned PFE-affected tooth; Type 2 PFE presents with a more varied pattern of infra-eruption distal to the most mesially affected tooth. An early possible treatment of isolated ankylosis of permanent first molars can be managed via the extraction of the offending ankylosed tooth; this will allow for normal eruption of the second and third molars. The failure of the second and third molars to fully erupt would be pathognomonic for PFE. This selective extraction pattern is a potential treatment for a patient who presents to the clinic before the eruption of the permanent second molars; however, the typical sequela for PFE affected individuals is unfortunately the high morbidity of
complex surgical intervention and multiple tooth extractions required to restore form and function (Figure 3).

From a treatment standpoint these cases are complicated; if traditional orthodontics is attempted the condition only worsens (Figure 1). Taken together, this supports that the diagnosis of PFE conveys a poor prognosis in the normal eruption and function of teeth. Recent investigations that have focused on PFE and other eruption disorders have simply done so from an etiologic perspective (i.e., no consideration as to how the etiology relates to the phenotype and proposed management has been combined for a holistic picture of the disorder). The discovery and association of PFE with the parathyroid one hormone receptor gene, PTH1R (discussed below), opens a new dimension of investigation; the role of the PTH1R gene alone may substantially contribute to our understanding of developmental processes beyond tooth eruption.8, 9, 16

**PTH1R and PFE**

Since the original description of PFE, investigations of its etiology have been completed by multiple researchers; a major commonality among these documented studies of PFE is the identification of mutations in the PTH1R gene. Currently, autosomal dominant mutations in the (PTH1R) gene have been identified as the only concrete cause of PFE.2,8, 9, 16, 17, 18, 30 This recent genetic discovery associated with PFE represents a breakthrough, creating a paradigm shift in diagnostic and clinical approaches. PFE and its connection with PTH1R not only reveals another dimension of inquiry into the aspects of dental eruption patterning, but it obviates the gaps in knowledge that exist in terms of how this gene may be integrated within the odontogenic code and what, if any relationship the PTH1R-associated dental phenotype has with the overall
systemic health. For example, the fact that PFE that solely expresses as a dental problem, now understood to be a genetic disorder caused by PTH1R, is implicitly connected to systemic processes that are also governed by this gene. Hence, the isolated genotype:phenotype relationship between PTH1R and PFE presents an interesting conundrum. A deeper understanding of the PTH1R gene may offer insights as to why this presents in clinically diverse conditions; such as Jansen’s Metaphyseal Chondrodysplasia (OMIM ID: 156400), Bloomstrand’s Lethal Chondrodysplasia (OMIM ID: 215045), Enchondromatosis (Ollier’s Disease) (OMIM ID: 166000), osteoarthritis, and PFE. Briefly, Jansen’s is caused by autosomal dominant heterozygous mutations in PTHR1 resulting in short-limbed dwarfism (Figure 4). Ollier’s disease is also correlated to an autosomal dominant mutation, which is characterized by multiple enchondromas located primarily in the metaphyseal shafts of long bones; having the ability to develop into chondrosarcomas. Bloomstand’s, however, exhibits a recessive PTHR1 mutative deactivation which results in early lethality, shortened limbs, and premature bone maturity (Figure 5). The variation of clinical presentations of both dominant and recessive autosomal mutations within the same gene make its inquiry that much more interesting, and exhibits why PFE, having more relatively benign effects, could be a potential model of PTH1R gene studies. Although studies completed in this project cannot fully answer these questions, they provide the impetus for future studies that will unravel these mysteries.

**PTH1R: A G-protein coupled receptor**

While the varied functions and interactions of PTH1R are indeed intriguing, one of the more interesting aspects of PTH1R is that it belongs to a very specific group of cell membrane-
bound G-protein coupled receptors (GPCR), called Class II (Family B) receptors that contain 7-transmembrane helices.\textsuperscript{11} In humans, PTH1R is located on chromosome 3 (3p22-p1.1), has 14 coding exons, 3 non-coding exons, and 3 promoter regions (Figure 6).\textsuperscript{28} Regardless of the species, PTH1R function is based on two key interactions: 1) the interaction between the c-terminus of the Parathyroid hormone(PTH)/Parathyroid hormone-related peptide (PTHrP) with the N-terminus of the receptor, and 2) the interaction between the N-terminus of the receptor with its transmembrane region.\textsuperscript{28} It is highly concentrated in the skeletal muscles, liver, kidneys, and bone and has the ability to bind to multiple ligands. Depending on which ligand it binds to (either PTH or PTHrp), it is responsible for a plethora of somatic functions (endochondral ossification, tooth eruption, or osteogenesis regulation). The PTH1R molecule is also a rather interestingly ‘promiscuous’ receptor, in that it can bind to a multitude of different G-proteins, which alter the type of action initiated via its signal transduction.\textsuperscript{33}

PTH1R primarily binds to the stimulatory subfamily of G-proteins (G\textsubscript{s}), which in turn stimulates the activation of adenylyl cyclase (AC) and the production of cAMP. cAMP then binds to the regulatory subunit of protein kinase A (PKA), which results in a fully active catalytic subunit.\textsuperscript{33} This subunit acts as an important mediator in gene transcription and also plays a large part in gene transactivation, through the action of PKA phosphorylizing the cAMP response element binding protein (CREB). PTH1R can also bind to the subclass G\textsubscript{q/11} protein, which results in the sequela of the activation of phospholipid C, inositol triphosphates (IP3), and diacylglycerol (DAG).\textsuperscript{33} This results in the release of intracellular calcium, and the production of protein kinase C (PKC). A third class of proteins that PTH1R can bind to is the subfamily on inhibitory G-proteins (G\textsubscript{i/o}), which bind to and inhibit AC, consequently reducing production of
cAMP. This pathway also activates the production of phospholipid C(PLC), resulting in increased intracellular calcium levels, via either calcium-channel mediated influx or intracellular calcium stores. Another interesting G-protein subfamily that PTH1R can bind to is the G_{12/13} group. This pathway occurs primarily in osteoblastic cells, and imitates activity through the activation of phospholipid D.

It is known that a majority of DNA is either ultimately translated into a protein with a primary, secondary, tertiary, and quaternary structure, or it indirectly affects protein function. It is also known that the sequence of the DNA affects the structures of the protein, and therefore expresses as numerous functions. Accordingly, alterations in the DNA sequence of PTH1R can directly affect the structure and behavior of the resultant protein. The literature has nicely illustrated that the 2\textsuperscript{nd} and 3\textsuperscript{rd} intracellular loops are the most important for G-protein binding, and that the N-terminus and 3\textsuperscript{rd} intracellular loop are most functionally sensitive to mutations. The importance of the 2\textsuperscript{nd} and 3\textsuperscript{rd} loops in connection to their responsibility surrounding G-protein binding has been used as a basis of interrogation in various labs, and it offers support towards the concept of competitive binding for G_{s} and G_{q/11}. It is also important to note that the C-terminus is most robust in terms of G-protein interaction. The fact that PHT1R has a direct pathogenic effect leading to PFE, and because of its relative reduced mortality as compared to other conditions caused by mutations in PTH1R (e.g. lethal dwarfism) it becomes an ideal model to further investigate the function of PTH1R at both ends of the spectrum – systemic and somatic effect versus localized effect resulting from different mutations. Through a careful review of the literature and our current findings, it is understood that alterations in molecular pathways that underlie normal eruption can result in an eruption
disorder; and that early detection and management of these situations provides the best chance at a successful treatment outcome.\textsuperscript{1,2} Inaccurate diagnosis will most certainly lead to suboptimal treatment choices, which ultimately hold negative consequences for the patient.

**Previous PTH/PTHrp and PTH1R Studies:**

PTH1R, PTH, and PTHrP have been the focus of numerous inquiries; our understanding of their functions and interactions are improving at a slow but steady pace. We have benefited from functional studies of the PTH/PTHrP/PTH1R pathway. For instance, Ouyang et al have shown that PTHrP plays an important role in cementum biomineralization.\textsuperscript{24} Not only is PTHrp and PTH1R crucial in the maintenance of calcium homeostasis, but each compound is found in significant concentrations in cementoblasts. While this demonstrates the important function of PthrP and PTH1R in cementogenesis, it is important to note that periodontal ligament (PDL) cells provide the osteoclast precursor cells needed to clear the eruption pathway.\textsuperscript{10} Studies have shown that within PDL cells, PTHrP increases the relative level of TNFSF-11 expression versus osteoprotegerin (OPG) through a cAMP/PKA-independent pathway, thereby increasing the amount of osteoclastogenesis.\textsuperscript{24} Collectively, this information reveals an obvious and intimate connection with the PTH1R pathway and tooth eruption, but the specific pathogenesis that leads to an eruption disorder versus a systemic disease is still elusive.\textsuperscript{10}

We can begin to understand the more enigmatic aspects of the role of PTH1R and tooth eruption by starting with an understanding of the interaction of the receptor to the ligand. Previous studies using mutated PTH1R and PTH revealed that the most important aspect of receptor activation was the interaction of the ligand with the transmembrane region of
Specifically, Shimizu and collaborators proved that a truncated ligand, as small as 14 amino acids, resulted in the production of similar basal cAMP levels—whether it interacted with the mutated PTH1R that was missing its extracellular N-terminus, or if it bound to the WT PTH1R. This further showed that binding and affinity assays with COS7 cells revealed that the most important factor for the activation of the receptor was the interaction of its transmembrane region with the ligand. Rickard completed an activity-based PTH1R assay identifying a small molecular ligand for the receptor that acts as a weak, micromolar agonist. This proposed allosteric mechanism offers a theoretical construct for future therapeutic approaches for diseases linked to PTH1R disorders, such as osteoporosis or osteoarthritis.

**Epidemiology**

The logical first step to further dissection and characterization of the clinical and molecular defect is to take advantage of the already vast database of eruption disorders in the UNC Department of Orthodontics. To date, no epidemiological studies have been completed to provide the exact incidence of PFE, but it has been estimated to occur in almost 1% of the world-wide population. The fact that this condition is often under-diagnosed and thereby improperly treated—suggests a prevalence that is likely higher than its estimated 1%. This modest estimate does not account for the more common clinical problem—delayed tooth eruption. Tooth eruption anomalies occur in a significant segment of the population causing problems such as inability to chew and digest food properly in addition to esthetic issues that affect the quality of life for affected individuals. While the effects of PFE are not nearly as dramatic compared to other disorders related to PTH1R mutations, the use of PFE as a model
for PTH1R study will allow for the attainment of an improved understanding surrounding the actions of PTH1R and PTH/PTHrP in the dental and somatic system.

The aims completed in this study further investigate the molecular genetic basis of primary failure of eruption (PFE) as well as characterize the corresponding clinical presentation. Our understanding of the pathogenesis of PFE in relationship to mutations in the PTH1R gene will aid us in elucidating the mechanism of PTH1R in the somatic system, the pathogenesis of a PTH1R mutation, and allow us to add to the body of knowledge defining the eruption process in general. We report here the completion of two aims that tested the hypothesis that PTH1R mutations result in a loss of function and that several potential genes cause PFE. Aim 1 utilized functional studies to determine the consequence of a functional PTH1R mutation (mut) compared to wildtype (WT), and Aim 2 evaluated the contribution of two candidate genes, BMP2 and TNFSF-11.

The collective body of work presented here reveals that differences exist in the expression (localization and intensity) of the PTH1R protein in the mutant vs WT. These studies represent a promising prelude to definitive quantitative and functional studies. We also sought to understand whether other genes known to be in the PTH1R pathway (Ingenuity Pathway Analysis) were found to harbor mutations in individuals with cleared clinical phenotype of PFE. This did reveal Single Nucleotide Polymorphisms (SNPs) in TNFSF-11 vs BMP-2, however further analyses, and more importantly WES, may reveal additional genes that contribute to the PFE phenotype. Taken together, these findings and the future studies that will derive from it are of paramount importance, because it enriches our current diagnostic regime of eruption disorders by utilizing genetic data. The future of medicine and dentistry will undoubtedly rely on the
precision of personal medicine; using genetic information in order to arrive at an accurate
diagnosis and to thereby develop an appropriate treatment plan.
CHAPTER ONE

INVESTIGATING THE PROTEIN EXPRESSION OF WT AND MUTANT PTH1R IN COS7 CELLS

Introduction

Eruption disorders represent multifactorial, and therefore clinically challenging entities to diagnose.\(^2\),\(^7\),\(^8\) The multifactorial aspect in combination with a limited understanding of the etiology and mechanism that leads to this problem translates into an elusive phenomenon. Specifically, the genetic basis of one category of eruption failure, Primary Failure of Eruption (PFE) (OMIM ID: 125350), has recently revealed the molecular basis of this disorder but the specific pathogenesis has yet to be elucidated.\(^2\),\(^9\) Moreover, it is known that autosomal recessive mutation in the very same gene that causes PFE, parathyroid hormone-1 receptor gene (\(PTH1R\)), leads to lethal conditions and PFE, which is limited to the dental units.\(^17\) One of the most fascinating aspects of \(PTH1R\) function is the juxtaposition of clinical presentations (i.e. lethal versus localized). Moreover, the \(PTH1R\) gene, which is known to regulate calcium homeostasis and bone metabolism, warrants further interrogation of this interesting receptor and the associated biologic/clinical phenomenon.

\textbf{\(PTH1R\): A G-protein coupled receptor (GPCR)}

\(PTH1R\) is a G-protein coupled receptor (GPCR) bound at the membrane of a cell and has the ability to bind either to PTH or PTHrP.\(^{15,17}\) It is found throughout the body, but is highly
The function of PTH1R is determined whether its activating ligand is PTH or PTHrP; these corresponding functions are responsible for a broad spectrum of systemic processes (endochondral ossification, tooth eruption, mammary gland development, or osteogenesis regulation). Moreover, the PTH1R receptor is also a rather interestingly ‘promiscuous’ receptor, in that it can bind to many different G-proteins, which alter the type of action initiated via its signal transduction pathway.

The PTH1R receptor consists of an N-terminus, C-terminus, 3 extracellular loops, 3 intracellular loops, and 7 transmembrane (TM) loops. The literature has shown that the 2nd and 3rd intracellular loops (attach TM3/4 and TM5/6, respectively) are the most important for efficient initial G-protein binding, and that the N-terminus and 3rd intracellular loop are most functionally sensitive to mutations. It is also important to note that the C-terminus is most robust for G-protein interaction. The GPCR mechanism represents an integral aspect of many drug delivery systems making the pharmacogenetic impact of future PTH1R discoveries significant. Since PHT1R has been proven to be causative in the development of PFE, the relatively milder phenotype resulting from a PTH1R mutation provides the potential to use PFE as a “low-morbidity level” model to further investigate the function of PTH1R. Accordingly, the localized defect observed in PFE versus the more severe and often lethal phenotype observed in Bloomstrand’s (OMIM ID: 215045) or Eiken’s Syndrome (OMIM: 600002), suggest a robust defect in general to further investigate the pathogenesis of
PTH1R (Figure 2.1 (http://www.eurorad.org/eurorad/case.php?id=1463) and Figures 2.2a/b ((Frazier-Bowers et al. 2013)). Based on the literature and our current findings, it is understood that alterations in molecular pathways that underlie normal eruption can result in an eruption disorder; and that early detection and management of these situations provides the best chance at a successful treatment outcome.\textsuperscript{1, 2} Inaccurate diagnosis will most certainly lead to suboptimal treatment choices, which ultimately hold negative consequences for the patient. The remaining question of how an understanding of the mechanism that leads to PFE can potentially improve diagnosis and treatment and these studies present a logical first step to accomplishing this.

**PTH/PTHrP and Dental Eruption**

In order to elucidate the function of PTH1R, it is imperative to understand the behaviors of its ligands. This is especially important in the inquiry of PFE and PTH1R because while more is understood about PTH1R and its activators in the skeletal and endocrine systems, less is known about its behavior in the dental tissues. PTHrP has been shown to be integral in the process of tooth eruption by its presence and activity in the enamel organ, specifically the stellate reticulum of the dental follicle.\textsuperscript{25} Studies have shown that the absence of PTHrP production of the dental follicle cells (via conditional-PTHrP knockout mice), initially normally developing teeth eventually become impacted and encapsulated by a bony crypt. This is due to the failure of the formation of an eruption pathway, and fusion of the cementum to the alveolar bone (ankylosis).\textsuperscript{25} It has been demonstrated that the cAMP/PKA pathway is largely responsible for the varying effects of PTHrP and PTH regulation of mineralized tissues.\textsuperscript{28} Due to the intricacy of up and downstream signal modulation and *cis*-element interactions, it is
supported that the signaling effect of PTHrP or PTH relies on the net outcome of cell’s integration into multiple signaling pathways. This explains why the activation of the Gs protein/cAMP/PKA pathway can result via PThrP or PTH, with each resulting in different downstream pathways, yet yielding a constitutively singular result. When concerning the dental unit, activation of the cAMP/PKA pathway (via Gs protein activation) by either ligand results in the progression of tooth development and eruption- while the disruption of the same pathway results in ankylosis (up-regulation of the biomineralization of cementoblasts) and a failure to erupt-secondary to a blocked eruption pathway.10

Similarly, in the skeletal system, the targeted loss of PTH1R is accompanied by impaired chondrocyte proliferation and accelerated maturation and calcification of chondrocytes, which mimics the loss of Gs (activation of cAMP and the PKA pathway).10 Clinically, inactivating mutations in PTH1R results in hypocalcaemia and Bloomstrand’s chondroplasia (OMIM ID: 215045). Constitutively activating mutations of PTH1R lead to hypercalcemia and Jansen’s metaphyseal chondroplasia (OMIM ID: 156400). Loss of parathyroid hormone (PTH) results in the aberrant formation of primary spongiosa of long bone and defective mineralization.20 This is in contrast with PFE (OMIM ID: 125350), which has numerous documented PTH1R mutations, yet it is isolated within the dental units, which increases the intrigue of the interactions between PFE, PTH1R, and PTH/PTHrP. These findings also point towards future studies involving further interrogation of PTH/PTHrP up/downstream signaling pathways and specific functional assays.

We therefore seek to understand the functional consequence of specific mutations in individuals that were identified with PFE. Our hypothesis is that PTH1R mutations identified in
PFE result in a loss of function. We speculate that this loss of function could be connected to an aberration in localization of the protein or G-binding. The studies presented in this report, taken together, reveal that differences do exist between the expressive localization of the WT versus mutant PTH1R. Further studies with specific binding assays will allow us to elucidate more precise mechanistic flaws that lead to PFE.

**Materials and Methods**

**Transformation and Plasmid Prep of E.Coli**

The pcDNA™3.1 (+) Mammalian Expression Vector (5.4kb) (Figure 2.3) was used to create the construct containing the wild type human PTH1R sequence, named TG682 7260. TG682 7260 was generously donated from the Gardella laboratory (Massachusetts General Hospital, Boston, Massachusetts) for the functional studies (Figure 2.4). Transformation of competent *Escherichia coli* DH5α cells (purchased from Invitrogen) was completed for the TG682 7260. Transformed E coli was grown to a logarithmic stage in LB broth (1.0% tryptone, 0.5% yeast extract, and 1% NaCl) and LB agar (1.0% tryptone, 0.5% yeast extract, 1% NaCl and 1.5% agar) medium were used. IPTG (100 mg/ml) and Ampicillin (100 mg/ml) for the preparation of agar plates for growth, incubation was done at 37°C. After transforming the E. coli cells, 50 uL of the cell suspension was plated overnight at 37°. A single clone was selected for mid-scale plasmid preparation (NucleoBond® Xtra Midi, Macherey-Nagel GmbH & Co. KG), and was performed as per the provided protocol. All media was prepared according to the manufacturer’s recommendations.
**Tissue Culture**

The COS7 cell line was generously donated by Dr. Sompop Bencharit and cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and 100 U/ml penicillin, and 100 µg/ml streptomycin. The cells were then typsinized and re-plated on 8-well glass chamber slides in preparation for transient transfection.

**Site-Directed Mutagenesis**

Site-Directed Mutagenesis was performed (Genscript ©) on the TG682 WT PTH1R vector to create the 1092delG PTH1R construct (Figures 2.5a and 2.5b). The mutated 1092delG vector was then transfected into COS7 cells for comparison with the WT PTH1R.

**Transfection of COS7 Cells**

Exponentially growing COS7 cells were plated on a glass 8-well chamber slide. Transfection was performed using FUGENE 6™ (Roche Applied Science) and Immunoflourence assay (IFA) studies were performed according to standard protocols. Briefly, cells were grown on a glass chamber slide, and grown to a density of 70% confluence prior to adding the DNA mixture, including 0.1 mg/ml of the vector containing the WT-PTH1R or mutant insert. The cells were transfected with either: the vector containing the human functional mutation (1092del G), or the wildtype PTH1R insert. The polyclonal anti-PTH1R antibody used for IFA was purchased from Abcam ©, and the secondary FITC 546 goat anti-rabbit antibody (Life Technologies ©, Thermo Fischer Brand Inc.) was generously donated from the Webster-Cyriaque laboratory. Transfection of COS7 cells with the pcDNA3.0 (+GFP insert) for 48 hours was completed as an approximation of transfection efficiency. The transfection efficiency was estimated to be 70% (Figure 2.6).
IFA Studies

Initial imaging of the WT PTH1R cells under a confocal microscope, illustrated that fact that transport vesicles were of the most notable prominence, with little evidence of cell membrane localization (Figure 2.7). This supported the selection of a 48-hours post transfection standard to evaluate WT vs Mutant PTH1R localization to the cell membrane.

After 24 and 48 hours, the transfected COS7 cells were fixed for 15 minutes with -20 °C 80% Acetone in PBS at 25°C. Nonspecific binding was blocked by incubation of the COS7 cells for 30 minutes in 1% albumin in PBS. The cells were incubated in primary FITC goat anti-rabbit antibody for 10 hours, washed, then incubated with secondary AlexaFluor 546 (Life Technologies, Grand Island, NY) for 1 hour in the dark. The nuclei were stained with Hoechst 33342 DNA-dye at 1/10,000 for 1 min (Life Technologies, Grand Island, NY). After a final wash within the chamber slides, cells were imaged using an inverted digital, wide-view, and confocal microscope. Preparation for publication was completed using Photoshop® (Adobe) software as described previously (Dibble, Bencharit et al, 2010).

In silico Analysis

In silico analysis was completed using software from Phyre™ software¹¹, followed by PyMol™ software for imaging (The PyMOL Molecular Graphics System, Version 1.7.4 Schrödinger, LLC.). This form of prediction analysis uses the known structure of a given protein based on protein X-ray crystallography.¹¹ The protein sequence containing the altered sequence of interest was interrogated by the software that uses previously scanned protein structures, their corresponding validated protein sequences, and the knowledge of molecular
interactions to fabricate a best-fit-model. The resulting prediction was provided in the form of a 3D image of a protein.\textsuperscript{11}

**Results**

**Phenotypic presentation of 1092delG and 966_997insC PTH1R mutations**

The 1092delG mutation, which resulted in a frameshift and truncated PTH1R protein, was selected for the functional studies described here for many reasons (Figure 2.10). From a clinical standpoint, this novel mutation was associated with the clinical finding of infraoccluded primary teeth, and was identified in a small nuclear family (N=2). The non-syndromic index case was a 7 years, 9 months old male who exhibited PFE affection of the permanent and primary teeth in the form of a right lateral posterior open bite, mild Class III skeletal malocclusion, and an early-onset dilaceration of a PFE-affected 1\textsuperscript{st} permanent molar (Figures 2.11a and 2.11b \cite{Rhoads et al, 2013}).\textsuperscript{29} Another mutation, the previously identified 966_997insC PTH1R mutation, was also selected to model the subsequent protein structure (Figure 2.12 \cite{Rhoads et al, 2013}). Similar to the 1092delG, another mutation, 996_997insC, was selected because of affection of the primary dentition with early-stage dilacerations of the PFE-affected 1\textsuperscript{st} permanent molar.\textsuperscript{12} This frameshift mutation segregated in a family as Type I PFE in affected individuals. Of those affected, two presented with one-jaw only patterning, and two with a bilateral maxillary and mandibular pattern. Unaffected individuals in this family (n=5) did not carry the 996_997insC mutation and did not reveal any signs of the dental eruption phenotype, indicating an autosomal dominant inheritance with complete penetrance.\textsuperscript{12}

Notably, systemic and/or skeletal problems were detected in this family. Five individuals indicated a previous diagnosis of early onset arthritis by a medical doctor manifesting in the
back and/or hip region around the second or third decade of life (Table 2.1). The functional alterations identified in our study were not refined enough to decipher how the development of osteoarthritis progression is related to the mutational pathology.

Variation in WT vs 1092delG PTH1R protein localization using IFA

There were observed differences between the expression of the WT and Mutant PTH1R as visualized by IFA. Although these differences were not specifically quantified, it was noted that the WT PTH1R (Figures 2.8a, b, and c) bound and concentrated itself at the membrane in higher levels than that of the mutant (Figure 2.9). This suggests that the mutant’s ability to fully bind to the cell membrane is diminished due to the mutation. The intensity of the WT stain was more pronounced, and the transport vesicles appeared more vibrant and concentrated, in comparison to the mutant.

Genotype: Protein Sequence (1092delG and 996_997insC PTH1R mutations)

The 1092delG PTH1R mutation results in an altered amino acid sequence extending from the transmembrane-5 (TM5) loop through transmembrane-7 (TM7), before shifting back into frame near the beginning of the c-terminus. The number of amino acids of the 1092delG protein is approximately 81 amino acids (AAs) short of the WT, although the c-terminus is conserved. The 996_997insC mutation resulted in a frameshift mutation that affected the regions of the transmembrane-4 (TM4) and TM5 loops. The protein was truncated; however its c-terminus was conserved. Most importantly, both of the aforementioned mutations alter the amino acids that constitute the 3rd intracellular loop, which are central to G-protein interaction. These important amino acids are Val\textsuperscript{384}, Leu\textsuperscript{385}, Thr\textsuperscript{387}, and Lys\textsuperscript{388}. Taken together, these findings support and validate these identified mutations and their respective protein alteration
sites for use in more specific, g-protein activation pathway interrogation. Figures 2.13, 2.14, and 2.15 illustrate the genotype: protein domain correlations, current database of PTH1R DNA: protein mutations, and a graphical depiction of the mutated regions of the PTH1R protein.

**In silico Predictions of WT PTH1R, 1092delG, and other functional PTH1R mutations**

We submitted WT and mutated protein sequences identified in our lab (1092delG, 1353-1 G>A, 966_997insC, 572delA) into Phyre2 ™ and visualized the resultant proteins via PyMol ™ (Figures 2.16a-e). All mutated predictions resulted in an altered, truncated protein with a slight alteration of the transmembrane c-terminus. However, within our current database of functional PTH1R mutations, the 1092delG mutation is the only one that caused a downstream alteration within the 6th TM loop. Additionally, while the c-terminus is conserved based on the amino acid sequence, the c-terminus of the 1092delG protein is truncated and altered to the greatest extent (Figure 2.15b). This is in comparison to the 996_997insC PTH1R mutation that led to a downstream alteration in the 3rd intracellular loop, and had a moderately altered c-terminus in comparison to the collection of current PTH1R mutations. All of the predictions yielded a reported confidence of 100%.

**Discussion**

**Reduction of Mutant PTH1R Cell Surface Presence**

While the potential alteration in G-protein coupling is a compelling argument, the functional study demonstrated that the mutated protein is less localized in the cell membrane as compared to the WT. This reduction in cell membrane localization supports the postulation that part of the consequence of the 1092delG mutation is the reduction of cell membrane localization, resulting in a decline of the appropriate signal transduction response to
PTH/PTHrP. Future studies that interrogate downstream effectors can further confirm whether there is an actual defect in the signal transduction pathway. An important place to begin such queries would be the enzymes critical for the G-protein signaling cascades (ie, AC, PLC, PKC, and PKA).

**In silico Analysis**

Computer modeling is a useful tool in the intellectual journey of improving our understanding of the molecular and structural consequence of PTH1R mutations, however, it has its limitations. In silico analysis, by its very nature, is a prediction of structure. It is a structural interpretation based on a limited database, albeit a very educated one. The reliability of an in silico analysis is centered on the reliability of its database, however, the absolute validity of our prediction results are strengthened by our functional studies. Indeed, the gestalt of our in silico analysis reveals differences in the protein structure that appear consistent with the location of the mutation but more importantly these studies can be used to support further and more pointed inquiries into the results of a PTH1R mutation.

**PTH1R Mutation Potentially Affects G-Protein Binding**

The in silico protein prediction of the WT v. the 1092delG, 1353-1 G>A, 966_997insC, 572delA predictions, resulted in truncated protein mutants with an altered intracellular c-terminus. However, the most remarkable of the protein predictions is the structural alteration of the 1092delG and 996_997insC PTH1R mutations. Although the c-terminus was conserved in both cases, each mutation led to downstream alterations that spanned key locations within the TM or intracellular loops; with each region being highly relevant for normal g-protein binding and signaling.\(^3\) We can therefore postulate that the alterations in the c-terminus, 3\(^{rd}\)
intracellular loop, and the TM6 loop creates an altered G-protein binding pattern. Furthermore, the fact that both of the identified mutations affected the very 4 amino acids critical for efficient G-protein coupling increases the probability of functional disruption. This is substantiated, in part, by our understanding that PTH1R can bind to different G-proteins, some of which, have antagonistic effects within the somatic and cell system. It is plausible that the TM, intracellular loop, and c-terminus alterations caused the PTH1R protein to bind to a G-protein that 1) has greater antagonistic activity towards the eruption process; 2) did not allow the receptor to bind to a protein at all, or 3) lead to an altered up or downstream regulatory pathway.

**Primary PFE Tooth Affection, 1092delG and 996_997insC**

The observation that both mutations represent unique cases in our database with PFE affection of primary teeth and the early stages of permanent 1st molar dilaceration is significant. These mutations are associated with the infraocclusion of primary teeth, and based on knowledge of the sequence they are the only mutations that affect key G-protein binding sites within locations of PTH1R. Therefore, isolating G-protein uncoupling occurring within these mutations could help identify the signaling pathway responsible for an integral part of eruption. Moreover, the affection of primary teeth potentially represents a more extreme manifestation of PTH1R malfunction; isolating the pathway of primary tooth eruption failure will aid in elucidating the pathway to permanent tooth eruption and eruption failure.

**G-Protein Conformational Change Alterations and Ligand Binding**

Since two mutations clearly affected TM3 and TM6 loops as discussed above, we considered their respective roles. It is known that TM3 and TM6 loops are critical in terms of
their altered G-protein affinity and basal signaling. Alterations in PTH1R conformation can also result in varying affinities for different ligands. Furthermore, PTH1R exists in two different G-protein related conformations: 1) RG, in which the receptor is bound to G-protein, and 2) Ro, in which the receptor is not coupled to a G-protein. PTH and PTHrP have been shown to bind similarly to the G-protein coupled PTH1R conformations, however PTH has a much greater binding affinity, than PTHrP in relation to the uncoupled G-protein PTH1R conformation. This confers that when PTH1R is in its Ro conformation, PTH has much greater signal production capability than PTHrP. Depending on the GPCR conformation, PTH and PTHrP will have different initial binding affinities to the receptor, as well as varying stability during its existence as the ligand-receptor complex. This concept is important to ponder when evaluating the potential effects of the 1092delG mutation and can help explain the different modes of ligand activity (endocrine v. paracrine). For instance, if the conformation of the receptor, due to the frameshift mutation, creates an inability able to bind effectively to the G-protein or the required ligand- a breakdown in the classical pattern of PTH1R function may be imminent.

Dimeric Oligomerization Activation/Deactivation of the Homodimeric PTH1R Complex

In the 1092delG mutation we can consider the findings of Xu et al. In their study, it was determined that the crystal structure of the ligand-free PTH1R extracellular domain (ECD) forms an α-helix, which mimicks PTH/PTHrP by effectively occupying the peptide binding groove of its opposing protomer. PTH binding was shown to disrupt receptor oligomerization via functional studies, as predicted by their model and a receptor reduced to a monomeric state by mutations in the ECD retained wild-type PTH binding and cAMP signaling ability. PTH1R therefore forms a constitutive dimer that is dissociated by the binding of a ligand. The Xu lab also showed that a
monomeric PTH1R is able to couple with, and activate, a G-protein.\textsuperscript{27} These findings are highly relevant to the results of our functional studies which showed that the diminished activity of one mutant PTH1R copy is likely tied to a decrease in G-protein activation. This also aligns with the varying degrees and types of PFE presentation because we suspect that if the overall activation of the coupled G-protein is attenuated, it is possible that the threshold for normal eruptive function is not met- which can result in the apparent temporal and spatial variations of eruption failure.

**Future Studies**

The functional studies described in this paper suggest that the mechanism of the clinical affection is likely partially due to the aberrant expression or localization of the PTH1R protein, but also represents a situation where a potentially large percentage of the mutation’s affect is loss of function; such as altered ligand binding or alterations in G-protein coupling. Based on these results we predict that this functional defect in the protein may act to disrupt the membrane binding, intracellular signaling, and G-protein binding; however, future studies are needed to interrogate downstream targets.

The use of bioluminescence (BRET) and fluorescence resonance energy transfer (FRET) techniques that would enable us to investigate the receptor oligomerization and interaction in living cells is one way to extend our interrogation. Reducing the amount of DNA used to transfect the COS7 cells in order to produce increased contrast of protein expression will provide a clearer image of localization. Additionally, the use of more specific confocal imaging, RT-PCR, specific binding assays, and quantitative Western blots are additional potential methods to further extrapolate data surrounding the function of PTH1R, and PTH1R in PFE.
Conclusions

1. The 1092delG PTH1R mutation results in the reduction of the protein’s ability to localize and bind to the cell membrane.

2. Identified amino acid alterations in the PTH1R transmembrane region, specifically the 3rd intracellular loop, affects the functional aspect of protein function.

3. Two (2) functional mutations affected TM6 and the third intracellular loops are specifically related to the eruption failure of primary teeth.

4. Further studies are needed to determine the functional effect of a PTH1R mutation, such as a RT-PCR, fractionation, a quantitative western blot, functional binding assays, bioluminescence (BRET), fluorescence resonance energy transfer (FRET) techniques, and transfection at a lower DNA concentration level.
CHAPTER TWO

MUTATIONAL ANALYSIS OF TNFSF-11 AND BMP2 IN PFE AFFECTED PATIENTS

Introduction

The presentation of eruption disorders among the human population presents the dental practitioner with a plethora of differential diagnoses, connected to a diverse set of phenotypes. Primary Failure of Eruption (PFE) (OMIM ID: 125350) was first described by Proffit and Vig, and is defined as a failure of the eruption mechanism itself\(^7\), which cannot be explained by a syndrome or a mechanical interference.\(^8\) A hallmark of PFE is that these teeth do not respond favorably to orthodontic traction and, in fact, attempted orthodontic treatment often results in a worsened malocclusion and increased open bite due to intrusion of adjacent teeth.\(^1,2,9\) PFE clinically presents as the infra-occlusion of affected teeth, resulting in a posterior open bite malocclusion (Figure 3.1 (Frazier Bowers et al, 2009)). Typically, all teeth distal to the most mesially affected tooth also fail to erupt.\(^9\) The diagnosis of PFE is critical as it dictates that treatment with continuous archwires should be avoided. Some successful treatment has been reported by multiple individual tooth osteotomies or selective individual tooth extractions followed by implant restorations to restore a functional occlusion.\(^8\) The connection between PFE and \textit{PTH1R} has been documented in multiple labs across the world.
**PTH1R and PFE**

Our current understanding of eruption disorders has been strengthened by human genetic studies which have highlighted mutations in parathyroid hormone receptor 1 (*PTH1R*) as a causative factor for familial cases of PFE.\(^1,2,8,9\) A study of nine family members revealed *PTH1R* as an autosomal dominant mutation associated with a PFE phenotype. All family members with PFE had a mutation in the *PTH1R* gene in this study, while those without PFE lacked this mutation.\(^8\) *PTH1R* mutations associated with PFE have *in silico* predictions that result in the formation of truncated proteins with a somewhat conserved c-terminus. Haploinsufficiency has been suggested to be part of the underlying cause of PFE, in which insufficient amounts of functional receptors are formed from the unaffected allele. Non-syndromic PFE patients do not exhibit any peripheral signs of the disease, and it may be hypothesized that this mutation causes a disruption confined to alveolar bone in the epithelial-to-mesenchymal (EMT) crosstalk signaling pathways that are necessary for normal bone resorption and apposition in tooth eruption.\(^8,9\) Taken together, this implicates genetic mutations in *PTH1R* as diagnostic and causative of PFE and is important in the context that many patients diagnosed with PFE and confirmed by the presence of a mutation in *PTH1R* were initially misdiagnosed with ankylosis.\(^1,2\) Both PFE and ankylosis preferentially affect molars and posterior teeth making them even more difficult to distinguish from one another.\(^2\)

**PFE: A Diagnostic Challenge**

The contrast between ankylosis and PFE are just examples of the complexity and overlapping nature of eruption disorders. Treatment decisions, and therefore the success of the
treatment, rely heavily on accurate diagnosis and treatment planning. The fact that we have a method to genetically and clinically diagnose individual disorders is a promising new frontier in medicine, due to the fact that the connection between the etiology and the clinical presentation of patients are not always as obvious. This can be seen specifically, in one family, in which five members carried the same mutation in $P\text{TH}1R$, but two affected individuals carrying this mutation were diagnosed incorrectly with ankylosis through percussion testing (see Figure 3.2). This scenario further reveals a gap in the knowledge and points to the difficulty in clinically distinguishing PFE from other eruption disorders (i.e. ankylosis or mechanical failure of eruption - MFE). PFE is a poorly understood problem of tooth eruption that with more investigation will improve our understanding of the biology of normal tooth eruption, but also the diagnosis of eruption failure.

**Mutational Analysis Studies of $P\text{TH}1R$**

The foundation for the mutational studies described herein was based on the database of functional and non-functional $P\text{TH}1R$ mutations previously identified and described in the Frazier-Bowers' Lab. Studies in this laboratory revealed that while six of twelve families with PFE segregate mutations in $P\text{TH}1R$, the remaining cohort lack functional mutations in $P\text{TH}1R$, further suggesting other gene(s) as causative of PFE. These findings also revealed a novel inheritance pattern in PFE, incomplete penetrance and therefore forms the basis for an additional research question, whether modifier genes contribute to PFE.
**Phenotypic Analysis of PFE**

PFE has been analyzed from a genetic and clinical perspective. Clinical analysis of photos and radiographs evaluated the following: jaw-affection, bilateral or unilateral affection, super- or infraosseous tooth position, and the clinical Type of PFE. Broadly, PFE is divided into two subtypes, Type 1 and Type 2. Type 1 PFE presents as a progressive, anterior-posterior lateral openbite that worsens distal to the most mesially positioned PFE-affected tooth; Type 2 PFE presents with a more varied pattern of infra-eruption distal to the most mesially affected tooth. The affected members in each family group had varied clinical PFE presentations. This variation was noted as both between and within familial segregating mutations. Of those families with functional mutations, four families were classified as Type I PFE, while the other two families were classified as either Type II or having a mixture of Type I and Type II presentations. All of the affected individuals who provided records were found to have an eruption failure that manifested during the supraosseous phase (i.e. the teeth had emerged partially through the bone), and unilateral versus bilateral eruption failure (i.e. manifesting on one side of the dental arches) occurred equally.

**The Interaction of BMP2, TNFSF-11, and PTHrP in Tooth Eruption**

PTHrP has been shown to be integral to tooth eruption through the observation of increased concentration of PTHrP levels in the dental follicle and stellate reticulum at the initiation of eruption. The coronal region of the dental follicle regulates osteoclastogenesis and the apical region is responsible for bone formation. CFS-1, localized in the superior region of the dental follicle, up-regulates osteoclast precursor cells and down-regulates osteoprotegerin (OGP), which is a soluble decoy receptor for RANK. Additionally, PTHrP, which is produced by
the stellate reticulum cells in a paracrine manner, up-regulates TNFSF-11 expression in dental follicle cells. This same hormone results in the production of vascular endothelial growth factor (VEGF), which up-regulates the producing of RANK receptors on osteoclast precursors. Taken together, this results in more available RANK receptors interacting with increased levels of TNFSF-11, leading to osteoclastogenesis and eruption pathway clearance. The apical region of the dental follicle has a relatively higher concentration of BMP2, which is responsible for alveolar bone formation via osteoblasts. PTHrP causes the apical dental follicle cells to up-regulate BMP2 production. Taken together, it was determined that BMP2 and TNFSF-11 were plausible candidate genes to investigate in terms for their potential role in PFE and eruption.

**Ingenuity Pathway Analysis (IPA)**

Ingenuity Pathway Analysis (IPA) was performed (Figure 3.3) to inform our understanding of high-priority candidate genes for PFE based on interactions with PTH1R. IPA revealed that multiple genes involved in craniofacial development interact with PTH1R (e.g. PTHR, TNFSF-11, TGFB1, BMP2, BMP6). As a result of this analysis and based on the literature, we chose to interrogate the role of BMP2 (20p12.3) and TNFSF-11 (13q14.11) (hRANKL) in the development of PFE. Our rationale to study these particular candidate genes was strengthened by the intimate connection between the periodontal ligament (PDL), TNFSF-11, PTH1R, and PTHrP/PTH.

**Materials and Methods**

**Ascertainment of Families and Diagnosis**

Approval for this study was granted by the Biomedical Institutional Review Board (IRB) at the University of North Carolina at Chapel Hill. Every adult participant, or parental guardian
in the case of minors, provided their consent to participate in this study. Typically, the index case was identified through a referring orthodontist. Fifteen cases were selected for analysis of BMP2 and TNFSF-11 based on the negative results of these probands following sequencing of the PTH1R gene. Probands lacking functional mutations in the PTH1R gene, despite clinical signs of PFE, ranged in age from 6 – 68 years. Previously collected phenotypic data was analyzed for characterization of non-functional genetic alterations. All probands (N=15) had pre-treatment clinical photographs, panoramic and cephalometric radiographs following the initial clinical assessment, as described below.

A positive PFE phenotype diagnosis was made based on at least one infraoccluded first molar using clinical data (ie radiographs, and/or examination at minimum). A clinical interview was completed for each affected individual and/or their family members to determine general health status and the elimination of any syndromic patients. Index cases (individuals of interest) were identified based on their affection with PFE, as determined from radiographic and clinical presentation, as well as their lack of a functional PTH1R mutation. DNA was extracted using Oragene Salivary Kit and using the manufacturer’s protocol.

**Phenotypic Analysis**

Clinical (phenotypic) information was collected and reviewed for the individuals harboring a nonfunctional PTH1R, TNFSF-11, or BMP2 mutation. The records assessed included a minimum of a panoramic radiograph and intraoral clinical photographs. The following information was gathered:

1) Unilateral or bilateral presentation of infraoccluded teeth

2) Arch involved (affected teeth present in the maxilla, mandible, or both)
3) Presence or absence of any other abnormal or noteworthy findings, including the significant involvement of primary teeth

Additionally, the type of PFE, Type I or II (determined by the degree of eruption of the second molars) was recorded.

The classification of PFE was recorded as either Type I or II, as previously described in the literature. These types are distinguished based on the timing of onset. Briefly, Type 1 PFE is characterized by a progressive posterior open bite, in which all teeth distal to the most mesial infra-occluded tooth are affected and do not erupt into occlusion. Type 2 PFE exhibits greater eruption potential, although still inadequate, for the more distal teeth, such as second molars.

A phenotypic analysis was completed for each individual that was clinically diagnosed with PFE in our cohort of inquiry. With the use of clinical photographs and radiographs, PFE affection was described as either unilateral or bilateral, and PFE pattern was described as either Type 1 or Type 2. All of the current PFE cases within this cohort are of European decent.

**Mutational Analysis of BMP2 and TNFSF-11**

Mutational analysis was performed following extraction and purification of DNA from saliva (Oragene, DNA Genotek, Toronto, Canada) for all individuals in this study. We amplified and sequenced all coding exons of *BMP2* (3 exons- only 2 coding exons) and *TNFSF-11* (4 exons), included identifying common synonymous SNPs and non-synonymous (deleterious) mutations. Sequencing was performed on 15 probands using designed primer sets. To include splice junctions in our analysis, primer sets were designed to delineate regions that included a minimum of 25 bases of intron sequence in addition to the exon sequence. Amplification was
performed using HotStart polymerase chain reaction (PCR) buffer and enzyme mix (Life Technologies/Invitrogen, Bethesda, MD) under the following conditions: 10 min 95°C activation/premelt, followed by 35 cycles of 30 s at 94°C melt, 30 s at 60°C anneal, and 3 min of 72°C extension. PCR products were purified using ExoSapIt (USB, Cleveland OH), and sequenced at the University of North Carolina at Chapel Hill Genome Analysis Core facility. Sequences were compared to wild type TNFSF-11 and BMP2 (accession NM_003701 and accession NC_000020.10) from Genbank release GRCh37 using the BLAST algorithm.

Ingenuity Pathway Analysis (IPA)

Based on Ingenuity Pathway Analyses (Ingenuity Systems, Inc.) several candidate genes have documented interactions with one another. The interactions that are presented in connection with an IPA are varied and vast. Therefore, it was determined to focus primarily on TNFSF-11 and BMP2 due to the strong association with PTH1R in the pathway analysis, as well as their involvement with the eruption process.

Results

Mutational Analysis

Analysis of both BMP2 and TNFSF-11 candidate genes in PFE-affected patients who did not carry a mutation in PTH1R (N=15) resulted in the identification of 3 intronic SNPs in TNFSF-11, without any compelling genetic alterations in BMP2, to-date. Two SNPs were located within Exon 5 and one was located within Exon 8.

The intronic SNPs in TNFSF-11 have been previously documented in NCBI: c.387+14G>A (21% minor allele frequency), c.533-34T>A (3.4% minor allele frequency), and c.220-75T>G (.042% minor allele frequency) (1000Genomes). The global minor allele frequency (MAF)
(reported by NCBI - dbSNP) is the minor allele frequency for each SNP ID in a default global population. Global MAF distinguishes common polymorphism from rare variants. The MAF is actually the second most frequently occurring allele value. The current default global population is generated from the 1000 Genomes phase 1 genotype data, which has a collection of 1094 worldwide individuals (released in the May 2011) (NCBI). The relatively rare allele of at least 2 SNPs identified in association with PFE, c.533-34T>A (3.4% minor allele frequency), and c.220-75T>G (.042% MAF) suggest the possibility of a functional role of these SNPs that do not represent common variants.

**Phenotypic Analysis**

After a comprehensive analysis of index cases, (Table 3.1) it was determined that 75% of reported TNFSF-11 SNP-containing genotypes of sequenced individuals presented with a bilateral, 2-jaw, supraosseous affection; of which both the premolars and molars were affected. A total of 75% of PFE affected individuals presented with a Type I pattern, and the remaining with a Type II pattern. However, only 50% of the bilaterally affected side had a Type I presentation. Out of our cohort of PFE affected, non-functional alterations; the majority (78%) presented with a bilateral affection, and 56% of individuals presented with Type 1 PFE (Chart 3.1). It was also noted, that no particular trend in affection appears to sequester with any SNP in particular (Chart 3.2 and 3.3).

**Discussion**

*BMP2* and *TNFSF-11* were prioritized for this project because *BMP2* is highly active in the basal region of the dental follicle and is responsible for root formation and apical bone apposition; while *TNFSF-11* has been found to be highly active in the superior region of the
dental follicle and is critical for bone resorption.\textsuperscript{5} Hence, choosing these two genes was supported by network pathway analysis, the current understanding of eruption pathways, and eruption studies in a rat model.\textsuperscript{5,28} The identification of 3 SNPs in $TNFSF-11$ represents continued progress in developing an understanding of $PTH1R$, PFE, and the eruption process. It is well understood, from the literature, pathway analyses and our genotype:phenotypes studies, that $PTH1R$ is not likely the sole gene responsible for eruption disorder, specifically PFE. Our interrogation of $TNFSF-11$ and $BMP2$ was based on their involvement in calcified tissue metabolism, tooth eruption, and $PTH1R$ interactions.

Currently, $TNFSF-11$ is the most promising in terms of our current findings. Although only putatively non-functional SNPs have been identified, it cannot be definitively determined how they affect the other genes in an up or downstream manner.\textsuperscript{19} Studies have shown that intronic mutations can cause alterations in genes both near and far from the intronic location. There are four different classes of pre-mRNA splicing defects that have been found to cause disease: cis-acting mutations that disrupt the use of constitutive splice-sites (most common cause of human diseases), cis-acting mutations that disrupt alternative splice-sites, trans-acting mutations that affect the basal splicing machinery, and lastly; trans-acting mutations that cause the disruption of splicing regulation (cause for cancers, myotonic dystrophy, etc).\textsuperscript{19}

This concept has also been validated in terms of Calpain-10 (diabetes) and BRCA1/2 (breast cancer). Intronic mutations can lead to splice-site alterations- which in turn, can lead to many different outcomes.\textsuperscript{19} Due to the fact that genes undergo alternative splicing patterns during replication; this allows different copies of slightly alternative proteins to be created from the same pre-mRNA. Keeping in mind that different cells and cell systems have varying
sensitivities to particular mutations, conditions, and varying protein product produced—of which range from redundant to problematic. The fact that one SNP identified, c.220-75T>G, represents a MAF of only .042% (ie low frequency in the population) strengthens the potential of further evaluation. In order to determine the effect of the intronic SNPs identified in TNFSF-11, we would need to pursue more eloquent studies, such as performing a miRNA assay, in order to determine the effect of the mutated intron—before it is removed from the protein sequence.

Future Study- Whole Exome Sequencing (WES)

Whole Exome Sequencing (exomic) is an efficient and powerful tool to identify novel genes that may contribute to PFE. WES was developed in recent years to accomplish extensive sequencing primarily targeted to rare genomic variation that leads to protein-coding changes. In this case, WES would be implemented to assess a single family in our database that harbors PFE clinical affection, but lacks a functional PTH1R mutation. Complete phenotypic characterization (based on a rigorous clinical rubric: Rhoads et al., 2013) has been completed for all of the individuals in the family, along with analysis of PTH1R. Collaboration has already been established with Dr. J. Evans (NCGENES Project) and his team, and the inclusion of our families/samples was approved. The following stipulations/assumptions have been made: in order to participate only one affected individual per family for WES (up to 5 five families) were provided; these individuals were deemed to have a genetic condition that does not show a mutation in genes known to be associated with their condition (i.e. PTH1R has already been excluded); participants in the NCGENES must consent to participate and attend two visits at the NCGENES central hub (Jim Evans clinical site).
Conclusions

1. Currently 3 intronic SNPs have been identified in *TNFSF-11*, which supports the inclusion of other genes to PFE and eruption, besides PTH1R.

2. *c.220-75T>G*, resents a MAF of only .042%, and further strengthens the need to continue interrogating theses SNPs due to the low frequency of occurrence in the global population.

3. There have been no *BMP2* alterations identified in PFE cohort, to-date.

4. Continued studies of candidate genes with WES and PCR (*TNFSF-11* and *BMP2*) are needed to conclude other genetic causes of PFE.

miRNA assay of current SNPs is needed to determine the functionality of present *TNFSF-11* mutations.
Table 2.1- Correlation of Genotype: phenotype of 4 families with segregating PFE. Family 1 shows four affected individuals carrying the mutation. One individual has PFE affected primary teeth. Family 2 shows incomplete penetrance with 2 individuals carrying mutation but not affected with the eruption phenotype. Two individuals were not available for testing and both reported eruption failure (*). One is able to note the familial segregation of each \textit{PTH1R} mutation observed. Family 4 demonstrates two PFE affected members carrying a mutation, with one having PFE affected primary teeth

<table>
<thead>
<tr>
<th>Family 1</th>
<th>PFE Affection</th>
<th>Publication</th>
<th>Mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>I:1</td>
<td>Type I</td>
<td>Frazier-Bowers et al. 2013</td>
<td>c.996_997insC</td>
</tr>
<tr>
<td>II:2</td>
<td>Type I</td>
<td>&quot;</td>
<td>c.996_997insC</td>
</tr>
<tr>
<td>II:3</td>
<td>Type I</td>
<td>&quot;</td>
<td>c.996_997insC</td>
</tr>
<tr>
<td>II:4</td>
<td>Type I-Primary</td>
<td>&quot;</td>
<td>c.996_997insC</td>
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</tbody>
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<table>
<thead>
<tr>
<th>Family 2</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>I:1</td>
<td>unaffected</td>
<td>Frazier-Bowers et al. 2013</td>
<td>c.572delA</td>
</tr>
<tr>
<td>II:2</td>
<td>unaffected</td>
<td>&quot;</td>
<td>c.572delA</td>
</tr>
<tr>
<td>II:4</td>
<td>Affected*</td>
<td>&quot;</td>
<td>c.572delA</td>
</tr>
<tr>
<td>III:1</td>
<td>Type I</td>
<td>&quot;</td>
<td>N/A</td>
</tr>
<tr>
<td>III:2</td>
<td>Affected*</td>
<td>&quot;</td>
<td>N/A</td>
</tr>
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<tr>
<th>Family 3</th>
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<tbody>
<tr>
<td>III:2</td>
<td>Type II</td>
<td>Frazier-Bowers et al. 2010a</td>
<td>c.1353-1 G&gt;A</td>
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<tr>
<td>II:2</td>
<td>Type I</td>
<td>&quot;</td>
<td>c.1353-1 G&gt;A</td>
</tr>
<tr>
<td>III:3</td>
<td>Type I</td>
<td>&quot;</td>
<td>c.1353-1 G&gt;A</td>
</tr>
<tr>
<td>III:4</td>
<td>Type II</td>
<td>&quot;</td>
<td>c.1353-1 G&gt;A</td>
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<tr>
<td>II:1</td>
<td>Type II-Primary</td>
<td>Rhoads et al. 2013</td>
<td>1092delG</td>
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<tr>
<td>I:2</td>
<td>Affected*</td>
<td>&quot;</td>
<td>1092delG</td>
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Table 3.1- Mutational Analysis Results of PFE affected individuals lacking functional PTH1R mutation. The results are shown in combination with SNP data from PTH1R (non-functional mutations) and phenotypic presentation.

<table>
<thead>
<tr>
<th>Family 1</th>
<th>PFE Status</th>
<th>Affection Pattern</th>
<th>PTH1R Genetic Alteration/ SNP</th>
<th>TNFSF-11 Genetic Alteration/ SNP</th>
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<tbody>
<tr>
<td>1</td>
<td>Unaffected</td>
<td>N/A</td>
<td>no alteration</td>
<td></td>
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<tr>
<td>2</td>
<td>Unaffected</td>
<td>N/A</td>
<td>no alteration</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Unaffected</td>
<td>N/A</td>
<td>no alteration</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Affected - Type I</td>
<td>Bilateral/ Supraosseous</td>
<td>c. 1389 T&gt;C, known variant</td>
<td></td>
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<th>Family 2</th>
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<tr>
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<th>Family 4</th>
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<tr>
<td>2</td>
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<td>3</td>
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<tr>
<td>1</td>
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</tbody>
</table>
Figures

Figure 1- Clinical photograph of child in the mixed dentition, presenting with a posterior lateral open bite- secondary to a lateral tongue thrust. The tongue acts as a mechanical impedance to tooth eruption.

Figure 2- Patient with a functional familial \textit{PTH1R} mutation, originally diagnosed as having ankylosis. As a result of mutational analysis of \textit{PTH1R} it was determined that PFE was in fact the correct diagnosis and that if orthodontic treatment was pursued, PFE affected teeth should be avoided.
Figure 3- Frontal intraoral photos of (A) before and (B) after-orthodontic treatment in a patient with a subsequently confirmed case of PFE. This patient had a positive family history of PFE, and harbored a familial autosomal dominant mutation in the PTH1R gene. After treatment with a continuous archwire, the PFE affected teeth ankylosed and resulted in a worsened posterior openbite.
Figure 4- Jansen’s Metaphyseal Chondrodysplasia is caused by autosomal dominant heterozygous mutations in \textit{PTHR1} resulting in short limbed dwarfism. (Taken from http://www.gfmer.ch/genetic_diseases_v2/gendis_detail_list.php?cat3=200)

Figure 5- Bloomstrand’s Lethal Chondrodysplasia exhibits a recessive \textit{PTHR1} mutative deactivation which results in early lethality, shortened limbs, and premature bone maturity. (Taken from http://jcem.endojournals.org/content/vol92/issue3/images)
Figure 6- The Human PTH1R gene is located on the short (p) arm of chromosome 3 between positions 22 and 21.1. The region is bracketed in red. (http://ghr.nlm.nih.gov/gene/PTH1R)

Figure 2.1- Bloomstrand’s Lethal Chondrodysplasia exhibits a recessive PTH1R mutative deactivation. This deactivation results in early lethality, shortened limbs, and very precocious bone maturity. The fetus does not usually carry to term. (http://www.eurorad.org/eurorad/case.php?id=1463)
Figure 2.2- PFE results from a autosomal dominant $PTH1R$ mutation. The classical presentation is a progressive anterior-posterior lateral openbite shown in 2.2a and 2.2b. PFE is unique to the $PTH1R$ mutations in that it only affects the dental units. (Frazier-Bowers et al. 2013)
Figure 2.3- Diagram of the pcDNA3.1 + vector used contain the WT PTH1R insert. It contains an ampicillin and neomycin vector to ensure selectability after transformation and transfection.
Figure 2.4- Vector constructed by the Gardella laboratory. The pcDNA 3.1 (+) vector was implemented to carry the WT PTH1R insert. The resultant vector was named TG682 7260.

Figure 2.5a- Nucleotide sequence segment of interest. The area highlighted in green denotes the section of interest for the site-directed mutagenesis of WT PTH1R.

```acttgagctc cgggaacaaa aagtggatca tccaGtgcc catcctggcc tccattgtgc```

Figure 2.5b- Nucleotide sequence segment of interest. The highlighted area of the sequence denotes the region of the 1092delG PTH1R mutation where a G was deleted. This sequence was used as the blueprint for the site-directed mutagenesis of the TG682 7260 WT PTH1R vector.

```acttgagctc cgggaacaaa aagtggatca tccaGtgcc catcctggcc tccattgtgc```
Figure 2.6- 48 hour post transfection of COS7 cells with pcDNA 3.0 (+GFP insert). This transfection was used as an approximation of transfection efficiency- which was determined to be 70%.
Figure 2.7- Confocal Image of 24 hours post-transfection of WT PTH1R/COS7 cell. This image illustrates the movement of the protein (fluorescence) from the nucleus, into the endoplasmic reticulum, and then into transport vesicles. Definitive cell membrane concentration had not yet been reached.
Figure 2.8a- Immunofluorescence assay (IFA) was utilized during visualization (Inverted digital microscope) of the PTH1R protein in terms of localization and expression. 48 hours post transfection with WT PTH1R v blank control, IFA demonstrated normal expression of PTH1R protein and an intact nucleus (visualized via DAPI).

Figure 2.8 b and c- 48 Hours Post-Transfection of WT PTH1R/COS7 cell (Inverted digital microscope). IFA was implemented to visualize protein expression, normal expression was founds, as well as a more intense illumination of transport vesicles and noted areas of PTH1R protein membrane intensity, in comparison with the mutant, imaged below. Figures b and c are both WT PTH1R, however, antibodies of different fluorescing wavelengths were used for initial imaging.
Figure 2.9- 48 Hours Post-Transfection of 1092delG PTH1R/COS7 Cell (Inverted digital microscope). IFA was implemented to visualize mutated PTH1R protein expression. Heavy perinuclear intensity can be noted, as well as relatively normal expression.

Figure 2.10- Chromatogram demonstrating the familial 1092delG in the PTH1R gene, linked to infraocclusion of primary teeth in an affected child.
Figure 2.11a- Panoramic radiograph demonstrating the involvement of both primary and permanent teeth in the affected patient who carries a 1092delG mutation in PTH1R. Note that the affected right mandibular 1st molar has the appearance of early-stage dilaceration in the region of the apical 3rd. similar to the patient bearing the 996_997insC PTH1R mutation, a dilaceration of an affected left permanent mandibular 1st molar is observed. (Rhoads et al, 2013)

Figure 2.11b- Intraoral clinical photographs of PFE presentation in a 7yo 9 mo patient whose 1092delG mutation was the foundation for the COS7 Cell functional study. This is the second case of PFE found to have a genetic linkage to a PTH1R mutation and affection of the primary dentition. Credit Dr. AB Hammond (Rhoads et al, 2013)
Figure 2.12- Chromatogram demonstrating the familial 996_997insC mutation in the PTH1R gene. This mutation is one of two mutations linked to infraocclusion of primary teeth in an affected child. (Rhoads et al, 2013)
Figure 2.13 - Exon:Domain Pairing of *hPTH1R*. Note that the functional exons are depicted, along with the denotation of the corresponding protein domains.
Figure 2.14- WT, 572G>A, 996_997insC, 1353delA, and 1092delG PTH1R Protein sequences with Transmembrane, extracellular, and intracellular loops labeled. Note the downstream involvement of the 3rd intracellular loop and the TM6 loop domain with 996_997insC and 1092delG PTH1R mutations, respectively.

**PTH1R Proteins in PFE Families**

- **HPTR1 WT**
  - WT: MGTARIAPGL ALLLCCPVL5 SAYALVDADD VMTKEEQIFL LHRAAQCEK RLKEVLQRP
  - 572GelA: REVFDRLGMI YTVGYSV5LA SLTVAVLILA YFRRLHCTRN YIHMHLFLSF MLRAVSIFVK
  - 996_997Ins: VEGLYLHSLI FMAFFSEKKY LWGFTVFVGW LP
  - 1353 G>A: WIIQVPILAS IVLNFILFIN IVRVLATKLR ETNAGRCDTR QQYR KLLKST LVLMPLFGVH
  - 1092delG: TLSSWPHHTP RSQGRSGKR CTMRCSTSPS RFDSLQSYT

- **HPTR1 1092delG**
  - WT: GAPGEVVAVP CPDYIYDFNH KGHAYRCDR NGSWELVPGH NRTANYSEC VKFLTNETRE
  - 572GelA: SWATCPWR P5P
  - 996_997Ins: VDHPGAHPGL HCAQLHPLHQ YRPGRHQAAD GQRRP
  - 1092delG: WIIQCPSPWLP CTSSSSSSISO GCSSPPSCGC RPRTPGVTHG SSTGSNSNPW WCSCPSLAST
  - 1353 G>A: YIVFMATPTY EVSGLWQVQ QMFYMELPNSF QGFVAAIYC VICAMEI KKSWSRTL
  - Deleted in PFE-01 (Exon 15 skip): TLSSWPHHTP RSQGRSGKR CTMRCSTSPS RFDSLQSYT *
**Extracellular Loop**

**Intracellular Loop**
Figure 2.15-This chart denotes the transmembrane loops and the locations of the functional mutations in PTH1R, identified in the Frazier-Bowers Laboratory. It is important to note that 996_997insC and 1092delG mutations affect the TM4-TM7 regions, including the 3rd intracellular loop. TM6 and the 3rd intracellular loop have been determined to be instrumental in G-protein affinity and binding.
Figure 2.16a- *In silico* prediction of WT PTH1R. Note the intact amino/carboxyl- tails, as well as the 7-transmembrane loops.

Figure 2.16b- *In silico* prediction of 572delA PTH1R mutation. This mutation resulted in an alteration in the region of TM1.
Figure 2.16c- *In silico* prediction of 996_997insC PTH1R mutation. This mutation lead to downstream alterations, including those within the 3\textsuperscript{rd} intracellular loop.
Figure 2.16d - *In silico* prediction of \textit{1092delG PTH1R} mutation. This mutation lead to downstream alterations, including the region of TM6 and 3\textsuperscript{rd} intracellular loops.
Figure 2.16e- *In silico* prediction of 1353-1G>A PTH1R mutation. This alteration affected a small region of the c-terminus.
Figure 3.1 - Initial radiograph of patient presenting with clinic sings of Primary Failure of Eruption (PFE) in the region of the lower left permanent molar. Affection of at least one permanent molar is a hallmark sign of PFE.

Figure 3.2 - Patient with a functional familial PTH1R mutation, initially diagnosed as having ankylosis. As a result of genetic testing- it was determined that PFE was the correct diagnosis and that if orthodontic treatment was pursued, PFE affected teeth should be avoided. (Frazier-Bowers et al, 2010)
Figure 3.3- Ingenuity Pathway Analysis (IPA) of the interaction of multiple genes with *PTH1R*. The genes of interest were *TNFSF-11* and *BMP2*. The interaction of other notable genes, such as *BMP4*, *RUNX2*, and *BMP6* are potential future candidate genes, and are possible hits expected through the Whole Exome Sequencing (WES) which is currently underway.
Figure 3.4- Chromatograms exhibit three TNFSF-11 SNPs c.387+14G>A (21% MAF), c.533-34T>A (3.4% MAF), and c.220-75T>G (.042% MAF). These SNPs are intronic, yet could still lead to downstream functional mutations. (1000 genomes©)
Charts

Chart 3.1- This chart displays the overall patterning of phenotypic expression in the cohort of comparable PFE patients with evident non-functional alterations in either *PTH1R* or *TNFSF-11*. The majority (78%) show bilateral affection and Type 1 patterning (56%).
Chart 3.2- This illustrates the distribution of individuals carrying non-functional alterations that are affected with either Type 1 or 2 PFE, within each mutation identified. A majority of the cohort demonstrated Type 1 affection.
Chart 3.3- Table demonstrating the distribution of Unilateral versus Bilateral Affection in PFE patients that had a non-functional alteration. It is easily seen that the vast majority presented with bilateral affection. It can be taken from both sets of cohort data, that bilateral, Type 1 affection is most prevalent in the present cohort of PFE patients with non-functional mutations.
REFERENCES


