COMPARATIVE GENE EXPRESSION ANALYSIS OF HUMAN DENTAL PULP CELLS FROM MAXILLARY AND MANDIBULAR TEETH

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A Thesis Submitted in Partial Fulfillment of the Requirements
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การวิเคราะห์เปรียบเทียบการแสดงออกของยีนของเซลล์เนื้อเยื่อโพรงฟันมนุษย์ที่ได้จากฟันบนและฟันล่าง

นายถิระ ฟ้าเรืองแสง

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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Field of Study	Geriatric Dentistry and Special Patients Care
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ถิระ ฟ้าเรืองแสง : การวิเคราะห์เปรียบเทียบการแสดงออกของยีนของเซลล์เนื้อเยื่อโพรงฟันมนุษย์ที่ได้จากฟันบนและฟันล่าง. (COMPARATIVE GENE EXPRESSION ANALYSIS OF HUMAN DENTAL PULP CELLS FROM MAXILLARY AND MANDIBULAR TEETH) อ.ที่ปรึกษาหลัก : รศ. ทญ. ดร.ทัณฑิริรา พรทวีทัศน์

วัตถุประสงค์ : จากลักษณะทางกายวิภาคและตำแหน่งของฟันในขากรรไกรบนและขากรรไกรล่าง มีความเป็นไปได้ที่ในเนื้อเยื่อโพรงฟันของฟันบนและฟันล่างจะมีลักษณะการแสดงออกของยีนที่แตกกต่างกัน การศึกษานี้จึงเกิดขึ้น เพื่อทำการประเมินและเปรียบเทียบลักษณะการแสดงออกของยีนในโพรงฟันบนและฟันล่างของมนุษย์ เพื่อหายีนที่มีการแสดงออกที่แตกต่างกันในฟันบนและฟันล่าง โดยใช้เทคนิคการจัดลำดับอาร์เอ็นเอ

วิธีการวิจัย : ฟันกรามน้อยและฟันกรามบน-ล่าง รวม 4 คู่ แต่ละคู่มาจากผู้ป่วยคนเดียวกัน รวมทั้งสิ้น 4 คน โดยแบ่งเป็นฟันกรามน้อย 2 คู่ ฟันกราม 2 คู่ นำเนื้อเยื่อโพรงฟันมาเพาะเลี้ยง และสกัดอาร์เอ็นเอส่งไปทำเทคนิคการจัดลำดับอาร์เอ็นเอ จากนั้นใช้โปรแกรมจัดเรียงและวิเคราะห์ข้อมูลอาร์เอ็นเอที่ได้ และใช้เทคนิคเรียลไทม์พีซีอาร์ เพื่อยืนยันผลที่ได้

ผลการวิจัย : ในเนื้อเยื่อโพรงประสาทฟัน มียีน 19,372 ยีน ที่มีการแสดงออก จากยีนทั้งหมด 27,914 ยีน โดยยีน *FN1, COL1A1, COL1A2, ACTB* และ *EEF1A1* มีการแสดงออกสูงที่สุด จากการทำเทคนิคการจัดลำดับอาร์เอ็นเอ แต่มีเฉพาะยีน *PITX1* ที่มีการแสดงออกในฟันล่างสูงอย่างมีนัยสำคัญทางสถิติกว่าในฟันบนโดยเฉลี่ย 89 เท่า (64 เท่าในฟันกรามน้อย และ 116 เท่า ในฟันกราม)

สรุปผล : ยีน *PITX1* มีการแสดงออกในฟันล่างสูงกว่าในฟันบนอย่างมีนัยสำคัญทางสถิติ และมีการระดับแสดงออกมากในฟันกรามเมื่อเทียบกับฟันกรามน้อย

สาขาวิชา	ทันตกรรมผู้สูงอายุและการดูแลผู้ป่วยพิเศษ	ลายมือชื่อนิสิต .....
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KEYWORD: maxilla, mandible, RNA-Sequencing, RNA profile, Cellular behavior, tooth
Thira Faruangsaeng : COMPARATIVE GENE EXPRESSION ANALYSIS OF HUMAN DENTAL PULP CELLS FROM MAXILLARY AND MANDIBULAR TEETH. Advisor: Assoc. Prof. THANTRIRA PORNTAVEETUS, D.D.S., M.Sc., Ph.D.

Objective : Given the anatomical and locational differences between the maxillary and mandibular teeth, it is possible that there are differences in gene expression patterns of dental pulp cells within those teeth. This study evaluates and compares the gene expression profiles of the maxillary and mandibular human teeth with the aim of finding differences in gene expression patterns of dental pulp cells by RNA-Sequencing technique.

Methods : The upper and lower premolar and molar teeth obtained from the same participant were employed. Two pairs of opposing premolar teeth and two pairs of opposing molar teeth were isolated, cultured, and subjected for RNA sequencing. RNA-Seq Alignment and RNA-Seq Differential Expression were used to analyze gene expression profile. Quantitative RT-PCR was performed to confirm the result obtained from RNA-Sequencing.

Results : RNA sequencing demonstrated that 19,372 genes out of 27,914 in total were expressed. The top expression genes were *FN1, COL1A1, COL1A2, ACTB* and *EEF1A1*. In DPSCs of posterior teeth (premolar and molar), only *PITX1* gene had a significant higher expression (exhibiting $2^{6.47}$ or 89-fold change) in the lower teeth compared to the upper teeth (64-fold change in premolar teeth and 116-fold change in molar teeth).

Conclusion : We showed that *PITX1* gene had a significant higher expression in the lower teeth compared to the upper teeth and this difference in *PITX1* level was more evident in the molars compared to premolars.

Field of Study:	Geriatric Dentistry and Special Patients Care	Student's Signature .....
Academic Year:	2020	Advisor's Signature .....

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Thira Faruangsaeng

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INTRODUCTION

Background and Rationale

There are many tooth types according to their oral masticatory functions such as cutting incisors, tearing canines, grinding premolars and molars. Continuously growing teeth are common in many animals (e.g., mouse) (1). Some organisms (e.g., sharks) can replace lost teeth throughout life (2, 3). However, human teeth don't have generating properties. Missing tooth compromises human health physically and psychiatrically. Biologists and tissue engineers are working together to investigate how to repair tooth dentin and create new tooth in different tooth types and morphologies. For this purpose, it would be useful to compare cellular physiology of teeth in different locations in the dental arch. Our understanding of these biological processes may serve as a foundation for the future design and fabrication of regenerated teeth.

Many studies have discovered fundamental signal pathways (e.g. TGFß, BMP, Wnt pathways) that are important for dentin repair and dentinogenesis, however, the whole process has not well understood (1, 4, 5). It is not known which proteins or genes regulate dentin repair, how dental pulp stem cells regenerate mature dentin tissue, or how the incisors, canine, premolar or molar are differently formed. Moreover, there is no studies comparing gene expression of dental pulp cells between maxillary and mandibular teeth.

RNA molecules that are copied from genes which ultimately direct protein synthesis are called *messenger RNA (mRNA)*. Transcription of genome-encoded information into mRNA and translation of mRNA into functional protein are the main layers of gene expression. Due to the existential need to adjust gene expression to both intracellular requirements and extracellular stimuli, both processes are subject to regulation at multiple levels. In general, transcription and

translation are still regarded as mutually independent processes, characterized by different timings, cellular locations, functional complexes, and mechanisms of action (6).

RNA sequencing (RNA-Seq) is the method that provides insight into the transcriptome of a cell (7, 8). Compared to microarray methods, RNA-Seq provides greater resolution and broad coverage of the transcriptome. Transcriptomic techniques have been particularly useful in identifying phenotypes and the functions of genes. Assembly of RNA-Seq reads is ideal for gene expression studies of non-existing or poorly developed genomic resources (9).

Given the anatomical and locational differences between the maxillary and mandibular teeth, it is possible that there are differences in gene expression patterns of dental pulp cells within those teeth. A few researches have revealed gene expression of human teeth (10-12). Pantalacci has studied and compared transcriptomes during the development of two morphologically distinct serial organs, the upper and lower first molars of the mouse. The identity of different brachial arches is regulated by **Hox**, **Pbx**, and **Otx** genes. It is hypothesized that the expression of **Dlx** genes regulates intra-BA identity (13). Depew and his team examined mice lacking expression of Dlx5 and Dlx6 gene. They concluded that loss of **Dlx5** and **Dlx6** resulted in a transformation of the lower jaw into an upper jaw and that cellular identity within an arch relied on a nested pattern of **Dlx** expression (14). However, no comparative study has yet been made between maxillary and mandibular teeth especially those obtained within the same individuals. The RNA expression is different between humans and animals and among different human subjects. To limit the variation using RNA sequencing to investigate gene expression, we employed the teeth with the same tooth type from the maxilla and mandible within the same individual. RNA-Seq technique will help us to determine differentially expressed genes between upper and lower teeth.

This study evaluated and compared the gene expression profiles of the maxillary and mandibular human teeth with the aim of finding differences in gene expression patterns of dental pulp cells. This information may be applicable to clinical problems such as eruption disturbance, repairing tooth defect, and tooth bioengineering. This will be the first fundamental study to unveil differential gene expression of similar teeth erupting from opposing jaw bones.

Future work can continue to explore the possibility of dentin tissue restoration in vivo and the regeneration of whole teeth, both in vivo and in vitro. It is believed that by continuous improvement of our understanding in these areas, we will be able to improve the way we diagnose and treat pathologies affecting mature human teeth, whether they arise from genetic or environmental factors, injury, or disease.

Research problem

There is a lack of knowledge on the differential expression of genes in dental pulp cells of maxillary and mandibular teeth in humans.

Research question

Does gene expression profile of human dental pulp cells from maxillary teeth differ from those from mandibular teeth?

Research objective

To analyze the differential gene expression of human dental pulp cells derived from maxillary and mandibular teeth.

Research hypothesis

H_0: Gene expression profiles of human dental pulp cells between maxillary and mandibular teeth are not significantly different.

H_1: Gene expression profiles of human dental pulp cells between maxillary and mandibular teeth are significantly different.

Keywords

Maxilla, Mandible, RNA-Sequencing, RNA profile, Cellular behavior

Field of research

Experimental research

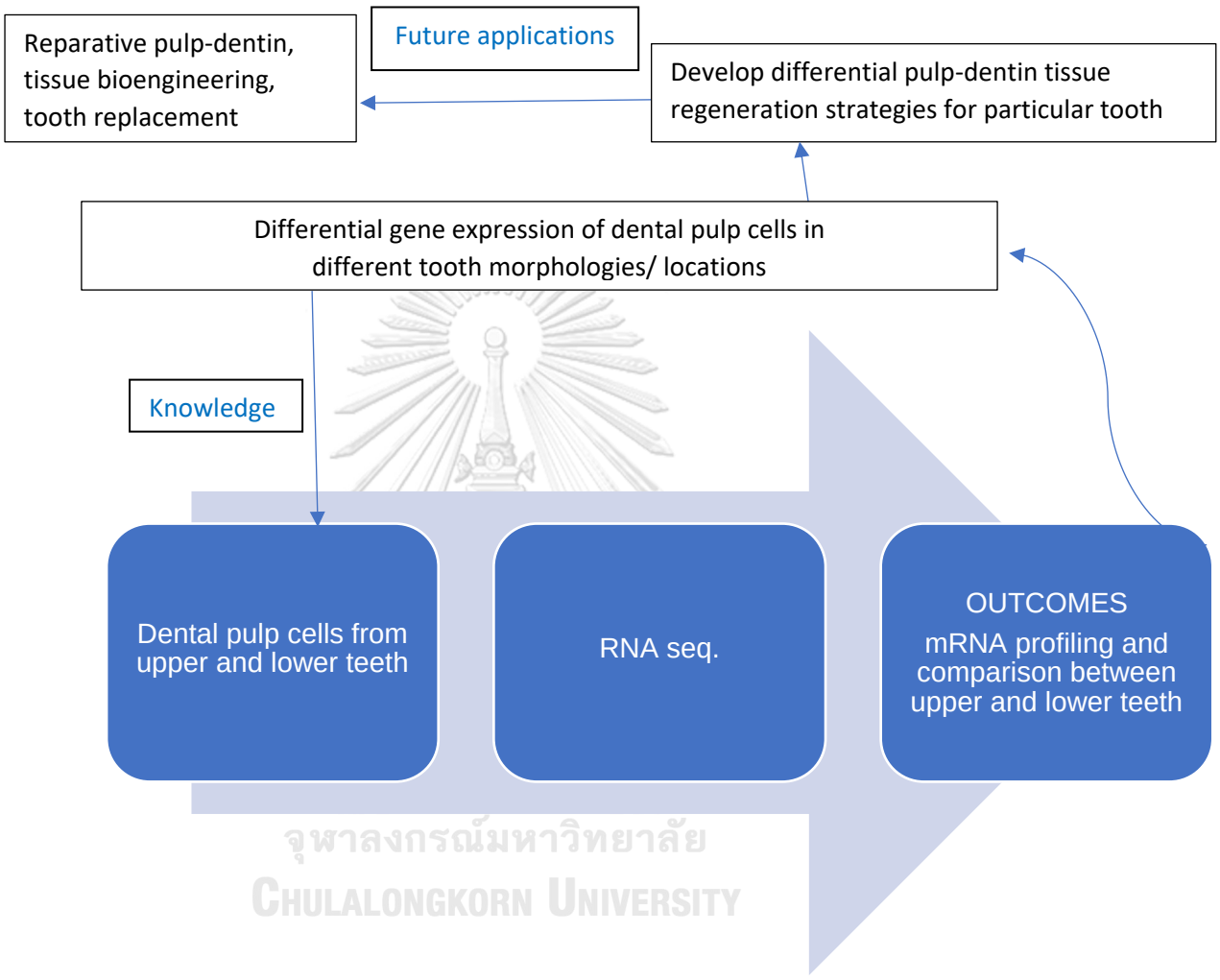
Type of research

Laboratory research

Expected outcome

This research will provide a comprehensive mRNA profile of human dental pulp cells and elucidate the genes that are potentially involved in DPSCs differentiation into maxillary and mandibular teeth. This result will change the way we understand tooth morphogenesis and it will give more information about genes expression in erupted teeth that is important for dentin bioengineering; the way to treat tooth loss and repair tooth damage.

Conceptual framework



LITERATURE REVIEW

Tooth

Humans depend on teeth for eating, communication, and physical appearance. One global health problem representing a burden to society and the economy is tooth loss. In 2015, 3.5 billion people present with untreated oral conditions and 276 million have complete loss of their natural teeth, mostly the elderly (15, 16). Major causes of tooth loss are dental caries, periodontal disease and genetically-driven disorders.

Current dental treatments such as tooth filling and fixed prosthesis are used to repair missing tooth structure while removable prosthesis and dental implant are used to replace missing tooth. Regenerative dentistry is a trending concept that uses stem cells to restore tooth structure. It requires an understanding of tooth development mechanisms and biological processes involved in tooth repair, which are important concepts applying for cell-based regeneration of damaged teeth in tooth bioengineering strategies (17).

Tooth development is controlled by complex reciprocal interactions between genes and cell signaling. During the first steps of differentiation, the developing tooth shares similar regulatory pathways with other ectodermal organs (18). However, human teeth do not display a regenerative capacity similar to other human epithelial appendages such as hairs and nails (19). A robust number of studies have investigated the origin, differentiation, organogenesis and regeneration of teeth. There has been a huge progress in recent years towards a much better understanding of the regulation of tooth development (20-23).

Calcified tissues of the tooth comprise enamel, dentin and cementum. The main component is the dentin which is produced from the odontoblasts (24). Dentine consists of collagen, hydroxyapatite, dentine sialophosphoprotein and dentine matrix protein. The upper

portion of the dentin is covered by a layer of enamel which is the hardest tissue of human body. It is produced by ameloblasts or oral epithelial cells. Enamel proteins are amelogenin (90%), ameloblastin, enamelin and tuftelin. The pulp, which is rich in fibroblast-like cells, blood vessels and nerves, is surrounded by the dentine. The root portion of the dentin is covered by cementum produced by cementoblasts. The tooth is attached with the alveolar bone socket by the periodontium consisting of periodontal ligament, cementum, alveolar bone and gingiva (25).

Ameloblasts, which produced enamel, undergo programmed cell death during tooth development and no longer exist in the mature enamel tissue. Thus, damaged enamel cannot be biologically repaired in teeth (26). Fluoride oral supplementation is currently used to re-mineralize eroded enamel (27).

A clinical goal towards a successful dental treatment requires the maintenance of a vital dental pulp. In early dental caries lesions, odontoblasts can survive and produced reactionary dentin to repair and produced reactionary dentin formation to repair and protect the pulp (28). But if the infectious lesion reaches the dental pulp, odontoblasts may not survive and be replaced by new odontoblasts. This can be achieved via the activation of stem cell proliferation cascades that can differentiate into odontoblasts and secrete reparative dentin (29). This dentin is laid in a form of a thin band of dentin (dentine bridge) that walls off the pulp from bacterial infection.

Odontogenic genes

The early markers for tooth formation are *LHX6* and *LHX7* which are expressed in oral portion of the first branchial arch. The fibroblast growth factor 8 (*FGF8*) is the major signaling molecule for inducing *LHX* genes expression. *LHX* genes are expressed in the ectomesenchyme throughout the tooth development process; *LHX7* is expressed along the proximal and distal part

of both maxilla and mandible while *LHX6* is only restricted at proximal part of maxilla and mandible. In mutant mice carrying at least one allele of *LHX6* or *LHX7*, the development of molar still happen but the double mutant mice show missing molar teeth due to arrested molar development at dental lamina stage (30).

Thickening of oral epithelium to produce the correct number and position of tooth germ is the later step after establishment of oral-aboral axis. *PAX9* is the early mesenchymal marker. *PAX9* is induced by *FGF8* but repressed by *BMP2* and *BMP4* (31, 32). The mice with *PAX9* mutation show absence of teeth resulting from arrested tooth formation at the bud stage (32).

PITX2 is another important gene during odontogenesis. *PITX2* is expressed in the proximal and distal parts of both maxillary and mandibular processes before odontogenesis and still expressed at the sites of tooth formation throughout odontogenesis (33). It is positively regulated by *FGF8* and negatively by *BMP4* (34). *LHX6* is induced by *PITX2* and also has a negative feedback to repress *PITX2* (35). Axenfeld-Reiger syndrome, a condition with tooth hypoplasia and hypodontia, is associated with the mutation of *PITX2* (36).

BARX1 gene is expressed in proximal part of maxilla and mandible where molar teeth develop while the distal part where incisor develop is the *BARX1* negative area (37). Apart from this gene, *ALX3*, *ISL1* and *MSX1* are also expressed in the incisor area (38). Inhibiting of *BMP4* in the distal area of mandibular arch results in molar teeth development instead of incisors because of the upregulation of *BARX1* and downregulation of *MSX1* (39).

DLX1 and *DLX2* are co-expressed in proximal part of both maxilla and mandible (33). The expression of *DLX* gene is induced by *FGF8* and *BMP4* (40). It is reported that *DLX1* and *DLX2* are required for upper molar development. The null mutation of both *DLX1* and *DLX2* in mice

lead to arrested formation of maxillary molars at epithelial thickening stage, however, the development of mandibular molars is still maintained (33).

PITX1 is expressed in mesenchyme and epithelium of developing incisors and molars throughout odontogenesis (33). It is first expressed in mesenchyme and transferred to epithelium at placode stage. It has synergistic interaction with *TBX1* and *BARX1*. Deletion of *PITX1* gene in mice causes abnormal tooth morphology of mandibular molars and a fusion of the first and second molars (41).

MSX2 is expressed dominantly in enamel free area such as epithelial rests of Malassez and HERs and it acts as an inhibitor of amelogenin promoter (42). *DLX2* is expressed in root epithelium during root formation and involved in cementogenesis and root formation (43). *DLX2* overexpression results in an increased cementum deposition and shortened roots (44). To date, most studies demonstrate tooth development in mouse embryos while that in post-natal/adult mice is scarce.

Odontoblasts are differentiated from ectomesenchymal cells that possess a cranial neural crest origin. During differentiation, odontoblasts secrete the organic matrix including dentin sialophosphoprotein (DSPP) and dentin matrix protein-1 (DMP-1), which are regarded as odontoblast differentiation markers (45). Many studies have demonstrated that odontoblast differentiation involves genes and signaling cues such as bone morphogenetic proteins (BMP), fibroblast growth factors (FGF) and wingless (WNT) signaling molecules as well as transcription factors such as *RUNX2* and *PAX9* (45-47). However, the exact molecular mechanisms underlying odontoblast differentiation are still unclear.

Dental pulp stem cells (DPSCs) have emerged as a promising source of cells for various applications in regenerative medicine. Currently, these cells are being investigated for repair of dental hard tissues like dentin and other body tissues such as bone and neurons. DPSCs are present in 'cell-rich zones' within the dental pulp and are considered to have similar characteristics as bone marrow mesenchymal stem cells (BM-MSCs) including self-renewal capability and multi-lineage differentiation (48, 49).

Previous studies investigated comparative gene profile between DPSCs and other types of MSCs. Those cells were found to share basic MSCs characteristics, however, they retained unique gene expression making each type of cells unique. For instance, DPSCs are primed towards neuro-ectoderm lineages compared with other cell lines (48, 50-52). It is believed that this phenomenon is due to unique molecular networks and regulatory pathways of DPSCs. To date, the knowledge of this fundamental cue of DPSCs is still insufficient. Hence, optimal conditions and signals, especially involving gene expression regulation governing the fate of DPSCs, need to be identified (19, 21, 52).

RNA-Sequencing technique

Nowadays, there are many methods to study gene expression, but the 3 popular methods are quantitative real time polymerize chain reaction, Microarray and RNA-sequencing technique. RNA sequencing (RNA-Seq) is a method approach for both discovering and profiling mRNAs to studied gene regulation in organisms that are not well-characterized at the genomic or transcriptomic levels whereas quantitative reverse transcription PCR (qRT-PCR) or microarray reagents are unavailable (7).

The quantitative reverse transcription PCR is commonly used to analyze the expression of few gene with known sequence and used to confirm result after microarray or RNA

sequencing. Microarray is used to measure the expression levels of a plenty of genes, but the disadvantage of microarray is the need for probe design sequence and the low dynamic range. RNA sequencing (RNA-Seq) technology offers several advantages compared to microarray analysis. It enables rapid profiling and deep investigation of the transcriptome, for any species (53). Compared to microarrays, RNA-Seq technology can detect a higher percentage of differentially expressed genes, especially genes with low expression. RNA seq is considerably expensive but the data achieved from RNA seq is enormous and very beneficial for genetic field (54).

Thus, expression profiling of mRNAs found in DPSCs can be carried out to uncover molecular signatures and regulatory pathways that could broaden our understanding of the roles of mRNAs for future biomedical applications. Ultimately, this can be used as a primordial approach to comprehend the DPSCs' biological progressions to ensure success when applied in stem cell therapy.

RESEARCH METHODOLOGY

Cell isolation and culture

The research protocol was submitted for approval to the Human Ethics Committee, Faculty of Dentistry, Chulalongkorn University (HREC-DCU 2019-062). Informed consent was obtained from participants. The 4 participants in this study were relatively healthy and did not have medical problem. Teeth scheduling for extraction according to a treatment plan such as extraction for orthodontics treatment were collected for cell isolation. Two pairs of lower and upper premolar teeth obtained from 16 year-old female patient and 23 year-old male patient and 2 pairs of lower and upper third molar teeth obtained from 22 year-old male patient and 27 year-old female patient were used. The data of teeth (tooth number, cusp, root) was recorded. Briefly, dental pulp tissues were gently removed and explanted. Cell isolation was performed by explant protocol. The culture media was changed every 3 days. Cells from passage 4 were used in the work.

RNA preparation and sequencing

RNA isolation was performed using RNeasy kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol with DNaseI treatment. RNA was eluted from the column using nuclease free water. Further, RNA quality was examined using a Bioanalyzer (Agilent 2100; Agilent Technologies, Santa Clara, CA, USA). Total RNA (1 μg) was used for mRNA library preparation. The RNA samples from two pairs of premolar teeth and two pairs of molar teeth were sent to Illumina Inc. (Seoul, Korea) to synthesize cDNA and performed following their protocols.

RNA-Seq Analysis

RNA-Seq Analysis was carried out using RNA-Seq Alignment and RNA-Seq Differential Expression program (Illumina Inc., Seoul, Korea.). The genes with log2 fold change (logFC) of ≤−2 or ≥2 (i.e. exhibiting 4-fold change or more), P-value ≤ 0.05 and false discovery rate (FDR) ≤ 0.05 from each pair of premolar teeth and molar teeth were selected and compared. The results were compared with RNA-Seq Analysis from all of 4 pairs.

Validation of RNA-Sey: Quantitative RT-PCR and related experiments

Quantitative RT-PCR analysis was performed to verify the results obtained from RNA-Seq Analysis. We used another 6 pairs of teeth in this qRT-PCR experiments. The specific genes with expression differences of 4-fold change or greater in 4 pairs of teeth from RNA-Seq Analysis were selected. Cq values were used to determine ΔCq values (ΔCq = Cq of the gene minus Cq of the control; actin), and differences in Cq values were used to quantify the relative amount of PCR product, expressed as the relative change by applying the equation $\Delta\Delta$Cq. For the statistical analysis, the Wilcoxon signed rank test (α<0.05) was performed to correlate the relative change with differential expression as detected by PCR. The PCR results were compared with the results from RNA-Seq analysis to validate the consistency. Identified genes of interests were further reviewed using the published literature to understand their roles and mechanisms.

Statistical Analysis

Statistical calculations were carried out using SPSS version 22 (IBM Corp., Armonk, NY, USA). Normality test: each group was checked for Quantitative RT-PCR results. Wilcoxon signed rank test was performed to determine the statistical significance for each comparison due to non-normal data distribution. *P*-value < 0.05 was considered statistically significant.

RESULT

Top expression genes

RNA-Seq Alignment program was used to analyze raw data and RNA-Seq Differential Expression program for data analysis after alignment according to the reference genome of Homo sapiens (UCSC hg19). Top-20 genes with the highest expression level in each tooth type were shown in Table 1-4. *FN1, COL1A2, ACTB* were the top three expression genes in the upper premolars (Table 1); *FN1, COL1A2, ACTB* in the lower premolars (Table 2), *FN1, COL1A2, ACTB* in the upper molars (Table 3), and *FN1, COL1A1, COL1A2* in the lower molars (Table 4). Overall, the *FN1, COL1A1, COL1A2, ACTB, EEF1A1* were the top expression genes of posterior teeth.

Table 1 20 top-expression genes in upper premolars

Gene	Tooth		
	Sample 1	Sample 2	Mean count
1) *FN1*	853954.7	866299.7	863429.6
2) *COL1A2*	536551.2	494620	514913.9
3) *ACTB*	465644.2	424213.7	443986.1
4) *EEF1A1*	350065.3	406808.1	387375.5
5) *TGFBI*	96093.95	550661.8	340760.8
6) *COL1A1*	542881.7	81018.95	294047.1
7) *VIM*	347757.2	197607.3	266072.5
8) *FTH1*	76591.95	389346.4	252911.8
9) *GREM1*	135423.3	255886	208786.4
10) *ACTG1*	232469.7	181235	205113.5
11) *IGFBP5*	123865.5	230236.4	182979.8
12) *COL6A3*	219547.6	150360.6	182289

13) THBS1	181930.2	162697.9	172412.9
14) PENK	197.1548	289768.2	158039.9
15) FLNA	222741	88166.41	151404.8
16) GAPDH	134745.4	140879.8	137898.6
17) PTGS1	39725.17	201251.9	128332.3
18) FSTL1	100987.7	133983	120746.6
19) FTL	56742.56	167780.7	114422.1
20) COL6A2	84064.44	137829.6	112922.8

Table 2 20 top-expression genes in lower premolars

Gene	Tooth		
	Sample 1	**Sample 2**	**Mean count**
1) FN1	731532.75	948013.14	839772.9
2) COL1A2	347285.89	720341.07	533813.5
3) ACTB	360057.80	348383.84	354220.8
4) COL1A1	356972.07	341019.60	348995.8
5) EEF1A1	263796.29	366186.36	314991.3
6) VIM	289188.06	270188.50	279688.3
7) TGFBI	50263.65	411307.27	230785.5
8) ACTG1	211085.99	193315.21	202200.6
9) FTH1	48299.71	350915.16	199607.4
10) THBS1	222719.50	163533.09	193126.3
11) GREM1	105511.83	267795.60	186653.7
12) PENK	26762.53	329865.62	178314.1
13) GAPDH	131272.26	216535.09	173903.7
14) IGFBP5	94387.68	250384.91	172386.3
15) COL6A3	118588.87	210611.13	164600.0

16) TIMP3	45765.42	253683.76	149724.6
17) FLNA	178216.23	108365.23	143290.7
18) FSTL1	80682.70	140907.00	110795.3
19) COL6A2	65960.32	129411.05	97685.7
20) COL5A2	58002.94	129550.85	93776.9

Table 3 20 top-expression genes in upper molars

Gene	Tooth		
	Sample 1	Sample 2	Mean count
1) FN1	989586.8	1011061	1006786
2) COL1A2	563030.8	437266.1	510880.9
3) ACTB	494335.6	417816.3	462643.4
4) EEF1A1	413496.3	317704.8	381988.4
5) COL1A1	287593.1	435163	355628.8
6) VIM	308996.5	378385.4	341845.8
7) ACTG1	258386.7	242629.4	252427.9
8) GREM1	280898.1	62583.58	198168.3
9) COL6A3	239727	132659.1	193142.9
10) GAPDH	203708.8	156111.9	182194.3
11) FLNA	129699.5	196587.9	160891.5
12) TGFBI	239372.5	56580.89	157917.2
13) FTH1	180418.5	110501.2	151807.3
14) PENK	220486.8	54093.46	147533.8
15) COL6A2	190618.9	95365.19	147446.3
16) THBS1	150190.9	124207.4	141744.6
17) IGFBP5	164621.3	75364.34	126801.1
18) TIMP3	156516.2	58080.13	115865.1

19) DKK3	117546.7	97518.97	109565.9
20) ITGB1	94351.16	124100.1	108884.1

Table 4 20 top-expression genes in lower molars

Gene	Tooth		
	Sample 1	Sample 2	Mean count
1) FN1	507727.5	594020	550001.9
2) COL1A1	296686.7	571735.2	423519.2
3) COL1A2	355850.7	487052.3	416950.9
4) ACTB	352479.6	426417	387886.7
5) EEF1A1	320701.7	389411.8	355100.3
6) VIM	342354.9	224251.5	292256.8
7) ACTG1	204374.5	239459.7	221354.8
8) IGFBP5	162427.9	245287.6	200383.1
9) GAPDH	209489.6	141546	178444.2
10) COL6A3	187409.2	133366	164130.6
11) TGFBI	211953.2	73935.89	150178.2
12) FLNA	128570.8	161127.9	144112.5
13) THBS1	77011.13	195886.3	128187
14) FTH1	112695.9	138679.7	125137.7
15) PKM	119262.2	110590.2	115709.6
16) DKK3	84158.7	140901.2	110018
17) COL6A2	139416.6	61789.05	103955.8
18) PENK	184167.9	224.5692	102578.1
19) SPARC	72350.34	137015.6	100980.4
20) ITGB1	73315.01	126422.9	96355.57

Premolar teeth

Premolar P1+P2

2 pairs of premolar teeth were aligned with RNA-Seq Alignment program. The aligned data from the first round and second round were used in differential analysis. RNA-Seq Differential Expression program was performed to analyze the data after alignment. The maxillary premolar teeth from P1 and P2 were used as a control group whereas the comparison group was the mandibular teeth. From 27,914 genes, after excluding genes with low counts, 16,999 genes were used for further analysis. 267 genes had true significance (*P*-value ≤ 0.05 and false discovery rate (FDR) ≤ 0.05) and 61 genes had log2 fold change (log_2FC) of ≤−2 or ≥2 (i.e. exhibiting 4-fold change or more) (Table 5). MA-plot and heatmap analysis are shown in Figure 1.

Table 5 Differential expression genes (Premolar teeth)

	Tooth		
	Pair 1 (P1)	**Pair 2 (P2)**	**P1+P2**
Assessed gene count	11908	15226	16999
Δ gene count	5983	4810	267
Δ gene count (log_2fold change ≤−2 or ≥2)	62	172	61

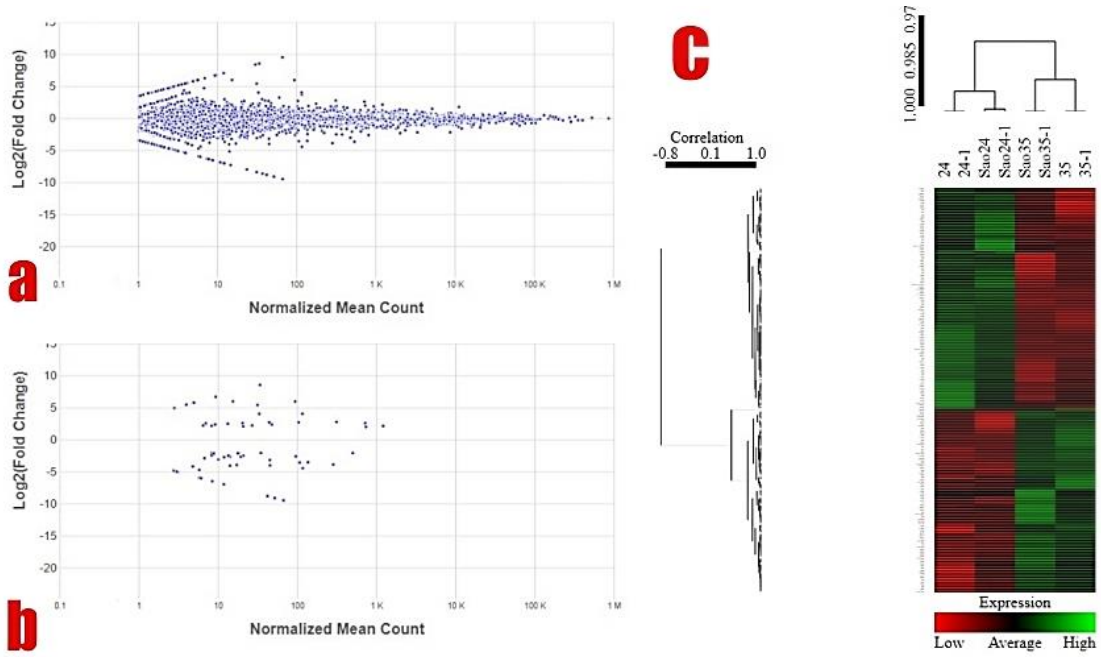


Figure 1 MA-plot and heatmap analysis indicated differences between two groups (maxillary and mandibular group) in P1+P2 (premolar).

(a) MA-plot provided an overall of the differential genes, with the log_2FC on the Y-axis and mean of normalized counts in X-axis. (b) MA-plot showed genes that pass the significant threshold ((FDR) ≤ 0.05 and log_2FC of ≤−2 or ≥2). (c) Hierarchical cluster heat maps of differential expressed genes in 2 repeats of the same sample (column 24 and 24-1 came from the same tooth as well as Sao24/Sao24-1, Sao35/Sao35-1, 35/35-1 represented the same tooth sample). One row in the heatmap represents one gene. The intensity of the color indicates the expression level, with green representing high expression level and red representing low expression level.

Premolar P1, P2 (first pair, second pair of premolar teeth)

The premolar teeth from the maxilla and mandible within the same individual were separately analyzed. From 27,914 genes, 11,908 genes (P1) and 15,226 genes (P2) were used for further analysis after ruling out genes with low counts. Of the 11,908 genes (P1), 5,983 genes had true significance (*P*-value ≤ 0.05 and false discovery rate (FDR) ≤ 0.05) and 62 genes had log2 fold change (log_2FC) of ≤−2 or ≥2 (i.e. exhibiting 4-fold change or more). In P2, 4,810 genes had true significance and 172 genes had log_2FC of ≤−2 or ≥2 (Table1). MA-plot is shown in Figure 2.

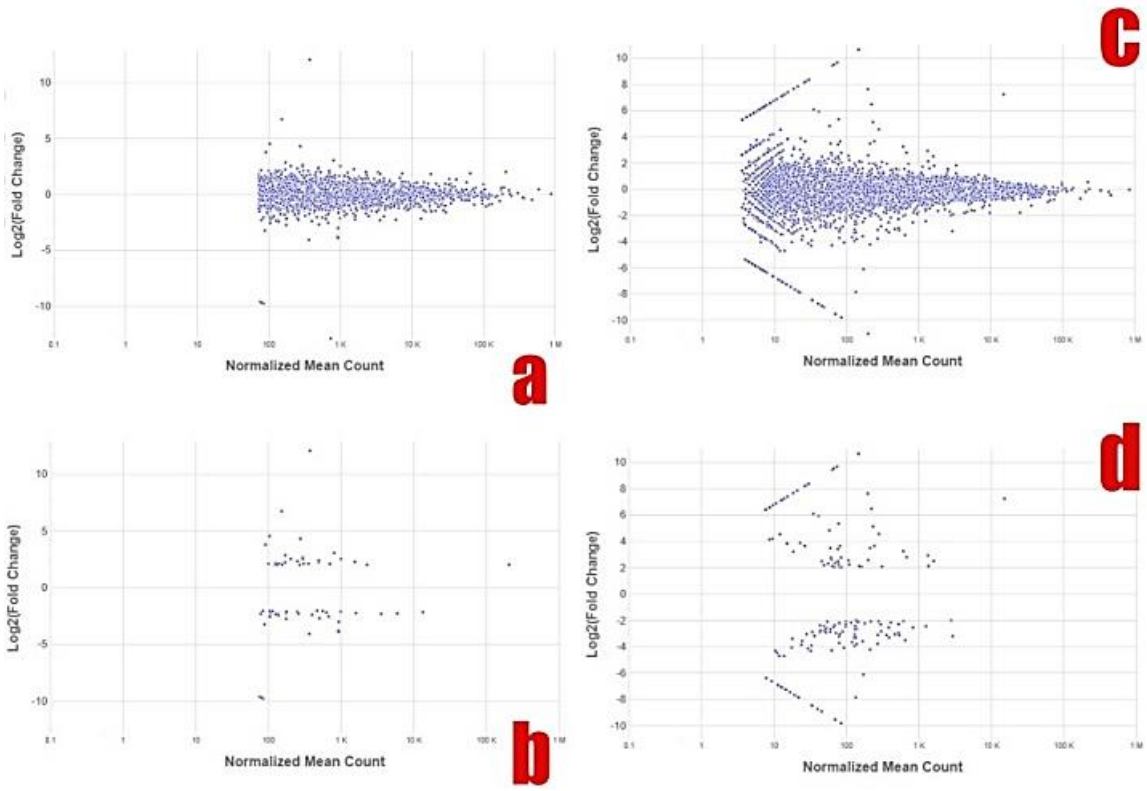


Figure 2 MA-plot analysis of premolar

(a) The MA-plot provided a global view of the differential genes in first pair of premolar teeth from the same individual (24/35). (b) Genes from first pair that passed the significant threshold and log_2FC of ≤−2 or ≥2 (c) Differential genes in second pair (Sao24/Sao35) (d) Genes from second pair that passed the significant threshold and log_2FC of ≤−2 or ≥2

When comparing P1, P2 and P1+P2, *PITX1* and *PITX2* genes were found to be differentially expressed between two groups (maxillary and mandibular group) (Figure 3). Heatmap of 2 DE genes (*P*-value ≤ 0.05 and false discovery rate (FDR) ≤ 0.05) indicated clear differences between two groups. Each row in the heatmap represents one gene, with the expression level ranging from high (green) to low (red) (Figure 4 and Figure 5). *PITX1* gene had higher expression (exhibiting 2^{6} or 64-fold change) in the mandibular group (lower teeth) compared to the maxillary group (upper teeth) (Table 6) while *PITX2* had higher expression (exhibiting $2^{3.49}$ or 11-fold change) in upper teeth more than lower teeth (Table 7).

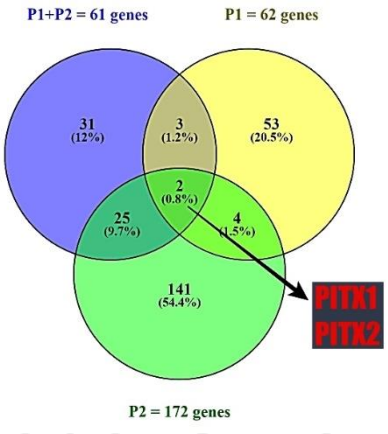


Figure 3 Venn diagram for DE genes of premolar teeth

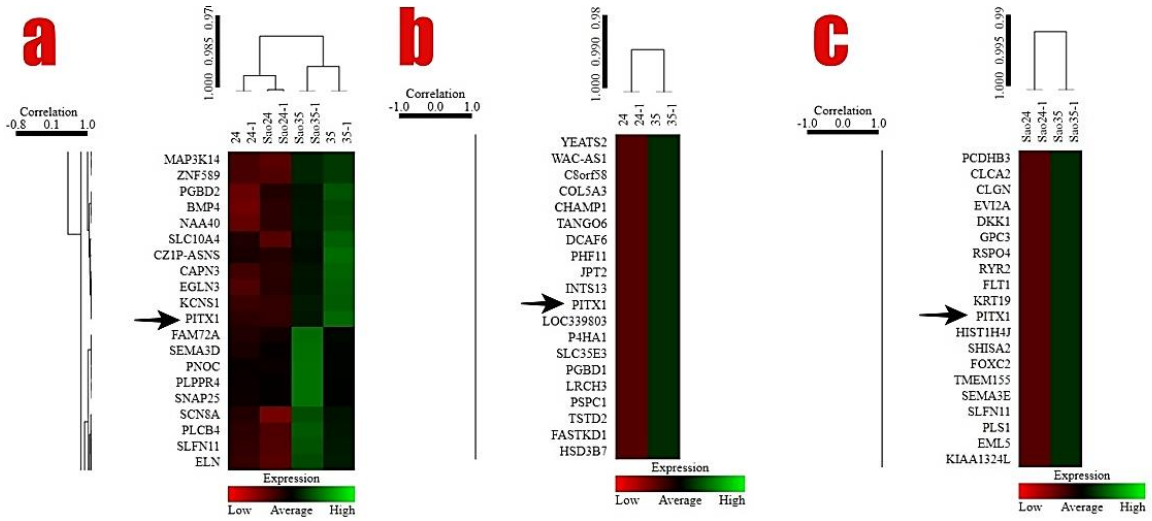


Figure 4 *PITX1* heatmap analysis of premolar

(a) Heatmap analysis from P1+P2 (b) Heatmap analysis from P1 (c) Heatmap analysis from P2

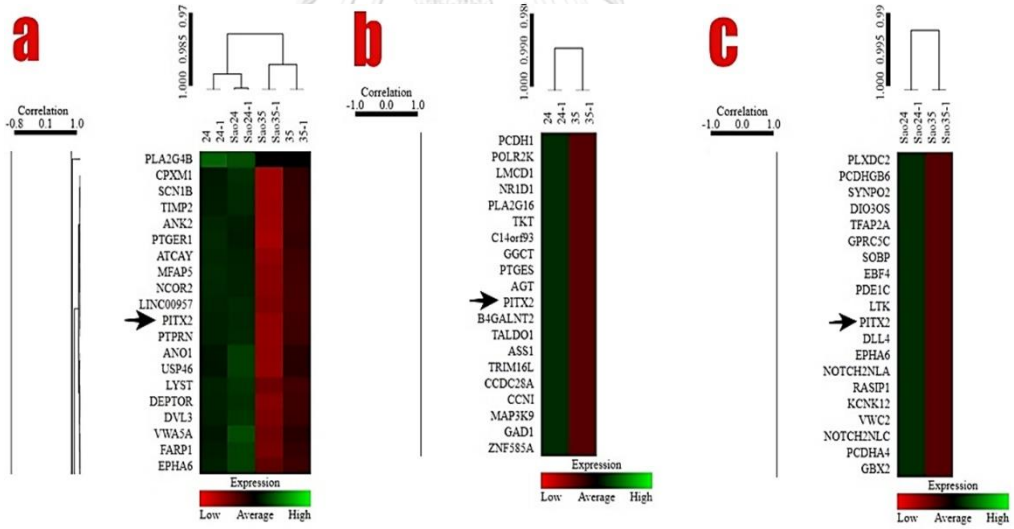


Figure 5 *PITX2* heatmap analysis of premolar

(a) Heatmap analysis from P1+P2 (b) Heatmap analysis from P1 (c) Heatmap analysis from P2

Table 6 *PITX1* Data (Premolar teeth)

	Tooth		
	Pair 1(P1)	**Pair 2(P2)**	**P1+P2**
Base mean	152.20	23.05	93.37
control	2.84	2.92	2.88
comparison	301.56	43.19	183.86
$\log_2$(Control count)	1.50	1.54	1.52
$\log_2$(Comparison count)	8.24	5.43	7.52
$\log_2$(Fold change)	6.73	3.89	6.00
p-value	0.0000*	0.0041*	0.0000*
p-adjust value (q)	0.0000**	0.0154**	0.0000**

*Denote significant at $\alpha \leq 0.05$, ** Denote significant at $q \leq 0.05$ (FDR≤0.05)

Table 7 *PITX2* Data (Premolar teeth)

	Tooth		
	Pair 1(P1)	**Pair 2(P2)**	**P1+P2**
Base mean	137.71	134.21	135.74
control	232.29	267.27	249.25
comparison	43.13	1.15	22.23
$\log_2$(Control count)	7.86	8.06	7.96
$\log_2$(Comparison count)	5.43	0.20	4.47
$\log_2$(Fold change)	-2.43	-7.86	-3.49
p-value	0.0000*	0.0000*	0.0002*
p-adjust value (q)	0.0000**	0.0000**	0.0213**

*Denote significant at $\alpha < 0.05$, ** Denote significant at $q < 0.05$ (FDR<0.05)

Molar teeth

Molar M1+M2

Raw data of 2 pairs of molars were aligned and analyzed. The maxillary molar teeth from M1 and M2 were used as a control group whereas a comparison group was mandibular teeth. From 27,914 genes, after excluding genes with low counts, 15,716 genes were used for further analysis. 15 genes had true significance (*P*-value ≤ 0.05 and false discovery rate (FDR) ≤ 0.05) and 11 genes had log2 fold change (log_2FC) of ≤−2 or ≥2 (i.e. exhibiting 4-fold change or more) (Table 8). MA-plot and heatmap analysis are shown in Figure 6.

Table 8 Differential expression genes (Molar teeth)

	Tooth		
	Pair 1(M1)	**Pair 2(M2)**	**M1+M2**
Assessed gene count	14771	11398	15716
Δ gene count	4312	2417	15
Δ gene count (log_2fold change ≤−2 or ≥2)	337	45	11

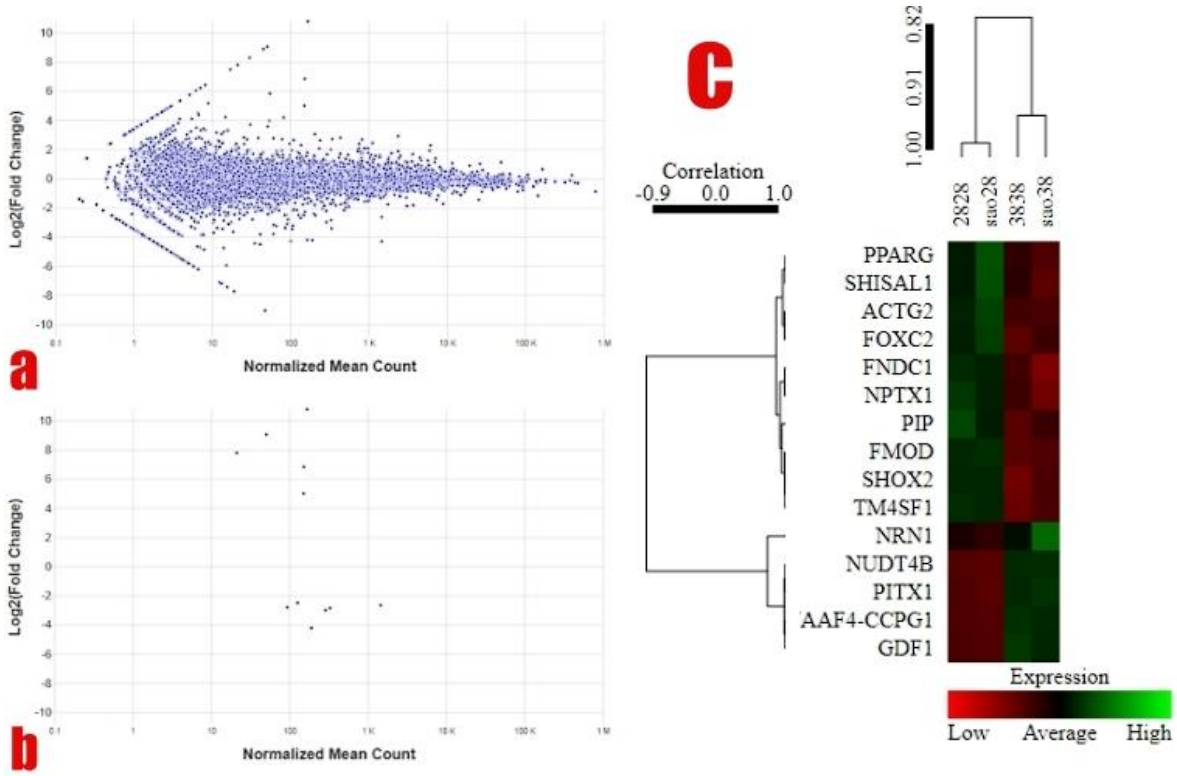


Figure 6 MA-plot and heatmap analysis indicated differences between the two groups (maxillary and mandibular group) in M1+M2 (molar).

(a) MA-plot provided an overall of the differential genes, with the log_2FC on the Y-axis and mean of normalized counts in X-axis. (b) MA-plot showed genes that pass the significant threshold ((FDR) ≤ 0.05 and log_2FC of ≤−2 or ≥2). (c) Hierarchical cluster heat maps of differential expressed genes. One row in the heatmap represents one gene. The intensity of the color indicates the expression level, with green representing high expression level and red representing low expression level.

Molar M1, M2 (first pair, second pair of molar teeth)

The molar teeth from the maxilla and mandible within the same individual were separately analyzed. From 27,914 genes, 14,771 genes (M1) and 11,398 genes (M2) were used for further analysis after ruling out genes with low counts. Of the 14,771 genes (M1), 4,312 genes had true significance (P-value ≤ 0.05 and false discovery rate (FDR) ≤ 0.05) and 337 genes had log2 fold change (log_2FC) of ≤−2 or ≥2 (i.e. exhibiting 4-fold change or more). In M2, 2,417 genes had true significant and 45 genes had log_2FC of ≤−2 or ≥2 (Table1). MA-plot is shown in Figure 7.

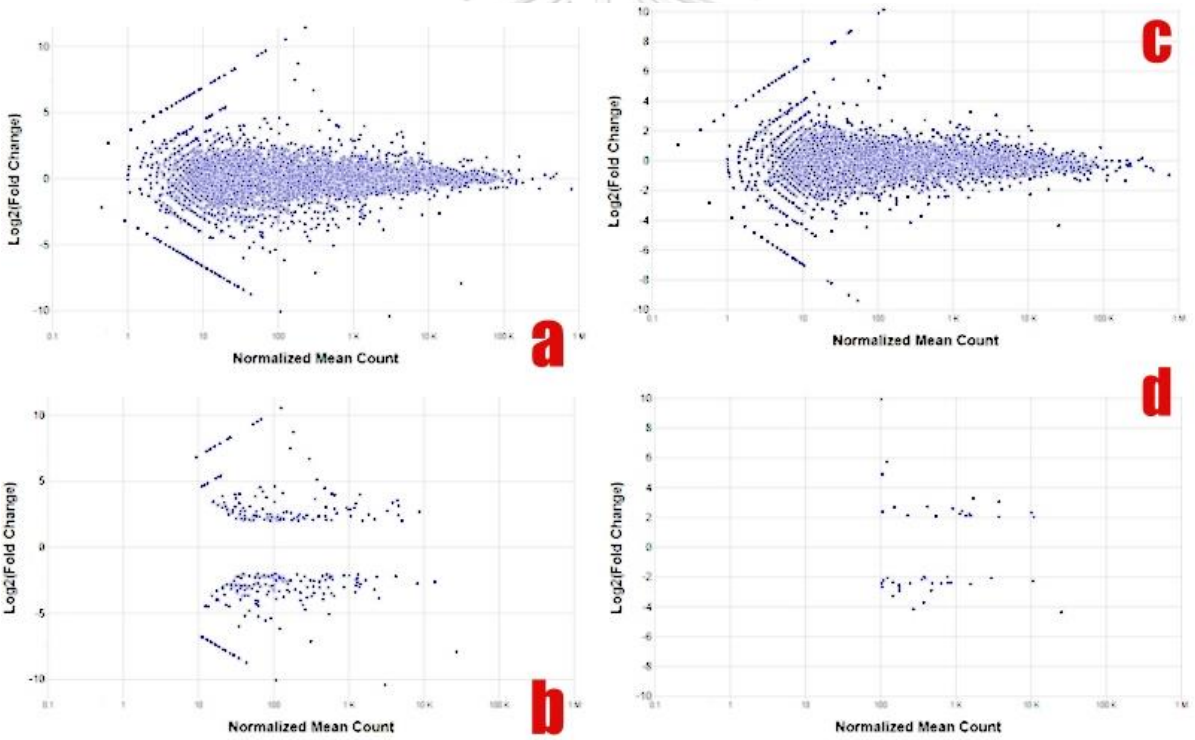


Figure 7 MA-plot analysis of molar

(a) The MA-plot provided a global view of the differential genes in first pair of molar teeth from the same individual (2828/3838). (b) Genes from first pair that passed the significant threshold and log_2FC of ≤−2 or ≥2 (c) Differential genes in second pair (Sao28/Sao38) (d) Genes from second pair that passed the significant threshold and log_2FC of ≤−2 or ≥2

Comparing M1, M2 and M1+M2, only *PITX1* genes were found to be differentially expressed between the two groups (maxillary and mandibular group) (Figure 8). Heatmap of 2 DE genes (P-value ≤ 0.05 and false discovery rate (FDR) ≤ 0.05) indicated clear differences between the two groups. Each row in the heatmap represents one gene, with the expression level ranging from high (green) to low (red) (Figure 9). *PITX1* gene had greater expression (exhibiting $2^{6.86}$ or 116-fold change) in the mandibular group (lower teeth) compared to the maxillary group (upper teeth) (Table 5).

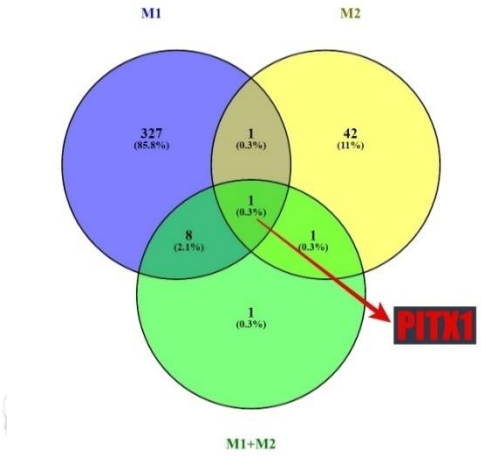


Figure 8 Venn diagram for DE genes of molar teeth

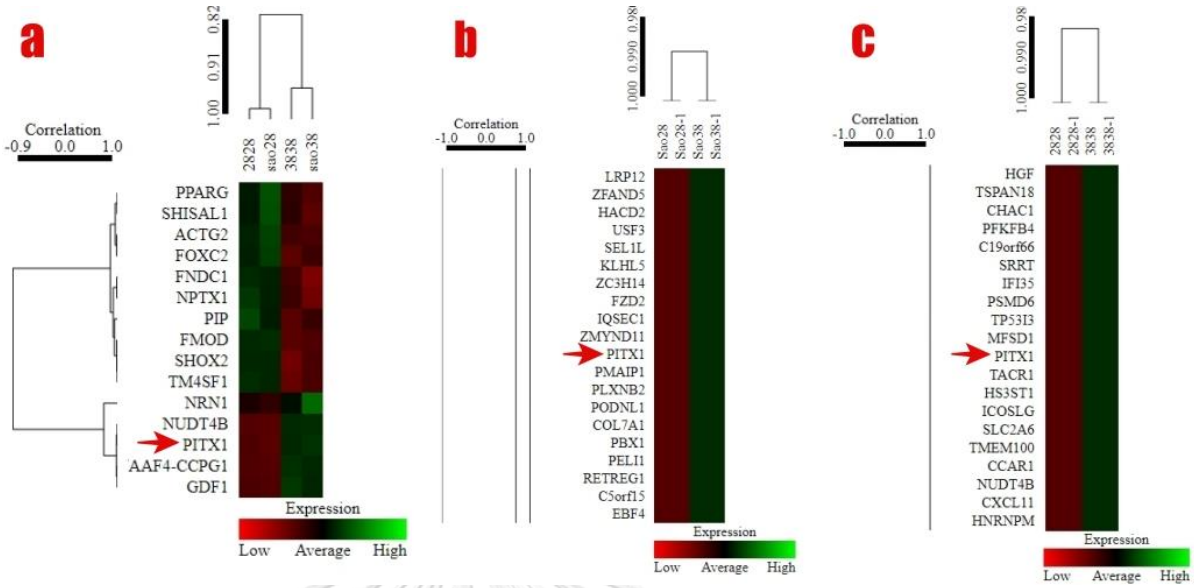


Figure 9 *PITX1* heatmap analysis of molar

(a) Heatmap analysis from M1+M2 (b) Heatmap analysis from M1 (c) Heatmap analysis from M2

Table 9 *PITX1* Data (Molar teeth)

	Tooth		
	Pair 1(M1)	**Pair 2(M2)**	**M1+M2**
Base mean	121.07	184.77	150.88
control	4.51	0.88	2.57
comparison	237.62	368.66	299.19
$\log_2$(Control count)	2.17	-0.18	1.36
$\log_2$(Comparison count)	7.89	8.53	8.22
$\log_2$(Fold change)	5.72	8.71	6.86
p-value	0.0000*	0.0000*	0.0000*
p-adjust value (q)	0.0000**	0.0000**	0.0000**

*Denote significant at $\alpha \leq 0.05$, ** Denote significant at $q \leq 0.05$ (FDR≤0.05)

Premolar + Molar teeth

Data of 4 pairs of teeth were analyzed. The maxillary teeth from P1, P2, M1 and M2 were used as control group whereas the comparison group was mandibular teeth. From 27,914 genes, after excluding genes with low counts, 19,372 genes were used for further analysis. 2 genes (*PITX1*, *DNAAF4-CCPG1*) had true significance (P-value ≤ 0.05 and false discovery rate (FDR) ≤ 0.05) and they had log2 fold change (log_2FC) of ≤−2 or ≥2 (i.e. exhibiting 4-fold change or more) (Table 10). MA-plot and heatmap analysis are shown in Figure 10 and 11.

Table 10 *PITX1* and *DNAAF4-CCPG1* Data

	Genes	
	PITX1	*DNAAF4-CCPG1*
Base mean	123.98	42.16
control	2.76	0.18
comparison	245.19	84.14
log_2(Control count)	1.47	-2.47
log_2(Comparison count)	7.94	6.39
log_2(Fold change)	6.47	8.86
p-value	0.0000*	0.0000*
p-adjust value (q)	0.0000**	0.0000**

*Denote significant at $\alpha \leq 0.05$, ** Denote significant at q≤0.05 (FDR≤0.05)

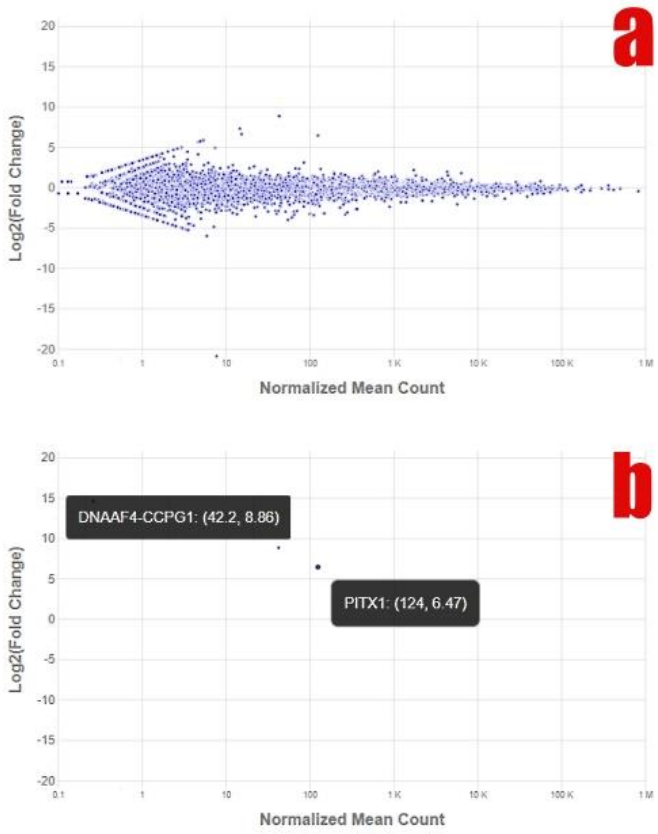


Figure 10 MA-plot indicated differences between the two groups (maxillary and mandibular group).
(a) MA-plot provided an overall of the differential genes, with the log_2FC on the Y-axis and mean of normalized counts in X-axis. (b) MA-plot showed genes that pass the significant threshold ((FDR) ≤ 0.05 and log_2FC of ≤−2 or ≥2).

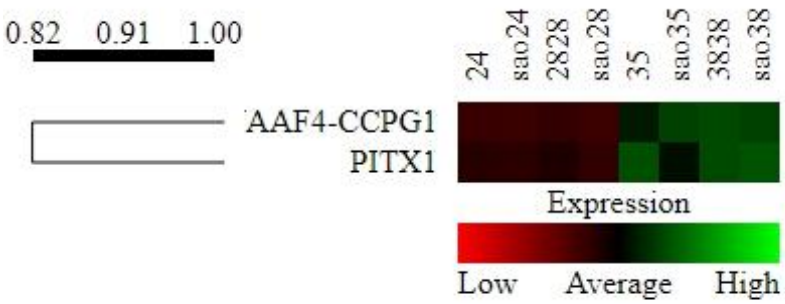


Figure 11 Hierarchical cluster heat maps of differential expressed genes in 4 pairs.
One row in the heatmap represents one gene. The intensity of the color indicates the expression level, with green representing high expression level and red representing low expression level.

P1, P2, M1, M2 (4 pairs, separately analyzed)

The teeth from the maxilla and mandible within the same individual were separately analyzed. From 27,914 genes, 11,908 genes (P1), 15,226 genes (P2), 14,771 genes (M1) and 11,398 genes (M2) were used for further analysis after ruled out genes with low counts. Only *PITX1* genes had true significance (*P*-value ≤ 0.05 and false discovery rate (FDR) ≤ 0.05) with log2 fold change (log_2FC) of ≤−2 or ≥2 (i.e. exhibiting 4-fold change or more) (Table 11). While *DNAAF4-CCPG1* did not have true significance (*P*-value ≤ 0.05 and false discovery rate (FDR) ≤ 0.05) in some pairs of teeth (Table 12).

Table 11 *PITX1* Data (Separately analyzed)

	Tooth			
	Pair 1(P1)	**Pair 2(P2)**	**Pair 3(M1)**	**Pair 4(M2)**
Base mean	152.20	23.05	121.07	184.77
control	2.84	2.92	4.51	0.88
comparison	301.56	43.19	237.62	368.66
log_2(Control count)	1.50	1.54	2.17	-0.18
log_2(Comparison count)	8.24	5.43	7.89	8.53
log_2(Fold change)	6.73	3.89	5.72	8.71
p-value	0.0000*	0.0041*	0.0000*	0.0000*
p-adjust value (q)	0.0000**	0.0154**	0.0000**	0.0000**

*Denote significant at $\alpha \leq 0.05$, ** Denote significant at q≤0.05 (FDR≤0.05)

Table 12 *DNAAF4-CCPG1* Data (Separately analyzed)

	Tooth			
	Pair 1(P1)	**Pair 2(P2)**	**Pair 3(M1)**	**Pair 4(M2)**
Base mean	13.73	63.43	60.62	40.85
control	0.17	0.18	0.17	0.21
comparison	27.29	126.68	121.07	81.49
$\log_2$(Control count)	-2.53	-2.48	-2.59	-2.26
$\log_2$(Comparison count)	4.77	6.99	6.92	6.35
$\log_2$(Fold change)	7.30	9.46	9.50	8.61
p-value	0.13	0.0000*	0.0000*	0.07
p-adjust value (q)	-	0.0000**	0.0000**	-

*Denote significant at $\alpha \leq 0.05$, ** Denote significant at $q \leq 0.05$ (FDR≤0.05)

Comparing P1, P2 and M1 and M2, only *PITX1* gene was found to be differentially expressed between the two groups (maxillary and mandibular group). Heatmap of 2 DE genes (P-value ≤ 0.05 and false discovery rate (FDR) ≤ 0.05) indicated clear differences between the two groups. Each row in the heatmap represents one gene, with the expression level ranging from high (green) to low (red) (Figure 12). *PITX1* gene had greater expression (exhibiting $2^{6.47}$ or 89-fold change) in the mandibular group (lower teeth) compared to the maxillary group (upper teeth).

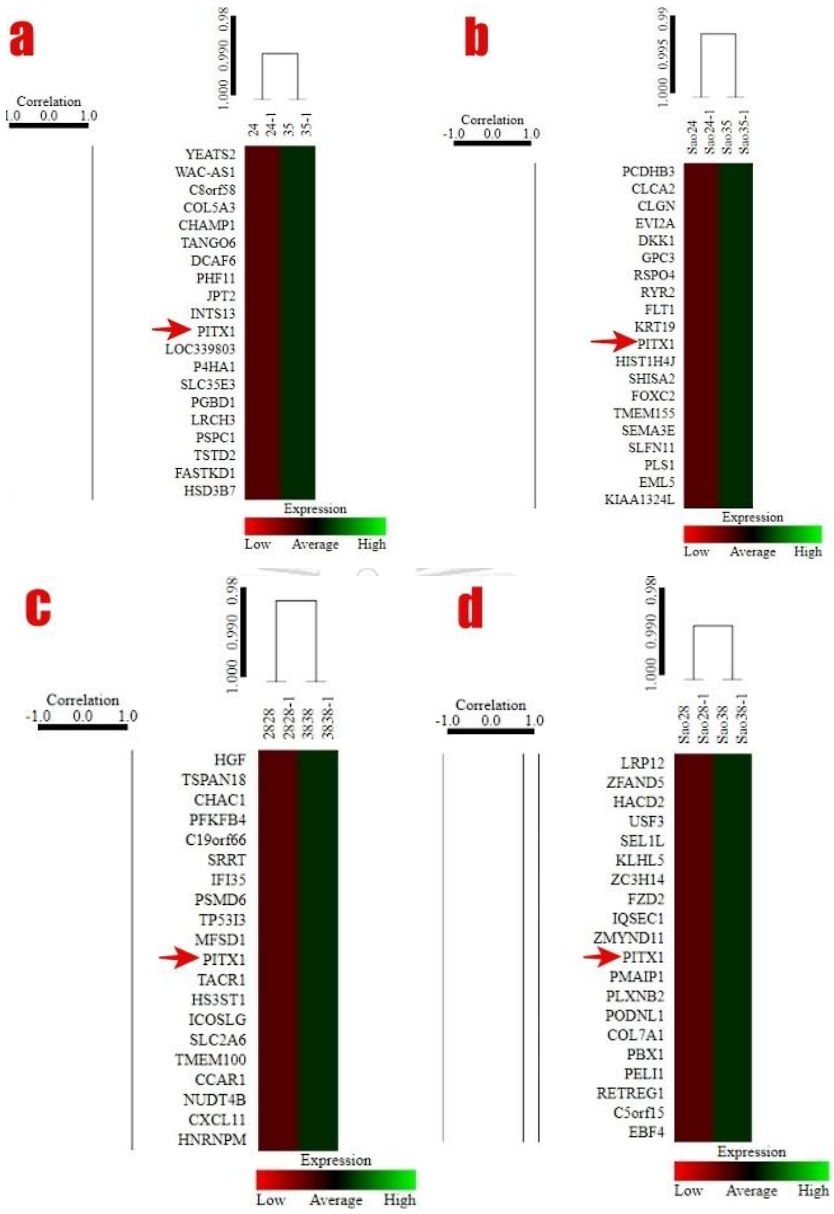


Figure 12 *PITX1* heatmap analysis from 4 pairs

(a) Heatmap analysis from P1 (b) Heatmap analysis from P2 (c) Heatmap analysis from M1 (d) Heatmap analysis from M2

Quantitative RT-PCR analysis

To further validate RNA-Sequencing experiments, *PITX1* gene was selected for quantitative RT-PCR analysis. From 6 pairs of teeth (3 pairs of premolars and 3 pairs of molars), the teeth from the maxilla and mandible within the same individual were separately analyzed. The results from quantitative RT-PCR analysis corresponded with the results from RNA-Sequencing technique (Table 13,14). Wilcoxon signed rank test was performed to determine the statistical significance. *PITX1* gene had higher expression (average fold change = 77) in the mandibular group (lower teeth) compared to the maxillary group (upper teeth) at p-value = 0.028 (Table 15).

Table 13 *PITX1* Data from quantitative RT-PCR of premolar teeth

	Tooth		
	Pair 1(P1)	**Pair 2(P2)**	**Pair 3(P3)**
$\Delta\Delta$Cq	-3.73	-6.19	-2.39
ΔCq (upper)	18.18	20.44	18.13
ΔCq (lower)	14.45	14.25	15.74
Fold change	13.27	73.00	5.22

Table 14 *PITX1* Data **from** quantitative RT-PCR of molar teeth

	Tooth		
	Pair 1(M1)	**Pair 2(M2)**	**Pair 3(M3)**
$\Delta\Delta$Cq	-7.40	-6.76	-4.90
ΔCq (upper)	18.17	21.06	18.25
ΔCq (lower)	10.77	14.30	13.35
Fold change	169.48	108.00	29.96

Table 15 Normality test and Wilcoxon signed rank test of *PITX1* Data from quantitative RT-PCR

Tests of Normality

	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	df	Sig.
upper	.388	6	.005	.710	6	.008
lower	.270	6	.195	.878	6	.261

a. Lilliefors Significance Correction

Wilcoxon Signed Ranks Test

Ranks

		N	Mean Rank	Sum of Ranks
lower - upper	Negative Ranks	6[a]	3.50	21.00
	Positive Ranks	0[b]	.00	.00
	Ties	0[c]		
	Total	6		

a. lower < upper

b. lower > upper

c. lower = upper

Test Statistics[a]

	lower - upper
Z	-2.201[b]
Asymp. Sig. (2-tailed)	.028

a. Wilcoxon Signed Ranks Test

b. Based on positive ranks.

Discussion

Tooth development is related to genetic and environmental factors. Genetics play a role in determination the shape, size, number and position of tooth (55, 56). Many studies in mice and laboratory have found many genes that are important for tooth development but there is still a lack of knowledge about gene expression in adult human teeth. The gene expression profile and complex regulatory mechanism still require elucidation.

This study investigated differential gene expression profile between upper and lower teeth by RNA-Sequencing technique. Compared to other techniques such as microarrays, RNA-Seq technique can detect a higher percentage of differentially expressed genes, especially genes with low expression. To limit the variation using RNA sequencing to investigate gene expression, the teeth with the same tooth type from the maxilla and mandible within the same individual were used. From the result, in DPSCs of posterior teeth, only *PITX1* gene had a significant higher expression in the mandibular teeth compared to the maxillary teeth in all 4 pairs. For *DNAAF4-CCPG1*, we did not further investigate because it did not have significance in some pairs of teeth.

PITX1 is a bicoid-related family homeoproteins. It is a crucial transcription factor that plays roles in the first branchial arch development. The *PITX1* null mutant chicks died immediately or shortly after birth due to shorten mandibular arch. In *PITX1* mutant mice, the shape and growth of hindlimb and mandible were seriously disturbed (57).

From the mouse embryos studies, during embryonic and postnatal development, *PITX1* is less expressed in the distal part of mandible where incisor develop but it is strongly expressed in the proximal part where molar develop. Micrognathia and mandibular molar deformation are

the result of *PITX1* deletion. *PITX1* expression persists in dental epithelium at all stage of odontogenesis and it interacts with *TBX1* and *BARX1*. Mutant mice with both maxilla and mandible deletion of *PITX1* had normal maxillary molar development while the mandibular molar had abnormal tooth morphology (41, 58).

Consistent with previous study in mice, our study found that *PITX1* gene showed higher expression in human lower teeth than upper teeth. According to our study, the differential expression of *PITX1* gene in lower premolar teeth was around 64 folds compared to that in upper premolar teeth. *PITX1* in the lower molar teeth was around 116 folds compare to upper molar teeth. There is a possibility that the more posterior site of opposing teeth, the difference in *PITX1* level between upper and lower teeth is more evident.

Quantitative RT-PCR was used to confirm the results obtained from RNA-Sequencing. The results from Quantitative RT-PCR were simultaneously with the results from RNA-Sequencing. The lower and upper teeth from the same individual were separately analyzed via Quantitative RT-PCR. *PITX1* in the lower teeth was higher expressed than the upper teeth in all 6 pairs. Average fold-change between lower teeth and upper teeth from Quantitative RT-PCR was around 77 while the results from RNA-Sequencing technique is around 89.

PITX2 is a member of the bicoid class of homeodomain transcription factors, which play important roles in embryonic development particularly eye, tooth, and abdominal organs, and establishment of left-right axis. *PITX2* is expressed in the dental epithelium. Variants in *PITX2* lead to Axenfeld-Rieger Syndrome, Type 1, anterior segment dysgenesis 4, and ring dermoid of cornea. Fan et al., 2019 studied and analyzed *PITX2*-related tooth agenesis in 18 *PITX2*-mutated patients and found that maxillary teeth agenesis was more evident compare with mandibular teeth especially in the upper anterior region (59). Similarly, our results show that *PITX2* gene had higher

expression (exhibiting around 11-fold change) in upper premolar teeth than that in lower premolar teeth, although no significance difference was observed between upper and lower molars.

DNAAF4-CCPG1 is ncRNA on chromosome 15 which is observed in read-through transcription between the neighboring dyslexia susceptibility 1 candidate 1 (*DYX1C1* or *DNAAF4*) and cell cycle progression 1 (*CCPG1*) genes. It is expected to subject for nonsense-mediated mRNA decay and unlikely to produce a protein product. The main function of NMD is to eliminate mRNA transcripts that contain premature stop codons to reduce error in gene expression (60). In our study, the *DNAAF4-CCPG1* showed higher expression in lower teeth compare to upper teeth but the results were not significant in some pairs of teeth.

From our result, the top expression gene in posterior teeth were *FN1, COL1A1, COL1A2, ACTB*, *EEF1A1*. The *FN1, COL1A1, COL1A2* genes are involved in tooth development. Fibronectin (*FN1*) is highly expressed in the odontoblasts and involved in extracellular matrix organization (61). In addition, *FN1* plays role in cell movement via actin organization in the primary tooth buds (62). The *FN1* is also a biomarker for head and neck squamous cell carcinoma due to its strong association with epithelial-mesenchymal transition and tumor invasion/metastasis (63).

COL1A1 (collagen type 1, alpha 1) has an important role in osteoblasts and odontoblasts activities (64, 65). Both *COL1A1* and *COL1A2* encodes for type 1 collagen which is the most common collagen type in humans (66). Mutations in these *COL1A1* and *COL1A2* are associated with osteogenesis imperfecta and dentinogenesis imperfecta (67). These suggest that FN1, COL1A1, and COL1A2, which are the essential genes during tooth development, could still play role in mature teeth.

The strength of this study is the upper and lower teeth obtained from the same individual that limit inter-subject variability of gene expression. The limitation of this study is we cannot obtain the other pairs of upper and lower teeth such as incisor and canine in the same individual. Our findings show that *PITX1* is dominantly expressed in the lower posterior teeth compared with the upper posterior teeth. However, the incisors and canines may have different gene expression pattern between upper and lower teeth.

This research provides new data of gene expression in the dental pulp cells isolated from the mature teeth. It can be used for comparison with that in developing teeth and in patients with congenital tooth defects, expanding the understanding of molecular physiology of dental pulp cells. Regenerative dentistry is relied on the understanding of biological mechanisms of tissue healing and repair. Regeneration of dentine–pulp complex involves the migration, proliferation, and differentiation of dental pulp cells in dentin formation and inflammatory response. The knowledge about gene profiling and cascade of signaling pathways in mature teeth paves a way to explore new dental treatment approach by targeting the interactions between tooth tissue and bioactive molecules.

This is the first study in human erupted and mature teeth showing differential gene expression between upper and lower teeth in the same individual. The present result may promote further studies to investigate the genes expression between upper and lower tooth in all tooth types (incisors, canine, premolars and molars) and the function/molecular roles of genes and regulatory mechanisms in human dental pulp cells. Moreover, these may advance knowledge genetic engineering, and this can be used as a primordial approach to comprehend the DPSCs' biological progressions to ensure success when applied in stem cell therapy.

Conclusion

In conclusion, application of RNA-Sequencing technique to study adult human dental pulp cells of maxillary and mandibular teeth, we demonstrated that *PITX1* gene had a significant higher expression in the mandibular posterior teeth compared to the maxillary posterior teeth and the difference in *PITX1* level between upper and lower teeth was more evident in the molars than the premolars.

REFERENCES

1. Chen Q, Zheng L, Zhang Y, Huang X, Wang F, Li S, et al. Special AT-rich sequence-binding protein 2 (Satb2) synergizes with Bmp9 and is essential for osteo/odontogenic differentiation of mouse incisor mesenchymal stem cells. Cell proliferation. 2021;54(4):e13016.
2. Martin KJ, Rasch LJ, Cooper RL, Metscher BD, Johanson Z, Fraser GJ. Sox2+ progenitors in sharks link taste development with the evolution of regenerative teeth from denticles. Proceedings of the National Academy of Sciences. 2016;113(51):14769-74.
3. Rasch LJ, Martin KJ, Cooper RL, Metscher BD, Underwood CJ, Fraser GJ. An ancient dental gene set governs development and continuous regeneration of teeth in sharks. Developmental Biology. 2016;415(2):347-70.
4. Zaugg L, Banu A, Walther A, Chandrasekaran D, Babb R, Salzlechner C, et al. Translation approach for dentine regeneration using GSK-3 antagonists. Journal of dental research. 2020;99(5):544-51.
5. Da Rosa W, Piva E, Da Silva A. Disclosing the physiology of pulp tissue for vital pulp therapy. International Endodontic Journal. 2018;51(8):829-46.
6. Buccitelli C, Selbach M. mRNAs, proteins and the emerging principles of gene expression control. Nature Reviews Genetics. 2020;21(10):630-44.
7. Metzker MLJNrg. Sequencing technologies—the next generation. 2010;11(1):31.
8. Bizuayehu TT, Lanes CF, Furmanek T, Karlsen BO, Fernandes JM, Johansen SD, et al. Differential expression patterns of conserved miRNAs and isomiRs during Atlantic halibut development. 2012;13(1):11.
9. Li J, Hou R, Niu X, Liu R, Wang Q, Wang C, et al. Comparison of microarray and RNA-Seq analysis of mRNA expression in dermal mesenchymal stem cells. Biotechnology letters. 2016;38(1):33-41.
10. Kang C-M, Kim S-O, Jeon M, Choi H-J, Jung H-S, Lee J-H. Comparison of Stemness and Gene Expression between Gingiva and Dental Follicles in Children. Stem cells international. 2016;2016.

11. Lee H-S, Lee J, Kim S-O, Song J-S, Lee J-H, Lee S-I, et al. Comparative gene-expression analysis of the dental follicle and periodontal ligament in humans. PloS one. 2013;8(12):e84201.

12. Song JS, Hwang DH, Kim S-O, Jeon M, Choi B-J, Jung H-S, et al. Comparative gene expression analysis of the human periodontal ligament in deciduous and permanent teeth. PLoS One. 2013;8(4):e61231.

13. Pantalacci S, Guéguen L, Petit C, Lambert A, Peterkovà R, Sémon M. Transcriptomic signatures shaped by cell proportions shed light on comparative developmental biology. Genome biology. 2017;18(1):29.

14. Depew MJ, Lufkin T, Rubenstein JL. Specification of jaw subdivisions by Dlx genes. Science. 2002;298(5592):381-5.

15. Kassebaum N, Smith A, Bernabé E, Fleming T, Reynolds A, Vos T, et al. Global, regional, and national prevalence, incidence, and disability-adjusted life years for oral conditions for 195 countries, 1990–2015: a systematic analysis for the global burden of diseases, injuries, and risk factors. Journal of dental research. 2017;96(4):380-7.

16. Righolt A, Jevdjevic M, Marcenes W, Listl S. Global-, regional-, and country-level economic impacts of dental diseases in 2015. Journal of dental research. 2018;97(5):501-7.

17. Volponi AA, Zaugg LK, Neves V, Liu Y, Sharpe PT. Tooth Repair and Regeneration. Current Oral Health Reports. 2018;5(4):295-303.

18. Thesleff I, Sharpe PJMod. Signalling networks regulating dental development. 1997;67(2):111-23.

19. Casagrande L, Cordeiro MM, Nör SA, Nör JE. Dental pulp stem cells in regenerative dentistry. Odontology. 2011;99(1):1-7.

20. Thesleff IJAJoMGPA. The genetic basis of tooth development and dental defects. 2006;140(23):2530-5.

21. Huang AHC, Chen YK, Lin LM, Shieh TY, Chan AWS. Isolation and characterization of dental pulp stem cells from a supernumerary tooth. Journal of Oral Pathology & Medicine. 2008;37(9):571-4.

22. Akmal M, Zarina ZI, Rohaya M, Sahidan S, Zaidah Z, Hisham ZS. Isolation and characterization of dental pulp stem cells from murine incisors. Journal of Biological Sciences. 2014;14(4):327-31.

23. Thesleff I. The genetic basis of tooth development and dental defects. Am J Med Genet A. 2006;140(23):2530-5.

24. Lumsden AJD. Spatial organization of the epithelium and the role of neural crest cells in the initiation of the mammalian tooth germ. 1988;103(Supplement):155-69.

25. Peyer B. Comparative odontology: University of Chicago Press; 1968.

26. Nanci A. Ten cate's oral histology-pageburst on vitalsource: development, structure, and function: Elsevier Health Sciences; 2007.

27. Ten Cate J. Review on fluoride, with special emphasis on calcium fluoride mechanisms in caries prevention. European journal of oral sciences. 1997;105(5):461-5.

28. Couve E, Osorio R, Schmachtenberg O. Reactionary dentinogenesis and neuroimmune response in dental caries. Journal of dental research. 2014;93(8):788-93.

29. Teaford MF, Smith MM, Ferguson MW. Development, function and evolution of teeth: Cambridge University Press; 2007.

30. Denaxa M, Sharpe PT, Pachnis V. The LIM homeodomain transcription factors Lhx6 and Lhx7 are key regulators of mammalian dentition. Developmental biology. 2009;333(2):324-36.

31. Hloušková A, Bonczek O, Izakovičová-Hollá L, Lochman J, Šoukalová J, Štembírek J, et al. Novel PAX9 gene polymorphisms and mutations and susceptibility to tooth agenesis in the Czech population. Neuroendocrinology Letters. 2015;36(5):101-06.

32. Peters H, Neubüser A, Kratochwil K, Balling R. Pax9-deficient mice lack pharyngeal pouch derivatives and teeth and exhibit craniofacial and limb abnormalities. Genes & development. 1998;12(17):2735-47.

33. Duverger O, Morasso MI. Role of homeobox genes in the patterning, specification, and differentiation of ectodermal appendages in mammals. Journal of cellular physiology. 2008;216(2):337-46.

34. Zhang YD, Zhi C, Song YQ, Chao L, Chen YP. Making a tooth: growth factors, transcription factors, and stem cells. Cell research. 2005;15(5):301-16.

35. Zhang Z, Gutierrez D, Li X, Bidlack F, Cao H, Wang J, et al. The LIM homeodomain transcription factor LHX6: a transcriptional repressor that interacts with pituitary homeobox 2 (PITX2) to regulate odontogenesis. Journal of Biological Chemistry. 2013;288(4):2485-500.

36. Intarak N, Theerapanon T, Ittiwut C, Suphapeetiporn K, Porntaveetus T, Shotelersuk V. A novel PITX2 mutation in non-syndromic orodental anomalies. Oral diseases. 2018;24(4):611-8.

37. Cobourne MT, Sharpe PT. Tooth and jaw: molecular mechanisms of patterning in the first branchial arch. Archives of oral biology. 2003;48(1):1-14.

38. Tucker A, Sharpe P. Molecular genetics of tooth morphogenesis and patterning: the right shape in the right place. Journal of dental research. 1999;78(4):826-34.

39. Tucker AS, Khamis AA, Sharpe PT. Interactions between Bmp-4 and Msx-1 act to restrict gene expression to odontogenic mesenchyme. Developmental dynamics: an official publication of the American Association of Anatomists. 1998;212(4):533-9.

40. Suryadeva S, Khan MB. Role of homeobox genes in tooth morphogenesis: a review. Journal of clinical and diagnostic research: JCDR. 2015;9(2):ZE09.

41. Mitsiadis TA, Drouin J. Deletion of the Pitx1 genomic locus affects mandibular tooth morphogenesis and expression of the Barx1 and Tbx1 genes. Developmental biology. 2008;313(2):887-96.

42. Molla M, Descroix V, Aïoub M, Simon S, Castañeda B, Hotton D, et al. Enamel protein regulation and dental and periodontal physiopathology in MSX2 mutant mice. The American journal of pathology. 2010;177(5):2516-26.

43. Lézot F, Thomas B, Greene SR, Hotton D, Yuan ZA, Castaneda B, et al. Physiological implications of DLX homeoproteins in enamel formation. Journal of cellular physiology. 2008;216(3):688-97.

44. Dai J, Si J, Ouyang N, Zhang J, Wu D, Wang X, et al. Dental and periodontal phenotypes of Dlx2 overexpression in mice. Molecular medicine reports. 2017;15(5):2443-50.

45. Sun Q, Liu H, Lin H, Yuan G, Zhang L, Chen ZJM, et al. MicroRNA-338-3p promotes differentiation of mDPC6T into odontoblast-like cells by targeting Runx2. 2013;377(1-2):143-9.

46. Tummers M, Thesleff IJJoEZPBM, Evolution D. The importance of signal pathway modulation in all aspects of tooth development. 2009;312(4):309-19.

47. Wang T, Xu ZJB, communications br. miR-27 promotes osteoblast differentiation by modulating Wnt signaling. 2010;402(2):186-9.

48. Gronthos S, Brahim J, Li W, Fisher L, Cherman N, Boyde A, et al. Stem cell properties of human dental pulp stem cells. Journal of dental research. 2002;81(8):531-5.

49. Gronthos S, Mankani M, Brahim J, Robey PG, Shi S. Postnatal human dental pulp stem cells (DPSCs) in vitro and in vivo. 2000;97(25):13625-30.

50. Abu Kasim NH, Govindasamy V, Gnanasegaran N, Musa S, Pradeep PJ, Srijaya TC, et al. Unique molecular signatures influencing the biological function and fate of post-natal stem cells isolated from different sources. 2015;9(12):E252-E66.

51. Shi S, Robey P, Gronthos S. Comparison of human dental pulp and bone marrow stromal stem cells by cDNA microarray analysis. Bone. 2001;29(6):532-9.

52. Karaöz E, Demircan PC, Sağlam Ö, Aksoy A, Kaymaz F, Duruksu G. Human dental pulp stem cells demonstrate better neural and epithelial stem cell properties than bone marrow-derived mesenchymal stem cells. Histochemistry and cell biology. 2011;136(4):455.

53. Wang Z, Gerstein M, Snyder MJNrg. RNA-Seq: a revolutionary tool for transcriptomics. 2009;10(1):57.

54. Li J, Hou R, Niu X, Liu R, Wang Q, Wang C, et al. Comparison of microarray and RNA-Seq analysis of mRNA expression in dermal mesenchymal stem cells. 2016;38(1):33-41.

55. Azzaldeen A, Watted N, Mai A, Borbély P, Abu-Hussein M. Tooth Agenesis; Aetiological Factors. Journal of Dental and Medical Sciences. 2017;16(1):75-85.

56. Cunha AS, Dos Santos LV, Marañón-Vásquez GA, Kirschneck C, Gerber JT, Stuani MB, et al. Genetic variants in tooth agenesis–related genes might be also involved in tooth size variations. Clinical Oral Investigations. 2020:1-12.

57. Mina M. Regulation of mandibular growth and morphogenesis. Critical Reviews in Oral Biology & Medicine. 2001;12(4):276-300.

58. Ramanathan A, Srijaya TC, Sukumaran P, Zain RB, Kasim NHA. Homeobox genes and tooth development: understanding the biological pathways and applications in regenerative dental science. Archives of oral biology. 2018;85:23-39.

59. Fan Z, Sun S, Liu H, Yu M, Liu Z, Wong SW, et al. Novel PITX2 mutations identified in Axenfeld–Rieger syndrome and the pattern of PITX2-related tooth agenesis. Oral diseases. 2019;25(8):2010-9.

60. Gene [Internet]. Bethesda (MD): National Library of Medicine (US) NCBI. DNAAF4-CCPG1 DNAAF4-CCPG1 readthrough (NMD candidate) [Homo sapiens (human)] ; 2004 – [2021-May-18]. Available from: https://www.ncbi.nlm.nih.gov/gene/100533483.

61. Kadler KE, Hill A, Canty-Laird EG. Collagen fibrillogenesis: fibronectin, integrins, and minor collagens as organizers and nucleators. Current opinion in cell biology. 2008;20(5):495-501.

62. Hu S, Parker J, Wright JT. Towards unraveling the human tooth transcriptome: the dentome. PloS one. 2015;10(4):e0124801.

63. Liu X, Meng L, Li X, Li D, Liu Q, Chen Y, et al. Regulation of FN1 degradation by the p62/SQSTM1-dependent autophagy–lysosome pathway in HNSCC. International Journal of Oral Science. 2020;12(1):1-11.

64. Winning L, El Karim IA, Lundy FT. A comparative analysis of the osteogenic potential of dental mesenchymal stem cells. Stem cells and development. 2019;28(15):1050-8.

65. Xiong Y, Fang Y, Qian Y, Liu Y, Yang X, Huang H, et al. Wnt production in dental epithelium is crucial for tooth differentiation. Journal of dental research. 2019;98(5):580-8.

66. Schröder A, Bauer K, Spanier G, Proff P, Wolf M, Kirschneck C. Expression kinetics of human periodontal ligament fibroblasts in the early phases of orthodontic tooth movement. Journal of Orofacial Orthopedics/Fortschritte der Kieferorthopädie. 2018;79(5):337-51.

67. Andersson K, Dahllöf G, Lindahl K, Kindmark A, Grigelioniene G, Åström E, et al. Mutations in COL1A1 and COL1A2 and dental aberrations in children and adolescents with osteogenesis imperfecta–a retrospective cohort study. PLoS One. 2017;12(5):e0176466.

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