Gene Expression in Human Dental Pulp Cells of Mandibular Second Premolar Teeth

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Abstract

Genes and signaling of the teeth are conserved during evolution and these similar gene networks regulate the development of other organs. The tooth has been an excellent model to study molecular mechanisms of organs, signaling networks, cellular heterogeneity, and adult tissue renewal. Human dental pulp cells (hDPCs) are pluripotent with a high capacity for differentiation and regeneration of dentin/pulp-like complex and extraoral tissues. To discover molecular characteristics of adult hDPSC, we performed a transcriptome analysis of hDPCs obtained from the premolar teeth to reveal gene expression profile, signaling pathways, and expression of odontogenesis genes. hDPCs of mandibular second premolar from two adult donors were isolated, cultured, and subjected for RNA sequencing. RNA-Seq Alignment, RNA-Seq Differential Expression, and Reactome program were used to analyze gene expression profile and pathways. The RNA sequencing demonstrated that 17,968 genes out of 27,914 in total were expressed in adult premolar teeth. The *FN1, COL1A2, ACTB, COL1A1, EEF1A1* were the top expression genes. The extracellular matrix pathway was the most involving pathway. The important genes for odontogenesis such as *BMP2, BMP4, MSX1, MSX2, TBX2* were still expressed in mature adult human teeth while *FGF3, FGF8, LHX7, ALX3, FOXI3* were not observed. We showed that the mature permanent teeth expressed tooth developmental genes especially those related to the extracellular matrix. Our findings provide new knowledge about RNA profiles and signaling networks in the permanent mandibular second premolar teeth.

Keywords**:** *Premolar tooth, RNA-Sequencing, RNA profile, Cellular behavior*

1. Introduction

Tooth development is controlled by complex reciprocal interactions between genes and cell signaling. During the initial steps of differentiation, the developing tooth shares similar regulatory pathways with other ectodermal organs. (Thesleff & Sharpe, 1997) Calcified tissues of the tooth comprise enamel, dentine, and cementum. The main component is the dentine which is produced from the odontoblasts. (Lumsden, 1988) Odontoblasts are differentiated from ectomesenchymal cells that possess a cranial neural crest origin. 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*. (Ramanathan, Srijaya, Sukumaran, Zain, & Kasim, 2018; Sun et al., 2013; Tummers, Thesleff, & Evolution, 2009; T. Wang, Xu, & communications, 2010)

Msx1 (Msh homeobox 1) and its main protein-protein interactor *Pax9* (paired box gene 9) are both transcription factors working together during odontogenesis. (Nakatomi et al., 2010) Msx1 and Pax9 mutation or deletion were associated with some oral phenotypes, including tooth agenesis. (Bonczek, Balcar, & Šerý, 2017) The transforming growth factor alpha (TGFa) regulates the transcription of genes, cell proliferation, differentiation, death, adhesion, migration, and positioning. Genetic variations in these genes can lead to tooth agenesis. (Phan et al., 2016; A. Vieira, Meira, Modesto, & Murray, 2004) Fibroblast growth factor (FGF) plays a significant role in craniofacial development and controls the balance among skeletal cell growth, differentiation, and apoptosis. (Xiong, Li, Cai, & Chen, 2017)

One of the most common congenitally missing teeth is the mandibular second premolar. (Rakhshan, 2015; Rølling & Poulsen, 2009) In the patients with second premolar missing, the primary second molars might be retained in some cases (Bergendal, 2008) while some cases required an intervention such as auto-transplant, (Bokelund, Andreasen, Christensen, & Kjær, 2013; Ok & Yilmaz, 2019), orthodontic treatment with space closure (Fines, Rebellato, & Saiar, 2003), or implants with an implant-supported restoration. (Borzabadi-Farahani, 2012; Eliášová, Marek, & Kamínek, 2014) Regenerative dentistry is a trending concept

that uses stem cells to restore tooth structure. For cell-based regeneration of damaged dental tissues using bioengineering strategies, it requires an understanding of molecular mechanisms and biological processes involved in tooth function and vitality (Volponi, Zaugg, Neves, Liu, & Sharpe, 2018)

This study investigated gene expression profiles of mandibular second premolars intending to reveal gene expression patterns of dental pulp cells by RNA-sequencing technique and demonstrate an expression of significant odontogenic genes (Cunha et al., 2020; Ramanathan et al., 2018) these findings could apply to dental tissue engineering and tackle clinical problems such as eruption disturbance and a tooth missing.

2. Objectives

To analyze gene expression profiles of dental pulp cells derived from mature mandibular second premolar teeth.

3. Materials and Methods

3.1 Cell isolation and culture

The research protocol was submitted for approval to the Human Ethics Committee, Faculty of Dentistry, Chulalongkorn University. Inform consents were obtained from participants. Teeth scheduling for extraction according to dental treatment plan were collected for cell isolation. The mandibular second premolar teeth obtained from 2 unrelated participants were employed. The data of teeth (tooth number, cusp, root) were pictured and recorded. Briefly, dental pulp tissues were gently removed and minced. Cell isolation was performed by explant protocol. Cells were maintained in Dulbecco's Modified Eagle's Medium (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Gibco), 2 mM L-glutamine (Invitrogen, Carlsbad, CA, USA), 100 Units/ml penicillin (Invitrogen), 100 µg/ml streptomycin (Invitrogen), and 250 ng/ml amphotericin B (Invitrogen) at 37°C in a humidified 5% CO_2 atmosphere. The culture medium was changed every 3 days. Cells from passage 4 were used for RNA isolation and analysis.

3.2 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 14 RNA (1 µg) were used for mRNA library preparation. The RNA samples were sent to Illumina Inc. to synthesize cDNA and performed following their protocols.

3.3 RNA-Seq analysis

RNA-Seq Analysis was carried out using RNA-Seq Alignment and RNA-Seq Differential Expression program (Illumina Inc.). Reactome program was used for pathway analysis.

4. Results and Discussion

4.1 Results

RNA-Seq Alignment program was used to analyzed raw data from 2 mandibular second premolar teeth for 2 rounds and the aligned data from the first round and second round were used simultaneously in differential analysis. RNA-Seq Differential Expression program was used for data analysis after alignment. Homo sapiens (UCSC hg19) was used as a reference genome. From total 27,914 genes, 9,946 genes were not expressed. From the remaining expressed 17,968 genes, top-20 genes with the most expression were shown in Table 1. MA-plot analysis was shown in Figure 1.

Table 1 list of 20 top-expression genes

Gene	Tooth		
	Sample 1	**Sample 2**	**Mean count**
1. COL1A2	347285.89	720341.07	533813.5
2. ACTB	360057.80	348383.84	354220.8
3. COL1A1	356972.07	341019.60	348995.8
4. EEF1A1	263796.29	366186.36	314991.3
5. VIM	289188.06	270188.50	279688.3
6. TGFBI	50263.65	411307.27	230785.5
7. ACTG1	211085.99	193315.21	202200.6/
8. FTH1	48299.71	350915.16	199607.4
9. THBS1	222719.50	163533.09	193126.3
10. GREM1	105511.83	267795.60	186653.7
11. PENK	26762.53	329865.62	178314.1
12. GAPDH	131272.26	216535.09	173903.7
13. IGFBP5	94387.68	250384.91	172386.3
14. COL6A3	118588.87	210611.13	164600.0
15. TIMP3	45765.42	253683.76	149724.6
16. FLNA	178216.23	108365.23	143290.7
17. FSTL1	80682.70	140907.00	110795.3
18. COL6A2	65960.32	129411.05	97685.7
19. COL5A2	58002.94	129550.85	93776.9

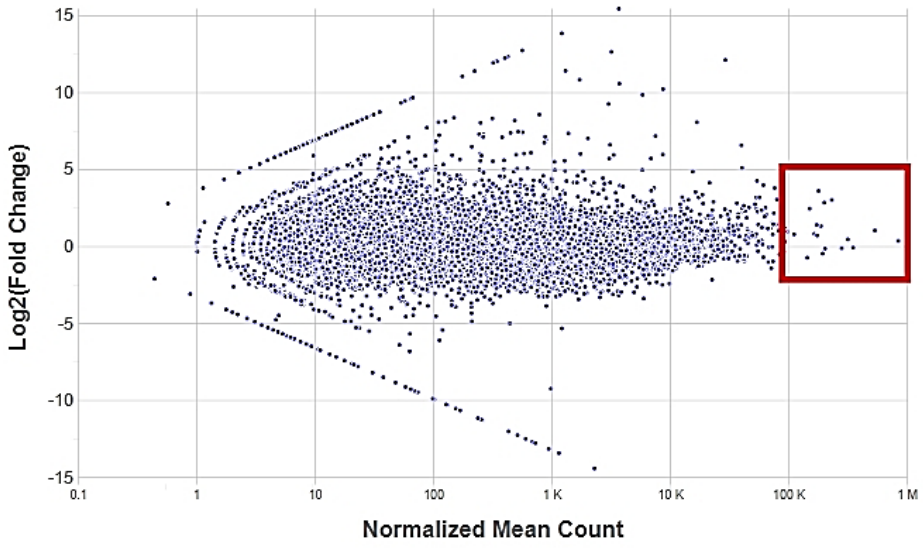

Figure 1 MA-plot provides an overall of the expression genes, with the log_2FC on the Y-axis and mean of normalized counts in X-axis (A red block shows top 20-ranked expressed genes)

From the top 20 genes expression, there were 8 major pathways involved according to the Reactome program analysis: 1) Extracellular matrix organization pathway, 2) Immune system, 3) Signal transduction, 4) Nervous system development, 5) Metabolism of proteins, 6) Hemostasis, 7) cell-cell communication, 8) vesicle-mediated transport (Figure 2, Table 2)

Table 2 Most related pathway from top 20 genes (Sort by p-value)

	Pathway	p-value	FDR*	Gene involvement
1.	Integrin cell surface interactions (E)**	2.67e-10	4.84e-08	*COL1A1, COL1A2, COL5A2, COL6A2, COL6A3, FN1, THBS1*
2.	Syndecan interactions (E)**	2.81e-09	2.53e-07	*COL1A1, COL1A2, COL5A2, FN1, THBS1*
3.	ECM proteoglycan (E)**	8.13e-09	4.88e-07	*COL1A1, COL1A2, COL5A2, COL6A2, COL6A3, FN1*
4.	Collagen chain trimerization(E)**	2.21e-08	9.96e-07	*COL1A1, COL1A2, COL5A2, COL6A2, COL6A3*
5.	Non-integrin membrane-ECM interactions (E)**	1.11e-07	3.99e-06	*COL1A1, COL1A2, COL5A2, FN1, THBS1*
6.	Assembly of collagen fibrils and other multimeric structures (E)**	1.76e-07	5.08e-06	*COL1A1, COL1A2, COL5A2, COL6A2, COL6A3*
7.	Collagen degradation (E)**	2.03e-07	5.08e-06	*COL1A1, COL1A2, COL5A2, COL6A2, COL6A3*
8.	Degradation of extracellular matrix (E)**	3.22e-07	6.53e-06	*COL1A1, COL1A2, COL5A2, COL6A2, COL6A3, FN1*
9.	Collagen biosynthesis and modifying enzymes (E)**	3.26e-07	6.53e-06	*COL1A1, COL1A2, COL5A2, COL6A2, COL6A3*
10.	MET activates PTK2 signaling	4.38e-07	7.89e-06	*COL1A1, COL1A2, COL5A2, FN1*
11.	Collagen formation (E)**	1.51e-06	2.10e-05	*COL1A1, COL1A2, COL5A2, COL6A2, COL6A3*
12.	Signaling by receptor Tyrosine kinases	1.56e-06	2.10e-05	*ACTB, ACTG1, COL1A1, COL1A2, COL5A2, COL6A2, COL6A3, FN1, THBS1*
13.	MET promotes cell motility	1.69e-06	2.10e-05	*COL1A1, COL1A2, COL5A2, FN1*
14.	GP1b-IX-V activation signaling	1.75e-06	2.10e-05	*COL1A1, COL1A2, FLNA*
15.	Extracellular matrix organization (E)**	2.22e-06	2.67e-05	*COL1A1, COL1A2, COL5A2, COL6A2, COL6A3, FN1, THBS1*
16.	Interleukin-4 and interleukin-13 signaling	2.85e-06	3.13e-05	*COL1A2, FN1, VIM*
17.	Cell-extracellular matrix interactions	6.88e-06	6.88e-05	*ACTB, ACTG1, FLNA*
18.	Platelet activation, signaling and aggregation	1.70e-05	1.70e-04	*COL1A1, COL1A2, FLNA, FN1, THBS1, TIMP3*
19.	Signaling by PDGF	2.13e-05	1.92e-04	*COL5A2, COL6A2, COL6A3, THBS1*
20.	Signaling by MET	2.33e-05	2.10e-04	*COL1A1, COL1A2, COL5A2, FN1*

*FDR = False Discovery Rate, **E = Extracellular matrix pathway

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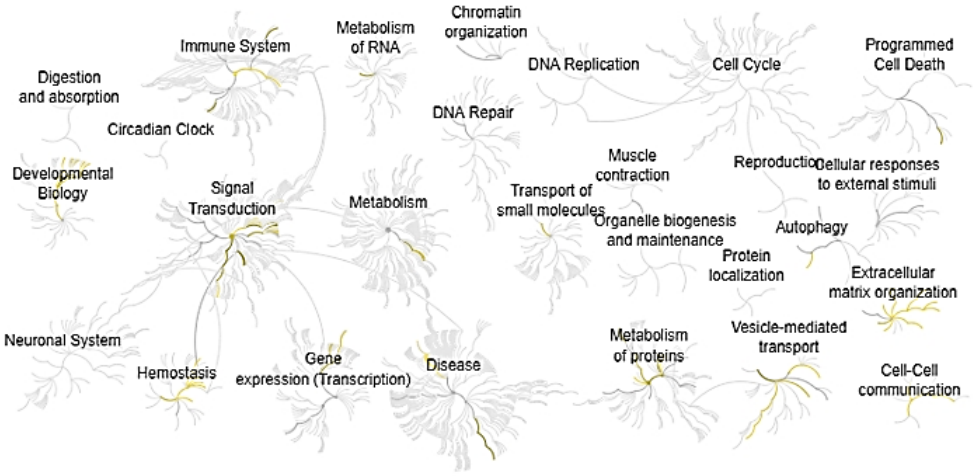

Figure 2 Significant pathways according to the top 20 expressed genes created by Reactome program. The related pathways are highlighted in yellow color.

Significant genes for odontogenesis

From the literature reviews, The genes that play an important role in odontogenesis consisting of *WNT6, WNT10A, WNT10B, PITX1, PITX2, FOXI3, LEF1, BMP2, BMP4, MSX1, MSX2, TBX2, PAX9, RUNX2, RUNX3, LHX6, LHX7, LHX8, BARX1, DLX1, DLX2, DLX3, DLX5, DLX6, ALX3, ALX4, TFGA, FGF3, FGF8, FGF10, FGF13, GLI2* and *GLI3* (Cunha et al., 2020; Ramanathan et al., 2018) were used for analysis. From 33 genes, 28 genes were expressed while 5 genes were not expressed in mature adult premolar teeth (Table 3, Figure 3).

4.2 Discussion

Tooth development is related to genetic and environmental factors. Genetics play a role in determining the shape, size, number and position of tooth. (Azzaldeen, Watted, Mai, Borbély, & Abu-Hussein, 2017; Cunha et al., 2020; Lee et al., 2012; Ramanathan et al., 2018) 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. In this study, we evaluate the gene expression profiles of the hDPCs of mandibular second premolar by RNA-sequencing technique and investigate the significant genes (*WNT6, WNT10A, WNT10B, PITX1, PITX2, FOXI3, LEF1, BMP2, BMP4, MSX1, MSX2, TBX2, PAX9, RUNX2, RUNX3, LHX6, LHX7, LHX8, BARX1, DLX1, DLX2, DLX3, DLX5, DLX6, ALX3, ALX4, TFGA, FGF3, FGF8, FGF10, FGF13, GLI2* and *GLI3*)(Cunha et al., 2020; Ramanathan et al., 2018) that play an important role in odontogenesis.

From 17,968 expressed genes, we targeted the top 20 expressed genes. Some genes are associated with tooth formation such as *FN1*, *COL1A1*, *COL1A2*, *TGFBI*, *IGFBP5*, and *TIMP3*. Fibronectin (*FN1*) showed a greater level in odontoblasts and the fibronectin protein is involving in the organization of extracellular matrix. (Kadler, Hill, & Canty-Laird, 2008) Furthermore, the studies in primary tooth buds showed that *FN1* was involved in cell movement via actin organization in cell cytoskeleton. (Hu, Parker, & Wright, 2015) The *FN1* is also a biomarker for head and neck squamous cell carcinoma because it has a strong association with EMT (Epithelial-mesenchymal transition) and tumor invasion/metastasis. (X. Liu et al., 2020)

Table 3 Expression of important odontogenic genes

Gene	Tooth		
	Sample 1	Sample 2	Mean count
1. *WNT6*	0.16	11.16	5.66
2. *WNT10A*	0.16	8.89	4.53
3. *WNT10B*	5.54	5.42	5.48
4. *PITX1*	38.20	313.35	175.77
5. *PITX2*	1.07	40.10	20.59
6. *FOXI3*	0	0	0
7. *LEF1*	507.46	506.12	506.79
8. *BMP2*	1189.62	9294.88	5242.25
9. *BMP4*	2327.84	3836.17	3082
10. *MSX1*	4167.62	4896.81	4532.21
11. *MSX2*	3395.72	3818.90	3607.31
12. *TBX2*	3778.70	4724.35	4251.52
13. *PAX9*	1049.14	464.40	756.77
14. *RUNX2*	1099.77	927.38	1013.57
15. *RUNX3*	1610.34	1453.59	1531.96
16. *LHX6*	9.92	47.69	28.80
17. *LHX7*	0	0	0
18. *LHX8*	949.28	694.47	821.88
19. *BARX1*	1045.63	2322.37	1684.00
20. *DLX1*	852.20	467.98	660.09
21. *DLX2*	418.82	158.11	288.47
22. *DLX3*	744.98	437.07	591.03
23. *DLX5*	2973.63	1772.74	2373.19
24. *DLX6*	556.31	388.40	472.36
25. *ALX3*	0	0	0
26. *ALX4*	66.05	79.58	72.81
27. *TGFA*	1.70	5.87	3.79
28. *FGF3*	0	0	0
29. *FGF8*	0	0	0
30. *FGF10*	2.69	27.92	15.30
31. *FGF13*	0.16	15.68	7.92
32. *GLI2*	566.44	456.07	511.26
33. *GLI3*	1102.78	1791.23	1447.00

COL1A2 encodes for alpha-2 type 1 collagen (the most common collagen type in humans) and plays role in collagen formation. (Schröder et al., 2018) *COL1A1* (collagen type 1, alpha 1) plays a crucial role in osteoblasts and odontoblasts' activities. (Winning, El Karim, & Lundy, 2019; Y. Xiong et al., 2019) Mutations in these genes (*COL1A1, COL1A2*) are associated with osteogenesis imperfecta and dentinogenesis imperfecta in humans. (Andersson et al., 2017)

TGFBI gene (transformed growth factor-beta-induced gene) encodes an extracellular matrix connective protein that can bind integrin to extracellular matrix protein and plays an essential role in embryonic development and many cell activities. (Thapa, Lee, & Kim, 2007) Many researchers have studied the relationship between overexpressed *TGFBI* and different kinds of tumors including oral squamous cell carcinoma. (B. Han et al., 2015; Ozawa et al., 2016; B.-j. Wang et al., 2019)

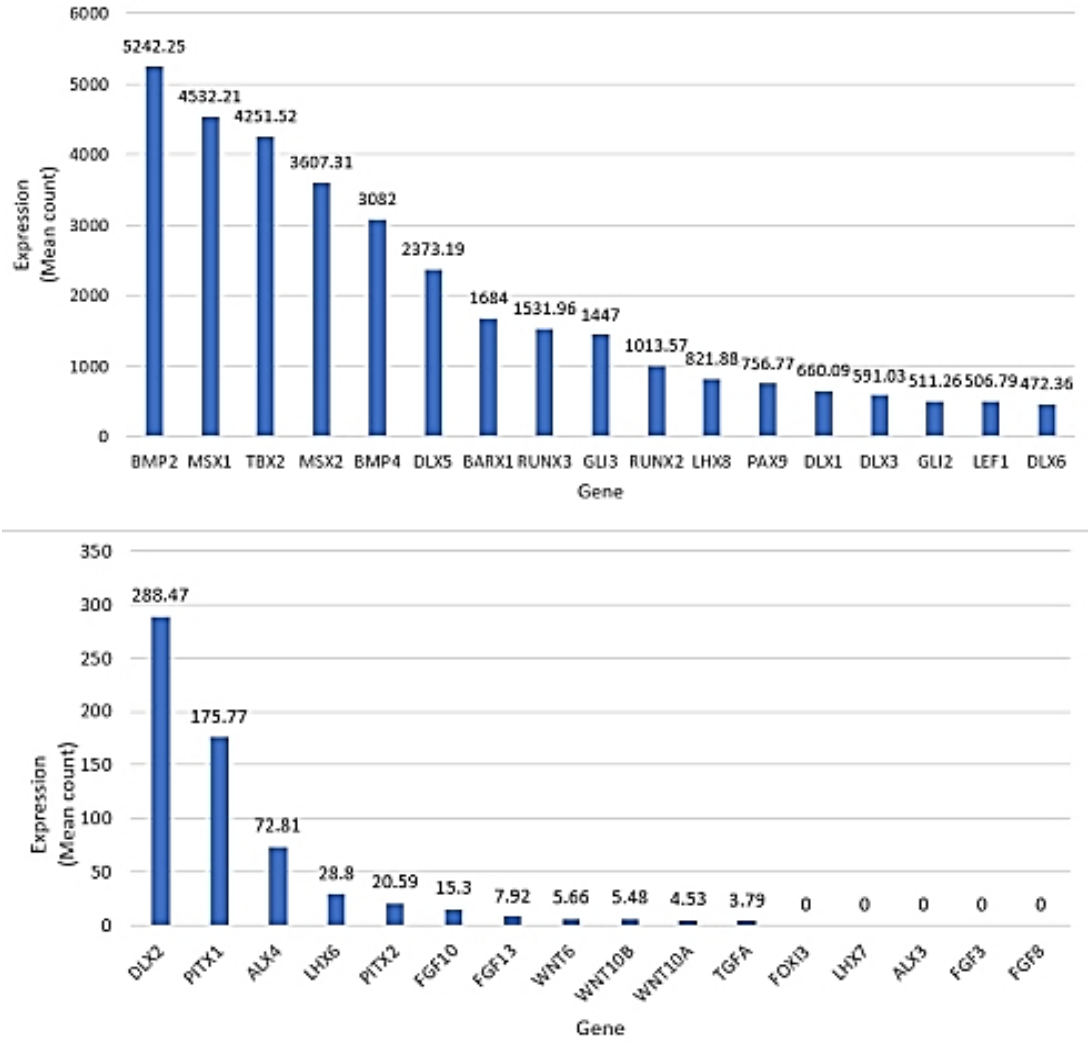

Figure 3 Bar charts show expression of selected genes, with the level of expression (mean count) on the Y-axis and the selected genes on X-axis

GREM1 is an inhibitor of osteogenesis (BMP antagonist). Inhibition of this gene results in an increased expression of *RUNX2*, *BSP*, *ALP*, and *OPN* (osteogenic markers) while overexpression of *GREM1* inhibited adipose-derived stem cells senescence and increased telomerase activity which had an anti-aging effect. (Liu et al., 2020) *IGFBP5* (Insulin-like growth factor binding protein 5), the member of IGFs family, has been shown to enhance the growth, remodeling, and repair of bone cells. In periodontitis patients, *IGFBP5* expression is significantly decreased in periodontal ligament and gingival crevicular fluid whereas *IGFBP5* overexpression could promote periodontal tissue regeneration and anti-inflammation of periodontal tissues. (Han et al., 2017; Liu et al., 2015)

TIMP3 is one member of tissue inhibitors of metalloproteinase (TIMPs). The balancing between TIMPs and MMPs regulates tissue remodeling, cell proliferation, cell cycle progression, and odontoblast/ameloblast differentiation. (Yoshiba et al., 2006; Yoshiba et al., 2003) *FLNA* gene, found in dental pulp, plays a role in cytoskeletal rearrangement in the differentiation of neural progenitors. (Gnanasegaran, Govindasamy, & Abu Kasim, 2016)

Expression of important genes for odontogenesis

WNTs and BMPs signaling pathways regulate the microenvironment and molecular cross-talk between stem cells in both humans and mice. (Mitsiadis & Graf, 2009) *WNT6* plays a significant role in human tooth development by promoting migration and differentiation of human dental pulp cells. (Li et al., 2014) *WNT10A* is related to cell-matrix interactions regulating odontoblast differentiation and cusp morphogenesis. (Yamashiro et al., 2007) *WNT10B* is particularly expressed in the dental epithelium. (Sarkar & Sharpe, 1999) *BMP2* and *BMP4* are expressed in odontoblasts and ameloblasts during embryonic tooth development until post-natal tooth cytodifferentiation. Deletion of *Bmp2* gene in early odontoblast affects molars and incisors, with thin dentin in a crown and reduced blood vessels/pericytes in dental pulp (Yang et al., 2012) while deletion of *BMP4* results in both odontogenesis and amelogenesis disrupted, with hypomineralized enamel and thin dentin. (Gluhak-Heinrich et al., 2010) In this study, *BMP2* and *Bmp4* have a high expression while *WNT6*, *WNT10A*, and *WNT10B* have a low expression. It is indicated that BMPs still have a role in normal dental pulp mature mandibular second premolar teeth more than WNTs.

FGF8 gene induces *BMP4* and *MSX1* expression in dental epithelium while *MSX1* triggers *BMP4* in the ectomesenchyme with a feedback loop. *BMP4* positive feedback loop can induce both *MSX1* and *MSX2*, on the other hand, *TBX2* negative feedback regulates *BMP4* and *MSX1* expression. (Saadi et al., 2013) This mechanism is crucial for the proliferation and determination of cranial neural crest cells until tooth differentiation. *MSX1* is expressed in the mesenchyme of all teeth from the dental placode stage to the bell stage in tooth development. *MSX2* expression is observed in both epithelial and mesenchymal compartments of all developing teeth. (Duverger & Morasso, 2008) From our results, *BMP4*, *TBX2*, *MSX1* and *MSX2* were still expressed in normal dental pulp cells of mature lower second premolars but *FGF8* was not expressed. Noticeably, *MSX1* and *MSX2* deletion resulted in a cessation of tooth development at the placode stage for both incisors and molars. (Bei, Stowell, & Maas, 2004; Duverger & Morasso, 2008) The missense mutation and frameshift insertion mutation of *MSX1* caused an absence of second premolars and third molars. (Abid, Simpson, Petridis, Cobourne, & Sharpe, 2017; Vastardis, Karimbux, Guthua, Seidman, & Seidman, 1996)

Distal-less (*DLX*) families are also related to tooth morphogenesis. They are expressed along proximo-distal axis of the branchial arch. The expression of *DLX1* and *DLX2* during tooth development are induced by *FGF8* and *BMP4*. At the molar area, *DLX1* and *DLX2* expression regulate the mesenchymal gene expression of both the maxillary and mandibular process but only *DLX2* is expressed in the distal epithelium of incisors area. (Suryadeva & Khan, 2015) The null mutation of both *Dlx1* and *Dlx2* in mice showed that the developing maxillary molars were arrest at epithelial thickening stage, however, the development of incisors and mandibular molars were still maintained. (Duverger & Morasso, 2008) At the early stage of tooth formation, *DLX5* and *DLX6* are expressed only proximal mesenchyme of mandibular process but later expressed in the mesenchyme of all teeth. (Duverger & Morasso, 2008) *DLX3* expressed in mesenchymal of developing tooth, inhibits proliferation of dental pulp cells through inactivation of WNTs signaling. (Zhan et al., 2018) The mutation of *DLX3* in humans affected the thickness and microhardness of enamel (amelogenesis imperfecta). (Kim et al., 2016)

PITX1 is expressed in mesenchyme and dental epithelium of developing incisors and molars throughout all stages of odontogenesis. It has synergistic interaction with *TBX1* and *BARX1*. Deletion of *Pitx1* gene in mice causing abnormal tooth morphology of mandibular molars. (Mitsiadis & Drouin, 2008) *PITX2* is a marker of tooth development. It is regulated positively by *FGF8* and negatively by *BMP4*. Axenfeld-Reiger syndrome, a condition with tooth hypoplasia and hypodontia, is associated with the mutation of *PITX2*. (Intarak et al., 2018) *LHX6* is induced by *PITX2* and *LHX6* has negative feedback to repress *PITX2*. (Zhang et al., 2013) *LHX6* and *LHX7* are expressed in the dental mesenchyme. *Lhx6* and *Lhx7* double mutant mice showed an absence of molar teeth but mutant mice with at least one allele of either *Lhx6* or *Lhx7* can develop molar teeth suggesting that two genes have an overlap function in odontogenesis. (Denaxa, Sharpe, & Pachnis, 2009)

PAX9, which is induced by *FGF8* and repressed by *BMP2/BMP4*, is the marker for determining the exact site for tooth germ appearances. (Hloušková et al., 2015) *GLI2* and *GLI3*, major component mediators of Sonic Hedgehog (Shh) signaling pathway, have also be related to tooth agenesis. (Vieira et al., 2013) FGFs family have important roles in craniofacial development but our results from mature teeth showed that FGFs had low expression (*FGF10*, *FGF13*) or were not expressed (*FGF3*, *FGF8*). *FGF3* is associated with upper

lateral incisors tooth formation. (Cunha et al., 2020) Deletion of *Fgf10* in mice causes a lack of cervical loop formation. (Harada et al., 2002)

The present results may promote further studies to investigate the gene expression in all tooth types (incisors, canine, premolar, molars) and the functions and molecular roles of genes and regulatory mechanisms in adult human dental pulp cells. Furthermore, these may advance knowledge about genetic engineering and stem-cell based therapy in regeneration medicine.

5. Conclusion

In conclusion, by applying RNA-seq to dental pulp cells of mandibular second premolar teeth, we identified and reported the top 20 expressed genes and the most significant involving pathways. Apart from these results, we also investigated the genes that have important roles in odontogenesis and showed that *BMP2, BMP4,* and *MSX1* were expressed whereas *FGF3, FGF8, LHX7, ALX3, and FOXI3* genes were not observed in the mature adult teeth.

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