Abstract. Although previous studies have demonstrated that dental pulp stem cells (DPSCs) from mature and immature teeth exhibit potential for multi-directional differentiation, the molecular and biological difference between the DPSCs from mature and immature permanent teeth has not been fully investigated. In the present study, 500 differentially expressed genes from dental pulp cells (DPCs) in mature and immature permanent teeth were obtained from the Gene Expression Omnibus online database. Based on bioinformatics analysis using the Database for Annotation, Visualization and Integrated Discovery, these genes were divided into a number of subgroups associated with immunity, inflammation and cell signaling. The results of the present study suggest that immune features, response to infection and cell signaling may be different in DPCs from mature and immature permanent teeth; furthermore, DPCs from immature permanent teeth may be more suitable for use in tissue engineering or stem cell therapy. The Online Mendelian Inheritance in Man database stated that Sonic Hedgehog (SHH), a differentially expressed gene in DPCs from mature and immature permanent teeth, serves a crucial role in the development of craniofacial tissues, including teeth, which further confirmed that SHH may cause DPCs from mature and immature permanent teeth to exhibit different biological characteristics. The Search Tool for the Retrieval of Interacting Genes/Proteins database revealed that SHH has functional protein associations with a number of other proteins, including Glioma-associated oncogene (GLI1), GLI2, growth arrest-specific protein 1, bone morphogenetic protein (BMP)2 and BMP4, in mice and humans. It was also demonstrated that SHH may interact with other genes to regulate the biological characteristics of DPCs. The results of the present study may provide a useful reference basis for selecting suitable DPSCs and molecules for the treatment of these cells to optimize features for tissue engineering or stem cell therapy. Quantitative polymerase chain reaction should be performed to confirm the differential expression of these genes prior to the beginning of a functional study.

Introduction

Dental pulp cells (DPCs) include unique mesenchymal stem cells called dental pulp stem cells (DPSCs). Previous studies have demonstrated that DPSCs have the potential for multi-directional differentiation into osteoblasts, odontoblasts, adipocytes, chondrocytes and neural-like cells in vitro and may be useful seed cells for tissue engineering or stem cell therapy in a number of disease conditions. DPSCs may be obtained easily from deciduous or permanent teeth that are discarded as medical waste. Previous studies have compared the difference between DPSCs from deciduous teeth and permanent teeth; however, differences between DPSCs from permanent teeth of immature and mature patients have not yet been described. A previous study established a number of DPC lines isolated from extracted wisdom teeth as a cell source for induced pluripotent stem (iPS) cells and the donor teeth of these DPC lines were divided into three developmental stages: Crown completed (CC), root forming (RF) and root completed (RC) (7). Teeth from 14-16 year old patients were defined as immature teeth (CC and RF), whereas teeth from 23-60 year old patients were defined as mature teeth (RC). The DPCs obtained from immature permanent teeth exhibited a much higher reprogramming potential compared with DPCs from mature permanent teeth (7). Furthermore, distal-less homeobox 4, a transcription factor that is highly expressed in DPCs from immature permanent teeth, significantly promoted human iPS cell generation as well as OCT3/4, sex-determining region Y box 2 and Kruppel-like factor 4 (7). These findings suggest that the biological characteristics of DPSCs differ in immature and mature permanent teeth. In the present study, the molecular characteristics of DPCs from immature and mature permanent teeth were compared based on bioinformatics analysis. The results of the present study may offer a basis for selecting a suitable preservation time for DPCs, as well as developing a suitable molecular intervention strategy for these cells to optimize features for tissue engineering or stem cell therapy.
Materials and methods

Use of the Gene Expression Omnibus (GEO) database to obtain differentially expressed genes in DPCs from mature and immature permanent teeth. Tamaoki et al (7) previously used an array to analyze gene expression profiles and investigate the effect of the developmental stage of teeth on global gene expression in human DPCs. The microarray data were deposited in the GEO online database (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE52853), the detailed information for which was described in a previous article and the online webpage (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE52853) (7). In the present study, differentially expressed genes in DPCs from mature and immature permanent teeth were acquired from the GEO database. The protocol was described as follows: i) Search 'dental pulp cells' in the GEO database (http://www.ncbi.nlm.nih.gov/gds/) to obtain the GSE52853 datasets (Title: Human Dental Pulp Cells Mature vs. Immature stages); ii) GEO2R analysis: Use GEO2R, one of GEO's own data analysis tools, to identify differentially expressed genes in DPCs from mature and immature permanent teeth. The default GEO2R statistical tests were applied. The expression value was stated with reference to that of mature permanent teeth and a moderated t-statistic was used to compare the expression of genes in DPCs from mature permanent teeth with those from immature permanent teeth. Genes that differed most significantly (P<0.001; logFC>1) were identified and were selected for further analysis (8). Some genes with P<0.001 and logFC>1 that serve a crucial role in tooth development were also included in the present study. In total, 500 differentially expressed genes were selected for further analysis.

Use of the Database for Annotation, Visualization and Integrated Discovery (DAVID) to evaluate 500 differentially expressed genes in DPCs from mature and immature permanent teeth. The DAVID bioinformatics database is an integrated online database that includes a number of powerful annotation resources, including Gene Ontology (GO), Biocarta and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases (9). In the present study, the DAVID bioinformatics database was used to analyze the protein associations, biological characteristics and functional characteristics of differentially expressed genes in DPCs from mature and immature permanent teeth. The protocol was as follows: i) Access DAVID (https://david.ncifcrf.gov/) and copy the 500 differentially expressed gene names into box A; ii) select the 'official gene symbol' as the gene identifier type and click the 'Submit List' button; iii) choose 'Homo sapiens' as annotations; and iv) select 'Start Analysis'. The modified Fisher's Exact test was used in the present study, and P<0.05 was considered to indicate a statistically significant difference.

Differentially expressed genes in DPCs from mature and immature permanent teeth may be divided into immunity, inflammation and cell signaling-associated biological process subgroups. Following DAVID analysis, the 500 differentially expressed genes in DPCs were divided into subgroups based on the GO term 'biological process'. The majority of these subgroups were separated into three groups, associated with immunity, inflammation and cell signaling. Genes in the immune-associated groups (Table I) were demonstrated to regulate lymphocyte activation and proliferation, antigen processing and negative regulation of immune system processes. A total of two genes, including cluster of differentiation 24 and SHH, existed in almost all subgroups. The 500 differentially expressed genes were enriched into different KEGG pathway subgroups based on DAVID analysis. A total of four genes, including γ-interferon-inducible lysosomal thiol reductase, human leukocyte antigen class II histocompatibility antigen (HLA)-DR β 1 chain, HLA DP β 1 chain and leukotriene A-4 hydrolase, were enriched into the 'Antigen processing and presentation' pathway (Fig. 1). Genes in the inflammation-associated groups (Table II) may act to regulate leukocyte migration, proliferation, activation and chemotaxis, mononuclear cell proliferation and the defense response. Genes in the cell signaling-associated groups (Table III)
regulate chemotaxis, cell-cell signaling transduction, cell surface receptor linked signal transduction and G-protein coupled receptor protein signaling pathways. These findings suggest that immune features, response to infection and signaling may be different in DPCs from mature and immature permanent teeth.

Table I. Differentially expressed genes in dental pulp cells from mature and immature permanent teeth were enriched into immunity associated subgroups based on ‘Biological Process’ terms analyzed using the Database for Annotation, Visualization and Integrated Discovery.

| Term                                   | Upregulated in IPT | Downregulated in IPT | Fold enrichment |
|----------------------------------------|--------------------|----------------------|-----------------|
| Regulation of lymphocyte activation    | CD24, LAG3         | INHBA, NCK2, CDKN1A, TNFRSF4, SHH | 3.677229        |
| Regulation of lymphocyte proliferation | CD24               | NCK2, CDKN1A, TNFRSF4, SHH | 4.683562        |
| Regulation of B cell activation        | CD24               | INHBA, CDKN1A, TNFRSF4 | 6.097814        |
| Negative regulation of lymphocyte activation | CD24, LAG3    | INHBA, SHH | 5.759046        |
| Antigen processing and presentation of peptide or polysaccharide antigen via MHC class II | CD24, CDKN1A, TNFRSF4 | IFI30, HLA-DRB5 | 8.330049        |
| Negative regulation of immune system process | CD24, LAG3  | INHBA, SHH | 7.067921        |

IPT, immature permanent teeth.

Figure 1. Differentially expressed genes (red stars) in dental pulp cells from mature and immature permanent teeth are enriched into an ‘antigen process and presentation pathway’ based on the Kyoto Encyclopedia of Genes and Genomes and analyzed by the Database for Annotation, Visualization and Integrated Discovery.

Genetic characteristics of SHH. The OMIM database analysis revealed that SHH is expressed in cellular projections, extracellular regions and plasma membranes and is associated with biological processes, including cell proliferation, cell differentiation and immune system processes. It was also demonstrated that, in the craniofacial region,
SHH mutation could lead to a number of deformities in mice (Table IV) or humans (Table V), including abnormal maxillary morphology, abnormal incisor morphology, tooth development arrest and single median maxillary incisor. These findings further confirm that SHH serves a crucial role in the development of teeth and may also cause DPCs from mature and immature permanent teeth to exhibit different biological characteristics.

Functional protein association networks of SHH. Based on STRING analysis, it was demonstrated that SHH has functional protein associations with a number of other proteins.
in mice and humans, including glioma-associated oncogene (GLI1), GLI2, growth arrest-specific protein 1 (GAS1), bone morphogenetic protein (BMP)2 and BMP4 (Fig. 2). These findings indicate that SHH may interact with other genes to regulate the biological characteristics of DPCs.

**Discussion**

Dental pulp tissues may be derived from migrating cranial neural crest cells during development; these tissues harbor various populations of multipotential stem cells and are useful cell resource for dental tissues, bone and neuron regeneration (1,11,12). DPSCs may be obtained from deciduous, inflamed deciduous, immature permanent or mature permanent teeth and the cells from each source may exhibit different biological characteristics (13). A previous study demonstrated that inflamed human dental pulp retains tissue regeneration potential (14). However, it has been suggested that inflamed DPSCs have reduced stemness and immunomodulatory properties and the expression of a number of molecules is different compared with non-inflamed DPSCs (15). This implies that inflamed DPSCs may not be a viable therapeutic cell source in cases of graft versus host disease and organ rejection (15). Previous findings have also demonstrated that DPSCs from deciduous teeth exhibit an upregulation in genes that participate in pathways associated with cell proliferation and the extracellular matrix including, fibroblast growth factor and tumor growth factor β (16-18). Although it has previously been reported that DPSCs from mature and immature teeth have the potential for multi-directional differentiation, the molecular and biological differences between DPSCs from mature and immature permanent teeth has not been fully

**Table IV. Craniofacial phenotypes in an SHH mutant mouse model.**

| Mouse genotype      | Mouse phenotypes                       |
|---------------------|----------------------------------------|
| Shh<sup>Dsh</sup>/Shh<sup>Dsh</sup> | AM                                     |
| Shh<sup>tm1Chg</sup>/Shh<sup>tm1Chg</sup> | ACM, ACBM, ANSM                        |
| Shh<sup>tm3(Cre)Chg</sup>/Shh<sup>tm3(Cre)Chg</sup> | ACM                                     |
| Shh<sup>Dsh</sup>/Shh<sup>+</sup> | ACMM, ANM, AFM, AIM, OBM, AMM          |
| Shh<sup>Hy</sup>/Shh<sup>+</sup> | CHP                                     |
| Shh<sup>tm1Amc</sup>/Shh<sup>tm2Amc</sup> | ACMM, AAP, AICM, ACMCM, SM, AABM, ATD, GRI, GRM, ADM, AEM, ANBM, APSF, PSH, CSP |
| Tg(KRT14-Cre)1Amc/0 (conditional) | AM                                      |
| Shh<sup>tm1Amc</sup>/Shh<sup>tm2Amc</sup> | ANM, AMSM, PSFM, CSP                   |

ACM, abnormal craniofacial morphology; ACBM, abnormal craniofacial bone morphology; ACMM, abnormal cranium morphology; ANM, abnormal neurocranium morphology; AFM, abnormal frontal bone morphology; AIM, abnormal intraparietal bone morphology; OBM, occipital bone foramen; AAP, absent alveolar process; AMM, abnormal maxilla morphology; AMSM, abnormal maxillary shelf morphology; AICM, abnormal incisor morphology; ACMCM, abnormal molar crown morphology; SM, small molars; AABM, abnormal ameloblast morphology; ATD, arrest of teeth development; GRI, growth retardation of incisor; GRM, growth retardation of molar; ADM, abnormal dentin morphology; AEM, abnormal enamel morphology; ANBM, abnormal nasal bone morphology; ANSM, abnormal nasal septum morphology; APSM, abnormal palate shelf fusion; PSFMM, palate shelf fail to meet at midline; PSH, palate shelf hypoplasia; CHP, cleft hard plate; CSP, cleft secondary palate; AM, absent mouth; SHH, Sonic hedgehog.

**Table V. Craniofacial phenotypes in patients with SHH-associated diseases.**

| Diseases associated with SHH | Human phenotypes                  |
|-----------------------------|-----------------------------------|
| Holoprosencephaly 3         | MF, MR, AN, PB, CC, HL            |
| Microphthalmia, isolated, with coloboma 5 | OC, AP, MP, BMP, IFC |
| Solitary median maxillary central incisor | CC, HL, AP, MP, SP, TULV, CUL, CP, TP, PMPP, SMMI, ARN, SN, CA, NNM, MS, ANB, AS, CB, ICB, MP |

MF, malar flattening; MR, midface retrusion; AN, abnormality of the nose; PB, proboscis; CC, cyclopia; HL, hypotelorism; OC, oral cleft; AP, anophthalmia; MP, microphthalmia; BMP, bilateral microphthalmia; IFC, irido-fundal coloboma; SP, short philtrum; TULV, tented upper lip vermilion; CUL, cleft upper lip; CP, cleft palate; TP, torus palatinus; PMPP, prominent median palate palate; SMMI, single median maxillary incisor; ARN, anteverted nares; SN, short nose; CA, choanal atresia; NNB, narrow nasal bridge; MS, midnasal stenosis; ABN, abnormality of the nasopharynx; AS, anosmia; CB, coloboma; ICB, iris coloboma; MP, microcephaly; SHH, Sonic hedgehog.
investigated. The results of the present study demonstrate that a number of differentially expressed genes in DPCs from mature and immature permanent teeth are associated with immunity, inflammation and cell signaling; furthermore, the findings herein suggest that immune features, response to infection and signaling may be different in DPCs from mature and immature permanent teeth. The differential expression patterns of genes also indicate that DPCs from immature permanent teeth may be more suitable for tissue engineering or stem cell therapy. In the present study, only one gene exhibited a statistically significant difference for the adjusted P (adj. P) value (adj. P < 0.05), suggesting that there may be some level of error. Therefore, the differentially expressed genes with a high logFC value should be focused on, and quantitative polymerase chain reaction (qPCR) should be performed to confirm differential expression prior to starting a functional study.

SHH, a mammalian homologue of the Drosophila secreted morphogen hedgehog, serves crucial role in the development of many tissues (19). Previous studies have demonstrated that SHH is expressed and serves key roles during all stages of tooth development, including tooth initiation, patterning and eruption (20-22). SHH mutation results in retarded grown, teeth malformation and mispatterned teeth in mice. The results of the present study demonstrate that SHH, which is differentially expressed in DPCs from mature and immature permanent teeth, is associated with many subgroups based on biological process GO terms. OMIM database analysis indicated that SHH serves a crucial role in the development of craniofacial tissues, suggesting that the differential expression of SHH may be responsible for the biological differences in DPCs from mature and immature permanent teeth. Previous studies have also reported that SHH protein could bind to Patched to release Smoothened to activate the downstream signaling cascade (23). GLI1-3, Huntington-interacting protein and GAS1 serve crucial roles in regulating the SHH pathway (24-26). In the present study, STRING database analysis revealed that SHH has functional protein associations with a number of other proteins, including GLI1, GLI2, GAS1, BMP2 and BMP4 in mice and humans. This suggests that SHH may interact with other genes to regulate the biological characteristics of DPCs.

In conclusion, 500 differentially expressed genes were identified in DPCs from mature and immature permanent teeth using the GEO online database. Based on bioinformatics analysis using the DAVID database, these genes were enriched into a number of subgroups associated with immune features, inflammation and cell signaling. These findings suggest that immune features, response to infection and signaling pathways may differ between DPCs from mature and immature permanent teeth and that DPCs from immature permanent teeth may be more suitable for tissue engineering or stem cell therapy. OMIM database analysis demonstrated that SHH serves crucial role in the development of craniofacial tissues, including teeth, and may be responsible for the biological differences in DPCs from mature and immature permanent teeth. The results of the present study may provide a basis for future functional experiments, including selecting suitable DPSCs for use in tissue engineering and stem cell therapy, as well as selecting intervention molecules to optimize the features of these tissues. qPCR may be required to confirm the differential expression prior to the launch of a functional study.

Competing interests

The authors declare that they have no competing interests.

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