An atlas of human dental pulp cells in multiple spatial and temporal levels based on single-cell sequencing analysis

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An atlas of human dental pulp cells in multiple spatial and temporal levels based on single-cell sequencing analysis

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Abstract

Background

The dental pulp plays a crucial role in the long-term maintenance of teeth function. The progress of endodontic treatment and pulp tissue regeneration engineering has therapeutic potential. But regulation mechanisms of tooth development or regeneration dependent on dental stem cells were still not fully understood. So, it is urgently needed bridge the gaps between basic and clinical research. With single cell sequencing technology being applied in medical research, landscapes of human dental pulp cells had been initially outlined. However, the specific cellular heterogeneity of dental pulp cells, especially dental stem cells in different spatial and temporal levels are remain unclear.

Methods

Gene expression profiles of human dental pulp cells from four teeth with different developmental periods analyzed by 10x Genomics single-cell RNA sequencing were studied and cluster analysis. Multiple spatial and temporal characteristics of subpopulation of cells were further studied by signal pathway analysis, trajectory analysis stemness analysis.

Results

In this study, transcriptomic expression-based clustering analysis showed that dental pulp cells at different developmental points exist differences in number, not cell types. Pathway analysis revealed that early in development, the pulp may perform more developmentally relevant functions such as synthetic translation, while later it begins to differentiate into specific directions or retains functional characteristics for homeostasis maintenance. The importance of the stem cell microenvironment in human tooth development was evident. Subpopulations in early development stage were clearly identified in fibroblasts, odontoblasts and mesenchymal stem cells. Particularly, differentially expressed gene analysis and stemness analysis defined three subpopulations of dental pulp stem cells with greater stemness and potential for

multiple differentiation. Combining the expression characteristics of the three cell subpopulations, some genes such as MIA, DGKI and so on that may contribute to the developmental differentiation of dental pulp stem cells were also uncovered

Conclusion

For the first time, the specific cellular heterogeneity of dental pulp cells, especially dental stem cells in different spatial and temporal level was proviede. Gene expression profile of early developing cells may help in cell screening for regenerative engineering and improve the success of dental pulp regeneration.

Key words:

Tooth development Single-cell RNA sequencing Spatial and temporal level Dental stem cells

Background：

The dental pulp being encapsulated in hard tissues plays an irreplaceable role in tooth formation, resistance to infection or trauma, and restoration of dentin. Previous studies have established a relatively clear framework of knowledge on pulp development, histopathology and function. Nevertheless, there are still no systematic and consistent conclusions regarding the development of tooth. Pulp revascularization and pulp regeneration are now being used in clinical practice based on research in pulp tissue engineering to maintain living pulp [1-3]. Seed cells, the key to pulp tissue engineering, are derived from diverse dental stem cells. Stem cells of dental origin contain human dental pulp stem cells (DPSC), stem cell from root apical papilla (SCAP), stem cells from human exfoliated deciduous (SHED) and so on[4-6]. These stem cells are named after the source of origin, and express an immunophenotype similar to human bone marrow stromal cells (BMSCs) in vitro rather than hematopoietic stem cells even though they localized in the microvasculature in vivo [7,8]. Some studies have also screened heterogeneous populations of dental pulp stem cells by surface markers such as CD146 and CD90 [9,10] and demonstrated their proliferative capacity and enhanced differentiated potential. However, the status and the role of human dental stem cells in vivo was still not fully characterized in current studies. The specific regulatory mechanism of pulp development and the heterogeneity of stem cells are remained to be clarified.

Recently, several studies have introduced single-cell RNA sequencing into human oral field and have elaborated the composition of human dental pulp cells in terms of gene expression [11-13]. Genes to identify different dental cell types were calculated by bioinformatics analysis, but it is challenging to decipher the similarities and differences of cell populations across various spatial and temporal and the role of intercellular communication.

In this study, we dissected information about dental pulp cells from a multiple-sample single-cell sequencing result consisted of four teeth, which one of them was collected from dental clinic and the other three were from GEO database. We compared the similarities and differences of pulp cell composition, number, function and cellular

communication in different samples. In particular, we performed detailed analysis and interpretation of fibroblasts, odontoblasts and mesenchymal stem cells (MSCs), and disclosed some intracellular variability within those cell populations. In particular, potential primitive stem cells and actively differentiated stem cell populations were identified, which would assist further explorations of dental pulp development and regeneration in the future.

Methods：

Sample collection & database source

The human teeth using in our research were extracted for orthodontic or other clinically needs in the Emergency Department of Peking University Hospital of Stomatology in 2020 and 2021. The teeth were all third molars in immature stages. This study was approved by the Biomedical Ethics Committee of Peking University School of Stomatology (PKUSSIRB-202060197). We also selected 3 samples' raw reads from Public database (GSM4365609, GSM4365610 and GSM4998458). Detailed information is detailed in Table supplement 1.

Single-cell suspension preparation

An immature pre-eruptive tooth germ was extracted from a 13-year-old boy and transported to laboratory in 4℃ α-MEM with 10% penicillin-streptomycin solution immediately. Soft dental pulp tissue was harvested and washed in phosphate-buffered saline (PBS) for 3 times following enamel and dentin was broken by sterilized pliers in super clean bench. Subsequently, the pulp tissue was shred into small pieces (<1 mm^3) by ophthalmic scissors and centrifuged at 4℃, 1200 rpm for 5 min. The precipitation was digested for 40 min in 3mg/ml Type 1 Collagenase (Sigma-Aldrich, USA) at 37℃, under agitation at 300 rpm in oscillation box, and blew by pipette gun 8-10 times every 8-10 min. The digestion was stopped by 2% FBS (ABW, Uruguay) and then filtered through a 40 μm cell strainer. The filtrate was centrifuged at 4℃, 1350 rpm, 10 min for 3 times and the supernatant was removed. Then, the pellet was re-suspended in PBS including 2% FBS and re-sieved through a 40 μm cell strainer.

Single-cell RNA sequencing

In order to improve cellular activity, we used 1×Red Blood Cell Lysis Solution at 4℃ to remove blood cells and flow cytometry sorting to lysis dead cells. We used the Chromium Single Cell 3′ Reagent Kits v3 to constructed cDNA library according to the manufacturer's protocols and sequenced on the Illumina Nova seq 6000 system in PE150Nova mode. The used sequencing volume was about 15Mb/cell. Cell quality control, detection, reference genome comparison, and gene expression matrix generation for cells were completed using Cell Ranger 4.0.0, the official analysis software of 10x Genomics. All sequencing work was done in cooperation with Beijing MicroRead Genetics Co., Ltd. (Beijing, China).

Quality control and analysis

Raw reads were processed with fastQC and fastp for removing low-quality reads. Poly-A tails and adaptor sequences were removed by cutadapt. Following quality control, reads were plotted to the reference genome GRCh38 (ensembl version 92 annotation) using STAR. Gene counts and UMI counts were obtained by featureCounts software. Expression matrix files for subsequent analyses were generated from gene counts and UMI counts. And the batch effect between our sequencing sample data and data from public database was removed by Harmony 9.

Cells were screened by gene count in the range of 300 and 7000 and UMI count under 30,000. Cells containing more than 10% mitochondrial content were excluded. We used Seurat v2.3 [14]for dimension-reduction and clustering. Gene expressions were normalized and scaled using NormalizeData and ScaleData. The top 2000 variable genes were picked by FindVariableFeautres for PCA analysis. Cells were grouped into clusters by FindClusters using the first 20 principle components and a resolution parameter of 1.0. For subclustering of various cell types, we set the resolution to 1.2. The UMAP algorithm was applied to perform two-dimensional visualization of cells. And Seurat FindMarkers selected genes as differentially expressed genes (DEGs) that were expressed in more than 10% of cells in a cluster with a mean fold change greater than 0.25 based on the Wilcox likelihood ratio test and default parameters. The cell type status of each cluster was determined based on the expression of typical markers found in DEGs, in conjunction with knowledge of the relevant literature. All data analysis work was done in cooperation with Singleron Biotechnologies (Nanjing, China)

Pathway enrichment analysis

To investigate the potential functions of different cell types, the Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis were performed with the "clusterProfiler" R package v4.0.2 [15]. Pathways with p_adj value less than 0.05 were deemed to be significantly enriched. For gene set variation analysis (GSVA) pathway enrichment analysis, the mean gene expression of each cell type was included as input data using the GSVA package v1.34.0 [16]. Protein-protein interactions (PPI) of DEGs were predicted based on known interactions of genes with relevant GO terms in the StringDB (1.22.0) [17].

Trajectory analysis

Pseudotime trajectory analysis was performed with Monocle2 [18]. To construct the trajectory, top 2000 highly variable genes were selected by Seurat v2.3 FindVairableFeatures, and dimensionality reduction was performed by DDRTree. The trajectory was visualized by plot_cell_trajectory.

CellPhoneDB

Cell-cell interaction (CCI) were estimated using Cellphone DB[19] version based on known ligand–receptor pairs. The number of reciprocals used to calculate the null distribution of the average expression of ligand-receptor pairs in random cell identities was set to 1000. Thresholds for individual ligand or receptor expression were determined based on the average log gene expression distribution of all genes in each

cell type. Predicted interaction pairs with p value < 0.05 and mean log expression > 0.1 were considered significant and visualized by circlize (0.4.10) R package.

Single-cell regulatory network inference and clustering (SCENIC) analysis
We used pyscenic (version 0.11.0) to conduct single-cell regulatory network analysis. The analysis was performed according to the protocol described in SCENIC working process [20]. The 'pyscenic grn' function was first used to generate co-expression gene regulatory networks by using the 'grnboost2' method. The AUCell analysis was further performed using 'pyscenic aucell' function with parameters 'rank_threshold' 5000, 'auc_threshold' 0.05 and 'nes_threshold' 3.

Single-cell entropy analysis
SLICE (version 0.99.0) [21] was used to assess the stemness of cells by gene expression entropy based on single-cell expression profiles. After removing ERCC spike-ins and ribosomal genes, a SLICE object was created to perform bootstrap calculation of single-cell gene entropy values by getEntropy function.

Sample preparation for histological evaluation
For histological evaluation, the molars or dental pulp from molars were fixed in 4% paraformaldehyde for 24 hours. Tooth with crown was decalcified in 17% EDTA solution for 1 years at 37℃. Then all samples were dehydrated in graded ethanol, embedded in paraffin and cut into sections for 5 μm.

Immunohistochemistry
Paraffin sections should be dewaxed by a series of ethanol solutions (100% to 70%) and washed with distilled water and PBS again. Antigen retrieval was carried out by heating at 70℃ in EDTA buffer pH 9 for 20 min. After cooling to room temperature, sections were washed in PBS solution 3 times and incubated in 3% hydrogen peroxide for 20 min to remove endogenous peroxidase. Then, 10% goat serum (ZSGB-BIO, China) was used for 20 min to avoid nonspecific reaction. The primary antibodies were incubated at 4℃ overnight. The specimens were incubated with biotin-conjugated IgG and horseradish peroxidase-conjugated streptavidin (ZSGB-BIO, China) for 20 min respectively next day. The sections then were visualized using 3-3-diaminobenzidine-tetrahydrochloride (ZSGB-BIO, China) and washed in distilled water. Finally, hematoxyline staining was added for nuclear staining. All sections were observed under the light microscope (Olympus, Japan).
The following antibodies were used in our study: DMP1 (1:50, Bioss, USA), CD146/MCAM (1: 200, Abcam, USA), CD90 (1:200, Abcam, USA), CD24 (1: 50, Santa cruz, USA).

Results：

Landscapes of multiple human dental pulps
We collected an immature third molar at the stage of early root development (shorter

than one third of the root completed) from a 13-year-old child and marked as Youth pulp 1 (Figure 1A, Table supplement.1). This pulp tissue was sequenced via 10X Genomics chromium platform, from which 12,620 cells were obtained. To explore the spatial and temporal characterization of genes, three set of single-cell RNA sequencing data (One sample from apical papilla of tooth with 2/3 root completed marked as Youth pulp 2 and two samples from pulp of mature teeth marked as Adult pulp 1&2) were also introduced into our study from the GEO public database (Figure 1A, Table supplement.1). After removal of batch effects and homogenization, we combined SingleR and the previous studies [11,12,22] to define all pulp cells into 8 diverse types (Figure 1B, E, and Table supplement.2). Where primarily, we used COL1A1 and DCN to note fibroblasts, DMP1 to note odontoblasts and MSCs were defined by THY1 and ACTA2. In terms of cell types, the four pulp tissues did not differ from each other (Figure supplement 1).

However, from the perspective of cell quantity, the proportions of various cells were distinct (Figure 1D, Table supplement.3). We harvested 46,428 cells in total. Fibroblasts and endothelial cells (ECs) were always the chief cell populations in each sample while heterogeneity of cell quantities occurred in other cell types. It was interesting that Youth pulp 1 tissue had more MPs and lymphocytes than MSCs. Regarded as a developing tissue, Youth pulp 2 had more MSCs. We also observed that in two adult mature teeth, cell composition varied. One got more ECs and glial cells, the other was relatively more represented on MSCs and ECs. Meanwhile, Odontoblasts, which closely related to dentin formation, accounted for a very small part.

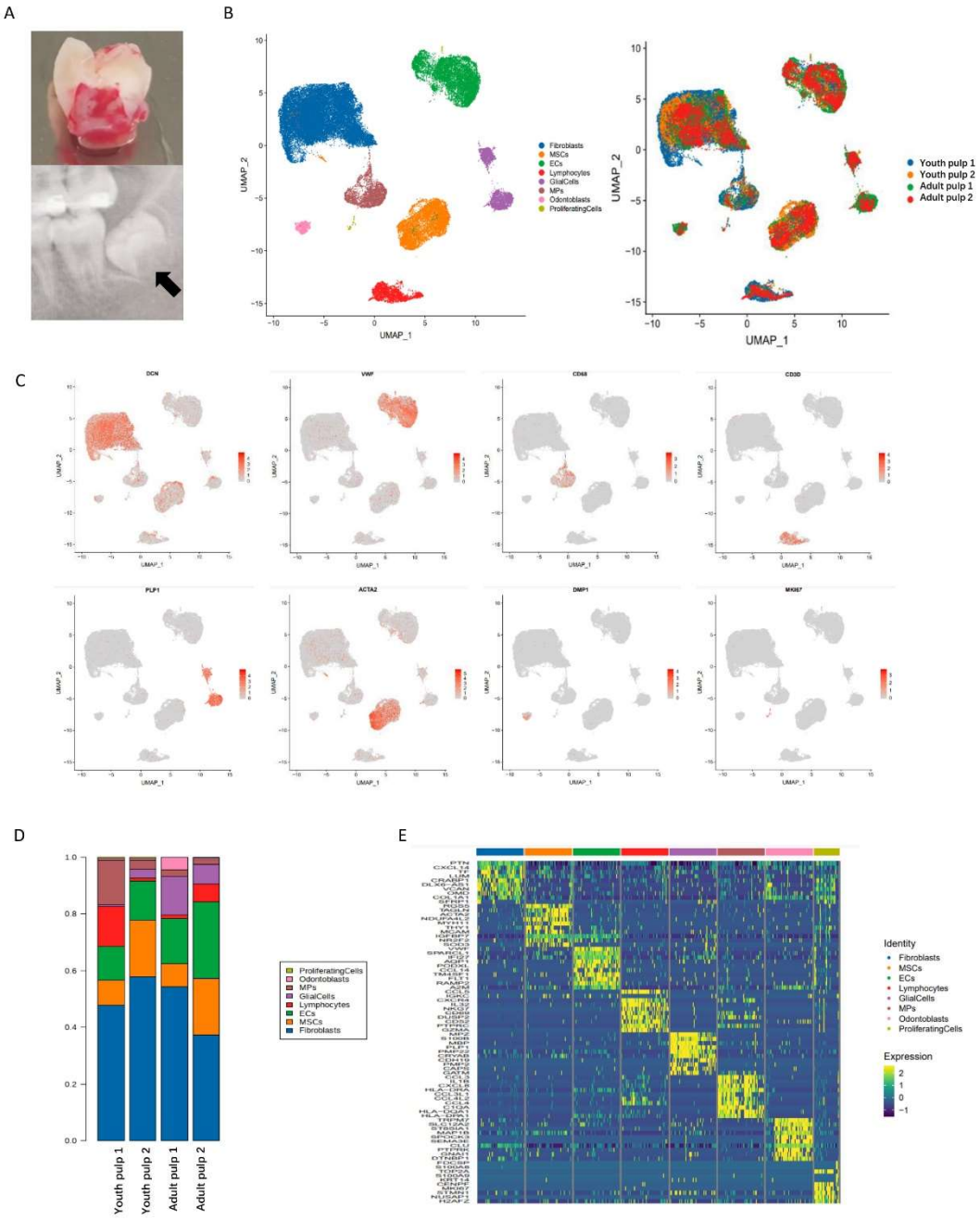

Figure 1. Landscape of multiple human dental pulp. A) Longitudinal section view and radiograph of collected tooth sample. B) Human pulp cell types and four sample distributions visualized in UMAP plots. C) Feature plots of unique marker genes in various cell clusters. D) Relative proportion of 8 cell types in four pulp tissues. E) Heatmap of the most identically expressed genes among each clusters.

Transcriptional differences and intercellular communications of human dental pulp cells in multiple spatial and temporal levels

Different cellular component means that there will be different molecular function and biological progress. We compared transcriptome expression differences cross the four samples and especially focused on comparing the pre-eruptive Youth pulp 1 with Youth pulp 2 and adult pulps. We can see that the DEGs in this sample are mainly enriched in the cellular component of ribosome and participated in translational initiation, protein localization and targeting (Figure 2A). It may also play a role in immune system such as “chemokine activity” and “MHC class II receptor activity”. The pulp produces collagen and protein for growth through transcription and translation prior to eruption, and the immune system begins to build early. The specific genes of Youth pulp 2 are more involved in organization of extracellular structure, osteoblast differentiation and ossification, showing it begins to or has differentiated into specific directions. And we find these genes relate to molecular function of binding with growth factor, integrin, and collagen (Figure 2B, supplement 2A). These should be linked to the completion of the development of the tooth root. Compared with the Youth pulp 1, adult pulps retain intercellular connections and adhesions (Figure 2B, supplement 2B). For mature adult pulp, homeostasis maintenance seems more important.

Cellphone DB was used to uncover the intercellular communications[19]. There are more different pulp cell types involved in Youth pulp 1, especially Fibroblasts, odontoblasts and MSCs (Figure 2C, D, supplement 2C). We then made diagram to reveal the ligand-receptor pair between different cell types (Figure 2E, F, and G). The related pathways of NOTCH 3 and BMP8A are specifically expressed in MSCs intercellular communication in Youth pulp 1, and in fibroblast-odontoblast crosstalk, TTR/AGER was significantly expressed. For Youth pulp 2, there are more appearances of NOTCH related pathways among ECs, MSCs, and Proliferating cells (Figure 2F). FGF1 and FGF2 also frequently appear. As for adult pulps, odontoblasts are observed actively in Adult pulp1 cell’s communication, while in the Adult pulp2, NOTCH/DLL-mediated signals frequently appeared in the crosstalk between ECs and MSCs. Both types of cells will still be active again after maturation. And members of chemokines and TNF-mediated pathways are positive signals in all four samples.

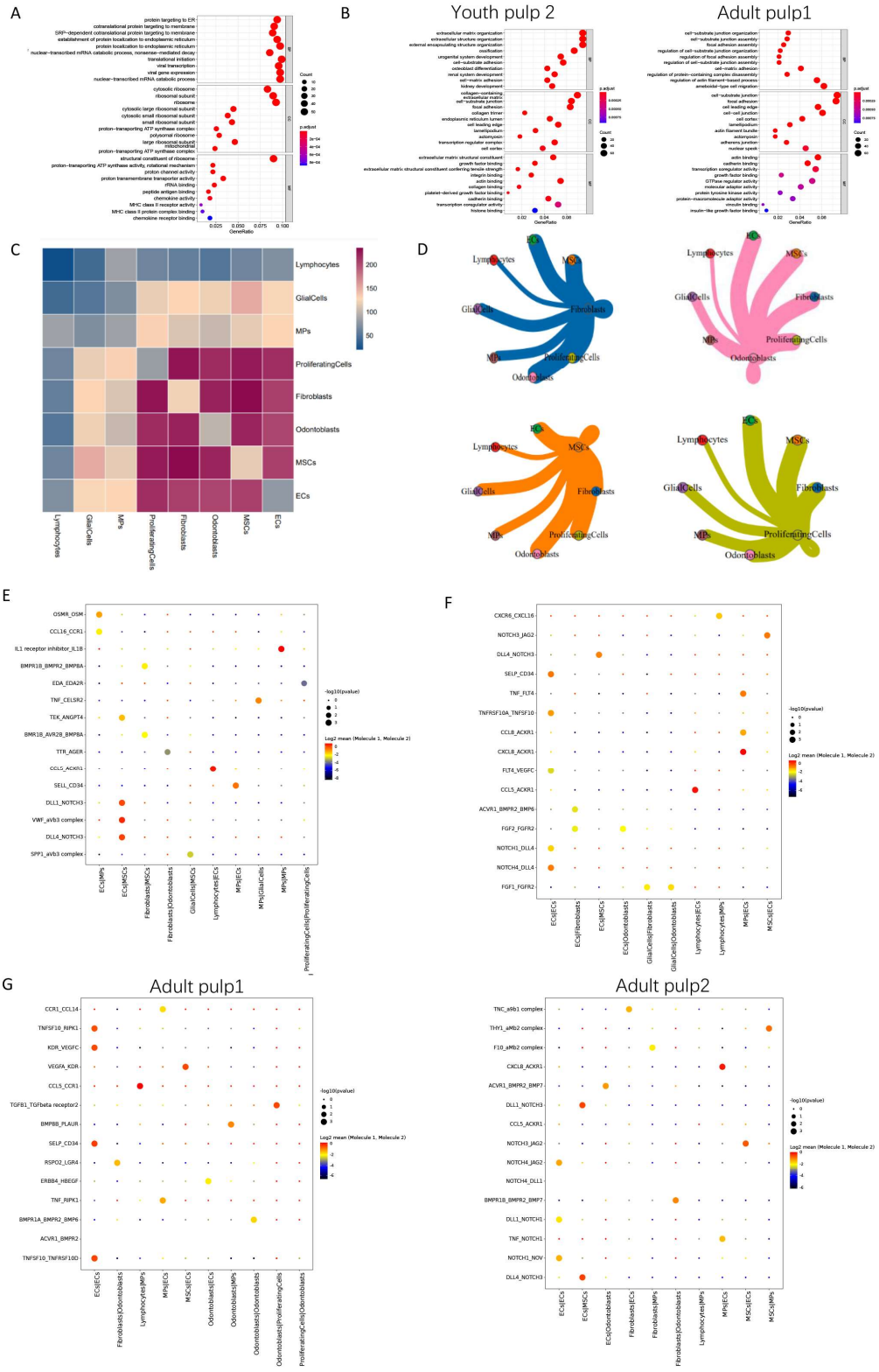

Figure 2. Transcriptional differences and intercellular communications of human dental pulp cells. A) Gene ontology pathway dotplot of Youth pulp 1. B) Gene pathway dotplot ontology pathway of Youth pulp 2 and Adult pulp 1. C) Heatmap of Youth pulp 1 demonstrating the number of pairs of the interaction between two cell types. D) Shell diagram of interactions between a single ligand cell and other receptor cells in Youth pulp 1, where network edge thicknesses are ligands and total number of receptor pairs. E) The top 15 pairs with significant differences in

interactions between different cells of Youth pulp 1. F) The top 15 pairs with significant differences in interactions between different cells of Youth pulp 2. G) The top 15 pairs with significant differences in interactions between different cells of two adult pulps.

Subpopulation characteristics of fibroblasts in multiple spatial and temporal

Fibroblasts forming the main part of the dental pulp are also known as pulp cells [23], consistently accounting for more than half of the population. After re-clustering the fibroblasts to show the cellular heterogeneity, we got five sub-clusters (Figure 3A). Almost all Fibro1 cells are distributed in the Youth pulp 1, which are identified by the gene of MIA and some gene related with ribosomal proteins, such as RPL17, EEF1G. Cluster 4 and 5 account for a large proportion of Youth pulp 2 and Adult pulp 2, respectively (Figure 3B, C). Cluster 4 exhibited a significant upregulation of gene DGKI and FBN2. And DAPL1, ribosome and ATP synthase genes (MT-RNR2, ATP5E, et al) located in mitochondrial are enriched in cluster 5. In cluster 2 and 3, we find high expression of IGFBP5, POSTN and EFNB2 (Figure 3C, D).

Exploring potential functional differences among subgroups used the Gene Set Variation Analysis (GSVA)[16]. We found that the cluster 2-4 may have more pathways related to “synaptic” and “neuropeptide receptor activity”, while cluster 1 own more about ribonuclease and RNA polymerase III complex, showing enrichment for pathways including various hydrolase activities, S100 protein binding and protein deneddylation. These pathways were also enriched in cluster 5 to some extent, but specifically shows the pathways involved in apoptotic process through peptidase activator activity and cysteine−type endopeptidase activity (Figure 3E).

We predicted Fibroblasts’ changes over time by constructing intercellular change trajectories (Figure 3F). The four samples distributed in different positions of the time trajectory, while Youth pulp 1 which is enriched of Fibro-1 located at the beginning of the proposed time. The two adult mature samples are indeed located at the end of two branches (Figure 3G). But in the cluster 2-5, there is no clear developmental sequence of pulp fibroblasts, maybe rather differences in function. (Figure supplement 3A). The expression levels of NBL1 and DHRS3 showed opposite trends in the proposed temporal trajectory, while IFI6 decreased and then increased (Figure 3H).

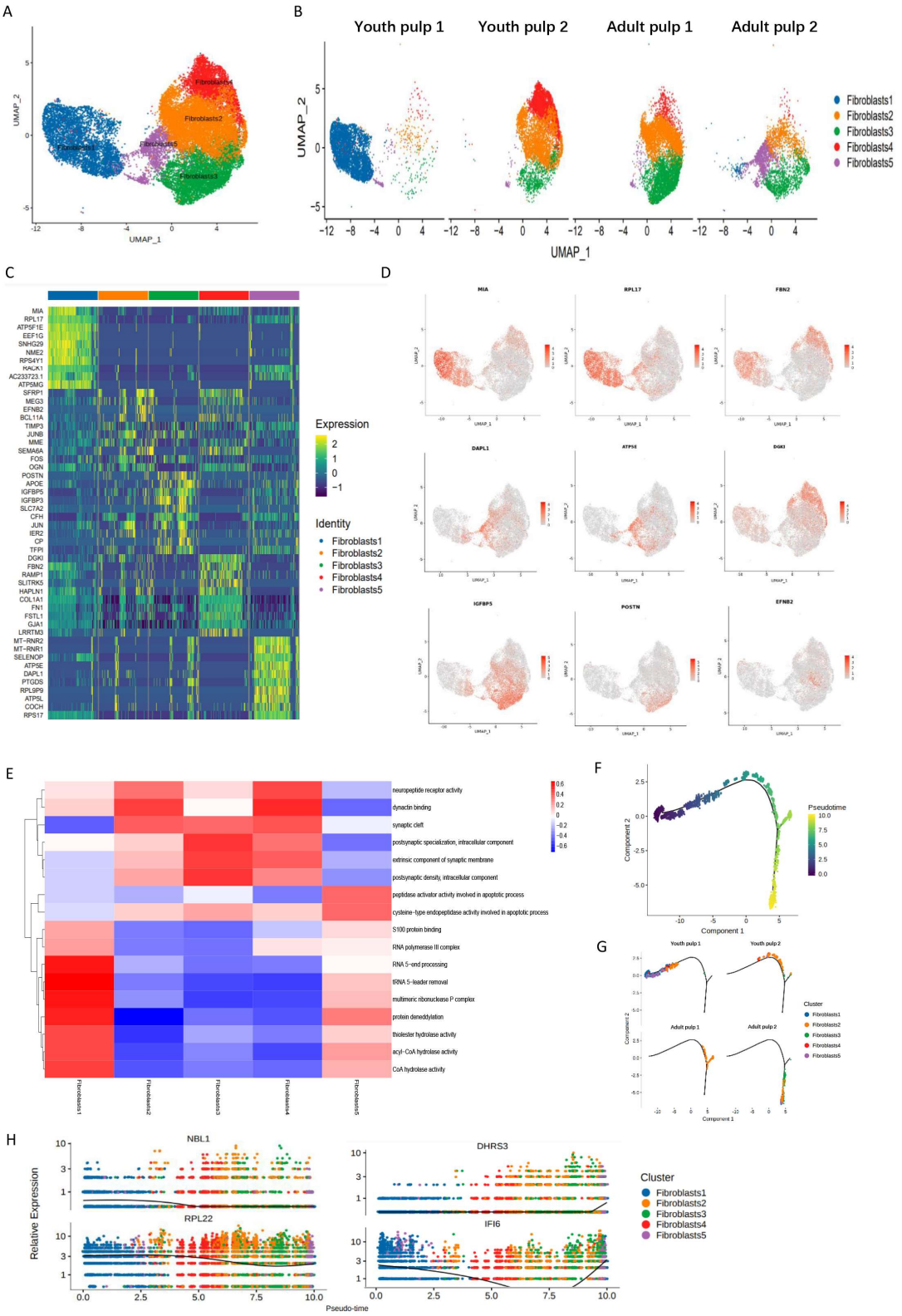

Figure 3. Subpopulation characteristics of fibroblasts in multiple spatial and temporal. A) UMAP plot of fibroblast subpopulation. B） UMAP displays of subgroups in four different samples. C) Heatmap of subset-specific markers. D) UMAP representation showing the marker genes in fibroblasts. E) Heatmap results of pathway enrichment for each subgroup. F) Plot of the proposed temporal order for all cells and visualized in descending order. The order of time indicates the differentiation of the pseudo-temporal sequences. G) The distribution of each sample in the

proposed time series trajectory is plotted, where different colors indicate the cell types in each sample. H) Variation in gene expression with pseudo-time.

Subpopulation characteristics of odontoblasts in multiple spatial and temporal

Odontoblasts are unique cells present in the dental pulp only that produce the mineralized dentin, specifically expressing DSPP and DMP1. When we try to explore this cell type, there are four sub-cluster divided (Figure 4A). It is clear that the developing dental pulp contains a unique class of dentin-forming cells. As for mature pulps, odontoblasts within the two samples were also not completely homogeneous. We can see that cluster 1 and 2 contributed to composition Adult pulp 1, while a new cluster 4 accounted for the majority of Adult pulp 2 (Figure 4B). Cluster 3, an earlier cell population, which was all contributed by developing youth pulps, expressed highly the gene DGKI, RRBP1 and VCAN. The older cells in cluster 1 and 2 have a large amount of SPOCK1, PHEX and LBH gene expression. What is the most different between clusters 1 and 2 is that there are more BMP7 but less WDR72 expressing in cluster 1 (Figure 4C, D). And the cluster 4 expresses IGKC and some ATP synthase. Despite the fact that these genes including NES, TRPM7 and PTN are expressed in all four subgroups, the variation in gene expression intensity at different developmental stages can be found in the violin plot (Figure 4D).

The pathway about negative regulation of "stress fiber assembly" and "actin filament bundle assembly" are enriched in cluster 1 and 2, but "nuclear cyclin−dependent protein kinase holoenzyme complex" and "cytoplasmic exosome" show high expression respectively in cluster 1 and 2. "nBAF complex", "proton channel activity", "collagen binding" and "protein binding involved in heterotypic cell−cell adhesion" are highly expressed in cluster 3. Pathway enrichment results to T cell relating and "angiogenesis involved in wound healing" suggest that cluster 4 from adult pulp 2 may be in an injury state (Figure supplement 4A).

The pseudotime developmental tree treated cluster 3 as the initial cell, locating mainly at the beginning and branch of the proposed timeline (Figure 4E). Cluster 1, 2 and 4 have been separated into two sides of the differentiation process because of the different potential situations they were in or simply due to differences in sample source. We can also observe the level of expression changes of different genes along the proposed timeline. Accompanied by a gradual increase in the S100A6 and CLU, the expression of COL1A2 and PTN begins to decline (Figure 4G).

Transcription factors, regulators of region-specific morphogenesis, are the key orchestrators of gene activity during development, and we used SCENIC to try to find the transcription factors that play an important role. THRB, DLX5, LHX8, TCF4 and ZNF530 are the five most specific regulatory molecules in developing cluster 3 (Figure 4H, supplement 2B). MEF2C and TBX3 act as important regulators of cluster 1 and may be of interest in maintaining homeostasis. MEF2C is a major transcription factor in the regulation of postnatal bone homeostasis [24], and when TBX3 undergoes mutation, the tooth exhibit various abnormalities [25]. Regulon "FOXO1" in cluster 4 was shown to be associated with the aging of dental pulp stem cells [26].

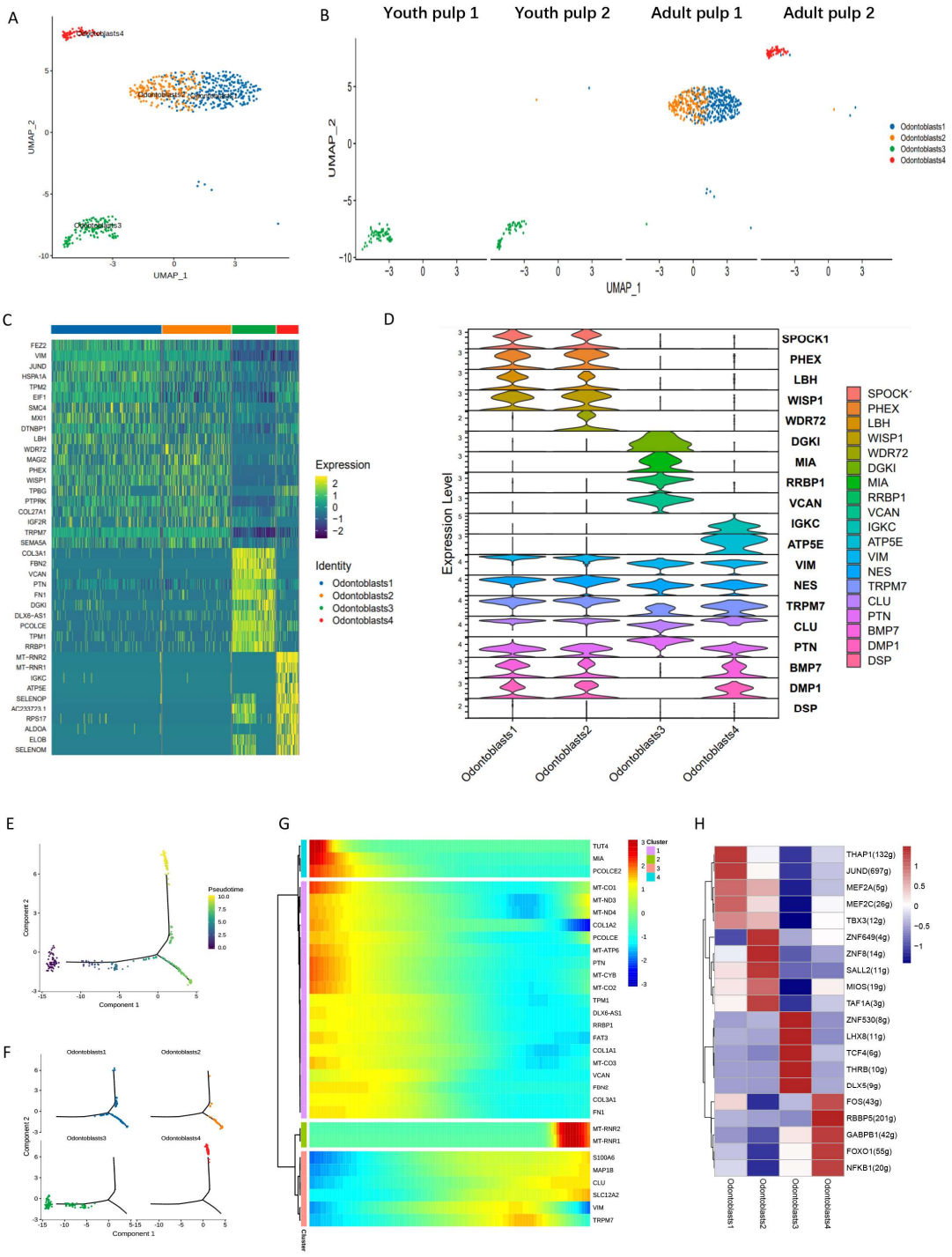

Figure 4. Subpopulation characteristics of odontoblasts in multiple spatial and temporal. A) UMAP plot of odontoblast subpopulation. B) UMAP displays of subgroups in four different samples. C) Heatmap of subset-specific markers. D) Violin plot representation showing the marker genes in odontoblasts. E) Pseudotime analysis of odontoblasts and the distribution of each subtype individually in the proposed time series trajectory. F) Clustered heatmap showing the dynamics of gene expression with pseudo-time changes. H) Top5 heatmap of AUC matrix clustering of regulon in each cell type. AUC: area under the curve

Subpopulation characteristics of MSCs in multiple spatial and temporal

Mesenchymal stem cells, as a class of cells with multiple differentiation potential and high regenerative capacity, are considered to be relevant to organ tissue development and repair in vivo[27]. We are accustomed to consider MCAM /CD146, THY1 /CD90 and CD24 as marker of dental mesenchymal stem cells [4,5,28] and they are located in the perivascular niche and apical papilla (Figure supplement 5) in human dental pulps. In our research, these cells are divided into five species in the re-clustering, which constitute four different samples in various proportions (Figure 5A). The Youth pulp 1 contains more subpopulation 3, 4 and 5 clearly, while the other three samples are more similar in composition (Figure 5B). THY1/CD90 is most differentially expressed in cluster 3, appeared in all samples except for some slight fluctuations in number. CD24 and TNC are specifically expressed in cluster 4. Some genes specifically expressed in odontoblasts and fibroblasts such as PTN, CLU, and VCAN are up-regulated in cluster 4 (Figure 5C, D). Cluster 5, in particular, is only present in large numbers in Young pulp 1 and show characteristics in the specific expression of some SEPTIN genes, while also highly expressing CCN2 and NME2. Cluster 1, was delineated for its specific expression in genes such as STEAP4 and ABCC9. Cluster 2 is separated from other subgroups because of the high expression of RERGL and MHY11 and the down-regulation of THY1/CD90. As for the other gene considered to be markers of oral MSCs, MCAM (CD146) is expressed in all subpopulations.

We then found clues and information about cell subpopulation function in pathway-level differential analysis, and got that cluster 4 is enriched with “polysomal ribosome”, “beta-catenin destruction complex”. “SAGA complex”, “SMN complex”, “dynein complex”, “polysaccharide binding” in cluster 5 are significantly expressed. Biosynthetic or metabolic process of dermatan sulfate, “BMP receptor binding” and other effects are concentrated in cluster 3 and 4 (Figure 5E).

The four subpopulations show the same distribution in the temporal trajectory, and the differentiated and developmental relationships between these subgroups could not be inferred based on monocle trajectory analysis. Therefore, we rediscovered a way, SLICE to explore the genealogy and differentiation status within MSCs subpopulations [21]. From the results of the entropy calculation, cluster 3-5 have higher entropy values and higher differentiation potential (Figure 5F).

Via the SCENIC, there are also some regulons calculated in each cluster to act significantly (Figure 5G). KLF9 and PRRX1 in cluster 3 are critical in cells development [29,30]. SOX21 and the odontogenic homeobox genes (DLX1, LHX8 and MSX2) are specific regulatory molecules in cluster 4. And in cluster 5, we get the regulon “NFIC”, it is essential in the development of tooth roots [31,32].

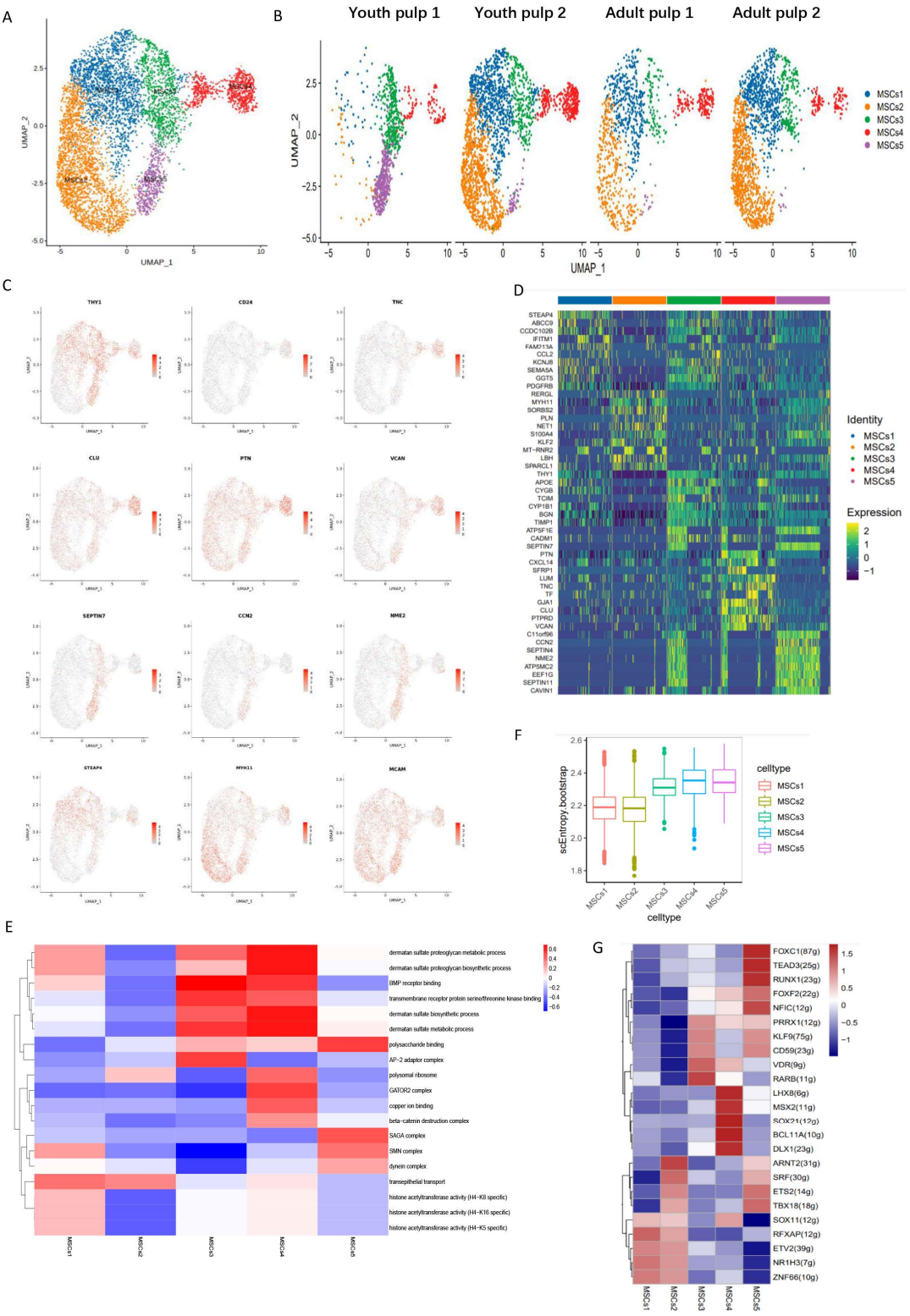

Figure 5. Subpopulation characteristics of MSCs in multiple spatial and temporal. A) UMAP plot of MSCs subpopulation. B) UMAP displays of subgroups in five different samples. C) UMAP representation showing the marker genes in MSCs. D) Heatmap of subset-specific markers. E) Heatmap results of pathway enrichment for each subgroup. F) Box line plot of entropy distribution of all cell subpopulations. G) Top5 heatmap of AUC matrix clustering of regulon in each cell type.

Discussion :

In our study, we combined our sequencing sample data with three published data from public database [11,12]to explore the gene expression characteristics of human dental pulp cells in different spatial and temporal levels.

We find that pulp cells at different developmental stage progresses showed variations only in number and proportion. Variations in the number of endothelial cells and Glial cells may be related to these developmental processes of the dental pulp. Previous studies have shown that endothelial cells appear in the bell phase and gradually increase with vascular and tooth development [33,34] and neural axons begin to enter the apical papilla progressively only after crown formation, although early innervation was present [35].But more samples are needed for verification. The high concentration MSCs found in Youth pulp 2 are highly proliferating to format dentin by differentiating into odontoblasts and/or are recruited to the connected pulp tissue [28,36]. And it is interesting that MPs are significantly more prevalent in the Young pulp 1 than in the other three samples, and the interaction of OSM/OSMR between MP and EC in Youth pulp 1 also showed significant differences. An article exploring the immune microenvironment of the mouse mandible has revealed that a population of MPs overexpressed the OSM gene while in traffic with MSCs and promoted osteogenic through this pathway [37]. So we presume that MPs and genes related may play a role in the development of teeth.

We provide in vivo evidence for the importance of the stem cell microenvironment in human tooth development. In our research, cells coordinate tooth development through a complex network of molecular regulation and interactions, such as NOTCH, FGF and BMP pathways, which have been proven in many studies [38-41]. These pathways appear repeatedly in ECs and Glial cells. The microenvironment in which stem cells reside in vivo and the exact origin of MSCs have received increasing attention in recent years. There has been proved that a large number of MSCs during tooth development, self-renewal and repair are generated from peripheral nerve-associated glial cells as well as peripheral vascular-associated pericytes [42,43]. Therefore, as cell populations closely related to the stem cell niche, ECs and Glial cells occur at high frequency in the network communication in our research can be expected. When we focus on two other specific cells in the dental pulp, fibroblasts and odontoblasts, we found that TTR and RSPO2 occur in the traffic, in addition to BMP and FGF. RSPO2 has been shown to indeed play a role in odontoblast differentiation [44], and TTR, binding and distributing thyroid hormones, remains to be further explored.

This work selected fibroblasts and odontoblasts for a detailed analysis and revealed their potential developmental characteristics. In our analysis, only one cluster in pre-eruptive Youth pulp 1 exhibited heterogeneity and demonstrated positive secretion characteristics to promote pulp development. Clusters 2-4 are present in varying proportions as more mature fibroblasts, stabilizing tissue homeostasis and preparing to resist external stimuli. Cluster 5, a suddenly appeared subgroup in one adult pulp, is presumed to be in an apoptosis-active state for some reason based on its expression. Odontoblasts can develop, differentiate and then secrete predentin-dentin components according to specific spatiotemporal patterns [41], which was verified by high-

throughput sequencing in our results. A total of four odontoblasts were defined in this study. We identified cluster 3, locating in immature youth pulps, as an earlier subpopulation in the dentin-forming phase, which may have a more vigorous secretory function. PTN is expressed in all odontoblasts, but at a higher level in cluster 3. Previous research has shown that PTN was expressed in the odontoblasts and in the basement membrane of organs that undergo epithelial-mesenchymal interactions [45]. The intensity of NES expression increased with the progression of odontoblasts differentiation, consistent with previous studies [46,47].We also identified genes that may be marker genes for early odontoblasts such as DGKI, RRBP1 and VCAN. In particular, Adult pulp 2, which showed specificity in the Fibroblasts subpopulation, appeared the same in the odontoblasts subclusters. The dentin-pulp complex is capable of repairing in answer to external damage after the occlusion is established [27] and we speculate that subpopulation in this sample may be in a state of repair response. The observation of some highly expressed in mature subpopulations but not in developmental subpopulations genes are associated with defective diseases, such as PHEX and WDR72 [48,49], possibly suggesting a more significant role in homeostasis maintenance compared to developmental promotion.

We focused on the heterogeneity of human dental pulp stem cell populations, and identified two populations of early and actively differentiated stem cells. In the past, we are accustomed to consider MCAM /CD146, THY1 /CD90 and CD24 as marker of dental mesenchymal stem cells [4,5,28], but their expression levels vary within stem cell populations. We can see MCAM is commonly expressed in all subgroups, but THY1 differs. As the pre-eruptive sample with the most advanced developmental stage of the four samples, Young pulp 1 contained cluster 3-5 that we considered to be the earlier stem cell population with high expression of THY1. There has been one study shown that THY1 presences in continuously growing mouse incisors with a dramatic decrease when growth rate homeostasis is established, but accelerates in damaged condition [50]. We hypothesize that $THY1^{+}$MSCs in human rapidly increase during dental pulp development and become quiescent after maturation. Cluster 5 upregulates the expression of SEPTIN7, and decreases significantly after post-eruptive stage. So this cluster may be a precursor cell population of cluster 3 and 4. And it has been demonstrated that SEPTIN as a cytoskeletal protein is important for development and differentiation [51,52], and as an earlier dental pulp stem cell population, it may show stronger proliferation and differentiation potential. Cluster 4 is presumed to differentiate into odontoblasts and fibroblasts based on it similar gene expression profile to them. The similarity in expression of TNC and CD24 in this cluster also adds credence to the differentiation characteristics of this cluster [53,54]. The function of cluster 1&2 with STEAP4 and MYH11 expressing specifically is yet to be further investigated. From the sample information combined with the subpopulation distribution, especially Youth pulp 1 and 2, it can be concluded that the dental papilla cells that support tooth development also differentiate into different stem cell populations with developmental stages and do not always remain the same.

For the first time, our study compared healthy pulpal characteristics at different times and sites, and the characteristics of the three types of cells that are important to

the pulp were also analyzed in depth. In particularly, we performed a cursory exploration of the stem cell spectrum and identified a potential early stem cell population, as well as stem cell population in an active state of differentiation. These findings may offer some promising new directions or goals. By the primary comparison, we found that in addition to complete and incomplete development, the fact that the teeth are in pre-eruption or post-eruption is also an important time point.

But the sample size was still small and affected by individual differences. Moreover, the results of functional enrichment based on database analysis as well as the results of the Monocle analysis did not match well in the present results, which may be related to the current peculiarities and limitations of dental development. Therefore, subsequent studies can obtain more comprehensive and continuous analysis by expanding sample size and establishing a complete temporal developmental time line. Basic experiments and targeted analyses can also be established in conjunction with multi-omics technologies to further reveal pulp development and disease mechanisms.

Conclusion

In summary, we compared four healthy pulpal characteristics at different times and sites, and the characteristics of the three specific types of pulp cells were analyzed in depth. In addition, the atlas of human dental pulp stem cells provide us a more comprehensive understanding and a potential basis for screening in pulp regeneration. But more work should be completed to validate the results of single-cell RNA sequencing.

Abbreviations

DPSC: Dental pulp stem cell
SCAP: Stem cell from root apical papilla
SHED: Stem cells from human exfoliated deciduous
BMSC: Bone marrow stromal cell
MSCs: Mesenchymal stem cells
PBS: Phosphate-buffered saline
DEGs: Differentially expressed genes
GO: Gene Ontology
KEGG: Kyoto Encyclopedia of Genes and Genomes
PPI: Protein-protein interactions
CCI: Cell-cell interaction
GSVA: Gene set variation analysis
SCENIC: Single-cell regulatory network inference and clustering

Declarations

Ethics approval and consent to participate

Our study was approved by the Biomedical Ethics Committee of Peking University School of Stomatology (PKUSSIRB-202060197). Each patient signed a written consent after full counseling.

Consent for publication

Written informed consent was obtained from the donors for recording/using their individual details (age, and gender) for research purposes.

Availability of data and materials

The data that support the findings of this study is being deposited in the Gene Expression Omnibus (GEO) database. The publicly available datasets of pulp cells were downloaded from GEO database with accession number GSM4365609, GSM4365610 and GSM4998458.

Competing interests

The authors declare no competing interests.

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Authors' contributions

Huihui Ren, Quan Wen and Yuming Zhao conceived and designed this study; Huihui Ren, Quan Wen and Qingxuan Zhao did the data analysis and interpretation; Huihui Ren, Qingxuan Zhao and Nan Wang recruited subjects and conducted the experiments; Huihui Ren wrote the manuscript. All authors read and approved final manuscript.

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Figure supplement 1

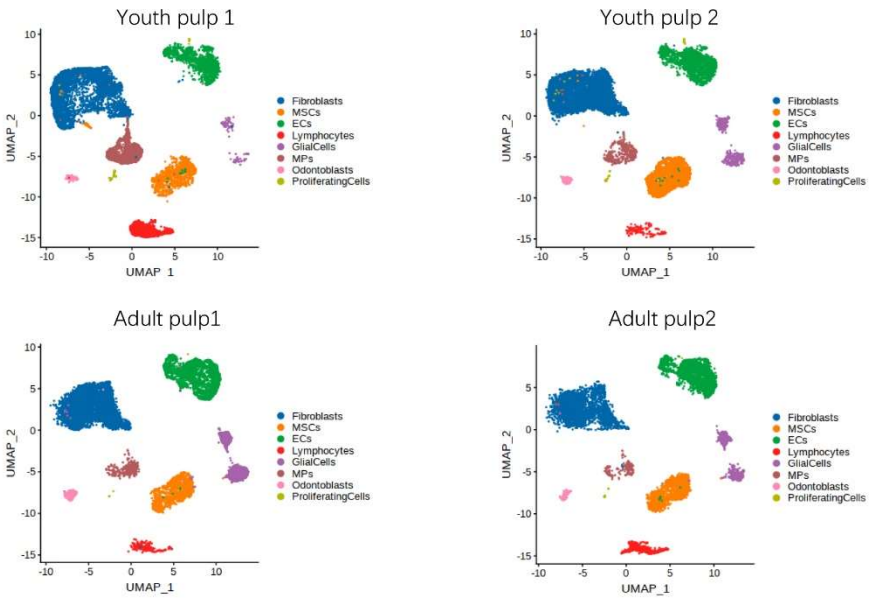

Figure supplement 1. Human pulp cell clusters in four samples separately.

Figure supplement 2

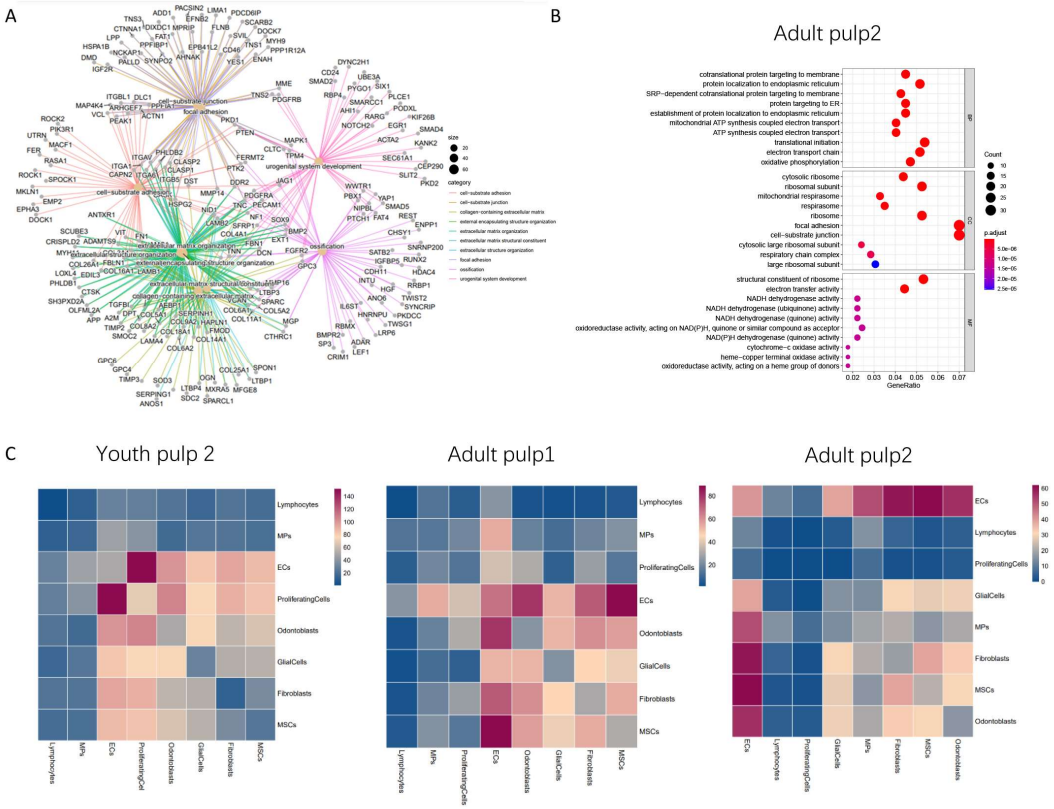

Figure supplement 2. A) Netplot diagram between genes and pathways in Youth pulp 2. B) Gene ontology pathway dotplot of adult pulp 2. C) Heatmaps of three samples demonstrating the number of pairs of the interaction between two cell types in different samples.

Figure supplement 3

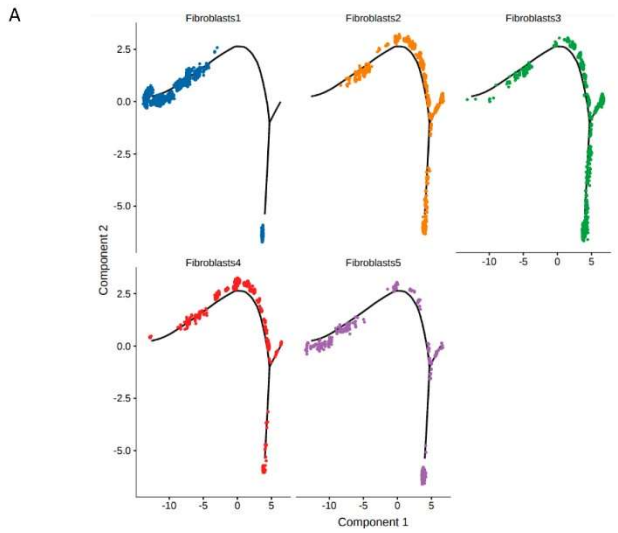

Figure supplement 3. A) Distribution plot of each Fibroblast type individually in pseudo-sequential trajectories.

Figure supplement 4

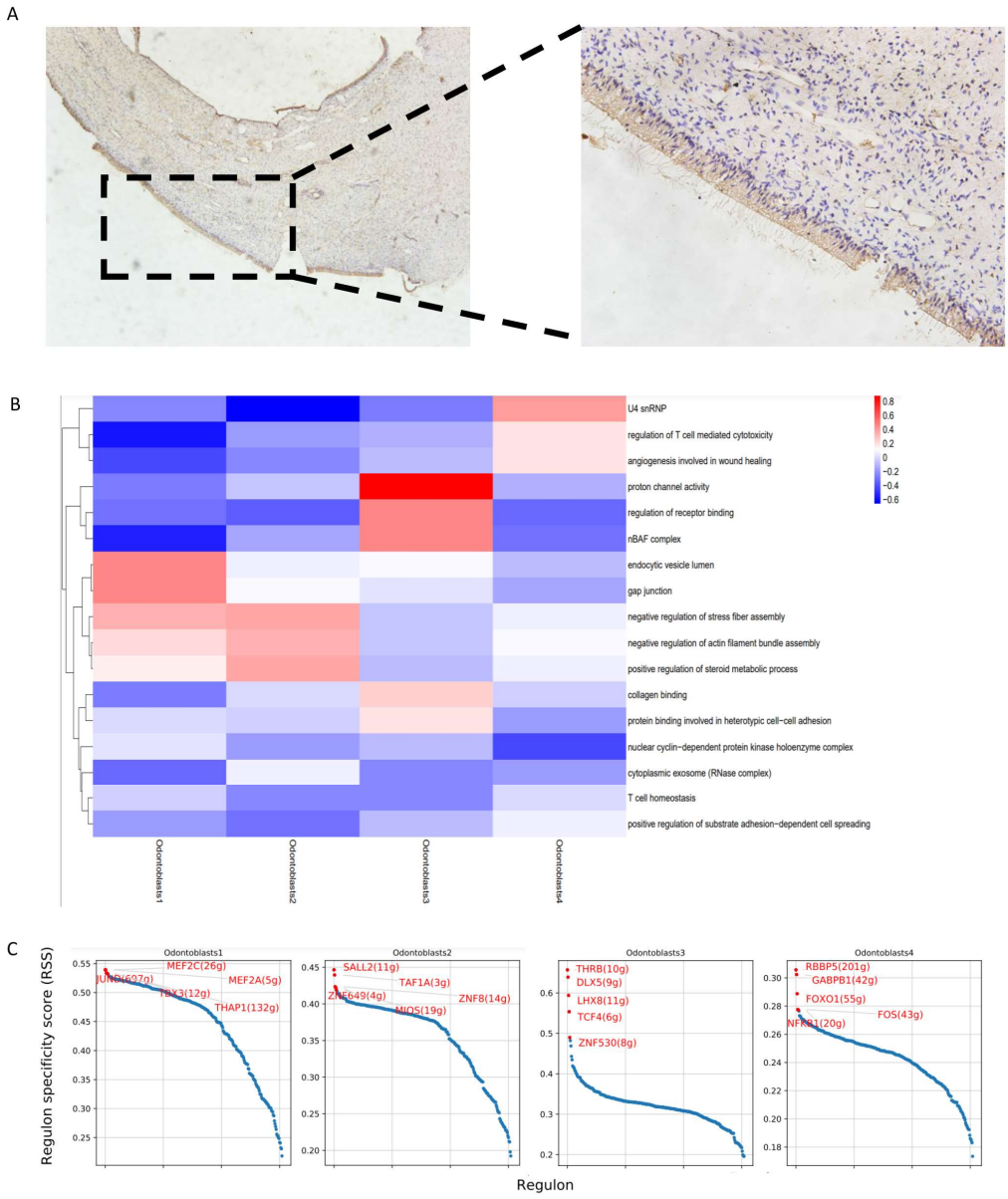

Figure supplement 4. A) Immunolocalization of the DMP1 antigen on odontoblasts in human dental pulp. B) Heatmap results of pathway enrichment for each odontoblast subgroup. C) Scatter plots of regulon specificity for each odontoblasts cell type, highlighting the highest top5 regulons.

Figure supplement 5

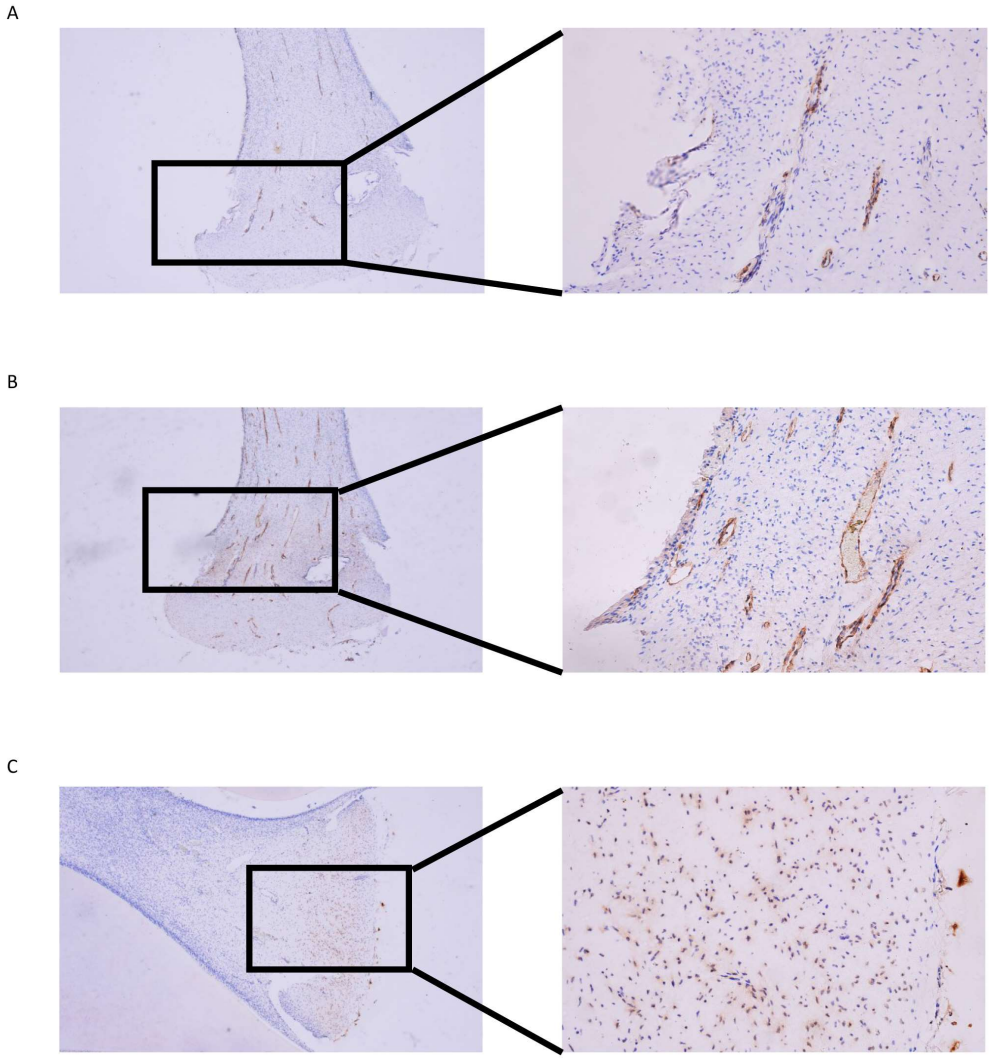

Figure supplement 5. A) Immunolocalization of the CD146 antigen on blood vessels in human dental pulp. B) Immunolocalization of the CD90 antigen in human dental pulp. C) Immunolocalization of the CD24 antigen in human dental apical papilla. Left: 200µm; Right: 50µm.

Table supplement.1

Sample tag	Sources	Characteristics
Youth pulp 1	Clinical Collection	Pulp of a third molar with less than 1/3 root completed (pre-eruptive) from a 13-year-old child
Youth pulp 2	Public Database (GSM4365609)	Apical papilla of a third molar with 2/3 root completed pulp
Adult pulp 1	Public Database (GSM4365610)	Adult mature pulp
Adult pulp 2	Public Database (GSM4998458)	Adult mature pulp

Table supplement.2

Cell type	Markers
Fibroblasts	LUM,DCN,COL1A1
Mesenchymal stem cells	THY1,ACTA2,NOTCH3,MYH11,FRZB
Endothelial cells	CDH5,PECAM1,VWF,EMCN
Proliferating Cells	TOP2A,MKI67,TUBA1B,TYMS
Lymphocytes	CD2,CD3D,TRAC,TRBC2,CD79A,NKG7,KLRD1
Glial Cell	PLP1,SOX10,COL28A1,SCN7A,GJC3,MBP,MPZ
Mononuclear phagocytes	LYZ,C1QA,MRC1,CD68,CD163,APOE, CSF1R, FCGR3A,CD14
Odontoblasts	TRPM7,DMP1,S100A13

Table supplement.3

	Youth pulp 1	Youth pulp 2	Adult pulp 1	Adult pulp 2	Total
Fibroblasts	5663	7092	7086	3456	23297
Mesenchymal stem cells	1061	2433	1056	1849	6399
Endothelial cells	1398	1693	2091	2512	7694
Proliferating Cells	71	67	4	21	163
Lymphocytes	1661	119	162	568	2510
Glial Cell	93	376	1759	643	2871
Mononuclear phagocytes	1849	381	304	163	2697
Odontoblasts	66	84	585	62	797
Total	11862	12245	13047	9274	46428

Table supplement.1 Samples information

Table supplement.2 Annotated genes of dental pulp cell types

Table supplement.3 Number of different cell types in each sample.