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Review

Unraveling the hidden complexity: Exploring dental tissues through single-cell transcriptional profiling

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ABSTRACT

Understanding the composition and function of cells constituting tissues and organs is vital for unraveling biological processes. Single-cell analysis has allowed us to move beyond traditional methods of categorizing cell types. This innovative technology allows the transcriptional and epigenetic profiling of numerous individual cells, leading to significant insights into the development, homeostasis, and pathology of various organs and tissues in both animal models and human samples. In this review, we delve into the outcomes of major investigations using single-cell transcriptomics to decipher the cellular composition of mammalian teeth and periodontal tissues. The recent single-cell transcriptome-based studies have traced in detail the dental epithelium-ameloblast lineage and dental mesenchyme lineages in the mouse incisors and the tooth germ of both mice and humans; unraveled the microenvironment, the identity of niche cells, and cellular intricacies in the dental pulp; shed light on the molecular mechanisms orchestrating root formation; and characterized cellular dynamics of the periodontal ligament. Additionally, cellular components in dental pulps were compared between healthy and carious teeth at a single-cell level. Each section of this review contributes to a comprehensive understanding of tooth biology, offering valuable insights into developmental processes, niche cell identification, and the molecular secrets of the dental environment.

Contents

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1. Introduction

Tooth development, a complex biological process, has been extensively studied. Advances in molecular biology, genetics, and tissue engineering have deepened our understanding of this intricate process. From embryonic tooth development to its postnatal eruption, cellular and molecular events guide the formation of functional dentition.

Odontogenesis involves tightly regulated steps resulting in distinct dental tissues and their surrounding periodontal tissues. These tissues include enamel, dentin, dental pulp, cementum, the periodontal ligament (PDL), and alveolar bone, each with unique properties and functions [1]. Tooth development begins with the initiation of dental placodes, which are specialized thickenings of the oral epithelium, leading to the formation of tooth germs, which are comprised of dental epithelium and cranial neural crest cell (CNCC)-derived mesenchymal components. The interactions between the two populations are essential for subsequent tooth morphogenesis. The tooth germ undergoes complex morphological changes during the bud and cap stages. The oral epithelium invaginates into the underlying mesenchyme, resulting in formation of three components: the enamel organ, dental papilla (DP), and surrounding dental follicle (DF). The enamel organ gives rise to enamel; cells in the inner enamel epithelium (IEE) in the structure differentiate into enamel-forming ameloblasts. The DP forms dentin and pulp. Odontoblasts, which are responsible for dentin formation, are differentiated from cells in the DP. Cells in the DF differentiate into cementoblasts that generate the cementum, osteoblasts that form the alveolar bone proper, and fibroblasts that form principal fibers of the PDL. The cementum, alveolar bone proper, and PDL collectively support functional teeth by anchoring them to bones.

Signaling molecules, such as fibroblast growth factors (FGFs), Wnt, bone morphogenetic proteins (BMPs), and Sonic hedgehog (Shh), play crucial roles in tooth development, regulating cell proliferation, differentiation, and tissue patterning [2]. The molecular regulation of tooth development involves a network of signaling pathways and transcription factors. Understanding the networks underlying tooth development will lead to the development of therapeutic approaches for dental anomalies, including regenerative dentistry.

Single-cell-level transcriptional profiling with single-cell RNA sequencing (scRNA-seq) has revolutionized our understanding of cellular diversity and dynamics in tissue development and disease progression [3]. Unlike traditional approaches, scRNA-seq reveals transcriptional profiles in individual cells, i.e., at a single-cell level. Various algorithms to analyze scRNA-seq datasets empower researchers to explore rare cell populations, to predict transition states between cell types, and to infer dynamic cellular interactions. The impact of scRNA-seq extends across multiple scientific disciplines. In developmental biology, scRNA-seq has illuminated the gene expression patterns during embryogenesis, unraveling intricate cell fate decisions and tissue formation [4,5].

In this review, with a particular focus on teeth and periodontal tissues, we summarize key findings in scRNA-seq studies, which have unraveled the cellular dynamics, networks of molecular signaling, and gene regulations at the single-cell level during embryonic and postnatal tooth development. This review proceeds in the following order:

- Analysis of mouse incisors
- Analysis of the mouse and human molar tooth germ
- Cellular characterization of the dental epithelium-ameloblast lineage
- Cellular characterization of the dental mesenchyme
- Identification of dental niche cells
- Cellular characterization of the human dental pulp
- Identification of molecular mechanisms underlying root formation
- Characterization of the periodontal ligament
- Comparison of dental pulps between healthy and carious teeth
- Conclusions and future perspectives

2. Analysis of mouse incisors

Rodents have been extensively utilized to study tooth development and human dental abnormalities, based on the biological similarities in key features of tooth development between rodents and human [6]. One particular focus has been the continuously growing incisors in mice, which allow the investigation of all stages of tooth development since enamel formation occurs throughout their life [7].

The conventional model of stem cell-driven homeostasis in mouse incisors posits that a few quiescent, slow-cycling stem cells are located in the proximal outer enamel epithelium (OEE) or stellate reticulum (SR); these give rise to transit-amplifying IEE cells, which then differentiate into ameloblasts as they move distally to form enamel [8,9] (Fig. 1). Recent scRNA-seq studies have provided insights into cellular dynamics in the growing teeth [10–13]; in particular, they have revolutionized our understanding of the above model.

In 2019, Sharir et al. elucidated the cellular hierarchy in the adult mouse incisor epithelium through scRNA-seq analysis [10]. Cells were initially classified into three major types based on the scRNA-seq data. Class 1 cells, enriched with actively cycling cells and localized to the IEE and the neighboring stratum intermedium (SI) region, were identified as a source of progenitor cells. These cells exhibited characteristics of self-renewal, expressed markers of various cell cycle phases, and displayed putative stem cell marker genes. Class 2 cells consisted of pre-ameloblasts and ameloblasts. These cells expressed genes characterizing secretory-stage ameloblasts, including *Amelx*, *Ambn*, *Enam*, and *Mmp20*, some of which were newly linked to ameloblast differentiation. Class 3 cells, composed of 9 clusters, localized within the remaining incisor epithelium or the non-ameloblastic cells. Distinct compartments of this class were labeled by enriched genes in each cell type, and gene set enrichment analysis highlighted active transcription and translation, suggesting the progenitor properties of this class. Moreover, the Notch pathway was significantly enriched in the SI of the Class 3 cells. Bioinformatic analysis of scRNA-seq data suggested that IEE, identified as Class 1, served as a reservoir for progenitor cells, giving rise to two other cell classes (Classes 2 and 3). RNA velocity and FateID analyses supported this lineage hierarchy, revealing differentiation biases in the Class 1 cells towards specific states in the Classes 2 and 3. Analysis of the proliferation kinetics supports the notion that proliferating progenitors in IEE differentiate into two populations: a larger one that populates the ameloblast layer and a smaller one that exits IEE and becomes SR and OEE cells. Thus, Sharir et al. shed new light on the cellular hierarchy governing incisor maintenance (Fig. 1).

They also investigated cellular dynamics during tooth repair after injury, which was induced by 5-fluorouracil (5-FU) treatment, causing cell cycle arrest and apoptosis. They found that at 3 days post-injury, proliferating cells increased and cell-cycle time decreased in the IEE during the recovery process. scRNA-seq demonstrated an increase in Class 1 cells and a decrease in Class 2 cells compared to the control, providing insights into the dynamic cellular hierarchy and regeneration mechanisms in the

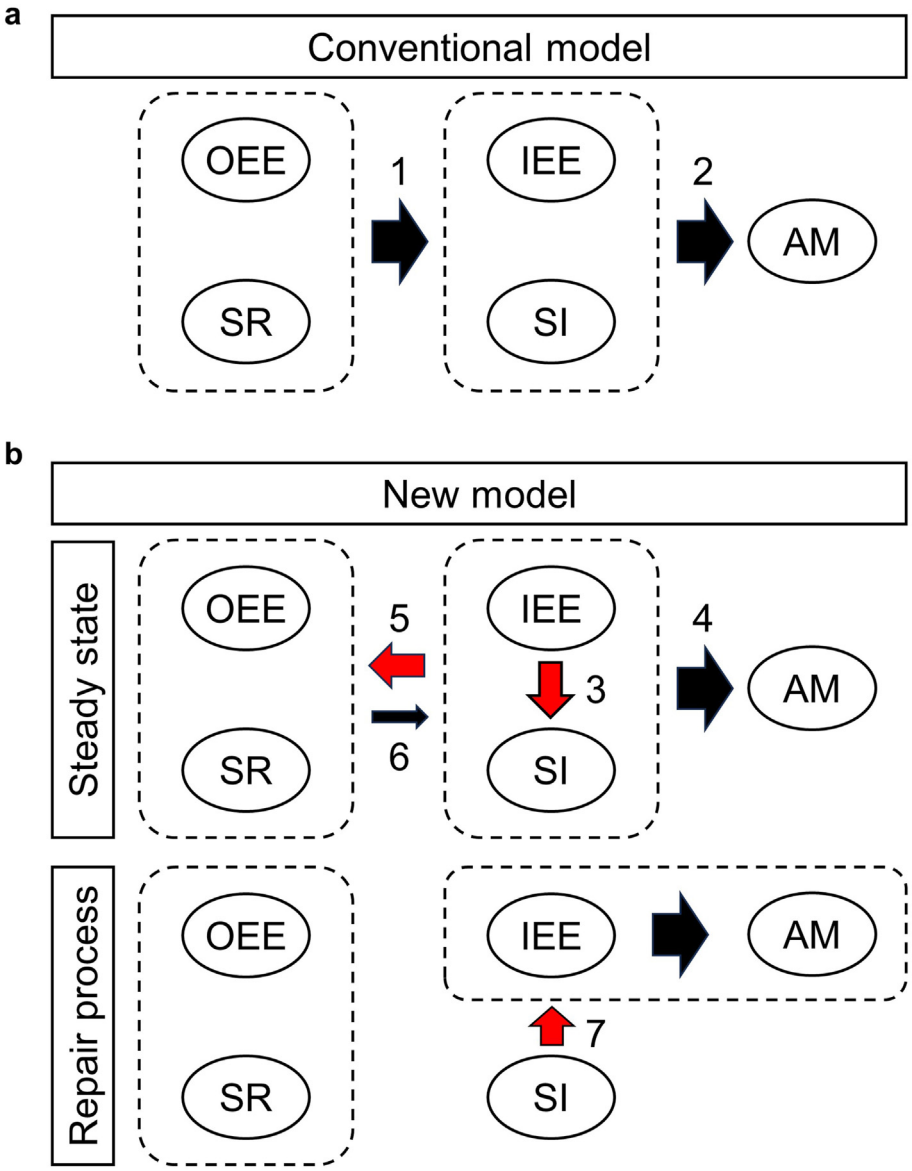

Fig. 1. Cellular trajectories of the mouse incisor epithelium. (a) A conventional model of the dental epithelium-ameloblast lineage specification in mouse incisors. The labial cervical loop consists of the four major cell types, the outer enamel epithelium (OEE), the stellate reticulum (SR), the stratum intermedium (SI), and the IEE; ameloblasts (AM) are derived from IEE cells. In the conventional model, the OEE and SR are thought to contain the incisor epithelial stem cells. The stem cell population gives rise to transit-amplifying cells in the IEE, which then differentiate into AM (1 and 2; thick black arrows). In this context, SI cells are thought to be derived from the OEE-SR population and support ameloblasts. (b) A new model proposed by recent scRNA-seq studies [10,11]. In the steady state, the IEE-SI population, in which SI is an intermediate population derived from IEE (3; red arrow), is a reservoir for progenitor cells. IEE-SI cells give rise to two lineages, AM (4; thick black arrow) and the OEE-SR population (5; red arrow); the trajectory to the former is more dominant, and the relationship between SR and OEE remains unclear. Given that the contribution of *Notch1*$^{+}$ SI descendants to ameloblasts is small [10], the IEE–SI–derived SR/OEE is likely to make a relatively small contribution to AM turnover in this context (6; thin black arrow). In the repair process, *Notch1*$^{+}$ SI largely contributes to the IEE-ameloblast population (7, red arrow) [10], suggesting direct recruitment of progenitors from the SI. It remains unclear whether the IEE-SI population still contribute to the OEE-SR population in the repair process.

incisor growth region. Lastly, they demonstrated conversion of *Notch1*-expressing SI cells into IEE and ameloblasts during the repair process as well as their contribution to SR, OEE, and ameloblast population in uninjured teeth, suggesting critical roles of *Notch1*-expressing SI cells for both steady-state and repair processes in mouse incisors (Fig. 1).

Chiba et al. examined dental epithelial cell types in mouse incisors using scRNA-seq [11]. Using a mouse model engineered to express *Keratin 14* (*Krt14*) promoter-driven RFP, they isolated epithelial cells from postnatal mouse incisors, which were subjected to scRNA-seq. The analysis revealed specific groups of cells—ameloblasts, IEE, OEE, SI, and SR—each displaying distinct gene expression patterns associated with particular cell types and developmental stages; cell cycle-related genes were highly expressed in IEE/OEE, whereas *Notch1* and *Notch2* were specifically expressed in SI and SR. These data partly support the findings by Sharir et al. Chiba et al. also identified new markers characterizing distinct epithelial cell subtypes: *Pttg1* (IEE and OEE), *Cldn10* (SI), *Atf3* (OEE and SR), and *Krt15* (OEE). The expression of these markers was confirmed by immunofluorescence in the P1 mouse molars. Lastly, they found that secretory stage ameloblasts expressing *Amel* and *Enam* were categorized into two subgroups, ameloblast (I) and ameloblast (II), which were characterized by the expression of *Dspp* and *Ambn*, respectively. Pseudo-time analysis revealed the

developmental trajectory from IEE and OEE clusters to SI, SR, and ameloblast clusters; differentiation genes were then grouped into four clusters (I)-(IV), according to their peak expression in the pseudo-time axis. The expression profiles of IEE/OEE marker *Tbx1*, ameloblast (I) marker *Dspp*, and ameloblast (II) marker *Ambn* correspond to those of groups (I)/(II), (III), and (IV), respectively. Based on these data, they suggested that *Dspp*-positive ameloblast (I) represented an early-differentiated ameloblast subpopulation, and *Ambn*-positive ameloblast (II) represented fully differentiated ameloblasts.

Krivanek et al. investigated epithelial and mesenchymal populations in mouse incisors [12]. They found 13 distinct clusters in the epithelium, including ameloblasts and a diverse group of stem/progenitor cells. The epithelial clusters showed several features. First, they represent sequential stages of ameloblast differentiation—namely, the *Shh* $^{+}$ pre-ameloblast, *Enam* $^{+}$ secretory, *Klf4*$^{+}$ maturation, and *Gm17660*$^{+}$ postmaturation stages. Second, a cluster of the *Thbd* $^{+}$ SI subpopulation (which they named the cuboidal layer of SI) expressed *Cygb*, *Nphs1*, and *Rhcg*, suggesting that these cells may play roles in maintenance of the functional interphase between blood vessel and metabolically active ameloblasts. Third, a cluster of stem and progenitor cells showed heterogeneity, as shown by, for example, the presence of *Acta2*$^{+}$, *Shh*$^{+}$, and long-lasting *Egr1*$^{+}$/*Fos1*$^{+}$ progenitors. Thus, these findings highlight the dynamic molecular identities within the mouse incisor epithelium. Regarding dental mesenchyme, they discovered two subtypes of dental follicle and three major populations within mouse incisors (odontoblasts, apical pulp, and distal pulp). The latter mesenchymal compartment was likely to function as an active pool of stem/progenitor cells. Additionally, they identified *Foxd1*$^{+}$ cells near the labial cervical loop, which have the ability to self-renew and give rise to periodontoblastic pulp cells and odontoblasts.

Krivanek et al. also compared the cellular composition between mouse incisors and nongrowing adult molars based on scRNA-seq data [12]. They found that the incisors and molars had similar cellular compositions, except for the absence of an epithelial population in the molars. The mesenchyme in the molars was more homogenous than that of the incisors and clustered into one group shared with the distal mouse incisor pulp. It is notable that the apical incisor pulp exhibited gene expression linked to regeneration and matrix production, whereas it was less apparent in the molar pulp, suggesting the potential regenerative abilities of the mouse incisor pulp, especially its apical portion, in response to damage. Lastly, through scRNA-seq on nongrowing and growing human wisdom teeth [12], they found similarities in cell types and lineage hierarchies between humans and mice. Human pulp cells showed substantial differences between growing apical papilla and non-growing molars, forming transcriptionally distinct subpopulations. Unique subpopulations in the periodontoblastic layer of human teeth, which had been morphologically defined as cell-free and cell-rich zones, were absent in mice, suggesting evolutionary divergence.

Chen et al. explored the roles of Runx2 in mesenchymal stem cell (MSC) populations and their homeostasis [13], complementing the aforementioned findings about an active pool of stem/progenitor cells in the incisor [12]. Their scRNA-seq analysis of 1-month-old mouse incisors identified distinct *Gli1*-positive cell clusters, which were previously found to be MSCs [14], at the dental epithelium, dental pulp, and the proximal region. Among them, a specific proximal subset showed prominent *Runx2* expression; the *Runx2*$^{+}$/*Gli1*$^{+}$ cells were present at the region adjacent to the cervical loop, close to transit-amplifying cells (TACs), in 1-month-old mice. Depleting *Runx2* in *Gli1*$^{+}$ cells in 1-month-old mice (*Gli1-Cre*^{ERT2};*Runx2*$^{flox/flox}$) led to phenotypic changes in the incisors, including a retarded growth rate, abnormal dentin formation, and defects in the PDL and alveolar bones; additional experiments suggested that these abnormalities in dental mesenchyme-derived tissues were independent of the effects of *Runx2* deficiency on ameloblasts. Lineage tracing analysis indicated that the *Runx2*$^{+}$/*Gli1*$^{+}$ cells modulated the TAC behavior (proliferation and differentiation) and thereby maintained the MSC niche. The function of the *Runx2*$^{+}$/*Gli1*$^{+}$ cells accounts for the incisor phenotypes caused by *Runx2* deletion in *Gli1*$^{+}$ cells. By bulk RNA-seq and subsequent functional analyses, they also found that insulin growth factor (IGF)-2 signaling in TACs is critical for the action of the *Runx2*$^{+}$/*Gli1*$^{+}$ cells, i.e., maintenance of the MSC niche; the *Runx2*$^{+}$/*Gli1*$^{+}$ cells secrete IGF binding protein 3 (IGFBP3) to maintain IGF-2 signaling in the niche.

3. Analysis of the mouse and human molar tooth germ

Hallikas et al. proposed the concept of “developmental keystone genes” that are involved in tooth development to varying degrees even though they are not necessarily essential for the process [15]. These genes were categorized into distinct groups based on a spectrum of phenotypic severities caused by their null mutations, providing functional insights into their roles in tooth development: (1) “progression” genes whose deletion causes developmental arrest of teeth, (2) “shape” genes whose deletion causes alteration of the tooth morphology, (3) “tissue” genes whose deletion causes defects in the tooth hard tissues, enamel and dentin, and (4) “dispensable” genes whose deletion has no phenotypic effects, although their combinatorial mutations may cause abnormalities. “Tissue” genes exert their functions at later stages compared to “progression” and “shape” genes. Therefore, the “tissue” genes and the “dispensable” genes were considered as control sets for the former two groups. In addition to the “developmental keystone genes,” Hallikas et al. also mentioned “initiation” and “eruption” genes, which are required for the initiation of tooth development and normal tooth eruption, respectively. Among the “developmental keystone genes,” “progression” genes emerged as central players in tooth development; bulk expression profiling of mouse molars (bud and cap stages) revealed that this group of genes were highly expressed at the bud stage [15]. This finding was further supported by scRNA-seq analysis in developing mouse molars (cap stage) [15]. Notably, specific signaling pathways such as FGF, Wnt, transforming growth factor (TGF)-β, Hedgehog (Hh), Notch, and Ectodysplasin (Eda) were enriched with these “progression” and “shape” genes, underscoring their significance in orchestrating the complex tooth developmental process [15].

As described above, the mouse serves as a model to study various stages of tooth development. Regarding the process in humans, the tooth germ of the third molar stands out as a correspondingly applicable model, as it is retained until adolescence or early adulthood in life. Shi et al. performed scRNA-seq analysis in human tooth germ of the growing third molar at different stages (developmental stages A and D [16]) [17]. Their analysis revealed a rich diversity of cell types in the human tooth germ, including immune cells, osteoblasts, osteoclasts, pericytes, endothelial cells, and *SOX9*$^{+}$ cell clusters. Within *SOX9*$^{+}$ cells, a subcluster expressing *CD24* (an apical pulp stem cell (APSC) marker) and another expressing *AMBN* (an ameloblast marker) and *CLU* (an epithelium-related gene) were identified. Osteoblasts were divided into immature (highly expressing *VIM*) and differentiated subpopulations (highly expressing *SPARC* and *GJA1*). In the dataset obtained by Shi et al., more than 80% of all cells were immune cells, half of which were T cells, suggesting their important functions in tooth development. Indeed, interaction analysis between different

cell types predicted strong communications between T cells and ameloblasts/osteoclasts via T cell subtype-distinct signaling pathways, between neutrophils and endothelial cells (ECs), and between B cells and dental cells, particularly ameloblasts. The research also delved into intercellular regulations of the self-renewal capacity of apical papilla stem cells (APSCs). Ligand-receptor analysis predicts the involvement of monocyte- (IL1B and IL1A), T cell- (TGFB1), and osteoblast-derived ligands (BMP4/5 and TGFB3), as well as the autocrine effects of BMP2 and BMP7, in APSC self-renewal. FGF receptor 1 (FGFR1) was a candidate for the receptor of these ligands, possibly targeting *MSX1* to regulate self-renewal genes in APSCs. In summary, this study offers comprehensive insights into the cellular composition and intercellular interactions within the human tooth germ, predicting the intricate processes at play during tooth development.

4. Cellular characterization of the dental epithelium-ameloblast lineage

Alghadeer et al. performed scRNA-seq on human tooth germs at their different developmental stages (9–22 gestational weeks) as well as developing salivary glands and jaws [18]. In the tooth sample data, major cell clusters included the dental mesenchyme, dental epithelium, odontoblasts, and ameloblasts. Dental epithelium-derived cells were then subclustered into 13 cell types: oral epithelium, dental epithelium, IEE, OEE, cervical loop, inner SI, outer SI, inner SR, outer SR, enamel knot, pre-ameloblasts, early ameloblasts, and secretary ameloblasts. Pseudotime analysis predicted a developmental trajectory of the dental epithelium lineages (Fig. 2). In the trajectory, the oral epithelium gives rise to dental epithelium, which then generates the two cell lineages, i.e., the OEE and enamel knot/SR lineages. OEE gives rise to the SI lineage and cervical loop; the latter differentiates into IEE, pre-ameloblasts, early ameloblasts, and secretary ameloblasts in that order. Based on spatial mapping of each cell type with multiplex *in situ* hybridization (ISH) as well as the single-cell transcriptome, the authors gained insights into potential roles of the enamel knot, SI and SR, cervical loop, and ameloblast lineage as follows.

The enamel knot is known to be an essential signaling center during tooth development, especially for determining crown shape [2]. The authors found that the enamel knot cluster appeared twice at 9–11 (an early cap stage) and 14–16 (an early bell stage) gestational weeks [18], potentially reflecting formation of the primary and secondary enamel knot, respectively, in the scRNA-seq data. In addition, gene ontology (GO) analysis revealed that the clusters were associated with morphogenesis- and appendage development-related genes [18], which supports the developmental significance of the enamel knot.

The transcriptome-based clustering identified the SI and SR, each with inner and outer populations; spatial mapping also demonstrated that the inner SI was the cell layer closer to the ameloblast lineage, whereas the outer SI was present as parallel

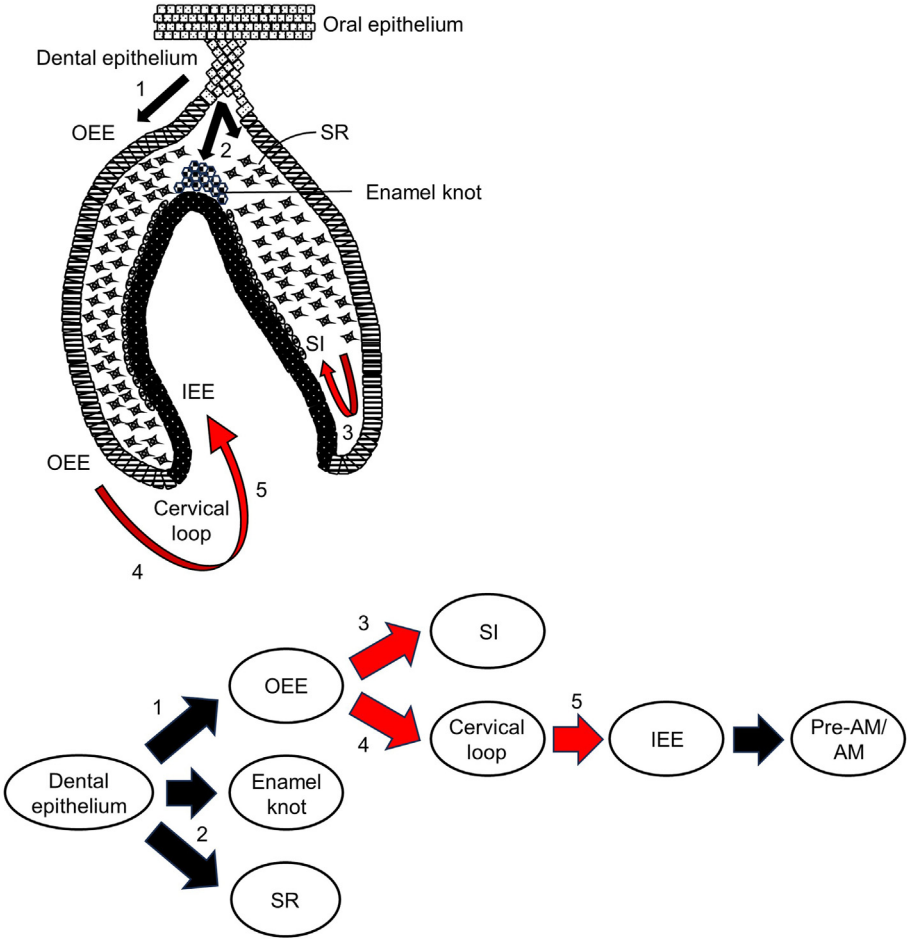

Fig. 2. Developmental trajectory of the dental epithelium. Cellular trajectory proposed by Alghadeer et al. [18] is illustrated in the upper panel and summarized in the lower panel. The oral epithelium gives rise to the dental epithelium, which differentiates into the OEE (1) and enamel knot/SR lineages (2). The OEE gives rise to the SI lineage (3) and cervical loop (4). The cervical loop differentiates into the IEE-ameloblast (AM) lineage (5).

cells adjacent to the inner SI [18]. The inner SI was associated with Hh and Wnt signaling, and the outer SI with TGF-β signaling. These signaling pathways may underlie the supporting function of the SI in ameloblast differentiation.

In the predicted trajectory, the OEE-derived cervical loop was a source of the ameloblast lineage; the authors also found a small *LGR6*$^{+}$ population in the cervical loop [18], which was shown to contain *Lgr5*$^{+}$ dental epithelial stem cells in mouse incisors [19]. These data support a role of the cervical loop in differentiation of the ameloblast lineage during crown expansion. In addition, the authors found that distinct steps of ameloblast differentiation that they identified were precisely marked by three genes: *VWDE*, *DSPP*^{low}, and *ENAM* mark pre-ameloblasts, early ameloblasts, and secretary ameloblasts, respectively [18].

Based on ligand-receptor analysis of their scRNA-seq data, Alghadeer et al. also predicted the signaling pathways that were most active at each step of human ameloblast development [18]: (1) oral epithelium-to-dental epithelium transition stage: BMPs, activin, and non-canonical Wnt (all secreted from the dental mesenchyme); (2) dental epithelium-to-OEE stage: canonical Wnt (from the dental epithelium and enamel knot), BMPs (from the dental mesenchyme), and FGF (from the dental mesenchyme); (3) OEE-to-IEE stage: BMPs (from the DP), canonical Wnt (from the inner SR and OEE), and activin (from the OEE); (4) IEE-to-pre-ameloblast stage: canonical Wnt (from the inner SR, inner SI, and IEE), non-canonical Wnt (from the inner SI, pre-odontoblasts, and IEE), and Hh (from the IEE); (5) pre-ameloblast-to-early ameloblast stage: Hh (from pre-ameloblasts), canonical Wnt (from pre-ameloblasts and the inner SR), BMPs (from pre-odontoblasts), and TGF-β (from the inner SR and outer SI); and (6) early ameloblast-secretary ameloblast stage: canonical Wnt (from early ameloblasts), epidermal growth factors (EGFs) (from the inner and outer SI), FGFs (from the inner and outer SI), and non-canonical Wnt (from the inner and outer SI, and odontoblasts). According to their findings, the authors developed a protocol for differentiating human induced pluripotent stem cells into pre- and early ameloblast-like cells.

It is worth noting that controversy exists over developmental trajectories of the dental epithelial cell lineage, when we compare the two models proposed by Sharir et al. [10] and Alghadeer et al. [18] The controversy lies on the difference in the cellular hierarchy within the lineage between the two models. Sharir et al. proposes that the IEE-SI population is the top of the hierarchy, acting as a reservoir of progenitors that gives rise to both ameloblasts and the SR/OEE lineage; they also demonstrate the conversion of SI cells into the IEE-ameloblast lineage during tooth repair [10] (Fig. 1b). On the other hand, Alghadeer et al. suggests that OEE, which arises as a distinct lineage from SR and enamel knot, gives rise to SI and cervical loop; the latter differentiates into the IEE-ameloblast lineage [18] (Fig. 2).

The controversy may result from differences in experimental approaches between the two studies. Alghadeer et al. investigated the human tooth germ at multiple developmental stages [18], whereas Sharir et al. specifically focused on mouse incisors [10]. Thus, the two models may reflect species-specific differences in dental epithelial development and may not directly correspond to each other. The two studies would rather shed light on two distinct aspects of the dental epithelium; Sharir et al. suggests the fast turnover of the mouse incisor epithelium, while Alghadeer et al. proposes an intricate developmental process, involving multiple cell types and signaling pathways, of the human dental epithelium. Collectively, as both studies imply the complex hierarchy and plasticity in the dental epithelium, further research is required to uncover a whole aspect of dental epithelial development.

5. Cellular characterization of the dental mesenchyme

The dental mesenchyme, which gives rise to the DP and DF of the tooth germ, is derived from CNCCs; after migrating into the oral region of the first pharyngeal arch, the postmigratory CNCCs are committed to the dental mesenchymal lineage [20].

Through scRNA-seq on developing mouse molars at the bud, cap, bell, and postnatal crown-root transition stages, Jing et al. have unveiled signature genes within the CNCC-derived dental mesenchyme, delineating different cellular domains and their contribution to mouse molar formation [20] (Fig. 3). At the bud stage (E13.5), they identified a *Tfap2b*$^{+}$/*Lhx6*$^{+}$ population as the dental mesenchyme, whose contribution to tooth formation was confirmed by a lineage tracing experiment using *Lhx6-Cre*^{ERT2};*tdTomato* mice. At the cap stage (E14.5), the previously known *Pax9*$^{+}$ mesenchymal cells, which give rise to all mesenchymal components in the postnatal mouse molars, were segregated into the DF (*Epha3*+/*Fxyd7*+/*Foxf1*+) and the DP (*Crym*+/*Egr3*+/*Fgf3*+) populations, which further evolve into four domains at the bell stage (E16.5): the lateral (*Lepr*$^{+}$/*Foxf1*$^{+}$/*Bmp3*$^{+}$) and apical (*Aldh1a2*$^{+}$/*Rasl11a*$^{+}$/*Sgk1*$^{+}$) domains of the DF, and the coronal (*Lmo1*$^{+}$/*Fgf3*$^{+}$/*Smpd3*$^{+}$) and apical (*Lhx6*$^{+}$/*Fst*$^{+}$/*Gldn*$^{+}$) domains of the DP. At P3.5, the DF consisted of lateral (*Bmp3*$^{+}$/*Tnmd*$^{+}$) and apical (*Smoc2*$^{+}$/*Slc1a3*$^{+}$) domains; the latter was shown to contribute the PDL and alveolar bone in the root furcation region. The DP had four distinct domains at P3.5: odontoblasts (*Phex*$^{+}$/*Ifitm5*$^{+}$), coronal DP (*Enpp6*$^{+}$/*Fabp7*$^{+}$), middle DP (*Nnat*$^{+}$/*Rab3b*$^{+}$), and apical DP (*Aox3*$^{+}$/*Tac1*$^{+}$). Among them, the *Aox3*$^{+}$/*Tac1*$^{+}$ apical DP contained progenitor populations that give rise to odontoblasts and dental pulp cells. The study also highlights the critical role of cell–cell interactions mediated by IGF signaling in PDL development. *Igf1* was expressed in the DF, and its receptor *Igf1r* was widely expressed in the molar. PDL development was compromised by deletion of *Igf1* and *Igf1r* in the lateral and apical DF, respectively. Lastly, the study predicted potential gene regulatory networks (GRNs) in the dental mesenchyme derivatives by identifying cell type-specific regulons. The DF was associated with CREB family members at the cap stage (E14.5) and then with KLF members at the bell stage (E16.5), whereas the DP was associated with ELF members at the cap stage and then with DLX members at the bell stage. At P3.5, Fox family members were enriched in mouse molars; loss-of-function analysis with mouse genetics revealed that FOXP4, which was expressed in both the DP and DF, was crucial for proper differentiation of the PDL lineage. Thus, this study demonstrated how cellular domains were established in the dental mesenchyme and how their cell fates were determined during mouse molar development (Fig. 3).

Wang et al. delve into the dental mesenchyme in molar and incisor tooth development to uncover its developmental and odontogenic potential by performing scRNA-seq on the E10.5, E11.5, E12.5, E13.5, E14.5, and E16.5 mouse tooth germs, providing six key findings [21], as explained below. First, clustering analysis on the integrated scRNA-seq datasets revealed distinct developmental paths for dental epithelial and mesenchymal cells. Second, marker gene expression analysis suggested that incisor and molar development was predetermined before the bud stage by distinguishing epithelial-Fgf8/mesenchymal-Barx1 signals for molars and epithelial-Bmp4/mesenchymal-Msx1 signals for incisors; this finding supports the idea that tooth-type determination is a complex process involving interactions between the neural crest-derived mesenchyme and early dental epithelium [22]. Third, they noted that dental mesenchymal cells have distinct paths for incisors and molars during development, predicting GRNs underlying the fate determination. At E12.5, the specific regulons included *Hand1*, *Alx1/3*, and *Pax3* for the incisor mesenchyme and

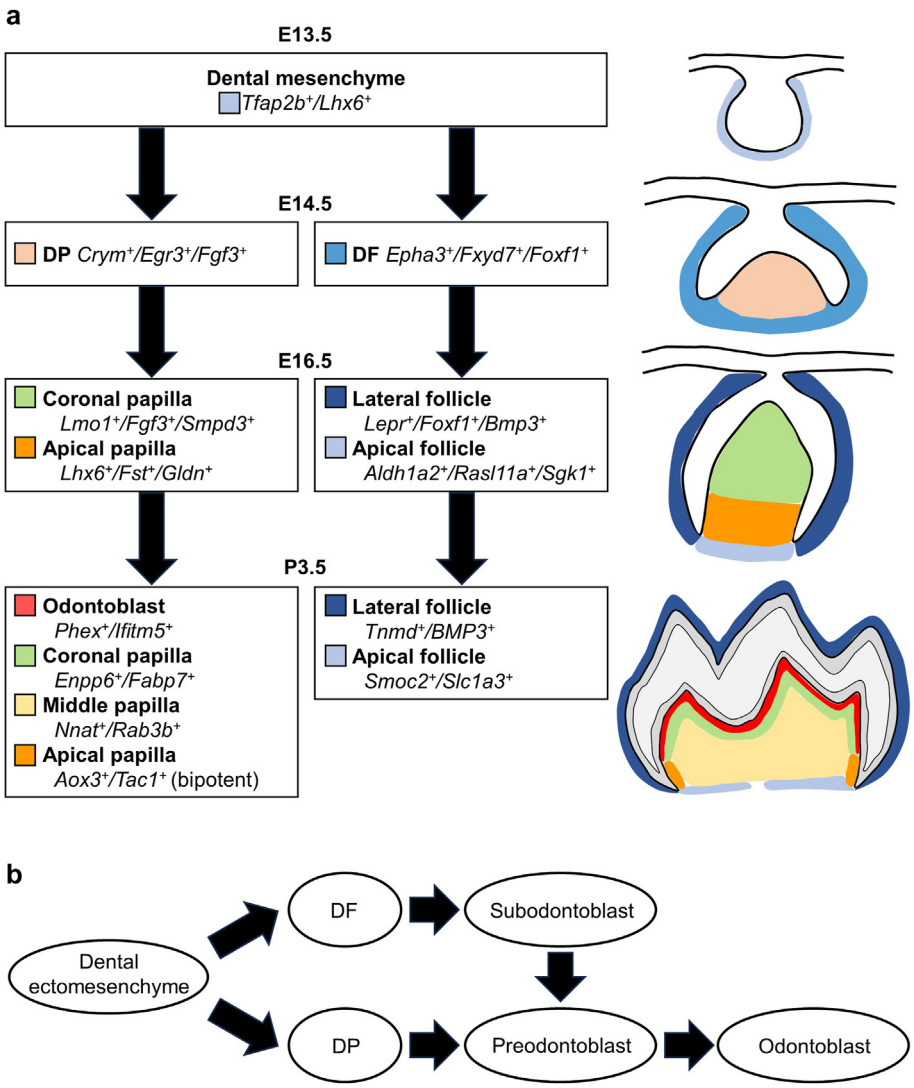

Fig. 3. Cellular dynamics and trajectory of the dental mesenchyme. (a) Cellular dynamics during mouse molar development, as presented by Jing et al. [20]. At the bud stage (E13.5), the dental mesenchyme is marked by *Tfap2b*$^+$ and *Lhx6*$^+$. Multiple lineage specification processes eventually generate the four DP domains and two DF domains until the crown-root transition stage (P3.5). Among them, the *Aox3*$^+$/*Tac1*$^+$ apical DP contains bipotent progenitor populations that give rise to odontoblasts and dental pulp cells. (b) Cellular trajectory proposed by Alghadeer et al. [18]. The dental ectomesenchyme generates the DP and DF. The DP is a major source of the odontoblast lineage. Interestingly, the DF is also predicted to be a source of subodontoblasts, which potentially differentiate into preodontoblasts.

Tbx15, *Lhx6*, and *Tfap2b* for the molar mesenchyme. Specific expression patterns of these regulons were confirmed in mesenchyme components of each tooth type by *in situ* hybridization. ATAC-seq (the assay for transposase-accessible chromatin with sequencing) analysis further suggests that distinct chromatin profiles underlie the tooth type-distinct regulon activities and marker gene expressions.

During tooth development, the odontogenic potential transits from the epithelium to the mesenchyme. The epithelium, known as the prebud epithelium, initially possesses the ability to initiate tooth formation, and the ability subsequently shifts to the mesenchyme, referred to as the postbud mesenchyme, at the bud stage [23]. In this regard, the fourth finding of Wang et al. was distinct sets of genes specific to each of the prebud epithelium and the postbud mesenchyme [21]. The prebud epithelium was characterized by known dental epithelial markers such as *Fgf8/9*, *Krt7/8/19*, *Lef1*, *Bmp4*, and *Shh*, along with genes associated with IGF signaling, Notch signaling, and various cellular processes including proliferation, migration, junction, and adhesion. The post-bud mesenchyme was characterized by *Fgf3*, *Fst*, *Dlx4*, *Igfbp3*, *Lgals7*, and *Loxl1*, which potentially play roles in FGF/Wnt-signaling pathways, insulin growth factors, cell adhesion, and the extracellular environment. Notably, the prebud epithelium genes are silenced at the postbud stage, and vice versa. These molecular differences highlight the contrasting characteristics between pre-bud epithelial cells and post-bud mesenchymal cells. Fifth, Wang et al. identified two GRNs regulated by *Barx1* and *Foxf1*, which possibly underlie the odontogenic potential of the postbud mesenchyme at the cap and bell stages, respectively. The two GRNs shared some genes related to osteoblast differentiation, dentin formation, and cell adhesion, but also had specific gene sets: *Barx1*-GRN was associated with Wnt signaling, ossification, and extracellular matrix organization, whereas *Foxf1*-GRN was associated with collagen fibril organization.

Lastly, they focused on the odontogenic abilities of *Cd24a* $^+$ cells and *Plac8*$^+$ ones, which were present in the upper DP and a top layer (preodontoblast layer) of the DP, respectively, in the E16.5 first molar germ (bell stage). In line with their previous report of CD24a$^+$ multipotent dental pulp regenerative stem cells (MDPSCs)

[24], the *Cd24a*$^{++}$ dental mesenchyme population, characterized by high expression of odontogenesis-related genes, showed tooth induction potential when combined with non-odontogenic epithelial cells; so did the *Plap8*$^{++}$ cells located in the top layer (preodontoblast layer) of the DP, when combined with a piece of the dental epithelium.

The DF is the condensed ectomesenchyme that surrounds the enamel organ and DP, playing a crucial role in tooth development and eruption [25]. The DF cells give rise to various cell types, including fibroblasts, osteoblasts, cementoblasts, and mesenchymal progenitors. A scRNA-seq study characterized the DF cells; Takahashi et al. characterized parathyroid hormone-related protein (PTHrP)-expressing DF cells during tooth development and proposed critical roles of PTHrP signaling in the cell fate determination of DF cells [26]. They performed scRNA-seq on mCherry-positive cells isolated from developing molars of PTHrP-mCherry knock-in mice; the mCherry-positive cells represent the PTHrP-expressing cells in the developing teeth. They found distinct cell clusters in the PTHrP-expressing population: odontoblasts/DP cells, fibroblasts, epithelial cells, DF cells, and transitional cells between epithelial and DF cells [26]. The data indicate cellular heterogeneity of PTHrP-expressing cells. Importantly, the PTHrP-expressing DF cluster displayed co-expression of *Pthlh* and *Pth1r*, genes encoding PTHrP and its receptor, respectively, suggesting the presence of PTHrP-PPR autocrine signaling in the DF cells. Subsequent analyses support the idea that the PTHrP-PPR autocrine signaling determines the proper cell fate of mesenchymal progenitors in the DF.

A study by Alghadeer et al., which was discussed above, also dissected the dental mesenchyme and its derivatives in human tooth development [18]. Dental mesenchyme-derived cells were subclustered into six cell types: the dental ectomesenchyme, DF, DP, subodontoblasts, preodontoblasts, and odontoblasts, each of which was transcriptionally defined. Trajectory analysis predicted that the DP and DF, derived from the dental ectomesenchyme, are two potential progenitor sources; the DP gives rise to preodontoblasts and subsequently odontoblasts, while the DF is the source of subodontoblasts, which have a capacity to transit to preodontoblasts (Fig. 3). The multiplex ISH-based spatial mapping of the identified clusters revealed the presence of sparse DF-type cells in the early dental pulp at the cap stage (13 gestational weeks), and the presence of subodontoblasts intermingled with preodontoblasts beneath the odontoblast layer at the late bell stage (19 gestational weeks). These observations support the lineage prediction from trajectory analysis, further suggesting that subodontoblasts may serve as a reserve. These dental mesenchyme clusters were validated by comparison with datasets from human [12] and mouse [20] molars.

6. Identification of dental niche cells

Pitx2 and Msx1 are developmentally important transcription factors (TFs) and play important roles in tooth development, as supported by their expression patterns in the developing tooth [27–29]. Hu et al. identified dental niche cells by utilizing double-fluorescence reporter mouse lines (*Pitx2*$^{P2A\text{-}copGFP}$ and *Msx1*$^{P2A\text{-}tdTomato}$) and scRNA-seq [30]. They initially demonstrated that *Pitx2* and *Msx1* specifically labeled and tracked the dental epithelium and dental mesenchyme, respectively. Importantly, the *Pitx2*$^{+}$ dental epithelium and *Msx1*$^{+}$ dental mesenchyme were sufficient components for ectopic tooth germ reconstruction, although their abilities were lost postnatally.

scRNA-seq at five developmental stages (E12.5, E14.5, E16.5, P1, and P7) identified five clusters in the *Pitx2*$^{+}$ epithelial cells and eight clusters in *Msx1*$^{+}$ mesenchymal cells. Within the *Msx1*$^{+}$ cell clusters, the dental niche cells were marked by the expression of *Cdkn1c*, *Postn*, and *C1qtnf3*, whereas the DP was marked by *Enpp1* and *Sdc1*. Intriguingly, these niche cells were found to be at an earlier progenitor stage compared to DP cells. Among the *Msx1*$^{+}$ populations, they next distinguished *Msx1*$^{+}$;*Sdc1*$^{-}$ or *Msx1*$^{+}$;*Enpp1*$^{-}$ dental niche cells from *Msx1*$^{+}$;*Sdc1*$^{+}$ or *Msx1*$^{+}$;*Enpp1*$^{+}$ DP cells. Notably, when combined with primary dental epithelial cells, the sorted dental niche cells demonstrated a greater capacity for reconstituting the tooth germ compared to the DP cells in both *in-vivo* kidney capsule and *in-vitro* organoid systems, highlighting the central role of the dental niche cells in the epithelial–mesenchymal interaction in tooth development.

Clustering and RNA velocity analyses of E12.5 scRNA-seq data suggested that *Msx1*$^{+}$;*Sox9*$^{+}$ mesenchymal cells were central in the differentiation into DP precursors and other niche cells. A lineage tracing experiment using *Sox9*$^{IRES\text{-}CreERT2}$*;tdTomato* mice supported that the *Msx1*$^{+}$;*Sox9*$^{+}$ dental niche cells, which first appeared in the dental niche at E12.5, directly contributed to the later stages of DP development and had the capacity to reconstruct teeth. Lastly, identification of a unique set of TFs activated in E16.5 *Msx1*$^{+}$;*Sox9*$^{+}$ dental niche cells suggested that the cells activate the tooth morphogenesis-related GRN upon their migration into the DP region. Overall, this study provides valuable insights into dental niche cells, which potentially govern the epithelial–mesenchymal interaction in tooth development.

7. Cellular characterization of the human dental pulp

The dental pulp, a specialized stromal tissue sealed in the pulp cavity, provides the microenvironment responsible for development, repair, and nutrient supply in teeth [31]. The tissue houses a diverse array of cells, including pulp cells, odontoblasts, mesenchymal cells, pericytes/endothelial cells, and immune cells, whose interactions have remained unclear.

By employing scRNA-seq, Yin et al. showed the complex cellular composition within the dental pulp and shed light on the intricate interactions among the cell types in this milieu [31]. They analyzed 12,114 cells extracted from human premolars for orthodontic treatment and identified 20 cell clusters in the normal pulp, encompassing diverse cell populations such as neural cells, red blood cells, immune cells, endothelial cells (ECs), glial cells, pulp cells, and dental pulp stem cells (DPSCs). They found several features of the distinct populations present in dental pulp. First, variations in proliferation activities were observed among clusters, with ECs and DPSCs exhibiting higher S-phase ratios. Second, ligand-receptor analysis suggested that pulp cells were key communicators, showing numerous ligand-receptor connections with other cell types, whereas T cells had limited interactions. Third, immune cells such as CD8^{+} T cells and M1 macrophages were present; the T cells were likely to communicate with macrophages and ECs. Fourth, DPSCs were classified into three clusters and characterized by the broad expression of *BGN*, *SOX4*, and *JAG1*, which were involved in stem cell activation and bone marrow MSC differentiation; cell communication analysis predicted that DPSCs most closely communicated with pulp cells, and also communicated with ECs, T cells, and DPSCs themselves, through several ligand-receptor interactions. Fifth, pulp cells demonstrated two distinct subtypes marked by the expression of a set of genes related to cell morphogenesis and differentiation. Lastly, ECs were predicted to closely communicate with pulp cells; key ligands and receptors were predicted for the communication. Thus, the study underscores the pivotal roles of pulp cells and DPSCs in orchestrating intercellular communication and maintaining tissue homeostasis within dental pulp.

Pagella et al. investigated gene expression in both the dental pulp and the periodontium by utilizing human third molars [32].

They obtained scRNA-seq data of 32,378 dental pulp cells and identified 15 distinct cell clusters, including MSCs, fibroblasts, odontoblasts, ECs, Schwann cells (ScCs), immune cells, epithelial-like cells, and erythrocytes. They characterized MSCs by the expression of *FRZB, NOTCH3, THY1*, and *MYH11* and found their localization around vessels and sub-odontoblastic areas. They also obtained scRNA-seq data of 2,883 cells from fresh periodontal tissues and identified 15 clusters that exhibited similarities to the dental pulp populations [32]. MSCs in periodontal tissues were characterized by the expression of the same set of markers as that in dental pulp, leading Pagella et al. to compare MSC populations between the two tissue types. MSCs from the two tissues showed similar gene expression profiles with some exceptions: Periodontal MSCs were characterized by higher expression of *CCL2*, collagen genes, and *SPARC* (osteonectin), whereas dental pulp MSCs showed higher expression of *CXCL14*, *RARRES1*, and *KRT18*. In a comparison of the distinct MSC niches between the dental pulp and periodontium, they found differences in the proportion of some cell types, especially fibroblasts and epithelial sections. Dental pulp had abundant fibroblasts, whereas the periodontium had fewer. Expression of collagen and MMP genes was higher in periodontal fibroblasts and MSCs compared to the dental pulp. Periodontium showed larger proportion of cells expressing epithelial markers such as *KRT5* and *KRT14*. In a comparison of cell types, ECs, erythrocytes, and MSCs showed the greatest similarity between the periodontium and dental pulp. They did not find major difference in the dynamics and differentiation trajectories of MSCs between the dental pulp and periodontium.

Lee et al. focused on human DPSCs (hDPSCs) and periodontal ligament stem cells (hPDLSCs) in adult human premolars [33]. They isolated hDPSCs and hPDLSCs from human premolars and confirmed their trilineage differentiation potential and stem cell marker expression; 6,530 hDPSCs and 3,341 hPDLSCs were subjected to scRNA-seq, leading to identification of three subpopulations with distinct gene expression profiles in each cell type. hDPSC clusters demonstrated osteogenic and odontogenic features, along with the expression of nerve cell-related genes, whereas hPDLSC clusters expressed myofibroblast-related genes and osteoblast-related genes. They also predicted divergent differentiation fates of the two populations; hDPSCs showed higher neurogenic and endogenic scores, while PDLSCs showed higher osteogenic, chondrogenic, and vascular endothelial growth factor family expression scores. These findings underscore the distinct characteristics and potentials of hDPSCs and hPDLSCs. Related to this work, Liang et al. identified a mouse skeletal stem cell (SSC) [34]-like population, which were characterized by $CD45^{-}Ter119^{-}Tie2^{-}CD51^{+}Thy^{-}6C3-Cd105^{-}CD200^{+}$, in PDL and dental pulp tissues of mouse incisors [35]; they also identified a human SSC [36]-like population in those tissues of human molars. The SSC-like cells showed cementogenic, odontogenic, and chondrogenic potentials as well as clonogenic capacities.

Pericytes are smooth muscle-like cells surrounding blood vessels in multiple organs. Pericytes have been shown to be a source of MSC precursors, eventually giving rise to mesodermal derivatives such as osteoblasts, odontoblasts, and myoblasts [37–39]. In this context, Yianni and Sharpe proposed an immunomodulatory role of dental pulp pericytes through bulk RNA-seq on a sorted pericyte population and scRNA-seq analyses in mouse incisor and molar pulp [40]. The RNA-seq analysis revealed that the transcriptional profiles were largely similar between molar and incisor pericytes, but immunological pathway-related genes were enriched in genes upregulated in the former; in particular, cytokine and chemokine signaling-related genes, such as *Ccl2/3/4/7/22* and *Cxcl1/2/9/10/12*, showed strong enrichment. The expression of cytokine/chemokine in molar pulp pericytes was also confirmed by scRNA-seq analysis. The analysis also identified subpopulations of the dental pulp pericytes; both molar and incisor pericytes were classified into three clusters, which potentially represented mature and immature pericytes. Lastly, *Tagln* was highly expressed in the dental pulp pericytes independently of tooth types (molars and incisors) and pericyte subpopulations (mature and immature cells). Yianni and Sharpe found that Sm22, which is encoded by *Tagln*, worked as a marker for the pericytes that had a capacity to give rise to odontoblasts.

8. Identification of molecular mechanisms underlying tooth root formation

The transcription factor Runx2 is crucial in tooth development, particularly in root formation [41]. By integrating bulk RNA-seq and scRNA-seq analyses, Wen et al. identified critical factors acting downstream of Runx2 in tooth root development [42]. They first found that *Runx2* expression overlapped with a subset of $Gli1^{+}$ cells, which are progenitor cells involved in tooth root development, and that progeny of the $Gli1^{+}$ cells contributed to the structure of the tooth root [41]. Deletion of the *Runx2* gene in $Gli1^{+}$ cells (*Gli1-CreERT2;*$Runx2^{flox/flox}$ mice) led to severe defects in root development, including shortened roots, thin root dentin, impaired odontoblast differentiation, and deficient formation of the periodontal ligament, cementoblasts, and alveolar bone. These data suggest that Runx2 plays indispensable roles in the differentiation of $Gli1^{+}$ progenitors during root formation.

By bulk RNA-seq, they identified differentially expressed genes in the apical halves of developing molars of *Gli1-CreERT2;*$Runx2^{flox/flox}$ mice compared to those of control mice. Integration of the bulk RNA-seq data and scRNA-seq data of whole molars of *Gli1-CreERT2;*$Runx2^{flox/flox}$ and control mice revealed that a set of genes enriched in a subset of DP cells were downregulated in *Gli1-CreERT2;*$Runx2^{flox/flox}$ molars; the subset was likely to be associated with root formation, as apical dental mesenchyme-related genes were expressed. Among the candidates for Runx2 targets, they focused on the Wnt inhibitor NOTUM and showed its involvement in the Runx2 action on root development.

9. Characterization of the periodontal ligament

The exploration of the PDL at the single-cell level has highlighted critical roles of several cell populations and molecules in the development, maintenance, and regeneration of the PDL. Gong et al. focused on the involvement of $Prrx1^{+}$ cells in development and regeneration of the molar PDL [43], given that $Prrx1^{+}$ populations were shown to participate in skeletal development and repair as well as PDL regeneration in mouse incisors. In development of mouse molars, the $Prrx1^{+}$ cells and their progeny are present within the dental mesenchyme, differentiating into odontoblasts and DP cells during root formation [43]. Turning to humans, scRNA-seq analysis of adult human molars revealed a comparable population of $PRRX1^{+}$ cells within the periodontal cells. One $PRRX1^{+}$ cell cluster showed the expression of MSC markers and perivascular markers, whereas another $PRRX1^{+}$ cluster revealed the expression of fibroblast signatures, suggesting that $PRRX1^{+}$ cells overlap with perivascular cells in the PDLSCs as well as PDL fibroblasts. *In vivo* lineage tracing in adult mice and *in vitro* loss-of-function studies support the potential involvement of PRRX1 and $PRRX1^{+}$ cells in facilitating blood vessel formation. Indeed, allograft tooth transplantation experiments in mice revealed that $PRRX1^{+}$ cells work as pericytes that participate in angiogenesis during PDL reconstruction. This series of data supports the findings by Bassir et al. [44], which demonstrated critical roles of $PRRX1^{+}$ cells in maintenance and regeneration of the PDL.

Another player in the PDL revealed by scRNA-seq is the transcription factor mohawk homeobox (Mkx), which has been shown to be crucial for both development and maintenance of the PDL [45]. A scRNA-seq analysis in the rat PDL illuminated the diverse range of cell populations coexisting within this dynamic tissue: pericytes, erythrocytes, macrophages, and osteoblasts, as well as various mesenchymal, epithelial, vascular endothelial, and neural cell types [46]. Of particular interest, the transcription factors *Mkx* and scleraxis (*Scx*), the latter of which is critical for ligament and tendon biology [47,48], exhibit distinct expression patterns within different MSC subclusters in the PDL; *Scx* was abundant in collagen-producing cells, whereas *Mkx* was abundant in cells producing oxytalan fibers and proteoglycan. Remarkably, a comparison of the scRNA-seq data between WT and *Mkx*$^{-/-}$ rat PDL indicated that *Mkx* deficiency triggered upregulation of osteogenesis-related genes in MSCs and osteoblast clusters, enamel formation-related genes in epithelial cell clusters, and inflammatory genes in macrophage clusters. These data suggest that (1) Mkx has distinct roles from Scx in development and maintenance of the PDL, and (2) Mkx has roles in suppressing mineralization and inflammation in the PDL, which potentially lead to prevention of ankylosis and periodontitis.

Periodontal ligament-associated protein-1 (*Plap1*), also known as its official gene symbol “asporin” (*Aspn*), was shown to function as a negative regulator of cytodifferentiation and mineralization of PDL fibroblasts [49–51]. Iwayama et al. revealed the fate of *Plap1*$^{+}$ populations with cellular dynamics of the PDL through scRNA-seq in mice [52]. They first confirmed that *Plap1* was a specific marker of the PDL fibroblast lineage (periodontal ligament cells including stem/progenitor cells: PDLCs). Lineage tracing of *Plap1*$^{+}$ PDLCs indicated that they not only maintain the PDL homeostasis, but also differentiate into osteoblasts and cementoblasts. In scRNA-seq on adult mouse PDL, the authors found distinct cell clusters including stromal, immune, epithelial, mural, and endothelial cells; the PDL stromal cell clusters were further divided into subclusters, in which *Plap1* and *Ibsp* were expressed in a mutually exclusive manner. RNA velocity analysis on the PDL stromal cell subclusters suggests that a *Plap1*^{high};*Ly6a*^{high} cell cluster is the source of the variety of cell populations in PDLCs. Lastly, the authors showed the contribution of the *Plap1*$^{+}$ PDLCs in periodontal tissue repair by using a mouse model. Thus, this study pinpoints the *Plap1*$^{+}$ population at the top of the PDLC lineage hierarchy and its contribution to maintenance and repair of the PDL.

10. Comparison of dental pulps between healthy and carious teeth

The investigation of dental pulp samples at the single-cell level has provided a comprehensive understanding of the cellular landscape within healthy teeth, teeth with enamel caries, and teeth with deep dental caries [53]. Diverse cell populations, including fibroblasts, odontoblasts, endothelial cells, hematopoietic stem cells (HSCs), and immune cells, were observed across all of the tissue conditions. Notably, B cells and CD103^{+} dendritic cells (DCs) were found exclusively in teeth with deep dental caries, although immune cells were present in all samples with different frequencies, highlighting the potential roles of the two immune cell types in the disease context.

The deep dental caries group exhibited distinct gene expression patterns, characterized by the upregulation of pro-inflammatory, anti-inflammatory, and regenerative genes. Inflammation- and immune response activation-related genes were upregulated in fibroblasts, odontoblasts, and HSCs of the deep dental caries group. Anti-inflammatory and regenerative genes were also upregulated in odontoblasts of the group, suggesting the activation of specific pathways associated with immune responses and tissue repair.

Moreover, the study predicted cell-cell interactions in the dental pulp microenvironment under the deep caries condition, emphasizing the interplay between B cells, DCs, macrophages, and other cell types. Although several interactions between odontoblasts and immune cells were present regardless of the tooth conditions, the following two interactions were found exclusively under the deep caries condition: TIMP1-CD63 interaction between odontoblasts and myeloid cells and CCL2-ACKR1 interaction between macrophages and ECs. These intricate interactions provide insights into the dynamic cellular processes occurring in response to dental caries and its progression.

11. Conclusions and future perspectives

Cutting-edge single-cell technology has led to significant strides in our understanding of tooth development. The studies reviewed here elevated our comprehension of distinct cell types in teeth and periodontal tissues, emphasizing their functions and delicate relationships with greater precision.

Current treatment strategies for lost tooth structures involve the restoration by synthetic filling materials, dentures, or implants. They are largely functional, but often suffer some drawbacks due to their insufficient abilities to recapitulate the physiological state of dental tissues. Regenerative therapies have drawn attention as a solution to the issue. In the dental field, such therapeutic approaches include the use of synthetic materials [54,55], biological components [56–63], or even whole bioengineered teeth/tooth organoids [64–66] to recover dental structures.

In this context, molecular- and cellular-level understanding of the development process is crucial, allowing us to identify potential molecular targets for regeneration of dental tissues. As reviewed in this paper, single-cell-level characterization of dental cell types, their developmental trajectories, and key signaling pathways underlying the process offers insights into molecular signatures of stem/progenitor populations and microenvironment during tooth development. As shown by Alghadeer et al. [18], those insights indeed lead to the development of stem-cell-based protocols for recapitulating a process of tooth development. Such protocols could contribute to generating stem cell-based tooth organoids, which would be invaluable not only for replacing lost teeth *in vivo*, but also for studying molecular mechanisms underlying tooth-related genetic diseases and potential therapeutic targets for the diseases *in vitro*. Thus, single-cell transcriptomics will contribute to development of novel diagnostic tools and therapeutic strategies for dental pathologies.

Despite the breakthroughs, we need to consider the immense challenges in applying scRNA-seq to dental tissues. First, human and rodent teeth, with their limited number of viable cells compared to tissues such as the skin, demand curation of cells from several individuals to overcome the issue of sample size. Second, it is essential to recognize the substantial heterogeneity observed in the developmental stages of the tooth germ [17]. Samples obtained from different individuals may not comprehensively encompass the entire process of development and maintenance of dental tissues; rather, they may reflect individual differences, particularly in the case of human samples and dynamically remodeled tissues like the periodontium or sensitive tissue like the dental pulp [32]. These issues complicate research design, analyses, and data interpretation. Third, the process of tissue dissociation into single cells deprives the isolated cells of their positional information; without the positional information, annotating dental cells often presents a major obstacle due to a limited set of bona-fide marker genes that characterize each population. Thus, it is necessary to confirm the

histological location of cells identified by scRNA-seq. Fourth, the sample preparation procedure may preferentially capture cells that have survived during the procedures. Thus, the proportions of different cell types in a given scRNA-seq dataset may not reflect their actual proportions in tissues. Granulocytes, in particular, are often misrepresented in scRNA-seq datasets due to their heightened sensitivity to degradation [53].

Despite these challenges, the use of scRNA-seq in dental research is rapidly expanding. Combining diverse methods to study epigenetics, protein expression profiles, and cell metabolism could provide a more complete understanding of tooth development. Spatial transcriptomics is especially promising for identifying specific cell types with their positional information in tissues [67]. The capture of sensitive cells could largely benefit from Live-seq, which preserves the cell viability [68]. These strategies will furnish an enriched understanding of the cellular and molecular mechanisms driving the development and regeneration of teeth.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Shinsuke Ohba reports a relationship with the Japan Society for the Promotion of Science (JSPS), Naito Foundation, and Takeda Science Foundation that include funding grants. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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