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Transit amplifying cells coordinate mouse incisor mesenchymal stem cell activation

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Stem cells (SCs) receive inductive cues from the surrounding microenvironment and cells. Limited molecular evidence has connected tissue-specific mesenchymal stem cells (MSCs) with mesenchymal transit amplifying cells (MTACs). Using mouse incisor as the model, we discover a population of MSCs neighboring to the MTACs and epithelial SCs. With Notch signaling as the key regulator, we disclose molecular proof and lineage tracing evidence showing the distinct MSCs contribute to incisor MTACs and the other mesenchymal cell lineages. MTACs can feedback and regulate the homeostasis and activation of CL-MSCs through Delta-like 1 homolog (Dlk1), which balances MSCs-MTACs number and the lineage differentiation. Dlk1’s function on SCs priming and self-renewal depends on its biological forms and its gene expression is under dynamic epigenetic control. Our findings can be validated in clinical samples and applied to accelerate tooth wound healing, providing an intriguing insight of how to direct SCs towards tissue regeneration.
issue development and regeneration largely rely on stem cells (SCs) that give rise to multiple cell lineages. In a healthy tissue, proper activation of SCs and preservation of SC reservoir are rigidly balanced to ensure that accurate SC homeostasis and differentiation are in place. In most cases, SCs reside in a specialized and dynamic microenvironment, also called the SC niche, and receive feedback cues notably from their surrounding environment including those from neighboring heterologous cell populations. The signaling that maintains SC niche have been thoroughly investigated. Both Wnt and Notch pathways have been implicated in regulation of SCs within muscle, intestinal epithelium, and interfollicular epidermis. Various cell types or even terminal differentiated cells re able to provide feedback loops to SCs. For instance, in the intestinal epithelium, Paneth cells play a key role in SC niche determination. In hair follicle keratinocytes, the transit amplifying cells (TACs) can orchestrate SC activity and tissue regeneration through the Sonic Hedgehog (SHH) pathway.

TACs are committed and proliferative direct lineage of primed SCs. For most of the mammalian organs, upon the completion of development or regeneration, the TACs vanish and tissue growth stops. TACs induction, maintenance, and differentiation therefore must be rigorously controlled and directed by molecular signaling and cellular microenvironment. In the case of regeneration, SCs can also be re-activated to produce new TACs when the tissues of interest receive stimulating (activating) signals, such as molecular cues for regeneration released by wounded tissues.

To date, most research focuses on epithelial tissues, such as intestinal epithelium, which have lifelong persistent TACs, while in hair follicles, keratinocytes contain periodic TACs that reenter the cell cycle. Discovering how the signaling pathways control TACs to SCs communications is challenging, partially due to the fact that most of tissues do not possess sufficient amounts of SCs, TACs, and differentiated cell populations at the same time, and importantly in close vicinity. However, in the tissues involved in epithelial–mesenchymal interactions, while the epithelial SCs and TACs are often investigated, the neighboring mesenchymal compartment’s SCs and niche have been less documented.

Mammalian tooth development involves dynamic epithelial–mesenchymal interactions. The mouse incisors undergo lifelong growth as a result of epithelial–mesenchymal interactions. Each mouse incisor tooth has a persisting epithelial SC-TAC zone at its posterior end of the tooth, named cervical loop (CL) based on the epithelial structure. Specifically, the CL epithelium has a defined SC niche located at the middle of the enlarged head like structure where cells highly express markers such as Sox2, Bmi1, and Lgr. The neighboring shoulder and upper front body epithelium admit TACs (Supplementary Fig. 1a). The latter are highly positive for Ki67 and the other markers such as Prom1 and SHH. Next to the epithelial SCs, TACs form a cluster of Ki67-positive cells that connect the SCs with differentiating cells named pre-ameloblasts, the cells start polarizing and deposit enamel matrix at the junction of CL-MSCs and TACs (Fig. 1a, c). Facing the epithelial TACs, the incisor mesenchyme also has a distinct epithelial mesenchymal junction (Fig. 1a, c). The epithelium TACs, the incisor mesenchyme also has a distinct epithelial–mesenchymal junction (Fig. 1a, c). The epithelium TACs, the incisor mesenchyme also has a distinct epithelial–mesenchymal junction (Fig. 1a, c). The epithelium TACs, the incisor mesenchyme also has a distinct epithelial–mesenchymal junction (Fig. 1a, c).

Besides the population of MTACs, recent evidence has revealed a mesenchymal SCs (MSCs) niche around the perineurovascular bundle (NVB) region where cells have been found to be label retaining and potentially with Glia origin as they are positive for Plp promoter-driven Cre, and Gli1 Cre and Thy-1 Cre. The NVB-MSCs can release progenitors that participate in tissue repair and replenish the undifferentiated mesenchyme upon injury. However, little experimental evidence has been shown that the MTACs are direct derivatives of the NVB-MSCs. Furthermore, the NVB SC niche is not able to contribute all of the differentiated cells in the incisor pulp. Together this hints at the co-existence of another of MSC population(s) contributing as the precursors of MTACs. Determining the existence of such population of MSCs, and potentially a distinct niche, remains a challenge, as does defining how MTACs could be maintained and communicate with MSCs within the mouse incisor.

In the present study, we discover an MSC population associating with the mouse incisor CL region and show that the MTACs can feedback to and control those MSCs through the Dlk1, a Notch pathway ligand, which is important in inducing MSCs to MTACs transition, lineage differentiation, and tissue regeneration, a mechanism that can potentially be translated into regenerative medicine.

Results

**Mouse incisor tooth harbors a distinct MSC population.** Given the mesenchymal cells contacting the opposing CL epithelial SCs were also positive for Gli1 Cre and essentially those cells were immediate neighbors of the MTACs (Fig. 1c), we asked whether they represented a special population of mesenchymal cells. We first performed immunostaining using specific antibodies against Gli1, Thy-1, or PDGFβ on postnatal day 7 (P7) CD1 mouse lower incisors and found that they were highly expressed in those cells, as in the NVB-MSCs (Fig. 1c, Supplementary Fig. 1a). Unlike the NVB-MSCs, this population of mesenchymal cells was negative for Sca1 and CD106 (Supplementary Fig. 1a). These observations could be further confirmed by micro-dissection followed by flow cytometry analysis (Supplementary Fig. 1b). We next profiled the expression of a panel of classical MSC and TAC markers, using laser capture microdissection (LCM; Supplementary Fig. 1c) and real-time RT-PCR (Fig. 1d, e). The results showed that indeed the population of mesenchymal cells highly expressed most of the general MSCs markers in mRNA levels, such as Ccdn3, Cdkn1a, SmarcA2, and Zbtb20 (Fig. 1d). Meanwhile the MTACs displayed a group of distinctly different markers such as Anhh, Cnca2, and Top2a (Fig. 1e). Conversely, NVB-MSCs expressed most of the MSC, and also MTAC markers (Fig. 1d, e), therefore, could not be molecularly dissected from MTACs based on mRNA expression (Fig. 1e). We thus named the MSCs population as CL-MSCs, in comparison to the NVB-MSCs.

We next performed additional immunofluorescent analysis and confirmed that SmarcA2 and Zbtb20 were two robust CL-MSCs markers (Fig. 1f). Essentially, SmarcA2 showed transitional expression overlapping with Ki67 at the junction of CL-MSCs and MTACs regions (Fig. 1g), which further hinted the linkage between CL-MSCs and MTACs. Similar to the flow cytometry data, CL-MSCs were found positive for CD73 and CD29 by immunofluorescent analysis (Supplementary Fig. 1d).

It has been reported that NVB-MSCs have a critical role in incisor homeostasis. To evaluate which MSC population could support the growth of CL, we performed CL organ culture (Fig. 2a). Interestingly, we found the NVB structures as observed by CD106 and Neural Filament staining had a short life in vitro, i.e. less than 3 days (Fig. 2b, c). Quite the contrary, the incisor CL-MSCs still persisted and expressed markers such as Thy-1 (Fig. 2d), and connected to the MTACs that are positive to Ki67 (Fig. 2e), therefore, contributed to the CL growth ex vivo (Fig. 2a–e).

**CL-MSCs are multipotent progenitors of odontoblasts.** To explore if CL-MSCs were the progenitors of odontoblasts,
Fig. 1 Identification of the CL-MSCs in the mouse incisor tooth. a Whole view of one P7 mouse lower incisor. Black arrowheads show approx. boundaries of indicated epithelial cells zones: ESCs epithelial stem cells, ETACs epithelial transit amplifying cells, MTACs, mesenchymal transit amplifying cells, and dentin layer. Light blue dotted line indicates epithelial-mesenchymal junction. b Stereo front view of a P7 incisor CL epithelium (left) and arbitrarily colored indexes for the ESCs (purple) and ETACs (green) regions (right). c Immunofluorescence analysis of indicated antigenic markers in P7 incisors. Nuclei were counterstained with DAPI. Light blue dotted line shows epithelial-mesenchymal junction while yellow arrowheads indicate the mesenchymal cells named CL-MSCs. NVB neurovascular bundle region. d, e Gene expression profiling using real time RT-PCR analysis on laser captured MTACs, CL-MSCs, and NVB-MSCs from $n=5$ biologically independent animals, using two panels of general MSCs (d) and MTACs (e) markers. Triplicated samples were used for each gene. Error bars represent standard deviation. Statistical analysis was performed with two-way ANOVA followed by Bonferoni correction. No asterisk: $p > 0.05$; one asterisk: $p < 0.05$; two asterisks: $p < 0.01$. f, g Immunofluorescence analysis using indicated markers. In g, squared region in the right panel is enlarged in the left one. Note the overlapped expression of SmarcA2 and Ki67 at the junction of CL-MSCs and MTACs. Light blue dotted line shows epithelial-mesenchymal junction while yellow arrowheads indicate the mesenchymal cells named CL-MSCs. Bars: a: 100 μm; b, c, f, g (left panel): 40 μm; g (right panel): 10 μm
differentiated tooth mesenchymal cells responsible for dentin formation, we performed chimeric-like tissue culture by recombinating freshly isolated CL-MSCs with devitalized dentin slice (Fig. 2f, g). We observed a rapid polarization of the CL-MSCs by producing branched protrusions into the dentin slices after only 4 days in culture (Fig. 2h, i). At day 7, while the tooth pulp cells only retained on the surface of the dentin slices (Fig. 2j), the CL-MSC-derived cells fully polarized and secreted Dentin Sialo Protein (DSP) and Dentin Matrix Protein 1 (DMP1) (Fig. 2k).

The application of SCs in tissue engineering and regenerative medicine often requires overcoming the major challenge of amplifying the cells in vitro without losing their pluripotency and...
differentiation capabilities. We established four independent lines of primary CL-MSCs and maintained them in culture in vitro for up to four passages using defined culture conditions (Supplementary Fig. 2a, b). Cultured CL-MSCs maintained MSC marker expression (Supplementary Fig. 2c). Cells responded to nutrient starvation and replenishment conditions by expressing MSC and MTAC markers, respectively (Supplementary Fig. 2d). Importantly, when co-cultured with dentin slices under differentiation conditions, even the passage 4 cells could give rise to odontoblast-like cells by forming protrusions into the dentin tubes and secreting DSP and DMP1 (Supplementary Fig. 2e). Additionally, we could confirm that in vitro, the CL-MSCs could also differentiate into adipocyte-, osteoblast-, and chondrocyte-like cells under proper differentiation conditions (Supplementary Fig. 3).

To follow the CL-MSCs fate, we traced and differentiated incisor MSCs. Based on our molecular marker profiling (Fig. 1c, Supplementary Fig. 1a), we adopted a lineage tracing strategy by crossing PDGFRβ Cre ERT2 mice with ROSA mT/mG mice (Fig. 3a). By injecting tamoxifen at P0 and 1, we could then trace the labeled cells persisting after birth. At P7, we found that most of the CL-MSCs and MTACs were labeled, and continuous labeling along CL-MSC and MTAC axis was also observed (Fig. 3b). Clusters of labeled cells were also detected within the odontoblast layer (Fig. 3b). At P30, when incisors have already erupted and functioning, the labeling was still persisting in the CL-MSCs, and again remained positive up to MTACs (Fig. 3c). It is noticeable that at P30 in the CL epithelial–mesenchymal junction, there was still an abundance of cells at the MTAC region (Fig. 3c, d). Consistent with the findings and similar to P7, CL-MSCs still express MSC markers such as PDGFβ, Thy-1, SmarcA2, and Zbtb20 at P30 (Fig. 3e–h). Altogether our data suggest that the population of the mesenchymal cells is indeed a distinct MSC population, which clearly contrasts to the MTACs but are different from the NVB-MSCs.

CL-MSCs and MTACs have contrasted Notch pathway profiles. Notch is a key signaling pathway in SC fate determination and niche maintenance in a number of biological systems such as nerve, muscle, mammary gland epithelium, and interfollicular epidermis. In murine incisor tooth, little information has been documented about Notch signaling activity although some data have revealed the expression of Notch1, 2, and 3.
mRNA therein. We therefore first evaluated the Notch activity in our system. Using Transgenic Notch Reporter (TNR) mice where enhanced green fluorescent protein (EGFP) is driven by four RBP-Jκappa binding sites and a minimal SV40 promoter and double immunofluorescent staining with an antibody directed against intracellular domain (ICD) of Notch1, we found that CL-MSC region harbored a high level of Notch signaling activity where a thin layer of cells located about 20 μm above the CL epithelial SC region was Notch1 ICD/EGFP-double positive (Fig. 4a). The MTAC region showed also positivity, yet weaker (Fig. 4a). Consistently, the mRNA expression of Notch1 and Notch2 gene products as well as their downstream molecular targets, i.e. Hes1, Hes5, Hey1, Hey2, and Heyl were significantly higher in the CL-MSCs than the MTACs as observed by real-time RT-PCR profiling (Fig. 4b). In contrast, Notch 2 ICD and Hes1 protein expression were found higher in MTACs than CL-MSCs (Fig. 4c), while Notch1 and its ICD expressions did not show major differences (Fig. 4a, c). The expression of Notch3 was restricted to endothelial cells present in the mesenchyme (Fig. 4c). Finally, the analysis of labial enamel–dentin junction in the tooth pulp revealed that Notch1 ICD was highly expressed in differentiated odontoblasts while Notch2 ICD was higher in the adjacent undifferentiated pulp cells (Fig. 4d). The Notch activation, illustrated by EGFP expression, was strong in both regions (Fig. 4d).

Loss of Notch causes MTACs premature differentiation. To understand the function of the Notch pathway in the CL-MSCs and MTACs, we crossed Collagen1a2 Cre with Rosa26R-LacZ reporter mice and found the LacZ staining specifically labeled some CL-MSCs cells but was absent from the incisor pulp and NVB-MSCs (Fig. 5a). We next investigated the consequence of losing Notch signaling on the CL-MSCs and subsequently in their descendants in the Collagen1a2 Cre × RBP-Jκappafl/fox mice. We performed immunostaining of DSP and DMP1 and found that unlike the wild-type (WT) mice, where DSP and DMP1 were mainly restrictedly expressed in the odontoblast layer, in the Collagen1a2 Cre × RBP-Jκappafl/fox mice the two proteins’ expression levels were highly elevated in the dental pulp cells (Fig. 5b). Particularly in those near odontoblast layers and DSP and DMP1 also started to be expressed by the MTACs (Fig. 5b). Hence losing RBP-Jκappa promoted early differentiation of MTACs. Consequently, we observed the mutant mice’s dentin consisted of two layers of tissue, an outer layer with relative normal dentin structure and an inner layer with highly disorganized mineralized tissues, further confirming an enhanced...
premature differentiation (Fig. 5c). Scanning electronic microscopy showed that in the mutant mice’s incisor’s second layer of dentin is a smear of disorganized dentin structure with ultrastructures entirely altered (Fig. 5d). Concurrently, the MTACs of the Collagen1a2 Cre × RBP-Jkappa^fl/fl^ mice was significantly reduced as well as the cell number of CL-MSCs (Fig. 5e). The finding that DSP and DMP1 were abnormally elevated in the absence of RBP-Jkappa in the MTACs and pulp cells suggested the possibility of RBP-Jkappa in performing a suppression role on the translation of DSPP and DMP1 genes. We then investigated the promoters of the two genes in a tooth pulp-derived cell line, MO6-G3 cells^35^, with chromatin immunoprecipitation (ChIP)
and confirmed that indeed the mouse DSP and DMP1 promoters harbored functional RBP-Jκappa-binding sites (Fig. 5f).

Therefore, these findings revealed that interrupting Notch signaling by deleting RBP-Jκappa in the CL-MSCs results in MTACs premature differentiation and abnormal dentin formation.

**Delta like 1 homolog (Dlk1) regulates MSC self-renewal.** To understand how the Notch signaling is modulated in different cellular compartments of the incisor mesenchyme, we next profiled Notch ligand expression by immunofluorescence and real-time RT-PCR analyses and found that Dlk1 was the only molecule that was exclusively expressed in the MTACs in the mesenchyme but not in the CL-MSCs (Fig. 6a–c). Dlk1 was also detected in pulp cells and odontoblasts, but weaker by comparison to MTACs (Fig. 6a–c). We also noticed epithelial SCs and TACs also expressed Dlk1 but again at a lower level comparing to the MTACs (Fig. 6a). The findings suggested that Dlk1 might have distinct roles in incisor MSC lineage patterning and differentiation. To determine whether Dlk1 is a key molecule in the incisor MSCs, we analyzed Dlk1 knockout (KO) mice36. Using Ki67 as the MTACs marker and SmarcA2 as the CL-MSCs marker, we then observed significantly reduced MTACs and increased CL-MSCs numbers (Fig. 6d–f). Hence in the absence of Dlk1, CL-MSCs’ activation was impeded. In the meantime, we also observed MTACs and tooth pulp cells starting expressing DSP and DMP1, suggesting a potential early differentiation (Fig. 6h), similar to the observation from the Collagen 1 α2 Cre × RBP-Jκappa conditional flox/flox mice (Fig. 5b). Micro CT analysis further confirmed that the dentin or dentin like structures of the Dlk1 KO mice was thicker than the WT mice (Fig. 6i). Therefore, losing Dlk1 could mirror tooth defects of canonical Notch null mice.

As a potent Notch ligand, Dlk1 can act either as a cell membrane-bound form or as a secreted one. Given the lack of endogenous Dlk1 expression in CL-MSCs, the latter can receive signals either as soluble form from MTACs and/or epithelial SCs, or as membrane-bound form through cell–cell contact with the neighboring epithelial SCs. To discern whether Dlk1 act on quiescent SC activation as a membrane bound or soluble form (or both), we grew MO6-G3 cells either on the Dlk1-coated dish or by adding soluble Dlk1 directly to the media. The effects of bound and free forms of Dlk1 differed: while bound form Dlk1 increased expression of the MSC markers (Fig. 7a), the free form increased those of MTAC marker expression (Fig. 7b). Also, soluble Dlk1 form enhanced cell growth enhanced cell growth (Fig. 7c).

To validate that Dlk1 has the function to induce SCs to exit quiescence, we introduced a cell cycle indicator system: a modified Ki67p-T2A-FUCCI system (Fig. 7d)37,38 into the MO6-G3 cells. The system allowed us to distinguish cell cycle phases being represented by different colors, and more importantly could facilitate the segregation of quiescent (G0, colorless) cells from those that have committed to cell cycle entry i.e. G1/early S; mCherry-hCdt positive or late S/G2/M mAG-hGem positive (Fig. 7e). With our system, we applied a classic cell cycle starvation and re-entry experiment. Under serum starvation for 4 days most cells entered G0 with reduced numbers in G1. The trend could be reversed by re-introducing serum (Fig. 7f). Therefore, with such setting, we observed adding Dlk1 onto the quiescent MO6-G3 cells could sufficiently trigger quiescent cells to enhance proliferation, i.e. to enter cell cycle.

To validate the significance of Dlk1 in the incisor teeth, we adopted adult mouse incisor clipping model25, which allowed us to trace the molecular changes of the stem cells upon stimulation. We first confirmed that in adult incisors, Notch1 and Notch2 expression remained the same as P7 (Supplementary Fig. 4a, Fig. 4c). After clipping, incisors grew faster than the control side (Supplementary Fig. 4b), with increased Dlk1 expression at the MTACs (Supplementary Fig. 4c). Together, using in vivo and in vitro approaches, we conclude Dlk1 is a significant molecule in CL-MSCs maintenance and activation.

**Dlk1 CpG islands are dynamically methylated.** Dlk1 is one of the known imprinted genes39,40 that marks early mesenchymal precursors during embryonic development41. It also has a unique role in the other systems such as self-renewal status maintenance of MSCs42,43. How Dlk1’s activation and deactivation are controlled at epigenetic level has not been explored. Dlk1 has two CpG islands, one at the 5′ beginning and the other at the 3′ end of the gene (Fig. 7g). We found in the incisor CL, two histone marks: H3K9me3 and H3K27me3 were both positive in CL-MSCs but controlled at epigenetic level has not been explored. Dlk1 CpG islands are dynamically methylated.

**Dlk1 regulates MSC lineage differentiation.** The effective role of Dlk1 in incisor MSCs activation and preservation prompted us to explore the possibility of translating the findings into potential regenerative medicine applications. The mouse and rat molar teeth are similar to human teeth in terms of development, biological structures, and pathological reactions. Unlike incisors, mouse molar tooth growth stops after the completion of root development and the tooth epithelium vanishes. However, the molar tooth’s pulp, a mesenchymal tissue sharing similar embryonic origin as incisor mesenchyme, has an apical bud region that contains the MSCs that can contribute to limited regeneration of dentin upon injury or in caries44. We found the
molar pulp apical bud cells also expressed SmarcA2 and Notch1 while the connecting differentiated odontoblasts expressed Notch2 and Dlk1 (Fig. 8a), suggesting that the molar MSCs might share with those of the incisor the same regulating molecular cascades. We therefore tested the capability of Dlk1 in inducing MSC lineage differentiation in the molar teeth using tooth germ organ culture. Consistently, in the presence of Dlk1, we observed the treated tooth germs received enhanced dentin deposition (Fig. 8b). In the MO6-G3 cells, Dlk1 could also induce the mouse DSPP gene’s 1.5 kb promoter expression in a dose-dependent manner (Fig. 8c).

We next analyzed the effect of Dlk1 overexpression on incisor and molar teeth using overexpressing Dlk1 in mesenchymal cells under rat Collagen I α1 promoter (we named as Col-Dlk1 Tg mice here). In adult mice, we observed the pulp of the Col-Dlk1 Tg molar teeth were entirely filled with dentin like structures (Fig. 8d), while in the incisor tooth we observed the CL-MSCs marker: SmarcA2 was significantly increased in the CL.
mesenchymal cells. In fact, almost the entire MTAC region was filled with Smarca2 positive cells (Fig. 8e) while the Ki67-positive cells were highly reduced (comparing Fig. 8e with Fig. 6d). In the Col-Dlk1 Tg mouse incisors, we also observed abnormal extra dentin formation (Fig. 8f, g). Together we proved Dlk1 is indeed a very potent factor in promoting MSC lineage differentiation and SC preservation. However constitutively expressing Dlk1 exhausted the MSCs pool at least in the molar teeth, but in the incisors Dlk1 overexpression enhanced CL-MSCs presence in parallel to exhausting the TAC pool. The different consequence of Dlk1 on incisor and molar teeth particularly in the Col-Dlk1 Tg mice could be explained by the fact the epithelial–mesenchymal interactions at the incisor CL region could maintain incisor progenitor cells; however, in the fully developed molar where the tooth epithelium was absent, Dlk1 functioned primarily as a trigger for the final differentiation of MSCs.

Translational application. We then strived to translate our findings in the mouse teeth into regenerative medicine. We found in the human teeth, under caries conditions (Fig. 9a), the site adjacent to the lesion showed that Notch2 ICD was initially expressed by the odontoblasts then the expression ceased (Fig. 9b). However, Dlk1 was always expressed by the odontoblasts or odontoblast like cells (Fig. 9b). Around the calcified pulp stone that represents abnormal tooth pulp cell differentiation and calcification, we also observed strong Notch2 ICD and Dlk1 expression (Fig. 9c). Hence the results suggest that Dlk1 has a potentially key role in the tooth pulp repair and regeneration. In vitro, in two independent primary human tooth pulp cell lines, we observed that Dlk1 could significantly induce DSPP and DMP1 mRNA expression in a dose-dependent manner (Fig. 9d). Finally, in an experimental tooth wound healing experiment (tooth pulp capping), where the rat molar pulp chamber was opened and covered either with a calcium hydroxide composition (Dycal®), a widely used clinical tooth pulp capping (covering the wound) regent or Dycal plus Dlk1 (Fig. 9e), after 14 days, we observed the Dlk1-treated tooth pulp underwent significant reparative dentin formation compared to the control (Fig. 9f). In addition, the Dlk1-treated pulp expressed higher DSPP and DMP1 (Fig. 9g). Therefore, Dlk1 is able to enhance tooth pulp healing through enhancing MSCs lineage differentiation and proliferation.

Discussion
A given SC niche has a specific location and composition within an individual tissue. It is generally accepted that solely one niche, or one kind of niche, exists in a tissue which can contribute to its homeostasis. Recently, search of SCs in the context of tissue regeneration has challenged this concept and highlighted the co-existence of distinct, and may be complementary niches within a tissue. The identification of intrinsic and extrinsic signals involved in SC activities could provide additional approaches with a therapeutic benefit. With this in mind, we report here that the mouse incisor tooth mesenchyme harbors a second niche named CL-MSC, besides the previously discovered NVB-MSC niche. CL-MSC region has a unique anatomical organization with a diameter of approximately 50–100 μm, which surrounds the epithelial CL structure.

Why does a mouse incisor mesenchyme need two distinct MSC niches? Given that both of them have distinct anatomical locations: CL-MSC niche directly contacts epithelial SCs, while NVB-MSC niche is associated with nerve-blood vessel bundles, they might respond differently, and independently, to extracellular cues such as NVB and epithelial signals. Although the CL-MSC and NVB-MSC niches have no sharp physical boundary, they share nonetheless the expression of similar SC marker such as Thy-1 and PDGFRβ. They are both positive for Gli1 Cre and neighbor their associated tissues separately: the NVB and the incisor tooth epithelium, the tissues both produce SHH.

While NVB-MSCs mainly contribute to the replenishment of injured incisor pulp, CL-MSCs, on the other hand, are more responsible for giving raise to endogenous cell lineages such as LTACs and odontoblasts, which require a dynamic crosstalk with the tooth epithelial SCs, TACs, and lineage differentiated cells such as pre- and mature ameloblasts. It is possible that CL-MSCs and NVB-MSCs originate from the same progenitors during development, but acquire different lineage potentials once they arrive at the designated anatomical destination. For example, both Ptp Cre and Pdgfrb Cre can label neural crest cells but Pdgfrb Cre appears can label broader scale of mesenchymal cells. This raises challenging questions such as do the two MSCs populations and/or niches have distinct and/or complementary roles in incisor development and wound healing? Do they share the same origin? From when and which stage do they start to acquire distinct phenotypes? Do LTAC population originate solely from CL-MSCs, or NVB-MSCs, or both? A detailed and specific lineage tracing analyses using Cre transgenics targeting individual MSCs populations might help to address these issues.

Unlike the incisor tooth, the molar tooth loses its epithelium after tooth eruption, and consequently, if any MSCs persist therein, those would be more like NVB-MSCs detected in the incisor tooth. A recent report have indeed suggested such a NVB-MSC niche in the molar teeth. In addition, we report here that the apical papilla of the molar tooth is highly positive for SmarcA2, a DNA dependent ATPase. How the incisor and molar NVB-MSCs differ or are similar to each other and the linkage of NVB-MSCs with apical papilla in the molar teeth remain as two intriguing questions requiring further studies. We propose based...
Fig. 7 MTACs feedback to CL-MSCs through Dlk1. a, b Real time RT-PCR analysis of the indicated markers in the MO6-G3 cells cultured under bound (a, coating the protein onto cell culture dish first before seeding cells) and free form of Dlk1 (b, by adding directly on top of cultured cells). Cells were starved for nutrients for 4 days before recovery or Dlk1 treatment for 24 h as indicated. The results were from n = 3 biologically independent samples. Two-way ANOVA followed by Bonferroni correction was performed. No asterisk: \( p > 0.05 \); *\( p < 0.05 \); **\( p < 0.01 \). c Colony areas coverage of the soluble Dlk1-reated cells as indicated in b. The results were from \( n = 3 \) biologically independent samples. d Design of a new Ki67p-T2A based FUCCI cell cycle indicator. e Representative images of MO6-G3 cells incorporated with the Ki67p-T2A-FUCCI cells. Images were taken under phase contrast setting. f Flow cytometry analysis of MO6-G3/Ki67p-T2A-FUCCI cells at different cell cycle phase under indicated culture condition. g Illustration of genomic location of mouse Dlk1 gene and its 5′ and 3′ CpG islands (green) and number. The positions of exons are indicated in purple. h Immunofluorescence analysis of H3k9me3 and H3k27me3 in the P7 mouse incisor. Nuclei were counterstained with DAPI. Yellow arrows mark clusters of odontoblasts that were EGFP positive. Light blue dotted lines indicate epithelial–mesenchymal junctions. i Methylation analysis of mouse Dlk1 gene CpG islands in the MO6-G3 cells at growing and starved conditions. Genomic DNA were extracted from \( n = 3 \) biologically independent samples. Two-way ANOVA followed by Bonferroni correction was performed. **\( p < 0.01 \). Error bars represent standard deviation. Bars: e: 10 μm; h: 100 μm.
on the natures of the tooth MSCs that the NVB-associated pericytes or SCs are more responsible for stimulated replenishing following injury or wear, while the epithelial-associated MSCs such as CL-MSCs contribute to general tissue homeostasis.

In most tissues, TACs have limited proliferation capabilities and directly define the number of terminally differentiated cells. The number of the TACs needs to be calculated precisely by the tissue to ensure an accurate number of differentiating cells are generated upon request. Hence the signals that direct TAC proliferation require precise fine tuning because insufficient activation of SCs results in developmental or tissue replenishment failure47, while over activation such as forcing SCs to enter cell cycle can exhaust the SC pool48. In hair follicles, epithelial SHH has a promoting role in SCs proliferation as well as in dermal signal regulation. We find that in incisor tooth, the mesenchymal Dlk1 is uniquely expressed by the MTACs but absent in the CL-MSCs. Dlk1 knockout mouse incisor has reduced CL-MSC number and premature differentiation, while Dlk1 overexpression mice develop increased CL-MSC pool, providing a good example for how one molecule can balance the TACs with SCs, particularly in a mesenchymal tissue.

Dlk1 an imprinted gene40 that has been originally discovered in pre-adipocytes with a unique role in mediating MSC differentiation into various cell lineages, including osteoblast, adipocyte, and chondrocyte32,43,49. Dlk1 has been shown either to activate50 or inhibit Notch pathway51,52, and the interaction

Fig. 8 Dlk1 can be applied in enhancing MSCs lineage differentiation. a Immunofluorescence analysis of indicated molecules in the P7 CD1 mouse first lower molar. AB apical bud, Ods odontoblasts. b Representative images for organ cultures of E16.5 mouse first lower molar for 4 days in the absence or presence of Dlk1. Black arrows indicate dentin layers. Note the increased deposition of dentin in the Dlk1-treated samples. c Mouse DSPP promoter luciferase analysis in the MO6-G3 cells after receiving Dlk1 treatment at different concentrations. *p < 0.05; **p < 0.01. Genomic DNA were extracted from n = 3 biologically independent samples. Paired Student’s t-test was performed. No asterisk: p > 0.05; *p < 0.05; **p < 0.01. d H&E images of the upper tooth crowns of the WT vs. Col-Dlk1 Tg mouse first lower molar. Note the mutant mouse’s dental pulp (DP) is fully mineralized. e SmarcA2 and Ki67 double staining on Col-Dlk1 Tg mouse incisor at P7. Light blue dotted lines indicate epithelial-mesenchymal junctions. f, g H&E (f) and immunofluorescence analysis (g) of WT vs. Col-Dlk1 Tg mouse incisor labial dentin and tooth pulp. Error bars represent standard deviation. Bars: a, b and d: 10 μm; e: 100 μm; f, g: 20 μm
between Dlk1 and Notch1 was demonstrated using a two-hybrid system. Here we shown that Dlk1 is co-expressed with Notch2 in the incisor MTACs, highlighting a potential existence of Dlk1-Notch2 regulation axis within MTAC population. The latter issue can be further dissected using approaches such as inducible Cre systems specifically targeting MTACs. Nonetheless, our study provides a functional insight into Dlk1’s role in the maintenance of MTAC status as an inhibitor of precocious differentiation. In addition, Dlk1 has different roles in MSCs preservation and activation depending on if it reaches to MSCs through surface ligand-receptor bonding or as a free diffusible form. Hence a given molecule could have a dual role on a SC population and its progeny.

It is important to note that in the murine incisor tooth the cellular complexity is beyond mesenchymal tissue. Both MTACs and CL-MSCs are in close vicinity to the epithelial compartment, where epithelial SCs and their progenies produced differentiated dental cells. Besides MTAC–CL-MSC communication, the mesenchymal–epithelial crosswalk can also be an essential feature to mediate the turnover of SCs and TACs associated with both cellular compartments. Our recent data have shown that, in addition to molecular signals such as SHH, the primary cilia...
found in the epithelial compartment are able to sense such crosswalk. In adult tissues, most of SCs are in a quiescent status and the majority their genes regulating their activation into TACs are silenced due to transcriptional suppression or epigenetic methylation. The pulp of a molar tooth being a good example. Deciphering key molecules involved in such SC activation as well those implicated in SC niche maintenance can provide a unique molecular toolbox for regulating the tissue regeneration. In the case of Dlk1 gene, we uncover that its 5' and 3' CpG islands are dynamically methylated and demethylated in the absence of the nutrients (e.g., serum), suggesting that specific molecular targets controlling the SC activation are potential tools to stimulate regeneration processes. Further investigations to determine whether similar switch persists in other molecular pathways are urgently needed.

The regeneration outcomes differ in the mesenchymal compartment between incisor and molar. For an incisor, it regenerates normal dentin and enamel at its posterior part, i.e. the region between incisor and molar. For an incisor, it regenerates normal tissue regeneration, further dissection of the implication and regulation mechanisms of CL-MSCs vs. NVB-MSCs can provide deeper insights into how to translate developmental biology knowledge into regenerative medicine.

Methods

Animals. All WT and transgenic animal breeding and operation procedures were approved by the institutional animal care and use committees at individual universities and in accordance with the guidelines and regulations for the care and use of laboratory animals, and compiled with all relevant ethical regulations for animal testing and research at each indicated countries and institutes, and received relevant ethical approvals. In details: CD1 mice at the University of Plymouth, UK and School of Stomatology, Capital Medical University, China; Collagen1a2 Cre, RBP-Jκα flox/flox, Transgenic Notch Reporter Mice at the University of Lausanne, Switzerland; Dlk1–/– mice at University of Castilla–La Mancha, Ciudad Real, Spain, Collagen 1 a1 Dlk1 Tg mice at the University of Southern Denmark, Denmark, mTG lineage tracing reporter mice at the University of Southern Denmark, Denmark, mT/mG at the Max Planck Institute for Molecular Biomedicine, Germany, and Wistar rats at Peking University, China.

PDGFβri and mTg lineage tracing. PDGFβri Cre ERT² x ROSA mTmG pups received intraperitoneal injections of 50 µg of tamoxifen (#T6364, Sigma) on postnatal days P0, P1 to induce Cre-mediated recombination. Samples were analyzed at P7 and P30. Tamoxifen stocks were prepared by dissolving 50 mg in 500 µl of ethanol and vortexing for 10 min before an equal volume of Kolliphor EL was added. One milligram aliquots were stored at −20 °C and dissolved in the required volume of phosphate-buffered saline (PBS) prior to injection. For analysis, P7 heads were frozen in liquid nitrogen, cryosectioned at 20 µm, and fixed paraffin-embedded (FFPE) samples were sectioned at a thickness of 10 µm, before being placed on top and the dish was covered with complete medium.

Immuno-fluorescence staining. For detail antibody information, please see Supplementary Table 1. Preparation of formalin-fixed paraffin-embedded (FFPE) samples is performed as described here; FFPE samples were sectioned at a thickness of 10 µm, and placed onto Superfrost slides (#Z692255; Sigma Aldrich). After drying overnight, deparaffinization was performed. Slides were heated to 55 °C for 20 min, before being twice washed in xylene (#534050; Sigma Aldrich) for 10 min. Slides were then washed in 100% industrial methylated spirits (IMS) (#23684.360, VWR) for 5 min, before being washed for 2 min in 95% IMS and then 70% IMS. Antigen retrieval was performed by microwaving the slides in a 0.01 M citrate buffer solution (citric acid and 0.05% Tween-20, #C2404 and P4164, respectively, Sigma Aldrich) for 1 min.

Once prepared all sample types were washed three times in PBST (PBS containing 0.1% Triton X-100 (Sigma Aldrich) for 5 min each) with 0.1% Triton X-100 (Sigma Aldrich) for 5 min each. Non-specific binding was blocked by incubation with PBST containing 5% Donkey Serum (#D9863, Sigma Aldrich), 0.25% cold water fish gelatine (#G7765; Sigma Aldrich), 0.1% ovalbumin bovine serum (#A2153; Sigma Aldrich) for 60 min. Primary antibodies were incubated overnight at 4 °C. Slides were washed thrice in PBST before incubation with secondary antibodies for 2 h at room temperature. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI, 2 µg/ml, #D9542; Sigma Aldrich) for 20 min before being dehydrated in 14% EDTA–steamed tooth was placed directly into 4% PFA solution in 10 mM PBS (#44175-035, Gibco) containing 10% fetal bovine serum (FBS, #F7524; Sigma Aldrich) and 1% penicillin–streptomycin (#SV30079.01; Hyclone) and filtered using a 0.22-µm filter, for 1 h at 37 °C. The tissues were then washed with 50 ml of methylcellulose (2% w/v, Methylcellulose Eagle’s medium (DMEM–F12, #13313-028; Gibco) with 20% FBS, 1% penicillin–streptomycin, and 1% l-asorbic acid (w/w) (#A4403, Sigma Aldrich). The extracted incisor CL-MSC mesenchyme was then placed on top of the dentin and cultured for 4–7 days. The dentin–mesenchyme samples were removed from culture and placed directly into 4% PFA solution in 10 mM PBS (#D9663; Sigma Aldrich), 0.25% cold water fish gelatine (#G7765; Sigma Aldrich) for 20 min at room temperature. After fixation, the samples were washed twice in PBS. Samples were then passed through serial solutions of 10%, 20%, and 30% sucrose (#S0389; Sigma Aldrich) in PBS for 20 min each, before being embedded in Tissue-Tek® O.C.T.™ (#{583; Sakura) and snap frozen by plunging into liquid nitrogen. Once frozen samples were stored at −80 °C before being sectioned at 15 µm and stained for immunofluorescent analysis. Alternatively, sections were used to visualize the actin cytoskeleton. Samples were cryosectioned at 15 µm thickness on a Leica CM1850 cryostat on Polysine® Microscope Adhesion Slides (#2800AM2N; Thermo Scientific).

Semisolid organ culture. Semisolid culture conditions were set up using culture media supplemented with agar14. Briefly, prewarmed DMEM–F12 supplemented with 20% FBS and 1% penicillin–streptomycin (complete medium) was mixed 1:10 with 2.5% (w/w) agar (#A1296; Sigma) dissolved in sterile water. Two milliliters of media/agar mix was added to each well of a six-well-plate and allowed to set at room temperature for 30 min, before tissue was placed on top and the dish was covered with complete medium.
imaging assembling and processing was conducted using Adobe Photoshop CC. Preparation and fixation of cells to be used for immunofluorescence (IF) analysis was performed by washing in HBSS, and then fixed in ice cooled 4% PFA solution in 10 mM PBS for 30 min. Preparation and fixation of frozen tissue is as follows; frozen tissue was cryosectioned at 15-µm thickness on a Leica CM1850 cryostat. Sections were mounted onto Polylysine™ Microscope Adhesion Slides and allowed to dry at 30°C for 30 min before fixing in ice cold acetone (#34850; Sigma Aldrich) or freshly made ice cooled 4% PFA solution in HBSS for 1 h at 37°C with frequent agitation. Following incubation, collagenase was neutralized by washing in HBSS three times with PBS ready for staining.

Hematoyxlin & eosin staining. Slides of FFPE-sectioned tissue are stained using hematoyxlin and eosin histological dyes according to the following protocol. FFPE samples were sectioned at a thickness of 10 µm, and placed onto Superfrost™ slides. After drying overnight, deparaffinization was performed. Slides were heated to 55 °C for 20 min. Subsequently slides were washed twice in xylens for 5 min. The tissue is then rehydrated through two changes of 100% IMS, followed by a 5 min wash in 95% IMS and then 100% IMS. In order to clear the sample, the slides were washed twice more in xylenes for 5 min before being mounted using Eukit, xylene-based mounting medium (#03989; Fluka).

Cell culture. Established mouse molar mesenchyme cell line MO6-G3 cells35 were cultured in DMEM containing 10% FBS, 1% penicillin–streptomycin, and 300 µg/ml Geneticin (#10133109; Gibco). Cervical loop mesenchyme cells were isolated as follows: incisors were dissected from postnatal day 30 CD1 mice shortly after death. The CL region was dissected under the direction and with the approval of the Ethical Committee of Beijing University. Cells were visualized using 0.01% crystal violet solution (#V5265; Sigma). Images were captured using a Leica DM1000 LED microscope with a Leica TCS SP8 attachment. The microscope ran LAS AF software from Leica (3.5.2.18963).

Hematoxylin & eosin staining. Slides of FFPE-sectioned tissue are stained using hematoyxlin and eosin histological dyes according to the following protocol. FFPE samples were sectioned at a thickness of 10 µm, and placed onto Superfrost™ slides. After drying overnight, deparaffinization was performed. Slides were heated to 55 °C for 20 min. Subsequently slides were washed twice in xylens for 5 min. The tissue is then rehydrated through two changes of 100% IMS, followed by a 5 min wash in 95% IMS and then 70% IMS, each for 2 min. The slides were briefly washed in distilled water before being stained for 8 min in Harris hematoxylin (#HHS16; Sigma). After staining was sufficient the slides were washed in tap water for 5 min. Differentiation of the stain was achieved by placing the slides in 1% acid alcohol (#5484; Sigma Aldrich) for 30 s, before a further 5 min tap water wash. Subsequent counterstaining was performed by washing the slides briefly in 95% IMS, followed by a 1-min incubation in 0.25% eosin Y solution (#230251; Sigma). After staining was complete, the tissue was dehydrated by passing the slides through two 5 min washes in 95% IMS and then 100% IMS. In order to clear the sample, the slides were washed twice more in xylenes for 5 min before being mounted using Eukit, xylene-based mounting medium (#03989; Fluka).

### Treatment of cultured cells with recombinant proteins.

Cells were treated with recombinant proteins either through direct addition of the protein to fresh media or via an indirect coating method. Direct protein treatment involved the addition of the protein into fresh un-supplemented or fully supplemented media as appropriate to the cell line. This fresh media was then placed onto the cells that had been cultured for 24 h. Media containing the protein was replaced every 48 h. The indirect binding method involved passing passages onto precooled dishes that had already been coated with protein. Dish preparation was performed as follows: six-well plates were coated by placing 10 µg/ml of goat anti-human IgG (#11886; Sigma) or Goat anti-Mouse IgG (#88642; Sigma) in HBSS into the dish. The dish was then incubated for 30 min at 37°C. Dishes were then washed five times in HBSS before being incubated with a 2% solution of BSA in HBSS overnight at 4°C. Following blocking 10 µg/ml of the recombinant protein is added to the blocking solution, and incubated for 2 h at 37°C. Following preparation of the dishes they are washed quickly in HBSS five times and immediately cells are plated into them.

### MSs multipotential differentiation assays.

MO6-G3 cells were cultured in DMEM/F12 containing 10% FBS and 1% penicillin–streptomycin. The explants were incubated for 60 min in 1% type I collagenase (#C0130; Sigma) in DMEM/F12–Hepes (1:1; Gibco) or freshly made ice cooled 4% PFA solution in HBSS for 30 min. Following incubation collagenase was neutralized with DMEM plus 20% FBS and 1% penicillin–streptomycin. Dish preparation was performed as follows: six-well plates were coated by placing 10 µg/ml of goat anti-human IgG (#11886; Sigma) or Goat anti-Mouse IgG (#88642; Sigma) in HBSS into the dish. The dish was then incubated for 30 min at 37°C. Dishes were then washed five times in HBSS before being incubated with a 2% solution of BSA in HBSS overnight at 4°C. Following blocking 10 µg/ml of the recombinant protein is added to the blocking solution, and incubated for 2 h at 37°C. Following preparation of the dishes they are washed quickly in HBSS five times and immediately cells are plated into them.

Cells were washed in HBSS, and then fixed in ice cooled 4% PFA solution in 10 mM PBS for 30 min. Preparation and fixation of frozen tissue is as follows; frozen tissue was cryosectioned at 15-µm thickness on a Leica CM1850 cryostat. Sections were mounted onto Polylysine™ Microscope Adhesion Slides and allowed to dry at 30°C for 30 min before fixing in ice cold acetone (#34850; Sigma Aldrich) or freshly made ice cooled 4% PFA solution in HBSS for 1 h at 37°C with frequent agitation. Following incubation, collagenase was neutralized with DMEM + 10% FBS was added to the cell suspension for 10 min, and cells were subsequently spun at 1 r.f.c. for 5 min. The supernatant was discarded and the pellet re-suspended in AminoMax basal media, 14% AminoMax supplement, 0.1% ascorbic acid, and 1% AA. CL-MSCs were amplified for 2 days in vitro before seeding into 96-well plates at a seeding density of 1 x 10^4 cells per well for osteogenic/adipogenic differentiation. Cell culture media was changed to StemXVivo osteogenic/adipogenic base media (#CCM007; R&D Biosystems) with 1% AA. Following 48 h of culture, osteogenic or adipogenic cell differentiation was induced by supplementing the base media with StemXVivo osteogenic supplement (#CCM008/CM009; R&D Systems) or 1% StemXVivo adipogenic supplement (#CCM011; R&D Systems) respectively. Media was replaced every 48 h. Following 21 days of culture cells were fixed in 4% PFA at RT for 30 min and washed twice in PBS ready for staining.

For chondrogenic differentiation 2 x 10^6 CL-MSCs were pelleted by centrifugation at 0.2 r.f.c. for 5 min. Media was replaced with 1 ml StemXVivo chondrogenic base media (#CCM005; R&D Systems), 1% StemXVivo chondrogenic supplement (#CCM006; R&D Systems), and 1% AA. Media was removed and replaced with complete Chondrogenic differentiation media every 2-3 days. Chondrogenic pellets were fixed in 4% PFA in 10 mM PBS (1:10,000; DAPI; #D9542; Sigma-Aldrich) mixed with 0.5 mM BODIPY for 20 min on a rocker at RT. Cells were washed 10 times with PBS before mounting with Dako fluorescence mounting medium (#53023; Aligent). Images were captured using a Leica DMi6000 confocal microscope with a Leica TCS SP8 attachment. The microscope ran LAS AF software from Leica (3.5.2.18963).

### Generation of Ki67p-T2A-Fucci cell cycle reporter.

The Fucci plasmids mAG-bGeminin(1/110)-pShi-EF-MCS and mCherry-hCd1t(30/120)-pShi-EF-MCS were kindly provided by Dr. Atsushi Miyawaki37. Open reading frames for mAG-bGeminin(1/110) and mCherry-hCd1t(30/120) were sub-cloned into pENTR-D-Topo plasmids (Life Technologies). Gibson Assembly (NEBuilder HiFi DNA Assembly Kit, New England Biolabs) was used to fuse the mAG-bGeminin (1/110)-pShi-EF-MCS and mCherry-hCd1t(30/120) separated by a ribosomal skip T2A sequence (T2A-Fucci) according to the manufacturer’s instructions. The coding region was verified by direct sequencing. Gateway recombination was then used to generate a lentiviral vector containing the 1.5 kb human K67 proximal promoter (K67p)38 upstream of T2A-Fucci open reading frame in the 2K7d lentiviral vector96. MO6-G3 were incubated with the lentiviral supernatant and observed as described in “Generation of Ki67p-T2A-Fucci cell cycle reporter”, and 10 µg/ml polybrene (Merieck) overnight under normal cell culture conditions. After 2 h, the viral supernatant was replaced with normal growth medium. Infected MO6-G3 cells were selected indefinitely using 10 µg/ml blasticidin (Sigma–Aldrich)96.

### Synchronization of cell cycle.

Twenty-four hours after initial seeding and culture under normal cell culture conditions (see above), cells were exposed to basal or full supplemented media. For MSs cell line, they were supplemented with new media, either maintaining the current supplement with or without supplements.

### Cell culture confluency assay.

Cells were seeded at equal densities into a six-well plate. In certain conditions siRNA was added to the culture at the point of replensing the supplements to the media. Cells were fixed in 10% formalin for 30 min at room temperature before being washed three times for 5 min each in HBSS. Cells were visualized using 0.01% crystal violet solution (V5265; Sigma). Images were taken of four distinct areas of each well using a Leica DM1000 LED microscope. Cell confluency was measured using Fiji (Image J 1.51n).

Flow cytometric and FACS analysis of cultured cells. K67-Fucci system infected MO6-G3 cells were harvested, fixed in 4% PFA in 10 mM PBS for 30 min at room temperature, and analyzed by flow cytometry using the BD FACSCount II SOR (Beckman Coulter). Data were acquired using blue laser (488 nm) for
mAzamiGreen signal and yellow-green laser (561 nm) for mCherry and analyzed using the FlowJo software v10.7 (Tree Star Inc.). Cultured cells were harvested and fixed using flow staining buffer (#FC001, R&D) for 15 min at room temperature. They were then immunolabeled with a panel of antibodies provided from the Mouse Mesenchymal Stem Cell Marker Antibody Panel kit (#CS018, R&D) for 30 min at room temperature. Afterward, they were washed and incubated with donkey secondary antibodies Alexa 488 anti-rat (#A21208; Life Technologies) for 30 min. Labeled cells were observed using a BD Accuri C6 (Beckman Coulter) and analysis with BD Accuri C6 Software (Accuri Cytometers Inc., version 1.0.264.21).

Chromatin immunoprecipitation. ChIP was performed on cells using the ChIP-IT high sensitivity kit (#53040; Active Motif) in accordance with the provided protocol. In summary the procedure was as follows; cells were fixed using Complete Cell Fixation Solution added directly to the culture media for 15 min at room temperature. Cells were then suspended using TrisPic, and collected by centrifugation at 1250 rcf for 3 min at 4°C. The pellet was resuspended and washed twice in ice-cold PBS. The resultant pellet was resuspended in Chroomatin Prep Buffer supplemented with protease inhibitor cocktail (PIC) and phenylmethylsulfonyl fluoride (PMSF) and incubated for 10 min on ice. Using a Dounce homogenizer the cells were homogenized, collected by centrifugation, and resuspended in fresh ChIP Buffer supplemented with PIC and PMSF. Chromatin was sheared using a Diagenode Bioruptor Pico (Diagenode) by sonication for 15 s followed by 30 s of rest for four cycles. Cell debris was removed by pelleting out this by centrifugation. Antibodies or control IgG was mixed with Blocker as outlined in Supplement Table 1 and antibody mix was mixed with ChIP Buffer and sonicated chromatin and incubated overnight at 4°C. Immunoprecipitation was performed by washing Protein G agarose beads in TE pH 8.8 and then mixing the washed beads to the antibody/chromatin mixture and incubating for 3 h at 4°C. Following incubation with the beads the solution was passed through a ChIP filtration membrane to collect the bound chromatin which was washed and eluted using the provided solutions. The cross-links within the eluted sample was reversed and the DNA purified by overnight incubation with Proteinase K at 37°C. The DNA was collected by heating the sample to 95°C for 8 min and then plugging into ice for 1 min to deactivate the proteinase K function. DNA Purification Wash Buffer and sodium acetate was added to the sample, which was then passed through a DNA Purification Column by centrifugation. The latter was washed with DNA Purification Wash Buffer, and DNA was eluted using DNA Purification Elution Buffer. Input DNA was prepared in the same manner, however, rather than column-based purification. Following proteinase K treatment, DNA was immunoprecipitated in 2-propanol (#34965; Fluka Analytical) with 1 µl GlycoBlue (#AM9515; Ambion), before being washed in 70% ethanol (#20821.321; VWR Chemicals) and resuspended in 0.1% diethylpyrocarbonate (#D5758; Sigma-Aldrich)-treated distilled water (DEPC).

LCM of frozen tissues. Frozen tissue was sectioned using a Leica CM1850 cryostat at a thickness of 20 µm. Sections were mounted onto PEN Membrane Glass Slides (#LCM052; Applied Biosystems). VFP blocks were sectioned on a Microm HM320 microtome at a thickness of 10 µm, and placed onto a membrane glass slide. All prepared slides were stained using 1% methyl green (#67060; Fluka Analytical) in 0.1% DEPC-treated distilled water, then washed three times for 30 s in 0.1% DEPC-treated distilled water before being kept for dry for 5 min. An ArcturusXT™ LCM instrument was used to perform LCM onto CapSure™ Macro LCM Chips (Sigma). LCM collected cryosections. The epithelium was outlined fluorescent-labeled cryosections. The epithelium was outlined using a Leica DMI6000 confocal microscope with a Leica TCS SP8 attachment at a scanning thickness of 1 µm per section. Microscope was running LAS AF software from Leica. These images were used to reconstruct the growth of the cells into the dentine tubules using Imaris (version 9.0.2; Bitplane). The three-dimensional (3D) reconstruction of the epithelium was performed by taking serial immunofluorescence-labeled cryosections. The epithelium was outlined from approximately 20 consecutive 20-µm-thick sagittal sections of the apical end of the mouse incisor.

Three-dimensional reconstruction. For dentin culture assay, immuno- fluorescence micrographs were captured using a Leica DMi6000 confocal microscope and a Leica TCS SP8 attachment at a scanning thickness of 1 µm per section. Microscope was running LAS AF software from Leica. These images were used to reconstruct the growth of the cells into the dentine tubules using Imaris (version 9.0.2; Bitplane). The three-dimensional (3D) reconstruction of the CL and surrounding transit amplifying mesenchyme region was performed by scanning microscopy. The epithelium was outlined from approximately 20 consecutive 20-µm-thick sagittal sections of the apical end of the mouse incisor.

Micro-computed tomography (micro-CT). Whole animal heads were scanned with a SkyScan-1072 desktop micro-CT system at a resolution of 9 µm. The generated data were then analyzed with Imaris software (version 9.0.2, Bitplane).

Scanning microscopy. Isolated incisor teeth were cut transversally at the tooth–bone junction with a No. 11 scalpel then fixed in 3% glutaraldehyde (Sigma-Aldrich) in PBS for 4 h then incubated in ethanol for 5 min each at a concentration of 50%, 70%, 90%, and 95%, and three times for 30 min 100%. Samples were then dried for 24 h, gold sputtered, and observed under a XL20 scanning microscope (Philips).

Data availability
The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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