



Early Determination of the Periodontal Domain by the Wnt-Antagonist *Frzb/Sfrp3*

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Odontogenesis results from the continuous and reciprocal interaction between cells of the oral epithelium and cranial neural crest-derived mesenchyme. The canonical Wnt signaling pathway plays a fundamental role in mediating these interactions from the earliest stages of tooth development. Here we analyze by *in situ* hybridization the expression patterns of the extracellular Wnt antagonist *Frzb/Sfrp3*. Although *Frzb* is expressed in dental mesenchymal cells from the earliest stages of odontogenesis, its expression is absent from a tiny population of mesenchymal cells immediately adjacent to the invaginating dental epithelium. Cell proliferation studies using BrdU showed that the *Frzb* expressing and *Frzb* non-expressing cell populations display different proliferative behavior during the initial stages of odontogenesis. Dil-mediated cell-fate tracing studies demonstrated that the *Frzb* expressing cells contribute to the formation of the dental follicle, the future periodontium. In contrast, the *Frzb* non-expressing cells give rise to the dental pulp. The present results indicate that *Frzb* is discriminating the presumptive periodontal territory from the rest of the dental mesenchyme from the very beginning of odontogenesis, where it might act as a barrier for the diffusion of Wnt molecules, thus regulating the activation of Wnt-dependent transcription within dental tissues.

Keywords: *Frzb/Sfrp3*, Wnt signaling, tooth development, cell fate, dental pulp, dental follicle, periodontium

INTRODUCTION

Odontogenesis is characterized by the sequential and reciprocal interactions between cells of the oral epithelium and the cranial neural crest-derived mesenchyme and proceeds through a series of well-defined morphological steps, namely bud, cap, bell, and cytodifferentiation/mineralization stages (Mitsiadis and Graf, 2009). Ectomesenchymal cells form two distinct and specialized tooth components, the dental papilla that gives rise to the pulp and the dentin-producing odontoblasts, and the dental follicle, which surrounds the developing dental epithelium and forms the periodontium (Mitsiadis and Graf, 2009; Krivanek et al., 2017).

All stages of tooth development are mediated by the exchange of a big variety of signaling molecules between homotypic and heterotypic cell populations (Mitsiadis and Graf, 2009; Mitsiadis and Luder, 2011; Jussila and Thesleff, 2012; Balic and Thesleff, 2015). Among these molecules, the secreted lipid-modified Wnt glycoproteins trigger the evolutionarily conserved Wnt signaling pathway, a molecular cascade important for the development of virtually all organs (Clevers, 2006). One previous study has shown that several extracellular Wnt ligands and Wnt inhibitors are expressed in specific stages and compartments during odontogenesis (Sarkar and Sharpe, 1999).

Although many reports addressed the role of the canonical Wnt/ β -catenin-mediated signaling in the formation and regeneration of dental tissues (Aurrekoetxea et al., 2012, 2016; Liu et al., 2014; Zhang et al., 2016; Babb et al., 2017) a unifying picture of its activity during odontogenesis is still missing (Tamura and Nemoto, 2016). The critical requirement of Wnt signaling during tooth development has been already evidenced, since the genetic loss of β -catenin, or the specific abrogation of β -catenin-dependent transcription, leads to arrested tooth formation at early stages (Liu et al., 2008; Cantù et al., 2017).

The action of the Wnt ligands in the extracellular matrix is regulated by physiologically secreted Wnt antagonists (or negative regulators) such as the ones belonging to the family of Secreted Frizzled Related Proteins (SFRPs), which possess a cysteine-rich domain homologous to the Wnt-binding domain of Frizzled (Frzb) receptors (Cruciat and Niehrs, 2013) and are implicated as tumor suppressors in several forms of cancer (Zimmerli et al., 2017).

Frzb (known as Frzb1 or Sfrp3, Secreted Frizzled Related Protein 3) was initially identified as a chondrogenic factor during bone morphogenesis (Hoang et al., 1996). It was subsequently shown to modulate the activity of XWnt8 during *Xenopus* dorsoventral axis development (Leyns et al., 1997) and to repress canonical Wnt signaling in other contexts (Person et al., 2005). Here we identify *Frzb* as a novel marker of the neural crest-derived mesenchymal cells that contribute to dental follicle formation, the future periodontium. *Frzb* expression at the earliest stages of odontogenesis allows distinguishing two dental mesenchymal cell populations with clearly defined developmental fates.

MATERIALS AND METHODS

Cell Proliferation Analysis

All animals were maintained and handled according to the Swiss Animal Welfare Law and in compliance with the regulations of the Cantonal Veterinary Office, Zurich (License 11/2014). *In vivo* cell proliferation in dental tissues was analyzed by immunohistochemistry for phosphorylated Histone 3 (pH3; rabbit Ab, 1:200; Upstate, Charlottesville, VA) and bromodeoxyuridine (BrdU). For the latter, a BrdU cell proliferation kit (Boehringer Mannheim, Germany) was used. Foster mothers were injected intraperitoneally with 5 mg/ml of BrdU in PBS at a concentration of 50 mg/kg body-weight, 60 min before embryos were sacrificed. BrdU-positive cells in developing teeth of E13–E15 embryos were analyzed on 14 μ m cryosections after staining with an anti-BrdU antibody. Immunohistochemistry was performed as described earlier (Mitsiadis et al., 2008). Cells were counted with the CellCounter Plugin, ImageJ. Statistical Analysis was performed with GraphPad Prism 7 (*t*-test).

Lineage Tracing Using DiI Labeling

DiI (1, 10, di-octadecyl-3, 3, 30,-tetramethylindo-carbocyanine perchlorate; Molecular probes cell tracker CM-DiI, C-7000) injection was performed to various locations of mesenchymal cells surrounding the dental epithelial ingrowths of cultured

mandible slices to monitor cell kinetics. Briefly, E13 mouse mandibles were carefully dissected out from the head, placed upon a chopping plate of a McIlwain tissue chopper (Mickle Laboratory Engineering Co., Ltd., Guilford, UK), orientated to obtain frontal sections and finally cut into 250 μ m thick slices. Slices containing the molar tooth buds were selected and cultured. DiI, which is highly lipophilic dye intercalating into the cell membranes, was dissolved in ethanol (EtOH) at 2.5 μ g/ μ l (stock solution) and then diluted 1–9 in 0.2 M sucrose. Thereafter, small amounts of DiI were injected using a mouth-controlled micropipette made from a 50 mm borosilicate glass into different areas of the condensing dental mesenchyme, either in mesenchymal cells contacting the molar bud epithelium or in condensing cells located at a more distant area from the tooth epithelium (Mitsiadis et al., 2008; Gruenbaum-Cohen et al., 2009).

Slice Cultures and Imaging

DiI labeled slices were placed upon Millipore filters coated with growth factor reduced Matrigel basement membrane matrix (BD Biosciences). The slices were completely encapsulated by an additional layer of Matrigel that served to structurally support the morphology of the explants during their development. The filters were supported above the culture medium by metal grids within Petri dishes. The culture medium was composed of Dulbecco's Minimum Essential Medium (DMEM) supplemented with 1% penicillin/streptomycin, 2 mM L-glutamine and 15% fetal calf serum (FCS). Slices were cultured up to 4 days in a 37°C/5% CO₂ air-jacketed incubator. After culturing, samples were fixed in 4% paraformaldehyde (PFA) for 30 min, washed with PBS and then embedded in wax and sectioned at 8 μ m.

The initial positions of the DiI injection and the subsequent location of the DiI-labeled cells were monitored throughout the culture period using a Leica dissecting microscope equipped with UV light (Leica Microsystems Ltd., Germany).

In Situ Hybridization

Frzb *in situ* hybridization probe was kindly provided by Prof. De Robertis (Leyns et al., 1997). The labeled probe was ethanol-precipitated, resuspended in 100 mM DTT, diluted in hybridization solution (60% deionized formamide, 20 mM Tris-HCl, 5 mM EDTA, pH 8, 0.3 M NaCl, 0.5 mg/ml yeast RNA, 5% dextran sulfate). *In situ* hybridization was performed according to standard procedures (Mitsiadis et al., 1995). Briefly, slides were incubated with the probe at 60°C. After intense washing, the slides were incubated in blocking solution (20% Normal Goat Serum) and anti-digoxigenin (DIG)-AP (alkaline phosphatase conjugate) Fab-fragment (Boehringer Mannheim, 1093 274) diluted 1:1,000 in blocking solution. The color reaction was developed using Nitro Blue Tetrazolium (NBT, Sigma N-6876) and 5-Bromo-4-Chloro-3-Indolyl Phosphate (BCIP, Sigma B-8503) in staining solution 2% NaCl, 5% MgCl₂, 10% Tris-HCl pH 9.5, 1% Tween-20. *In situ* hybridization immediately followed by BrdU immunohistochemistry was performed in cryosectioned slides of E13–E15 mouse embryos to show the correlation between *Frzb* expression and cell proliferation (Mitsiadis et al.,

2008). No hybridization signal was detected with the sense probe at these developmental stages.

RESULTS

Frzb Is Expressed in a Subpopulation of Dental Mesenchymal Cells

To determine the potential role of *Frzb* in odontogenesis, we analyzed its expression pattern during the early stages of mouse tooth development (Figure 1A). We monitored the expression of *Frzb* in the developing mouse tooth germs from embryonic day 11 (E11; initiation stage) to E15 (cap stage). Intense hybridization signal was observed in the mesenchyme of the mandible during the tooth initiation period (E11) (Figure 1B). During the dental epithelial invagination to the underlying mesenchyme (early bud stage, E12), *Frzb* mRNA was restricted in mesenchymal cells located at the areas of molar (Figures 1C,D) and incisor (Figure 1E) formation. At this stage, the hybridization signal was strikingly absent from a layer of mesenchymal cells nearby the epithelium (Figures 1C–E, red asterisk). However, *Frzb* was strongly expressed in mesenchymal cells that are not in close contact with the dental epithelium (Figures 1C–E). This observation was confirmed by transcript localization at E13 (late bud stage) (Figures 1F,G). At the cap stage (E14–E15), *Frzb* hybridization signal was absent from the cells composing the dental papilla, while *Frzb* expression was strong in the peripheral regions of the developing tooth germ (Figures 1H,I).

Differential Proliferation of Presumptive Dental Papilla and Follicle Cells

We then wondered whether this distinct *Frzb* expression pattern in the developing dental mesenchyme could correlate with dissimilar proliferative behavior between these two cell populations (*Frzb* expressing and *Frzb* non-expressing cells). To test this hypothesis, pregnant females were injected with BrdU and tooth germs of E13–E15 embryos were analyzed for cell proliferation. At E13 (bud stage), two territories could be observed according to BrdU immunoreactivity. Cell proliferation was significantly lower in the mesenchyme nearby the epithelium (Figure 2A, red asterisk), which does not express *Frzb* (*Frzb*⁻, Figure 2G), when compared to the mesenchyme that is most distant from the epithelium (Figure 2A; green arrowheads) and expresses *Frzb* (*Frzb*⁺, Figures 2G,J). At the subsequent cap stage (E14–E15), the proliferation status of the two cell populations switched: abundant mitotic activity was observed in mesenchymal cells forming the dental papilla (Figures 2B,C; green arrowheads) outside the *Frzb* expression domain (*Frzb*⁻, Figures 2H,I). In contrast, proliferative activity was sporadically detected in the forming dental follicle (Figures 2B,C; green arrowheads) that expresses *Frzb* (*Frzb*⁺, Figures 2H–J). To confirm the differential proliferation status between cells of the dental follicle (*Frzb*⁺ cells) and dental papilla (*Frzb*⁻ cells), we stained E13–E15 tooth germs for phospho-Histone H3 (pH3), which marks cells in active mitosis (M phase) (Figures 2D–F). pH3 immunohistochemistry showed that *Frzb*⁺ cells of the dental follicle proliferate significantly more than *Frzb*⁻ cells

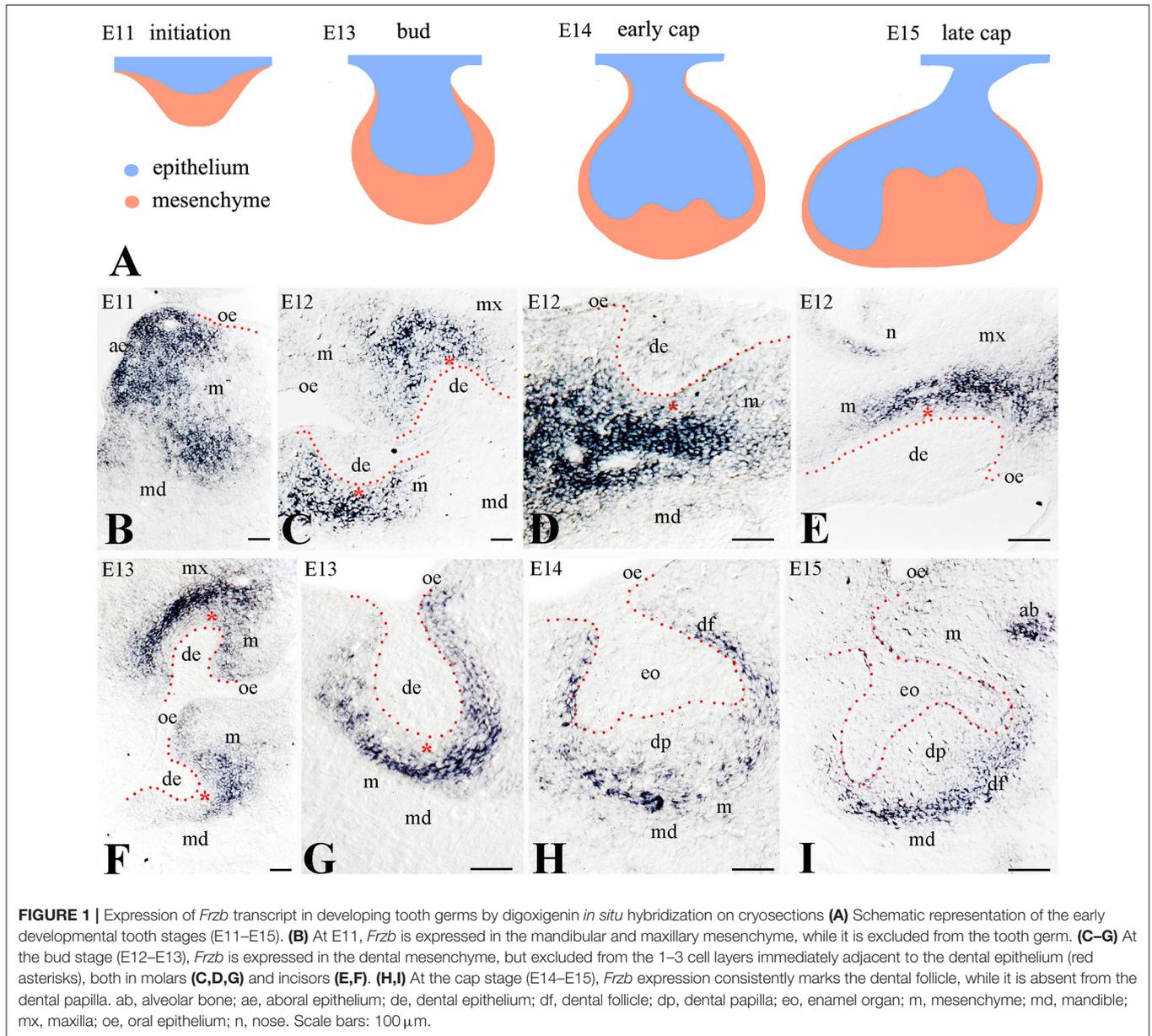
of the presumptive dental papilla at E13 (Figures 2D,K). A proliferative switch occurred at E14–E15, when *Frzb*⁺ cells of the follicle display a significantly lower mitotic activity than *Frzb*⁻ cells of the papilla (Figures 2E,I,K).

Frzb Expressing Cells Mark the Presumptive Dental Follicle

To test if the *Frzb* expressing cells selectively contribute to the formation of dental follicle, we labeled subsets of the *Frzb* expressing (Figure 3A) and non-expressing (Figure 3E) mesenchymal domains with DiI at the bud stage and followed the fate of DiI-positive cells (Figures 3B–D,F–H). DiI-positive cells that were located in the territory of *Frzb* expression participated in the formation of the dental follicle of the cap (2 days of culture) and early bell (4 days of culture) staged teeth (Figures 3B–D). As DiI does not allow simultaneous labeling of the entire *Frzb* expression domain, we marked different sub-regions in a significant number of experiments ($n > 10$). In all experiments realized, DiI staining was never observed neither in the dental papilla compartment of these developing teeth, nor in other tissues formed far away from the tooth germ (Figures 3C,D,I,J). Conversely, DiI labeled cells in direct contact with the dental epithelium (*Frzb*⁻ cells) contributed to dental papilla formation at the cap (2 days of culture) and early bell (4 days of culture) stages ($n > 10$; Figures 3F–H). Sections of the tooth germs of the early bell stage indicated dental papilla cells stained with DiI (Figures 3G,H,K,L). Since DiI injection was performed in both tooth mesenchymal cell populations in this experiment, DiI labeled cells were present in both dental follicle and dental papilla cells. Due to the intrinsic limitations of this technique we rarely obtained DiI labeling in dental epithelial cells when injection was performed in mesenchymal cells immediately adjacent to dental epithelial bud (Figures 3E,F,I).

DISCUSSION

Cranial neural crest-derived mesenchymal cells play a crucial role in tooth formation and are recognizable since the earliest stages of odontogenesis due to their positivity to classical neural crest or dental mesenchyme-specific markers such as *Pax9* (Bonczek et al., 2017), *Barx1* (Mitsiadis et al., 1998), and *midkine* (*MK*) (Mitsiadis et al., 1995). These cells have been historically considered as a homogeneous population of cells; nevertheless, the dental mesenchyme generates highly specialized adult soft tissues, such as the pulp and the periodontium (Jiménez-Rojo et al., 2014; Otsu et al., 2014; Mitsiadis et al., 2015). It is therefore of great interest the discovery of new markers that would allow distinguishing the subsets of mesenchymal cells possessing different differentiation commitments. Here we identify *Frzb* as a novel dental mesenchymal marker. Importantly, *Frzb* is specifically expressed from the earliest tooth developmental stages in mesenchymal cells that are not in direct contact with the dental epithelium. This specific expression pattern only partially overlaps with the expression domain of other well-established dental mesenchymal markers, such as *Pax9* (Figure 4A). One previous report described the expression of



Frzb in the dental mesenchyme (Sarkar and Sharpe, 1999). However, the radioactive *in situ* hybridization technique that was used for the detection of the transcripts did not offer sufficient resolution to distinguish the absence of *Frzb* mRNA expression in the cell layers immediately adjacent to the dental epithelium. Based on *Frzb* expression we could identify two functionally distinct cell domains: one expressing *Frzb* that will give rise to the dental follicle, and another non-expressing *Frzb* that will form the dental papilla. This important finding shows for the first time the presence of two functionally distinct cell populations in the tooth mesenchyme and their commitment for generating specialized dental structures of the tooth at very early developmental stages (**Figure 4B**). During early odontogenesis (E11–E12), the thickened dental

epithelium is surrounded by mesenchymal cells that condense and actively proliferate (Mitsiadis et al., 2003; Mitsiadis and Graf, 2009). These combined activities of cell migration, adhesion and proliferation within the mesenchyme are controlled by signaling and cell adhesion molecules such as fibroblast growth factors, midkine, syndecan. Dental mesenchyme is important for the tooth shape determination that involves alternated and well-orchestrated proliferations of distinct cell populations throughout odontogenesis. Initially, active proliferative events in the territory that will give rise to dental follicle/periodontium correlate with *Frzb* expression. This initial process of active cell proliferation within the *Frzb* expression domain determines the size of the periodontal domain that is important for proper tooth germ development and its integration in the growing

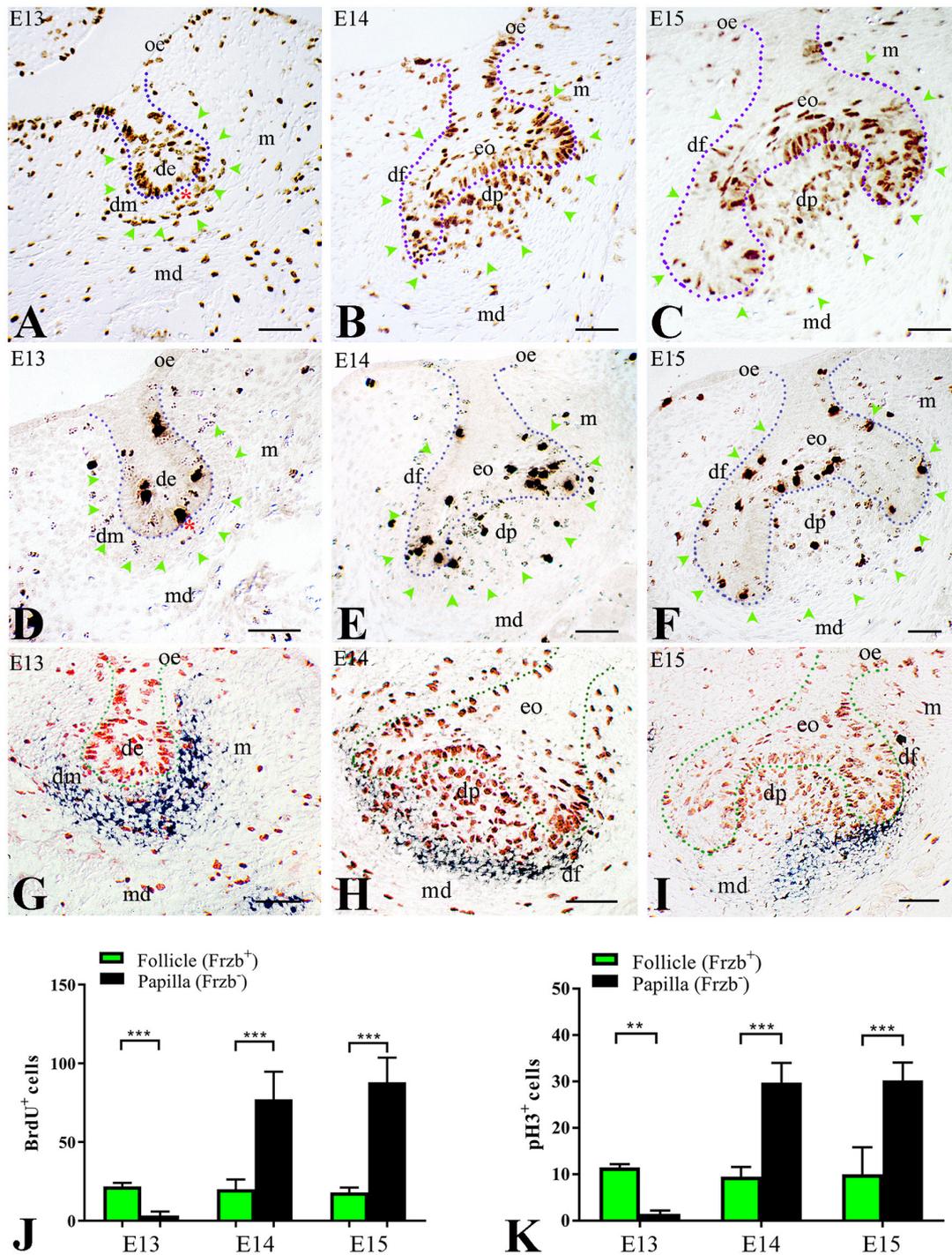
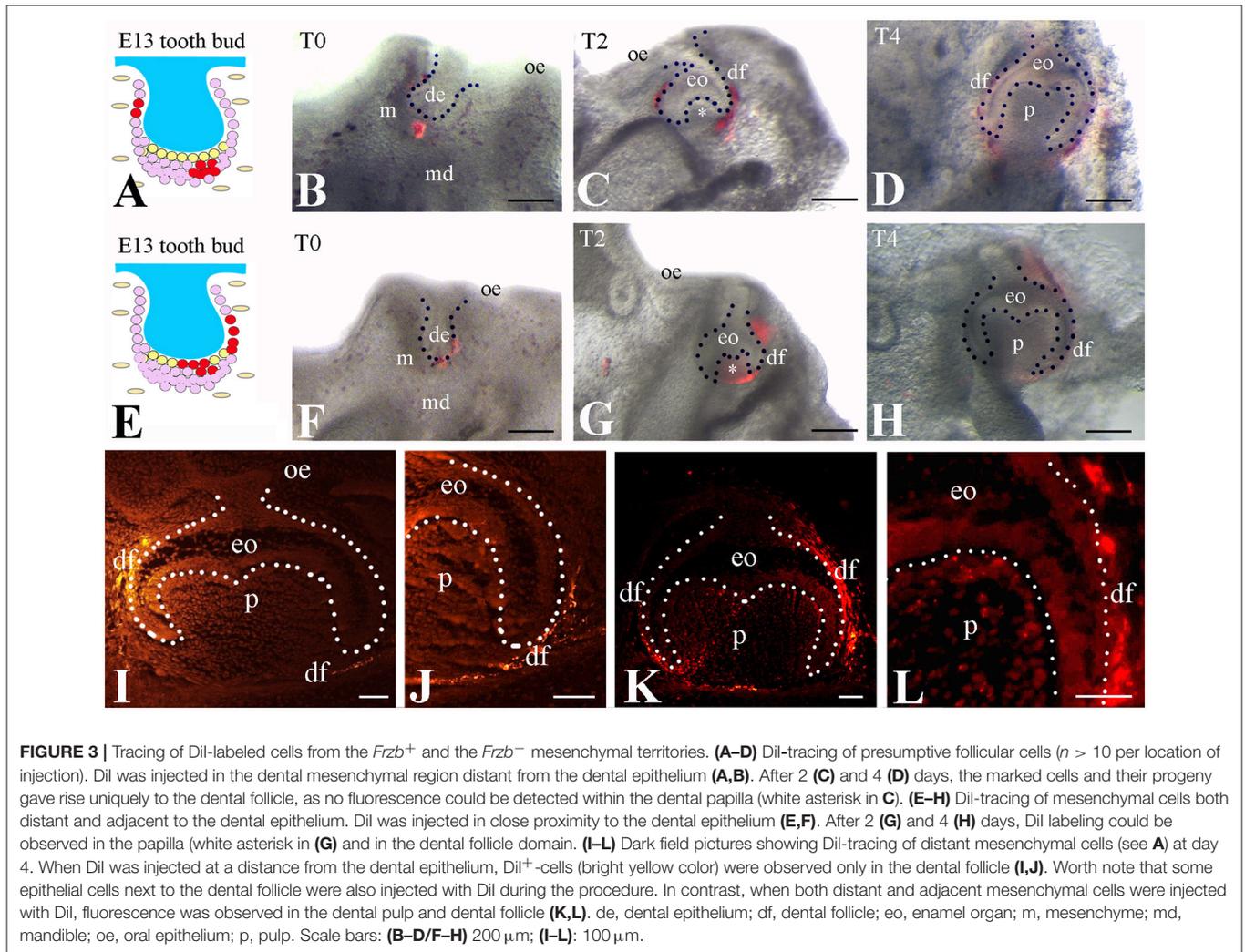


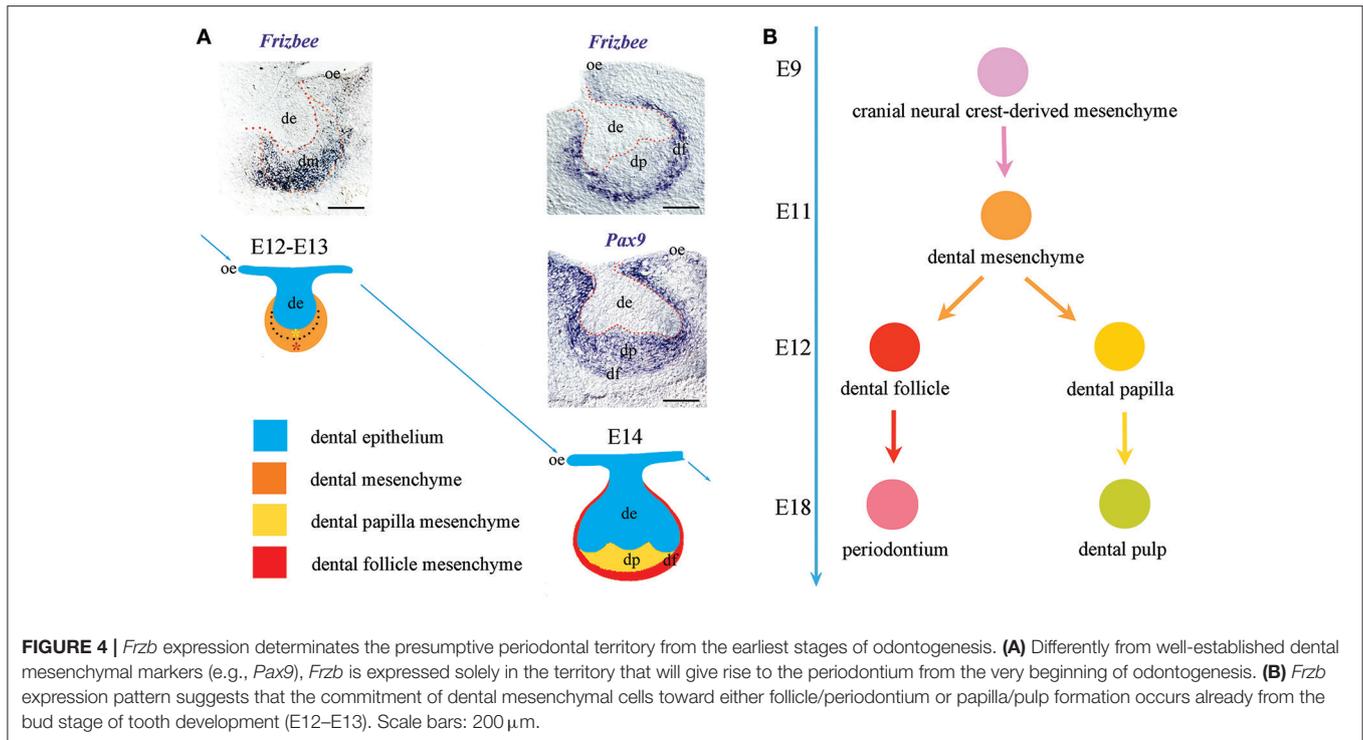
FIGURE 2 | Differential proliferative behavior of dental mesenchymal populations. **(A)** BrdU immunostaining showing intense proliferation in the presumptive dental follicle region (green arrowheads), and little proliferation in the presumptive dental papilla (red asterisk). **(B,C)** At E14–E15, cell proliferation is concentrated in the dental papilla, while it is very limited in the dental follicle (green arrowheads). **(D)** At E13, phosphorylated Histone H3 (pH3) immunostaining indicates cell mitosis in the presumptive dental follicle region (green arrowheads), and little mitotic activity in the presumptive dental papilla (red asterisk). **(E,F)** At E14 and E15, pH3 immunolabelling shows intense mitotic activity in dental papilla and moderate in dental follicle. **(G–I)** Combined BrdU immunostaining (red) and *Frzb* *in situ* hybridization (blue) shows the correlation between cell proliferation and *Frzb* expression within the dental follicle and papilla at E13 **(G)**, E14 **(H)** and E15 **(I)**. **(J)** Quantification of BrdU⁺ cells in dental follicle and dental papilla at E13, E14, and E15. $n = 5$ vs. 5. **(K)** Quantification of pH3⁺ cells in the dental follicle and dental papilla at E13, E14, and E15 ($n = 5$ vs. 5). de, dental epithelium; df, dental follicle; dm, dental mesenchyme; dp, dental papilla; eo, enamel organ; m, mesenchyme; md, mandible; oe, oral epithelium. Scale bars: 100 μ m. ** $p < 0.001$; *** $p < 0.0001$.



surrounding environment (e.g., alveolar bone). Subsequently, once *Frzb* expressing cells have delimited the dental papilla domain, cells from this tiny *Frzb*-negative territory start to actively proliferate in order to increase the tissue size. This process is accompanied by morphological rearrangements within the growing epithelium that dictate the tooth shape.

The differential proliferative activities between dental pulp cells and dental follicle cells are not only limited to development. In our previous studies, we have shown that when pulp and follicle cells are co-cultured, they never intermingle and they assume an organization reminiscent of the *in vivo* situation, with follicle cells surrounding and engulfing pulp cells (Schiraldi et al., 2012). These cell populations appear to have a clear genetic memory and compete for their own territory even *in vitro*. Our results indicate that these two cell populations are functionally and molecularly discriminated from the earliest stages of odontogenesis, thus providing a developmental basis to their clear and persistent peculiarity. Future studies are required to understand the molecular mechanisms underlying the early specification and fate of these cell populations.

In this regard, *Frzb*-based genetic lineage tracing fluorescent analysis on transgenic mouse models might be instrumental to understand the exact contribution of *Frzb*-expressing cells to tooth development as well as to the formation and the regeneration of the periodontium. Since *Frzb* is an extracellular Wnt antagonist, it is tempting to speculate that the restricted expression of *Frzb* to a subset of mesenchymal cells could generate an asymmetric barrier whose function is to limit the activation of the Wnt cascade only in certain cells. It is plausible, in fact, that distinct differentiation fates require cells to be Wnt-responsive, while others necessitate the inhibition of this pathway, as it occurs for the early development of the mouse heart (Marvin et al., 2001) and the eye lens (Cantù et al., 2014; Cvekl and Ashery-Padan, 2014). In this scenario, *Frzb* might restrict the activity of the secreted Wnt ligands to the oral epithelium and to the presumptive dental papilla. Consistently, many genes encoding for Wnt ligands are specifically expressed from the oral epithelium throughout tooth development (Sarkar and Sharpe, 1999) and genetic and molecular evidence indicates that the epithelium must remain responsive to canonical Wnt



signaling. When a mutated, transcriptionally silent, version of β -catenin is exclusively expressed in the epithelium (i.e., epithelial cells become unresponsive to canonical Wnt signaling), tooth development stops abruptly (Cantù et al., 2017). Intriguingly, *Frzb* expression coincides with the dental mesenchymal territory that receives the growing neurons (Pagella et al., 2014). Indeed, from the earliest stages of odontogenesis, nerve fibers grow toward the tooth germ and progressively innervate the dental follicle. On the contrary, these nerves do not penetrate the dental papilla mesenchyme until the late stage of tooth mineralization (Pagella et al., 2014). It is thus possible that *Frzb* expression could regulate tooth innervation and probably morphogenesis. Indeed, increasing evidence indicates that innervation plays an active role in organ morphogenesis and development (Kumar and Brockes, 2012; Pagella et al., 2014). Taken together the present results suggest that the developing dental follicle necessitates a specific inhibition of the activity of the Wnt ligands. Conditional knockout and overexpression studies will constitute fundamental approaches to determine the exact role of *Frzb* in odontogenesis.

Both the dental pulp (formed by the *Frzb* non-expressing dental papilla cells) and the periodontal ligament (formed by

the *Frzb* expressing dental follicle) contain stem cells, the dental pulp and the periodontal ligament stem cell populations, which hold the promise of regenerative approaches (Huang et al., 2009; Mitsiadis et al., 2011; Jiménez-Rojo et al., 2014). The present findings increase the arsenal of markers that permits the specific isolation of mesenchymal cells with desired differentiation potential by the combined use of mesenchymal markers. An increased knowledge of the regulation of mesenchymal dental cells will allow the future development of novel treatments for dental tissue repair and regeneration.

AUTHOR CONTRIBUTIONS

TM designed the project, performed the experiments, interpreted the data, and wrote the manuscript; PP and CC contributed to the interpretation of the data and wrote the manuscript.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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