FGF signaling sustains the odontogenic fate of dental mesenchyme by suppressing β-catenin signaling

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SUMMARY
Odontoblasts and osteoblasts develop from multipotent craniofacial neural crest cells during tooth and jawbone development, but the mechanisms that specify and maintain their respective fates remain largely unknown. In this study, we used early mouse molar and incisor tooth germs that possess distinct tooth-forming capability after dissociation and reaggregation in vitro to investigate the mechanism that sustains odontogenic fate of dental mesenchyme during tooth development. We found that after dissociation and reaggregation, incisor, but not molar, mesenchyme exhibits a strong osteogenic potency associated with robustly elevated β-catenin signaling activity in a cell-autonomous manner, leading to failed tooth formation in the reaggregates. Application of FGF3 to incisor reaggregates inhibits β-catenin signaling activity and rescues tooth formation. The lack of FGF retention on the cell surface of incisor mesenchyme appears to account for the differential osteogenic potency between incisor and molar, which can be further attributed to the differential expression of syndecan 1 and NDST genes. We further demonstrate that FGF signaling inhibits intracellular β-catenin signaling by activating the PI3K/Akt pathway to regulate the subcellular localization of active GSK3β in dental mesenchymal cells. Our results reveal a novel function for FGF signaling in ensuring the proper fate of dental mesenchyme by regulating β-catenin signaling activity during tooth development.

KEY WORDS: β-catenin signaling, FGF, Tooth development

INTRODUCTION
A variety of craniofacial organs and tissues, such as the Meckel’s cartilage, maxillary and mandible bone, trigeminal ganglion and dentin-producing odontoblasts, derive from craniofacial neural crest cells (Chai et al., 2000; Chung et al., 2009). Despite originating from the same progenitor population, craniofacial bone and tooth exhibit distinct developmental, morphological and histological characteristics (Lumsden, 1988; D’Souza et al., 1999; Chai et al., 2000; Zhang et al., 2005; James et al., 2006). It is well established that multiple signaling pathways, including Wnt, TGFβ/BMP, HH and FGF signaling, are involved in regulating every step of tooth development (Theis and Mecklenburg, 2002; Tummers and Theis, 2009), but the mechanisms that specify and ensure the odontogenic fate in dental mesenchyme remain largely unknown.

FGF signaling has been implicated in regulating tooth development at several distinct steps. FGF signaling might be involved in the specification of odontogenic fate in both dental epithelial and dental mesenchyme, as evidenced by Fgfr8 expression in the presumptive dental epithelium and its induction of Pitx2 and Pax9, the earliest molecular markers of the dental epithelium and mesenchyme, respectively, to determine the tooth-forming site (Neubüser et al., 1997; Trumpp et al., 1999; St Amand et al., 2000). At the bud stage, epithelial FGF4 and FGF8 are likely to activate Fgf3 in the dental mesenchyme through the mediation of Msx1 and Runx2, and FGF3 in turn, possibly together with FGF10, acts back on the dental epithelium to induce/maintain Shh expression in the enamel knot (Bei and Maas, 1998; Kettunen et al., 2000; Aberg et al., 2004). At the cap stage, expression of several FGFs in the enamel knot stimulates cell proliferation in the dental epithelium, leading to epithelial folding and cusp patterning (Jernvall et al., 1994; Jernvall and Thesleff, 2000). Furthermore, releasing FGF signaling from suppression by Sprouty factors leads to tooth formation in the diastema region, indicating a potential role for FGF signaling in the regulation of odontogenic fate (Klein et al., 2006; Li et al., 2011b).

The essential role of canonical Wnt (Wnt/β-catenin) signaling in tooth development has been well documented (Liu and Millar, 2010). Many Wnt ligands are expressed in the developing tooth, predominantly in the epithelial component, with WNT5A, a non-canonical Wnt, in the mesenchyme (Dassule and McMahon, 1998; Sarkar and Sharpe, 1999). These Wnt ligands appear to act in both intra- and intertissue manners to regulate tooth development. Epithelial deletion of Catnb (Catnb1 – Mouse Genome Informatics), the gene encoding β-catenin, or Gpr177 (Wls – Mouse Genome Informatics), the product of which is required for secretion of Wnts, leads to an arrest of tooth development at the bud or early cap stage (Liu et al., 2008; Zhu et al., 2013). A similar developmental defect was also observed in mice lacking Catnb in the dental mesenchyme (Chen et al., 2009). Conversely, constitutive activation of β-catenin signaling in oral epithelium induces ectopic tooth formation (Järvinen et al., 2006; Liu et al., 2008). Although β-catenin signaling activity is present in the dental mesenchyme of the E12.5 incisor (Fujimori et al., 2010), such activity has never been reported in the incisor mesenchyme beyond E12.5 and was not detected in developing molar mesenchyme using several Wnt/β-catenin signaling reporter mouse lines, including BATGAL, TOPGAL and TCF/Lef-lacZ mice (Liu et al., 2008), suggesting that Wnt/β-catenin activity is maintained at a very low level, if any, in the dental mesenchyme. Elevated Wnt/β-catenin signaling results in the formation of bone-like tissues in the dental pulp (Chen et al., 2009; Li et al., 2011a). Thus, a finely tuned level of Wnt/β-catenin signaling is essential for proper tooth development.

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In this study, we investigated the mechanisms underlying our previous finding that early molar and incisor tooth germs exhibit distinct tooth-forming capability after dissociation and reaggregation in vitro (Song et al., 2006).

**MATERIALS AND METHODS**

**Animals**

BATGAL mice (Maretto et al., 2003) were obtained from Jackson Laboratories and were crossed onto the CD-1 background. All wild-type mice were CD-1 background and purchased from Charles River. Animals and procedures used in this study were approved by the Institutional Animal Care and Use Committee of Tulane University.

**Tissue recombination, organ culture, bead implantation and subrenal culture**

Embryonic day (E) 13.5 or E14.5 embryos were collected from timed pregnant mice. To prepare tooth reaggregates, mandibular incisor or molar germs from one litter of embryos were isolated and pooled, respectively, then treated with 0.25% trypsin in 1 mM EDTA at 37°C for 5 minutes, and then dispersed into a single-cell suspension by mechanical aspiration with a micropipette. About 1×10^6 cells from either the incisor or molar pool were added to a 1.5-ml Eppendorf tube, centrifuged at 3000 rpm (550 g) for 5 minutes, and incubated at 37°C and 5% CO₂ for 1 hour to allow the formation of a firm cell pellet. Cell pellets were removed from Eppendorf tubes, placed in Trowell type organ culture in DMEM supplemented with 20% FBS overnight prior to being subjected to subrenal culture as described previously (Zhang et al., 2003; Song et al., 2006).

To prepare tooth reaggregates with exchanged dental epithelial cells, isolated incisor and molar germs were treated with 2 mg/ml dispase at 37°C for 30 minutes, washed with DMEM containing 20% FBS, and then dental epithelia were separated from dental mesenchyme with the aid of fine forceps. Incisor mesenchyme was pooled together with molar epithelia and vice versa, and pooled dental tissues were further treated with 0.25% trypsin in 1 mM EDTA at 37°C for 2 minutes to generate the single-cell suspension and tooth reaggregates as described above.

Protein-soaked bead preparation and implantation in tooth reaggregates or intact dental mesenchyme were performed as reported previously (Li et al., 2011b). Affi-Gel Blue agarose beads or heparin beads (100-200 μm in diameter; Bio-Rad) were used as carriers for FGF8 (0.5 mg/ml), FGF4 (4 μg/ml), FGF3 (0.5 mg/ml), FGF9 (0.5 mg/ml), FGF10 (0.5 mg/ml), DKK1 (0.4 mg/ml) and WNT10B (0.1 mg/ml) (all from R&D Systems).

For the heparinase treatment experiment, E14.5 BATGAL molar germs were isolated and treated with dispase, and the epithelia were removed, as described above. The remaining dental mesenchyme was dispersed into single-cell suspension and pelletted. Cell pellet containing ~1×10^6 cells was resuspended in 0.5 ml heparinase buffer (New England Biolabs). The final concentration of heparinases was adjusted as follows: heparinase I (150 μg/ml), heparinase II (10 unit/ml) and heparinase III (40 unit/ml). The cell suspension was incubated at 37°C for 1 hour before reaggregation and organ culture.

**Histology, in situ hybridization and X-Gal staining**

Samples for histological analysis were harvested and fixed in 4% paraformaldehyde (PFA)/PBS. Ossified samples were subjected to Decalifier I (Leica Biosystems) for demineralization for a week, and then dehydrated through graded ethanol, cleared with xylene, embedded in paraffin, and sectioned at 10 μm for standard Hematoxylin/Eosin (H&E) staining (Presnell and Schreibman, 1997).

For in situ hybridization, samples were harvested in ice-cold PBS and fixed in 4% PFA/PBS at 4°C overnight prior to dehydration through graded ethanol and embedding in paraffin. Samples were sectioned at 10 μm and subjected to non-radioactive in situ hybridization as described (Yu et al., 2005b). At least three samples were used for each probe.

For whole-mount X-Gal staining, samples were fixed with 4% PFA/PBS at room temperature for 20 minutes and then stained for β-galactosidase activity according to a standard procedure (Chai et al., 2000). For section X-Gal staining, samples were fixed in 4% PFA/PBS at 4°C overnight, passed through 15% and 30% sucrose series, embedded in O.C.T. compound (Tissue-Tek) and cryosectioned at 10 μm. Sections were then subjected to standard X-Gal staining (Chai et al., 2000).

**Immunostaining and immunoblotting**

For immunohistochemical staining, samples were fixed in Z-Fix (Anatech) at room temperature for 2 hours, dehydrated with 15% and 30% sucrose series, embedded in O.C.T. and cryosectioned at 10 μm. Immunohistochemical staining was conducted according to the manufacturer’s instruction using the following antibodies: mouse monoclonal anti-FGF3 antibody (Santa Cruz), rat anti-mouse syndecan 1 antibody (BD Pharmingen), rat monoclonal antibodies against heparan chondroitin sulfate, heparan dermatan sulfate and heparan keratan sulfate, respectively (Antibodies-online), biotinylated rabbit anti-mouse antibody (Vector Laboratories), horseradish peroxidase-coupled goat anti-rabbit IgG (Sigma), and Alexa Fluor 488 goat anti-rat antibody (Molecular Probes).

For immunocytochemical staining, lower incisor and molar germs were treated with dispase and epithelia removed. Dental mesenchyme was collected and treated with trypsin to make a single-cell suspension as described above. Suspended dental mesenchymal cells were placed onto cell culture dishes and cultured in DMEM supplemented with 20% FBS for 6-12 hours, and then fixed with Z-Fix for 20 minutes. Immunocytofluorescence was performed using primary antibodies against β-catenin (Millipore), GSK3β Y216 (Abcam) and GSK3βα (Abcam). Alexa Fluor 488 and Alexa Fluor 546 goat anti-rabbit IgG (Molecular Probes) were used as secondary antibodies.

Immunoblotting was performed as described previously (Iwata et al., 2006). Rabbit polyclonal antibodies against P-Akt Ser473 and total Akt (Cell Signaling) and mouse monoclonal antibodies against β-actin and FGF3 (Santa Cruz) were used as primary antibodies. IRDye 800cw goat anti-rabbit IgG and IRDye 800cw donkey anti-mouse IgG were used as secondary antibodies (Li-Cor).

**Quantitative RT-PCR**

For quantitative (q) RT-PCR analysis, samples were subjected to RNA extraction using the RNAqueous-4PCR Kit (Ambion). The high capacity cDNA Reverse Transcription Kit (Applied Biosystems) was used for cDNA synthesis. qPCR was carried out on the 7500 Fast Real-Time PCR System (Applied Biosystems) with gene-specific primers and SYBR Green. Values were normalized to Gapdh using the 2^-ΔΔCT method. Data from at least three independent experiments or samples for each gene were used for analysis.

**RESULTS**

**Incisor mesenchymal cells adopt an osteogenic fate in tooth germ reaggregates**

We reported previously that E13.5 mouse molar germ, after dissociation and reaggregation, is able to form a well differentiated tooth organ, whereas incisor germ fails (Song et al., 2006) (Fig. 1A,B). We followed up on this observation to investigate the underlying mechanism. We first tested if failed tooth formation in incisor reaggregates results from a loss of odontogenic competence in the dental epithelial cells after dispersion by exchanging dissociated epithelial cells and mesenchymal cells between E13.5 incisor and molar germ. Reaggregates composed of incisor epithelial and molar mesenchymal cells formed teeth (n=9/10), but reaggregates constituted by incisor mesenchymal and molar epithelial cells failed (n=0/10) and generated bony structures and keratinized cysts after 2 weeks in subrenal culture (Fig. 1C,D). These observations indicate that the incisor mesenchyme loses its odontogenic capability to instruct dispersed dental epithelial cells to form teeth.

Gene expression assays demonstrate the expression of mesenchymal odontogenic markers, including Pax9, Bmp4 and Msx1, in mesenchymal cells surrounding the reorganized dental epithelial structures in molar reaggregates but not in incisor...
reaggregates after 3 days in culture (Fig. 1E-J). By contrast, osteogenic markers, including \( \text{Runx2} \), osteocalcin (\( \text{Bglap} \) – Mouse Genome Informatics) and osterix (\( \text{Sp7} \) – Mouse Genome Informatics), were activated in mesenchymal cells of incisor reaggregates but not in molar reaggregates (Fig. 1K-N; data not shown), which was further confirmed by qPCR assay (Fig. 1Q). These expression patterns persisted in incisor and molar reaggregates after 5 days in subrenal culture (data not shown). The retained expression of \( \text{Pitx2} \), a dental epithelial molecular marker, in the reorganized dental epithelial masses in both incisor and molar reaggregates indicates their odontogenic fate (Fig. 1O,P). These results suggest a deviation of odontogenic fate and the rapid adoption of osteogenic fate in the incisor mesenchyme after dissociation and reaggregation.

**Wnt/\( \beta \)-catenin signaling is robustly activated in mesenchymal cells of incisor reaggregates**

We next investigated the molecular basis for the adoption of osteogenic fate in mesenchymal cells of incisor reaggregates. Wnt/\( \beta \)-catenin signaling plays a crucial role in promoting osteogenic fate and the maturation of osteoblasts (Clément-Lacroix et al., 2005; Gaur et al., 2005). We suspected that dissociation and reaggregation of incisor tooth germ led to the activation of \( \beta \)-catenin signaling and subsequent osteogenesis. \( \beta \)-catenin signaling activity is restricted to the dental epithelium of E13.5 incisor and molar, as detected by the \( \text{BATGAL} \) transgenic reporter (Fig. 2A,B). Using the \( \text{BATGAL} \) reporter mice, we found that after 3 days in subrenal culture, robust \( \text{BATGAL} \) activity could be detected in both epithelial and mesenchymal cells in E13.5 incisor reaggregates (Fig. 2D; \( n=14/14 \)). By contrast, \( \text{BATGAL} \) activity was detected only in the reorganized dental epithelial structures in molar reaggregates (Fig. 2C; \( n=20/20 \)). However, in molar reaggregates after 5 days in subrenal culture, \( \text{BATGAL} \) activity was detected in some mesenchymal cells that were not associated with the forming tooth but also expressed osteocalcin (Fig. 2E,F), indicating an association of active \( \beta \)-catenin signaling with osteogenic fate.

We examined whether molar epithelial cells played an inhibitory role in suppressing \( \beta \)-catenin signaling in molar reaggregates. E13.5 \( \text{BATGAL} \) incisor and molar mesenchyme without dental epithelium were dissociated and reaggregated. Robust \( \text{BATGAL} \) activity was detected in the incisor but not in the molar mesenchymal reaggregate as early as 2 hours in organ culture (Fig. 2E,F), suggesting the existence of a mechanism to inhibit \( \beta \)-catenin signaling in the molar mesenchyme.
FGF3 sustains odontogenic fate and rescues tooth formation in incisor reaggregates by suppressing β-catenin signaling

We next investigated the mechanism that leads to the differential activation of β-catenin signaling in the incisor and molar mesenchyme after dissociation and reaggregation. Since Wnt ligands are expressed predominantly in the dental epithelium and the expression of several Wnt antagonists was found at much higher levels in incisor than in molar mesenchymal cell reaggregates after 12 hours in organ culture (Fig. 2I), we reasoned that β-catenin signaling is activated in a cell-autonomous manner in incisor mesenchymal reaggregates. Since FGF signaling may be involved in odontogenic specification (Neubüser et al., 1997; Trumpp et al., 1999; Kettunen et al., 2000; St Amand et al., 2000; Mandler and Neubüser, 2001), and Fgf3 is expressed in the molar mesenchyme but at an extremely low level in incisor mesenchyme at E13.5 as determined by in situ hybridization and qRT-PCR (Fig. 3A,B,I), we tested if FGF3 could sustain the odontogenic fate by implanting FGF3-soaked beads into E13.5 incisor germ reaggregates. Surprisingly, tooth formation was observed in FGF3-supplemented incisor reaggregates (48%, n=11/23) after 2 weeks in subrenal culture (Fig. 3D). As a negative control, BSA-soaked beads failed to have any such effect (n=0/20; Fig. 3C). In addition, we found that exogenously applied FGF3 did not affect tooth formation in molar reaggregates (n=11/11; data not shown). Consistent with the observation that loss of odontogenic fate is accompanied by rapid osteogenesis in the incisor mesenchyme, FGF3-soaked beads inhibited osteogenesis, as assessed by the dramatically reduced expression of Runx2 and osterix in incisor mesenchymal cell reaggregates as compared with the BSA controls (Fig. 3E-H).

Because Runx2 and osterix are the direct targets of β-catenin signaling and their expression is inhibited by FGF3 in incisor reaggregates, we hypothesized that FGF signaling might prevent osteogenesis by inhibiting β-catenin activity in the dental mesenchyme. We applied FGF3-soaked beads to isolated E13.5 incisor mesenchyme and found an inhibition of BATGAL activity after 12 hours in organ culture, as compared with BSA controls (Fig. 4A,B). As several other FGFs are also expressed in the early developing tooth, we further tested whether they have a similar inhibitory effect by applying FGF4-, FGF8-, FGF9- and FGF10-soaked beads to E13.5 incisor mesenchyme explants. FGF4 and FGF8, but not FGF9 and FGF10, were able to inhibit BATGAL activity (Fig. 4C-F; data not shown). The fact that DKK1-soaked beads were not able to inhibit ectopic activation of β-catenin signaling in incisor mesenchyme explants suggests a ligand-independent activation through an intracellular regulatory mechanism (Fig. 4F). Although exogenously applied WNT10B, one of the canonical Wnts expressed in the dental epithelium, was able to induce BATGAL expression in E10.5 limb mesenchyme without epithelium, at which time BATGAL activity is restricted in the apical ectodermal ridge (Noda et al., 2012), WNT10B-soaked beads failed to induce BATGAL expression in E13.5 molar mesenchyme (Fig. 4G-I). These observations further support the existence of an intracellular repressive mechanism in the dental mesenchyme. Indeed, at the cellular level, in contrast to E13.5 molar mesenchymal cells in which β-catenin exhibited...
cytoplasmic localization (Fig. 4J–J/H11033; 93%, from seven independent
experiments), E13.5 incisor mesenchymal cells showed intense
nuclear accumulation of β-catenin (Fig. 4K–K/H11033; 88.1%, from seven
independent experiments). However, application of FGF3 (250
ng/ml) to cell culture prevented the nuclear accumulation of
β-catenin in incisor mesenchymal cells (Fig. 4L–L/H11033; 54.8%, from three
independent experiments; *P<0.005 compared with untreated group),
indicating that FGF3 inhibits β-catenin signaling by regulating its
subcellular localization. It is interesting to note that the nuclear size
of incisor mesenchymal cells is much smaller than that of molar
mesenchymal cells in cell culture, but becomes enlarged after FGF3
treatment (Fig. 4J–J/H11032, K–K/H11032, L–L/H11032). Although the biological importance of
the changes in nuclear size is unknown, these observations suggest
an involvement of FGF and β-catenin signaling in the regulation of
nuclear size.

differential expression of syndecan 1 and NDST
genes is associated with distinct FGF3 retention
capability between the incisor and molar
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Whereas Fgf3 expression was not detectable by in situ hybridization
in E13.5 incisor (Fig. 3), its expression level in the incisor
mesenchyme at E14.5 became comparable to that in E14.5 molar
mesenchyme (Fig. 5A,B). However, despite Fgf3 expression, teeth
still failed to form in E14.5 incisor germ reaggregates (Fig. 5E).
Interestingly, similar to E13.5 incisor mesenchymal reaggregates,
E14.5 incisor mesenchymal reaggregates also showed significantly

Fig. 3. FGF3 rescues tooth formation and inhibits rapid osteogenesis
in incisor reaggregates. (A,B) Fgf3 expression is not detectable in the
incisor (A) but is seen in the molar mesenchyme (B). (C,D) FGF3-soaked
beads but not BSA-soaked beads rescue tooth formation in E13.5 incisor
reaggregates after 2 weeks in subrenal culture. (E-H) FGF3 beads but not
BSA beads inhibit Runx2 and osterix expression in E13.5 incisor
mesenchymal cell reaggregates after 3 days in culture. (I) Real-time RT-
PCR results show relative levels of Fgf3 expression in E13.5 incisor and
molar germs. **P<0.01 (Student’s t-test); error bars indicate s.d. B, bead.
Scale bars: 200 μm.

Fig. 4. FGF signaling inhibits β-catenin signaling in dental
mesenchymal cells. (A–F) FGF3 (B), FGF4 (C) and FGF8 (D) soaked beads
prevent ectopic activation of Wnt/β-catenin signaling in isolated E13.5
BATGAL incisor mesenchyme, but BSA (A), FGF10 (E) and DKK1 (F) soaked
beads fail to do so. Note that Wnt/β-catenin activity is not inhibited by
FGF3, FGF4 or FGF8 in the symphystic portion (asterisk) of Meckel’s
cartilage. Red dashed line encircles the implanted beads. (G–I) WNT10B-
but not BSA-soaked beads induce BATGAL expression in E10.5 limb bud mesenchyme (G,H), but WNT10B beads cannot induce
BATGAL activity in E13.5 molar mesenchyme after 12 hours in organ
culture (I). (J–L) Immunocytochemical staining shows localization of β-
catenin in the cytoplasm of E13.5 molar mesenchymal cells (J–K’), and
nuclear accumulation of β-catenin in E13.5 incisor mesenchymal cells (K–
K’/H11033) after 12 hours in cell culture. Addition of FGF3 to cell culture prevents
nuclear accumulation of β-catenin in incisor mesenchymal cells (L–L’;
arrrows point to cells with nuclear localization of β-catenin).

Differential expression of syndecan 1 and NDST
genes is associated with distinct FGF3 retention
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Fig. 5. Failed FGF3 retention is associated with ectopic activation of β-catenin signaling in incisor reaggregates. (A,B) In situ hybridization shows Fgf3 expression in the mesenchyme of E14.5 molar (A) and incisor (B). (C,D) X-Gal staining shows elevated Wnt/β-catenin signaling in E14.5 BATGAL incisor mesenchymal cell reaggregate (D) but not in E14.5 molar mesenchymal cell reaggregate (C) after 2 hours in culture. (E) H&E staining shows lack of tooth formation in an E14.5 incisor reaggregate. (F-H) Immunohistochemical staining shows retention of FGF3 in E14.5 molar reaggregate (F) but not in E14.5 incisor reaggregate (G) and lack of positive signaling in the negative control of molar reaggregate (H). (I-L) In situ hybridization shows lack of Fgf3 expression in E14.5 molar reaggregate after 3 days in subrenal culture (I) and in E14.5 incisor reaggregate after 3 days (J) and 5 days (L) in subrenal culture. However, Fgf3 expression was detected in mesenchymal cells immediately adjacent to a reorganized epithelial structure in an E14.5 molar reaggregate after 5 days in culture (K). (M,N) Immunohistochemical staining shows differential expression of syndecan 1 in E14.5 molar (M) and incisor (N). (O,P) X-Gal staining shows activation of β-catenin signaling in E14.5 BATGAL molar mesenchymal cell reaggregate after treatment with heparinas (P) but not in control (O). (Q,R) E14.5 molar reaggregates failed to form tooth (Q) and did not express Fgf3 after heparinase treatment. (S) Western blotting shows the presence of FGF3 protein in intact E14.5 molar germ (lane 1) and retention of FGF3 in E14.5 molar reaggregates after 1 hour (lane 2) and 3 days (lane 4) in culture but not in heparinase-treated reaggregates after 1 hour in culture. Dashed lines demarcate dental epithelial structures. Scale bars: 200 μm.

Elevated BATGAL activity after 2 hours in culture, as compared with E14.5 molar mesenchymal reaggregates (Fig. 5C,D). We wondered whether the activation of β-catenin signaling and failure of tooth formation result from a lack of de novo Fgf3 expression in incisor germ reaggregates. In situ hybridization showed that Fgf3 expression was lost and was never re-established in E14.5 incisor reaggregates after 3 and 5 days in culture (Fig. 5J,K). However, de novo Fgf3 expression was not detected in E14.5 molar reaggregates until 5 days in culture (Fig. 5J,K), suggesting that the rapid activation of β-catenin signaling in incisor reaggregates is not a consequence of failed de novo synthesis of FGF3. Since both E14.5 incisor and molar germs express Fgf3, we next examined whether incisor and molar mesenchymal cells have different capabilities in retaining FGF3 protein in reaggregates. Immunohistochemical studies revealed the presence of FGF3 in the mesenchymal compartment of E14.5 molar reaggregates after 2 days in culture when de novo activation of Fgf3 had not yet begun (Fig. 5F). By contrast, no retained FGF3 was detected in E14.5 incisor reaggregates (Fig. 5G).

Heparan sulfate proteoglycans (HSPGs) play crucial roles in the transport and reception of secreted factors, and are known to promote FGF signaling by enriching FGF ligands, preventing them from degrading, and facilitating their binding to receptors (Lin, 2004; Häcker et al., 2005). We asked whether there is a differential expression of HSPGs in the incisor and molar that could account for the distinct FGF retention capability. We performed immunohistochemical staining on E14.5 incisor and molar teeth to examine a number of HSPGs, including heparan chondroitin sulfate, heparan keratan sulfate and heparan dermatan sulfate, as well as syndecan 1, which has the highest expression level among several syndecans in the developing tooth (Thesleff et al., 1988; Vainio et al., 1989; Vainio et al., 1991; Vainio and Thesleff, 1992; Bai et al., 1994). Although heparan chondroitin sulfate, heparan keratan sulfate and heparan dermatan sulfate are among the richest HSPGs in the developing embryo, none was expressed in incisor or molar germs (data not shown). However, syndecan 1 was found to be highly expressed in the molar mesenchyme, but was absent or expressed at a very low level in the incisor mesenchyme despite its expression in the surrounding tissues (Fig. 5M,N). These results suggest that the higher level of syndecan 1 in the molar mesenchyme protects FGF3 from degradation by enzyme treatment and from diffusing into suspension during dissociation and reaggregation.

To determine whether HSPGs play a role in suppressing β-catenin signaling by facilitating FGF signaling in dental mesenchymal cells, we treated dissociated E14.5 BATGAL molar mesenchymal cells with heparinases before reaggregation. An elevated BATGAL activity was detected in heparinase-treated molar mesenchymal reaggregates (n=11/11) after 12 hours in culture, as compared with control reaggregates (n=2/10) (Fig. 5O,P). Moreover, heparinase-treated molar mesenchymal cells failed to form a tooth (n=0/8) after reaggregation with dissociated molar epithelial cells (Fig. 5Q), as
FGF sustains odontogenic fate

Development

FGF signaling regulates the subcellular localization of active GSK3β and β-catenin in dental mesenchymal cells by activating the PI3K/Akt pathway

We next sought to determine the mechanism through which FGF3 prevents nuclear accumulation of β-catenin in dental mesenchymal cells. GSK3β acts a negative modulator of β-catenin signaling by phosphorylating β-catenin for degradation. We investigated the immunocytochemical localization of both inactive and active forms of GSK3β in dissociated E13.5 incisor and molar mesenchymal cells in culture. Whereas the inactive form (p-GSK3βS9) is similar to localized in the cytoplasm of incisor and molar mesenchymal cells (data not shown), the active form (p-GSK3βY216) showed distinct subcellular localizations. In the majority of molar cells, p-GSK3βY216 was found predominantly in the cytoplasm (Fig. 7A–C), but in the incisor cells p-GSK3βY216 was localized exclusively in the nuclei (Fig. 7B–D; 88%, from five independent experiments). Since the degradation of β-catenin by p-GSK3βY216 requires the coordination of AXIN2 and APC, which reside only in the cytoplasm (Logan and Nusse, 2004; Ciani and Salinas, 2005), the nuclear localized p-GSK3βY216 in the incisor mesenchymal cells is incapable of degrading β-catenin and modulating β-catenin signaling negatively. Treatment of incisor mesenchymal cells with FGF3 (250 ng/ml) in cell culture changed the subcellular localization of p-GSK3βY216, with 46% (from three independent experiments) of cultured incisor mesenchymal cells exhibiting cytoplasmic localization of p-GSK3βY216 (Fig. 7C–C), indicating that FGF3 promotes the cytoplasmic localization of active GSK3β.

It was reported previously that FGF signaling promotes both the nuclear export and activation of GSK3β through the PI3K/Akt pathway in mouse embryonic stem cells (mESCs) (Bechard and Dalton, 2009; Singh et al., 2012). To determine if similar mechanisms are employed in dental mesenchymal cells, we first performed western blotting to examine the activity levels of the PI3K/Akt pathway. Similar levels of active Akt (P-AktS473) and total Akt (Pan-Akt) were found in the intact incisor and molar mesenchyme at both E13.5 and E14.5 (Fig. 8A). However, after 4 hours in cell culture, active Akt was completely absent from the incisor mesenchymal cells, but was retained in molar mesenchymal cells (Fig. 8B). Addition of FGF3 (250 ng/ml) to incisor mesenchymal cell culture resumed the expression of active Akt (Fig. 8B).

We further determined whether the active PI3K/Akt pathway regulates the subcellular localization of active GSK3β (p-GSK3βY216) in dental mesenchymal cells by immunocytochemical assay. Dissociated E13.5 molar mesenchymal cells retained the cytoplasmic localization of p-GSK3βY216 in ~94.2% (from three experiments) of cells after 12 hours in cell culture, whereas after 4 hours in cell culture, active Akt was completely absent from the incisor mesenchymal cells, but was retained in molar mesenchymal cells (Fig. 8B). Addition of FGF3 (250 ng/ml) to incisor mesenchymal cell culture resumed the expression of active Akt (Fig. 8B).

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DISCUSSION

Elevated β-catenin signaling in the dental mesenchyme is detrimental to normal odontogenesis

Multiple Wnt ligands are expressed in the dental epithelium of the developing tooth and have been demonstrated to act in an intra-
epithelial manner to regulate early tooth development (Zhu et al., 2013). These epitheliaically expressed Wnts also act on dental mesenchyme and form a Wnt-BMP feedback circuit with mesenchymally expressed BMP4 to mediate epithelial-mesenchymal interactions during early tooth development (O’Connell et al., 2012). The requirement of β-catenin signaling in the dental mesenchyme for early tooth development was manifested by the arrested molar development at the bud stage and the splitting of the incisor placode in mice carrying tissue-specific inactivation of 

**Fig. 7. FGF3 regulates cytoplasmic localization of active GSK3β in dental mesenchymal cells.** Immunocytochemical staining reveals (A-A') cytoplasmic localization of active GSK3β in E13.5 molar mesenchymal cells and (B-B') nuclear localization of active GSK3β in E13.5 incisor mesenchymal cells. (C-C') In the presence of FGF3, active GSK3β becomes cytoplasmic (arrows) in incisor mesenchymal cells.

**Fig. 8. FGF signaling regulates the subcellular localization of active GSK3β in dental mesenchymal cells by activating the PI3K/Akt pathway.** (A) Western blotting assay shows similar levels of total Akt (Pan-Akt) and activated Akt (P-Akt) in E13.5 and E14.5 incisor and molar mesenchyme. (B) Western blotting shows unaltered level of P-Akt in E13.5 molar mesenchymal cells after 4 hours in culture, but the complete absence of P-Akt in incisor mesenchymal cells. P-Akt was retained in incisor mesenchymal cells after 4 hours in cell culture in the presence of FGF3. (C-F) Immunocytochemical staining shows cytoplasmic localization of GSK3β (C-C') and β-catenin (E-E') in E13.5 molar mesenchymal cells after 12 hours in cell culture, and nuclear localization (arrows) of GSK3β (C-C') and β-catenin (E-E') in molar mesenchymal cells in the presence of the PI3K/Akt pathway inhibitor BEZ235.

**FGF signaling inhibits β-catenin signaling in the dental mesenchyme**

During development and physiological processes, Wnt signaling is precisely regulated by a number of modulators at intra- and extracellular levels (Clevers and Nusse, 2012). In addition, the intensity of Wnt/β-catenin signaling is also regulated by its crosstalk with other signaling pathways. In the developing tooth, several extracellular Wnt antagonists, including Dkk1, Sfrps and SOSTDC1, are expressed (Leimeister et al., 1998; Laurikkala et al., 2003; Fjeld et al., 2005), and loss of Smad4 in the dental mesenchyme results in downregulation of the Wnt inhibitors DKK1 and SFRP1, leading to elevated β-catenin activity and subsequent formation of bone-like structure (Li et al., 2011a). In the present study, we show that exogenously applied DKK1 failed to prevent ectopic activation of β-catenin signaling in the incisor mesenchyme and exogenously applied WNT10B could not induce a canonical signaling response in the molar mesenchyme, indicating the
existence of an intracellular regulatory mechanism of β-catenin activity in the dental mesenchyme. These observations also explain why β-catenin activity is maintained at a very low level, if any, in the dental mesenchyme, despite expression of multiple canonical Wnts in the dental epithelium.

We further show that FGFs, including mesenchymally expressed FGF3 and epithelium-derived FGF4 and FGF8, suppress β-catenin activity in the incisor mesenchyme. Remarkably, application of exogenous FGF3 not only inhibited β-catenin activity and osteogenesis in incisor reaggregates but also resumed odontogenic capability in terms of tooth formation in the reaggregates. Certainly, because mechanochemocontrol of mesenchymal condensation has been shown to be crucial for tooth development (Mammoto et al., 2011), a contribution of mesenchymal condensation by FGF signaling to tooth formation cannot be ruled out.

These results suggest a novel function for FGF signaling in regulating odontogenic fate by attenuating β-catenin signaling through the prevention of β-catenin nuclear localization. Since epithelium-derived FGF4 and FGF8 could also inhibit β-catenin signaling activity in the dental mesenchyme and as other FGFs, such and Fgf9 and Fgf10, are co-expressed in the developing tooth (Kettunen and Thesleff, 1998; Kettunen et al., 2000), the lack of a tooth defect in the Fgf null mouse might be attributed to functional redundancy between these FGFs (Mansour et al., 1993). This could also explain why tooth forms in the tissue recombinants of an intact dental epithelium and FGF-free incisor mesenchymal reaggregate (data not shown).

**FGF signaling inhibits mesenchymal β-catenin signaling through activating the PI3K/Akt pathway**

Since Wnt ligands are expressed predominantly in the dental epithelium of developing tooth germ, the ectopic activation of β-catenin signaling in incisor mesenchymal reaggregates without dental epithelium appears to result from the intracellular relief of β-catenin activity suppression. This point is further supported by the fact that exogenously applied DKK1 failed to prevent activation of β-catenin signaling in isolated incisor mesenchyme and by the failure of exogenous WNT10B to induce β-catenin signaling in molar mesenchyme. It was reported previously that FGF signaling activates canonical Wnt activity by inhibiting GSK3β via the PI3K/Akt pathway in tumorigenesis (Katoh and Katoh, 2006). However, FGF signaling can also suppress β-catenin signaling by activating GSK3β via the PI3K/Akt pathway in mESCs (Singh et al., 2012). In the latter system, the accumulation of active GSK3β (p-GSK3βY216) in the nucleus promotes the differentiation of mESCs, whereas the activated PI3K/Akt pathway relocates the active GSK3β into the cytoplasm and promotes cell proliferation (Bechard and Dalton, 2009).

In this study, we show that in the dental mesenchymal cells FGF signaling suppresses β-catenin signaling by maintaining the active GSK3β in the cytoplasm via activation of the PI3K/Akt pathway. This is evidenced by the increased level of p-Akt\(^{S473}\) and the translocation of p-GSK3βY216 from the nucleus to the cytoplasm in the dissociated incisor mesenchymal cells in the presence of FGF3. Inhibition of the PI3K/Akt pathway facilitates the importation of both active GSK3β and β-catenin into the nucleus, leading to activation of β-catenin signaling. However, whether other FGF-mediated pathways, such as the Erk/Mek pathway, also contribute to the repression of β-catenin signaling and whether FGF signaling regulates non-canonical Wnt signaling in the dental mesenchymal cells warrant further investigation.

**Differential expression of syndecan 1 and NDST genes confers different osteogenic potency on incisor and molar mesenchyme after dissociation and reaggregation**

Our studies show that despite Fgfb3 expression in the mesenchyme of both E14.5 incisor and molar germs, FGF3 was retained on the cell surface of molar mesenchyme but not incisor mesenchyme after dissociation and reaggregation. The retention of FGF3 in molar reaggregates appears to sustain the odontogenic fate and allows odontogenesis, but the lack of FGF3 retention leads to activation of β-catenin signaling and deviates odontogenic fate in incisor reaggregates. This distinct capability for FGF retention could be attributed to the differential expression of syndecan 1 in the incisor and molar mesenchyme. Several syndecans, which are the major cell membrane HSPGs, are expressed in the developing tooth, with syndecan 1 exhibiting the highest expression level (Thesleff et al., 1988; Vainio et al., 1989; Vainio et al., 1991; Vainio and Thesleff, 1992; Bai et al., 1994). The requirement of syndecan 1 for FGF signaling has been reported in mammalian cortical development, epithelial-mesenchymal transition and tumorigenesis (Stepp et al., 2002; McDermott et al., 2007; Wang et al., 2012). However, syndecan 1 null mice do not exhibit a tooth development defect, suggesting functional compensation from other syndecans (Alexander et al., 2000; Stepp et al., 2002). The higher level of syndecan 1 expression in the molar mesenchyme appears to be crucial for FGF3 retention in reaggregates. In addition, NDSTs also regulate FGF signaling during organogenesis, as the heparan sulfate chains provide resistance to enzyme digestion and high FGF binding affinity to the core proteoglycan (Pan et al., 2006; Pan et al., 2008; Hu et al., 2009). The higher level of NDST expression in the molar mesenchyme, as compared with that in the incisor mesenchyme, could further confer higher heparan sulfation of HSPGs, including syndecan 1, in the molar mesenchyme and contribute to FGF3 retention (Lin, 2004; Hécker et al., 2005). Thus, the higher levels of syndecan 1 and NDSTs are responsible for FGF3 retention in molar reaggregates. This notion is further supported by the fact that overdigestion with trypsin or treatment with heparanases resulted in activation of β-catenin signaling, lack of FGF3 retention, and failed tooth formation in molar reaggregates.

In summary, we have shown that elevated β-catenin signaling is associated with the fate change of dental mesenchymal cells, and FGF signaling is able to sustain the odontogenic fate by suppressing intracellular β-catenin signaling. The interplay between FGF and β-catenin signaling appears to regulate the proper fate of craniofacial neural crest cells during tooth and jawbone formation.

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**Competing interests statement**

The authors declare no competing financial interests.

**Author contributions**

Y.C., C.L. and Y.Z. conceived and designed the experiments. C.L. and Y.Z. initiated the project. C.L. carried out most of the experiments. S.G. performed...
qPCR. C.S. and Z.S. performed histological and in situ hybridization assays. W.Y. helped to conduct western blotting and immunocytochemical assays. Y.C., C.L. and S.G. analyzed the data. Y.C. and C.L. wrote the manuscript.

References


