IGF-I Mediates EphrinB1 Activation in Regulating Tertiary Dentin Formation

Journal of Dental Research
2017, Vol. 96(10) 1153–1161
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for Dental Research 2017
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DOI: 10.1177/0022034517708572
journals.sagepub.com/home/jdr

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Abstract

Eph receptors belong to a subfamily of receptor tyrosine kinases that are activated by membrane-spanning ligands called ephrins. Previously, we demonstrated that the ephrinB1-EphB2 interaction regulates odontogenic/osteogenic differentiation from dental pulp cells (DPCs) in vitro. The goal of this study was to identify the molecular mechanisms regulated by the EphB2/ephrinB1 system that govern tertiary dentin formation in vitro and in vivo. During tooth development, ephrinB1, and EphB2 were expressed in preodontoblast and odontoblasts at postnatal day 4. EphrinB1 was continuously expressed in odontoblasts and odontoblastic processes until the completion of tooth eruption. In addition, ephrinB1 was expressed in odontoblastic processes 2 wk following tooth injury without pulp exposure, whereas EphB2 was expressed in the center of pulp niches but not odontoblasts. In a model of tooth injury with pulp exposure, ephrinB1 was strongly expressed in odontoblasts 4 wk postinjury. In vitro studies with human and mouse DPCs treated with calcium hydroxide (CH) or mineral trioxide aggregate (MTA) showed an increased expression of insulin-like growth factor 1 (IGF-I). Experiments using several inhibitors of IGF-I receptor signaling revealed that inhibiting the Ras/Raf-1/MAPK pathway inhibited EphB2 expression, and inhibiting the PI3K/Akt/mTOR pathway specifically inhibited ephrinB1 gene expression. Tooth injury in mice with odontoblast-specific IGF-I receptor ablation exhibited a reduced tertiary dentin volume, mineral density, and ephrinB1 expression 4 wk following injury. We conclude that the IGF-I/ephrinB1 axis plays significant roles in the early stages of tooth injury. Further research is needed to fully understand the potential of targeting ephrinB1 as a regenerative pulp therapy.

Keywords: dentinogenesis, pulp biology, stem cell(s), odontogenesis, cell signaling, histochemistry

Introduction

Dental pulp is important not only for tooth viability but also for maintaining the capacity to generate the tertiary dentin matrix in response to injury (Qureshi et al. 2014). Thus, vital pulp therapy is aimed at maintaining the viability of the cells in that niche and stimulating tissue repair—namely, cell differentiation and tertiary dentin formation.

Numerous clinical and animal studies have shown that growth factors, including transforming growth factors (TGFs) (Bègue-Kirn et al. 1992; Nie et al. 2006), bone morphogenetic proteins (BMPs) (Bègue-Kirn et al. 1992; Chen et al. 2008), fibroblast growth factors (FGFs) (Sagomonyants and Mina 2014), and insulin-like growth factor 1 (IGF-1) (Li et al. 1998; Wang et al. 2012), can induce the differentiation of pulp progenitor/stem cells into odontoblasts, which results in dentin formation. Moreover, a combination of growth factors such as TGF- β and FGF2 (He et al. 2008) or IGF-1 and platelet-derived growth factor-BB (Denholm et al. 1998) can synergistically induce the proliferation and differentiation of dental pulp cells (DPCs) and the formation of mineralized nodules.

Eph receptors belong to a subfamily of receptor tyrosine kinases that are activated by membrane-spanning ligands called ephrins. Interactions between Eph receptors and ephrins at the cell membrane trigger bidirectional signaling. The extracellular domains of ephrins on one cell activate the tyrosine

kinase domain of Eph receptors on the adjacent cell; the intracellular domains of ephrins then engage the Src family kinases and other effector molecules (Gerety and Anderson 2002; Yu et al. 2003; Salvucci et al. 2006; Stokowski et al. 2007; Klein 2009; Vermeer et al. 2012). The molecular mechanisms that regulate reparative dentinogenesis are not fully understood. We previously found that EphB2-ephrinB1 interactions play important roles in odontoblast proliferation and differentiation of DPCs (Wang et al. 2013). Here, we investigated how the ephrinB1-EphB2 interaction regulates primary dentin formation during tooth development and tertiary dentin formation in response to injury. We found that ephrinB1 and EphB2 are

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A supplemental appendix to this article is available online.

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upregulated by direct pulp capping materials via IGF-1 signaling in vitro and that ephrinB1 is also expressed during tertiary dentin formation in a manner regulated by the IGF-1 pathway in vivo.

Materials and Methods

Animals

C57BL/6J (B6) mice were purchased from Jackson Laboratories. The odontoblast-specific IGF-1 receptor (DMP1-*Igf1r*) mouse model was described previously (Liu et al. 2016). Weaned mice were allocated randomly into cages according to their sex. Mice were housed 2 to 5 animals per cage in a facility with 12-h light-dark cycles and free access to food and water. All animal protocols were approved by the New York University Institutional Animal Care and Use Committee (IACUC).

Tooth Injury

Mice (1–2 mo old) were anesthetized with ketamine HCl (62.5 mg/kg) and xylazine (12.5 mg/kg) in sterile phosphate-buffered saline by intraperitoneal injection and then mounted on a jaw retraction board. For tooth injury without pulp exposure, the occlusal surfaces of the right maxillary and right mandibular first molars were injured halfway into the dentin layer using a No. 2 round bur drill, air-dried, and sealed with Cavit (Premier). For tooth injury with pulp exposure, the occlusal surfaces of the right maxillary first molars were injured until pulp was exposed using a No. 2 round bur drill with water spray, air-dried, and sealed with glass ionomer cement (Prime-Dent). The right injured maxillae were used for histology, and the right injured mandibles were used for micro-computed tomography (CT) or the analysis of gene expression. The left side of the maxillae was used as a control (noninjured site) for histology or the analysis of gene expression. For tooth injury without pulp exposure, mouse heads were collected at 2 d, 2 wk, and 4 wk following tooth injury. For tooth injury with pulp exposure, mouse heads were collected at day 0, 2 wk, and 4 wk. The heads of DMP1-*Igf1r* or control mice were collected at 4 wk following tooth injury.

Culture of Mouse/Human DPCs

Mouse DPCs were harvested from 1- to 2-mo-old male C57BL/6 or DMP1-*Igf1r* mice, minced in phosphate-buffered saline (PBS), and incubated in PBS containing 3 mg/mL collagenase type I (Sigma-Aldrich) and 4 mg/mL trypsin (Sigma-Aldrich) for 30 min at 37°C. Cells were cultured on plates with differentiation media (α -minimum essential medium [α -MEM] supplemented with 20% fetal bovine serum, 2 mM L-glutamine, and 100 U/mL penicillin-streptomycin; Life Technologies, Grand Island, NY) and 50 μ g/mL L-ascorbic acid (Sigma-Aldrich). Human DPCs were purchased from Lonza and cultured in DPC growth medium, including Basal Medium and SingleQuots Kit (Lonza) for 10 d according to the manufacturer's

instructions. For the last 4 d of the culture period, 5 mM β -glycerol phosphate (EMD Millipore) was added to the media for mineralization. A final concentration of 1 μ g/mL of calcium hydroxide (CH) was made by adding 1 μ L of 1 mg/mL calcium hydroxide stock into the 12-well plate with 1 mL media, as described previously (Wang et al. 2013). Cells were treated with 10 mg/mL mineral trioxide aggregate (MTA) as described by Moghaddame-Jafari et al. (2005). Mouse and human recombinant IGF-1 was purchased from Peprotech.

Inhibitors for Cell Cultures

Mouse or human DPCs were cultured on 12-well plates with differentiation media and then preincubated with a MEK inhibitor (5 μ M PD98059; Cell Signaling Technology for 1 h), a PI3K inhibitor (10 μ M LY294002; Cell Signaling Technology) for 1 h, or an mTOR inhibitor (200 nM NVP-BEZ235; Cell Signaling Technology) for 6 h. Following incubation, the cells were treated with or without CH for 24 h.

Quantitative Real-Time Polymerase Chain Reaction

Total RNA was isolated from DPCs or mouse mandibular teeth following tooth injury using TRIzol (Thermo Fisher Scientific) and reverse transcribed to complementary DNA with TaqMan Reverse Transcription Reagents (Thermo Fisher Scientific) according to the manufacturer's instructions. The sequences were amplified by adding complementary DNA to the polymerase chain reaction (PCR) mixture containing each primer and Platinum SYBR Green qPCR SuperMix uracil-DNA glycosylase (UDG) (Thermo Fisher Scientific). The reactions were preincubated at 50°C for 2 min for decontamination of deoxyuridine (dU)-containing DNA by UDG and then incubated at 95°C for 2 min to inactivate UDG and activate Taq. The PCR program continued with 49 cycles of denaturation at 95°C for 15 s followed by annealing and elongation of the primers at 60°C for 30 s. All samples were normalized to β -actin. The primer sequences are shown in Appendix Table 1.

Immunohistochemistry

For paraffin sections, the maxillae were fixed in 4% paraformaldehyde and decalcified in 10% EDTA. Paraffin sections were cut longitudinally. For frozen sections, the heads were fixed in 4% paraformaldehyde and decalcified in 10% EDTA. Frozen sections were cut coronally. Immunohistochemical staining was performed using the ABC staining system (Santa Cruz Biotechnology) according to the manufacturer's instructions. The sections were incubated with anti-ephrinB1 (Santa Cruz Biotechnology) and anti-EphB2 (Santa Cruz Biotechnology) antibodies or normal immunoglobulin G (IgG) as negative control at 4°C followed by biotin-labeled anti-IgG. Staining was completed with a 5-min incubation with 3,3'-diaminobenzidine (DAB).

Figure 1. EphrinB1/EphB2 is expressed in odontoblasts during tooth formation. (A) Mouse heads at postnatal day 2, day 4, 2 wk, and 4 wk. Frozen or paraffin sections were processed and the expression of ephrinB1 (A, B, E, F, I, J, M, and N) and EphB2 (C, D, G, H, K, L, O, and P) in the upper first molar was assessed. DP, dental pulp; OB, odontoblasts. Low magnification (A, C, E, G, I, K, M, and O; 100 μ m) and higher magnification views (B, D, F, H, J, L, N, and P; scale bars: 10 μ m) are shown. (B) The expression of EphB2 in the upper first molar of 4-wk-old mice. The higher magnification view is inset into the low magnification image.

Statistical Analysis

All results are expressed as the means $\pm$ standard error of the mean (SEM) of triplicate measurements; all experiments were repeated at least 3 times. Statistical analyses were performed using Student's *t* tests or 1-way analysis of variance (ANOVA) with Tukey's honestly significant difference (HSD) tests.

Results

EphrinB1 Is Expressed in Odontoblasts in the Developing and Mature Tooth

To explore the time points at which ephrinB1 and EphB2 are detected in developing and mature teeth, mandibles were dissected from postnatal 2-d, 4-d, 2-wk, and 4-wk-old male C57BL/6 mice. The mandibles were fixed and then processed for immunostaining for ephrinB1 and EphB2 (Fig. 1A). EphrinB1 was detectable in preodontoblasts and odontoblasts as early as 4 d postnatally. Expression then persisted in the odontoblastic layers and even in the mature molars at 4 wk. EphB2 was transiently expressed in odontoblasts at 4 d (Fig. 1A-G, H). At 4 wk, EphB2 was expressed in the center of the pulp and the perivascular area (Fig. 1A-O, B) but not in odontoblasts (Fig. 1A-P).

EphrinB1 Is Upregulated Following Tooth Injury

To study whether ephrinB1/EphB2 plays a role in dentinogenesis, a tooth injury model with or without pulp exposure was used. Tooth injury was induced in the right maxillary first molars by drilling until pulp was exposed and then sealing with glass ionomer cement. Three weeks following injury, RNA extraction from molars revealed a $\sim$ 2-fold increase in *ephrinB1* and *IGF-1* gene expression but not in *ephrinB2*, *EphB2*, or *IGF1R*. Furthermore, the expression of the inflammatory-cytokines interleukin-1 (*IL-1*) and tumor necrosis factor- α (*TNF- α*) was increased in the injured tooth compared with the intact tooth (Fig. 2A). Molars from mice with and without pulp exposure were collected and evaluated by hematoxylin and eosin staining and immunohistochemistry for ephrinB1 and EphB2 after the operation (Fig. 2B, C; Appendix Fig. 1A, B). In the control teeth without tooth injury, odontoblasts were aligned under the predentin layer (Fig. 2B-A, B), and ephrinB1 was located in the odontoblasts and subodontoblastic layer (Fig. 2B-C, D). At day 2, the odontoblasts under the injured cusps maintained their characteristic features, although they were slightly disorganized (Fig. 2B-E, F). The expression of ephrinB1 in odontoblasts was increased compared with control (Fig. 2B-G, H). At week 2, intense ephrinB1 expression was observed in odontoblasts, including their cellular processes

Figure 2. Expression of ephrinB1 after tooth injury with or without pulp exposure. **(A)** Left (noninjury) and right (injury) mandibular molars were extracted 3 wk following injury. Gene expression was measured using real-time reverse transcriptase polymerase chain reaction. * $P < 0.05$ versus control (left side). **(B)** The occlusal surfaces of the right maxillary first and second molars on the right side were injured halfway into the dentin and sealed using Cavit. The maxillae were collected at 2 d, 2 wk, and 4 wk following tooth injury without pulp exposure. Hematoxylin and eosin-stained (A, B, E, F, I, J, M, and N) and ephrinB1-stained (C, D, G, H, K, L, O, and P) sections of control (A–D, left side of maxilla, noninjured site) and injured teeth (right side of maxilla) at day 2 (E–H), week 2 (I–L), and week 4 (M–P) are shown. High-magnification views (B, D, F, H, J, L, N, and P) of the boxes are inset in the low-magnification images (A, C, E, G, I, K, M, and O). Scale bars: 250 μ m (A, E, I, and M), 100 μ m (C, G, K, and O), or 20 μ m (B, D, F, H, J, L, N, and P). **(C)** The occlusal surfaces of the right maxillary first molars were injured until pulp was exposed, sprayed with water, air-dried, and sealed with glass ionomer cement. Maxillae were collected at day 0, week 2, and week 4 following tooth injury with pulp exposure. Hematoxylin and eosin-stained (A, B, E, F, I, and J) and ephrinB1-stained (C, D, G, H, K, and L) sections of injured teeth (right side of the maxilla) at day 0 (A–D), week 2 (E–H), and week 4 (I–L) are shown. High-magnification views (B, D, F, H, J, and L) are of the regions highlighted by boxes in the low-magnification images (A, C, E, G, I, and K). AB, alveolar bone; DP, dental pulp; OB, odontoblasts; TD, tertiary dentin. Scale bars: 100 μ m (A, C, E, G, I, and K), or 20 μ m (B, D, F, H, J, and L).

(Fig. 2B–K, L), which correlated with the beginning of tertiary dentin deposition (Fig. 2B–I, J). Four weeks after the operation, the deposition of tertiary dentin was increased under the

afflicted cusps. Some cellular inclusions were also observed embedded in the calcified matrix (Fig. 2B–M, N). EphrinB1 immunostaining in the odontoblast and subodontoblast layers

Figure 3. Direct pulp-capping materials induce *ephrinB1* and *EphB2* gene expression in dental pulp cells (DPCs). The expression of *ephrinB1* and *Igf-1* in mouse DPCs harvested from 1- to 2-mo-old C57BL/6 mice that had been treated with vehicle, (A, D) CH, and (B, E) MTA for the indicated times. (C, F) The expression of *ephrinB1* and *Igf-1* in human DPCs following treatment with calcium hydroxide (CH) or mineral trioxide aggregate (MTA) for 24 h. * $P < 0.05$ versus control. # $P < 0.03$ versus control. DPSC, dental pulp mesenchymal stem cell; IGF-I, insulin-like growth factor I.

was decreased in intensity compared at week 2 (Fig. 2B-O, P). After tooth injury with pulp exposure, odontoblasts were disrupted under the injury site (Fig. 2C-A, B), and *ephrinB1* was expressed in the subodontoblastic layer (Fig. 2C-C, D). At week 2, tertiary dentin deposition was observed at the injury site with a disorganized odontoblastic layer (Fig. 2C-E, F), but the expression of *ephrinB1* was weak and located at the subodontoblastic layer (Fig. 2C-G, H). At 4 wk post injury, the deposition of tertiary dentin was increased under the injured cusps, and odontoblasts aligned at the surface of the dental pulp (Fig. 2C-I, J). Intense *ephrinB1* expression was observed in odontoblasts and their cellular processes (Fig. 2C-K, L). *EphB2* was weakly expressed in dental pulp and the perivascular area following tooth injury both with and without pulp exposure (Appendix Fig. 1A and Appendix Fig. 1B) but not in the odontoblasts under injured cusps. Furthermore, *ephrinB1* but not *EphB2* was detected in the odontoblast cellular zone and at the border of the primary and tertiary dentin following tooth injury, suggesting that *ephrinB1* plays roles in the response to tooth injury.

CH and MTA, Which Are Used in the Clinic for Direct Pulp Capping, Induce *EphrinB1* and *EphB2* Gene Expression in DPCs

Mouse DPCs isolated from the first and second molars were cultured in odontoblast differentiation media for 14 d and stimulated with CH (1 μ g/mL) or MTA (10 mg/mL). Cells were

harvested at the indicated time points, and the gene expression of *ephrinB1* and *EphB2* was measured. CH significantly increased *ephrinB1* (by ~30 fold) and *EphB2* (by ~4 fold) gene expression 24 h following treatment (Fig. 3A). Treatment with MTA also increased *ephrinB1* gene expression but much earlier: it increased at 4 h (by ~20-fold) and peaked at 24 h, where it was increased by ~120-fold compared with untreated cells (Fig. 3B). MTA-induced *EphB2* gene expression increased by 2-fold 4 h following treatment, peaked at 16 h, and decreased thereafter. Both CH and MTA increased *ephrinB1* by 24 h following treatment; the expression of *EphB2* was much lower than that of *ephrinB1* following CH treatment. However, MTA induced a much higher *EphB2* gene expression (by ~140-fold) at 16 h. These data were also obtained in human DPCs (Lonza) cultured in odontoblast differentiation media (Fig. 3C), where both CH and MTA increased *ephrinB1* and *EphB2* expression 24 h following treatment.

To further explore how CH and MTA affect primary odontoblast gene expression, PCR arrays were performed 4 and 24 h following treatment. *IGF-1* expression was significantly increased ~3-fold by CH and MTA treatment (Appendix Fig. 2). The PCR array data were validated in time courses in mouse DPCs showing an approximate 9-fold increase in *IGF-1* gene expression 24 h following treatment (Fig. 3D, E) and in human DPCs, where *IGF-1* expression was increased by approximately 3-fold following CH and MTA treatment (Fig. 3F). *IGF-1* gene expression was correlated with the expression of the odontoblast differentiation marker *nestin* (Fig. 3F).

Figure 4. Insulin-like growth factor I (IGF-I) induces *ephrinB1* and *EphB2* gene expression in dental pulp cells (DPCs). Mouse (A) and human (B) DPCs were treated with 50 ng/mL recombinant IGF-I for 24 h. Gene expression was measured using real-time reverse transcriptase polymerase chain reaction. * $P < 0.02$ versus control. (C) Mouse DPCs were preincubated with PD98059 or LY294002 for 1 h and then treated with calcium hydroxide (CH) for 24 h. * $P < 0.05$. (D) Mouse DPCs were preincubated with NVP-BEZ235 for 6 h and then treated with CH for 24 h. * $P < 0.05$. Mouse (E) and human (F) DPCs were preincubated with a MEK inhibitor, a PI3K inhibitor, or an mTOR inhibitor with or without CH for 24 h. * $P < 0.05$, # $P < 0.05$ versus CH-induced *ephrinB1* expression; § $P < 0.05$ versus CH-induced *nestin* expression. DPSCs, dental pulp mesenchymal stem cells; rIGF-I, recombinant insulin-like growth factor I; mRNA, messenger RNA.

IGF-I Treatment Induces *EphrinB1* and *EphB2* Expression in Mouse and Human DPCs

Primary odontoblast cultures from mouse and human DPCs were treated with recombinant IGF-I (50 ng/mL) for 24 h. In mouse DPCs, IGF-I increased *ephrinB1*/*EphB2* gene expression by approximately 9- and 6-fold, respectively, 24 h following treatment (Fig. 4A). In human DPCs, IGF-I increased

ephrinB1/*EphB2* gene expression by approximately 2-fold. This was accompanied by increases in *type-1 collagen* and dentin sialophosphoprotein (*Dspp*) expression (Fig. 4B).

IGF-I binds to a tyrosine kinase receptor, the IGF-I receptor (IGF-1R), and activates 2 major signaling pathways: the Ras/Raf/MAPK pathway and the PI3K pathway. Several inhibitors were used to explore which of the pathways dominates the effects of IGF-I effects on *ephrinB1*/*EphB2* gene expression.

CH-induced *ephrinB1* gene expression was significantly inhibited by LY294002, a PI3K inhibitor, and PD98059, a MEK inhibitor. *EphB2* expression was only inhibited by PD98059 (Fig. 4C). These data suggest that IGF-1 regulates *ephrinB1* gene expression via both the PI3K/AKT and MEK pathways and *EphB2* gene expression via the MEK pathway. In addition, CH-induced *ephrinB1* (but not *EphB2*) gene expression was significantly inhibited by NVP-BEZ235 (Fig. 4D), an mTOR inhibitor (downstream of AKT signaling pathway). CH-induced *osterix* and *nestin* expression was abolished by MEK, PI3K, and mTOR inhibitors in mouse DPCs (Fig. 4E). In human DPCs, CH-induced *ephrinB1* and *nestin* expression was inhibited by MEK, PI3K, and mTOR inhibitors (Fig. 4F). The expression of *EphB2* and *osterix* was inhibited only by the MEK inhibitor.

Inactivation of the IGF-1R in Preodontoblasts Impairs Dentinogenesis In Vivo

DMP1-*Igf1r* mice were used to study the interactions between IGF-1 and ephrinB1 in vivo. DMP1-*Igf1r* mice exhibit *igf-1r* gene ablation in preodontoblasts (Liu et al. 2016). Micro-CT analysis of the molars (Fig. 5A) and incisors (Fig. 5B) of DMP1-*Igf1r* mice revealed a significantly reduced dentin volume and an increased pulp volume. In addition, the CH-induced *ephrinB1*, *EphB2*, *IGF-1*, and *nestin* gene expression observed in DPCs isolated from control mice was not evident in DPCs isolated from DMP1-*Igf1r* mice (Fig. 5C). DMP1-*Igf1r* mice were also used to confirm whether the IGF-1/ephrinB1 axis is activated following tooth injury in vivo. DMP1-*Igf1r* mice exhibited a reduced dentin volume (~40%) and mineral density (~10%) 4 wk following tooth injury (Fig. 5D). Hematoxylin and eosin staining revealed the presence of a newly formed tertiary dentin matrix under the injury site in control mice (Fig. 5E-A, B), whereas the thickness of the tertiary dentin matrix was reduced in DMP1-*Igf1r* mice (Fig. 5E-C, D). The expression of ephrinB1 was also assessed using immunohistochemistry; the data revealed that the expression of ephrinB1 was decreased in the odontoblastic layer of DMP1-*Igf1r* mice (Fig. 5E-G, H) compared with control mice (Fig. 5E-E, F). The expression of *EphB2* was not detected in odontoblasts in control or DMP1-*Igf1r* mice (Appendix Fig. 3).

Discussion

The role of ephrinB1 in the development of long bones was established by Xing et al. (2010) using a transgenic mouse model in which ephrinB1 was overexpressed in osteoblast progenitors driven by the collagen 1 α 3.6 promoter, which led to an increased bone mass. In contrast, the conditional ablation of ephrinB1 in mesenchymal cells using the Col1 α 2-iCre promoter resulted in significant skull defects and reduced bone formation (Xing et al. 2010; Cheng et al. 2013). Further studies of the conditional ablation of ephrinB1 in the myeloid lineage revealed that ephrinB1 is a negative regulator of osteoclast activity in vivo (Cheng et al. 2012). Overall, it was established that ephrinB1 is essential for craniofacial development, bone formation,

and bone regeneration. Although ephrinB1 plays important roles in tissue mineralization, its roles in the dental pulp niche have not been investigated. Our previous report indicated that EphB2/ephrinB1 interactions play important roles in odontoblast proliferation and differentiation in vitro (Wang et al. 2013). Here, we show that ephrinB1 and EphB2 are expressed in the odontoblasts of molars at the beginning of dentin formation and that ephrinB1 is expressed continuously during tooth maturation in vivo. The expression of ephrinB1 and EphB2 is upregulated by direct pulp-capping materials (CH and MTA) in vitro, and ephrinB1 but not EphB2 is upregulated following tooth injury in vivo. Furthermore, IGF-1 acts upstream of ephrinB1, which plays important roles in dentinogenesis.

IGF-1 plays a crucial role in tooth development, including periodontal ligament homeostasis and the elongation of tooth formation (Fujiwara et al. 2005; Rath-Deschner et al. 2009). Magnucki et al. (2013) demonstrated that IGF-1 and IGF-R1 are involved in the differentiation of dental pulp mesenchymal stem cells (DPSCs). IGF1 and IGF-R1 are highly expressed in stromal precursor antigen 1 (STRO-1)-positive DPSCs. Recently, it was shown that IGF-1 induced the proliferation and differentiation of human DPSCs via the MAPK pathway (Lv et al. 2016). The current study revealed that IGF-1 is upregulated by direct capping materials in mouse and human DPCs in vitro and following tooth injury in mice in vivo. In addition, recombinant IGF-1 stimulates *ephrinB1* and *EphB2* gene expression in mouse and human DPCs, whereas knocking down IGF-1R in DPCs decreases *ephrinB1* and *EphB2* expression in response to CH. Finally, the IGF-1-mediated Ras/Raf-1/MAPK pathway stimulates both *ephrinB1* and *EphB2* gene expression, whereas the PI3K/Akt/mTOR pathway specifically activates *ephrinB1* gene expression.

Following injury or damage in many tissues, canonical Wnt/ β -catenin signaling is activated and stimulates the cellular-based repair process (Whyte et al. 2012). In a mouse tooth injury model, treatment in the pulp cavity with Wnt3A or glycogen synthase kinase (GSK) antagonists led to an organized tubular dentin structure in the reparative dentin formation (Hunter et al. 2015; Neves et al. 2017). In addition, a tooth injury treated with vascular endothelial growth factor exhibited an increase in the number of blood vessels and the volume of repaired dentin (Zhang et al. 2016). Here, we show that DMP1-*Igf1r* mice had impaired tertiary dentinogenesis following tooth injury, suggesting that IGF-1 also plays an important role in tertiary dentin formation. Furthermore, we show that ephrinB1 expression, which is essential for the reparative process, is decreased following tooth injury in DMP1-*Igf1r* mice. Clearly, ephrinB1 is not solely regulated by IGF-1 in odontoblasts, but it likely plays a role in ephrinB1 induction following tooth injury in vivo. Translating these findings from mouse DPCs to human DPCs will allow us to acquire preliminary data for developing future translational approaches using IGF-1 and ephrinB1 to induce tertiary dentin formation in the clinic.

In conclusion, the in vitro and in vivo evidence shown in this study demonstrates that the IGF-1/ephrinB1 axis plays an important role in odontogenic differentiation and reactionary dentin formation.

Figure 5. The insulin-like growth factor I (IGF-I)/ephrinB1 axis plays an important role in dentinogenesis in vivo. The lower jaws were harvested from 1-mo-old wild-type (control) or DMP1-Igf1r mice and subjected to micro-computed tomography. **(A)** The apical end of the incisors was used to determine the volume of dentin or pulp. **(B)** The first molar (mesial side to the distal side) was used to determine volume of dentin or pulp. $n = 5$ /group, $*P < 0.05$ versus control. **(C)** Mouse dental pulp cells (DPCs) harvested from the molars of 1- to 2-mo-old wild-type or DMP1-Igf1r mice and treated with calcium hydroxide (CH) for 24 h. The gene expression of ephrinB1, EphB2, Igf-1, and nestin was evaluated using real-time reverse transcriptase polymerase chain reaction. $*P < 0.05$ versus control; $#P < 0.05$ versus CH-treated cells from control mice. **(D)** Tertiary dentin in the first molar (mesial side to distal side) following tooth injury was used to measure the dentin volume and density. $n = 8$ (control), $n = 5$ (DMP1-Igf1r). $*P < 0.05$ versus control. The image of the mineral density was created using CTan software. The arrow indicates tertiary dentin formation in control and DMP1-Igf1r mice. **(E)** Hematoxylin and eosin (HE)-stained (A–D) and ephrinB1-stained (E–H) sections of injured teeth from control (A, B, E, and F) and DMP1-Igf1r (C, D, G, and H) mice 4 wk after the operation. High-magnification views (B, D, F, and H) show the regions highlighted with boxes in the low-magnification images (A, C, E, and G). DP, dental pulp; OB, odontoblasts; TD, tertiary dentin. Scale bars: 100 μ m (A, C, E, and G) or 20 μ m (B, D, F, and H). **(F)** Schematic summary: (1) Direct pulp-capping materials induce Igf-1 gene expression in DPCs. (2) IGF-1 binds to IGF-1 receptor (IGF-IR) and then activates the (3) Ras/Raf1/MAPK or (4) PI3K/Akt/mTOR pathways. (3) Ras/Raf1/ERK stimulates the expression of EphB2 and ephrinB1, whereas (4) the PI3K/AKT/mTOR pathway enhances ephrinB1 gene expression in DPCs.

Author Contributions

S. Matsumura, contributed to data acquisition and analysis, drafted and critically revised the manuscript; A. Quispe-Salcedo, contributed to data acquisition, drafted and critically revised the manuscript; C.M. Schiller, J.S. Shin, B.M. Locke, contributed to data acquisition, drafted the manuscript; S. Yakar, contributed to conception, design, and data analysis, drafted and critically revised the manuscript; E. Shimizu, contributed to conception, design, data acquisition, analysis, and interpretation, drafted and critically revised the manuscript. All authors gave final approval and agree to be accountable for all aspects of the work.

Acknowledgments

This study was supported by National Institutes of Dental and Craniofacial Research grant R01-DE025885 and National Institutes of Diabetes and Digestive and Kidney Diseases grant R01-DK100246. The authors declare no potential conflicts of interest with respect to the authorship and/or publication of this article.

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