Enhanced Dentinogenesis of Pulp Progenitors by Early Exposure to FGF2

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Abstract

Members of the fibroblast growth factor (FGF) family play essential and important roles in primary and reparative dentinogenesis. Although there appears to be a general agreement on the effects of FGF signaling on the proliferation of pulp cells, there are conflicting results regarding its effects on odontoblast differentiation. We recently examined the effects of continuous exposure of dental pulp cells to FGF2 and showed that the effects of FGF2 on differentiation of progenitor cells into odontoblasts were stage specific and dependent on the stage of cell maturity. The purpose of this study was to gain further insight into cellular and molecular mechanisms regulating the stimulatory effects of FGF2 on odontoblast differentiation. To do so, we examined the effects of early and limited exposure of pulp cells from a series of green fluorescent protein (GFP) reporter transgenic mice that display stage-specific activation of transgenes during odontoblast differentiation to FGF2. Our results showed that early and limited exposure of pulp cells to FGF2 did not have significant effects on the extent of mineralization but induced significant increases in the expression of *Dmp1* and *Dspp* and the number of DMP1-GFP⁺ and DSPP-Cerulean⁺ odontoblasts. Our results also showed that the stimulatory effects of FGF2 on odontoblast differentiation were mediated through FGFR/MEK/Erk1/2 signaling, increases in *Bmp2*, and activation of the BMP/BMPR signaling pathway. These observations show that early and limited exposure of pulp cells to FGF2 alone promotes odontoblast differentiation and provides critical insight for applications of FGF2 in dentin regeneration.

Keywords: odontoblasts, dental pulp cells, BMP, green fluorescent protein, dentin matrix protein 1 (DMP1), dentin sialophosphoprotein (DSPP)

Introduction

Reparative dentinogenesis in response to various injuries involves the recruitment and proliferation of progenitor/stem cells to the site of injury and their differentiation into a second generation of odontoblasts or “odontoblast-like” cells (Smith et al. 2012). These odontoblast-like cells synthesize the reparative dentin (also referred to as osteodentin) immediately below the site of damage to preserve pulp vitality (Smith et al. 2012). Many aspects of reparative dentinogenesis are similar to those regulating primary dentinogenesis, including the differentiation of odontoblast-like cells from progenitors/stem cells, which is dependent on multiple signaling molecules, including fibroblast growth factors (FGFs). During primary dentinogenesis, FGFs derived from primary and secondary enamel knots regulate differentiation of dental papilla cells at the tip of the cusps and in close proximity to the epithelial-mesenchymal interface (Thesleff et al. 2001). However, reparative dentinogenesis depends on factors including FGFs sequestered in the dentin matrix and pulp-supportive tissues (Sloan and Waddington 2009; About 2011; Smith et al. 2012).

It has been shown that FGF2 is involved in proliferation, homing, and migration of healthy and inflamed dental pulp cells (Kim et al. 2012; Kim et al. 2014). FGF2 induced vascular invasion (Kim et al. 2012) and upregulated the expression of the embryonic stem cell markers in the dental pulp cells (Osathanon et al. 2011). However, effects of FGFs on

mineralization, odontoblast differentiation, and expression of dentin sialophosphoprotein (*Dspp*; expressed at high levels by odontoblasts) remain controversial. Several studies have shown the inhibitory effects of FGF2 on odontoblast differentiation and expression of *Dspp* (Kim et al. 2012; Kim et al. 2014). Others have shown that FGF2 alone did not induce odontoblast differentiation but, when combined with TGF- β 1, induced differentiation of dental pulp cells into odontoblast-like cells and enhanced effects of TGF- β 1 on odontoblast differentiation (He et al. 2008; Kim et al. 2012). Other studies have reported that FGF2 stimulates *Dspp* expression in vitro, and the application of FGF2 to exposed pulp induces formation of calcified bridges containing cells expressing dentin matrix protein 1 (DMP1; expressed at high levels by functional

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odontoblasts and osteocytes; Kim et al. 2012; Mathieu et al. 2013; Kim et al. 2014).

We have used a series of green fluorescent protein (GFP) reporter transgenic mice that display stage-specific activation of transgenes during odontoblast differentiation *in vivo* and *in vitro* to gain a better understanding of the progression of progenitor cells in the odontoblast lineage (Balic et al. 2010; Balic and Mina 2011; Sagomonyants and Mina 2015). These studies showed that 2.3-GFP and 3.6-GFP transgenes identify cells at early stages of odontoblast differentiation (polarizing odontoblasts that lack expression of *Dmp1* and *Dspp*; Balic et al. 2010), whereas DMP1-GFP and DSPP-Cerulean transgenes identify postmitotic functional and fully differentiated odontoblasts within mineralized nodules, respectively (Balic and Mina, 2011; Sagomonyants and Mina 2014, 2015). Using these transgenic animals, we have recently reported that the effects of FGF2 on differentiation of progenitor cells into odontoblasts are stage specific and dependent on the stage of cell differentiation/maturity (Sagomonyants and Mina 2015). Continuous exposure of pulp cells to FGF2 inhibited mineralization and revealed both stimulatory and inhibitory effects of FGF2 on expression of markers of odontoblast differentiation and various transgenes (Sagomonyants and Mina 2015). During the proliferation phase of *in vitro* growth, FGF2 transiently increased expression of markers of odontoblast differentiation, including *Dspp* and *Dmp1*. Additional exposure to FGF2 during the differentiation/mineralization phase of *in vitro* growth decreased the extent of mineralization and expression of markers of odontoblast differentiation. The purpose of our present study was to gain further insight into cellular and molecular mechanisms regulating the stimulatory effects of FGF2 on odontoblast differentiation by examining the effects of early and limited exposure of pulp cells to FGF2.

Materials and Methods

Primary Dental Pulp Cultures

All experimental protocols involving animal tissues in the present study were approved by the Institutional Animal Care and Use Committee of the University of Connecticut Health Center. The coronal portions of the pulps from first and second molars were isolated from 5- to 7-d-old hemizygous pOBCol3.6GFP (referred to as 3.6-GFP), pOBCol2.3GFP (referred to as 2.3-GFP), DMP1-GFP, DSPP-Cerulean, and nontransgenic mouse pups and prepared for cultures as described previously (Balic et al. 2010; Balic and Mina 2011; Sagomonyants and Mina 2015). Low molecular weight (18 kDa) bovine FGF2 (R&D systems, Inc., Minneapolis, MN, USA) and vehicle (0.1% bovine serum albumin) were added to the cultures at the final concentration of 20 ng/mL during the proliferation phase of *in vitro* growth (days 3 to 7) and is referred to as early and limited exposure. At day 7, when cells reached confluence, all cultures were grown in the mineralization-inducing medium containing 50 µg/mL of fresh ascorbic acid and 4mM β-glycerophosphate without FGF2 for additional 7 to 14 d. Medium was changed every other day.

Inhibition of Signaling Pathways

The FGFR inhibitor SU5402 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), the MEK/Erk1/2 inhibitor U0126 (Promega Corporation, Madison, WI, USA), and the BMP/BMPR inhibitor noggin (PeproTech, Rocky Hill, NJ, USA) were dissolved in dimethyl sulfoxide or bovine serum albumin and added at various concentrations indicated in Figure 4 to the media between days 3 and 7. Medium containing inhibitors and FGF2 was changed every other day.

Detection and Quantification of Mineralization in Cultures

Mineralization in live and fixed cultures was examined by xylenol orange and von Kossa silver nitrate staining, respectively, as described previously (Balic et al. 2010).

Immunocytochemistry

Cultures were processed for immunocytochemistry for detection of DSPP-Cerulean and phospho-Erk1/2 using anti-GFP (Invitrogen, Grand Island, NY, USA) and rabbit anti-mouse phospho-Erk1/2 (Cell Signaling, Boston, MA, USA) antibodies, respectively, as previously described (Sagomonyants and Mina 2015).

Digital Imaging and Epifluorescence Analysis of Cell Cultures

At different time points, the mean fluorescence intensity in culture wells was measured as previously described (Kuhn et al. 2010; Sagomonyants and Mina 2015).

RNA Extraction and Analysis

Total RNA was isolated using TRIzol reagent (Invitrogen), followed by cDNA synthesis. Gene expression was examined by TaqMan or SYBR Green quantitative polymerase chain reaction analyses using the primers and conditions shown in Appendix Tables 1 and 2 as previously described (Sagomonyants and Mina 2015).

Fluorescence-Activated Cell Sorting and Cell Cycle Analysis

Cultures from various transgenic animals were processed for fluorescence-activated cell sorting (FACS) analysis by a BD LSR-II FACS cytometer (BD Biosciences, San Jose, CA, USA) at various time points as previously described (Sagomonyants and Mina 2015). Percentages of GFP⁺ and GFP⁻ cells were determined with BD FACSDiva 6.2 software. Pulp cells from nontransgenic littermates served as control. FACS and cell cycle analysis were performed on pulp cells from 2.3-GFP pups as previously described (Balic et al. 2010; Sagomonyants and Mina 2015).

Statistical Analysis of Data

Results represent mean $\pm$ SEM of at least 3 independent experiments. Statistical analysis was performed by GraphPad Prism 6 software using 1-way analysis of variance with Bonferroni's multiple-comparison posttest or unpaired 2-tailed Student's *t* test. Statistical significance was determined at $P \leq 0.05$.

Results

Effects of FGF2 on Mineralization in Primary Dental Pulp Cultures

Staining of live and fixed cultures using xylenol orange and von Kossa, respectively, showed that the extent of mineralization in FGF2-treated cultures was similar to that in control at days 10 and 14 and slightly higher than that in control at day 21 (Fig. 1A, B). FGF2-treated cultures showed increased expression of all markers of mineralization and odontoblast differentiation as compared with control at day 7 (Fig. 1B). The most marked increases were in the expression of *Dmp1*, followed by increases in the expression of *Dspp*, bone sialoprotein (*Bsp*), and osteocalcin (Fig. 1B). Increases in the expression of *Dmp1* and *Dspp* in FGF2-treated cultures were detected as early as 12 and 24 h after treatment, respectively (Fig. 1C).

Analysis between days 10 to 21 showed that expression of type I collagen, *Bsp*, osteocalcin, and *Dmp1* in FGF2-treated cultures was either similar or slightly higher than that in control (Fig. 1B). Expression of *Dspp* in FGF2-treated cultures was significantly higher (~ 3.4 to 7.3 -fold) than that in control at all time points (Fig. 1B).

Effects of FGF2 on Expression of Transgenes in Primary Dental Pulp Cultures

FACS analysis between 24 and 96 h after treatment showed slight increases in the percentages of 2.3-GFP⁺ and 3.6-GFP⁺ cells (proliferative cells at early stages of odontoblast differentiation) in FGF2-treated cultures as compared with respective controls (Appendix Table 3).

FGF2 markedly increased the percentage of DMP1-GFP⁺ cells (postmitotic functional odontoblasts) as early as 24 h (~ 4 -fold) with further increases at 96 h (~ 7 - to 8 -fold) as compared with control (Appendix Table 3).

Epifluorescent analysis of cultures at day 7 confirmed the slight (~ 1.5 -fold) increases in the intensity of 2.3-GFP and 3.6-GFP transgenes and marked increases (~ 38 -fold) in the intensity of DMP1-GFP transgene in FGF2-treated cultures as compared with respective controls (Fig. 2A, Appendix Fig. 1, and data not shown). Furthermore, these analyses showed that the intensity of expression of all 3 transgenes in FGF2-treated cultures at days 10 to 21 was similar to the respective controls (Fig. 2A and data not shown).

Pulp cultures from DSPP-Cerulean transgenic mice showed increases in the percentage of postmitotic DSPP-Cerulean⁺ differentiated odontoblasts (~ 17 -fold) and intensity of DSPP-Cerulean transgene expression at all time points in FGF2-treated

cultures as compared with control (Fig. 2A, Appendix Fig. 1, and Table 3).

The increases in the percentage of various GFP⁺ cells and in the intensity of GFP⁺ in these cultures could be due to activation of transgenes in GFP⁺ cells and/or proliferation of existing GFP⁺ cells. DNA content/cell cycle analysis showed slight increases (~ 1.3 to 1.6 fold) in the rates of proliferation of 2.3-GFP⁺ and 3.6-GFP⁺ populations in FGF2-treated cultures as compared with respective controls (Appendix Fig. 2 and Table 4), indicating that increases in these populations were related to cell proliferation. However, the lack of proliferation in postmitotic DMP1-GFP⁺ and DSPP-Cerulean⁺ cells (Balic and Mina 2011; Sagomonyants and Mina 2015) indicated that marked increases in the percentage of these populations were most likely related to activation of these transgenes in new cells (cells at earlier stages of differentiation) and not related to their proliferation.

These observations indicated that early and limited exposure of pulp cultures to FGF2 resulted in accelerated differentiation of early progenitors at day 7 (marked increases in the number of DMP1-GFP⁺ and DSPP-Cerulean⁺ cells), followed by further increases in the number of DSPP-Cerulean⁺ odontoblasts at days 10 to 21.

Effects of FGF2 on Expression of Selected Genes in Primary Dental Pulp Cultures

Accelerated differentiation in FGF2-treated cultures at day 7 was further studied by examining the expression of additional markers expressed at distinct stages of odontoblast differentiation in vivo. Ameloblastin (*Ambn*) and FGF receptor 2c (*Fgfr2c*) have been shown to be transiently expressed by preodontoblasts and cells at early stages of odontoblast differentiation, respectively (Begue-Kirn et al. 1998; Kettunen et al. 1998). However, *Bono1* and *Fgfr1c* were shown to be expressed at high levels by functional and fully differentiated odontoblasts and not by early progenitors (Kettunen et al. 1998; James et al. 2004).

Quantitative polymerase chain reaction analysis of whole (unsorted) cultures at day 7 showed increased expression of *Bono1* and *Fgfr1c* as well as *Dmp1* and *Dspp* (markers of more advanced stages of odontoblast differentiation) and decreased expression of *Ambn* and *Fgfr2c* (markers of early stages of odontoblast differentiation) in FGF2-treated cultures as compared with control (Fig. 2B).

Reverse transcription polymerase chain reaction analysis confirmed the differential expression of *Fgf*s during differentiation of pulp cultures in vitro and showed increases in the levels of expression of *Fgfr1c* and decreases in the levels of *Fgfr2c* in FGF2-treated cultures as compared with control as early as 48 h after treatment (Appendix Fig. 3A). These results are consistent with accelerated differentiation of pulp cells in FGF2-treated cultures as compared with control.

Effects of FGF2 on FACS-sorted 2.3-GFP⁺ and 2.3-GFP⁻ Populations

Pulp cultures contain heterogeneous cell types (odontoprogenitors, osteoprogenitors, and a small number of stem cells) and

cells at different stages of differentiation (Balic et al. 2010; Balic and Mina 2011). This heterogeneity makes it difficult to identify cell populations responsive to the stimulatory effects of FGF2. Our previous observations showed that FACS-sorted 2.3-GFP⁺ and 2.3-GFP⁻ populations represented proliferative cells enriched in early progenitors and undifferentiated cells, respectively (Balic et al. 2010). On the basis of these observations, we examined the effects of FGF2 on relatively homogeneous populations of FACS-sorted 2.3-GFP⁺ and 2.3-GFP⁻ cells ($\geq 98\%$ purity of isolated populations; Appendix Fig. 3B).

Early and limited exposure of 2.3-GFP⁺ and 2.3-GFP⁻ cultures to FGF2 did not have significant effects on mineralization (Fig. 3A) but resulted in increased expression of *Dmp1*, *Dspp*, *Bonol*, and *Fgfr1c* and decreased expression of *Ambn* and *Fgfr2c* at day 7 (Fig. 2B). In both populations, FGF2-treated cultures showed earlier detection of *Dspp* expression and continuous and marked increases in the expression of *Dmp1* and *Dspp* as compared with respective controls (Fig. 3B). These

fluorescence for XO was measured with cultures from the preodontoblastic Q705 cell line (Priam et al. 2005) that lacks mineralization potential, and these values were subtracted from respective XO measurements. Expression of all genes except *Dspp* was normalized to that in VH-treated cultures at day 7, which is arbitrarily set to 1 and is indicated by the dashed line. Expression of *Dspp* was normalized to that in VH-treated cultures at day 10, which is arbitrarily set to 1 and is indicated by the dashed line. Results in all histograms represent mean $\pm$ SEM of at least 3 independent experiments; * $P \leq 0.05$ relative to VH at each time point. (C) Histograms showing increases in the expression of *Dmp1* and *Dspp* in FGF2-treated cultures were detected as early as at 12 and 24 h, respectively. In these histograms, expression of *Dmp1* was normalized to that in VH-treated cultures at 12 h, which is arbitrarily set to 1 and is indicated by the dashed line. Expression of *Dspp* was normalized to that in FGF2-treated cultures at 96 h, which is arbitrarily set to 1 and is indicated by the dashed line. Results represent mean $\pm$ SEM of at least 3 independent experiments; * $P \leq 0.05$ relative to VH at each time point. FGF2, fibroblast growth factor 2; ND, not detected; VH, vehicle; XO, xylenol orange.

Figure 1. Effects of early and limited exposure to FGF2 on the extent of mineralization and the expression of markers of mineralization and dentinogenesis in primary dental pulp cultures. (A) Representative images of the same areas in cultures analyzed under brightfield (BF; upper row) and epifluorescent light using TRITC red filter for detection of XO staining (middle row). The lower row shows representative cultures stained with von Kossa for each time point. Early and limited exposure to FGF2 did not inhibit the mineralization in pulp cultures. Scale bar = 200 μ m. (B) Histograms showing changes in the intensity of XO staining (in absolute values) and the expression of markers of mineralization and dentinogenesis in VH- and FGF2-treated cultures. Early and limited exposure to FGF2 increased the intensity of XO at day 21 and the expression of all markers of mineralization and dentinogenesis as compared with control. The intensity of XO staining was measured at 570/610-nm wavelength (excitation/emission) and at a gain of 80. Background

Figure 2. Effects of early and limited exposure to FGF2 on expression of various transgenes and various markers in primary dental pulp cultures. **(A)** Histograms showing changes in the intensity of expression of 2.3-GFP, DMP1-GFP, and DSPP-Cerulean transgenes in VH- and FGF2-treated cultures at various time points. FGF2 increased the intensity of expression of various transgenes at day 7. Between days 10 and 21, the intensity of the expression of 2.3-GFP and DMP1-GFP in FGF2-treated cultures is similar to that in the respective controls. However, the intensity of expression of DSPP-Cerulean in FGF2-treated cultures is significantly higher than that in the respective controls at all time points. The intensity of 2.3-GFP transgene was measured at 483/525-nm (excitation/emission) and 500/540-nm wavelengths for DMP1-GFP transgenes at a gain of 80. Background fluorescence for GFP was measured with dental pulp cultures from nontransgenic littermates, and these values were subtracted from respective GFP measurements. DSPP-Cerulean⁺ cells were detected with an anti-GFP antibody, and Hoechst 33342 was used to visualize nuclei. The intensity of staining with anti-GFP antibody was measured at 500/540 nm wavelength and gain 80. Background fluorescence for GFP was measured with BMSC cultures from the DSPP-Cerulean littermates stained with anti-GFP antibody, and these values were subtracted from respective GFP measurements. Results are expressed in absolute values and represent mean $\pm$ SEM of at least 3 independent experiments; * $P \leq 0.05$ relative to VH at each time point. **(B)** Histograms showing the expression of various markers in unsorted and 2.3-GFP⁺ and 2.3-GFP⁻ FACS-sorted populations. Note the increases in the expression of *Dmp1*, *Dspp*, *Bono1*, and *Fgfr1c* as well as decreases in the expression of *Fgfr2c* and *Ambn* in FGF2-treated cultures as compared with respective controls. In these experiments, cultures derived from 2.3-GFP transgenic mice were treated with VH or FGF2 between days 3 to 7 and then processed for FACS sorting, RNA isolation, and quantitative polymerase chain reaction analysis. Expression of all genes except *Dspp* was normalized to that in VH-treated unsorted cultures, which is arbitrarily set to 1 and is indicated by the dashed line. Expression of *Dspp* was normalized to that in FGF2-treated unsorted cultures, which is arbitrarily set to 1 and is indicated by the dashed line. Results in all histograms represent mean $\pm$ SEM of at least 3 independent experiments; * $P \leq 0.05$ relative to VH at each time point. DSPP, dentin sialophosphoprotein; DMP1, dentin matrix protein 1; FACS, fluorescence-activated cell sorting; FGF2, fibroblast growth factor 2; GFP, green fluorescent protein; ND, not detected; VH, vehicle.

observations indicated that FGF2 accelerated differentiation of both populations (early progenitors and undifferentiated cells) into functional and fully differentiated odontoblasts.

Involvement of FGFR/MEK/Erk1/2 Signaling in Stimulatory Effects of FGF2 on Expression of *Dmp1* and *Dspp* in Primary Dental Pulp Cultures

Previous studies indicated that effects of FGFs on proliferation, differentiation, and expression of markers of mineralization in osteoblasts were mediated through FGFR and MEK/

Erk1/2 signaling (Marie 2012; Marie et al. 2012). Thus, the roles of FGFR/MEK/Erk1/2 signaling pathway in mediating the stimulatory effects of FGF2 on expression of *Dmp1*, *Dspp*, and the number of DSPP-Cerulean⁺ odontoblasts were examined by using SU5402 and U0126, respectively. In these experiments, primary pulp cultures were treated with inhibitors, with or without FGF2, during the proliferation phase of in vitro growth (days 3 to 7).

SU5402 and U0126 decreased FGF2-mediated increases in the expression of *Dmp1* and *Dspp* in a concentration-dependent manner at all time points (Fig. 4) and attenuated FGF2-mediated formation of DSPP-Cerulean⁺ odontoblasts at 96 h (Appendix Fig. 4A). Immunocytochemical analysis of pulp cultures showed preferential nuclear localization of phospho-Erk1/2 protein (Kodiha et al. 2009) in FGF2-treated cultures as compared with control, indicating activation of MEK/Erk1/2 signaling by FGF2 (Appendix Fig. 4B).

The inhibitory effects of SU5402 and U0126 on *Dmp1* and *Dspp* expression were not related to marked differences in the cellularity of the cultures, evidenced by the lack of detectable differences in the intensity of crystal violet staining (data not shown).

Involvement of BMP Signaling in Mediating Stimulatory Effects of FGF2 on *Dmp1* and *Dspp* in Primary Dental Pulp Cultures

Although SU5402 and U0126 down-regulated FGF2-mediated increases in *Dmp1* and *Dspp*, their levels did not reach levels in the respective controls,

suggesting that FGF2-mediated increases in *Dmp1* and *Dspp* involved other signaling pathways. Our recent study showed that FGF2 rapidly and significantly increased expression of components of BMP signaling, including *Bmp2*, in dental pulp cultures (Sagomonyants and Mina 2014), suggesting that the stimulatory effects of FGF2 on odontoblast lineage may be mediated by crosstalk between the FGF/FGFR and BMP/BMPR signaling pathways.

SU5402 and U0126 decreased FGF2-mediated increases in expression of *Bmp2* (Fig. 4), and noggin (a specific inhibitor of BMP/BMPR signaling) markedly decreased FGF2-mediated increases in *Dmp1* expression and completely abolished

FGF2-mediated increases in *Dspp* expression (Fig. 4) and formation of DSPP-Cerulean⁺ odontoblasts at 96 h (Appendix Fig. 4B).

Discussion

Our results showed that early and limited exposure (during the proliferation phase of in vitro growth) of pulp progenitors to FGF2 only slightly increases in the extent of mineralization and percentages of cells at early stages of differentiation (2.3-GFP⁺ and 3.6-GFP⁺ cells). However, FGF2 accelerated and enhanced the differentiation of functional odontoblasts from earlier progenitors, leading to significant increases in the levels of *Dspp* and the number of DSPP-Cerulean⁺ odontoblasts (Fig. 5). FGF2-treated cultures displayed earlier and increased expression of *Dmp1* and *Dspp* as well as increased percentages of functional and fully differentiated odontoblasts (DMP1-GFP⁺ and DSPP-Cerulean⁺ cells).

The increases in expression of various markers of differentiation and dentinogenesis during the first 7 d in cultures exposed to early and limited FGF2 were similar to changes in

these experiments, primary pulp cultures from 2.3-GFP transgenic mice were first grown in culture conditions supporting their proliferation and expansion in the absence of FGF2. At day 7, 2.3-GFP⁺ and 2.3-GFP⁻ cells were separated, and isolated populations were reanalyzed. Both populations were replated at the same density as unsorted cells and cultured in the absence or presence of FGF2 as described for unsorted cultures. (B) Histograms showing increased expression of *Dmp1* and *Dspp* in FGF2-treated cultures in both populations at all time points. Expression of *Dmp1* in 2.3-GFP⁺ and 2.3-GFP⁻ populations was normalized to that in VH-treated 2.3-GFP⁺ cultures at day 7, which is arbitrarily set to 1 and is indicated by the dashed line. Expression of *Dspp* in both populations was normalized to that in VH-treated 2.3-GFP⁺ cultures at day 10, which is arbitrarily set to 1 and is indicated by the dashed line. Results in all histograms represent mean ± SEM of at least 3 independent experiments; *P ≤ 0.05 relative to the respective controls at each time point. FACS, fluorescence-activated cell sorting; FGF2, fibroblast growth factor 2; GFP, green fluorescent protein; ND, not detected; VH, vehicle; XO, xylenol orange.

Figure 3. Effects of early and limited exposure to FGF2 on mineralization and expression of *Dspp* and *Dmp1* in FACS-sorted 2.3-GFP⁺ and 2.3-GFP⁻ populations. (A) Representative images of the same areas in cultures at various time points analyzed under brightfield (BF; upper row) and epifluorescent light using filters for GFPem and TRITC red for detection of GFP (second upper row) and XO (third upper row), respectively. Scale bar = 200 μm. The lower row shows representative cultures stained with von Kossa for each time point. Note the differences in the onset and extent of mineralization in 2.3-GFP⁻ and 2.3-GFP⁺ populations indicating that 2.3-GFP⁻ population contained undifferentiated cells giving rise to few mineralized nodules, whereas 2.3-GFP⁺ population was enriched in polarizing odontoblasts giving rise to a sheet of mineralized matrix. In

Figure 4. Effects of inhibition of the FGFR, MEK/Erk1/2, and BMP signaling pathways on expression of *Dmp1*, *Dspp*, and *Bmp2* in primary dental pulp cultures. SU5402 and U0126 decreased FGF2-mediated increases in *Dmp1*, *Dspp*, and *Bmp2* in a concentration-dependent manner at all time points. Noggin markedly decreased FGF2-mediated increases in *Dmp1* and completely abolished FGF2-mediated increases in *Dspp*. In these experiments, pulp cultures were prepared and treated with VH, FGF2, and FGF2 in combination with SU5402 (5, 10, 20 μ M), U0126 (5, 10, 20 μ M), or noggin (100, 200, 300 ng/mL) during the proliferation phase of in vitro growth (0 to 96 h of treatment). Medium and inhibitors were changed every other day. In all histograms, expression of *Dmp1* and *Bmp2* was normalized to that in VH-treated cultures at 48 h, which is arbitrarily set to 1 and is indicated by the dashed line. In all histograms, expression of *Dspp* was normalized to that in FGF2-treated cultures at 96 h, which is arbitrarily set to 1 and is indicated by the dashed line. Results in all histograms represent mean $\pm$ SEM of at least 3 independent experiments; * $P \leq 0.05$ relative to VH at each time point. FGF2, fibroblast growth factor 2; ND, not detected; VH, vehicle.

cultures exposed to FGF2 continuously (Sagomonyants and Mina 2015). Yet, increases in *Dspp* and *Dmp1* expression and formation of DMP1-GFP⁺ and DSPP-Cerulean⁺ cells between days 10 and 21 (differentiation/mineralization phase of in vitro growth) in cultures exposed to early and limited FGF2 were opposite to the inhibitory effects in cultures exposed to FGF2 continuously (Sagomonyants and Mina 2014). This provides further evidence for stage specificity of the effects of FGF2 on early progenitors versus cells at a more advanced stage of differentiation.

Our results also showed that stimulatory effects of FGF2 on odontoblast differentiation (expression of *Dmp1* and *Dspp* and percentage of DSPP-Cerulean⁺ cells) were mediated by FGFR/MEK/Erk1/2 signaling, increases in expression of *Bmp2*, and activation of BMP/BMPR signaling.

The roles of MEK/Erk1/2 signaling in regulating the expression of *Dspp* and *Dmp1* in our study are supported by others (Ye et al. 2006; Yao et al. 2011; Zhang et al. 2012; Zhao et al. 2012). The roles of FGFR/MEK/Erk1/2 signaling in odontoblast differentiation and the crosstalk between the FGF/FGFR and BMP/BMPR signaling pathways in the stimulatory effects of FGF2 on odontoblast differentiation in our study are consistent with previously reported roles of these pathways in osteoblasts (Fakhry et al. 2005; Spector et al. 2005; Ye et al. 2006; Yao et al. 2011; Marie 2012; Marie et al. 2012; Zhang et al. 2012; Zhao et al. 2012).

Several studies have indicated roles of canonical (Smad-mediated) BMP signaling on *Dspp* expression and odontoblast differentiation (Cho et al. 2010; Li et al. 2011; Qin et al. 2012; Yang et al. 2012). However, BMP/BMPR signaling is also mediated by the noncanonical (Smad-independent) pathway, including MEK/Erk1/2 (Miyazono et al. 2010), shown to be involved in BMP2-induced differentiation of SVF4 dental follicle cells (Zhao et al. 2002). Binding motifs of AP-1, one of the downstream effectors of the MEK/Erk1/2 pathway, have been identified in the regulatory regions of *Dspp* and *Dmp1* and shown to regulate their expression (Feng et al. 1998; Narayanan et al. 2002; Chen et al. 2008).

The temporal expression of *Dmp1* and *Dspp* in our studies is consistent with several lines of evidence indicating that, during dentinogenesis, DMP1 regulates DSPP expression. In developing teeth, the expression of *Dmp1* is detected earlier than that of *Dspp*, and both *Dspp* and *Dmp1* knockout (KO) mice display dentin hypomineralization (Qin et al. 2007). *Dspp* expression is reduced in the dentin of *Dmp1* KO mice, and

Figure 5. Summary of the effects of early and limited exposure of dental pulp cultures to FGF2. During the proliferation phase of in vitro growth (first 7 d in culture), pulp cells undergo proliferation and contain early progenitors. Following addition of the mineralization-inducing medium at day 7, these cells undergo differentiation and give rise to an extensive amount of mineralized matrix (differentiation/mineralization phase of in vitro growth). The first sign of mineralization is around day 10 with significant increases in the extent of mineralization thereafter. In these cultures, *Dmp1* and *Dspp* are expressed at low levels at days 7 and 10, respectively. DMP1-GFP⁺ and DSPP-Cerulean⁺ cells are detected at days 7 and 10, respectively, with increases thereafter. Early and limited exposure of pulp cultures to FGF2 resulted in slight increases in the extent of mineralization. In FGF2-treated cultures, *Dmp1* expression and DMP1-GFP⁺ cells were detected earlier. FGF2-treated cultures also showed increases in the levels of *Dmp1* and percentage of DMP1-GFP⁺ cells as early as 24 h after treatment as compared with control. The levels of *Dmp1* and intensity of DMP1-GFP transgene between days 10 and 21 in FGF2-treated cultures were similar to those in control. In FGF2-treated cultures, *Dspp* and DSPP-Cerulean⁺ cells were detected earlier and at higher levels. FGF2-treated cultures showed continuous increases in the levels of *Dspp* and percentage of DSPP-Cerulean⁺ cells at all time points as compared with control. DSPP, dentin sialophosphoprotein; DMP1, dentin matrix protein 1; FGF2, fibroblast growth factor 2; GFP, green fluorescent protein.

Dspp KO mice do not show alteration in the expression of *Dmp1*. Transgenic expression of *Dspp* rescued the tooth and alveolar bone defects of *Dmp1* KO mice (Qin et al. 2007; Gibson et al. 2013). In vitro studies showed that *Dmp1* significantly upregulated *Dspp* promoter activities in a mesenchymal cell line (Gibson et al. 2013).

Our observations suggest that reported contradictory positive and negative effects of FGF2 on odontoblast differentiation and *Dspp* expression are related to differences in the maturity of cells exposed to FGF2. Our study provides evidence for the stimulatory roles of FGF signaling in odontoblast differentiation by early progenitors and provides insight into the complex interaction between pulp cells and FGF2 for improved strategies for applications of FGF2 in dentin regeneration.

Author Contributions

K. Sagomonyants, M. Mina, contributed to conception, design, data acquisition, analysis, and interpretation, drafted and critically revised the manuscript; P. Maye, contributed to data acquisition,

analysis, and interpretation, critically revised the manuscript; I. Kalajzic, contributed to conception, design, data acquisition, and interpretation, critically revised the manuscript. All authors gave final approval and agree to be accountable for all aspects of the work.

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References

- About I. 2011. Dentin regeneration in vitro: the pivotal role of supportive cells. *Adv Dent Res*. 23(3):320–324.
- Balic A, Aguila HL, Mina M. 2010. Identification of cells at early and late stages of polarization during odontoblast differentiation using pOBCol3.6GFP and pOBCol2.3GFP transgenic mice. *Bone*. 47(5):948–958.
- Balic A, Mina M. 2011. Identification of secretory odontoblasts using DMP1-GFP transgenic mice. *Bone*. 48(4):927–937.
- Begue-Kim C, Krebsbach PH, Bartlett JD, Butler WT. 1998. Dentin sialoprotein, dentin phosphoprotein, enamelysin and ameloblastin: tooth-specific molecules that are distinctively expressed during murine dental differentiation. *Eur J Oral Sci*. 106(5):963–970.
- Chen S, Gluhak-Heinrich J, Martinez M, Li T, Wu Y, Chuang HH, Chen L, Dong J, Gay I, MacDougall M. 2008. Bone morphogenetic protein 2 mediates dentin sialophosphoprotein expression and odontoblast differentiation via NF- κ B signaling. *J Biol Chem*. 283(28):19359–19370.
- Cho YD, Yoon WJ, Woo KM, Baek JH, Park JC, Ryoo HM. 2010. The canonical BMP signaling pathway plays a crucial part in stimulation of dentin sialophosphoprotein expression by BMP-2. *J Biol Chem*. 285(47):36369–36376.
- Fakhry A, Ratisoontorn C, Vedhachalam C, Salhab I, Koyama E, Leboy P, Pacifici M, Kirschner RE, Nah HD. 2005. Effects of FGF-2/-9 in calvarial bone cell cultures: differentiation stage-dependent mitogenic effect, inverse regulation of BMP-2 and noggin, and enhancement of osteogenic potential. *Bone*. 36(2):254–266.
- Feng JQ, Luan X, Wallace J, Jing D, Ohshima T, Kulkarni AB, D'Souza RN, Kozak CA, MacDougall M. 1998. Genomic organization, chromosomal mapping, and promoter analysis of the mouse dentin sialophosphoprotein (*Dspp*) gene, which codes for both dentin sialoprotein and dentin phosphoprotein. *J Biol Chem*. 273(16):9457–9464.
- Gibson MP, Zhu Q, Wang S, Liu Q, Liu Y, Wang X, Yuan B, Ruest LB, Feng JQ, D'Souza RN, et al. 2013. The rescue of dentin matrix protein 1 (DMP1)-deficient tooth defects by the transgenic expression of dentin sialophosphoprotein (DSPP) indicates that DSPP is a downstream effector molecule of DMP1 in dentinogenesis. *J Biol Chem*. 288(10):7204–7214.
- He H, Yu J, Liu Y, Lu S, Liu H, Shi J, Jin Y. 2008. Effects of FGF2 and TGF β 1 on the differentiation of human dental pulp stem cells in vitro. *Cell Biol Int*. 32(7):827–834.
- James MJ, Jarvinen E, Thesleff I. 2004. Bono 1: a gene associated with regions of deposition of bone and dentine. *Gene Expr Patterns*. 4(5):595–599.
- Kettunen P, Karavanova I, Thesleff I. 1998. Responsiveness of developing dental tissues to fibroblast growth factors: expression of splicing alternatives of FGFR1, -2, -3, and of FGFR4; and stimulation of cell proliferation by FGF-2, -4, -8, and -9. *Dev Genet*. 22(4):374–385.
- Kim J, Park JC, Kim SH, Im GI, Kim BS, Lee JB, Choi EY, Song JS, Cho KS, Kim CS. 2014. Treatment of FGF-2 on stem cells from inflamed dental pulp tissue from human deciduous teeth. *Oral Dis*. 20(2):191–204.
- Kim SG, Zhou J, Solomon C, Zheng Y, Suzuki T, Chen M, Song S, Jiang N, Cho S, Mao JJ. 2012. Effects of growth factors on dental stem/progenitor cells. *Dent Clin North Am*. 56(3):563–575.
- Kodiha M, Banski P, Stochaj U. 2009. Interplay between MEK and PI3 kinase signaling regulates the subcellular localization of protein kinases ERK1/2 and Akt upon oxidative stress. *FEBS Lett*. 583(12):1987–1993.
- Kuhn LT, Liu Y, Advincula M, Wang YH, Maye P, Goldberg AJ. 2010. A nondestructive method for evaluating in vitro osteoblast differentiation on biomaterials using osteoblast-specific fluorescence. *Tissue Eng Part C Methods*. 16(6):1357–1366.
- Li J, Huang X, Xu X, Mayo J, Bringas P Jr, Jiang R, Wang S, Chai Y. 2011. SMAD4-mediated WNT signaling controls the fate of cranial neural crest cells during tooth morphogenesis. *Development*. 138(10):1977–1989.
- Marie PJ. 2012. Fibroblast growth factor signaling controlling bone formation: an update. *Gene*. 498(1):1–4.
- Marie PJ, Miraoui H, Severe N. 2012. FGF/FGFR signaling in bone formation: progress and perspectives. *Growth Factors*. 30(2):117–123.
- Mathieu S, Jeanneau C, Sheibat-Othman N, Kalaji N, Fessi H, About I. 2013. Usefulness of controlled release of growth factors in investigating the early events of dentin-pulp regeneration. *J Endod*. 39(2):228–235.
- Miyazono K, Kamiya Y, Morikawa M. 2010. Bone morphogenetic protein receptors and signal transduction. *J Biochem*. 147(1):35–51.
- Narayanan K, Ramachandran A, Hao J, George A. 2002. Transcriptional regulation of dentin matrix protein 1 (DMP1) by AP-1 (c-fos/c-jun) factors. *Connect Tissue Res*. 43(2–3):365–371.
- Osathanon T, Nowwarote N, Pavasant P. 2011. Basic fibroblast growth factor inhibits mineralization but induces neuronal differentiation by human dental pulp stem cells through a FGFR and PLC γ signaling pathway. *J Cell Biochem*. 112(7):1807–1816.
- Priam F, Ronco V, Locker M, Bourd K, Bonnefoix M, Duchene T, Bitard J, Wurtz T, Kellermann O, Goldberg M, et al. 2005. New cellular models for tracking the odontoblast phenotype. *Arch Oral Biol*. 50(2):271–277.
- Qin C, D'Souza R, Feng JQ. 2007. Dentin matrix protein 1 (DMP1): new and important roles for biomineralization and phosphate homeostasis. *J Dent Res*. 86(12):1134–1141.
- Qin W, Yang F, Deng R, Li D, Song Z, Tian Y, Wang R, Ling J, Lin Z. 2012. Smad 1/5 is involved in bone morphogenetic protein-2-induced odontoblastic differentiation in human dental pulp cells. *J Endod*. 38(1):66–71.
- Sagomonyants K, Mina M. 2014. Biphasic effects of FGF2 on odontoblast differentiation involve changes in the BMP and Wnt signaling pathways. *Connect Tissue Res*. 55 Suppl 1:53–56.
- Sagomonyants K, Mina M. 2015. Stage-specific effects of fibroblast growth factor 2 on the differentiation of dental pulp cells. *Cells Tissues Organs*. 199(5–6):311–328.
- Sloan AJ, Waddington RJ. 2009. Dental pulp stem cells: what, where, how? *Int J Paediatr Dent*. 19(1):61–70.
- Smith AJ, Smith JG, Shelton RM, Cooper PR. 2012. Harnessing the natural regenerative potential of the dental pulp. *Dent Clin North Am*. 56(3):589–601.
- Spector JA, Mathy JA, Warren SM, Nacamuli RP, Song HM, Lenton K, Fong KD, Fang DT, Longaker MT. 2005. FGF-2 acts through an ERK1/2 intracellular pathway to affect osteoblast differentiation. *Plast Reconstr Surg*. 115(3):838–852.
- Thesleff I, Keranen S, Jernvall J. 2001. Enamel knots as signaling centers linking tooth morphogenesis and odontoblast differentiation. *Adv Dent Res*. 15:14–18.
- Yang W, Harris MA, Cui Y, Mishina Y, Harris SE, Gluhak-Heinrich J. 2012. Bmp2 is required for odontoblast differentiation and pulp vasculogenesis. *J Dent Res*. 91(1):58–64.
- Yao N, Li S, Jiang Y, Qiu S, Tan Y. 2011. Amelogenin promotes odontoblast-like MDPC-23 cell differentiation via activation of ERK1/2 and p38 MAPK. *Mol Cell Biochem*. 355(1–2):91–97.
- Ye L, Peng L, Tan H, Zhou X. 2006. HGF enhanced proliferation and differentiation of dental pulp cells. *J Endod*. 32(8):736–741.
- Zhang H, Liu S, Zhou Y, Tan J, Che H, Ning F, Zhang X, Xun W, Huo N, Tang L, et al. 2012. Natural mineralized scaffolds promote the dentinogenic potential of dental pulp stem cells via the mitogen-activated protein kinase signaling pathway. *Tissue Eng Part A*. 18(7–8):677–691.
- Zhao M, Xiao G, Berry JE, Franceschi RT, Reddi A, Somerman MJ. 2002. Bone morphogenetic protein 2 induces dental follicle cells to differentiate toward a cementoblast/osteoblast phenotype. *J Bone Miner Res*. 17(8):1441–1451.
- Zhao X, He W, Song Z, Tong Z, Li S, Ni L. 2012. Mineral trioxide aggregate promotes odontoblastic differentiation via mitogen-activated protein kinase pathway in human dental pulp stem cells. *Mol Biol Rep*. 39(1):215–220.