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FGF Signaling Prevents the Terminal Differentiation of Odontoblasts

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Abstract

Members of the fibroblast growth factor (FGF) family play essential and important roles in primary and reparative dentinogenesis, with conflicting results regarding their effects on odontoblast differentiation. Our recent studies showed that the effects of FGF2 on cells in odontoblast lineage were stage-specific and depended on the stage of cell maturity. Continuous exposure of pulp cells to FGF2 inhibited odontoblast differentiation, whereas early and limited exposure of pulp cells to FGF2 resulted in marked increases in odontoblast differentiation. The purpose of this study was to evaluate the cellular and molecular mechanisms regulating the inhibitory effects of FGF2 on odontoblast differentiation. To do so, we examined the effects of the addition of FGF2 during the differentiation/mineralization phase of the in vitro growth of pulp cultures derived from a series of green fluorescent protein reporter transgenic mice that display stage-specific activation of transgenes during odontoblast differentiation. Our results showed that this treatment first stimulated the differentiation of remaining progenitors in pulp cultures into functional odontoblasts but prevented their differentiation into mature odontoblasts. In addition, this treatment inhibited expression of markers of osteogenesis. Furthermore, we demonstrated that the inhibitory effects of FGF2 on odontoblast differentiation were mediated through activation of FGFR/MEK/Erk1/2 signaling and downregulation of bone morphogenetic protein signaling, with negative and positive roles in the expression of *Dmp1* and *Dspp*, respectively, during the advanced stage of odontoblast differentiation.

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

Introduction

The fibroblast growth factor (FGF) family consists of 22 members that can be divided into 3 distinct subfamilies: canonical FGFs (1 to 10, 16 to 18, 20, 22), hormone-like FGFs (15/19, 21, 23), and intracellular FGFs (11 to 14; Itoh and Ornitz 2008). The canonical and endocrine subfamilies comprise 18 secreted signaling proteins that bind to and activate 4 receptor tyrosine kinase molecules (FGF receptors [FGFRs]; Ornitz and Itoh 2015). These FGFs bind to the extracellular domain of FGFRs, cause receptor dimerization, and induce phosphorylation of tyrosine residues in their intracellular domain. The activated FGFR recruits target proteins to its cytoplasmic tail and modifies these proteins, leading to activation of downstream signaling pathways, including STAT, MAPK, phosphatidylinositol 3-kinase/AKT, and phospholipase C-gamma/protein kinase C pathways (Itoh and Ornitz 2008; Ornitz and Itoh 2015). These pathways regulate distinct biological processes, including cell proliferation, differentiation, survival, and matrix production. The FGFR intracellular signaling is also regulated by several inhibitory molecules, including GRB2 and Sprouty proteins (Itoh and Ornitz 2008; Ornitz and Itoh 2015).

Canonical FGFs are differentially expressed in most tissues of the developing embryo, where they function as essential regulators of the earliest stages of development (Ornitz and Itoh 2015). In the developing teeth, FGFs derived from primary and secondary enamel knots regulate differentiation of dental papilla cells in their proximity into odontoblasts (Thesleff et al. 2001). Canonical FGFs are also expressed in postnatal and adult tissues, where they play essential roles in growth, repair, and regeneration (Ornitz and Itoh 2015). In the adult teeth, canonical FGFs sequestrated in the dentin matrix and pulp-supportive tissues play essential roles in reparative dentinogenesis (Smith 2000; Sloan and Waddington 2009).

Several studies have shown the roles of FGFs in proliferation, upregulation of the expression of embryonic stem cell markers, homing and migration of dental pulp cells, and vascular invasion (Osathanon et al. 2011; Kim et al. 2012; Kim et al.

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A supplemental appendix to this article is available online.

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2014). However, effects of FGFs on mineralization, odontoblast differentiation, and expression of dentin sialophosphoprotein (*Dspp*; expressed at high levels by odontoblasts) remain elusive, as both inhibitory and stimulatory effects have been reported (Kim et al. 2012; Kim et al. 2014).

With 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 (Balic et al. 2010; Balic and Mina 2011; Sagomonyants et al. 2015), we have examined the effects of FGF2 on primary pulp cultures and showed that effects of FGF2 on differentiation of progenitor cells into odontoblasts were stage-specific and depended on the stage of cell differentiation/maturity (Sagomonyants and Mina 2014; Sagomonyants et al. 2015). Continuous exposure of pulp cells to FGF2 inhibited odontoblast differentiation (Sagomonyants et al. 2015), whereas early and limited exposure of pulp cells to FGF2 resulted in significant increases in odontoblast differentiation, shown by increases in the expression of *Dmp1, Dspp*, and the number of DMP1-GFP$^+$ and DSPP-Cerulean$^+$ odontoblasts (Sagomonyants et al. 2015). Our results also showed that the stimulatory effects of FGF2 on odontoblast differentiation were mediated through FGFR/MEK/Erk1/2 signaling, increases in bone morphogenetic protein 2 (*Bmp2*) and activation of the BMP/BMPR signaling pathway.

The purpose of our present study was to gain further insight into cellular and molecular mechanisms regulating the inhibitory effects of FGF2 on odontoblast differentiation by examining the effects of late 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 University of Connecticut Health Center. The coronal portions of the pulp 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 2014; Sagomonyants et al. 2015). At day 7, when cells reached confluence, cultures were grown in mineralization-inducing medium containing 50 μg/mL of ascorbic acid and 4mM β-glycerophosphate in the presence of 20 ng/mL of low molecular weight (18 kDa) bovine FGF2 (R&D Systems, Inc.) or vehicle (VH; 0.1% bovine serum albumin), referred to as *late exposure* (days 7 to 21). Medium was replenished every other day.

Inhibition of Signaling Pathways

The FGFR inhibitor SU5402 (Santa Cruz Biotechnology), the MEK/Erk1/2 inhibitor U0126 (Promega Corporation), and the BMP/BMPR inhibitor Noggin (PeproTech) were dissolved in dimethyl sulfoxide (DMSO) or 0.1% bovine serum albumin in phosphate-buffered saline and added at various concentrations to the media between days 7 and 14. Medium containing inhibitors and FGF2 was replenished every other day.

Detection and Quantification of Mineralization in Cultures

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

Immunocytochemistry

Cultures were processed for immunocytochemistry with anti-GFP antibody (Invitrogen) and rabbit anti-mouse phospho-Erk1/2 and phospho-SMAD1/5 antibodies (Ser463/465, clone 41D10; Cell Signaling) as previously described (Sagomonyants and Mina 2014).

Digital Imaging and Epifluorescence Analysis of Cell Cultures

At different time points, the mean fluorescence intensity in culture wells was measured with a fluorescent plate reader, as described previously (Sagomonyants et al. 2015)

RNA Extraction and Analysis

Total RNA was isolated with TRIzol reagent (Invitrogen), followed by cDNA synthesis. Gene expression was examined by TaqMan real-time polymerase chain reaction analysis with primers and conditions, as described previously (Sagomonyants et al. 2015).

Flow Cytometric Sorting

Cultures derived from 2.3-GFP transgenic animals were processed for flow cytometric sorting (fluorescence-activated cell sorting [FACS]). Pulp cells were grown under control culture conditions for 7 d. At day 7, cells were detached and processed for FACS based on GFP expression, as described previously (Balic et al. 2010; Sagomonyants et al. 2015). Upon separation, reanalysis confirmed that the purity of isolated 2.3-GFP$^+$ and 2.3-GFP$^-$ populations was >98%. Live GFP$^+$ and GFP$^-$ cells were collected into DMEM with 20% fetal bovine serum, recounted, and replated at the same density as the primary cultures (8.75×10^4 cells/cm^2). Cultures were treated with VH or FGF2 (20 ng/mL) between days 7 and 14 and processed for various analyses, as described for unsorted cultures.

Statistical Analysis of Data

Results represent mean ± SEM of at least 3 independent experiments. Statistical analysis was performed by GraphPad Prism 6 software with 1-way analysis of variance with the 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 the Late Exposure to FGF2 on Mineralization and Odontoblast Differentiation

Late exposure of pulp cultures to FGF2 (days 7 to 21) markedly inhibited mineralization at all time points, as shown by XO and von Kossa staining (Fig. 1A, Appendix Fig. 1A).

Late exposure to FGF2 also resulted in changes in the expression of markers of odontoblast and osteoblast differentiation as well as the expression of various transgenes. There were transient increases in the levels of expression of Osteocalcin, *Dmp1*, and *Dspp* (~2- to 3-fold) at day 10. The levels of Osteocalcin and *Dmp1* were similar to control at day 14 but markedly decreased as compared with control at day 21. The levels of *Dspp* were decreased at days 14 and 21 as compared with control (Fig. 1B). In FGF2-treated cultures, the intensity of the expression of DMP1-GFP and DSPP-Cerulean transgenes and the percentage of DSPP-Cerulean$^+$ odontoblasts were increased at day 10, followed by decreases at days 14 to 21 as compared with control (Fig. 1C, Appendix Fig. 1B, Appendix Table 1).

FGF2-treated cultures also showed decreased expression of Type I collagen and *Bsp* and the intensity of expression of 2.3-GFP transgene between days 10 and 21 as compared with control (Fig. 1B–C, Appendix Fig. 1B).

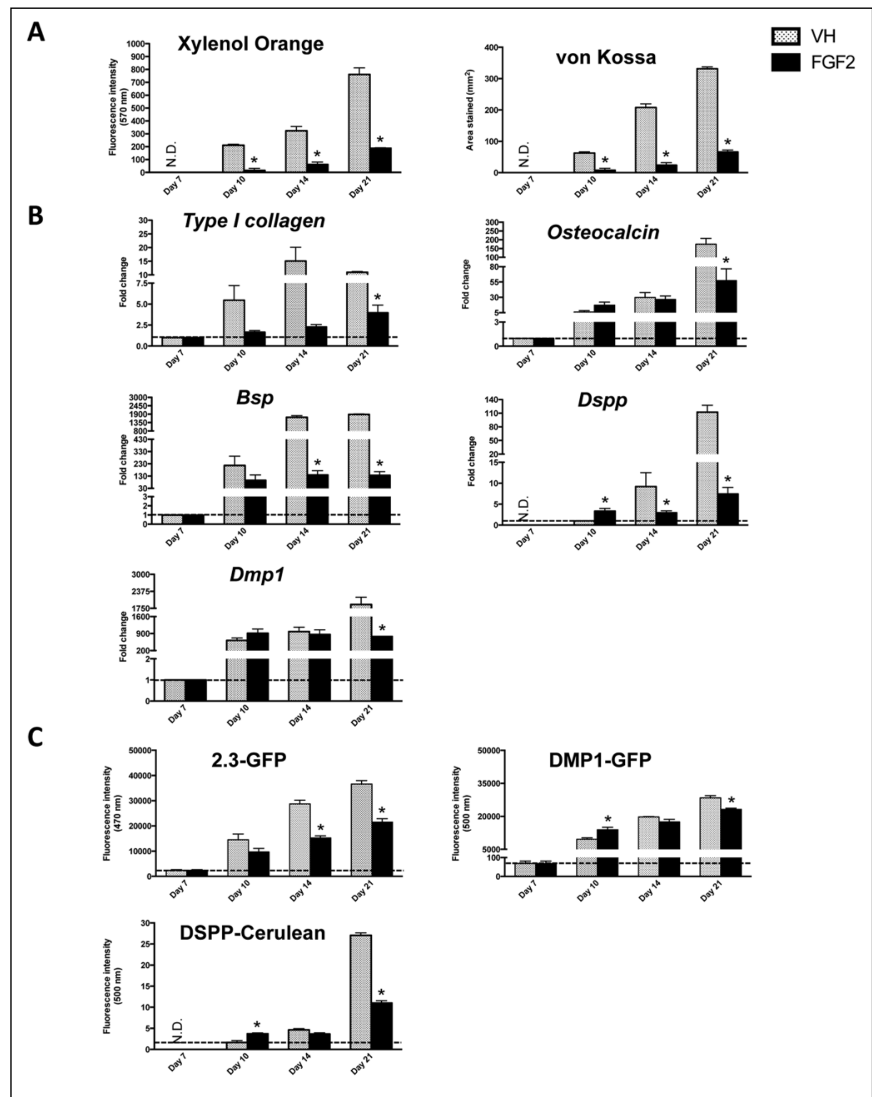

Figure 1. Effects of late exposure to FGF2 on odontoblast differentiation and mineralization. Primary dental pulp cultures were grown in the absence or presence of 20 ng/mL of FGF2 between days 7 and 21. (**A**) Histograms showing decreased mineralization, indicated by the intensity of xylenol orange staining (in absolute values) and the area of von Kossa staining (in mm^2) in FGF2-treated cultures as compared with VH-treated cultures. (**B**) Expression of markers of differentiation, analyzed by real-time polymerase chain reaction at various time points. Expression of all genes except for *Dspp* was normalized to VH at day 7, which is set to 1 and is indicated by the dashed line. The expression of *Dspp* was normalized to VH at day 10, which is set to 1 and is indicated by the dashed line. Note the decreases in the expression of Type I collagen and *Bsp* at all time points in FGF2-treated cultures as compared with VH-treated cultures. Also note the transient increase in the expression of Osteocalcin, *Dmp1*, and *Dspp* at day 10, followed by their decreased expression at days 14 and 21 in FGF2-treated cultures as compared with VH-treated cultures. Also note that, despite decreases in their levels of expression as compared with VH, the expression levels of *Bsp, Dmp1*, and *Dspp* in FGF2-treated cultures between days 10 and 21 remained unchanged. (**C**) The intensity of 2.3-GFP, DMP1-GFP, and DSPP-Cerulean transgene expression was examined at various time points. The results are expressed in absolute values, and the dashed line indicates levels of expression in VH-treated cultures at day 7. Note the transient increases in the intensity of the expression of DMP1-GFP and DSPP-Cerulean transgenes at day 10. Results of all experiments represent mean ± SEM of at least 3 independent experiments. $^*P \leq 0.05$ relative to VH at each time point. FGF, fibroblast growth factor; GFP, green fluorescent protein; ND, not detected; VH, vehicle.

Recovery of Differentiation and Expression of Various GFP Reporters in FGF2-Treated Cells

As shown in Figure 1B–C, despite decreases at days 14 and 21 as compared with the respective controls, the intensity of expression of DMP1-GFP and DSPP-Cerulean transgenes and levels of expression of *Dmp1* and *Dspp* in FGF2-treated cultures remained relatively unchanged. These observations suggested that FGF2 prevented the differentiation of functional odontoblasts into fully differentiated/mature odontoblasts. To test this possibility, we examined the effects of withdrawal of FGF2 on the differentiation of pulp cells. In these experiments, dental pulp cells were exposed to FGF2

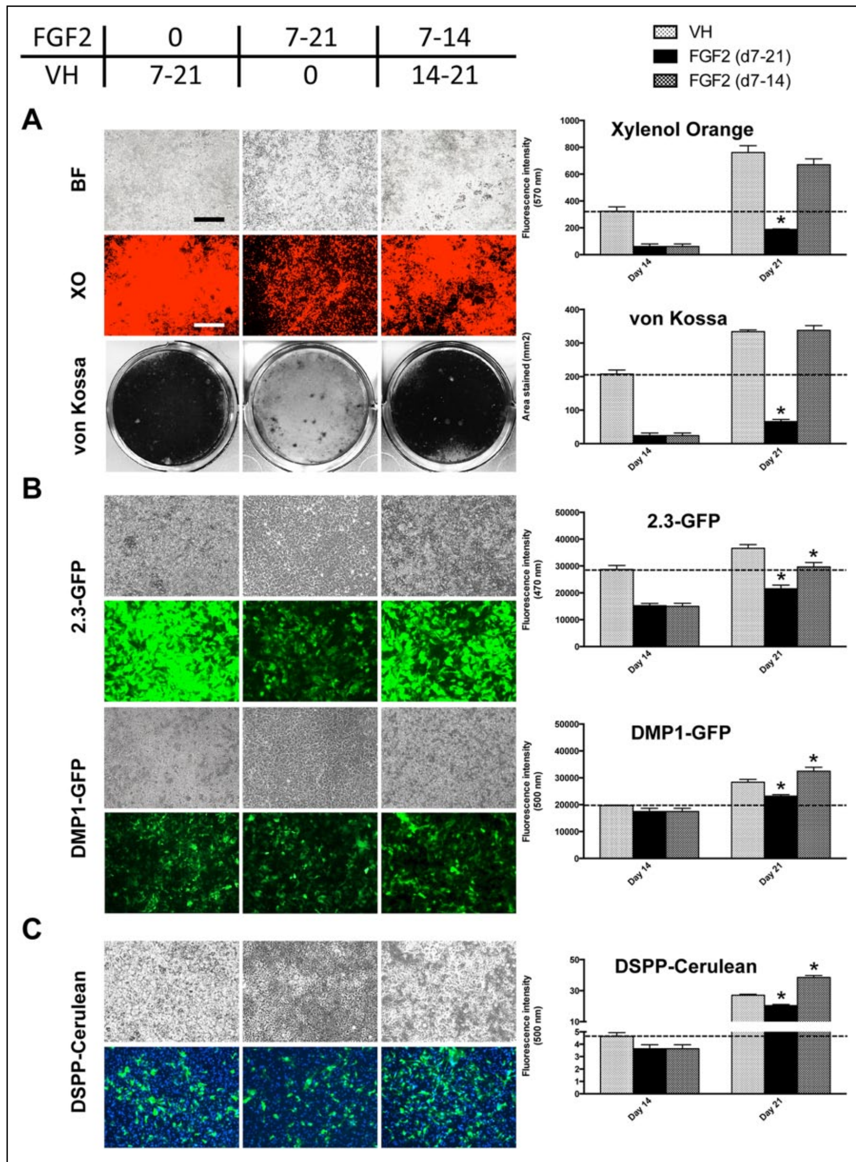

Figure 2. Effects of withdrawal of FGF2 on mineralization and expression of transgenes in primary dental pulp cultures. Cultures were grown in the absence or presence of FGF2 between days 7 and 14. At day 14, FGF2 was withdrawn, and cells were cultured for additional 7 d. (**A**) Images of the same areas of cultures analyzed at day 21 under bright field (upper row) and epifluorescence for xylenol orange (XO) staining (middle row). The lower row shows representative cultures stained with von Kossa assay. Histograms showing changes in the intensity of XO staining (in absolute values) and areas stained with von Kossa (in mm^2) in VH- and FGF2-treated cultures at days 14 and day 21. Note that the intensity of XO staining and area of von Kossa staining after withdrawal of FGF2 were similar to those in VH-treated cultures. Scale bar = 200 µm. (**B**) Images of the same areas of cultures analyzed at day 21 under bright field (upper row) and epifluorescent light through filters for GFPemd (for detection of the 2.3-GFP transgene) or GFPtpz (for detection of the DMP1-GFP transgene; lower row). Histograms showing changes in the intensity of 2.3-GFP and DMP1-GFP transgene expression in VH- and FGF2-treated cultures at days 14 and 21. Note that the intensity of 2.3-GFP transgene after withdrawal of FGF2 did not reach the levels in VH-treated cultures. However, the intensity of DMP1-GFP transgene expression after withdrawal of FGF2 was higher than that in VH-treated cultures. (**C**) Images of the same areas of cultures analyzed under bright field (upper row) and epifluorescent light through filters for GFPtpz and DAPI (for detection of the DSPP-Cerulean transgene and Hoechst 33342, respectively) at day 21. DSPP-Cerulean$^+$ odontoblasts were detected with anti-GFP antibody. The lower row represents the overlay image of Hoechst and DSPP-Cerulean. Histograms show changes in the intensity of DSPP-Cerulean transgene expression in VH- and FGF2-treated cultures at days 14 and 21. The intensity of DSPP-Cerulean transgene expression after withdrawal of FGF2 was higher than that in VH-treated cultures. Results of all experiments represent mean ± SEM of at least 6 independent experiments. $^*P \leq 0.05$ relative to VH at each time point. FGF, fibroblast growth factor; GFP, green fluorescent protein; VH, vehicle.

between days 7 and 14 and then grown in control medium (without FGF2) for an additional 7 d. The effects of withdrawal of FGF2 on the extent of mineralization and odontoblast differentiation in these cultures were compared with control cultures (not exposed to FGF2) and cultures exposed to FGF2 between days 7 and 21.

Withdrawal of FGF2 for 7 d allowed rapid and complete recovery of mineralization (Fig. 2A). The intensity of DSPP-Cerulean and DMP1-GFP transgenes and the percentage of DSPP-Cerulean$^+$ odontoblasts in these cultures were significantly higher than those in the respective VH-treated controls (Fig. 2B–C, Appendix Table 1). The levels of expression of *Dspp* and *Dmp1* were only slightly higher than those in VH-treated controls (Fig. 3).

In these cultures, the levels of *Bsp* reached those in VH-treated controls, whereas the intensity of the expression of 2.3-GFP transgene and the levels of Type I collagen and Osteocalcin did not (Fig. 2B–C and Fig. 3).

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 mesenchymal stem cells) at different stages of differentiation (Balic et al. 2010; Balic and Mina 2011). This heterogeneity makes it difficult to understand the underlying mechanisms of the positive and negative effects of FGF2 on mineralization and odontoblast differentiation. To gain a better understanding of the response of cells at different stages of differentiation to FGF2, we examined the effects of FGF2 on relatively homogeneous populations of FACS-sorted 2.3-GFP$^+$ and 2.3-GFP$^-$ cells (Appendix Fig. 2A) that represent proliferative cells enriched in early progenitors and undifferentiated cells, respectively (Balic et al. 2010).

Late exposure to FGF2 resulted in markedly decreased mineralization as early as day 10 in both 2.3-GFP$^-$ and 2.3-GFP$^+$ populations (Appendix Fig. 2B). In both populations, late exposure to FGF2 decreased the levels of Type I

collagen, *Bsp*, and Osteocalcin (Fig. 4). Interestingly, FGF2 markedly increased the levels of *Dmp1* and *Dspp* in the 2.3-GFP⁻ population at days 10 and 14 as compared with control (Fig. 4). However, in the 2.3-GFP⁺ population, FGF2 resulted in transient increases in the levels of expression of *Dmp1* and *Dspp* at day 10, followed by decreases in their levels at day 14 as compared with control (Fig. 4). However, the levels of *Dmp1* and *Dspp* in FGF2-treated cultures at day 14 were similar to those at day 10 (Fig. 4).

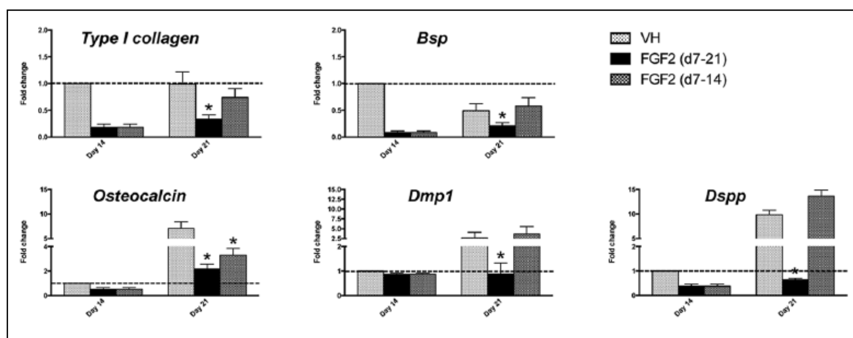

Figure 3. Effects of withdrawal of FGF2 on the expression of markers of odontoblast and osteoblast differentiation in primary dental pulp cultures. Cultures were grown in the absence or presence of FGF2 between days 7 and 14. At day 14, FGF2 was withdrawn, and cells were cultured for additional 7 d. Expression levels of all genes were normalized to VH at day 14, which is set to 1 and is indicated by the dashed line. Note that the levels of expression of *Dspp* and *Dmp1* after withdrawal of FGF2 were slightly higher than that in VH-treated cultures. Results represent mean ± SEM of at least 5 independent experiments. $^{*}P \leq 0.05$ relative to VH at each time point. FGF, fibroblast growth factor; VH, vehicle.

Activation of FGFR/MEK/Erk1/2 Signaling Mediates the Inhibitory Effects of FGF2 on Mineralization and Expression of Dmp1 *and* Dspp *in Primary Dental Pulp Cultures*

The roles of the FGFR/MEK/Erk1/2 signaling pathway in mediating the inhibitory effects of FGF2 on mineralization, the expression of markers of mineralization, and odontoblast differentiation during later stages of differentiation were examined with SU5402 and U0126, inhibitors of FGFR and MEK/Erk1/2, respectively. In these experiments, primary pulp cultures were treated with inhibitors, with or without FGF2, during the mineralization phase of in vitro growth (days 7 to 14) and evaluated at day 14.

Immunocytochemical analysis of pulp cultures showed preferential nuclear localization of phospho-Erk1/2 protein in FGF2-treated cultures as compared with control, indicating activation of MEK/Erk1/2 signaling by FGF2 (Appendix Fig. 3A). Addition of SU5402 and U0126 alone to pulp cultures did not have significant effects on the extent of mineralization (Fig. 5A) or the levels of expression of *Dmp1* (Fig. 5B) but did increase the levels of *Dspp* (Fig. 5B) and the percentage of DSPP-Cerulean⁺ cells (Appendix Table 2), revealing the negative roles of FGFR and MEK/Erk1/2 on the expression of *Dspp* at late stages of odontoblast differentiation (Fig. 5B).

SU5402 and U0126 reversed the FGF2-induced decreases in mineralization (Fig. 5A), the levels of *Dspp* and *Dmp1* (Fig. 5B), and the percentage of DSPP-Cerulean⁺ cells (Appendix Table 2), suggesting that the FGF2-mediated inhibition of odontoblast differentiation was partially mediated by the reactivation of FGFR/MEK/Erk1/2 signaling with negative roles in terminal differentiation of odontoblasts.

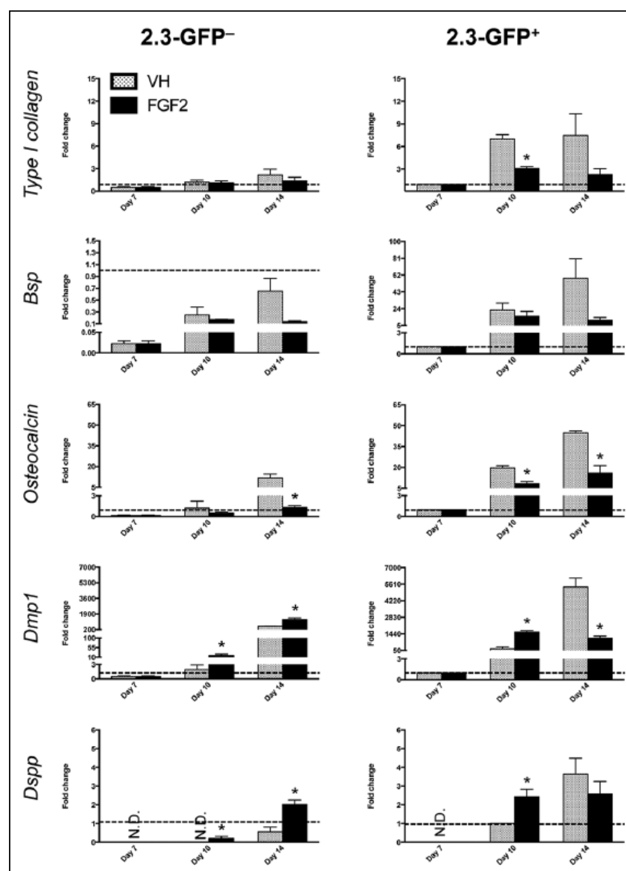

Figure 4. Effects of late exposure to FGF2 on the expression of differentiation markers in FACS-sorted 2.3-GFP⁺ and 2.3-GFP⁻ populations. Primary pulp cultures derived from 2.3-GFP transgenic mice were subjected to FACS sorting to separate homogeneous populations of 2.3-GFP⁻ and 2.3-GFP⁺ cells, as described in the Materials and Methods section and shown in Appendix Figure 2A. In both 2.3-GFP⁻ and 2.3-GFP⁺ populations, expression of Type I collagen, *Bsp*, Osteocalcin, and *Dmp1* was normalized to VH-treated cultures in the 2.3-GFP⁺ population at day 7, which is set to 1 and is indicated by the dashed line. Expression of *Dspp* in both 2.3-GFP⁻ and 2.3-GFP⁺ populations was normalized to VH-treated cultures in 2.3-GFP⁺

population at day 10. In the 2.3-GFP⁻ population, FGF2 increased the levels of *Dmp1* and *Dspp* at days 14 and 21. In the 2.3-GFP⁺population, FGF2 increased the levels of *Dmp1* and *Dspp* only at day 14. Results represent mean ± SEM of at least 3 independent experiments. $^{*}P \leq 0.05$ relative to the respective VH-treated controls at each time point. FACS, fluorescence-activated cell sorting; FGF, fibroblast growth factor; GFP, green fluorescent protein; ND, not detected; VH, vehicle.

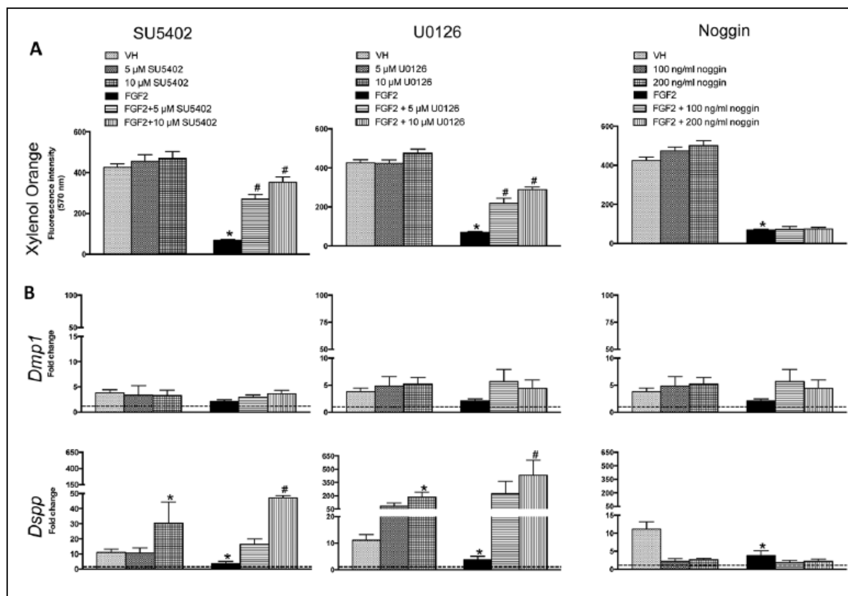

Figure 5. Effects of the inhibition of FGFR, MEK/Erk1/2, and BMP signaling on mineralization and gene expression in primary dental pulp cultures. Cultures were treated with VH and 20 ng/mL of FGF2 alone or in combination with inhibitors of the FGFR (SU5402), MEK/Erk1/2 (U0126) and BMP (Noggin) signaling pathways between days 7 and 14. All assays were performed at day 14. (**A**) Histograms showing changes in mineralization, indicated by the intensity of xylenol orange staining (in absolute values). Note that SU5402 and U0126 but not Noggin reversed the FGF2-induced decreases in mineralization in a concentration-dependent manner. (**B**) Histograms showing changes in the levels of expression *Dmp1* and *Dspp*, analyzed by real-time polymerase chain reaction. SU5402 and U0126 were used at the concentration of 5 and 10 μM, and noggin was used at the concentration of 100 and 200 ng/mL. Expression levels of all genes were normalized to VH at day 10, which is set to 1 and is indicated by the dashed line. SU5402, U0126, and noggin reversed the FGF2-induced decreases in expression of *Dmp1*. SU5402 and U0126 but not noggin also reversed the FGF2-induced decreases in expression of *Dspp*. Results of all experiments represent mean ± SEM of values from at least 3 independent experiments. $^{*}P \leq 0.05$ relative to VH. $^{\#}P \leq 0.05$ relative to FGF2-treated cultures. FGF, fibroblast growth factor; VH, vehicle.

Downregulation of BMP Signaling Is Also Involved in the Inhibitory Effects of FGF2 on Terminal Differentiation of Primary Dental Pulp Cultures

Our previous studies have shown that stimulatory effects of FGF2 on odontoblast differentiation included increases in the expression of *Bmp2* and activation of the BMP/BMPR signaling pathway. Therefore, we examined the involvement of the crosstalk between FGF and BMP signaling in mediating the inhibitory effect of FGF2 on odontoblast differentiation. To do that, primary pulp cultures were treated with Noggin, a specific inhibitor of BMP/BMPR signaling, with or without FGF2, between days 7 and 14.

Late exposure of pulp cells to FGF2 did not cause nuclear localization of phospho-SMAD1/5 protein (Appendix Fig. 3B). Slightly decreased levels of *Bmp2* and inhibition of FGFR but not MEK/Erk1/2 signaling reversed the FGF2-induced decreases in the levels of *Bmp2* (Appendix Fig. 4).

Noggin alone or with FGF2 did not affect the extent of mineralization (Appendix Fig. 5A) and did not reverse the FGF2-induced decreases in the expression of *Dmp1* and *Dspp* (Appendix Fig. 3) or the percentage of DSPP-Cerulean^{+} cells (Appendix Table 2). These treatments led to further decreases in the levels of expression of *Dmp1, Dspp*, and *Bmp2* (Fig. 5 and Appendix Fig. 3), indicating the positive roles of BMP signaling in their expression.

These observations suggest that inhibition of terminal differentiation of odontoblasts by FGF/FGFR signaling may be in part related to decreases in activity of BMP signaling with positive roles on the expression of *Dmp1, Dspp*, and *Bmp2*.

Discussion

In this study, we demonstrated that exposure of pulp cells to FGF2 during the mineralization phase of in vitro growth (days 7 to 21) inhibited mineralization and changed the expression of markers of odontoblast differentiation. These changes included transient increases in the expression of *Dmp1, Dspp*, DMP1-GFP, and DSPP-Cerulean transgenes (markers of functional odontoblasts) at day 10, followed by decreases in their expression at days 14 and 21 as compared with control. Our further studies on FACS-sorted populations showed that this treatment resulted in continuous increases in expression of *Dspp* and *Dmp1* in the undifferentiated 2.3-GFP^{-} population and transient increases in their expression in the 2.3-GFP^{+} population. Similar to the observation in the whole pulp cultures, the transient increases in the expression of *Dmp1* and *Dspp* in 2.3-GFP^{+} populations were followed by decreases in their expression at day 14. These observations indicated that exposure of dental pulp cells to FGF2 during the differentiation/mineralization phase of in vitro growth first stimulated the differentiation of remaining undifferentiated odontoprogenitors into functional odontoblasts. The decreases in the expression of markers of odontoblast differentiation in the cultures from whole pulp and 2.3-GFP^{+} (not 2.3-GFP^{-}) population indicated that FGF2 prevented the differentiation of functional odontoblasts into mature odontoblasts.

These observations with our previous studies (Sagomonyants and Mina 2014; Sagomonyants et al. 2015) provide clear evidence for stage specificity of the effects FGF2 on cells in odontoblast lineage. In dental pulp, FGF2 promoted the formation of functional odontoblasts from undifferentiated and early progenitors through the FGFR/MEK/Erk1/2 and BMP/BMPR signaling pathways (Sagomonyants et al. 2015). Unlike its effects on early progenitors, additional exposure to FGF2 inhibited further differentiation of functional odontoblasts to fully differentiated odontoblasts. The inhibitory effects of FGF2 on the terminal differentiation of odontoblast was mediated through 1) activation of the FGF/FGFR/MEK/Erk1/2 signaling

pathway with negative roles at final stages of differentiation and 2) downregulation of BMP/BMPR signaling with positive roles at the final stages of differentiation.

The stage-specific positive and negative roles of the FGFR/MEK/Erk1/2 and BMP/BMPR signaling pathways on early progenitors versus more mature cells in odontoblast lineage in present and previous studies (Sagomonyants and Mina 2014; Sagomonyants et al. 2015) are similar to stage-specific roles of RUNX2 during odontoblast differentiation, suggesting that *Runx2* may be one of the mediators of both the positive and negative effects of FGF2 on odontoblast differentiation.

RUNX2 regulates the expression of members of the SIBLING family (small integrin-binding ligand N-linked glycoprotein), including *Dspp* and *Dmp1* (Camilleri and McDonald 2006; Qin et al. 2007; Prasad et al. 2010; Bruderer et al. 2014), and is a target for several signaling pathways that regulate its phosphorylation, activation, and stabilization (Park et al. 2010; Bruderer et al. 2014; Vimalraj et al. 2015). RUNX2 phosphorylation occurs in the nucleus and is generally mediated by ERK/MAPK kinases as well as BMP-mediated Smads (Park et al. 2010; Bruderer et al. 2014; Vimalraj et al. 2015). In teeth, *Runx2* is expressed in preodontoblasts but not in more differentiated odontoblasts (D'Souza et al. 1999; Yamashiro et al. 2002; Camilleri and McDonald 2006). RUNX2 regulates the expression of DSPP through multiple *Runx2*-binding sites (Chen et al. 2005; Camilleri and McDonald 2006). In vitro studies have shown that forced overexpression of *Runx2* increased expression of DSPP in preodontoblast cell lines but reduced its expression in mature odontoblast cell lines (Gaikwad et al. 2001; Chen et al. 2005; Camilleri and McDonald 2006).

Transgenic mice overexpressing *Runx2* in odontoblasts at early and late stages of differentiation with 2.3-kb Col1a1 and *Dspp* promoter fragments confirmed the stage-specific positive and negative roles of Runx2 during odontoblast differentiation (Miyazaki et al. 2008; Li et al. 2011). In these animals, the expression of *Dspp* was upregulated in immature odontoblasts and downregulated in more mature odontoblasts. In both transgenic animals, sustained expression of *Runx2* inhibited terminal differentiation of odontoblasts, indicating positive and negative roles of RUNX2 during the early and late stages of odontoblast differentiation (Miyazaki et al. 2008; Li et al. 2011).

Another interesting observation in our study was the differences in the response of markers of odontoblast differentiation and osteoblast differentiation to late exposure to FGF2. Unlike its effects on *Dmp1* and *Dspp*, late exposure of whole pulp cultures and sorted 2.3-GFP$^+$ and 2.3-GFP$^-$ populations to FGF2 resulted in continuous decreases in the expression of Type I collagen, *Bsp* (a differentiation marker for osteoblasts and not odontoblasts), and Osteocalcin (a marker of mature osteoblasts; Bouleftour et al. 2016). Furthermore, withdrawal of FGF2 did not allow complete recovery of expression of Type I collagen and Osteocalcin. The inhibitory effects of late exposure to FGF2 on these transcripts are different from the stimulatory effects of early exposure to FGF2 (Sagomonyants and Mina 2014; Sagomonyants et al. 2015). These differences may also be related to RUNX2 activity. Transgenic mice that overexpressed *Runx2* with 2.3-kb *Col1a1* displayed abnormalities in bones, including reduced numbers of mature osteoblasts and reduced numbers of osteocytes (Liu et al. 2001; Geoffroy et al. 2002; Kanatani et al. 2006; Bruderer et al. 2014). A more recent study also showed that *Runx2* deficiency in osteoblasts disrupted osteoblast function (Adhami et al. 2014).

In summary, our study provides evidence for the inhibitory roles of FGF signaling in terminal differentiation of odontoblasts and provides insight into the complex interaction between pulp cells and FGF2. These findings also suggest that in dental pulp, positive and negative effects of the FGF2 on early and late stages of odontoblast differentiation, respectively, might be mediated by RUNX2. Further studies are therefore needed to elucidate the possible associations between the FGF signaling pathway and RUNX2 in determining the differentiation fate of progenitor cells in dental pulp.

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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