

8-2014

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## Recommended Citation

Sagomonyants, Karen and Mina, Mina, "Biphasic Effects of FGF2 on Odontoblast Differentiation Involve Changes in the BMP and Wnt Signaling Pathways" (2014). *Articles - Research*. 264.  
[http://digitalcommons.uconn.edu/uchres\\_articles/264](http://digitalcommons.uconn.edu/uchres_articles/264)



Published in final edited form as:

Connect Tissue Res. 2014 August ; 55(0 1): 53–56. doi:10.3109/03008207.2014.923867.

## Biphasic effects of FGF2 on odontoblast differentiation involve changes in the BMP and Wnt signaling pathways

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### Abstract

Odontoblast differentiation during physiological and reparative dentinogenesis is dependent upon multiple signaling molecules, including Fibroblast Growth Factors (FGFs), Bone Morphogenetic Proteins (BMPs) and Wingless/Integrated (Wnt) ligands. Recent studies in our laboratory showed that continuous exposure of primary dental pulp cultures to FGF2 exerted biphasic effects on the expression of markers of dentinogenesis. In the present study we examined the possible involvement of the BMP and Wnt signaling pathways in mediating the effects of FGF2 on dental pulp cells. Our results showed that stimulatory effects of FGF2 on dentinogenesis during the proliferation phase of growth were associated with increased expression of the components of the BMP (*Bmp2*, *Dlx5*, *Msx2*, *Osx*) and Wnt (*Wnt10a*, *Wisp2*) pathways, and decreased expression of an inhibitor of the Wnt signaling, *Nkd2*. Further addition of FGF2 during the differentiation/mineralization phase of growth resulted in decreased expression of components of the BMP signaling (*Bmp2*, *Runx2*, *Osx*) and increased expression of inhibitors of the Wnt signaling (*Nkd2*, *Dkk3*). This suggests that both BMP and Wnt pathways may be involved in mediating the effects of FGF2 on dental pulp cells.

### Keywords

FGF; BMP; Wnt; odontoblast; gene expression

### Introduction

Fibroblast growth factors (FGFs) are a family of growth factors shown to play an essential role in development, repair and regeneration of damaged skeletal tissues, including cartilage, bone and tooth (1). The FGF family contains 22 members, which elicit their effects through interaction with four highly conserved transmembrane tyrosine kinase receptors (FGFR1–4) in concert with heparin or heparan sulfate proteoglycans (1). Among FGFs, FGF2 is widely expressed in the cells of the odontoblast lineage (2), suggesting a role in odontoblast proliferation and differentiation.

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**Declaration of interest statement.** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

Our recent studies have demonstrated that continuous exposure of primary dental pulp cultures to FGF2 during both proliferation and differentiation/mineralization phases of growth (days 3–14) decreased mineralization and expression of markers of dentinogenesis at days 10 and 14. However, these inhibitory effects were preceded by a transient stimulatory effect of FGF2 on expression of *Dmp1* and *Dspp* at day 7, suggesting that FGF2 exerted biphasic effects on odontoblast differentiation *in vitro* (manuscript in preparation).

In the present study we examined changes in the expression of components of the Bone Morphogenetic Proteins (BMPs) and Wingless/Integrated (Wnt) signaling pathways, known regulators of odontoblast differentiation, in response to FGF2 during proliferation and differentiation/mineralization phases of growth.

## Materials and Methods

### Primary dental pulp cultures

Primary dental pulp cultures were prepared from the coronal portions of pulps from unerupted first and second molars of 5–7-day-old mouse pups as described previously (3). Cells were first grown for 7 days in medium supporting their proliferation (proliferation phase) and then for additional 7 days in mineralization-inducing medium (differentiation/mineralization phase). Cells were exposed to 20 ng/ml low molecular weight (18 kDa) bovine FGF2 (R&D systems, Inc., Minneapolis, MN) or vehicle (VH, 0.1% BSA fraction V in PBS) between days 3–14 of the culture.

### RNA extraction and quantitative PCR (qPCR) analysis

Total RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's protocol and treated with RNase-free DNase to eliminate genomic DNA. Gene expression in cultures was examined by TaqMan or SYBR Green qPCR analysis using manufacturer's recommended conditions (Applied Biosystems, Branchburg, NJ). The list of primers is included in Supplemental Table 1. Controls in these experiments included elimination of Superscript II Reverse Transcriptase from the reactions performed using *Gapdh* primers.

### Statistical analysis

Statistical analysis was performed by GraphPad Prism 6 software using unpaired two-tailed Student *t*-test. In all graphs, values represented mean  $\pm$  SEM of at least three independent experiments, and a *p*-value  $\leq 0.05$  was considered statistically significant.

## Results

In control dental pulp cultures, mineralization and expression of markers of dentinogenesis increased after induction of mineralization at days 10 and 14 (data not shown). Our results demonstrated that control cultures also displayed increases in the expression levels of *Bmp2*, *Runx2* and *Osx* during days 10 and 14 (Figure 1).

We next examined changes in the expression of selected components of the BMP signaling in response to FGF2. FGF2-treated cultures showed marked increases in the expression of *Bmp2* and more modest increases in the expression of *Runx2* and *Osx* at day 7 as compared

to the control. Further exposure to FGF2 resulted in decreases in the expression of these transcripts at days 10 and 14 as compared to the control (Figure 1).

Examination of gene expression during the first 7 days showed that FGF2 markedly (~3–6-fold) and rapidly (within ~12 hrs after addition) increased the levels of *Bmp2* but not *Bmp4*. Expression of *Dlx5*, *Msx2* and *Osx* was increased around 48–96 hrs after addition of FGF2. There were no significant differences in the levels of *Runx2* in the control and FGF2-treated cultures (Figure 2).

Next we examined effects of FGF2 on expression of components of the Wnt signaling. Mineralization and dentinogenesis in the control cultures were associated with relatively unchanged levels of expression of the *Wnt10a* ligand (Figure 1). However, expression of *Nkd2*, an inhibitor of the Wnt signaling (4), and *Dkk3* was significantly increased in more advanced stages of culture differentiation (Figure 1). Addition of FGF2 to these cultures increased expression of *Wnt10a* and *Dkk3* at day 7 as compared to the control, but decreased expression of *Nkd2* (Figure 1). Further exposure to FGF2 resulted in increased expression of *Wnt10a* and decreased expression of *Nkd2* and *Dkk3* at days 10 and 14 as compared to the control (Figure 1). Although the role of *Dkk3* in regulation of the Wnt signaling remains controversial (5), in our studies the pattern of its expression during the differentiation/mineralization phase of growth was similar to that of *Nkd2*, suggesting that *Dkk3* may function as an inhibitor of the Wnt signaling.

Further analysis during the first 7 days (Figure 2) showed that FGF2 increased expression of *Wnt10a* and *Wisp2*, another component of the Wnt signaling, at 48–96 hrs as compared to the control. Expression of *Dkk3* also exhibited an increase at 48–96 hrs after treatment that was not statistically significant ( $p > 0.05$ ). Expression of *Nkd2* was decreased in FGF2-treated cultures at 12–96 hrs as compared to the control.

## Discussion

Our previous studies demonstrated that continuous exposure of dental pulp cells to FGF2 resulted in biphasic effects on odontoblast differentiation, including stimulatory effects during the proliferation phase of growth, and inhibitory effects during the differentiation/mineralization phase of growth (manuscript in preparation). In our present study we examined the possible involvement of the BMP and Wnt signaling pathways in FGF2-induced changes in dentinogenic differentiation of pulp cells.

BMP signaling is a potent regulator of odontoblast differentiation (6), which interacts with the FGF signaling to mediate its effects (7). Our results showed that progression of mineralization and dentinogenesis in control cultures was associated with increases in the expression of *Bmp2*, *Runx2* and *Osx*, suggesting that the BMP signaling could act as a positive regulator of odontoblast differentiation. We also showed that decreases in mineralization and dentinogenesis by FGF2 were associated with decreases in the expression of *Bmp2*, *Runx2* and *Osx* at days 10 and 14.

In control cultures, expression of components of the BMP signaling was relatively unchanged during the first 7 days. In contrast, FGF2 rapidly upregulated expression of *Bmp2*

(~12 hrs after treatment), whereas expression of *Dlx5*, *Msx2* and *Osx* was increased at later time points. The increases in the expression of these genes during the first 7 days were associated with increased expression of *Dmp1* and *Dspp* by FGF2 (data not shown). Taken together, these observations suggest that the stimulatory and inhibitory effects of FGF2 on dentinogenic differentiation of dental pulp cells involved changes in the BMP signaling.

Wnt is also one of the essential signaling pathways regulating tooth development and odontoblast differentiation (8). The interactions between the Wnt and FGF signaling pathways are well documented (9). In contrast to the BMP signaling, effects of the Wnt signaling include stimulation of early odontoblast differentiation and inhibition of terminal odontoblast differentiation.

Our results showed that mineralization and dentinogenesis in the control cultures were associated with increases in the expression of *Nkd2* and *Dkk3*, inhibitors of the Wnt signaling, at days 10 and 14. In addition, FGF2-induced decreases in mineralization and dentinogenesis at days 10 and 14 were associated with lower levels of *Nkd2* and *Dkk3*, but higher levels of *Wnt10a*. Furthermore, *Wnt10a* and *Wisp2* expression was increased and the expression of *Nkd2* was decreased by FGF2 during the first 7 days. This suggests that the increased activity of the Wnt signaling in response to FGF2 stimulates early odontoblast differentiation but inhibits late odontoblast differentiation. This is in agreement with previous results showing that Wnt signaling stimulates formation of polarized and functional odontoblasts, but inhibits their terminal differentiation (10).

In conclusion, our results suggest that the BMP and Wnt signaling pathways are involved in mediating both stimulatory and inhibitory effects of FGF2 on dentinogenic differentiation of dental pulp cells. Better understanding of the molecular mechanisms underlying these effects of FGF2 on dentinogenesis could provide critical information for the development of improved treatments for vital pulp therapy and dentin regeneration.

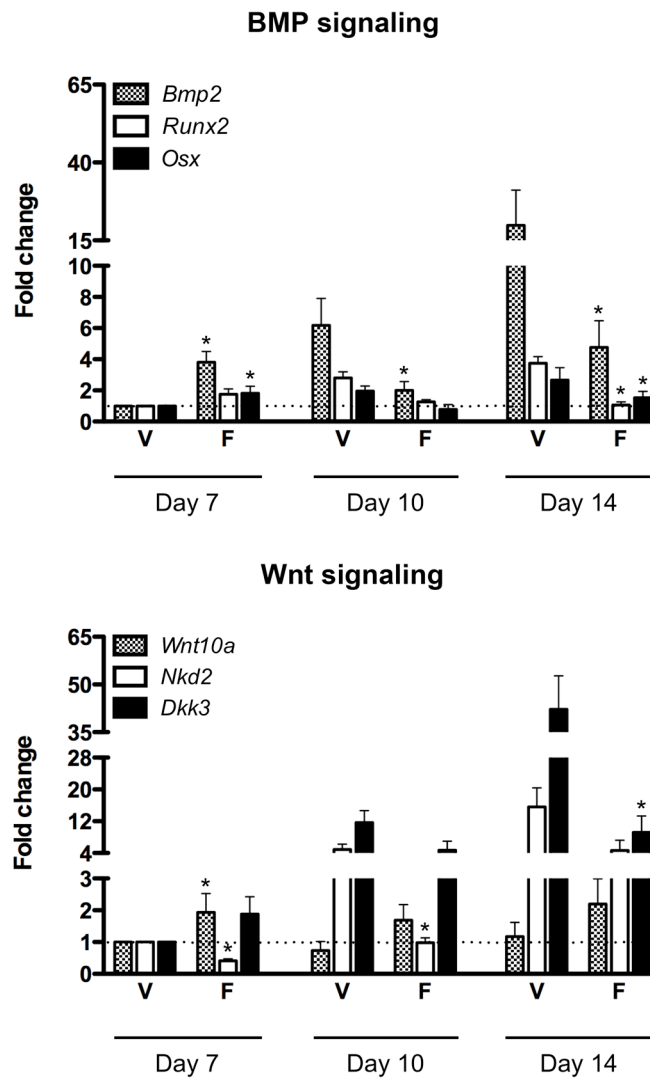
## Acknowledgments

The authors would like to thank Mrs. Barbara Rodgers, Mrs. Trushna Desai and Mr. John Glynn for technical assistance in this project. This work was supported by grant # R01-DE016689 and T90-DE022526.

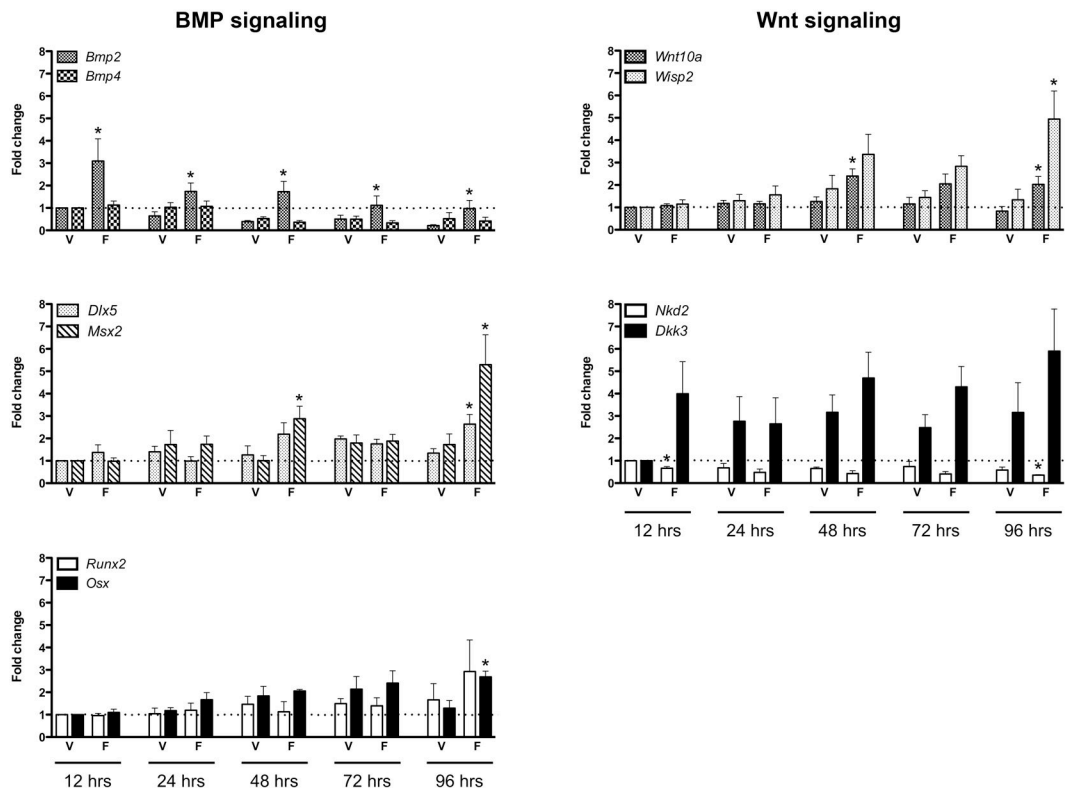
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**Figure 1. Effects of FGF2 on the expression of selected components of the BMP and Wnt signaling pathways during the differentiation/mineralization phase of growth**  
 Expression of all genes was normalized to VH at day 7 that was arbitrarily set to 1 and is indicated by the dashed line. Abbreviations: V: vehicle-treated (control) cultures; F: FGF2-treated cultures.



**Figure 2. Effects of FGF2 on the expression of selected components of the BMP and Wnt signaling pathways during the proliferation phase of growth**

Expression of all genes was normalized to VH at 12 hrs that was arbitrarily set to 1 and is indicated by the dashed line. Abbreviations: V: vehicle-treated (control) cultures; F: FGF2-treated cultures.