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The Role of BMP Signaling in Mediating Stimulatory Effects of FGF-FGFR Signaling on Odontoblast Differentiation in Dental Pulp

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The Role of BMP Signaling in Mediating Stimulatory Effects of FGF-FGFR Signaling on Odontoblast Differentiation in Dental Pulp

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Odontoblast differentiation is dependent on multiple signaling molecules, including growth factors stored in the extracellular dentin matrix. Our previous studies have demonstrated that Fibroblast Growth Factor 2 (FGF2) exerted stage-specific effects on odontoblast differentiation in primary dental pulp cultures. FGF2 stimulated differentiation of functional odontoblasts expressing high levels of *Dmp1* from early progenitors but inhibited the terminal differentiation of functional odontoblasts to fully differentiated odontoblasts expressing high levels of *Dspp*. These stimulatory effects involved activation of FGFR and ERK1/2, and increased levels of expression of *Bmp2*, *Runx2*, and *Osx* (components of BMP signaling) suggesting the involvement of BMP signaling in FGF2-induced *Dspp* expression. Therefore, the overall goal of the studies outlined in this thesis was to examine the role of BMP signaling in stimulatory effects of FGF signaling on odontoblast differentiation of dental pulp cells. We showed that BMP2 did not affect the extent of mineralization but rapidly (within ~12-24 hrs) stimulated expression of *Dspp* and intensity of DSPP-Cerulean transgene in a concentration-dependent manner without affecting the percentage of DSPP-Cerulean^{+} odontoblasts. In contrast to *Dspp*, BMP2 rapidly (within ~24-48 hrs) decreased expression of *Dmp1*, *Bsp*, DMP1-mCherry, and BSP-GFP in a

concentration-dependent manner. Inhibition of the BMP and FGF signaling pathways by noggin and SU5402, respectively, did not have marked effects on the extent of mineralization but decreased expression of *Dspp* stimulated by these growth factors. These inhibitory effects were long-lasting and observed up to 14 days after removal of the growth factors. Additional experiments in primary bone marrow stromal cells cultures demonstrated that early and limited exposure to BMP2 did not affect mineralization but decreased expression of *Dmp1*, *Bsp*, and respective transgenes. Taken together, our results demonstrated that activation of the canonical BMP signaling pathway during the proliferation phase of *in vitro* growth resulted in significant increases in the expression of *Dspp*, and these increases were mediated via reciprocal interaction of the BMP and FGF signaling pathways. These data will further elucidate the perspectives of using both FGF2 and BMP2 in dentin regeneration applications.

CHAPTER I. Introduction

Tooth Development and Odontoblast Differentiation

Odontoblast differentiation during *in vivo* tooth development is a complex and multistep process, involving epithelial-mesenchymal interactions [1,2]. During early stages of dentinogenesis, cells originating from the neural crest migrate toward the paraxial mesenchyme of the first branchial arch, where they contribute to the formation of tooth buds. These neural crest-derived dental papilla cells receive signals from dental epithelium and become *preodontoblasts*. Preodontoblasts undergo the minimum number of cell cycles before they become competent to respond to external stimuli, such as growth factors or trauma, and become *polarized odontoblasts*. In addition to morphological changes, these cells undergo functional changes, as they are the first cells within the odontoblast lineage to secrete Type I collagen (a major component of unmineralized predentin) as well as Types V and VI collagen [1,3].

During further differentiation, polarized odontoblasts differentiate into *functional* (or *secretory*) *odontoblasts*, which in addition to Type I collagen start secreting various non-collagenous components (NCPs) of unmineralized predentin, including members of the SIBLING family, such as BSP, DMP1, and DSPP. Functional odontoblasts eventually become *fully differentiated* (or *mature*) *odontoblasts* secreting mineralized dentin matrix [1,3,4].

Dentin

Dentin is a mineralized tissue resulting from the formation of a specific extracellular

matrix (ECM) by the highly polarized postmitotic odontoblasts located at the periphery of the dental pulp [5]. Dentin is first deposited as a layer of unmineralized matrix called predentin, which lines its innermost (pulpal) portion. Predentin is composed primarily of Type I collagen and is biochemically similar to osteoid in bone. Predentin gradually undergoes mineralization, as various NCPs are incorporated into the mineralization front, forming mineralized dentin matrix [6]. This is a matrix-controlled process, which requires the presence of Type I collagen serving as a scaffold for NCPs [3].

Mature dentin is composed of approximately 70% inorganic (mineral) material, 20% organic material and 10% water by weight (40-45%, 30% and 20-25% by volume, respectively). This makes dentin slightly harder than bone and softer than enamel [3,6].

The organic component consists primarily of Type I collagen, which constitutes approximately 90% of all dentin proteins, and is one of the earliest markers of odontoblast differentiation [3,6]. Type I collagen by itself does not induce mineralization, however, it serves as an organic scaffold to retain various NCPs and accommodate a large proportion (~56%) of the mineral in the holes and pores of the fibrils [6-8]. In addition to Type I collagen, small amounts of Types III and V collagen (~3%) are also present in the dentin of very young animals or during defective collagen synthesis [3]. The importance of the correct collagen structure in dentin is seen in patients with dentinogenesis imperfecta (DGI) type I caused by mutations in the Type I collagen gene, which clinically resembles DGI type II and III caused by *Dspp* mutations [6,9-11].

Inorganic component of dentin is primarily represented by hydroxyapatite (HA)

[$Ca_5(PO_4)_3OH$] [6]. The mineral phase appears first within the matrix vesicles as single crystals seeded by phospholipids of the vesicle membrane. These crystals grow rapidly and later fuse with adjacent crystals to form a continuous layer of the mineralized matrix. Following mineral seeding, NCPs produced by odontoblasts regulate mineral deposition [6].

Dental Pulp

Dental pulp is a connective tissue that resides within the pulp chamber of a tooth. Dentin is an immediate tissue that contacts the dental pulp cells. Due to slow but continuous deposition of physiological secondary dentin, the space occupied by pulp is gradually reduced. Thus, the communication between pulp and periapical tissues occurs only via the apical foramen, which contains blood vessels and nerves [12]. Although dental pulp shares many properties with other connective tissues in the body, its unique localization imposes several special constrains on its development, maintenance and response to injury [13].

Several zones within the pulp can be distinguished based on their location. Subjacent to the layer of odontoblasts is the cell-free zone (also called zone of Weil), which is the area that is relatively free of cells. More deeply, there is a cell-rich zone, which is relatively rich in cells (fibroblasts, undifferentiated mesenchymal cells, immune cells, etc.) and blood capillaries. Due to the presence of undifferentiated cells that are frequently occupying the perivascular area, this zone is presumably the source of cells that will give rise to newly formed odontoblast-like cells after injury or death of primary odontoblasts. Finally, pulp proper (or pulp core) is also rich in cells (fibroblasts, undifferentiated mesenchymal cells, immune cells) and blood vessels [13].

Decreased numbers of pulp cells are observed with aging in humans and rodents [12].

Besides cells and blood vessels, dental pulp contains extracellular matrix, which typically includes various proteins, such as collagen, glycosaminoglycans, proteoglycans, and fibronectin [12,13]. An important feature of dental pulp is the lack of DSP and DPP proteins, suggesting the lack of mature odontoblasts [12].

The considerable capacity of dental pulp for dentin regeneration and repair and identification of stem/progenitor cells in dental pulp capable of giving rise to new odontoblast-like cells make dental pulp cells a valuable model for examining the mechanisms regulating the sequential steps involved in odontoblast differentiation.

Bone Morphogenetic Proteins (BMPs)

Definition and Classification:

First indirect evidence of the existence of BMPs was provided by Urist's rabbit studies in 1965 [14], but it was not until the 1970s that their osteogenic potential was characterized [15,16]. These molecules were called "bone morphogenetic proteins" due to their ability to target a group of cells capable of responding to their osteogenic-inducing effects. To date, BMPs represent the largest subfamily of the transforming growth factor β (TGFβ) superfamily of growth factors. Currently, over 25 distinct BMPs have been identified [17,18], which can be classified into four subfamilies: (i) BMP2 and 4; (ii) BMP5, 6, 7, 8a, and 8b; (iii) BMP9 and 10; and (iv) BMP12,

13, and 14 [19,20]. These proteins are surprisingly conserved among various species, and during 700 million years of evolution, the increased number of components of BMP signaling occurred as a result of genomic duplications or chromosomal translocation [21,22]. Although in more primitive species, such as *Drosophila*, BMPs control dorsoventral body patterning and imaginal disk formation, and mammalian BMP functions are much more diverse, these molecules share at least 75% homology with each other [23].

In humans, genes encoding BMPs are located on several chromosomes. BMP proteins and the respective receptors are expressed during early embryonic development and continue during later organogenesis [24]. These proteins are involved in various physiological events, including regulation of bone formation, kidney and heart development, vasculogenesis, glucose metabolism, development of oocytes and spermatogenesis, and others [17,25]. Therefore, deletion of BMPs, such as *Bmp2* or *Bmp4*, in mice results in embryonic lethality due to multiple defects of germ layer formation and patterning, and *Bmp7* and *Bmp11* knockout mice die shortly after birth due to multiple defects in organ formation [25].

Secretion:

BMPs are synthesized as large (400-500 aa) precursors containing an N-terminal signal peptide directing secretion, a prodomain for proper folding, and a C-terminal mature peptide. These precursors are proteolytically cleaved by serine endoproteases to form active BMP ligands (50-100 aa) containing seven cysteine intramolecular disulfide bonds (cysteine knots). These bonds are essential for dimerization of BMP monomers to form biologically active homo- or

heterodimers (except BMP3, GDF9, and BMP15, which lack the seventh cysteine bond). Secretion of BMPs into the extracellular space can occur either in matrix vesicles or in association with their own ECM fibrils [17].

The Role of BMPs in Bone Formation and Skeletal Development:

The role of BMP signaling in the development of skeletal tissues and bone homeostasis has been demonstrated in various studies [21,26,27]. The role of BMPs in patterning the skull and regulating the skeletogenic fates of neural crest-derived mesenchyme has recently been shown, suggesting a stage-specific regulation of the fate of chondro-osteoprogenitor cells in facial mesenchyme [28]. *In vivo*, expression of *Bmp4* has been detected in differentiating osteoblasts [29], whereas expression of *Bmp1* and *Bmp2* has been localized to mature/differentiated osteoblasts [29,30]. Overexpression of *Bmp4* in osteoblasts under the control of a 2.3-kb fragment of *Col1a1* promoter resulted in increased SMAD signaling and impaired bone formation at least in part due to increased osteoclast formation [31]. Transgenic mice overexpressing *Noggin*, a BMP antagonist [32], under the control of a 1.7-kb fragment of rat *Bglap1* promoter displayed severely impaired bone formation and bone mineral density, and multiple long bone fractures [33]. Overexpression of another BMP antagonist, *Sost*, resulted in reduced bone formation *in vivo* and decreased mineralization in BMSC cultures *in vitro* [34]. In addition, both *Bmp2* and *Bmp4* promoters contain several binding motifs for *Runx2*, a transcription factor essential for bone formation [35]. Interestingly, *Bmp2* is required for osteoblast differentiation in response to WNT signaling. In addition, $Prx1^{+}$ preosteoblasts are the target of BMP2, and it acts to induce these cells via activation of *Runx2* and *Osx* [36].

During long bone fracture healing, expression of BMP2, BMP4, and BMP7 was upregulated, whereas expression of BMP antagonists (PRDC, SOST, SMAD7, GREM1, and CERBERUS) was downregulated [37], suggesting the importance of BMP signaling in the commitment of osteoprogenitor cells to mature and produce mineralized bone matrix. Interestingly, tissue-specific inactivation of *Bmp2* in *Bmp2*$^{c/c}$*;Prx1Cre*$^{-/-}$ mice resulted in minor alterations in skeletal formation, delayed ossification, reduced bone mineral density, and significantly delayed bone fracture healing [38].

In addition, multiple BMPs are expressed in bone metastases [19] and have been implicated in the osteoclast lineage functions [39]. Furthermore, a complex regulation of BMPs during osteogenesis by micro-RNAs, including but not limited to miRNA-133/135, 141/200a, 208/370, and 20a, has been recently demonstrated, suggesting a more complicated regulation of osteogenesis by BMPs than it has been considered in the past [40]. In addition, BMPs mediate their effects on osteogenesis by triggering apoptosis in the limb buds, thus allowing osteoprogenitor cells to populate this area [41].

In vitro studies have demonstrated that continuous exposure of osteoblast cells to BMP2 stimulates their proliferation and differentiation [42,43]. Exposure of adipose-derived stem cells (ADSCs) transfected with the *Bmp2* construct to mineralization-inducing medium significantly increased the extent of mineralization and expression of *Runx2* [44]. Expression levels of *Bmp2* and *Bmp4* were continuously increasing, as the BMSC cultures continued differentiating under mineralization-inducing conditions [45].

However, not all BMPs possess the osteogenic-inducing properties. For example, BMP1 is unrelated to other BMPs, are thought to not regulate osteogenesis and acts as an enzyme to process cleavage of procollagen fibrils and full-length NCPs [46]. Nevertheless, recent case report studies showed that inactivating mutations of *Bmp1* resulted in osteogenesis imperfecta phenotype, suggesting that BMP1 can act as a positive regulator of osteogenesis, possibly though regulation of processing of various ECM proteins [47]. Another member of the BMP family, BMP3, has been shown to act as a negative regulator of bone formation via upregulation of a *Sost* gene and subsequent inhibition of the Wnt signaling pathway [48].

The Role of BMPs in Tooth Development and Odontoblast Differentiation:

The role of BMPs during tooth development has been well documented [20]. BMPs have been implicated in the regulation of tooth number, size, morphology, and differentiation [49-51]. Expression of BMPs has been detected during all stages of tooth development. In the dental epithelium, expression of *Bmp2*, *Bmp4*, and *Bmp7* has been detected as early as E10-12, suggesting that these BMPs can function as mediators of the odontogenic potential from dental epithelium to dental mesenchyme. In the dental mesenchyme and odontoblasts, expression of *Bmp1*, *Bmp2, Bmp3*, and *Bmp7* has been detected at the late bell stage (E16-19 and P1), when predentin and dentin started to be deposited [30,52-57]. Expression of BMP4 has been localized to the dental epithelium, dental mesenchyme, preodontoblasts, and at lower levels to odontoblasts, suggesting that BMP4 can be important during early odontoblast differentiation [55,58]. The E17 dental papillae cultured in the presence of exogenous BMP2 resulted in increased production of

extracellular matrix [59]. Mice overexpressing *Runx2* in odontoblasts under the control of β-catenin promoter impaired odontoblast differentiation, suggesting that BMP signaling can be important in the regulation of odontoblast differentiation by Wnt signaling [60]. Furthermore, deletion of *Bmp2* or *Bmp4* from cells expressing a 3.6-kb fragment of *Col1a1* promoter reduced the rate of dentin formation, impaired dentin architecture, and decreased expression of *Dlx3*, *Dlx5*, and *Osx* transcription factors and *Dmp1* and *Dspp* [61,62]. Tissue-specific inactivation of *Bmp4* in dental mesenchyme in *Bmp4$^{f/f}$;Wnt1Cre* mice resulted in inhibition of SMAD1/5 signaling and delayed/inhibited tooth development [63].

Similar to the bone literature, BMP1 has been thought to play insignificant roles during tooth development and odontoblast differentiation. However, recent studies have demonstrated that inactivating mutations of *Bmp1* in *Col1a1-Cre;Bmp1$^{flox/flox}$;Tll1$^{flox/flox}$* mice resulted in increased predentin thickness, thinner dentin, impaired odontoblast morphology, and decreased expression of *Dspp* [30]. In addition, expression of BMP1 has been localized to odontoblast-like cells *in vitro* and in the areas of reparative osteodentin formation in proximity to carious lesions *in vivo* [64].

In vitro studies have shown that exposure of MD10-F2 immortalized preodontoblasts to BMP2 significantly and rapidly (within 12 hrs) upregulated expression of *Dspp* via the NF-Y signaling pathway [65]. Similarly, rapid (within 6-24 hrs) and concentration-dependent upregulation of *Dspp* by BMP2 has been demonstrated in human dental pulp cells [66]. In addition, upregulation of *Dspp* by BMP2 in MDPC-23 cells involved activation of *Dlx5* and *Runx2* via activation of SMAD1/5 signaling [67]. Continuous exposure of stem cells from exfoliated

deciduous teeth (SHED) [68], dental papilla cells [69] or stem cells of apical papilla (SCAP) [70] to exogenous BMP2 significantly stimulated odontoblast differentiation. Continuous adenovirus-driven overexpression of *Bmp2* [71] or *Bmp7* [72] in human dental pulp cells significantly stimulated odontoblast differentiation. Similarly, three-dimensional chitosan/dental pulp culture transfected with *Bmp7* displayed significantly increased mineralization and expression of markers of odontoblast differentiation [73]. Transfection of SCAP cells with *Bmp2* resulted in fast (within 24 hrs) and significant expression of *Dspp*, *Dmp1*, and *Alp*, and the extent of mineralization [74]. Exposure of integrin $\alpha7^+$ mesenchymal stem cells to BMP4 during the differentiation phase of *in vitro* growth resulted in markedly stimulated odontoblast differentiation [75]. Overexpression of miR-135a, an inhibitor of BMP signaling, in E14 tooth germs has suppressed the expression of BMPR-1A and BMPR-1B and led to impaired tooth formation [76]. Stimulation of odontoblast differentiation by BMP2 occurred via upregulation of both canonical (SMAD1/5/8) and non-canonical (MAPK) signaling pathways, including Erk1/2 [77,78], p38 [79,80] and JNK [81].

Despite multiple reports on the stimulatory effects of BMPs on odontoblast differentiation, the limitations of these studies include a lack of approach to study possible stage-specific effects of BMPs. Most, if not all, of these studies, involve exposure of target cells to exogenous BMP ligands or *Bmp2*-transfected constructs only during the differentiation/mineralization phase of *in vitro* growth. Another limitation of these studies includes the fact that cells are exposed to mineralization-inducing medium with the addition of BMPs immediately or very shortly after the establishment of the culture, thus minimizing examination of the effects of BMPs on odontogenesis before production of the mineralized dentin-like matrix.

BMP Receptors (BMPRs)

Structure:

BMP receptors are transmembrane serine/threonine kinases composed of two subunits, referred to as Types I and II receptors [21,22,82,83]. These proteins are conserved in evolution [84] and contain an extracellular ligand binding domain and an intracellular serine/threonine kinase domain. They are classified based on the presence (type I) and absence (type II) of the glycine-serine (GS)-rich region located between the transmembrane and kinase domains and preceding the kinase domain, and a short region termed L45 loop within its kinase domain [85]. There are four types of Type I receptors, including activin-receptor-like kinase 1 (ALK1), ALK2, BMPR1B (also called ALK3) and BMPR-1B (also called ALK6) and three types of Type II receptors (ActR-II, ActR-IIb, and BMPR-II) [86]. Even though both types of receptors differ substantially in their structure, they share ~78% amino acid homology [20,87,88].

Based on a similarity in their structure and function, Type I receptors can also be classified into BMPR-I (ALK3 and ALK6) and (ii) ALK-1 groups (ALK1 and ALK2). The third group of Type I receptors, TβR-I (ALK4, ActR-IB, ALK5/TβR-I, and ALK7) are not activated by BMPs. These differences lead to the differential expression of these receptors in tissues and their ligand-binding specificity. For example, BMPR-IA and ALK2 are widely expressed in a variety of tissues, whereas BMPR-IB and ALK1 show a much more restrictive pattern of expression [88-90].

Ligand-binding specificity of Type I receptors depends on specific Type II receptor that forms heterotetramer (see details below). For example, BMP2 and BMP4 ligands bind to BMPR-IA and BMPR-IB, whereas BMP6 and BMP7 bind to ALK2 and with a much lower affinity to BMPR-IB. BMP9 and BMP10 bind to ALK1 and ALK2. BMPR-II is specific to BMP ligands, whereas ActR-II and ActR-IIB are shared by activitins, BMPs, and myostatin. When dimerized with BMPR-I, BMPR-II affects the binding specificity of BMP ligands to the receptors [89-91].

A monomeric BMP ligand binds to a tetramer of two Type I and II receptors. The Type II receptor is constitutively active even in the absence of a ligand, whereas Type I receptor has FKBP12, a molecule which is bound to the unphosphorylated Type I receptor and suppresses its activity. Activation of Type I receptor requires ligand binding, ligand-receptor oligomerization, and transphosphorylation of its GS-box via Type II receptor [85]. Phosphorylation of the Type I receptor at the GS domain releases the FKBP12 inhibitor and activates Type I receptor and activates intracellular signaling pathways [20,87].

Activation:

The activation of BMP receptors has been described in details in various review publications [17,21,27,89]. Binding of BMP ligands to their respective BMP receptors (in particular, BMPR-I [91]) triggers a cascade of intracellular events, including canonical (SMAD-dependent) and non-canonical (SMAD-independent) signaling pathways. Activation of the canonical pathway is characterized by phosphorylation of SMAD1, 5, and 8 transcription factors (also

called receptor SMADs, or R-SMADs), which destabilizes interaction with SMAD anchor protein Endofin and facilitates interaction of SMAD1/5/8 with SMAD4 (also called Co-SMAD) in the cytoplasm. This complex then translocates to the nucleus to regulate transcription of target genes. Non-canonical pathway is characterized by activation of various non-SMAD signaling pathways, including MAPK, PI3K/Akt, and others [18,19,25,83,92].

In addition to this, recent studies have demonstrated that integrins are important for modulation (both positive and negative) of BMP signaling [93]. Furthermore, proteoglycans can regulate the activity of BMPs and Noggin by modulation their bioavailability in the ECM, and this regulation has been shown to be important for osteoblast differentiation [94]. Various other diffusible and membrane/matrix-associated proteins have BMP-binding properties [95].

The importance of BMPRs in development has been demonstrated in the literature. Deletion of *Bmpr-1a* or *Alk2* resulted in embryonic death due to defects in multiple organs. Mice lacking *Bmpr-1b* are vital but display multiple defects in appendicular skeleton and retina, whereas mice lacking *Actr-2b* die shortly after birth due to defective cardiovascular system formation [25]. In addition, mice lacking components of SMAD signaling are embryonically lethal due to multiple defects in gastrulation or organ formation (*Smad1*, *Smad4*, *Smad5*, and *Smad7*) or viable but display multiple defects in cardiovascular and pulmonary systems (*Smad6* and *Smad8*) [25].

The Role of BMPRs in Bone Formation and Skeletal Development:

The role of BMPR signaling has been discussed in details in several review publications, and its deregulated activity has been involved in multiple human genetic skeletal disorders [92,96].

Expression of BMPR-IA, BMPR-IB, and BMPR-II is detected at high levels in the bone [97]. Transgenic mice overexpressing a dominant negative form of *Bmpr1a* receptor under the control of 2.3-kb *Col1a1* promoter displayed inhibition of osteoblast SMAD signaling, reduced skeletal formation and impaired bone mineral density *in vivo* and inhibition of osteoblast differentiation *in vitro* [98]. Tissue-specific expression of the truncated *Bmpr-1b* in these mice resulted in impaired skeletal formation, delayed ossification, and inhibition of SMAD1 expression [98]. During fracture healing, expression of BMPR-1A and BMPR-2 was upregulated [37]. Interestingly, tissue-specific deletion of *Bmpr2* in *Prx1*-expressing osteo-chondroprogenitors resulted in unaffected SMAD1/5/8 signaling and increased bone formation and bone mass, suggesting that increased BMP utilization of Type II receptor occurs at the expense of activin signaling [99]. Furthermore, deletion of *Bmpr1a* gene from *Dmp1*-expressing cells resulted in increased proliferation of $Sp7^{+}$ preosteoblasts and increased bone mass via mTORC1 signaling [100].

In vitro studies have shown that expression of both *BmprIa* and *Bmpr2* increased after induction of mineralization in differentiating BMSC cultures [101]. Similarly, expression of *Bmpr1a* and *Bmpr1b* was continuously increasing as the BMSC cultures continued differentiating in mineralization-inducing conditions [45]. Inhibition of *Bmpr1b* by siRNA reduced BMP2-induced expression of markers of mineralization in osteoblast cultures [102]. Overexpression of *Noggin* under the control of the 2.3-kb fragment of *Col1a1* promoter

significantly impaired both bone formation and bone resorption, suggesting that regulation of bone mass by BMP signaling is mediated through modulation of bone remodeling [31]. Inactivation of *Bmpr1a* using *SF3b4* expression vector resulted in reduced osteogenic differentiation of C2C12 mouse myoblast cells [103]. Overall, these results suggest that BMPRs act as positive regulators of bone formation.

The Role of BMPRs in Tooth Development and Odontoblast Differentiation:

Several reports have demonstrated the importance of BMPRs in tooth development. Conditional overexpression of *Noggin* in *K14Cre;pNog* mice resulted in the loss of *Bmp4* and *Msx1* expression in the dental mesenchyme, significant downregulation of p38 and ERK1/2 (but not SMADs), and arrest in tooth development at E12.5, which was rescued by simultaneous activation of the BMP and WNT signaling pathways [104]. Condition deletion of *Smad4* in $Osr2^{Cre}Smad4$ mice resulted in marked inhibition of *Osx* and *Phex* in odontoblasts and impaired dentin formation [105]. Tissue-specific deletion of *Bmpr1a* in the dental epithelium in *K14-Cre;* $Bmpr1a^{cl/cl}$ mice resulted in the arrest in tooth development by E16.5. Interestingly, the same mice also displayed impaired hair follicle development, suggesting the importance of BMPR signaling in tissues, where epithelial-mesenchymal interactions play an essential role in their formation [106]. At all stages of tooth development, expression of *Bmpr1a* in developing teeth has been localized to dental epithelium, dental mesenchyme, and odontoblasts, suggesting that BMP signaling plays a role at various stages of odontoblast differentiation [107]. Furthermore, tissue-specific deletion of *Bmpr1a* in the dental mesenchyme of *Wnt1Cre;Bmpr1a* mice resulted in significantly decreased proliferation of dental mesenchyme cells and arrest in tooth development

at E16.5 [107]. In addition, BMP4-induced expression of *Msx1* in developing teeth required the presence of SMAD1/5/8 [108].

Consistent with the expression of BMP ligands in cells of odontoblast lineage, expression of BMPR-IA has been localized to epithelial and mesenchymal components of developing teeth [109], and expression of BMPR-IB and BMPR-II has been detected in dental mesenchyme and odontoblasts [58,110]. *In vitro* studies have shown that expression of *Alk-2*, *Alk-3*, *Alk-5*, and *Bmpr2* in differentiating dental pulp cultures [111]. Taken together, these observations suggest that BMPRs may play an important role during tooth development and odontoblast differentiation.

The Role of BMPR Signaling in Reparative Dentinogenesis:

The first report in the literature demonstrating the successful application of growth factors for induction of reparative dentinogenesis was by Nakashima in 1990, who demonstrated that crude fraction of BMPs extracted from the canine bone matrix and implanted into the cavity of amputated dental pulp stimulated the formation of tubular-like tertiary dentin in partially amputated pulps of adult canine teeth [112]. Capping of amputated pulp with BMP2 for 2-3 weeks in dogs resulted in the formation of substantial amounts of mixed tubular dentin and osteodentin [113]. The combination of inactivated and demineralized dentin matrix powder with either BMP2 or BMP4 results in stimulation of reparative dentinogenesis in canine teeth [114]. Active formation of reparative dentin is observed in the areas of implantation of scaffolds loaded with BMP7 in exposed pulp cavities in rats or miniature pigs [115-117]. Capping of freshly exposed pulp chambers with BMP7 and collagen powder for 6 weeks in monkeys resulted in the formation of reparative

dentin, which was absent in control (no BMP7) group [118]. Continuous exposure of dental pulp pellet cultures to BMP2 resulted in significant increases in expression of *Dmp1*, *Dspp*, and *Phex*, markers of odontoblast differentiation. Implantation of dental pulp pellet pre-treated with BMP2 into the canine amputated pulp resulted in the formation of osteodentin [119]. Increased expression of BMP2 in regenerative dentin *in vivo* has been observed along with activated SMAD1/5/8 signaling [120,121].

Fibroblast Growth Factors (FGFs)

Description and classification:

FGF ligands are small polypeptides, which have been identified in both invertebrates and vertebrates [122]. In vertebrates, 22 highly conserved members of the FGF family have been currently identified, and they constitute one of the largest and well-studied families of polypeptide growth factors. Most recent studies suggest that no other FGF genes could be identified in the complete human genome sequence [123]. In humans and mice, FGFs are found scattered throughout the genome, however, some of them (FGF3, 4, and 19, or FGF6 and 23) are clustered. The increased number of FGF members occurred during expansion from one of few archeo-FGF2 to eight pro-FGFs and during chromosomal translocation or duplication [122,124,125].

Vertebrate FGFs can be classified into several subfamilies based on their evolutionary, biochemical, and functional properties. Based on the phylogenetic analysis (i.e. evolutionary relationship), all FGFs are grouped into seven evolutionary divergent subfamilies according to

their sequence homology and function: FGF A (FGF1 and 2), FGF B (FGF3, 7, 10, and 22), FGF C (FGF4, 5, and 6), FGF D (FGF8, 17, and 18), FGF E (FGF9, 16, and 20), FGF F (FGF11-14) and FGF G (FGF15, 19, 21, and 23) [123,125-127]. Human FGF15 and mouse FGF19 have not been identified [122,123]. By the mechanisms of their action, all vertebrate FGFs can be classified into canonical, or paracrine, FGFs (FGF1/2/5, FGF3/4/6, FGF7/10/22, FGF8/17/18, and FGF9/16/20), fibroblast homologous factors, or intracrine FGFs, (FHFs, FGF11/12/13/14), and hormone-like, or endocrine, FGFs (hFGFs, FGF15 or 19/21/23) [128,129].

Expression of FGF ligands has been detected in virtually all tissues and organs. However, many of them have a unique pattern of expression and stage of development (embryonic vs. postnatal). For example, some FGFs (FGF3, 4, 8, 15, 17, and 19) are expressed only during embryonic development, whereas others (FGF1, 2, 5-7, 9-14, 16, 18, and 20-23) are expressed during both embryonic and postnatal development [122]. In teeth, expression of FGFs has been detected in cells of dental epithelium, odontoblasts, and cells of the subodontoblastic layer but not in the underlying dental papilla/pulp cells [130-137].

Specific patterns of FGF expression in certain types of tissue (for example, epithelial vs. mesenchymal) could also be due to the interaction with FGF receptors expressed exclusively in a specific type of the tissue. Functions of various FGFs during development and the phenotypes of their loss in vertebrates have been discussed in details in several review papers [123,126,138,139].

Structure:

FGFs contain a partially conserved core of 120–130 amino acids (~17-34 kDa in vertebrates), and in humans, they share ~30-60% homology [140]. In 1991, several independent laboratories revealed three-dimensional structure of FGF2 [141-143]. These studies have demonstrated that FGF2 is composed entirely of the β-trefoil structure that contains four-stranded β-sheets arranged in a pattern with approximate threefold internal symmetry (resembling trigonal pyramid), and linked to each other by hydrogen bonds. Two β-strands (β-strands 10 and 11) contain several basic amino acid residues that form the primary heparin-binding site of FGF2. Other 10 β-strands are involved in interaction with FGF receptors and nuclear translocation [122,144]. Although FGF2 contains four cysteine residues in its amino sequence, biochemical analyses have demonstrated that they do not form intramolecular disulfide bonds [145]. In addition, two inverse arginine-glycine-aspartate (RGD) sequences are identified within FGF2, and they are shown to be important for FGF2-mediated cell adhesion and proliferation [146].

Secretion:

Several review papers have summarized the current knowledge about secretion of various FGF ligands [122,123,147,148]. Most of FGFs are typical growth factors, which are synthesized in the cells and then released into the extracellular space to mediate their biological effects through binding to FGF receptors on the surface of the same (autocrine) and/or neighboring (paracrine) cells. A majority of FGFs (FGFs 3-8, 10, 15, 17-19, and 21-23) contain signal peptides within their N-terminus, which allows them to get secreted through the endoplasmic reticulum (ER)/Golgi and later translocate into the extracellular space, where they function as conventional

growth factors by binding to FGFRs. Although FGF9, 16, and 20 lack the N-terminal signal peptide, they are nevertheless secreted through the ER/Golgi pathway (due to their non-cleaved N-terminal hydrophobic sequence). In contrast, LMW FGF2 (as well as FGF1 and FHFs) does not have signal peptides, and is not secreted. Nevertheless, FGF2 mediates its signaling through binding to the transmembrane FGF receptors, suggesting that FGF2 should be translocated to the extracellular space. For a long time, it has been considered that FGF2 is stored in the cytosol and can be released from the damaged cells. However, more recent studies have demonstrated that FGF2 can also be released from intact cells by unconventional ER/Golgi-independent mechanisms. Possible mechanisms of FGF2 secretion into the extracellular space have been discussed in details in these review papers [149,150].

The Role of FGFs in Bone Formation and Skeletal Development:

The important roles of FGF ligands have been well documented in the literature [151,152]. Global knockout of *Fgf2* results in a significant reduction in bone formation *in vivo* and *in vitro*, and decreased expression of markers of mineralization (*Type I collagen*, *Osteocalcin*) in cultured BMSCs *in vitro* [153-155]. These decreases are in part due to the decreased number of osteoprogenitors [153]. In addition, *Fgf2*$^{-/-}$ mice display significantly impaired anabolic effects of PTH on osteoblast proliferation, differentiation and apoptosis [156,157]. BMSCs isolated from *Fgf2*$^{-/-}$ mice have a substantially decreased capacity to form mineralized nodules *in vitro*, and these decreases are completely reversed by exogenous FGF2 [158].

Transgenic mice constitutively overexpressing human *Fgf2*, which encodes all FGF2

isoforms, display various skeletal alterations, including dwarfism, shortening of long bones (~20-30% reduction), severe chondrodysplasia [159,160], markedly decreased proliferation of the growth plate chondrocytes, and increased apoptosis of chondrocytes and calvarial osteoblasts [160]. Further studies have demonstrated that these mice have significantly decreased bone formation *in vivo* and mineralization in BMSC cultures *in vitro* [161]. However, transgenic mice overexpressing *Fgf2* under the control of the Col3.6 promoter (Col3.6–18-kDa FGF2-IRES-GFPsaph mice) exhibit significantly increased bone formation *in vivo* [155]. BMSC cultures derived from transgenic mice overexpressing high molecular weight (HMW) *Fgf2* under the control of the 3.6-kb *Col1a1* promoter (Col3.6-HMWFgf2) display accelerated tibial fracture healing *in vivo* [162], increased osteogenic differentiation during early stages of their differentiation (1 week), however display significantly decreased osteogenic differentiation during later stages of the culture (2-3 weeks) [163]. Inhibition of FGFR signaling by SU5402 at these later time points significantly increases the extent of mineralization and levels of expression of markers of mineralization [163].

Multiple studies have demonstrated the stimulatory effects of FGF2 on osteoblasts during the proliferation phase of their growth (prior to induction of differentiation). Exposure of primary osteoblast cultures to FGF2 markedly increases the extent of mineralization [164] and expression of markers of mineralization [164,165]. Exposure of human BMSCs to FGF2 markedly increases ALP activity [166]. Exposure of human BMSCs to FGF2 markedly increases the extent of mineralized tissue *in vitro* and after their implantation on the back of the mice *in vivo* [167]. Early and transient (between days 1-2) exposure of MC3T3-E1 cells to FGF2 results in a slightly increased formation of mineralized tissue [168].

Various studies using primary BMSCs or osteoblast cell lines have demonstrated rapid stimulatory effects of FGF2 on the expression of markers of mineralization. Exposure of rat ROS17/2.8 osteoblast-like cells to FGF2 induces rapid upregulation of *Bsp* mRNA and RUNX2 protein via activation of the Erk1/2/AP-1 signaling pathway [169]. Exposure of MC-4 osteoblast-like cells or BMSCs to FGF2 markedly increases expression levels of *Dmp1* and other osteocyte-associated markers (*E11*, *Cx43*, *Phex*, *Sost*), but decreases the expression of *Alp* and ALP activity [170]. Exposure of MLO-Y4 osteocyte-like cells to FGF2 rapidly (within ~8 hrs after exposure) stimulates expression of *Dmp1* and other osteoblast/osteocyte-associated genes (*Mgp*, *Slc20a1*) [171]. FGF2 rapidly (within ~12 hrs after exposure) increases expression of *Osteocalcin* mRNA in MC-4 osteoblast-like cells [172].

These stimulatory effects of FGF2 on osteogenic differentiation are mediated, at least in part, by the enrichment of these cultures with osteoprogenitors prior to induction of their osteogenic differentiation. Stimulatory effects of FGF2 on the proliferation of osteoprogenitor cells have been demonstrated in multiple studies [167,173-179]. In addition, several studies have demonstrated anti-apoptotic effects of FGF2 on osteoprogenitor cells [180,181], suggesting that promoting of cell survival could also be an important mechanism of the enrichment of cultures with osteoprogenitors.

These results correlate with the high levels of *Fgf2* expression in the mesenchymal progenitor cells of the developing mouse calvaria and low levels of expression in the calvarial bone [182]. This suggests that FGF2 may be required for commitment of undifferentiated progenitors towards the osteoblast lineage. However, its downregulation is required for

maturation of osteoblasts.

FGF Receptors (FGFRs)

Description:

Paracrine FGF ligands trigger cascades of intracellular events regulating various cell functions through interaction with transmembrane FGF receptors [148]. To date, four FGFR transmembrane tyrosine kinase receptors (FGFR1, FGFR2, FGFR3, and FGFR4), which share a high degree of homology, have been identified in humans and mice [148,183-185]. A fifth related receptor, FGFR5 (also known as FGFRL1), is soluble and although it can bind FGFs, it has no tyrosine kinase domain [186-188].

The full-length FGFRs (~800 aa), like other typical tyrosine kinase receptors, consist of an extracellular ligand-biding domain, a single-pass transmembrane domain, and a split intracellular tyrosine kinase domain. The extracellular region consists of a signal peptide and three immunoglobulin-like domains (IgI, IgII, IgIII). A unique feature of FGFRs is the presence of an acidic, serine-rich domain in the linker between IgI and IgII, termed the acid box (AB) domain. Cysteine residues present within each Ig-like domain form an intramolecular disulfide bond to maintain the tertiary structure of the receptor [126]. Ig-like domains play various but distinct roles during interactions with FGF ligands. The IgI and AB domains are thought to play a role in receptor autoinhibition, whereas the IgII and IgIII domains are necessary and sufficient

for ligand binding and specificity [126].

The diversity of FGF receptors is enhanced by the existence of multiple alternative splice sites that can result in the generation of numerous isoforms. Alternative mRNA splicing of the second half of the IgIII domain of *Fgfr1-Fgfr3* genes specifies the sequence of the C-terminus of IgIII domain, resulting in either the IIIb or the IIIc isoform of the FGFR with different ligand-receptor binding specificity. In contrast, no alternative mRNA splicing of *Fgfr4* gene has been demonstrated [126], however, two *Fgfr4* isoforms resulted from alternative splicing of intron 17 have been described [189]. Thus, in vertebrates, seven FGFR proteins (FGFR1IIIb, FGFR1IIIc, FGFR2IIIb, FGFR2IIIc, FGFR3IIIb, FGFR3IIIc, and FGFR4) differing in their ligand-binding specificity are generated from four *Fgfr* genes [126,185].

High affinity of FGF ligands to specific FGF receptors suggests that different splicing isoforms of FGFRs may have a tissue-specific pattern of expression and/or developmental stage-specific functions. For example, *Fgfr1* is expressed predominantly in cells and tissues of the mesenchymal origin, whereas *Fgfr2* is expressed predominantly in tissues of the ectodermal origin [190,191]. Expression of *Fgfr3* is localized to the epithelium of the neural tube during early mouse development and later is detected in various regions of the brain and central nervous system and at very high levels in the cartilage rudiments of developing bone [192]. Expression of *Fgfr4* is detected in the endoderm of the developing gut at E14.5 of mouse development [193].

FGF ligands produced in either epithelial or mesenchymal tissue activate receptors of the opposite tissue specificity, thus resulting in directional epithelial-mesenchymal signaling [126,194-

[196]. For example, FGF2, 4, 5, 6, 8, 9, 17, 19, and 20 bind to the IIIc isoform with a much higher affinity as compared to the IIIb isoform [194,197]. In contrast, FGF3, 7, 10 and 22 bind to the IIIb isoform with a much higher affinity as compared to the IIIc isoform [194,197]. Although almost all FGF ligands bind to epithelial or mesenchymal FGFRs with different affinities, certain ligands (such as FGF1) bind to both IIIb and IIIc isoforms with the same affinity [126,194,197]. Although splicing of the extracellular domain controls ligand specificity, no evidence that this affects intracellular signaling exists [198]. For a summary of FGFR isoforms binding specific FGF ligands, please read a review by Saichaemchan *et al*. [199].

Activation:

Binding of FGFs to the FGFRs results in their dimerization and autophosphorylation, and triggers a cascade of intracellular events, including recruitment of docking and signaling proteins at the plasma membrane [139,185]. Upon binding to FGF ligands, FGF receptors undergo a series of changes leading to their activation. After binding of an FGF ligand and HSPGs, FGF receptors undergo dimerization leading to conformational changes in their structure, activation of the intracellular kinase domain, and subsequent phosphorylation of the tyrosine kinase domains and intracellular C-terminus [198,200]. Interestingly, recent studies have also demonstrated that FGFRs can form phosphorylated dimers even in the absence of FGF ligands [201]. The complexity of interactions between FGF ligands and receptors has allowed identification of novel regulators and the design of multiple inhibitors of FGF/FGFR signaling [199,202-204].

Interactions with heparin or heparan sulfate proteoglycans (HSPGs):

An important feature of paracrine FGFs is the interaction between FGFs and heparin or heparan sulfate proteoglycans (HSPGs) [205]. It has been established that heparin is required for FGF ligands to effectively activate FGFR in cells deficient in HSPGs or unable to synthesize them, or in cells pretreated with heparin/HSPG-degrading enzymes. The importance of HSPGs for FGF/FGFR signaling has first been demonstrated by studies, which show that mutations of *Sgl* and *Sfl*, genes essential for biosynthesis and modification of HSPGs, greatly decrease the ability of FGF signaling to activate MAPK [206]. These and other studies have demonstrated that a unique feature of FGF-FGFR interactions is that FGFs are unable to activate FGFRs without the cooperation of HSPGs [207]. The identification of heparin/HSPGs as an active and essential component of FGF/FGFR signaling complex suggests that FGF activity and specificity can be modulated not only at the transcriptional and translational levels, but also at the level of "bioavailability" [144]. Thus, HSPGs control a gradient of FGF distribution in the tissues and act as co-receptors of FGF signaling [208].

The role of FGFRs in Bone Formation and Skeletal Development:

Important roles of FGFRs in skeletal formation have been revealed in various congenital craniofacial and skeletal disorders [209]. Expression of *Fgfrs* has been localized to the mesenchymal cells of limb buds and cranial sutures, hypertrophic chondrocytes, osteoblasts, and osteocytes [210-214], suggesting that FGFR signaling plays an important role in bone formation [215]. This has been further demonstrated in multiple human and animal studies, where deregulated activities of FGFRs lead to the development of various skeletal syndromes. Inactivating mutations of *Fgfr1* lead to Pfeiffer syndrome, Kallmann syndrome, and osteoglophonic dysplasia

[216-219]. Similarly, animal models have shown that FGFR1 acts as a positive regulator of bone formation [220-222]. Similar to those of *Fgfr1*, inactivating mutations of *Fgfr2* lead to various skeletal abnormalities, including Apert syndrome [223,224], Crouzon syndrome [225,226], Pfeiffer syndrome [217,227], Jackson-Weiss syndrome [226], bent bone dysplasia [228], and others. Animal models have further shown that FGFR2 acts as a positive regulator of bone formation [221,229,230].

In contrast to FGFR1 and FGFR2, mutations of FGFR3 resulted only in few human craniofacial and skeletal disorders, including hypochondria, achondroplasia, and thanatophoric dysplasia [231]. Deletion of *Fgfr3* from cells of osteoclast lineage in *Fgfr$^{-f/f}$;Lysozyme-Cre* mice resulted in increased skeletal mass due to impaired osteoclast-mediated bone resorption [232]. At least in part, the negative regulation of bone mass by FGFR3 can be mediated through increased degradation of BMPR-1a [233].

The current role of FGFR4 in craniofacial and skeletal bone formation is currently not clear due to a limited number of studies. The expression of FGFR4 has been detected at high levels in the periosteal and endosteal osteoblasts *in vivo* and within or in proximity to mineralized nodules *in vitro* [214].

The Role of FGFR Signaling in Tooth Development and Odontoblast Differentiation:

The role of FGF/FGFR signaling in tooth development has been summarized by Li *et al*. [234]. Expression of *Fgf2* during mouse embryonic development is localized to the dental mesenchyme and dental papilla cells [133]. In the developing mouse root structures, FGF2 is

localized to the differentiating odontoblasts [132]. Intense expression of the FGF2 protein is observed in the layer of differentiating and mature odontoblasts, dentin matrix, and dental papilla cells immediately below the odontoblast layer [131,136]. These results indicate that FGF2 may be important during various stages of odontoblast differentiation, especially in early odontoprogenitors.

Previous studies have demonstrated a specific pattern of expression of FGFRs in cells at early and more advanced stages of their differentiation. Similar to cells of the osteoblast lineage, expression of *Fgfr1c* increases from undifferentiated dental pulp cells and preodontoblasts to postmitotic secretory/functional odontoblasts, however, *Fgfr2c* is expressed in dental follicle but not in odontoblasts [135]. Expression of *Fgfr3* is primarily localized to the dental papilla cells but not to more mature cells [135]. Overall, these studies demonstrate a differential pattern of FGFR1 and FGFR2 expression during odontoblast differentiation *in vivo* and suggest that they act as important regulators of intracellular signaling in undifferentiated cells and cells committed to the odontoblast lineage.

In contrast to multiple studies on the effects of *Fgf2* deletion or overexpression on bone formation, very little information is available on dentinogenesis, as most of the studies have examined the tissue-specific deletions of *Fgf2* and/or *Fgfrs* only in the dental epithelium. Conditional inactivation of *Fgfr1* under the control of a Keratin 14 promoter (*K14-Cre;Fgfr1fl/fl* mice) leads to abnormal ameloblast structure and enamel defects in both molars and incisors by 8 weeks of age, whereas odontoblasts appear to be unaffected [235]. Tissue-specific inactivation of *Fgfr2* in the epithelium results in markedly decreased levels of DMP1 and DSP

proteins [236]. Conditional inactivation of *Fgfr2IIIb* in the dental epithelium using *Nkx3.1-Cre* results in the defects in enamel, impaired odontoblast organization, and reduced expression levels of *Dmp1* and *Dspp* [236]. Injections of BMS-645737, an inhibitor of both FGFR and VEGFR2, lead to degeneration and necrosis of odontoblasts and formation of thinner dentin in rat incisors [237].

Transgenic mice expressing a dominant-negative chimeric FGFR2 protein (dnFGFR-HFc mice) display a lack of tooth buds by E18.5 [238], whereas homozygous mice lacking the *IIIb*-specific isoform of *Fgfr2* (*Fgfr2IIIb*$^{-/-}$ mice) display an arrest in tooth development beyond the bud stage (E13.5) [239]. Targeted inactivation of the *IIIb*-specific isoform of *Fgfr2* in *Fgfr2IIIb*$^{-/-}$ ^{lacZ} mice display tooth agenesis by E16.5 [240]. Specific deletion of *Fgfr2* in the dental epithelium in *K14-Cre;Fgfr2*$^{fl/fl}$ mice results in a delay in tooth formation at the bud stage, most likely due to the decreased proliferation of dental epithelium cells [241]. Similarly, targeted deletion of *Fgfr2IIIb* from the mouse germline using *Cre* recombinase results in the arrest of tooth development at the bud stage [239].

Overall, these results have demonstrated that FGFRs are important for tooth development. However, the exact role of FGF/FGFR signaling in the regulation of odontoblast differentiation during tooth development is yet to be further investigated.

The Role of FGFR Signaling in Reparative Dentinogenesis:

Both dentin matrix and dental pulp cells contain FGF2, and its large amounts can be

released from the dental pulp fibroblasts or endothelial cells upon their injury [242-245].

Beads loaded with FGF2 led to the formation of numerous groups of polarized cells without deposition of the mineralized matrix [246]. In other studies, the formation of the mineralized tissue in the area of pulp exposure in response to FGF2 has been observed. However, this mineralized tissue does not contain dentinal tubules, suggesting the formation of osteodentin [247]. More recent studies have expanded our understanding of the role of FGF2 in dentin regeneration. Using FGF2-carrying gelatin hydrogel microspheres and collagen sponges as a scaffold, Kikuchi *et al*. demonstrated the formation of the DSP^{+} mineralized tissue in the area of pulp exposure 21 days after surgery [248]. However, authors conclude that the regenerated dentin-like tissue is a porous aggregate composed of dentin-like particles, suggesting the formation of a tissue other than physiological tertiary dentin, which is non-porous. Using the same model, formation of $DMP1^{+}/Nestin^{-}$ osteodentin on the surface of the regenerated pulp by FGF2 has been demonstrated, whereas no effect is observed at lower concentrations [249]. Overall, these observations suggest that a controlled release of FGF2 can induce the formation of reparative dentin *in vivo*, however further studies are needed to optimize the concentrations of FGF2, duration of exposure and scaffold materials for a more controlled release of FGF2.

Dental pulp cells isolated from different species display marked increases in proliferation upon treatment with FGF2, suggesting that it stimulated odontoprogenitor cells [250-254]. Similarly, FGF2-soaked beads markedly increase cell proliferation of E11 dental mesenchyme *ex vivo* [135]. Concentration-dependent increases in cell proliferation in response to FGF2 have also been demonstrated [244,255].

Rat incisor pulp cells cultured on Type I collagen-coated gel culture exhibit a pronounced increase in the expression of *Dspp* [254]. Continuous exposure of hTERT-immortalized human pulp cells to FGF2 between days 0-14 stimulates their dentinogenic differentiation and increases expression of *Dmp1* and *Dspp* [256].

In addition, some *ex vivo* studies suggest that FGF2 in a combination with other growth factors can be involved in regulation of odontoblast polarization and functional activity. The combination of FGF2 and BMP4 potentiates FGF2-induced increases in the expression of *Dspp* [254]. Cultured mouse dental papillae (E17 bell stage) exposed to either FGF2 or TGFβ1 for 6 days do not exhibit visible morphological changes or predentin formation. However, a combination of these growth factors induces cell polarization and leads to intense secretion of extracellular matrix, suggesting stimulation of functional differentiation of odontoblasts [257,258].

In contrast to the positive effects described above, several studies have demonstrated the negative effects of FGF2 on dentinogenesis. Continuous exposure of dental pulp cells isolated from various species to FGF2 increases proliferation and significantly decreases mineralization, ALP activity, and expression of various markers of dentinogenesis, including *Dspp* [244,250-253,255,259]. Interestingly, in contrast to immortalized pulp cells continuously exposed to 50 ng/ml FGF2 and exhibited reduced dentinogenic differentiation, lower concentrations of FGF2 (1-10 ng/ml) positively regulate expression of *Dmp1* and *Dspp* [256], further raising a possibility of concentration-dependent effects of FGF2 on dentinogenesis.

Although extended exposure of differentiating human dental pulp cells to FGF2 greatly decreases the extent of mineralization, withdrawal of FGF2 almost completely reverses this inhibitory effect [244]. This suggests that cells continuously exposed to FGF2 do not de-differentiate or die, but rather retain their dentinogenic potential and are capable of differentiating into mature odontoblasts upon withdrawal of FGF2.

Mouse tooth germs (E17 bell stage) exposed to FGF2 for 4 days display marked decreases in dentin formation and expression of *Dspp* and *Alp*, whereas *Fgf2*-specific antisense oligodeoxynucleotides exert an opposite effect. Interestingly, authors note that the same treatment of the tooth germs isolated at the earlier stage of their development (E15 cap stage) results in some stimulatory effects on expression of *Dspp* and *Alp*. This may suggest that FGF2 stimulates dentinogenic differentiation of less mature odontoblasts and inhibits dentinogenic differentiation of more mature odontoblasts [260].

Overall, these results suggest that FGF2 exerts both positive and negative effects on dentinogenic differentiation of dental pulp cells. It is important to note that cells continuously exposed to FGF2 remain capable of differentiating into mature odontoblasts, suggesting that FGF2 prevents terminal differentiation of odontoprogenitor cells. However, studies demonstrating stage-specific effects of FGF2 on odontoprogenitor cells have not been reported.

Interactions Between the BMPR and FGFR Signaling Pathways

Interactions between the FGF and BMP signaling pathways during skeletal development

have been well documented [261]. In cultured chick calvarial osteoblasts, expression of *Bmp2* was markedly upregulated by FGF2 and FGF9 [221]. *Fgf2*$^{-/-}$ transgenic mice displayed markedly reduced levels of *Bmp2* in neonatal calvarial osteoblasts, the decreased ability of exogenous BMP2 to induce mineralization in osteoblast cultures *in vitro* [262], and markedly reduced SMAD1/5/8 signaling [263], suggesting a role of endogenous FGF2 in regulating BMP2 expression. *Ex vivo* exposure of fetal calvarial sutures to FGF18-soaked beads resulted in increased expression of *Fgfr1*, *Fgfr2*, accelerated osteogenesis, and increased expression of markers of mineralization that occurred through upregulation of BMP2 [264]. Exposure of cultured rat calvarial osteoblasts to FGF2 or gain-of-function mutation of *Fgfr2* resulted in marked inhibition of BMP inhibitor *Noggin* leading to a phenotype similar to that of human craniosynostosis [265]. On the other hand, dominant-negative *Fgfr2* had a stimulatory effect on noggin [265] and markedly reduced the expression of *Bmp2* [221,265,266]. Exposure of murine calvarial organ culture to FGF2 resulted in marked increases in the expression of *Bmp2* that was mediated though *Runx2* upregulation [267]. Exposure of cultured undifferentiated mesenchymal cells from calvarial sutures with FGF2 resulted in significant increase in the expression of *Bmp2* [268]. In addition, BMP2-induced expression of *Runx2* and *Osx* in osteoblasts was inhibited by ablation of *Fgfr1*, suggesting that FGF signaling is important in mediating stimulatory effects of BMPs on its downstream targets [269]. Implantation of beads loaded with FGF18 into the coronal sutures stimulated osteogenesis and markedly increased expression of *Bmp2*, and noggin reversed these effects [264] FGF2 antagonized retinoic acid-mediated upregulation of *Bmpr-1b* [270], and constitutive activation of *Bmpr-1b* restored FGF2-mediated inhibition of mineralization [271].

Similarly, several knockout studies have demonstrated the ability of the BMP and FGF

signaling pathways to modulate the expression of each other and other components of the respective signaling pathways during tooth development *in vivo* (for review, please see Balic and Thesleff [2]). The interplay between the FGF and BMP signaling pathways is important for proper tooth development and morphology [51]. Both signaling pathways are necessary for activation of *Pax9* and *Msx1* transcription factors, downstream targets of BMP signaling, in the presumptive dental mesenchyme of developing teeth. Expression of *Fgf3* in dental mesenchyme is not detected in *Msx1* knockout mice during very early stages of tooth development, and even though the teeth in these mice develop normally, these data suggest that *Msx1* is necessary for expression of mesenchymal *Fgf3* [272]. Overall, these data suggest that both the BMP and FGF signaling pathways are essential for reciprocal interactions between the epithelium and mesenchyme during early tooth development.

Exposure of murine dental pulp cultures to FGF2 significantly increases the expression of *Runx2* and *Bmp2* during the proliferation phase of *in vitro* growth [273]. The stimulatory effects of FGF2 on *Bmp2* expression occur within 12-24 hrs, suggesting a direct stimulatory effect [274]. Furthermore, inhibition of BMP signaling by noggin markedly decreases or inhibits FGF2-mediated increases in *Dmp1* and *Dspp* [274]. These interactions between the FGF and BMP signaling pathways are shown to be mediated by various intracellular mediators, including Erk1/2 [275], *Runx2* [262,264,267], and *Foxc1* [276].

A number of studies have utilized the approach of controlled and stage-specific exposure of target cells/tissues to both FGF2 and BMP2. The rationale behind this approach is a proliferative capacity of FGF2 and a differentiation potential of BMP2, suggesting that early

exposure of target cells to FGF2 would increase the number of progenitors exposed to the differentiation-inducing potential of BMP2 [277]. In addition, this approach has been used to induce the differentiation potential of osteoblasts derived from ageing mice and characterized by a decreased capacity to proliferate and produce mineralized bone-like matrix [278]. Interestingly, the expression of FGF2 (and subsequent expression of β-catenin, a downstream target of Wnt signaling) in human bone cells significantly decreases with age, suggesting that the activity of these pathways is important for maintaining a bone-forming capacity of osteoblast progenitor cells [279]. This approach has created a foundation for the development of multilayer biomimetic coatings for sequential delivery of various growth factors, including FGF2 and BMP2 [280-282].

Considering important roles of BMP2 and FGF2, advances in basic science research make these growth factors suitable various dental clinical applications [283-286 287,288].

CHAPTER II. Specific Aims

Specific Aim #1: To Examine the Effects of Early and Limited Exposure to BMP2 on Odontoblast and Osteoblast Differentiation.

A. Examine the effects of early and limited exposure to BMP2 on mineralization, expression of markers of mineralization and dentinogenesis, and expression of various transgenes (BSP-GFP, DMP1-mCherry, and DSPP-Cerulean) in primary dental pulp cultures.

Specific Aim #2: To Examine the Interplay Between the BMP and FGF Signaling Pathways on Odontoblast Differentiation in Primary Dental Pulp Cultures.

A. Examine the effects of SU5402, a specific inhibitor of FGF/FGFR signaling, on changes induced in dental pulp by early and limited exposure to BMP2.

B. Examine the effects of noggin, a specific inhibitor of BMP/BMPR signaling, on changes induced in dental pulp by early and limited exposure to FGF2.

CHAPTER III. Materials and Methods

Primary dental pulp cultures.

The coronal portions of the pulps from first and second molars were isolated from 5-7-day-old hemizygous BSP-GFP, DMP1-mCherry, DSPP-Cerulean, and non-transgenic pups, as described previously [289]. All mice were maintained in the CD1 background. After isolation, 8.75×10^4 cells/cm^2 were grown first in Dulbecco's modified Eagle's medium (DMEM), 20% fetal bovine serum (FBS), 2 mM L-glutamine, 40 U/ml penicillin, 40 μg/ml streptomycin, and 0.1 μg/ml Fungizone (Invitrogen). Three days later, the medium was changed to DMEM containing 5% FBS. At day 7, mineralization was induced by addition of medium containing Minimum Essential Medium alpha (αMEM), 5% FBS, with 50 μg/ml fresh ascorbic acid and 4 mM β-glycerophosphate. The medium was changed every other day.

Primary bone marrow stromal cell (BMSC) cultures.

BMSCs were prepared from femurs and tibiae of 5-7-day-old pups as described before [289]. Briefly, single cell suspension was prepared from flushed marrows, plated at a density of 6.5×10^5 cells/cm^2 and grown in αMEM containing 10% FBS, 40 U/ml penicillin, and 40 μg/ml streptomycin. Three days later, the medium was changed to the medium containing αMEM and 5% FBS. At day 7, when the cells became confluent, the medium was switched to the mineralization-inducing medium containing αMEM, 5% FBS, with 50 μg/ml fresh ascorbic acid and 4 mM β-glycerophosphate. The medium was changed every other day.

Treatment of primary cultures with growth factors.

To examine the effects of BMP2 and FGF2, human recombinant BMP2 (catalog #355-BM-010/CF., R&D systems, Inc., Minneapolis, MN) and low molecular weight (18 kDa) bovine FGF2 (catalog #133-FB, R&D systems, Inc., Minneapolis, MN) were used. The growth factors were added to the cultures with fresh culture medium every other day during either the proliferation phase of *in vitro* growth (between days 3-7, referred to as early and limited exposure) or during the differentiation/mineralization phase of *in vitro* growth (between days 7-21, referred to as late and limited exposure). For experiments involved BMP2 and FGF2, 4mM HCl and 0.1% BSA fraction V in PBS, respectively, were used as vehicle controls.

Inhibition of signaling pathways.

The FGFR inhibitor SU5402 [290,291] (catalog #sc-204308, Santa Cruz Biotechnology, Santa Cruz, CA) was dissolved in DMSO (5 μM stock solution) and added to the cultures at various concentrations (5, 10 and 20 μM). The BMP/BMPR inhibitor noggin [292-294] (catalog #250-38, PeproTech, Rocky Hill, NJ) was dissolved in 0.1% BSA fraction V in PBS (50 μg/ml, or 1.08 pM, stock solution), and added to the cultures at final concentrations of 100, 200, and 300 ng/ml (2.16, 4.32, and 6.47 pM, respectively).

Detection and quantification of mineralization in primary cultures.

Mineralization in live cultures was examined and quantified by Xylenol Orange (XO) staining, as described previously [289]. The mean epifluorescence intensity of XO staining was measured using a multidetection monochromator microplate reader (Safire2, Tecan, Research Triangle Park, NC), as described previously [289]. Fluorometric measurements were performed at 570/610 nm wavelength (excitation/emission) and gain of 80. The entire area of each well was

read at a scan density of 6×6 regions (a high sensitivity flash mode). Background fluorescence for XO was measured using unstained dental pulp cultures at the identical time points and subtracted from respective XO measurements.

Immunocytochemistry.

Pulp cells derived from the DSPP-Cerulean transgenic mice were treated with VH or BMP2 and processed for immunocytochemistry, as described previously [289]. Cells were fixed with 3.7% formaldehyde in PBS for 4 minutes at room temperature (RT), incubated with 0.5% Triton X in PBS for 10 minutes at RT, and blocked with 3% milk for 1 hr at RT. For detection of DSPP-Cerulean, cultures were incubated with 1:1000 dilution of anti-GFP Alexa Fluor 488 conjugated antibody (Invitrogen) in 0.3% Triton X in PBS overnight at +4°C. In these cultures, the anti-GFP antibody binds specifically to the Cerulean fluorescent protein to enhance its visualization.

The percentage of DSPP-Cerulean$^+$ odontoblasts in cultures was calculated as the ratio of cells stained with the anti-GFP antibody (DSPP-Cerulean$^+$ odontoblasts) to the total number of Hoechst$^+$ cells. Approximately $20x10^3$ Hoechst$^+$ nuclei were counted from 20-60 different representative areas of the culture. Negative controls included (i) primary BMSC cultures derived from DSPP-Cerulean littermates and stained with anti-GFP antibody and (ii) primary dental pulp cultures derived from DSPP-Cerulean littermates without the addition of anti-GFP antibody.

For detection of phospho-SMAD1/5/8, cultures were established as described above. At

day 3, the culture medium was switched to serum-free DMEM, and cells were incubated for 5 hrs. After that, serum-free medium was replaced with the fresh medium containing BMP2, FGF2, or BMP4 for 1 hr. After that, the cells were incubated with 1:200 dilution of rabbit anti-mouse phospho-SMAD1/5/8 primary antibody (Cell Signaling, Boston, MA). All samples were then incubated with 1:400 secondary Alexa Fluor 488 goat anti-rabbit antibody (Invitrogen) for 1 hr at RT. The nuclei were stained with 1.0 μg/ml Hoechst 33342 dye (Molecular Probes, Invitrogen) for 15 minutes at RT. After staining, coverslips were mounted using Dako Fluorescent Mounting Medium (Dako North America, Inc., Carpinteria, CA) and visualized under the microscope.

Detection and quantification of mineralization in primary cultures.

GFP expression in cell cultures at various time points was examined using Zeiss AxioObserver Z.1 microscope equipped with the AxioCam MRc digital camera and appropriate filters. Exposure times were adjusted for optimum imaging and kept consistent for each time point of the culture. Panoramic images of larger areas of the cultures were obtained using a computer-controlled motorized imaging workstation and Zeiss AxioObserver Z.1 microscope.

Epifluorescence intensity of GFP.

The mean epifluorescence intensity of BSP-GFP transgene in each well was measured as described for XO staining. The fluorometric measurement was performed at 500/540 nm wavelength and gain 80. Background fluorescence for GFP was measured using dental pulp cultures from non-transgenic littermates, and these values were subtracted from respective GFP measurements.

Fluorometric measurements were also obtained in DSPP-Cerulean cultures stained with the anti-GFP antibody (500/540 nm wavelength and gain 80) and Hoechst 33342 dye (343/483 nm wavelength and gain 70). Background fluorescence for GFP was measured using pulp cultures from non-transgenic littermates stained with the anti-GFP antibody, and these values were subtracted from respective GFP measurements.

RNA extraction and quantitative PCR (qPCR) analysis.

Total RNA was isolated using TRIzol reagent (Life Technologies, Carlsbad, CA) according to the manufacturer's protocol and treated with RNase-free DNase to eliminate genomic DNA. Isolated RNA was reverse transcribed by Superscript II Reverse Transcriptase with Oligo$(dT)_{12–18}$ primers (Life Technologies, Grand Island, NY). Gene expression in the cultures was examined by TaqMan qPCR analysis using the $2^{-\Delta\Delta CT}$ method, as described previously [295-297]. For all reactions, 9 ng of cDNA was combined with 5 µl TaqMan Universal PCR Master Mix (Applied Biosystems, Branchburg, NJ), 2.5 µl H_2O and 0.5 µl TaqMan primers (total 10 µl). TaqMan primers for *Bsp*, *Dmp1*, *Dspp*, *Gapdh*, and *Osteocalcin* were purchased from Applied Biosystems (Table 1). We defined the acceptable range of CT values representing gene expression to be between 10 and 35 cycles, according to manufacturer's recommendations (Applied Biosystems, Branchburg, NJ).

Statistical analysis.

Statistical analysis was performed by GraphPad Prism 7 software (GraphPad Software, Inc, La Jolla, CA) using one-way ANOVA analysis with the Tukey's multiple comparison post-

test or unpaired two-tailed Student *t*-test. In all experiments, values represent mean ± SEM of at least three independent experiments, and a *p-value $\leq$ 0.05 was considered statistically significant.

Tables.

Table 1. Primers used for TaqMan qPCR reactions.

Gene ID	Assay ID
Bsp	Mm01208381_g1
Dmp1	Mm00803831_m1
Dspp	Mm00515666_m1
Gapdh	Mm99999915_g1
Osteocalcin	Mm03413826_mH

CHAPTER IV. Effects of Early and Limited Exposure to BMP2 on Odontoblast and Osteoblast Differentiation

Introduction

Bone morphogenetic proteins (BMPs) are a family of signaling molecules that belong to the transforming growth factor β (TGFβ) superfamily of growth factors. Since their first identification and characterization in the 1960-70s [14,15], multiple studies have demonstrated essential roles of BMPs in embryonic development, organogenesis, fracture healing, and reparative processes [24]. To date, over 25 distinct members of the BMP family have been identified in almost all tissues and organs [17,18]. BMPs mediate their effects through interaction with highly conserved transmembrane serine/threonine kinase receptors (BMPRs), referred to as Type I and II receptors [21,82]. BMP signaling through BMPRs is also regulated by multiple local regulators, including integrins [93], proteoglycans [94], and various membrane or matrix-associated proteins [95].

Previous studies have demonstrated important regulatory roles of BMP signaling during skeletal development and bone formation *in vivo* and *in vitro*. Deregulated BMP signaling is associated with multiple human genetic skeletal disorders [21,26,27,92,96]. *In vivo*, expression of *Bmp2* and *Bmp4* has been localized to differentiated and differentiating osteoblasts, respectively [29]. Similarly, expression of BMP receptors (BMPR-IA, BMPR-IB, and BMPR-II) has been detected at high levels in the bone [97]. Overexpression of BMP antagonist noggin under the control of a

1.7-kb fragment of the rat *Bglap1* promoter has led to severe abnormalities in bone formation and bone mineral density and multiple long bone fractures [33]. Overexpression of a dominant-negative form of *Bmpr1a* receptor under the control of a 2.3-kb murine *Col1a1* promoter inhibits SMAD signaling in osteoblasts and impairs skeletal formation *in vivo* and osteoblast differentiation *in vitro* [98]. Other *in vitro* studies have shown that exposure of BMSC, ADSC or osteoblast cultures to exogenous BMPs during the differentiation phase of *in vitro* growth results in increased osteoblast differentiation and formation of a mineralized bone-like matrix [42-44]. However, some BMPs, such as BMP3, negatively affect bone formation via regulation of Wnt signaling [48]. Taken together, these resulted suggested that a majority of BMPs play a positive role in bone formation.

Various studies have also shown essential roles of BMP signaling in tooth development and odontoblast differentiation [20]. In the dental mesenchyme and odontoblasts, expression of *Bmp1*, *Bmp2*, *Bmp3*, and *Bmp7* has been detected at a late bell stage (E16-19 and P1), when deposition of predentin and dentin starts [30,52-57,267]. Furthermore, deletion of *Bmp2* or *Bmp4* from cells expressing a 3.6-kb fragment of *Col1a1* promoter reduced the rate of dentin formation, impaired dentin architecture, and decreased expression of *Dlx3*, *Dlx5*, and *Osx* transcription factors and *Dmp1* and *Dspp* [61,62]. Tissue-specific inactivation of *Bmp4* in dental mesenchyme in *Bmp4$^{f/f}$;Wnt1Cre* mice resulted in inhibition of SMAD1/5 signaling and delayed/inhibited tooth development [63]. Inactivating mutations of *Bmp1* in *Col1a1- Cre;Bmp1$^{flox/flox}$;Tll1$^{flox/flox}$* mice resulted in increased predentin thickness, thinner dentin, impaired odontoblast morphology, and decreased expression of *Dspp* [30]. In addition, exposure of dental pulp cells [66,71-73], SHED cells [68] or SCAP cells [70,74] to exogenous BMP2 or *Bmp2*-expressing vectors significantly stimulated

odontoblast differentiation. Taken together, these studies suggest that BMP signaling acts as a positive regulator of odontoblast differentiation through activation of various intracellular signaling pathways.

Previous studies in our laboratory have demonstrated that exposure of primary dental pulp cultures to fibroblast growth factor 2 (FGF2) during the proliferation phase of *in vitro* growth (referred to as short and limited exposure) does not have significant effects on the extent of mineralization but induces significant increases in the expression of *Dmp1* and *Dspp* and the number of DMP1-GFP^{+} and DSPP-Cerulean^{+} odontoblasts. Our results have also shown that the stimulatory effects of FGF2 on odontoblast differentiation are mediated through activation of FGFR/MEK/Erk1/2 signaling, increases in *Bmp2*, *Runx2*, and *Osx* [273,274], and activation of the BMP/BMPR signaling pathway. These observations suggest that stimulatory effects of FGF signaling on odontoblast differentiation could be mediated through induced BMP signaling. Therefore, the goal of the experiments outlined in this thesis was to examine the effects of early and limited exposure of primary dental pulp cultures to BMP2 on odontoblast differentiation and examine the possible involvement of BMP signaling in stimulatory effects of FGF2 on odontoblast differentiation.

Results.

Signaling pathways activated by BMPs in primary dental pulp cultures.

Previous studies in our laboratory have shown that when placed in primary culture, pulp cells from unerupted molars proliferate rapidly and reach confluence around day 7 (proliferation phase of *in vitro* growth). 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 phase of *in vitro* growth). The first sign of mineralization appears around day 10 with significant increases in the extent of mineralization thereafter. At day 21 almost the entire culture dish is covered with a sheet of mineralized tissue [274].

Using this well-characterized culture system, we first examined signaling pathways activated by BMP2 in primary dental pulp cultures. In these experiments, the cultures were exposed to 50 ng/ml BMP2, 50 ng/ml BMP4 or 20 ng/ml FGF2 and processed for immunohistochemistry using antibodies against phosphorylation of SMAD1/5/8 and MAPK ERK1/2 proteins as described in Materials and Methods. These experiments showed that the cultures treated with BMP2 and BMP4 displayed marked increases in the number of $pSMAD^{+}$ nuclei and intensity of the GFP signal as compared to VH-treated cultures (Figure 1A). These increases in the number of $pSMAD^{+}$ nuclei and intensity of the GFP signal were similar between BMP2 and BMP4 treated cultures. In contrast to BMP-treated cultures, FGF2-treated cultures did not display increases in the number of $pSMAD^{+}$ nuclei (Figure 1A). BMP2 or BMP4

treatment did not exert any increases in the number of pERK1/2^{+} nuclei, whereas their number appeared to be somewhat increased in FGF2-treated cultures (Figure 1B). These observations demonstrate that in primary dental pulp cultures BMP2 regulates its effects through activation of the canonical (SMAD-dependent) signaling pathway.

Effects of early and limited exposure to BMP2 on mineralization and expression of markers of odontoblast differentiation in primary dental pulp cultures.

We next examined the effects of early and limited exposure of dental pulp cultures to various concentrations of BMP2 during the proliferation phase of *in vitro* growth. Xylenol Orange (XO) staining showed that at all concentrations of BMP2 did not exert significant effects on the extent of mineralization at any time point (Figure 2A-B and Table 2).

Quantitative PCR analysis showed that BMP2 affected the expression of markers of odontoblast differentiation in a concentration-dependent manner (Figure 2C and Table 3). At day 7 the expression of *Bsp* (~1.7-5.6-fold) and *Dmp1* (~12.0-23.0-fold) was significantly decreased in BMP2-treated cultures at day 7 as compared to control. On the other hand, although *Dspp* expression was not detected in the control cultures at day 7, its expression was stimulated by 20 and 50 ng/ml of BMP2. Expression of *Ocn* was not significantly affected by BMP2 at day 7. These changes in the expression of *Bsp*, *Dmp1*, and *Dspp* in BMP2-treated cultures were detected as early as 12-36 hrs after treatment (Figure 3 and Table 4). At days 10-21 the levels of expression of *Bsp* and *Ocn* in BMP2-treated cultures were similar to those in control cultures

(Figure 2C and Table 3). At these time points, BMP2-treated cultures displayed decreased levels of *Dmp1* (~1.2-1.5-fold) and increased levels of *Dspp* (~1.4-3.2-fold) as compared to control.

In addition, we observed that expression of *Runx2*, a downstream target of canonical (SMAD-dependent) BMP signaling, was significantly increased by BMP2 (up to ~2.0-fold) as early as 36 hrs after exposure (Figure 3 and Table 4).

Effects of early and limited exposure to BMP2 on expression of transgenes in primary dental pulp cultures.

To further examine underlying mechanisms of the effects of BMP2 on dental pulp cells, we utilized pulp cells from a series of transgenic mice that display stage-specific activation of fluorescent proteins during odontoblast differentiation *in vivo* and *in vitro*. DMP1-mCherry and DSPP-Cerulean transgenes were used as markers for functional and fully differentiated odontoblasts [274], and BSP-GFP transgene was used as a marker for osteoblasts, as its expression has not been detected in dental pulp or odontoblasts *in vivo* (*manuscript in preparation*).

Our results demonstrated that changes in the intensity of expression of BSP-GFP and DMP1-mCherry transgenes in BMP2-treated cultures were similar to that of respective endogenous *Bsp* and *Dmp1* transcripts. At day 7, the intensity of BSP-GFP transgene expression was decreased as compared to control, followed by similar levels of intensity between BMP2-treated and control cultures at later time points (Figure 4A-B and Table 5). The intensity of

DMP1-mCherry transgene expression was decreased in BMP2-treated cultures as compared to control at all time points (Figure 4C and Table 6). As compared to control, cultures exposed to BMP2 did not display significant changes in the percentage of DSPP-Cerulean^{+} odontoblasts (Figure 5A), however BMP2 significantly increased the intensity of DSPP-Cerulean transgene (Figure 5B and Table 7).

Effects of late and limited exposure to BMP2 on mineralization and expression of markers of odontoblast differentiation in primary dental pulp cultures.

We also examined the effects of late and limited exposure of pulp cultures to BMP2 on odontoblast differentiation. In these studies, BMP2 was added to the primary dental pulp cultures during the differentiation phase of *in vitro* growth between days 7-21. XO staining showed that exposure to BMP2 resulted in slight increases in the extent of mineralization at days 10 (~1.5-fold), 14, and 21 as compared to control (~1.1-fold) (Figure 6A-B and Table 8).

Quantitative PCR analysis (qPCR) showed continuous increases in the expression of markers of mineralization and odontoblast differentiation (*Bsp*, *Ocn*, *Dmp1*, and *Dspp*) in the control cultures between days 7-21 (Figure 6C and Table 9). BMP2-treated cultures displayed decreased levels of *Bsp* (~1.2-1.6-fold) and *Dmp1* (~2.4-fold) as compared to control cultures. Expression of *Ocn* in BMP2-treated cultures was similar to that in control, but the expression of *Dspp*, a marker of odontoblast differentiation, was increased at days 10, 14, and 21 as compared to control (~1.6-2.4-fold). Taken together, these observations showed the exposure of primary

dental pulp cultures to BMP during the differentiation/mineralization phase of *in vitro* growth resulted in increased levels of expression of *Dspp* but decreased expression of *Dmp1* and *Bsp*.

Effects of early and limited exposure to BMP2 on mineralization, expression of markers of osteoblast differentiation and transgenes in primary bone marrow stromal cell cultures.

Our previous studies showed that primary dental pulp cultures contained progenitors capable of giving rise to both osteoblasts and odontoblasts [298]. This makes it difficult to distinguish the effects of BMP2 on cells of osteoblast *vs.* odontoblast lineages and raises the possibility that some of the effects of BMP2 on dental pulp cultures could be due to its effects on osteoblasts rather than on odontoblasts.

To distinguish between the effects of early and limited exposure to BMP2 on cells of odontoblast *vs.* osteoblast lineages, we examined the effects of BMP2 on BMSC cultures, as they do not contain odontoprogenitors and are used routinely to examine mineralization and osteoblast differentiation *in vitro*.

Our results showed that early and limited exposure of BMSC to BMP2 resulted in no significant changes in the extent of mineralization as compared to control (Figure 7A-B and Table 10). BMP2-treated cultures displayed concentration-dependent decreases in the intensity of the expression of *Bsp* and BSP-GFP (markers of early stages of osteoblast differentiation) and *Dmp1* and DMP1-mCherry (markers of late stages of osteoblast differentiation) in a

concentration-dependent manner (~1.3-6.2-fold) (Figure 8A-D and Tables 11-13).

Involvement of FGF/FGFR signaling in changes induced by early and limited exposure of pulp cells to BMP2.

Our results showed that although early and limited exposure of pulp cultures to BMP2 did not affect mineralization, it induced changes in the expression of various markers of odontoblast and osteoblast differentiation. These changes included decreases in the expression of *Bsp* and *Dmp1* as well as increases in the expression of *Dspp* at day 7. As the next step, we examined the roles of the FGF/FGFR signaling pathway in mediating the stimulatory effects of BMP2 on the expression of *Dspp* and its inhibitory effects of *Dmp1*, *Bsp*, and *Ocn* by using SU5402. In these experiments, primary pulp cultures were treated with FGF/FGFR signaling inhibitor SU5402 in the presence or absence of 50 ng/ml BMP2 during the proliferation phase of *in vitro* growth. At day 7, all cultures were grown in control mineralization-inducing medium.

Our results showed that early and limited exposure of dental pulp cultures to 5 and 10 μM SU5402 alone did not significantly affect the extent of mineralization at any time point, whereas 20 μM SU5402 significantly decreased it at day 21 (~1.4-fold) as compared to control (Figure 9A-B and Table 14).

Quantitative PCR analysis showed that early and limited exposure of dental pulp cultures to SU5402 resulted in markedly decreased the expression of *Bsp* (up to ~2.9-fold), *Dmp1* (up to ~8.5-fold), and *Ocn* (up to ~3.8-fold) (Figure 9C and Table 15) at day 7. On the other hand, there were no significant changes in the levels of expression of *Bsp* and *Dmp1* between days 10-21 in treated cultures as compared to control. Despite the withdrawal of SU5402, there were marked

decreases in the levels of expression of *Ocn* (up to ~3.8-fold) and *Dspp* (up to ~6.4-fold) at days 14 and 21 in treated cultures as compared to control (Figure 9C and Table 15). Effects of SU5402 on the intensity of expression of BSP-GFP and DMP1-mCherry transgenes were similar to those on respective endogenous transcripts (Figure 10A-C and Tables 16 and 17).

Cultures exposed to SU5402 and 50 ng/ml of BMP2 showed no significant effects on the extent of mineralization as compared to BMP2 alone (Figure 11A-B and Table 18). Quantitative PCR analysis showed that prior to induction of mineralization, the combination of SU5402 and BMP2 did not reverse the decreases in the expression of *Bsp*, *Dmp1*, and *Ocn* but was able to reverse the stimulatory effects of BMP2 on *Dspp* (Figure 11C and Table 19). Similar effects of the combination of SU5402 and BMP2 were observed on expression of BSP-GFP and DMP1-mCherry transgenes (Figure 12A-C and Tables 20 and 21). These observations together suggest roles of FGF/FGFR signaling in mediating the stimulatory effects of BMP2 on *Dspp*. However, the BMP2-induced decreases in the levels of expression of other markers of mineralization were not mediated through FGF/FGFR signaling.

Involvement of BMP/BMPR signaling in changes induced by early and limited exposure of pulp cells to FGF2.

Results outlined above have suggested that the interplay between BMP and FGF signaling pathways can be important in regulation of BMP2-induced increases in *Dspp* expression. To further examine the role of this interplay in pulp cultures, we exposed them to

various concentrations of the BMP/BMPR signaling inhibitor noggin between days 3-7.

Xylenol Orange staining demonstrated that noggin decreased the extent of mineralization at day 10 (up to ~2-fold), but not at days 14 and 21 as compared to control (Figure 12A-B and Table 22). Quantitative PCR analysis demonstrated that prior to induction of mineralization, noggin significantly decreased the expression of *Bsp*, *Dmp1*, and *Ocn* in a concentration-dependent manner (up to ~3.5-fold), whereas no *Dspp* expression was observed in any cultures (Figure 13C and Table 23). After induction of mineralization, noggin decreased the expression of *Dspp* (up to ~3.3-fold at day 10) and *Ocn* (up to ~2.6-fold at day 21), whereas the expression of *Bsp* and *Dmp1* remained similar to that in control cultures (Figure 13C and Table 23). The inhibitory effects of noggin on the expression of *Bsp* and *Dmp1* prior to induction of mineralization and the lack of effects on the expression of these genes after induction of mineralization were reflected in the similar changes in the intensity of BSP-GFP and DMP1-mCherry transgenes (Figure 14A-C and Tables 24 and 25).

Next, we examined the effects of inhibition of BMP signaling by noggin in the presence of FGF2 during the proliferation phase of *in vitro* growth. In the presence of FGF2, noggin did not exert significant effects on the extent of mineralization, however, 300-ng/ml concentration resulted in the most considerable inhibitory effect (up to 1.9-fold) (Figure 15A-B and Table 26). Quantitative PCR analysis showed that prior to induction of mineralization FGF2 markedly increased the expression of *Bsp* (~2.3-fold), *Dmp1* (~215-fold), *Ocn* (~4.6-fold), and *Dspp* (~3.64-fold) as compared to control (Figure 15C and Table 27). Addition of noggin reversed these stimulatory effects of FGF2 to the levels comparable to control (Figure 15C and Table 27).

After induction of mineralization, noggin significantly decreased FGF2-induced expression of *Dspp* (up to ~7.6-fold at days 10-21) and *Ocn* (up to ~6.8-fold at days 14 and 21), whereas *Bsp* and *Dmp1* displayed no significant changes (Figure 15C and Table 27). Effects of noggin in the presence of FGF2 on the expression of BSP-GFP and DMP1-mCherry transgenes were similar to those on respective endogenous transcripts (Figure 16A-C and Tables 28 and 29).

Discussion.

Our previous studies demonstrated that FGF2 exerted stage-specific effects on odontoblast differentiation. FGF2 stimulated differentiation of functional odontoblasts expressing high levels of *Dmp1* from early progenitors but inhibited the terminal differentiation of functional odontoblasts to fully differentiated odontoblasts expressing high levels of *Dspp* [289]. Our further studies showed that the stimulatory effects of FGF2 on the differentiation of functional odontoblasts from early progenitors involved activation of FGFR and ERK1/2 and increased levels of expression of *Bmp2*, *Runx2*, and *Osx*, which are components of BMP/BMPR signaling [273]. The inhibitory effects of FGF2 of terminal differentiation of functional odontoblasts into fully differentiated odontoblasts were related to reactivation of FGFR/ERK1/2 signaling and downregulation of BMP/BMPR signaling [299].

In our present study, we examined the roles of BMP/BMPR signaling in the differentiation of early progenitors by exposing the primary dental pulp cultures to BMP2 between days 3-7 (during the proliferation phase of *in vitro* growth). Our observation showed that BMP2 did not affect the extent of mineralization, decreased the levels of expression of *Bsp* and *Dmp1*, and significantly increased the levels of *Dspp*. The lack of significant changes in the number of DSPP-Cerulean^{+} odontoblasts in BMP2-treated cultures as compared to control indicated that increases in *Dspp* were related to increased transcriptional activity of *Dspp* but not to the increased number of odontoblasts. These results are consistent with previously reported stimulatory effects of BMP signaling on the expression of *Dspp in vivo* [30,61,62] and *in vitro* [65,66,69,70,74,300].

Previous studies also showed that these stimulatory effects of BMP2 on odontoblast differentiation and *Dspp* expression were mediated through activation of both canonical [67,77,78,120,121] and non-canonical [77,79-81] signaling pathways. These stimulatory effects were rapid and occurred within 6-24 hrs after exposure [65-67,74].

In our study we showed that exposure of dental pulp cells to BMP2 resulted in rapid (~1 hr) activation of the canonical but not non-canonical pathway evidenced by enhanced nuclear localization of pSMAD1/5/8 (but not pERK1/2). These observations are in agreement with previous studies showing rapid upregulation of pSMAD1/5/8 by BMP2 in the dental pulp [78] and odontoblast-like cells [67,301]. We also showed that the increases in the expression of *Dspp* by BMP2 were observed as early as 12 hrs after exposure, suggesting a direct interaction between the activated SMAD complex with regulatory elements of *Dspp*. This is in agreement with the recent study demonstrating that the *Dspp* promoter contains four SMAD binding elements (SBEs) for SMAD1/5/8 and SMAD4 proteins, explaining rapid (~12 hrs) upregulation of *Dspp* in mDPC6T dental papilla cells. The same study showed that a mutation of SBEs within the promoter markedly decreased *Dspp* expression [302].

We further observed that early exposure to BMP2 resulted in decreased expression of *Bsp* and *Dmp1* as early as 36-48 hrs after exposure, and these decreases were observed up to day 21 of the culture. Considering that *Bsp* and *Dmp1* are expressed at early and late stages of osteoblast differentiation, respectively, and *Bsp* is exclusively expressed by osteoblasts, these observations suggest that early and limited exposure of dental pulp cells to BMP2 negatively regulates

osteoprogenitors in the dental pulp. Thus, this reveals significant differences in the response of odontoprogenitors and osteoprogenitors to BMP2 (stimulatory and inhibitory, respectively). In addition, decreased expression of *Dmp1* and DMP1-GFP during differentiation/mineralization phase of *in vitro* growth by BMP2 could result from increased odontoblast differentiation and decreased expression of *Dmp1* and DMP1-GFP in mature odontoblasts [303].

We further explored the possible mechanisms underlying decreased expression levels of *Bsp* and *Dmp1* in BMP2-treated pulp cultures. Previous studies have demonstrated that BMP2 stimulates expression of *Dmp1* in dental pulp [78] and SCAP cells [74]. In addition, DSP-induced expression of *Dmp1* in mDPC6T dental papilla cells was inhibited by SMAD1/5/8 siRNA [302]. Our previous studies have shown that inhibition of BMP signaling by noggin resulted in marked decreases in FGF2-stimulated expression of *Dmp1* [274]. Taken together, these results suggest that BMP2 stimulates expression of *Dmp1* in odontoprogenitors.

Effects of BMP signaling on *Dmp1* and *Bsp* expression in cells of osteoblast lineage are conflicting and report positive [268,304], negative [99,100,305] or the absence [306] of effects.

Since the negative effects of BMP2 on *Dmp1* and *Bsp* in our experiments were observed within 36-48 hrs, it raises a possibility of the presence of binding elements within the promoters of these genes regulated by SMAD proteins or SMAD-associated transcription factors, such as RUNX2. This is in agreement with previous studies showing the presence of RUNX2 binding sites within *Dmp1* and *Bsp* promoters [307,308] and suggests that effects of BMP2 on *Dmp1* and *Bsp* could be mediated through SMAD/RUNX2 signaling.

Thus, our experiments suggest that roles of BMP signaling in the regulation of odontoprogenitors and osteoprogenitors can be different. In addition, this could also suggest the differences in the phenotype of osteoprogenitor cells residing in the dental pulp versus bone or bone marrow. Using primary BMSC cultures, we demonstrated that early and limited exposure to BMP2 resulted in decreased expression of *Bsp* and *Dmp1*, suggesting that this treatment prevents maturation of early osteoprogenitors.

Although maturation-suppressing effects of BMP2 on early osteoprogenitors have not been reported in the literature, they are similar to the maturation-suppressing effects of FGF2 on osteoprogenitors reported in our previous study [289]. In addition, these effects of BMP2 and FGF2 on early osteoprogenitors are consistent with the suppressing effects on osteoprogenitors by *Runx2* reported previously. Therefore, *Runx2* can be a converging point essential for reciprocal regulation of both BMP and FGF signaling pathways. This is in agreement with previous reports demonstrating that BMP2-induced nuclear co-localization of RUNX2 and pSMAD1/5/8 (but not pERK1/2) is impaired in *Fgf2*$^{-/-}$ mice and is dependent on the presence of FGF2 [262,263]. In addition, FGF2-stimulated expression of *Bmp2* in MC3T3-E1 osteoblast-like cells is mediated by RUNX2, as deletion of *Runx2* markedly impairs this stimulation [267].

Taken together, our results suggest that increased odontoblast differentiation of early progenitors in BMP2-treated dental pulp cultures could be due to the increased expression of *Runx2*. On the other hand, increased expression of *Runx2* in dental pulp or BMSC cultures can lead to increased formation of committed early osteoprogenitors, which express lower levels of

Dmp1 and *Bsp*, markers of mature osteoblasts/osteocytes.

Our further results demonstrated that inhibition of BMP signaling by noggin decreased FGF2-induced expression of *Dspp* and other markers of mineralization up to day 21 of culture (14 days after removal of noggin and FGF2). This suggests that BMP signaling is involved in mediating stimulatory effects of FGF2 on *Dspp* expression. In addition, these results indicated that the early and limited exposure of pulp cells to noggin was sufficient to exert long-lasting inhibitory effects on *Dspp* expression.

Similarly, inhibition of FGF signaling by SU5402 decreased BMP2-induced expression of *Dspp* and other markers of mineralization up to day 21 of culture (14 days after removal of SU5402 and BMP2). This suggests that FGF signaling is involved in mediating stimulatory effects of BMP2 on *Dspp* expression. Similar to noggin, early and limited exposure of pulp cells to SU5402 exerted long-lasting inhibitory effects on *Dspp* expression.

These results indicated that there is a reciprocal interaction between the BMP and FGF signaling pathways in their positive regulation of *Dspp* expression in dental pulp cultures. In addition, they are consistent with the previously reported results demonstrating important roles of the BMP and FGF signaling pathways in the reciprocal regulation of their activity during tooth development and odontoblast differentiation [2,51,272]. Similarly, interactions between the BMP (both canonical and non-canonical) and FGF signaling pathways have been well documented during skeletal development [221,261-271]. Taken together, these data suggest that both the BMP and FGF signaling pathways are essential for the reciprocal interactions during early

tooth development and odontoblast differentiation.

Exposure of murine dental pulp cultures to FGF2 significantly increased the expression of *Runx2* and *Bmp2* during the proliferation phase of *in vitro* growth [273]. The stimulatory effects of FGF2 on *Bmp2* expression occurred within 12-24 hrs, suggesting a direct stimulatory effect [274]. Furthermore, inhibition of BMP signaling by noggin markedly decreased or inhibited FGF2-mediated increases in *Dmp1* and *Dspp* [274].

Comparison between the effects of early and limited exposure of primary dental pulp and bone marrow stromal cell cultures to BMP2 *vs.* FGF2.

Our present observations of the effects of BMP2 on odontoblast differentiation of dental pulp cells reveal some similarities and differences as compared to those of FGF2 previously reported by us [273,289]. Neither FGF2 nor BMP2 stimulated the formation of the mineralized matrix as compared to control. In addition, our previous observations showed that stimulatory effects of FGF2 involved marked and rapid (within 12-24 hrs) increases in the expression of *Dspp* and *Dmp1* and formation of DMP1-GFP$^+$ functional odontoblasts. In our present studies, exposure to BMP2 led to rapid increases in the expression of *Dspp*, however, we did not observe any increases in the formation of DMP1-mCherry$^+$ functional odontoblasts. In fact, BMP2 significantly decreased the expression of *Dmp1* as compared to control. In addition, BMP2-stimulated odontoblast differentiation occurred through their increased transcriptional activity, whereas FGF2-stimulated odontoblast differentiation occurred through both increased transcriptional activity and the increased percentage of DSPP-Cerulean$^+$ odontoblasts.

In BMSC cultures, early and limited exposure to FGF2 resulted in decreased expression of *Col1a1*, *Bsp*, and *Ocn* at day 7, whereas expression of *Dmp1* and DMP1-GFP transgene was significantly increased. BMSC cultures exposed to BMP2 showed decreased expression of *Bsp*, *Dmp1* and, to a lesser extent, *Ocn*. This further displays similarities in the early effects of FGF2 and BMP2 on osteoprogenitor cells.

Even though the physiological basis of stimulation of dentinogenesis and inhibition of osteogenesis in undifferentiated pulp cells/progenitors is not clear, it may be important during reparative dentinogenesis. Reparative dentinogenesis animal studies have shown that FGF2 stimulates the formation of osteodentin, which represents bone-like tissue expressing DSP and DMP1 but negative for Nestin [309]. The formation of the mineralized tissue in the area of pulp exposure in response to FGF2 has been observed, however, this mineralized tissue does not contain dentinal tubules, further suggesting formation of osteodentin [247]. Using FGF2-carrying gelatin hydrogel microspheres and collagen sponges as a scaffold, Kikuchi *et al*. demonstrated formation of the DSP^{+} mineralized tissue in the area of pulp exposure 21 days after surgery [248]. The regenerated tissue, however, represented a porous aggregate composed of dentin-like particles, whereas physiological reparative dentin is non-porous. Using the same model, formation of $DMP1^{+}$/$Nestin^{-}$ osteodentin on the surface of the regenerated pulp by FGF2 has been demonstrated [249].

In contrast, exposure of undifferentiated pulp cells during reparative dentinogenesis to BMP2 resulted in the formation of tubular dentin-like tissue called orthodentin. Crude BMP

fraction stimulated the formation of tubular-like tertiary dentin in partially amputated pulps of adult canine teeth [112]. Capping of amputated pulp with BMP2 [113,114], BMP4 [114] or BMP7/OP-1 [115-118] resulted in formation of mixed osteodentin and tubular orthodentin. These results suggest that formation of dentin-like orthodentin by BMP2 could be due to differential regulation of commitment of undifferentiated pulp cells to odontogenic *vs.* osteogenic lineages.

Summary and conclusions.

In conclusion, our results demonstrated that early and limited exposure of dental pulp cells to BMP2 stimulated odontoblast differentiation evidenced by increased expression of *Dspp* and DSPP-Cerulean transgene. We further showed that stimulatory effects of BMP2 on *Dspp* expression were mediated via reciprocal interaction with FGF/FGFR signaling. In addition, we provided *in vitro* evidence that this treatment resulted in a significant decrease in the expression of markers of osteoblast differentiation (*Bsp*, *Dmp1*, BSP-GFP, and DMP1-mCherry) in both dental pulp and BMSC cultures. These effects were largely independent of interactions with FGF/FGFR signaling.

These results revealed similarities and differences between the effects of early and limited exposure of dental pulp cells to BMP2 and FGF2 with a possible explanation of the differences between these growth factors and formation of reparative dentinogenesis *in vivo*. These findings provide critical information for the development of improved treatments for vital pulp therapy and dentin regeneration.

Figures.

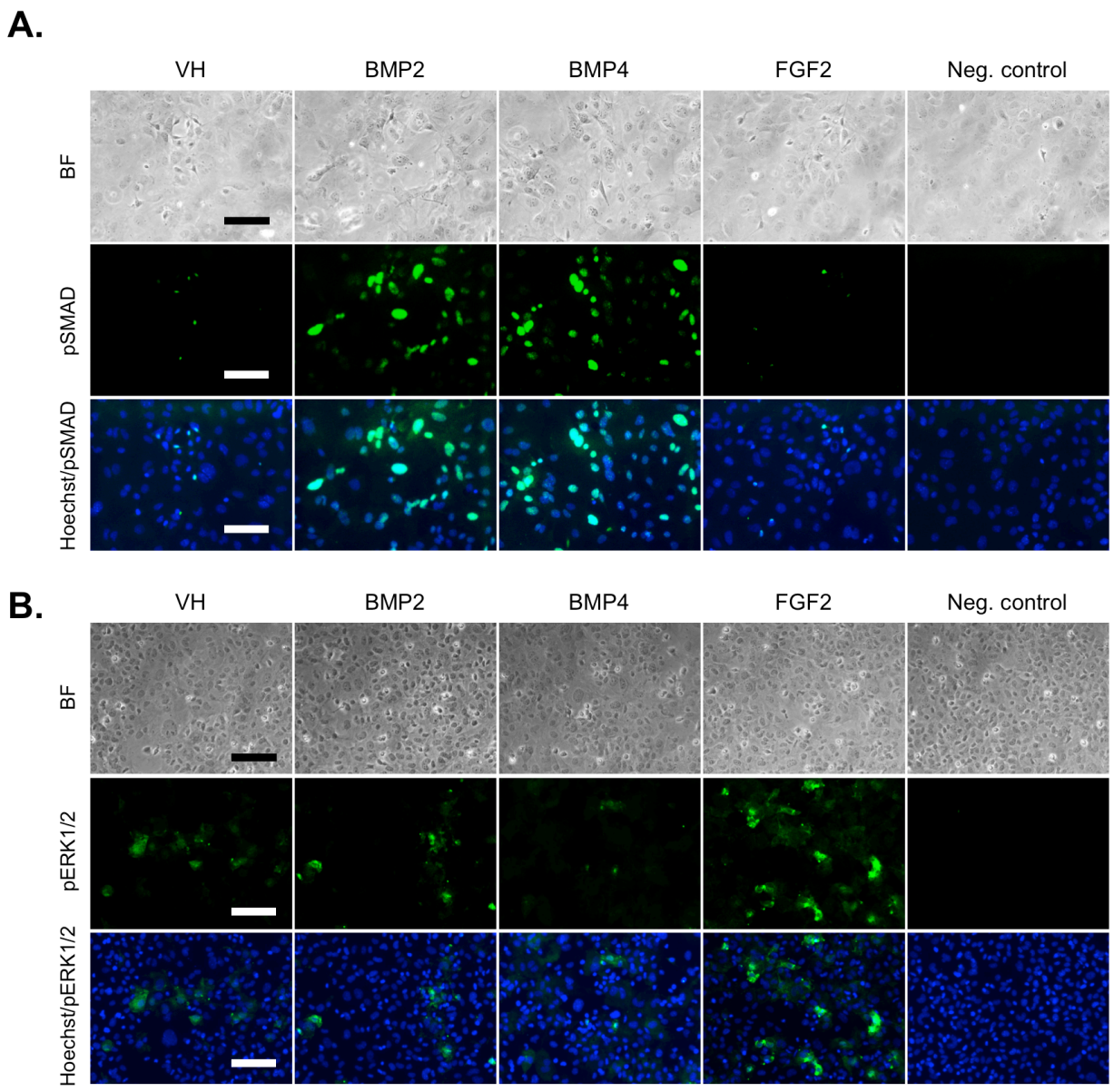

Figure 1. Effects of early and limited exposure to BMP2 on phosphorylation of SMAD1/5/8 and ERK1/2 proteins in primary dental pulp cultures.

Pulp cultures derived from non-transgenic 5-7-day-old pups were exposed to 50 ng/ml BMP2, 50 ng/ml BMP4 or 20 ng/ml FGF2 at day 3 for 60 minutes as described in the Materials and Methods. Representative images of the same area taken under brightfield (BF, upper row), epifluorescent light using filters for detection of pSMAD1/5/8 (Panel A, middle row) or pERK1/2 (Panel B, middle row). Bottom rows in Panels A and B represent overlaid images of phosphorylated proteins with Hoechst 33342 (Hoechst/pSMAD and Hoechst/pERK1/2,

respectively). Negative control (Neg. Control) included dental pulp cells exposed to 50 ng/ml BMP2 with the omission of primary antibody. Scale bar for images in Panel A and B is 100 and 200 μm, respectively.

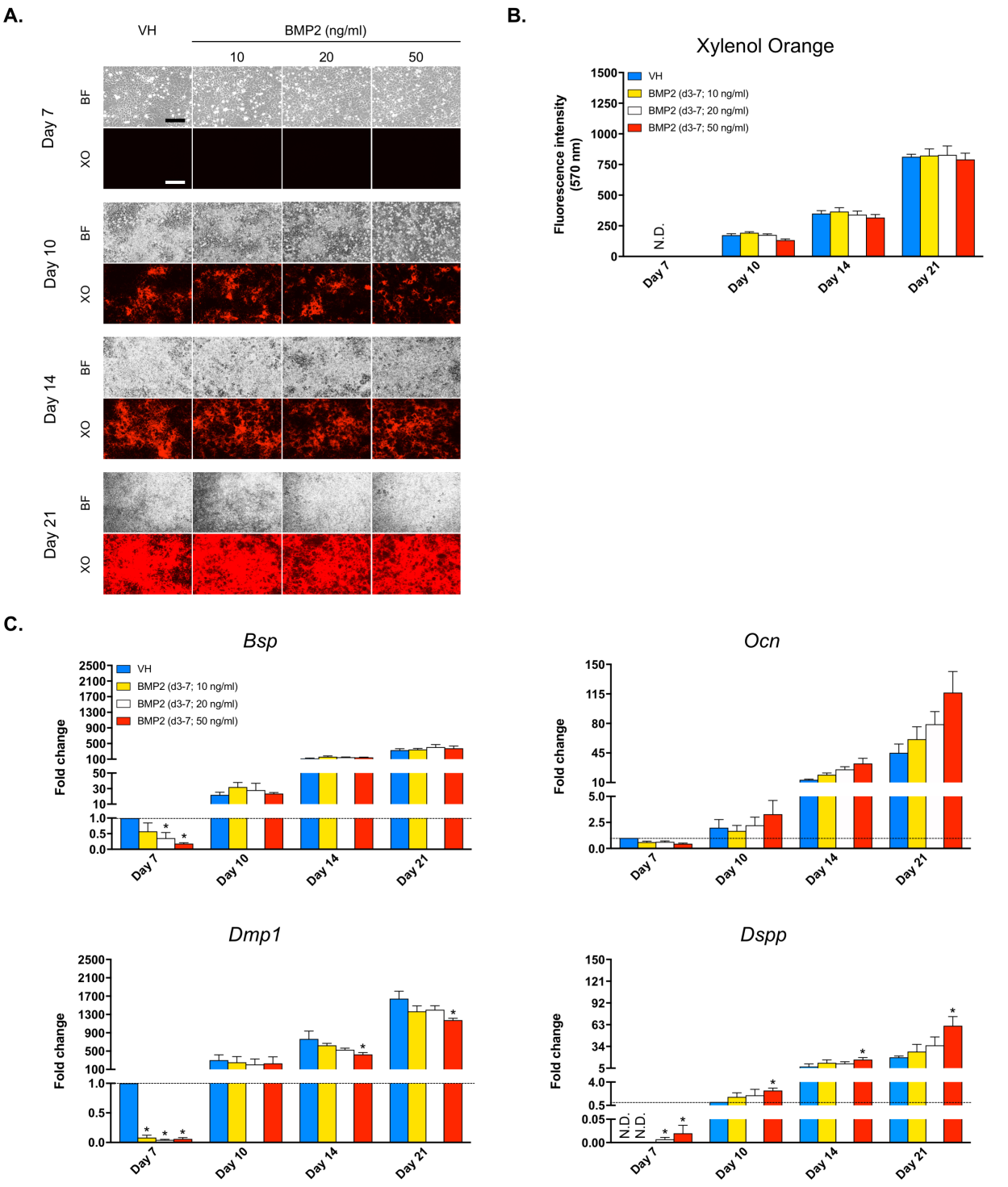

Figure 2. Effects of early and limited exposure to BMP2 on the extent of mineralization and expression of markers of mineralization and odontoblast differentiation in primary dental pulp cultures.

Primary dental pulp cultures derived from 5-7-day-old pups were treated with VH or 10, 20 or 50 ng/ml BMP2 between days 3-7 as described in the Materials and Methods. Starting day 7 all

cultures were grown under the mineralization-inducing culture conditions in the absence of BMP2 for additional 14 days (until day 21 of the culture).

A. Representative images of the same areas in cultures at various time points analyzed under brightfield (BF, upper row) and epifluorescent light using TRITC Red filter for detection of Xylenol Orange staining (XO, lower row). Scale bar is 200 μm.

B. The histogram represents the intensity of XO staining at various time points.

C. Quantitative PCR (qPCR) analysis of expression of *Bsp*, *Ocn*, *Dmp1*, and *Dspp*.

Results represent mean ± SEM of at least three independent experiments; $*p \leq 0.05$ relative to control at each time point.

Expression levels of *Bsp*, *Osteocalcin*, and *Dmp1* were normalized to VH at day 7, which is arbitrarily set to 1 and is indicated by the dashed line. Expression levels of *Dspp* were normalized to VH at day 10, which is arbitrarily set to 1 and is indicated by the dashed line.

N.D. = not detected; VH = vehicle; *Bsp = Bone sialoprotein*; *Ocn = Osteocalcin*; *Dmp1 = Dentin matrix protein 1*; *Dspp = Dentin sialophosphoprotein*.

Early and limited exposure to any concentration of BMP2 did not exert significant effects on the extent of mineralization as compared to control. However, BMP2 decreased the expression of *Bsp* in a concentration-dependent manner at day 7 but had no effect at later time points as compared to control. Expression of *Dmp1* was decreased in a concentration-dependent manner at day 7 and then remained lower as compared to control in cultures exposed to 50 ng/ml BMP2 (at days 14 and 21). There was a trend for increased expression of *Ocn* in BMP2-treated cultures, which did not reach statistical significance. Expression of *Dspp* was significantly increased in BMP2-treated cultures in a concentration-dependent manner at all time points as compared to control.

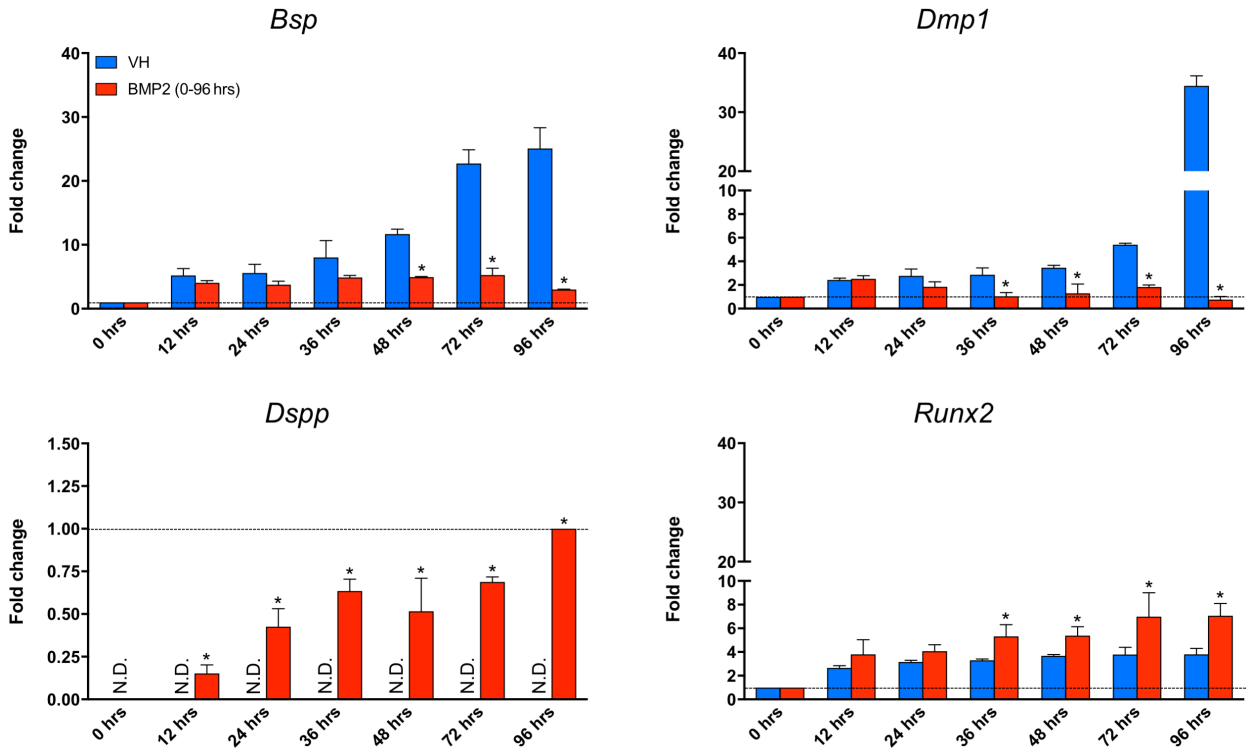

Figure 3. Effects of early and limited exposure to BMP2 on the early expression of *Bsp*, *Dmp1*, *Dspp*, and *Runx2* in primary dental pulp cultures.

Pulp cultures were treated with VH or 50 ng/ml BMP2 starting day 3 (0 hrs) and processed for qPCR analysis for *Bsp*, *Dmp1*, and *Dspp* at various time points. Expression of *Bsp*, *Dmp1*, and *Runx2* was normalized to VH at 0 hrs, which is arbitrarily set to 1 and is indicated by the dashed line. Expression of *Dspp* was normalized to the BMP2-treated culture at 96 hrs, which is arbitrarily set to 1 and is indicated by the dashed line.

Results represent mean ± SEM of at least three independent experiments; *$p \leq 0.05$ relative to control at each time point.

N.D. = not detected; VH = vehicle; *Bsp = Bone sialoprotein*; *Dmp1 = Dentin matrix protein 1*; *Dspp = Dentin sialophosphoprotein*; *Runx2 = Runt-related transcription factor 2.*

Early and limited exposure to BMP2 significantly decreased expression of *Bsp* and *Dmp1* and significantly increased expression of *Runx2* as early as 36-48 hrs after exposure as compared to control. Expression of *Dspp* was not detected in control cultures at any time point, whereas its expression was detected in BMP2-treated cultures as early as 12 hrs after exposure.

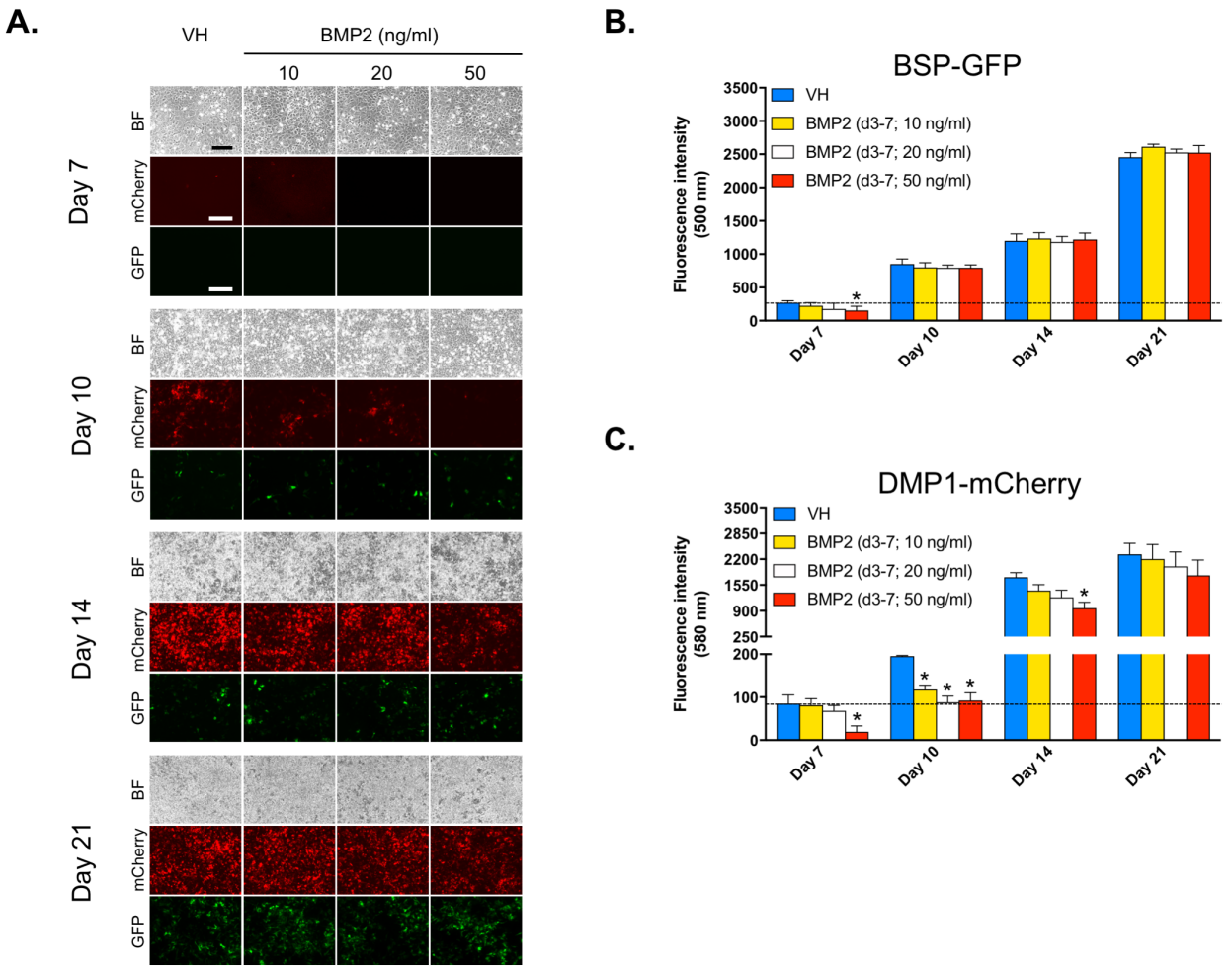

Figure 4. Effects of early and limited exposure to BMP2 on the expression of BSP-GFP and DMP1-mCherry transgenes in primary dental pulp cultures.

Primary dental pulp cultures derived from 5-7-day-old BSP-GFP and DMP1-mCherry transgenic mice were treated with VH or BMP2 between days 3-7 as described in Materials and Methods.

A. Representative images of the same areas in cultures at different time points analyzed under brightfield (BF, upper rows) and epifluorescent light using mCherry filter for detection of DMP1-mCherry (mCherry, middle row) and GFPtpz filter for detection of BSP-GFP (GFP, lower row).

B. The histograms represent epifluorescence intensity of BSP-GFP transgene at various time points.

C. The histograms represent epifluorescence intensity of BSP-GFP and DMP1-mCherry transgene at various time points.

Results represent mean ± SEM of at least three independent experiments; *$p \leq 0.05$ relative to control at each time point.

Early and limited exposure to BMP2 slightly decreased the intensity of expression of BSP-GFP transgene at days 7 and 10, however, these changes did not reach statistical significance. No effect on BSP-GFP was detected in BMP2-treated cultures at later time points (days 14 and 21) as compared to control.

Expression of DMP1-mCherry transgene was significantly decreased in BMP2-treated cultures in a concentration-dependent manner at days 7 and 10. At day 14, only 50 ng/ml BMP2 significantly decreased expression of DMP1-mCherry. No significant differences were observed at day 21 as compared to control.

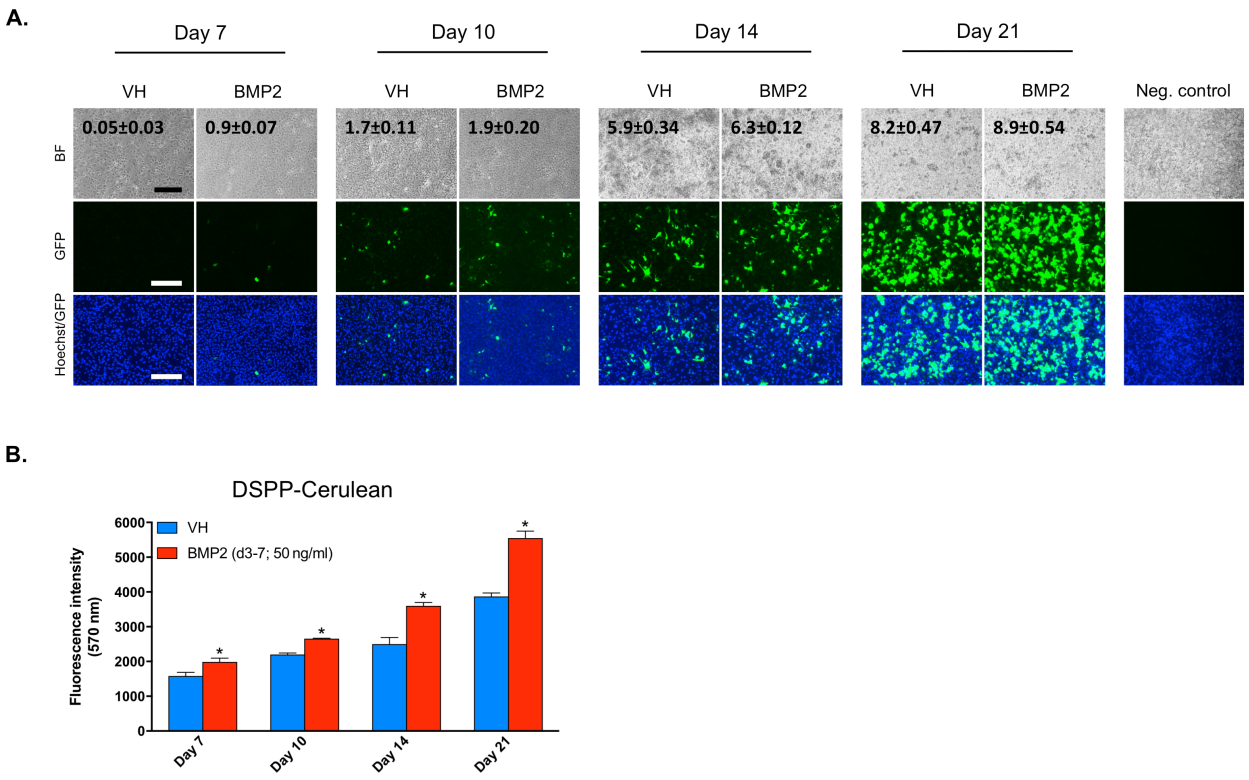

Figure 5. Effects of early and limited exposure to BMP2 on the percentage of DSPP-Cerulean$^+$ odontoblasts in primary dental pulp cultures.

Cultures derived from 5-7-day-old DSPP-Cerulean transgenic mice were treated with VH or 50 ng/ml BMP2 between days 3-7. Cultures were processed for immunocytochemistry at days 7, 10, 14, and 21 as described in the Materials and Methods.

A. The percentage of DSPP-Cerulean$^+$ odontoblasts was calculated as a ratio between Cerulean$^+$ cells (visualized by anti-GFP antibody) and the total number of Hoechst$^+$ cells. Dental pulp cultures established from DSPP-Cerulean transgenic mice and processed for immunocytochemistry without the addition of antibody at day 21 served as a negative control (Neg. control, without Ab).

B. The histogram represents epifluorescence intensity of DSPP-Cerulean transgene at various time points.

Representative images of the same area taken under brightfield (BF, upper row), epifluorescent light using filters for GFPtpz (GFP, middle row) and overlaid Hoechst/GFP (Hoechst/GFP, lower row) filters. Scale bar is 200 μm.

Results represent mean ± SEM of at least three independent experiments; *$p \leq 0.05$ relative to control at each time point.

Early and limited exposure to BMP2 did not affect the percentage of DSPP-Cerulean$^+$

odontoblasts but increased the intensity of DSPP-Cerulean transgene as compared to control.

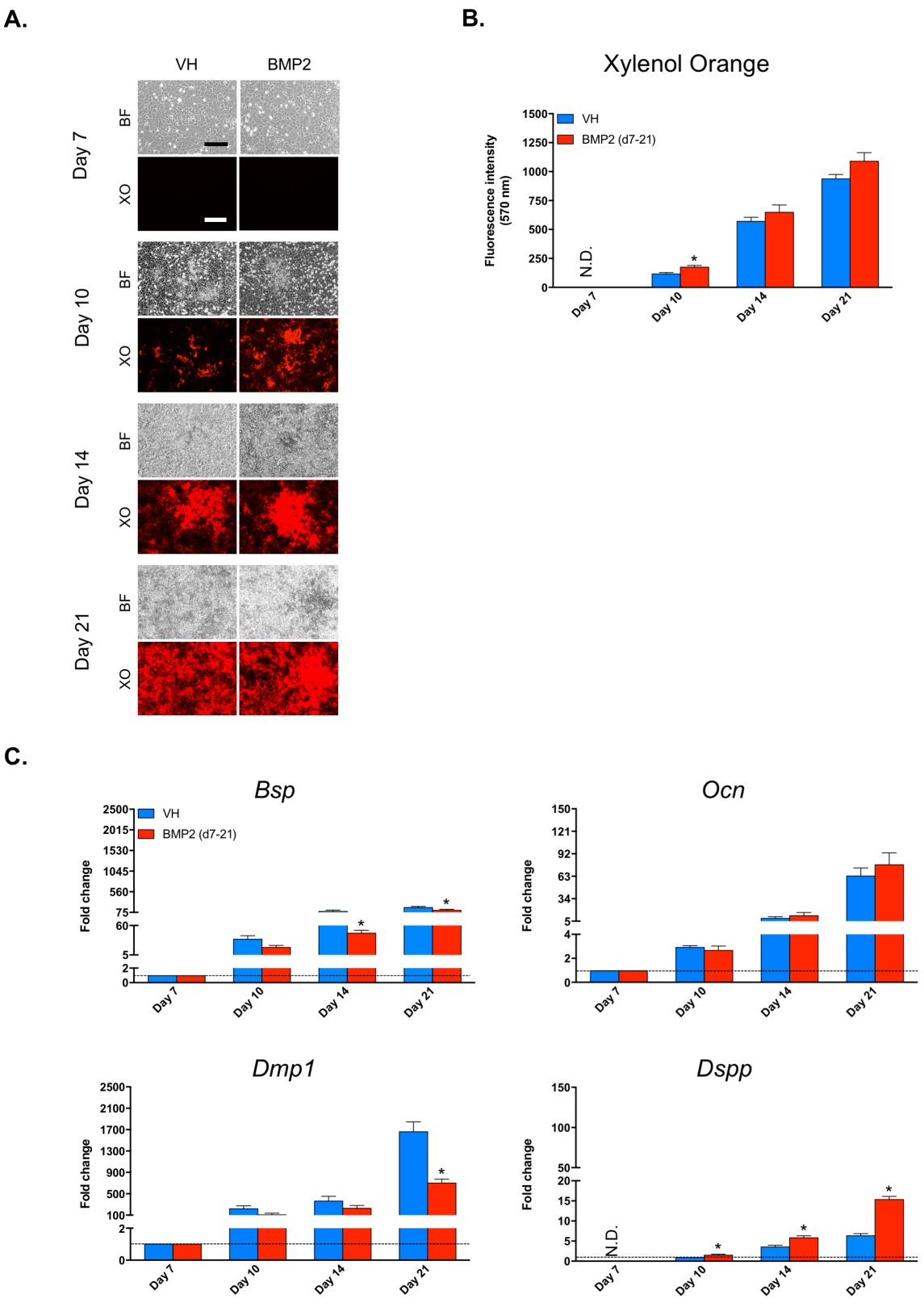

Figure 6. Effects of late and limited exposure to BMP2 on the extent of mineralization and expression of markers of mineralization and odontoblast differentiation in primary dental

pulp cultures.

A. Representative images of the same areas in cultures at various time points analyzed under brightfield (BF, upper row) and epifluorescent light using TRITC Red filter for detection of Xylenol Orange staining (XO, lower row). Scale bar is 200 μm.

B. The histogram shows the intensity of XO staining at various time points.

C. Quantitative PCR (qPCR) analysis of expression of *Bsp*, *Ocn*, *Dmp1*, and *Dspp*.

Expression of markers of mineralization and odontoblast differentiation in the VH (control) and BMP2-treated cultures was analyzed at days 7, 10, 14 and 21. Expression levels of *Bsp*, *Osteocalcin*, and *Dmp1* were normalized to VH at day 7, which is arbitrarily set to 1 and is indicated by the dashed line. Expression levels of *Dspp* were normalized to VH at day 10, which is arbitrarily set to 1 and is indicated by the dashed line.

Results represent mean ± SEM of three independent experiments; $*p \leq 0.05$ relative to control at each time point.

N.D. = not detected; VH = Vehicle; BMP2 = Bone morphogenetic protein 2; *Bsp = Bone sialoprotein*; *Ocn = Osteocalcin*; *Dmp1 = Dentin matrix protein 1*; *Dspp = Dentin sialophosphoprotein*.

Late and limited exposure to BMP2 significantly increased the extent of mineralization as compared to control at day 10 but not at days 14 and 21. This treatment decreased the expression of *Bsp* (at days 14 and 21) and *Dmp1* (at day 21) and had similar expression levels as compared to control. Expression of *Dspp* was significantly increased in BMP2-treated cultures at all time points as compared to control.

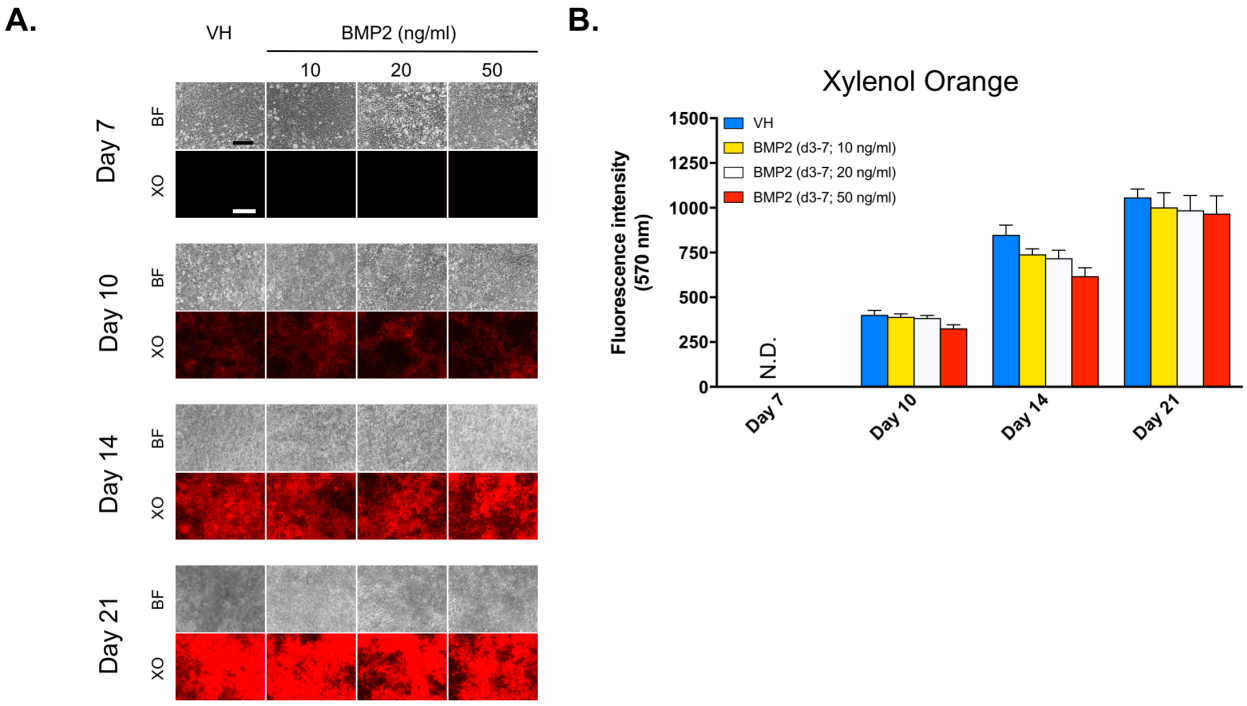

Figure 7. Effects of early and limited exposure to BMP2 on the extent of mineralization in primary BMSC cultures.

Primary BMSC cultures derived from 5-7-day-old pups were grown in the presence of VH or BMP2 at days 3-7 as described in the Materials and Methods.

A. Representative images of the same areas in cultures at various time points analyzed under brightfield (BF, upper row) and epifluorescent light using TRITC Red filter for detection of XO staining (XO, lower row). Scale bar is 200 μm.

B. The histogram represents the intensity of fluorescence of XO staining at various time points.

Results represent mean ± SEM of three independent experiments; *$p \leq 0.05$ relative to control at each time point.

N.D. = not detected; VH = vehicle; BMP2 = Bone morphogenetic protein 2.

Early and limited exposure of BMSC cultures to BMP2 resulted in no changes in the extent of mineralization as compared to control.

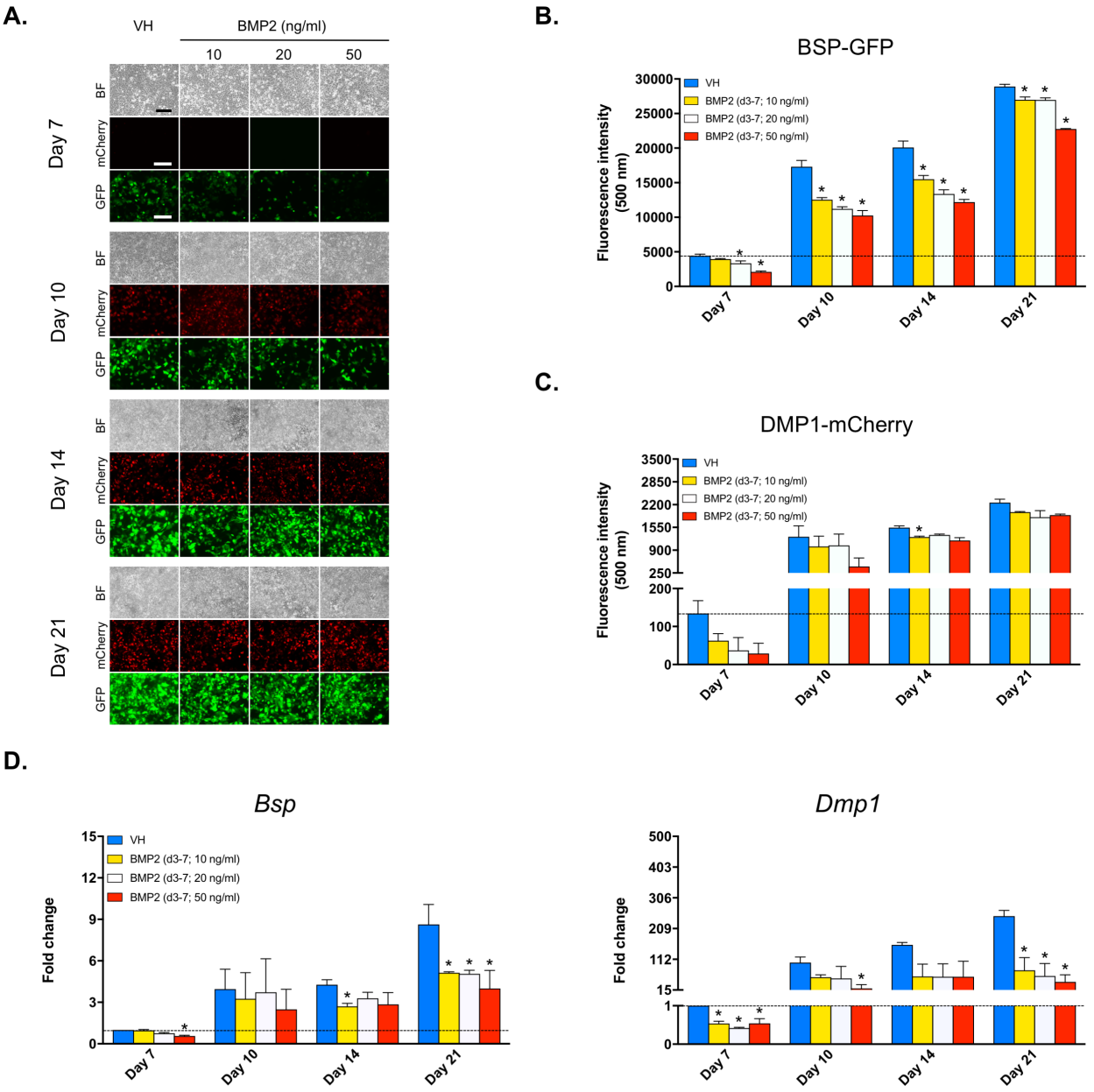

Figure 8. Effects of early and limited exposure to BMP2 on the expression of BSP-GFP and DMP1-mCherry transgenes and expression of *Bsp* and *Dmp1* transcripts in primary BMSC cultures.

A. Representative images of the same areas in cultures at various time points analyzed under brightfield (BF, upper row) and epifluorescent light using Topaz and TRITC Red filters for detection of DMP1-mCherry (middle row) and BSP-GFP (lower row) transgenes. Scale bar is 200 μm.

B. The histogram represents the intensity of fluorescence of BSP-GFP transgene at various time points.

C. The histogram represents the intensity of fluorescence of DMP1-mCherry transgene at various time points.

D. The histograms represent expression levels of *Bsp* and *Dmp1* transcripts at various time points.

Results represent mean ± SEM of three independent experiments; *$p \leq 0.05$ relative to control at each time point.

N.D. = not detected; VH = vehicle; BMP2 = Bone morphogenetic protein 2. *Bsp = Bone sialoprotein*; *Dmp1 = Dentin matrix protein 1*.

Early and limited exposure of BMSC cultures to BMP2 resulted in decreased expression of BSP-GFP transgene in a concentration-dependent manner as compared to control. BMP2 treatment reduced expression of DMP1-mCherry transgene, but these decreases largely did not reach statistical significance.

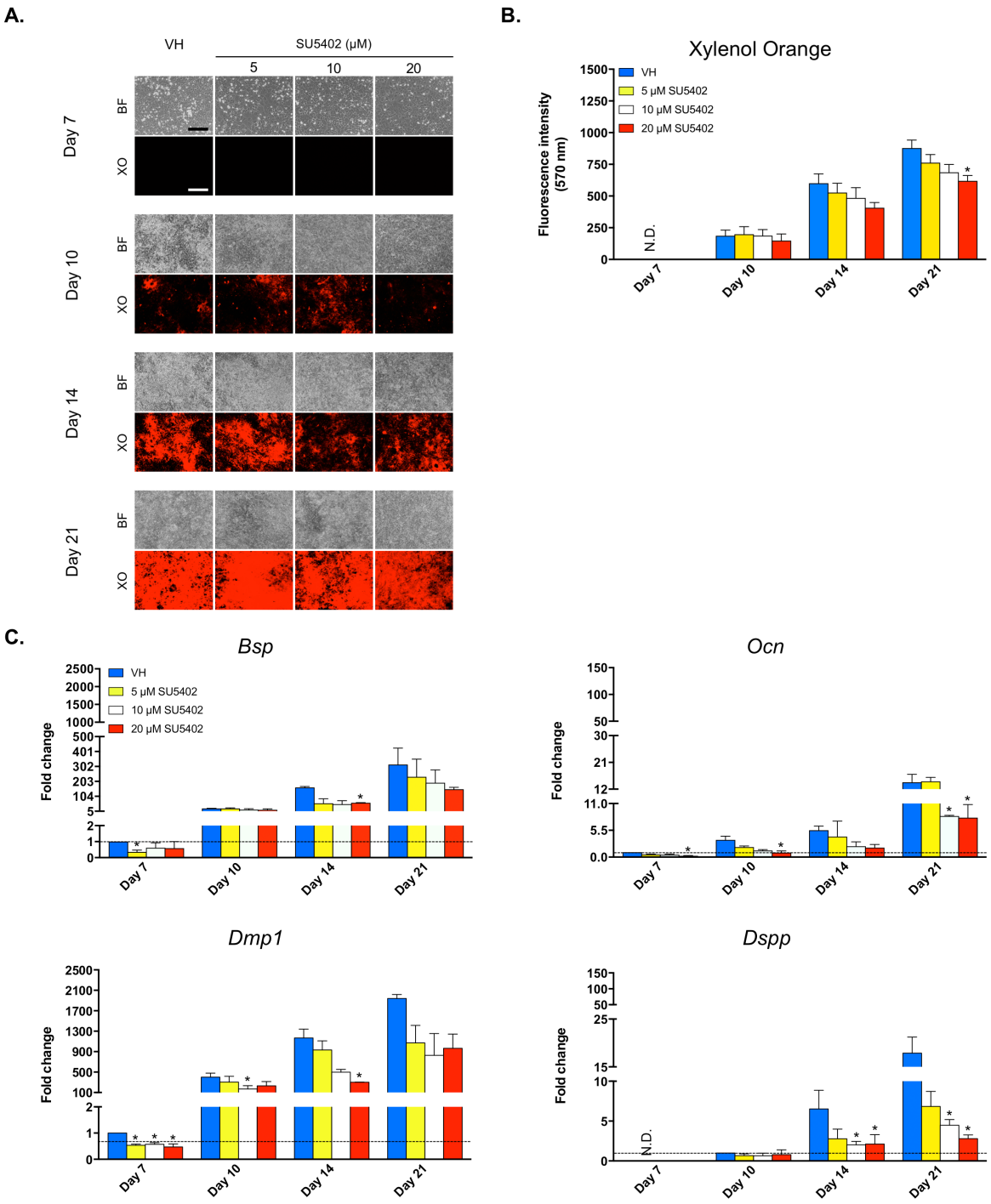

Figure 9. Effects of early and limited exposure to SU5402 on the extent of mineralization and expression of markers of mineralization and odontoblast differentiation in primary dental pulp cultures.

Primary dental pulp cultures derived from 5-7-day-old pups were treated with VH or 5, 10, and 20 μM SU5402 between days 3-7 as described in the Materials and Methods. Starting day 7 all cultures were grown under the control mineralization-inducing culture conditions for additional

14 days (until day 21 of the culture).

A. Representative images of the same areas in cultures at various time points analyzed under brightfield (BF, upper row), and epifluorescent light using TRITC Red filter for detection of Xylenol Orange staining (XO, lower row). Scale bar is 200 μm.

B. The histogram represents the intensity of XO staining at various time points.

C. Quantitative PCR (qPCR) analysis of expression of *Bsp*, *Ocn*, *Dmp1*, and *Dspp*.

Results represent mean ± SEM of at least three independent experiments; $*p \leq 0.05$ relative to control at each time point.

Expression levels of *Bsp*, *Osteocalcin*, and *Dmp1* were normalized to VH at day 7, which is arbitrarily set to 1 and is indicated by the dashed line. Expression levels of *Dspp* were normalized to VH at day 10, which is arbitrarily set to 1 and is indicated by the dashed line.

N.D. = not detected; VH = vehicle; *Bsp = Bone sialoprotein*; *Ocn = Osteocalcin*; *Dmp1 = Dentin matrix protein 1*; *Dspp = Dentin sialophosphoprotein*.

Early and limited exposure to SU5402 resulted in decreased mineralization at day 21 by 20 μM concentration as compared to control. Despite a trend for decreased mineralization in SU5402-treated cultures at day 14, no statistical significance was observed.

Prior to induction of mineralization at day 7, SU5402 decreased the expression of *Bsp*, *Ocn*, and *Dmp1* in a concentration-dependent manner as compared to control. No *Dspp* expression was detected under any treatment. After induction of mineralization, SU5402 significantly decreased expression of *Bsp*, *Dmp1*, *Ocn*, and *Dspp* at various time points as compared to control.

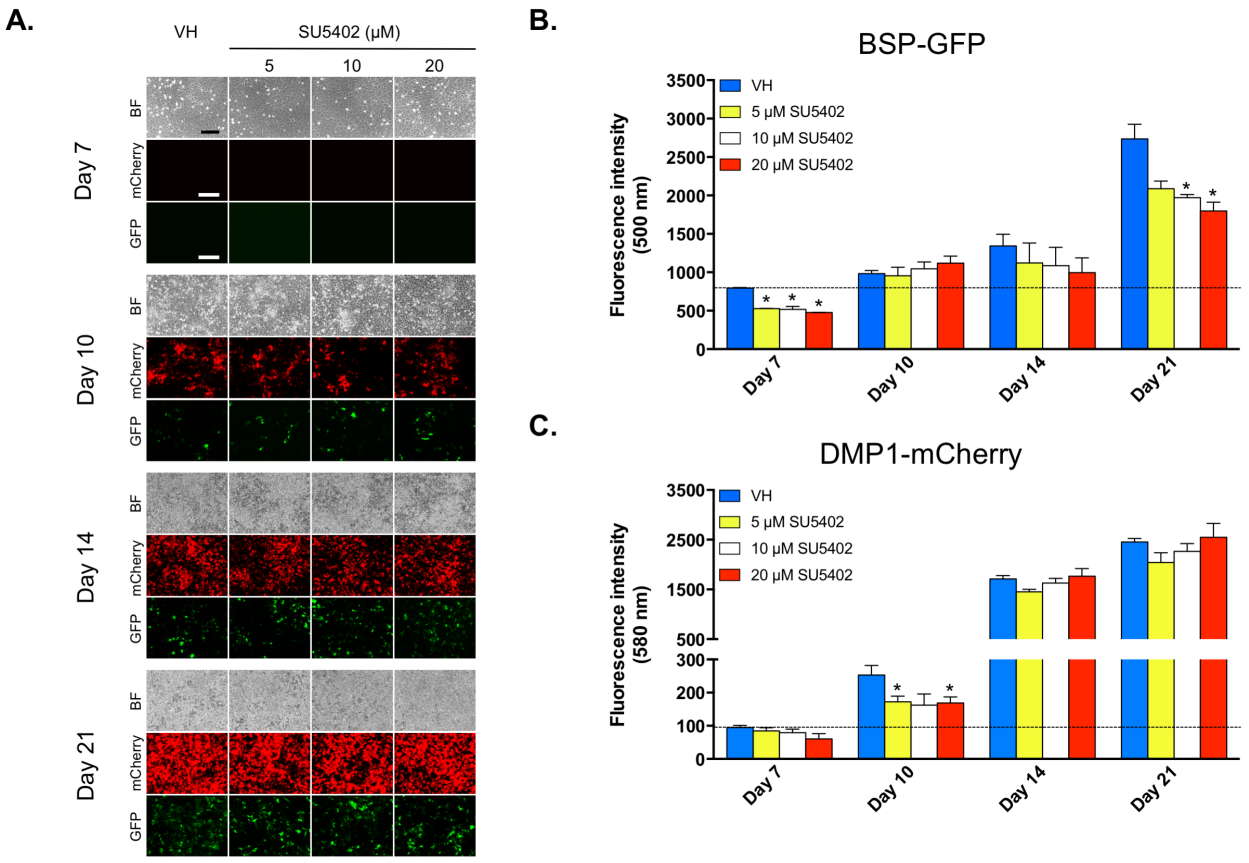

Figure 10. Effects of early and limited exposure to SU5402 on the expression of BSP-GFP and DMP1-mCherry transgenes in primary dental pulp cultures.

Primary dental pulp cultures derived from 5-7-day-old BSP-GFP and DMP1-mCherry transgenic mice were treated with VH or SU5402 between days 3-7 as described for Figure 9.

A. Representative images of the same areas in cultures at different time points analyzed under brightfield (BF, upper rows) and epifluorescent light using the mCherry filter for detection of DMP1-mCherry (mCherry, middle row) and GFPtpz filter for detection of BSP-GFP (GFP, lower row).

B. The histogram represents epifluorescence intensity of BSP-GFP transgene at various time points.

C. The histogram represents epifluorescence intensity of DMP1-mCherry transgene at various time points.

Results represent mean ± SEM of at least three independent experiments; *$p \leq 0.05$ relative to control at each time point.

Prior to induction of mineralization, SU5402 decreased expression of BSP-GFP ($p \leq 0.05$) and DMP1-mCherry ($p > 0.05$) as compared to control. After induction of mineralization, SU5402

decreased the intensity of expression of BSP-GFP at day 21 and DMP1-mCherry at day 10 as compared to control.

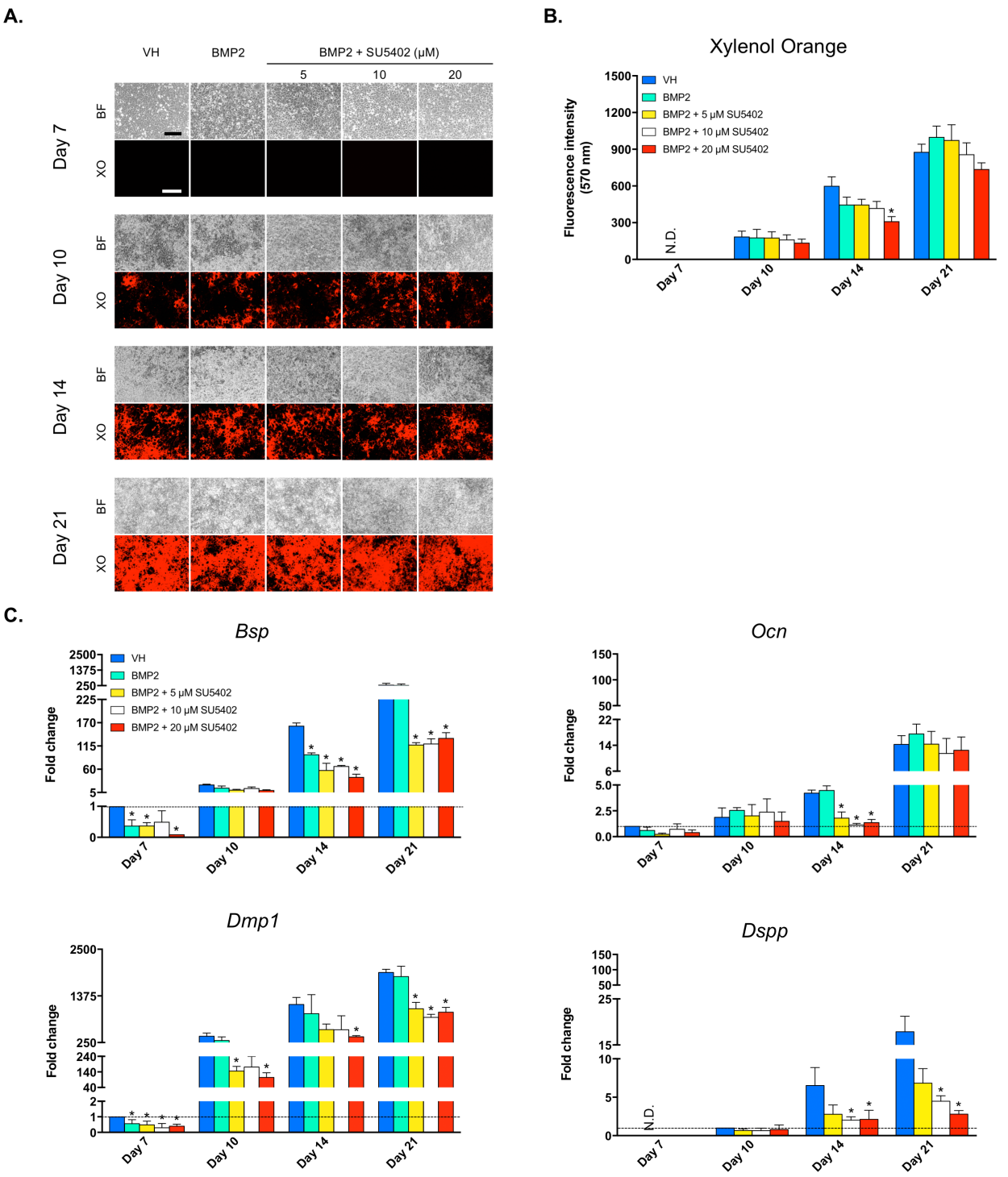

Figure 11. Effects of early and limited exposure to SU5402 in the presence of BMP2 on the extent of mineralization and expression of markers of mineralization and odontoblast differentiation in primary dental pulp cultures.

Primary dental pulp cultures derived from 5-7-day-old pups were treated with VH or 5, 10, and 20 μM SU5402 in the presence of 50 ng/ml BMP2 between days 3-7 as described in the

Materials and Methods. Starting day 7 all cultures were grown under the control mineralization-inducing culture conditions for additional 14 days (until day 21 of the culture).

A. Representative images of the same areas in cultures at various time points analyzed under brightfield (BF, upper row), and epifluorescent light using TRITC Red filter for detection of Xylenol Orange staining (XO, lower row). Scale bar is 200 μm.

B. The histogram represents the intensity of XO staining at various time points.

C. Quantitative PCR (qPCR) analysis of expression of *Bsp*, *Ocn*, *Dmp1*, and *Dspp*.

Results represent mean ± SEM of at least three independent experiments; $*p \leq 0.05$ relative to control at each time point.

Expression levels of *Bsp*, *Osteocalcin*, and *Dmp1* were normalized to VH at day 7, which is arbitrarily set to 1 and is indicated by the dashed line. Expression levels of *Dspp* were normalized to VH at day 10, which is arbitrarily set to 1 and is indicated by the dashed line.

N.D. = not detected; VH = Vehicle; BMP2 = Bone morphogenetic protein 2; *Bsp* = *Bone sialoprotein*; *Ocn* = *Osteocalcin*; *Dmp1* = *Dentin matrix protein 1*; *Dspp* = *Dentin sialophosphoprotein*.

Early and limited exposure to BMP2 had no significant effects on the extent of mineralization as compared to control. In the presence of BMP2, SU5402 decreased the extent of mineralization as compared to control at day 14 and by 20 μM concentration only.

Prior to induction of mineralization at day 7, BMP2 decreased the expression of *Bsp*, *Dmp1*, *Ocn*, and *Dspp* as compared to control. Exposure of cultures to both SU5402 and BMP2 either did not affect BMP2-mediated decreases (for *Bsp*, *Dmp1*, and *Ocn*) or further enhanced them (for *Dspp*). After induction of mineralization, BMP2 decreased the expression of *Bsp*, increased expression of *Dspp*, and had no significant effects on the expression of *Bsp* and *Ocn*. Exposure of cultures to both SU5402 and BMP2 further enhanced BMP2-mediated decreases in *Bsp* (at days 14 and 21), *Ocn* (at day 14), *Dmp1* (at days 10-21), and *Dspp* (at days 10-21) as compared to control.

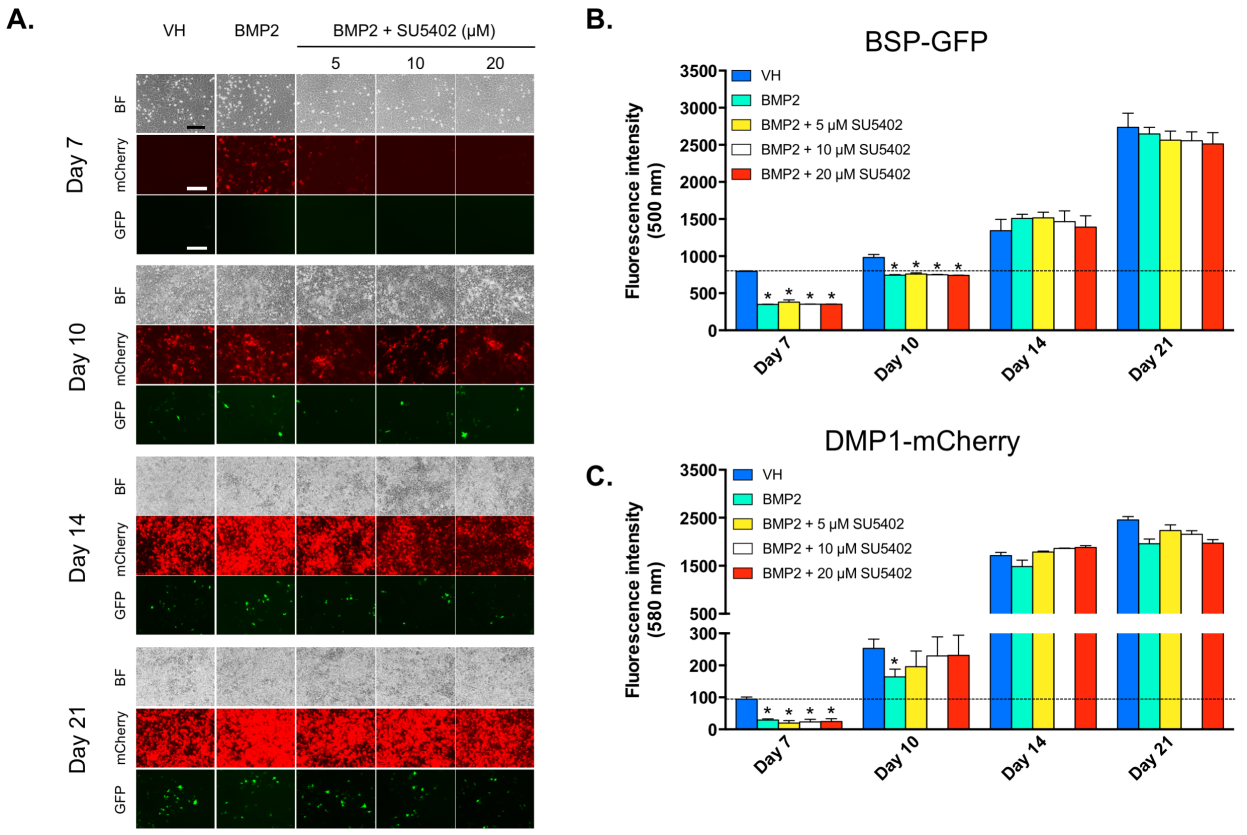

Figure 12. Effects of early and limited exposure to SU5402 in the presence of BMP2 on the extent of mineralization and expression of markers of mineralization and odontoblast differentiation in primary dental pulp cultures.

Primary dental pulp cultures derived from 5-7-day-old pups were treated with VH or 5, 10, and 20 μM SU5402 as described for Figure 11.

A. Representative images of the same areas in cultures at various time points analyzed under brightfield (BF, upper row), and epifluorescent light using TRITC Red filter for detection of Xylenol Orange staining (XO, lower row). Scale bar is 200 μm.

B. The histogram represents the intensity of XO staining at various time points.

C. Quantitative PCR (qPCR) analysis of expression of *Bsp*, *Ocn*, *Dmp1*, and *Dspp*.

Results represent mean ± SEM of at least three independent experiments; *$p \leq 0.05$ relative to control at each time point.

Prior to induction of mineralization at day 7, BMP2 significantly decreased the intensity of both BSP-GFP and DMP1-mCherry transgenes as compared to control. After induction of mineralization, BMP2 significantly decreased intensity of BSP-GFP and DMP1-mCherry at day 10 but not at days 14 and 21 as compared to control. Exposure of cultures to both BMP2 and SU5402 had an inhibitory effect similar to that of BMP2 but did not further enhance it.

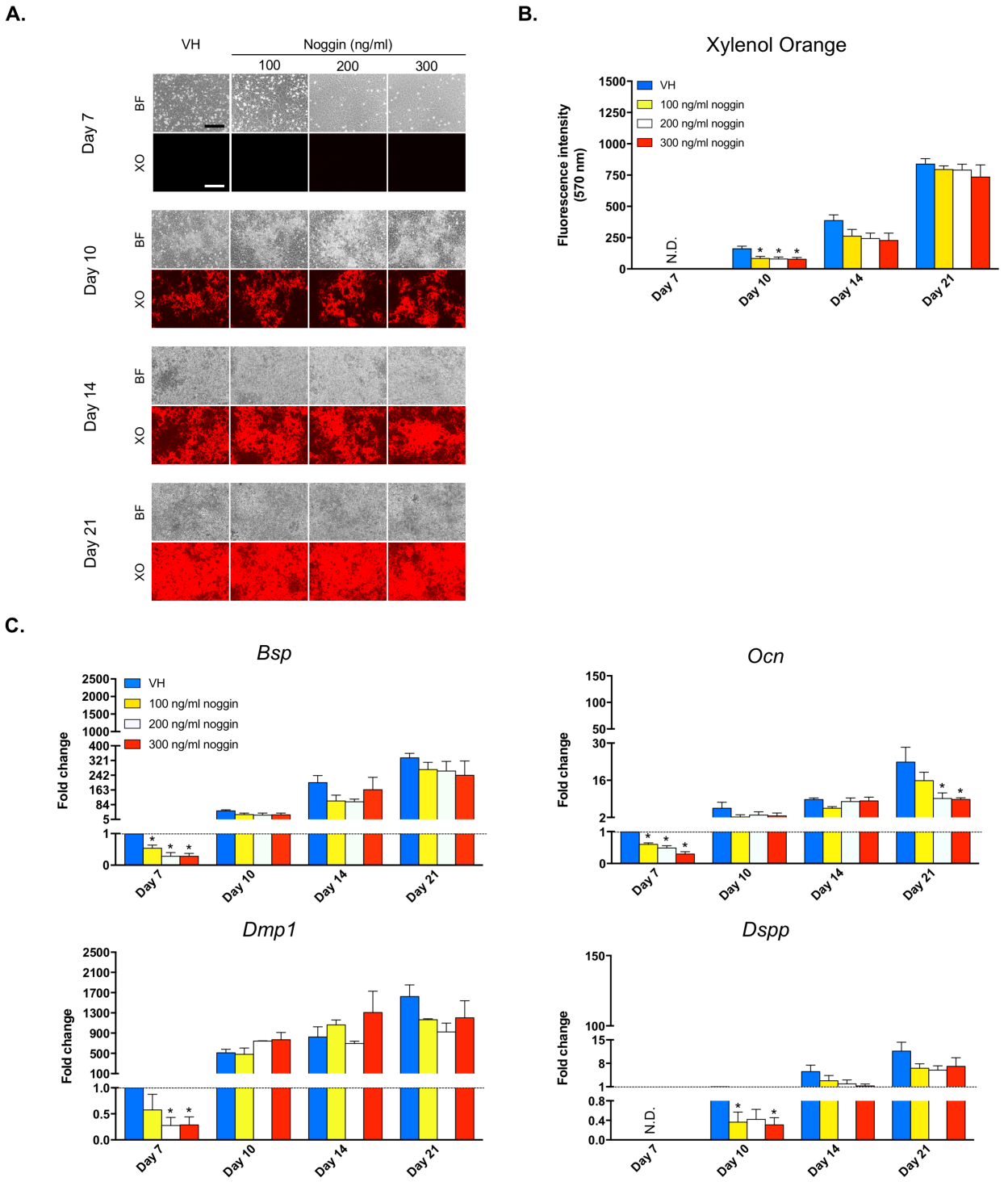

Figure 13. Effects of early and limited exposure to noggin on the extent of mineralization and expression of markers of mineralization and odontoblast differentiation in primary dental pulp cultures.

Primary dental pulp cultures derived from 5-7-day-old pups were treated with VH or 100, 200, and 300 ng/ml noggin between days 3-7 as described in the Materials and Methods. Starting day

7 all cultures were grown under the control mineralization-inducing culture conditions for additional 14 days (until day 21 of the culture).

A. Representative images of the same areas in cultures at various time points analyzed under brightfield (BF, upper row), and epifluorescent light using TRITC Red filter for detection of Xylenol Orange staining (XO, lower row). Scale bar is 200 μm.

B. The histogram represents the intensity of XO staining at various time points.

C. Quantitative PCR (qPCR) analysis of expression of *Bsp*, *Ocn*, *Dmp1*, and *Dspp*.

Results represent mean ± SEM of at least three independent experiments; $*p \leq 0.05$ relative to control at each time point.

Expression levels of *Bsp*, *Osteocalcin*, and *Dmp1* were normalized to VH at day 7, which is arbitrarily set to 1 and is indicated by the dashed line. Expression levels of *Dspp* were normalized to VH at day 10, which is arbitrarily set to 1 and is indicated by the dashed line.

N.D. = not detected; VH = vehicle; *Bsp = Bone sialoprotein*; *Ocn = Osteocalcin*; *Dmp1 = Dentin matrix protein 1*; *Dspp = Dentin sialophosphoprotein*.

Early and limited exposure to all concentrations of noggin decreased the extent of mineralization at day 10 as compared to control. Despite a trend for decreased mineralization in noggin-treated cultures at days 14 and 21, no statistical significance was observed.

Prior to induction of mineralization at day 7, noggin decreased the expression of *Bsp*, *Ocn*, and *Dmp1* in a concentration-dependent manner as compared to control. No *Dspp* expression was detected under any treatment. After induction of mineralization, noggin significantly decreased expression of *Dspp* at day 10 and expression of *Ocn* at day 21 as compared to control. Noggin exerted no significant effects on expression of *Bsp* and *Dmp1* at days 10-21 as compared to control.

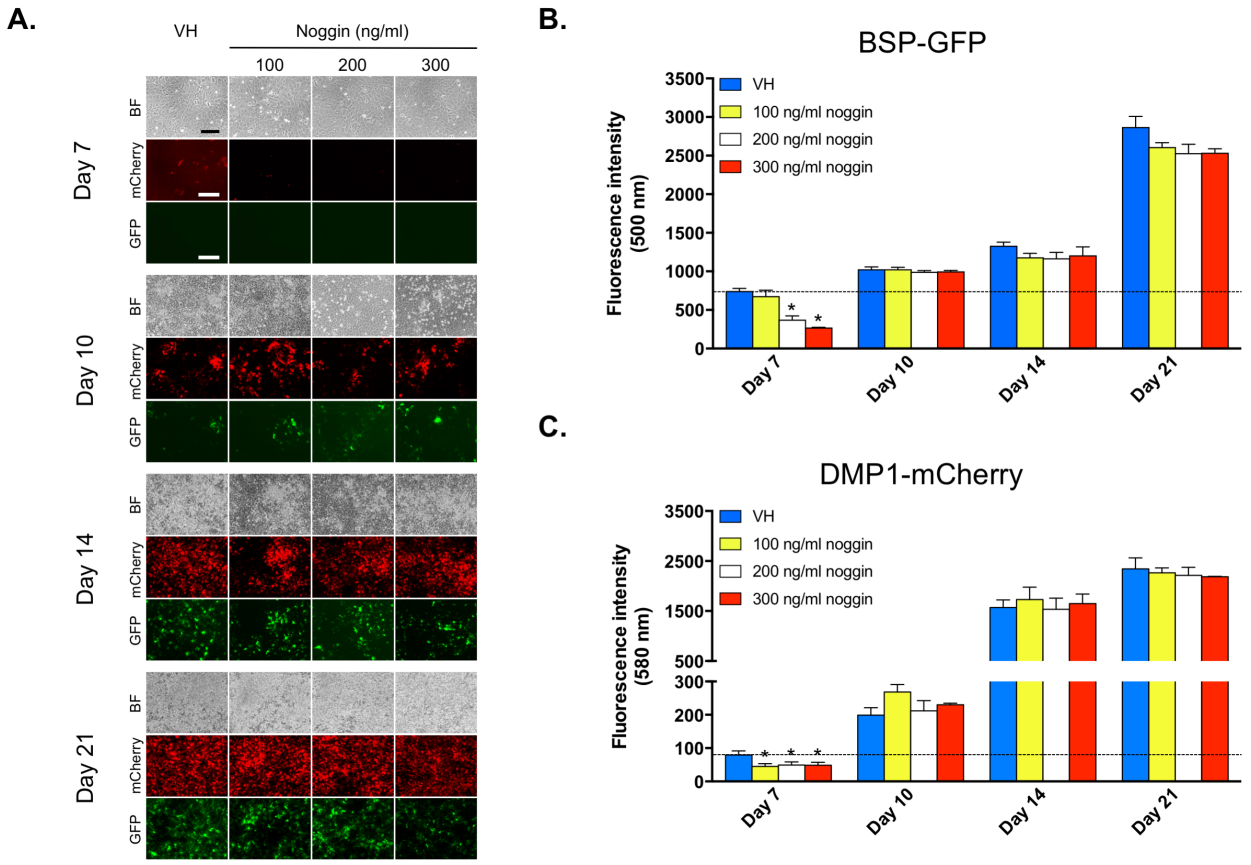

Figure 14. Effects of early and limited exposure to noggin on the expression of BSP-GFP and DMP1-mCherry transgenes in primary dental pulp cultures.

Primary dental pulp cultures derived from 5-7-day-old BSP-GFP and DMP1-mCherry transgenic mice were treated with VH or noggin between days 3-7 as described for Figure 13.

A. Representative images of the same areas in cultures at different time points analyzed under brightfield (BF, upper rows) and epifluorescent light using the mCherry filter for detection of DMP1-mCherry (mCherry, middle row) and GFPtpz filter for detection of BSP-GFP (GFP, lower row).

B. The histogram represents epifluorescence intensity of BSP-GFP transgene at various time points.

C. The histogram represents epifluorescence intensity of DMP1-mCherry transgene at various time points.

Results represent mean ± SEM of at least three independent experiments; $*p \leq 0.05$ relative to control at each time point.

Early and limited exposure to noggin significantly decreased the intensity of expression of BSP-GFP and DMP1-mCherry transgene at day7 but not at later time points as compared to control.

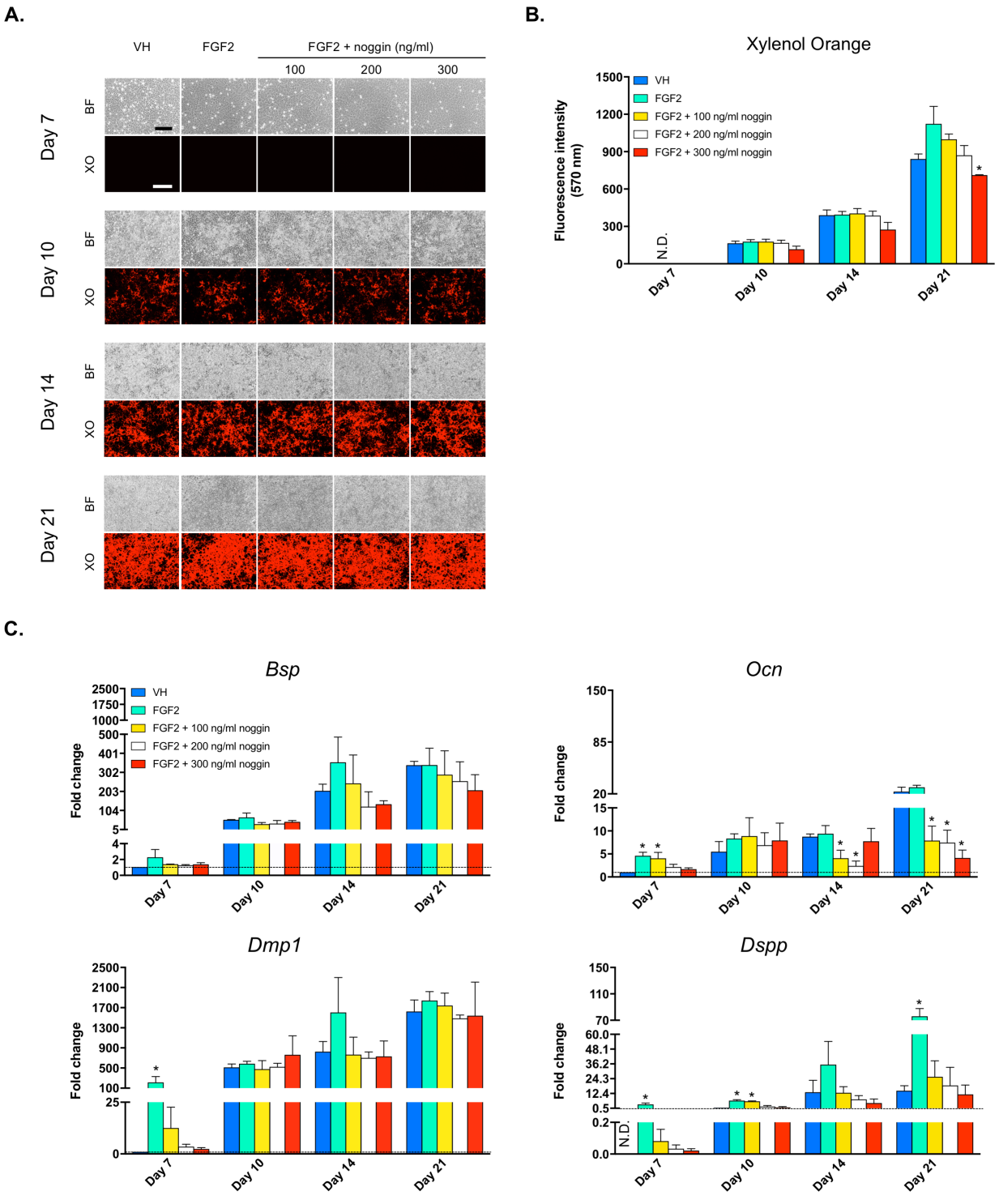

Figure 15. Effects of early and limited exposure to noggin in the presence of FGF2 on the extent of mineralization and expression of markers of mineralization and odontoblast differentiation in primary dental pulp cultures.

Primary dental pulp cultures derived from 5-7-day-old pups were treated with VH or 100, 200,

and 300 ng/ml noggin in the presence of 20 ng/ml FGF2 between days 3-7 as described in the Materials and Methods. Starting day 7 all cultures were grown under the control mineralization-inducing culture conditions for additional 14 days (until day 21 of the culture).

A. Representative images of the same areas in cultures at various time points analyzed under brightfield (BF, upper row), and epifluorescent light using TRITC Red filter for detection of Xylenol Orange staining (XO, lower row). Scale bar is 200 μm.

B. The histogram represents the intensity of XO staining at various time points.

C. Quantitative PCR (qPCR) analysis of expression of *Bsp*, *Ocn*, *Dmp1*, and *Dspp*.

Results represent mean ± SEM of at least three independent experiments; $*p \leq 0.05$ relative to control at each time point.

Expression levels of *Bsp*, *Osteocalcin*, and *Dmp1* were normalized to VH at day 7, which is arbitrarily set to 1 and is indicated by the dashed line. Expression levels of *Dspp* were normalized to VH at day 10, which is arbitrarily set to 1 and is indicated by the dashed line.

N.D. = not detected; VH = vehicle; FGF2 = Fibroblast Growth Factor 2; *Bsp = Bone sialoprotein*; *Ocn = Osteocalcin*; *Dmp1 = Dentin matrix protein 1*; *Dspp = Dentin sialophosphoprotein*.

Early and limited exposure to FGF2 significantly increased the extent of mineralization at day 21 as compared to control. In the presence of FGF2, noggin decreased the extent of mineralization at day 21 and at 300 ng/ml concentration only.

Prior to induction of mineralization at day 7, FGF2 markedly increased the expression of *Bsp*, *Dmp1*, *Ocn*, and *Dspp* as compared to control. After induction of mineralization, FGF2 increased expression of *Dspp* at days 10-21 but did not have significant effects on expression of other transcripts. In the presence of FGF2, noggin decreased expression of *Bsp*, *Dmp1*, *Ocn*, and *Dspp* at day 7, and expression of *Dspp* at days 14-21 and expression of *Ocn* at days 14 and 21 as compared to control.

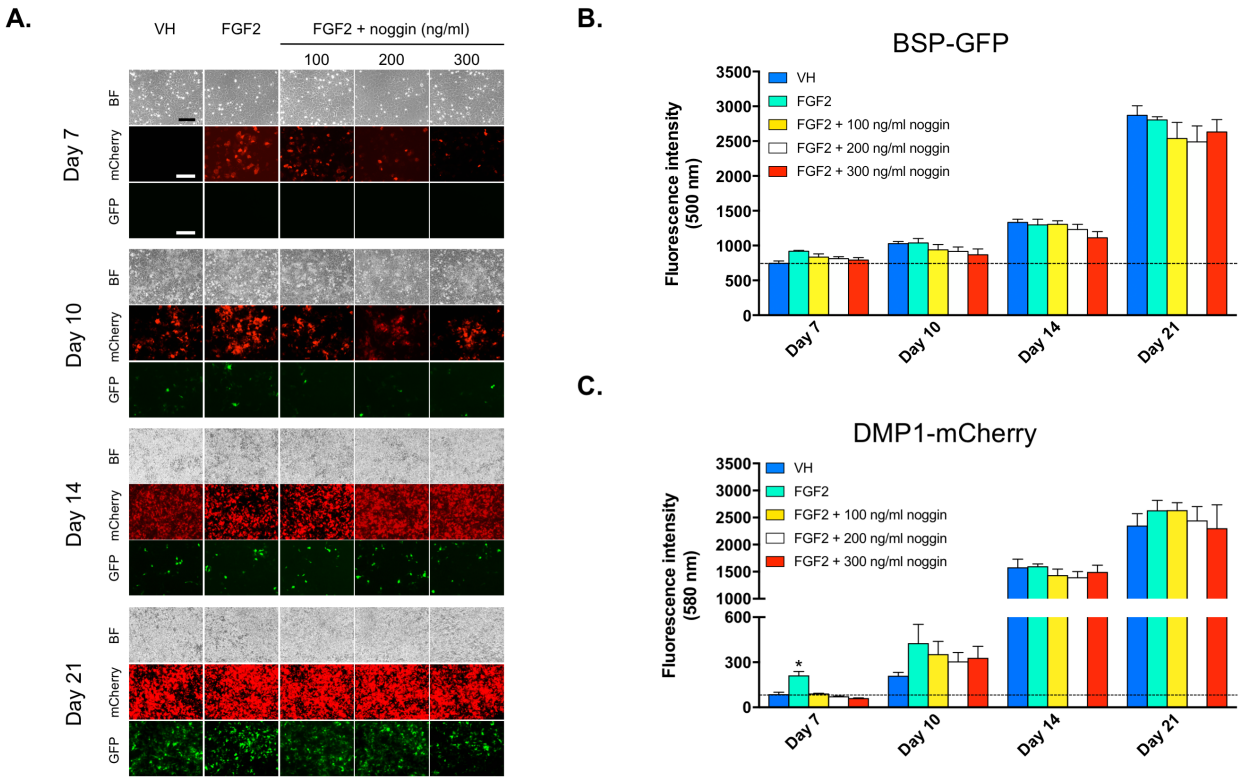

Figure 16. Effects of early and limited exposure to noggin in the presence of FGF2 on the expression of BSP-GFP and DMP1-mCherry transgenes in primary dental pulp cultures.

Primary dental pulp cultures derived from 5-7-day-old BSP-GFP and DMP1-mCherry transgenic mice were treated with VH or noggin between days 3-7 as described for Figure 11.

A. Representative images of the same areas in cultures at different time points analyzed under brightfield (BF, upper rows) and epifluorescent light using the mCherry filter for detection of DMP1-mCherry (mCherry, middle row) and the GFPtpz filter for detection of BSP-GFP (GFP, lower row).

B. The histogram represents epifluorescence intensity of BSP-GFP transgene at various time points.

C. The histogram represents epifluorescence intensity of DMP1-mCherry transgene at various time points.

Results represent mean ± SEM of at least three independent experiments; *$p \leq 0.05$ relative to control at each time point.

Prior to induction of mineralization, FGF2 significantly increased the intensity of expression of BSP-GFP and DMP1-mCherry as compared to control. Noggin decreased FGF2-induced increases in the intensity of both transgenes to the level comparable with that in control. After induction of mineralization, all cultures had similar levels of intensity of both transgenes.

Table 2. Concentration-dependent effects of early and limited exposure to BMP2 on intensity of Xylenol Orange staining in primary dental pulp cultures.

Treatment		Day of culture	Epifluorescence intensity	Fold change
Control		**7**	N.D.	–
		10	168.75 ± 15.28	1
		14	336.20 ± 28.15	1
		21	814.33 ± 26.99	1
BMP2 (ng/ml)	**10**	**7**	N.D.	–
		10	186.25 ± 10.66	~1.10
		14	367.80 ± 36.24	~1.09
		21	810.58 ± 57.90	~1.00
	20	**7**	N.D.	–
		10	170.25 ± 10.79	~1.01
		14	325.00 ± 33.13	~0.97
		21	812.58 ± 79.25	~1.00
	50	**7**	N.D.	–
		10	124.00 ± 10.55	~0.73
		14	310.20 ± 26.98	~0.92
		21	769.08 ± 52.27	~0.94

Results are expressed in absolute values and represent mean ± SEM of at least three independent experiments; $*p \leq 0.05$ relative to control at each time point. Fold changes represent BMP2 value divided by the control value for each time point.

N.D. = not detected.

Table 3. Concentration-dependent effects of early and limited exposure to BMP2 on the expression of markers of mineralization and odontoblast differentiation in primary dental pulp cultures.

Control

		Gene	CT	ΔCT	-ΔΔCT	Fold change
Control	**Day 7**	*Gapdh*	19.88 ± 0.42	–	–	–
		Bsp	25.70 ± 0.27	5.81 ± 0.47	0	1
		Dmp1	30.68 ± 0.44	10.79 ± 0.66	0	1
		Osteocalcin	20.90 ± 0.61	1.01 ± 0.60	0	1
		Dspp	40 ± 0.00	20.11 ± 0.41	N.D.	N.D.
	Day 10	*Gapdh*	18.91 ± 0.32	–	–	–
		Bsp	20.30 ± 0.50	1.39 ± 0.52	4.42 ± 0.20	22.01 ± 2.74
		Dmp1	21.68 ± 0.25	2.77 ± 0.22	8.02 ± 0.45	301.64 ± 94.87
		Osteocalcin	19.24 ± 0.17	0.33 ± 0.30	0.68 ± 0.47	1.91 ± 0.67
		Dspp	27.55 ± 1.55	8.64 ± 1.42	0	1
	Day 14	*Gapdh*	19.44 ± 0.81	–	–	–
		Bsp	18.39 ± 0.59	-1.04 ± 0.42	6.86 ± 0.12	117.17 ± 9.65
		Dmp1	0.28 ± 0.22	1.30 ± 0.94	9.49 ± 0.30	765.16 ± 139.55
		Osteocalcin	16.72 ± 1.02	-2.70 ± 0.63	3.72 ± 0.07	13.30 ± 0.64
		Dspp	25.52 ± 1.44	6.08 ± 0.92	2.56 ± 0.51	7.28 ± 2.84
	Day 21	*Gapdh*	20.25 ± 0.41	–	–	–
		Bsp	17.71 ± 0.06	-2.54 ± 0.36	8.35 ± 0.13	330.64 ± 31.08
		Dmp1	20.37 ± 0.82	0.12 ± 0.70	10.66 ± 0.13	1644.36 ± 134.76
		Osteocalcin	15.85 ± 0.61	-4.39 ± 0.89	5.41 ± 0.29	45.19 ± 8.54
		Dspp	24.62 ± 1.24	4.38 ± 1.39	4.26 ± 0.11	19.44 ± 1.46

BMP2-treated (10 ng/ml)

		Gene	CT	ΔCT	-ΔΔCT	Fold change
BMP2 (10 ng/ml)	**Day 7**	*Gapdh*	20.40 ± 0.21	–	–	–
		Bsp	27.30 ± 0.36	6.91 ± 0.56	-1.09 ± 0.51	0.35 ± 0.14
		Dmp1	36.05 ± 1.13	15.65 ± 0.98	-4.86 ± 1.50	0.08 ± 0.03
		Osteocalcin	22.15 ± 0.55	1.75 ± 0.46	-0.73 ± 0.16	0.61 ± 0.06
		Dspp	38.46 ± 0.55	18.06 ± 0.53	N.D.	N.D.
	Day 10	*Gapdh*	18.80 ± 0.34	–	–	–
		Bsp	19.67 ± 0.32	0.87 ± 0.55	4.94 ± 0.24	32.02 ± 4.76
		Dmp1	22.03 ± 0.34	3.23 ± 0.03	7.55 ± 0.68	254.47 ± 102.84
		Osteocalcin	19.19 ± 0.19	0.26 ± 0.50	0.75 ± 0.29	1.79 ± 0.38
		Dspp	26.81 ± 0.97	8.01 ± 1.01	0.64 ± 0.42	1.77 ± 0.50
		Gapdh	19.51 ± 0.61	–	–	–

	Day 14	*Bsp*	17.98 ± 0.71	-1.53 ± 0.42	7.34 ± 0.13	164.28 ± 15.60
		Dmp1	21.02 ± 0.16	1.51 ± 0.71	9.28 ± 0.08	625.37 ± 36.68
		Osteocalcin	16.28 ± 1.07	-3.22 ± 0.65	4.24 ± 0.13	19.19 ± 1.78
		Dspp	24.74 ± 1.57	5.23 ± 1.04	3.41 ± 0.45	12.18 ± 3.24
	Day 21	*Gapdh*	19.90 ± 0.46	–	–	–
		Bsp	17.25 ± 0.02	-2.62 ± 0.44	8.43 ± 0.09	348.00 ± 22.73
		Dmp1	19.56 ± 0.80	0.38 ± 069	10.40 ± 0.10	1368.07 ± 98.39
		Osteocalcin	15.24 ± 0.63	-4.83 ± 0.78	5.85 ± 0.29	61.32 ± 11.97
		Dspp	24.36 ± 0.87	4.05 ± 1.05	4.60 ± 0.38	27.10 ± 7.78

BMP2-treated (20 ng/ml)

		Gene	CT	ΔCT	-ΔΔCT	Fold change
BMP2 (20 ng/ml)	**Day 7**	*Gapdh*	20.00 ± 0.35	–	–	–
		Bsp	27.62 ± 0.26	7.62 ± 0.55	-1.80 ± 0.54	0.35 ± 0.14
		Dmp1	35.40 ± 0.11	15.40 ± 0.44	-4.60 ± 0.30	0.04 ± 0.00
		Osteocalcin	21.69 ± 0.52	1.69 ± 0.44	-0.67 ± 0.16	0.63 ± 0.07
		Dspp	36.37 ± 0.90	16.37 ± 0.90	N.D.	N.D.
	Day 10	*Gapdh*	18.83 ± 0.35	–	–	–
		Bsp	20.04 ± 0.62	1.21 ± 0.75	4.60 ± 0.49	28.12 ± 7.18
		Dmp1	22.57 ± 0.46	3.74 ± 0.24	7.06 ± 0.88	210.40 ± 96.01
		Osteocalcin	18.91 ± 0.08	0.08 ± 0.38	0.94 ± 0.40	2.17 ± 0.66
		Dspp	26.73 ± 0.91	7.90 ± 0.96	0.75 ± 0.48	2.01 ± 0.73
	Day 14	*Gapdh*	19.46 ± 0.62	–	–	–
		Bsp	18.05 ± 0.52	-1.41 ± 0.51	7.22 ± 0.05	149.93 ± 6.07
		Dmp1	21.21 ± 0.30	1.75 ± 0.72	9.04 ± 0.08	528.74 ± 29.72
		Osteocalcin	15.82 ± 0.97	-3.63 ± 0.68	4.65 ± 0.14	25.62 ± 2.55
		Dspp	24.68 ± 1.61	5.22 ± 1.22	3.42 ± 0.28	11.30 ± 1.92
	Day 21	*Gapdh*	20.10 ± 0.45	–	–	–
		Bsp	17.24 ± 0.05	-2.82 ± 0.43	8.64 ± 0.18	408.14 ± 53.70
		Dmp1	19.79 ± 0.76	0.34 ± 0.61	10.45 ± 0.07	1402.17 ± 71.97
		Osteocalcin	15.00 ± 0.50	-5.23 ± 0.71	6.25 ± 0.22	79.01 ± 12.26
		Dspp	24.01 ± 0.99	3.65 ± 1.05	4.99 ± 0.39	35.44 ± 9.11

BMP2-treated (50 ng/ml)

		Gene	CT	ΔCT	-ΔΔCT	Fold change
BMP2 (50 ng/ml)	**Day 7**	*Gapdh*	19.88 ± 0.28	–	–	–
		Bsp	28.21 ± 0.11	8.33 ± 0.30	-2.51 ± 0.22	0.18 ± 0.02
		Dmp1	34.59 ± 0.18	15.07 ± 0.19	-4.28 ± 4.46	0.05 ± 0.01
		Osteocalcin	22.04 ± 0.49	2.16 ± 0.45	-1.14 ± 0.15	0.46 ± 0.04
		Dspp	35.55 ± 0.53	15.67 ± 0.66	N.D.	N.D.

	Day 10	*Gapdh*	19.20 ± 0.39	–	–	–
		Bsp	20.45 ± 0.27	1.25 ± 0.47	4.56 ± 0.06	23.70 ± 1.07
		Dmp1	22.96 ± 0.64	3.77 ± 0.38	7.02 ± 1.02	232.92 ± 115.35
		Osteocalcin	19.13 ± 0.38	-0.06 ± 0.13	1.08 ± 0.72	2.96 ± 1.25
		Dspp	26.42 ± 1.35	7.23 ± 1.28	1.42 ± 0.16	2.72 ± 0.30
	Day 14	*Gapdh*	19.67 ± 0.57	–	–	–
		Bsp	18.29 ± 0.51	-1.38 ± 0.41	7.19 ± 0.10	146.64 ± 6.21
		Dmp1	21.73 ± 0.19	2.06 ± 0.57	8.73 ± 0.10	428.15 ± 32.26
		Osteocalcin	15.71 ± 1.04	-3.95 ± 0.64	4.97 ± 0.21	32.48 ± 5.13
		Dspp	24.31 ± 1.63	4.64 ± 1.25	4.01 ± 0.18	16.48 ± 2.20
	Day 21	*Gapdh*	20.22 ± 0.45	–	–	–
		Bsp	17.49 ± 0.17	-2.72 ± 0.41	8.53 ± 0.17	378.66 ± 47.27
		Dmp1	20.26 ± 1.06	0.59 ± 0.69	10.20 ± 0.04	1175.12 ± 33.56
		Osteocalcin	14.47 ± 0.27	-5.78 ± 0.60	6.80 ± 0.23	116.59 ± 20.34
		Dspp	23.40 ± 1.06	2.77 ± 1.28	5.87 ± 0.26	61.55 ± 10.06

Results represent mean ± SEM of at least three independent experiments; *$p \leq 0.05$ relative to the control at each time point.

All fold changes are relative to VH at day 7, except *Dspp*, which is relative to VH at day 10.

N.D. = not detected.

Table 4. Time-dependent effects of early and limited exposure to FGF2 on expression of *Bsp*, *Dmp1*, *Dspp,* and *Runx2* in primary dental pulp cultures.

Control

Hours after treatment	Gene	CT	ΔCT	-ΔΔCT	Fold change
0	*Gapdh*	19.49 ± 0.14	–	–	–
	Dmp1	33.11 ± 0.57	13.62 ± 0.44	0	1
	Dspp	37.47 ± 1.65	17.98 ± 1.78	–	N.D.
	Bsp	28.22 ± 0.01	8.73 ± 0.13	0	1
	Runx2	25.99 ± 0.02	7.88 ± 0.86	0	1
12	*Gapdh*	19.59 ± 0.27	–	–	–
	Dmp1	32.18 ± 0.60	12.60 ± 0.33	1.03 ± 0.11	2.05 ± 0.15
	Dspp	36.73 ± 0.95	17.14 ± 1.22	–	N.D.
	Bsp	25.97 ± 0.36	6.38 ± 0.09	2.35 ± 0.22	5.20 ± 0.77
	Runx2	24.33 ± 0.02	6.47 ± 0.93	1.41 ± 0.07	2.66 ± 0.13
24	*Gapdh*	19.98 ± 0.40	–	–	–
	Dmp1	32.17 ± 1.06	12.18 ± 0.66	1.44 ± 0.22	2.77 ± 0.41
	Dspp	36.85 ± 0.48	16.87 ± 0.88	–	N.D.
	Bsp	26.27 ± 0.02	6.29 ± 0.38	2.44 ± 0.25	5.59 ± 0.95
	Runx2	24.55 ± 0.14	6.22 ± 0.91	1.66 ± 0.05	3.16 ± 0.10
36	*Gapdh*	19.68 ± 0.29	–	–	–
	Dmp1	31.82 ± 0.94	12.14 ± 0.66	1.48 ± 0.21	2.86 ± 0.42
	Dspp	37.42 ± 0.32	17.74 ± 0.03	–	N.D.
	Bsp	25.49 ± 0.19	5.81 ± 0.48	2.92 ± 0.35	8.01 ± 1.87
	Runx2	24.31 ± 0.03	6.16 ± 0.83	1.72 ± 0.03	3.29 ± 0.08
48	*Gapdh*	19.72 ± 0.23	–	–	–
	Dmp1	31.55 ± 0.73	11.84 ± 0.50	1.79 ± 0.06	3.45 ± 0.15
	Dspp	37.34 ± 0.43	17.62 ± 0.20	–	N.D.
	Bsp	24.90 ± 0.03	5.19 ± 0.20	3.54 ± 0.07	11.67 ± 0.56
	Runx2	24.32 ± 0.07	6.00 ± 0.83	1.88 ± 0.03	3.68 ± 0.08
72	*Gapdh*	19.34 ± 0.04	–	–	–
	Dmp1	30.03 ± 0.72	10.69 ± 0.77	2.93 ± 0.33	8.05 ± 1.78
	Dspp	35.67 ± 0.36	16.32 ± 0.40	–	N.D.
	Bsp	23.96 ± 0.27	4.23 ± 0.23	4.50 ± 0.10	22.73 ± 1.52
	Runx2	24.57 ± 0.12	5.98 ± 0.70	1.90 ± 0.16	3.79 ± 0.43
	Gapdh	19.95 ± 0.34	–	–	–
	Dmp1	28.47 ± 0.73	8.52 ± 0.39	5.11 ± 0.05	34.46 ± 1.20

96	*Dspp*	36.84 ± 1.31	16.89 ± 0.97	–	N.D.
	Bsp	23.93 ± 0.08	4.09 ± 0.26	4.64 ± 0.13	25.09 ± 2.28
	Runx2	24.41 ± 0.01	5.97 ± 0.73	1.91 ± 0.13	3.80 ± 0.35

BMP2 (50 ng/ml)

Hours after treatment	Gene	CT	ΔCT	-ΔΔCT	Fold change
0	*Gapdh*	19.49 ± 0.14	–	–	–
	Dmp1	33.11 ± 0.57	13.62 ± 0.44	0	1
	Dspp	37.47 ± 1.65	17.98 ± 1.78	–	N.D.
	Bsp	28.22 ± 0.01	8.73 ± 0.13	0	1
	Runx2	25.99 ± 0.02	7.88 ± 0.86	0	1
12	*Gapdh*	19.91 ± 0.11	–	–	–
	Dmp1	32.22 ± 0.65	12.31 ± 0.55	1.31 ± 0.11	2.50 ± 0.19
	Dspp	34.20 ± 0.27	14.28 ± 0.38	-2.81 ± 0.35	0.15 ± 0.04
	Bsp	26.64 ± 0.07	6.72 ± 0.04	2.01 ± 0.09	4.04 ± 0.26
	Runx2	24.32 ± 0.16	6.03 ± 1.20	1.85 ± 0.34	3.81 ± 0.87
24	*Gapdh*	19.65 ± 0.30	–	–	–
	Dmp1	32.44 ± 0.98	12.79 ± 0.68	0.83 ± 0.24	1.83 ± 0.30
	Dspp	32.41 ± 0.17	12.76 ± 0.47	-1.28 ± 0.26	0.43 ± 0.07
	Bsp	26.48 ± 0.02	6.84 ± 0.28	1.89 ± 0.15	3.76 ± 0.40
	Runx2	24.24 ± 0.12	5.87 ± 0.72	2.01 ± 0.14	4.07 ± 0.39
36	*Gapdh*	19.98 ± 0.05	–	–	–
	Dmp1	33.12 ± 1.16	13.14 ± 1.11	0.49 ± 0.67	1.71 ± 0.70
	Dspp	32.12 ± 0.57	12.15 ± 0.62	-0.67 ± 0.11	0.63 ± 0.05
	Bsp	26.42 ± 0.15	6.45 ± 0.20	2.28 ± 0.07	4.87 ± 0.24
	Runx2	24.11 ± 0.14	5.49 ± 1.05	2.39 ± 0.19	5.33 ± 0.70
48	*Gapdh*	19.48 ± 0.25	–	–	–
	Dmp1	33.58 ± 1.26	13.10 ± 1.51	0.53 ± 1.07	2.31 ± 1.28
	Dspp	32.03 ± 0.58	12.54 ± 0.33	-1.07 ± 0.40	0.52 ± 0.14
	Bsp	24.47 ± 0.40	6.42 ± 0.15	2.31 ± 0.02	4.94 ± 0.07
	Runx2	24.09 ± 0.15	5.47 ± 1.00	2.41 ± 0.14	5.38 ± 0.53
72	*Gapdh*	19.94 ± 0.38	–	–	–
	Dmp1	32.71 ± 0.92	12.78 ± 0.54	0.85 ± 0.11	1.81 ± 0.13
	Dspp	31.96 ± 0.40	12.02 ± 0.77	-0.54 ± 0.04	0.69 ± 0.02
	Bsp	26.26 ± 0.03	6.37 ± 0.35	2.36 ± 0.22	5.26 ± 0.77
	Runx2	23.94 ± 0.13	5.14 ± 0.55	2.74 ± 0.31	6.98 ± 1.44
	Gapdh	19.83 ± 0.21	–	–	–

96	*Dmp1*	33.99 ± 1.04	14.16 ± 0.83	-0.54 ± 0.39	0.74 ± 0.19
	Dspp	31.31 ± 0.52	11.48 ± 0.73	0	1
	Bsp	26.82 ± 0.11	7.15 ± 0.11	1.58 ± 0.02	3.00 ± 0.05
	Runx2	23.85 ± 0.18	5.08 ± 0.71	2.80 ± 0.15	7.06 ± 0.73

Expression of *Dmp1* was normalized to untreated sample at 0 hrs, whereas *Dspp* was normalized to BMP2-treated cultures at 96 hrs.

Results represent mean ± SEM of at least three independent experiments; $*p \leq 0.05$ relative to control at each time point.

N.D. = not detected.

Table 5. Concentration-dependent effects of early and limited exposure to BMP2 on expression of BSP-GFP transgene in primary dental pulp cultures.

Treatment		Day of culture	BSP-GFP	Fold change
Control		**7**	271.00 ± 23.96	1
		10	852.00 ± 62.44	1
		14	1199.00 ± 86.31	1
		21	2345.00 ± 56.40	1
BMP2 (ng/ml)	10	**7**	225.00 ± 36.38	~0.83
		10	800.00 ± 58.73	~0.94
		14	1236.00 ± 69.72	~1.03
		21	2612.00 ± 32.29	~1.07
	20	**7**	176.00 ± 72.02	~0.65
		10	795.00 ± 34.48	~0.93
		14	1186.00 ± 64.63	~0.99
		21	2526.00 ± 41.62	~1.02
	50	**7**	154.00 ± 52.29	~0.57
		10	796.00 ± 34.88	~0.93
		14	1221.00 ± 78.59	~1.02
		21	2525.00 ± 87.31	~1.03

Results are expressed in absolute values and represent mean ± SEM of at least three independent experiments; $*p \leq 0.05$ relative to control at each time point. Fold changes represent BMP2 value divided by the control value for each respective time point.

Table 6. Concentration-dependent effects of early and limited exposure to BMP2 on expression of DMP1-mCherry transgene in primary dental pulp cultures.

Treatment		Day of culture	DMP1-mCherry	Fold change
Control		**7**	85.00 ± 16.57	1
		10	196.00 ± 1.22	1
		14	1747.00 ± 90.68	1
		21	2324.00 ± 228.22	1
BMP2 (ng/ml)	10	**7**	81.00 ± 12.50	~0.96
		10	118.00 ± 8.59	~0.60
		14	1409.00 ± 121.03	~0.81
		21	2206.00 ± 300.49	~0.95
	20	**7**	68.00 ± 10.52	~0.80
		10	88.00 ± 11.54	~0.45
		14	1240.00 ± 145.34	~0.71
		21	2016.00 ± 299.17	~0.87
	50	**7**	19.00 ± 11.33	~0.23
		10	92.00 ± 14.63	~0.47
		14	966.00 ± 122.13	~0.55
		21	1793.00 ± 314.58	~0.77

Results are expressed in absolute values and represent mean ± SEM of at least three independent experiments; $*p \leq 0.05$ relative to control at each time point. Fold changes represent BMP2 value divided by the control value for each respective time point.

Table 7. Effects of early and limited exposure to BMP2 on epifluorescence intensity of DSPP-Cerulean transgene in primary dental pulp cultures.

Day of the culture	VH	BMP2 (d3-7)	Fold change
7	1582.00 ± 91.95	1986.00 ± 93.91	~1.26
10	2199.00 ± 31.41	2659.00 ± 11.34	~1.21
14	2501.00 ± 135.41	3601.00 ± 83.20	~1.44
21	3872.00 ± 68.59	5551.00 ± 172.59	~1.43

Results are expressed in absolute values and represent mean ± SEM of at least three independent experiments; *$p \leq 0.05$ relative to control at each time point. Fold changes represent BMP2 value divided by the control value for each respective time point.

Table 8. Effects of late and limited exposure to BMP2 on intensity of Xylenol Orange staining in primary dental pulp cultures.

Day of the culture	VH	BMP2 (d7-21)	Fold change
7	N.D.	N.D.	–
10	119.34 ± 7.86	178.00 ± 10.20	~1.49
14	574.00 ± 29.32	652.00 ± 53.62	~1.14
21	940.00 ± 30.28	1093.00 ± 57.54	~1.16

Results are expressed in absolute values and represent mean ± SEM of at least three independent experiments; *$p \leq 0.05$ relative to control at each time point. Fold changes represent BMP2 value divided by the control value for each time point.

N.D. = not detected.

Table 9. Effects of late and limited exposure to BMP2 on the expression of markers of mineralization and osteoblast differentiation in primary BMSC cultures.

Control

Day of the culture	Gene	CT	ΔCT	-ΔΔCT	Fold change
7	*Gapdh*	19.05 ± 0.21	–	–	–
	Bsp	25.21 ± 0.17	6.16 ± 0.11	0	1
	Dmp1	32.50 ± 0.12	13.45 ± 0.11	0	1
	Dspp	38.52 ± 0.47	19.47 ± 0.47	N.D.	N.D.
	Osteocalcin	20.56 ± 0.36	1.51 ± 0.20	0	1
10	*Gapdh*	19.13 ± 0.10	–	–	–
	Bsp	20.20 ± 0.10	1.07 ± 0.21	5.09 ± 0.21	35.18 ± 4.72
	Dmp1	24.88 ± 0.46	5.75 ± 0.37	7.69 ± 0.27	219.59 ± 43.15
	Dspp	27.60 ± 0.34	8.47 ± 0.44	0	1
	Osteocalcin	19.09 ± 0.06	-0.03 ± 0.15	1.55 ± 0.04	2.93 ± 0.09
14	*Gapdh*	18.50± 0.15	–	–	–
	Bsp	18.02 ± 0.03	-0.47 ± 0.18	6.63 ± 0.27	105.21 ± 20.46
	Dmp1	23.52 ± 0.53	5.02 ± 0.38	8.42 ± 0.27	363.75 ± 72.88
	Dspp	25.12 ± 0.37	6.62 ± 0.52	1.84 ± 0.10	3.63 ± 0.27
	Osteocalcin	16.86 ± 0.06	-1.63 ± 0.13	3.15 ± 0.21	9.21 ± 1.30
21	*Gapdh*	19.07 ± 0.12	–	–	–
	Bsp	17.61 ± 0.06	-1.45 ± 0.10	7.61 ± 0.11	198.43 ± 15.48
	Dmp1	21.84 ± 0.26	2.77 ± 0.16	10.68 ± 0.12	1660.43 ± 150.68
	Dspp	24.87 ± 0.37	5.79 ± 0.49	2.67 ± 0.07	6.40 ± 0.36
	Osteocalcin	14.63 ± 0.21	-4.44 ± 0.33	5.95 ± 0.19	63.74 ± 8.18

BMP2-treated between d7-21

Day of the culture	Gene	CT	ΔCT	-ΔΔCT	Fold change
7	*Gapdh*	19.05 ± 0.21	–	–	–
	Bsp	25.21 ± 0.17	6.16 ± 0.11	0	1
	Dmp1	32.50 ± 0.12	13.45 ± 0.11	0	1
	Dspp	38.52 ± 0.47	19.47 ± 0.47	N.D.	N.D.
	Osteocalcin	20.56 ± 0.36	1.51 ± 0.20	0	1
10	*Gapdh*	18.36 ± 0.11	–	–	–
	Bsp	20.26 ± 0.23	1.89 ± 0.11	4.26 ± 0.17	19.70 ± 2.47
	Dmp1	25.25 ± 0.37	6.88 ± 0.48	6.56 ± 0.40	105.29 ± 25.22
	Dspp	26.19 ± 0.45	7.82 ± 0.33	0.64 ± 0.12	1.58 ± 0.13

	Osteocalcin	18.48 ± 0.23	0.11 ± 0.11	1.40 ± 0.15	2.68 ± 0.27
14	*Gapdh*	18.21 ± 0.10	–	–	–
	Bsp	18.84 ± 0.25	0.63 ± 0.15	5.52 ± 0.11	46.45 ± 3.79
	Dmp1	23.88 ± 0.43	5.67 ± 0.36	7.77 ± 0.24	229.43 ± 41.69
	Dspp	24.12 ± 0.47	5.91 ± 0.53	2.55 ± 0.08	5.90 ± 0.34
	Osteocalcin	16.17 ± 0.24	-2.03 ± 0.34	3.54 ± 0.30	12.57 ± 2.90
21	*Gapdh*	19.05 ± 0.10	–	–	–
	Bsp	18.16 ± 0.15	-0.88 ± 0.14	7.04 ± 0.13	133.91 ± 11.89
	Dmp1	23.06 ± 0.33	4.01 ± 0.23	9.43 ± 0.11	699.56 ± 59.37
	Dspp	23.57 ± 0.40	4.52 ± 0.48	3.94 ± 0.50	15.43 ± 0.54
	Osteocalcin	14.33 ± 0.29	-4.71 ± 0.37	6.23 ± 0.23	78.27 ± 12.14

All fold changes are relative to control at day 7, except for *Dspp*, which is relative to VH at day 10. Results represent mean ± SEM of at least three independent experiments; *$p \leq 0.05$ relative to control at each time point.

N.D. = not detected.

Table 10. Concentration-dependent effects of early and limited exposure to BMP2 on intensity of Xylenol Orange staining in primary BMSC cultures.

Treatment		Day of culture	Xylenol Orange	Fold change
Control		**7**	N.D.	–
		10	402.00 ± 23.12	1
		14	849.00 ± 47.81	1
		21	1057.00 ± 38.77	1
BMP2 (ng/ml)	10	**7**	N.D.	–
		10	392.00 ± 15.31	~0.97
		14	718.00 ± 39.57	~0.87
		21	1002.00 ± 66.62	~0.95
	20	**7**	N.D.	–
		10	384.00 ± 13.87	~0.95
		14	718.00 ± 39.57	~0.85
		21	986.00 ± 67.93	~0.93
	50	**7**	N.D.	–
		10	327.00 ± 18.00	~0.81
		14	619.00 ± 47.93	~0.73
		21	968.00 ± 80.05	~0.92

Results are expressed in absolute values and represent mean ± SEM of at least three independent experiments; $*p \leq 0.05$ relative to control at each time point. Fold changes represent BMP2 value divided by the control value for each respective time point.

N.D. = not detected.

Table 11. Effects of early and limited exposure to BMP2 on the expression of markers of mineralization and osteoblast differentiation in primary BMSC cultures.

Control

		Gene	CT	ΔCT	-ΔΔCT	Fold change
Control	**Day 7**	*Gapdh*	20.78 ± 0.15	–	–	–
		Bsp	19.35 ± 0.03	-1.43 ± 0.15	0	1
		Dmp1	29.05 ± 0.85	8.26 ± 0.67	0	1
	Day 10	*Gapdh*	21.61 ± 0.22	–	–	–
		Bsp	28.29 ± 0.81	-3.31 ± 0.54	1.88 ± 0.39	3.95 ± 1.02
		Dmp1	23.22 ± 0.23	1.62 ± 0.49	6.65 ± 0.18	101.82 ± 12.32
	Day 14	*Gapdh*	21.48 ± 0.74	–	–	–
		Bsp	17.96 ± 1.14	-3.52 ± 0.24	2.09 ± 0.09	4.27 ± 0.25
		Dmp1	22.45 ± 0.28	0.97 ± 0.62	7.30 ± 0.05	157.36 ± 5.56
	Day 21	*Gapdh*	20.70 ± 0.27	–	–	–
		Bsp	16.17 ± 0.01	-4.52 ± 0.32	3.09 ± 0.17	8.63 ± 1.03
		Dmp1	21.01 ± 0.93	0.31 ± 0.59	7.95 ± 0.08	247.96 ± 12.92

BMP2-treated (10 ng/ml)

		Gene	CT	ΔCT	-ΔΔCT	Fold change
BMP2 (10 ng/ml)	**Day 7**	*Gapdh*	21.04 ± 0.18	–	–	–
		Bsp	19.65 ± 0.44	-1.38 ± 0.22	-0.05 ± 0.07	0.97 ± 0.04
		Dmp1	30.20 ± 0.34	9.16 ± 0.56	-0.90 ± 0.10	0.54 ± 0.04
	Day 10	*Gapdh*	21.32 ± 0.30	–	–	–
		Bsp	18.48 ± 1.20	-2.84 ± 0.83	1.40 ± 0.68	3.25 ± 1.34
		Dmp1	23.81 ± 0.16	2.49 ± 0.52	5.77 ± 0.15	55.16 ± 5.64
	Day 14	*Gapdh*	19.77 ± 0.16	–	–	–
		Bsp	16.91 ± 0.27	-2.87 ± 0.07	1.43 ± 0.08	2.71 ± 0.15
		Dmp1	22.64 ± 0.39	2.87 ± 0.19	5.39 ± 0.86	57.60 ± 27.90
	Day 21	*Gapdh*	20.11 ± 0.32	–	–	–
		Bsp	16.32 ± 0.23	-3.79 ± 0.17	2.36 ± 0.02	5.13 ± 0.15
		Dmp1	22.32 ± 0.48	2.22 ± 0.19	6.05 ± 0.58	77.39 ± 28.32

BMP2-treated (20 ng/ml)

		Gene	CT	ΔCT	-ΔΔCT	Fold change
BMP2 (20 ng/ml)	**Day 7**	*Gapdh*	20.86 ± 0.18	–	–	–
		Bsp	19.83 ± 0.45	-1.04 ± 0.23	-0.40 ± 0.07	0.76 ± 0.04
		Dmp1	30.39 ± 0.40	9.53 ± 0.62	-1.26 ± 0.05	0.42 ± 0.02

	Day 10	*Gapdh*	21.06 ± 0.15	–	–	–
		Bsp	18.14 ± 0.76	-2.92 ± 0.95	1.49 ± 0.80	3.72 ± 1.72
		Dmp1	24.22 ± 0.11	3.16 ± 0.30	5.11 ± 0.97	51.37 ± 26.95
	Day 14	*Gapdh*	19.98 ± 0.15	–	–	–
		Bsp	16.84 ± 0.11	-3.14 ± 0.29	1.71 ± 0.14	3.30 ± 0.31
		Dmp1	22.96 ± 0.09	2.98 ± 0.27	5.29 ± 0.94	56.64 ± 29.02
	Day 21	*Gapdh*	20.08 ± 0.20	–	–	–
		Bsp	16.32 ± 0.14	-3.77 ± 0.10	2.34 ± 0.05	5.06 ± 0.18
		Dmp1	22.88 ± 0.10	2.79 ± 0.14	5.47 ± 0.81	59.28 ± 27.79

BMP2-treated (50 ng/ml)

BMP2 (50 ng/ml)		Gene	CT	ΔCT	-ΔΔCT	Fold change
	Day 7	*Gapdh*	21.07 ± 0.23	–	–	–
		Bsp	20.47 ± 0.53	-0.60 ± 0.25	-0.83 ± 0.10	0.56 ± 0.04
		Dmp1	30.26 ± 0.63	9.19 ± 0.90	-0.92 ± 0.23	0.54 ± 0.09
	Day 10	*Gapdh*	20.49 ± 0.22	–	–	–
		Bsp	18.04 ± 1.10	-2.45 ± 0.83	1.01 ± 0.68	2.49 ± 1.03
		Dmp1	24.77 ± 0.31	4.28 ± 0.04	3.99 ± 0.71	19.84 ± 8.45
	Day 14	*Gapdh*	20.07 ± 0.07	–	–	–
		Bsp	17.20 ± 0.38	-2.87 ± 0.47	1.44 ± 0.32	2.85 ± 0.61
		Dmp1	23.50 ± 0.58	3.42 ± 0.66	4.84 ± 1.33	56.76 ± 34.63
	Day 21	*Gapdh*	19.78 ± 0.09	–	–	–
		Bsp	16.43 ± 0.30	-3.35 ± 0.19	1.92 ± 0.35	4.00 ± 0.92
		Dmp1	22.99 ± 0.12	3.21 ± 0.02	5.06 ± 0.65	40.34 ± 16.07

Results represent mean ± SEM of at least three independent experiments; *$p \leq 0.05$ relative to the control at each time point.

All fold changes are relative to VH at day 7.

N.D. = not detected.

Table 12. Concentration-dependent effects of early and limited exposure to BMP2 on intensity of BSP-GFP transgene in primary BMSC cultures.

Treatment		Day of culture	Xylenol Orange	Fold change
Control		7	4430.00 ± 204.78	1
		10	17303.00 ± 829.56	1
		14	20095.00 ± 799.77	1
		21	28905.00 ± 257.47	1
BMP2 (ng/ml)	10	7	3939.00 ± 78.79	–
		10	12533.00 ± 282.08	~0.89
		14	15505.00 ± 458.05	~0.72
		21	26988.00 ± 331.55	~0.93
	20	7	3331.00 ± 311.62	–
		10	11206.00 ± 285.93	~0.75
		14	13377.00 ± 523.14	~0.67
		21	26957.00 ± 253.39	~0.93
	50	7	2091.00 ± 111.92	–
		10	10257.00 ± 646.65	~0.47
		14	12190.00 ± 350.99	~0.61
		21	22764.00 ± 62.60	~0.79

Results are expressed in absolute values and represent mean ± SEM of at least three independent experiments; $*p \leq 0.05$ relative to control at each time point. Fold changes represent BMP2 value divided by the control value for each respective time point.

Table 13. Concentration-dependent effects of early and limited exposure to BMP2 on intensity of DMP1-mCherry transgene in primary BMSC cultures.

Treatment		Day of culture	DMP1-mCherry	Fold change
Control		**7**	134.00 ± 24.14	1
		10	1282.00 ± 221.68	1
		14	1541.00 ± 38.18	1
		21	2258.00 ± 71.01	1
BMP2 (ng/ml)	10	**7**	63.00 ± 12.97	~0.47
		10	1006.00 ± 209.30	~0.79
		14	1274.00 ± 19.80	~0.83
		21	1985.00 ± 13.89	~0.88
	20	**7**	37.00 ± 24.02	~0.28
		10	1036.00 ± 229.46	~0.81
		14	1333.00 ± 19.46	~0.86
		21	1843.00 ± 130.84	~0.82
	50	**7**	29.00 ± 19.07	~0.22
		10	888.00 ± 147.70	~0.69
		14	1177.00 ± 56.30	~0.76
		21	1901.00 ± 20.71	~0.84

Results are expressed in absolute values and represent mean ± SEM of at least three independent experiments; $*p \leq 0.05$ relative to control at each time point. Fold changes represent BMP2 value divided by the control value for each respective time point.

Table 14. Concentration-dependent effects of early and limited exposure to SU5402 on intensity of Xylenol Orange staining in primary dental pulp cultures.

Treatment		Day of culture	Epifluorescence intensity	Fold change
Control		**7**	N.D.	–
		10	187.00 ± 38.68	1
		14	601.00 ± 63.36	1
		21	878.00 ± 51.86	1
SU5402 (μM)	**5**	**7**	N.D.	–
		10	199.00 ± 51.69	~1.06
		14	527.00 ± 64.31	~0.81
		21	763.00 ± 51.95	~0.87
	10	**7**	N.D.	–
		10	188.00 ± 41.44	~1.01
		14	485.00 ± 69.88	~0.81
		21	687.00 ± 51.00	~0.78
	20	**7**	N.D.	–
		10	149.00 ± 44.84	~0.80
		14	407.00 ± 36.57	~0.75
		21	620.00 ± 34.72	~0.71

Results are expressed in absolute values and represent mean ± SEM of at least three independent experiments; *$p \leq 0.05$ relative to control at each time point. Fold changes represent noggin value divided by the control value for each time point.

N.D. = not detected.

Table 15. Concentration-dependent effects of early and limited exposure to SU5402 on the expression of markers of mineralization and odontoblast differentiation in primary dental pulp cultures.

		Gene	CT	ΔCT	-ΔΔCT	Fold change
Control	**Day 7**	*Gapdh*	18.57 ± 0.10	–	–	–
		Bsp	26.03 ± 0.22	7.44 ± 0.31	0	1
		Dmp1	33.07 ± 0.40	14.49 ± 0.50	0	1
		Osteocalcin	22.80 ± 0.35	4.22 ± 0.26	0	1
		Dspp	40.00 ± 0.00	21.43 ± 0.10	N.D.	N.D.
	Day 10	*Gapdh*	18.25 ± 0.13	–	–	–
		Bsp	21.13 ± 0.39	2.88 ± 0.27	4.56 ± 0.05	23.69 ± 0.78
		Dmp1	24.12 ± 0.42	5.87 ± 0.29	8.62 ± 0.20	401.96 ± 55.60
		Osteocalcin	20.69 ± 0.35	2.44 ± 0.47	1.79 ± 0.22	3.53 ± 0.53
		Dspp	33.57 ± 0.83	15.31 ± 0.97	0	1
	Day 14	*Gapdh*	18.74 ± 0.01	–	–	–
		Bsp	18.83 ± 0.26	0.10 ± 0.27	7.35 ± 0.04	162.73 ± 4.73
		Dmp1	23.05 ± 0.34	4.32 ± 0.35	10.17 ± 0.15	1168.36 ± 121.10
		Osteocalcin	20.89 ± 0.34	2.15 ± 0.33	2.07 ± 0.07	4.21 ± 0.21
		Dspp	31.45 ± 1.35	12.71 ± 1.34	2.60 ± 0.38	6.52 ± 1.66
	Day 21	*Gapdh*	18.94 ± 0.18	–	–	–
		Bsp	18.18 ± 0.13	-0.77 ± 0.05	8.21 ± 0.37	315.11 ± 76.53
		Dmp1	22.51 ± 0.72	3.57 ± 0.54	10.92 ± 0.04	1939.58 ± 54.68
		Osteocalcin	19.36 ± 0.27	0.41 ± 0.45	3.81 ± 0.19	14.30 ± 1.90
		Dspp	30.13 ± 0.58	11.81 ± 0.76	4.13 ± 0.19	17.87 ± 2.38

		Gene	CT	ΔCT	-ΔΔCT	Fold change
SU5402 (5 μM)	**Day 7**	*Gapdh*	18.51 ± 0.08	–	–	–
		Bsp	27.53 ± 0.05	9.02 ± 0.03	-1.58 ± 0.35	0.35 ± 0.08
		Dmp1	33.91 ± 0.49	15.41 ± 0.41	-0.92 ± 0.09	0.53 ± 0.03
		Osteocalcin	23.60 ± 0.44	5.09 ± 0.13	-0.87 ± 0.13	0.55 ± 0.05
		Dspp	40.00 ± 0.00	21.49 ± 0.08	N.D.	N.D.
	Day 10	*Gapdh*	18.18 ± 0.18	–	–	–
		Bsp	21.07 ± 0.35	2.89 ± 0.16	4.55 ± 0.15	23.65 ± 2.45
		Dmp1	24.53 ± 0.29	6.35 ± 0.10	8.14 ± 0.40	303.70 ± 79.84
		Osteocalcin	21.38 ± 0.05	3.20 ± 0.14	1.02 ± 0.12	2.05 ± 0.11
		Dspp	34.15 ± 0.43	15.97 ± 0.62	-0.65 ± 0.34	0.67 ± 0.15
	Day 14	*Gapdh*	18.33 ± 0.10	–	–	–
		Bsp	20.12 ± 0.15	1.80 ± 0.26	5.64 ± 0.57	58.01 ± 20.86
		Dmp1	22.98 ± 0.40	4.65 ± 0.30	9.84 ± 0.20	931.76 ± 127.70

		Osteocalcin	21.58 ± 0.70	3.25 ± 0.80	0.97 ± 0.54	2.24 ± 0.77
		Dspp	32.32 ± 1.33	13.99 ± 1.43	1.32 ± 0.47	2.78 ± 0.86
	Day 21	*Gapdh*	18.54 ± 0.22	–	–	–
		Bsp	18.32 ± 0.02	-0.22 ± 0.24	7.66 ± 0.56	233.69 ± 82.22
		Dmp1	23.05 ± 0.38	4.51 ± 0.16	9.99 ± 0.34	1070.23 ± 242.86
		Osteocalcin	18.90 ± 0.13	0.36 ± 0.35	3.86 ± 0.09	14.62 ± 0.94
		Dspp	31.14 ± 1.02	12.60 ± 1.25	2.71 ± 0.29	6.84 ± 1.34

		Gene	CT	ΔCT	-ΔΔCT	Fold change
SU5402 (10 μM)	**Day 7**	*Gapdh*	19.18 ± 0.43	–	–	–
		Bsp	27.52 ± 0.44	8.34 ± 0.87	-0.91 ± 0.56	0.62 ± 0.22
		Dmp1	34.48 ± 0.04	15.31 ± 0.39	-0.82 ± 0.11	0.57 ± 0.42
		Osteocalcin	24.44 ± 0.47	5.26 ± 0.03	-1.04 ± 0.22	0.50 ± 0.08
		Dspp	40.00 ± 0.00	20.82 ± 0.43	N.D.	N.D.
	Day 10	*Gapdh*	17.79 ± 0.12	–	–	–
		Bsp	21.37 ± 0.84	3.58 ± 0.73	3.86 ± 0.41	15.73 ± 4.25
		Dmp1	25.01 ± 0.19	7.13 ± 0.08	7.27 ± 0.42	167.93 ± 46.56
		Osteocalcin	21.59 ± 0.01	3.81 ± 0.12	0.42 ± 0.14	1.35 ± 0.13
		Dspp	33.96 ± 0.26	16.17 ± 0.38	-1.03 ± 1.07	0.78 ± 0.43
	Day 14	*Gapdh*	18.37 ± 0.01	–	–	–
		Bsp	20.28 ± 0.17	1.91 ± 0.18	5.53 ± 0.41	51.81 ± 16.38
		Dmp1	23.91 ± 0.40	5.54 ± 0.39	8.95 ± 0.11	498.15 ± 38.11
		Osteocalcin	21.75 ± 0.59	3.37 ± 0.60	0.85 ± 0.35	1.91 ± 0.44
		Dspp	32.71 ± 0.73	14.33 ± 0.74	0.98 ± 0.21	2.02 ± 0.30
	Day 21	*Gapdh*	18.32 ± 0.24	–	–	–
		Bsp	18.31 ± 0.08	-0.01 ± 0.16	7.45 ± 0.48	194.20 ± 59.74
		Dmp1	23.34 ± 0.16	5.02 ± 0.08	9.47 ± 0.58	827.48 ± 301.14
		Osteocalcin	19.47 ± 0.01	1.15 ± 0.23	3.07 ± 0.02	8.43 ± 0.11
		Dspp	31.49 ± 0.88	13.17 ± 1.12	2.15 ± 0.16	4.48 ± 0.50

		Gene	CT	ΔCT	-ΔΔCT	Fold change
SU5402 (20 μM)	**Day 7**	*Gapdh*	18.72 ± 0.33	–	–	–
		Bsp	27.40 ± 0.86	8.68 ± 1.19	-1.24 ± 0.88	0.59 ± 0.29
		Dmp1	34.34 ± 0.09	15.62 ± 0.24	-1.13 ± 0.24	0.47 ± 0.08
		Osteocalcin	24.87 ± 0.41	6.15 ± 0.08	-1.92 ± 0.18	0.27 ± 0.03
		Dspp	40.00 ± 0.00	21.28 ± 0.33	N.D.	N.D.
	Day 10	*Gapdh*	18.12 ± 0.45	–	–	–
		Bsp	21.89 ± 0.40	3.78 ± 0.85	3.55 ± 0.54	14.47 ± 4.93
		Dmp1	24.85 ± 0.43	6.74 ± 0.89	7.75 ± 0.39	231.70 ± 59.40
		Osteocalcin	22.56 ± 0.31	4.44 ± 0.14	-0.21 ± 0.40	0.93 ± 0.24

		Dspp	34.46 ± 0.34	16.34 ± 0.11	-1.03 ± 1.07	0.78 ± 0.43
	Day 14	*Gapdh*	18.71 ± 0.20	–	–	–
		Bsp	20.22 ± 1.00	1.51 ± 0.30	5.93 ± 0.02	60.97 ± 0.68
		Dmp1	24.96 ± 0.31	6.26 ± 0.51	8.24 ± 0.01	301.39 ± 1.61
		Osteocalcin	22.43 ± 0.57	3.72 ± 0.36	0.50 ± 0.10	1.42 ± 0.10
		Dspp	33.21 ± 0.51	14.50 ± 0.31	0.81 ± 0.65	2.12 ± 0.84
	Day 21	*Gapdh*	18.72 ± 0.21	–	–	–
		Bsp	18.93 ± 0.02	-0.21 ± 0.23	7.24 ± 0.09	151.25 ± 9.03
		Dmp1	23.36 ± 0.01	4.64 ± 0.20	9.85 ± 0.30	964.32 ± 197.22
		Osteocalcin	20.02 ± 0.11	1.30 ± 0.10	2.93 ± 0.36	8.08 ± 1.92
		Dspp	32.58 ± 0.99	13.85 ± 0.78	1.46 ± 0.17	2.80 ± 0.33

Results represent mean ± SEM of at least three independent experiments; *$p \leq 0.05$ relative to the control at each time point.

All fold changes are relative to VH at day 7, except *Dspp*, which is relative to VH at day 10.

N.D. = not detected.

Table 16. Concentration-dependent effects of early and limited exposure to SU5402 on intensity of BSP-GFP transgene in primary dental pulp cultures.

Treatment		Day of culture	Epifluorescence intensity	Fold change
Control		**7**	793.00 ± 7.35	1
		10	982.00 ± 38.28	1
		14	1343.00 ± 139.82	1
		21	2736.00 ± 173.58	1
SU5402 (μM)	5	**7**	528.00 ± 1.19	~0.67
		10	956.00 ± 91.33	~0.97
		14	1123.00 ± 212.17	~0.84
		21	2089.00 ± 80.63	~0.76
	10	**7**	517.00 ± 32.16	~0.65
		10	1045.00 ± 72.46	~1.07
		14	1086.00 ± 196.56	~0.81
		21	1971.00 ± 33.83	~0.72
	20	**7**	477.00 ± 1.00	~0.60
		10	1119.00 ± 75.26	~1.14
		14	995.00 ± 157.09	~0.74
		21	1798.00 ± 94.52	~0.97

Results are expressed in absolute values and represent mean ± SEM of at least three independent experiments; $*p \leq 0.05$ relative to control at each time point. Fold changes represent BMP2 value divided by the control value for each respective time point.

Table 17. Concentration-dependent effects of early and limited exposure to SU5402 on intensity of DMP1-mCherry transgene in primary dental pulp cultures.

Treatment		Day of culture	Epifluorescence intensity	Fold change
Control		**7**	93.00 ± 6.27	1
		10	253.00 ± 26.47	1
		14	1713.00 ± 58.30	1
		21	2452.00 ± 65.48	1
SU5402 (μM)	**5**	**7**	85.00 ± 7.56	~0.91
		10	173.00 ± 13.86	~0.68
		14	1454.00 ± 41.12	~0.85
		21	1859.00 ± 53.97	~0.76
	10	**7**	79.00 ± 8.66	~0.85
		10	162.00 ± 37.35	~0.64
		14	1627.00 ± 77.11	~0.95
		21	1916.00 ± 7.38	~0.78
	20	**7**	61.00 ± 12.61	~0.65
		10	169.00 ± 14.66	~0.67
		14	1765.00 ± 127.42	~1.03
		21	1918.00 ± 4.11	~0.80

Results are expressed in absolute values and represent mean ± SEM of at least three independent experiments; $*p \leq 0.05$ relative to control at each time point. Fold changes represent BMP2 value divided by the control value for each respective time point.

Table 18. Concentration-dependent effects of early and limited exposure to SU5402 in combination with BMP2 on intensity of Xylenol Orange staining in primary dental pulp cultures.

Treatment		Day of culture	Epifluorescence intensity	Fold change
Control		**7**	N.D.	–
		10	187.00 ± 38.68	1
		14	601.00 ± 63.36	1
		21	878.00 ± 51.86	1
BMP2		**7**	N.D.	–
		10	179.00 ± 57.15	~0.96
		14	448.00 ± 52.71	~0.74
		21	1001.00 ± 72.20	~1.14
BMP2 + SU5402 (µM)	5	**7**	N.D.	–
		10	178.00 ± 41.98	~0.95
		14	448.00 ± 37.48	~0.74
		21	975.00 ± 102.17	~1.11
	10	**7**	N.D.	–
		10	163.00 ± 32.90	~0.87
		14	419.00 ± 47.70	~0.70
		21	858.00 ± 71.10	~0.98
	20	**7**	N.D.	–
		10	137.00 ± 25.01	~0.73
		14	312.00 ± 33.27	~0.52
		21	738.00 ± 41.32	~0.84

Results are expressed in absolute values and represent mean ± SEM of at least three independent experiments; *$p \leq 0.05$ relative to control at each time point. Fold changes represent value obtained in a treated culture divided by control value for each time point. N.D. = not detected.

Table 19. Concentration-dependent effects of early and limited exposure to SU5402 in combination with BMP2 on expression of markers of mineralization and odontoblast differentiation in primary dental pulp cultures.

		Gene	CT	ΔCT	-ΔΔCT	Fold change
Control	**Day 7**	*Gapdh*	18.57 ± 0.10	–	–	–
		Bsp	26.03 ± 0.22	7.44 ± 0.31	0	1
		Dmp1	33.07 ± 0.40	14.49 ± 0.50	0	1
		Osteocalcin	22.80 ± 0.35	4.22 ± 0.26	0	1
		Dspp	40.00 ± 0.00	21.43 ± 0.10	N.D.	N.D.
	Day 10	*Gapdh*	18.25 ± 0.13	–	–	–
		Bsp	21.13 ± 0.39	2.88 ± 0.27	4.56 ± 0.05	23.69 ± 0.78
		Dmp1	24.12 ± 0.42	5.87 ± 0.29	8.62 ± 0.20	401.96 ± 55.60
		Osteocalcin	20.69 ± 0.35	2.44 ± 0.47	1.79 ± 0.22	3.53 ± 0.53
		Dspp	33.57 ± 0.83	15.31 ± 0.97	0	1
	Day 14	*Gapdh*	18.74 ± 0.01	–	–	–
		Bsp	18.83 ± 0.26	0.10 ± 0.27	7.35 ± 0.04	162.73 ± 4.73
		Dmp1	23.05 ± 0.34	4.32 ± 0.35	10.17 ± 0.15	1168.36 ± 121.10
		Osteocalcin	20.89 ± 0.34	2.15 ± 0.33	2.07 ± 0.07	4.21 ± 0.21
		Dspp	31.45 ± 1.35	12.71 ± 1.34	2.60 ± 0.38	6.52 ± 1.66
	Day 21	*Gapdh*	18.94 ± 0.18	–	–	–
		Bsp	18.18 ± 0.13	-0.77 ± 0.05	8.21 ± 0.37	315.11 ± 76.53
		Dmp1	22.51 ± 0.72	3.57 ± 0.54	10.92 ± 0.04	1939.58 ± 54.68
		Osteocalcin	19.36 ± 0.27	0.41 ± 0.45	3.81 ± 0.19	14.30 ± 1.90
		Dspp	30.13 ± 0.58	11.81 ± 0.76	4.13 ± 0.19	17.87 ± 2.38

		Gene	CT	ΔCT	-ΔΔCT	Fold change
BMP2 (50 ng/ml)	**Day 7**	*Gapdh*	18.40 ± 0.23	–	–	–
		Bsp	27.46 ± 0.65	6.06 ± 0.87	-1.62 ± 0.56	0.38 ± 0.13
		Dmp1	33.88 ± 0.02	15.49 ± 0.01	-1.00 ± 0.49	0.56 ± 0.18
		Osteocalcin	23.59 ± 0.11	5.19 ± 0.34	-0.97 ± 0.59	0.60 ± 0.22
		Dspp	33.14 ± 1.29	14.75 ± 1.06	0.57 ± 0.10	1.49 ± 0.10
	Day 10	*Gapdh*	18.09 ± 0.41	–	–	–
		Bsp	21.58 ± 0.17	3.48 ± 0.58	3.96 ± 0.26	16.05 ± 2.88
		Dmp1	24.41 ± 0.20	6.32 ± 0.20	8.17 ± 0.30	301.20 ± 60.04
		Osteocalcin	22.05 ± 0.20	3.96 ± 0.61	0.26 ± 0.87	1.66 ± 0.81
		Dspp	31.83 ± 1.19	13.74 ± 0.79	1.58 ± 0.17	3.03 ± 0.35
	Day 14	*Gapdh*	18.21 ± 0.23	–	–	–
		Bsp	19.08 ± 0.12	0.87 ± 0.35	6.57 ± 0.04	95.10 ± 2.49
		Dmp1	23.00 ± 0.27	4.79 ± 0.04	9.70 ± 0.54	947.78 ± 332.96

		Osteocalcin	20.28 ± 0.59	2.07 ± 0.36	2.15 ± 0.10	4.47 ± 0.32
		Dspp	30.72 ± 1.49	12.51 ± 1.26	2.80 ± 0.30	7.27 ± 1.47
	Day 21	*Gapdh*	18.66 ± 0.28	–	–	–
		Bsp	17.98 ± 0.25	-0.68 ± 0.03	8.12 ± 0.29	288.74 ± 56.32
		Dmp1	22.32 ± 0.08	3.66 ± 0.36	10.83 ± 0.14	1842.55 ± 175.77
		Osteocalcin	18.77 ± 0.35	0.11 ± 0.08	4.11 ± 0.18	17.53 ± 2.16
		Dspp	28.15 ± 0.71	9.49 ± 0.43	5.82 ± 0.53	64.17 ± 21.40

BMP2 + SU5402 (5 µM)		Gene	CT	ΔCT	-ΔΔCT	Fold change
	Day 7	*Gapdh*	18.63 ± 0.07	–	–	–
		Bsp	27.52 ± 0.66	8.89 ± 0.58	1.45 ± 0.27	0.38 ± 0.07
		Dmp1	34.36 ± 0.02	15.74 ± 0.05	-1.24 ± 0.55	0.49 ± 0.17
		Osteocalcin	25.22 ± 0.76	6.59 ± 0.83	-2.36 ± 0.57	0.23 ± 0.08
		Dspp	35.43 ± 1.41	16.80 ± 0.27	-1.49 ± 0.52	0.40 ± 0.13
	Day 10	*Gapdh*	17.76 ± 0.21	–	–	–
		Bsp	21.70 ± 0.02	3.94 ± 0.23	-3.50 ± 0.09	11.35 ± 0.67
		Dmp1	25.09 ± 0.08	6.32 ± 0.20	-1.24 ± 0.55	145.90 ± 20.89
		Osteocalcin	21.81 ± 0.56	4.05 ± 0.77	0.17 ± 1.03	1.76 ± 0.95
		Dspp	32.39 ± 0.96	14.62 ± 0.75	0.70 ± 0.21	1.66 ± 0.24
	Day 14	*Gapdh*	18.00 ± 0.18	–	–	–
		Bsp	19.65 ± 0.43	1.65 ± 0.61	5.80 ± 0.29	57.89 ± 11.50
		Dmp1	23.40 ± 0.08	4.79 ± 0.04	9.09 ± 0.25	562.72 ± 94.18
		Osteocalcin	21.47 ± 0.08	3.47 ± 0.10	0.75 ± 0.35	1.79 ± 0.42
		Dspp	31.86 ± 0.95	13.86 ± 0.77	1.46 ± 0.18	2.79 ± 0.35
	Day 21	*Gapdh*	18.50 ± 0.29	–	–	–
		Bsp	19.06 ± 0.02	0.56 ± 0.27	-6.88 ± 0.04	118.00 ± 3.49
		Dmp1	22.95 ± 0.36	4.45 ± 0.65	10.04 ± 0.15	1064.11 ± 110.65
		Osteocalcin	18.94 ± 0.26	0.44 ± 0.03	3.79 ± 0.29	14.37 ± 2.80
		Dspp	29.17 ± 0.04	10.68 ± 0.25	4.64 ± 1.21	44.56 ± 26.12

BMP2 + SU5402 (10 µM)		Gene	CT	ΔCT	-ΔΔCT	Fold change
	Day 7	*Gapdh*	19.06 ± 0.49	–	–	–
		Bsp	28.01 ± 0.73	8.95 ± 1.22	-1.51 ± 0.91	0.50 ± 0.25
		Dmp1	34.04 ± 0.22	14.96 ± 0.27	-0.49 ± 0.23	0.73 ± 0.12
		Osteocalcin	24.34 ± 0.21	5.28 ± 0.70	-1.05 ± 0.96	0.71 ± 0.37
		Dspp	34.79 ± 0.73	15.73 ± 0.24	-0.42 ± 0.72	0.94 ± 0.40
	Day 10	*Gapdh*	18.21 ± 0.24	–	–	–
		Bsp	21.75 ± 0.11	3.54 ± 0.13	3.91 ± 0.19	15.21 ± 1.94
		Dmp1	25.41 ± 0.17	7.20 ± 0.07	7.29 ± 0.43	171.09 ± 48.49
		Osteocalcin	22.05 ± 0.56	3.85 ± 0.80	0.38 ± 1.05	2.06 ± 1.12

		Dspp	32.71 ± 0.86	14.50 ± 0.62	0.81 ± 0.33	1.85 ± 0.42
	Day 14	*Gapdh*	17.88 ± 0.22	–	–	–
		Bsp	19.24 ± 0.52	1.36 ± 0.29	6.08 ± 0.02	67.61 ± 1.02
		Dmp1	23.57 ± 0.01	5.69 ± 0.21	8.80 ± 0.71	558.14 ± 238.04
		Osteocalcin	21.94 ± 0.17	4.06 ± 0.39	0.16 ± 0.14	1.14 ± 0.11
		Dspp	32.78 ± 0.22	14.90 ± 0.45	0.41 ± 0.51	1.50 ± 0.49
	Day 21	*Gapdh*	18.43 ± 0.20	–	–	–
		Bsp	18.96 ± 0.22	0.53 ± 0.41	6.91 ± 0.10	120.61 ± 8.00
		Dmp1	23.19 ± 0.21	4.75 ± 0.41	9.74 ± 0.13	856.01 ± 53.07
		Osteocalcin	19.26 ± 0.89	0.83 ± 0.70	3.40 ± 0.44	11.49 ± 3.30
		Dspp	30.03 ± 0.10	11.60 ± 0.29	3.71 ± 1.25	24.27 ± 14.43

		Gene	CT	ΔCT	-ΔΔCT	Fold change
BMP2 + SU5402 (20 μM)	**Day 7**	*Gapdh*	18.39 ± 0.12	–	–	–
		Bsp	29.21 ± 0.20	10.82 ± 0.32	-3.38 ± 0.01	0.10 ± 0.001
		Dmp1	34.29 ± 0.04	15.90 ± 0.17	-1.41 ± 0.23	0.40 ± 0.09
		Osteocalcin	24.35 ± 0.40	5.96 ± 0.52	-1.74 ± 0.78	0.39 ± 0.18
		Dspp	37.50 ± 1.77	19.11 ± 1.89	N.D.	N.D.
	Day 10	*Gapdh*	18.00 ± 0.37	–	–	–
		Bsp	22.06 ± 0.05	4.06 ± 0.42	3.38 ± 0.10	10.44 ± 0.71
		Dmp1	25.84 ± 0.16	7.84 ± 0.20	6.65 ± 0.30	104.92 ± 20.84
		Osteocalcin	22.26 ± 0.29	4.26 ± 0.65	-0.03 ± 0.91	1.39 ± 0.70
		Dspp	32.84 ± 0.97	14.84 ± 0.61	0.47 ± 0.35	1.47 ± 0.34
	Day 14	*Gapdh*	18.38 ± 0.10	–	–	–
		Bsp	20.46 ± 0.05	2.08 ± 0.15	5.36 ± 0.17	41.62 ± 4.75
		Dmp1	24.27 ± 0.33	5.89 ± 0.43	8.60 ± 0.07	390.20 ± 19.09
		Osteocalcin	22.20 ± 0.13	3.82 ± 0.03	0.41 ± 0.23	1.36 ± 0.21
		Dspp	33.50 ± 0.47	15.12 ± 0.38	0.20 ± 0.59	1.34 ± 0.49
	Day 21	*Gapdh*	18.90 ± 0.36	–	–	–
		Bsp	19.28 ± 0.05	0.38 ± 0.41	7.06 ± 0.10	134.02 ± 8.87
		Dmp1	23.45 ± 0.02	4.56 ± 0.38	9.93 ± 0.16	983.64 ± 80.26
		Osteocalcin	19.56 ± 0.27	0.66 ± 0.09	3.56 ± 0.35	12.50 ± 2.91
		Dspp	30.41 ± 0.34	11.52 ± 0.69	3.80 ± 1.65	36.51 ± 23.86

Results represent mean ± SEM of at least three independent experiments; *$p \leq 0.05$ relative to the control at each time point.

All fold changes are relative to VH at day 7, except *Dspp*, which is relative to VH at day 10.

N.D. = not detected.

Table 20. Concentration-dependent effects of early and limited exposure to SU5402 in combination with BMP2 on intensity of BSP-GFP transgene in primary dental pulp cultures.

Treatment		Day of culture	Epifluorescence intensity	Fold change
Control		**7**	793.00 ± 7.35	1
		10	982.00 ± 38.28	1
		14	1343.00 ± 139.82	1
		21	2736.00 ± 173.58	1
BMP2		**7**	349.00 ± 2.68	~0.44
		10	758.00 ± 14.11	~0.76
		14	1508.00 ± 47.16	~1.12
		21	2647.00 ± 72.24	~0.97
BMP2 + SU5402 (μM)	5	**7**	380.00 ± 26.06	~0.48
		10	758.00 ± 14.11	~0.76
		14	1516.00 ± 65.20	~1.13
		21	2565.00 ± 103.82	~0.97
	10	**7**	351.00 ± 3.62	~0.44
		10	749.00 ± 3.15	~0.76
		14	1466.00 ± 117.33	~1.09
		21	2555.00 ± 97.75	~0.93
	20	**7**	351.00 ± 2.95	~0.44
		10	739.00 ± 0.75	~0.75
		14	1392.00 ± 124.50	~1.04
		21	2511.00 ± 124.41	~0.92

Results are expressed in absolute values and represent mean ± SEM of at least three independent experiments; *$p \leq 0.05$ relative to control at each time point. Fold changes represent value obtained in a treated culture divided by the control value for each time point.

Table 21. Concentration-dependent effects of early and limited exposure to SU5402 in combination with BMP2 on intensity of DMP1-mCherry transgene in primary dental pulp cultures.

Treatment		Day of culture	Epifluorescence intensity	Fold change
Control		**7**	93.00 ± 6.27	1
		10	253.00 ± 26.47	1
		14	1713.00 ± 58.30	1
		21	2452.00 ± 65.48	1
BMP2		**7**	29.00 ± 2.66	~0.31
		10	164.00 ± 17.30	~0.65
		14	1484.00 ± 93.86	~0.87
		21	1958.00 ± 71.43	~0.80
BMP2 + SU5402 (μM)	5	**7**	20.00 ± 6.34	~0.21
		10	196.00 ± 48.51	~0.78
		14	1628.00 ± 91.78	~0.95
		21	1975.00 ± 105.13	~0.80
	10	**7**	23.00 ± 6.91	~0.25
		10	230.00 ± 48.38	~0.91
		14	1733.00 ± 109.15	~1.01
		21	2037.00 ± 96.36	~0.84
	20	**7**	24.00 ± 7.56	~0.26
		10	231.00 ± 51.48	~0.91
		14	1773.00 ± 109.15	~1.03
		21	2046.00 ± 86.65	~0.83

Results are expressed in absolute values and represent mean ± SEM of at least three independent experiments; *$p \leq 0.05$ relative to control at each time point. Fold changes represent value obtained in a treated culture divided by the control value for each time point.

Table 22. Concentration-dependent effects of early and limited exposure to noggin on intensity of Xylenol Orange staining in primary dental pulp cultures.

Treatment		Day of culture	Epifluorescence intensity	Fold change
Control		**7**	N.D.	–
		10	165.00 ± 16.04	1
		14	390.00 ± 38.69	1
		21	841.00 ± 35.34	1
Noggin (ng/ml)	**100**	**7**	N.D.	–
		10	88.00 ± 11.48	~0.53
		14	266.00 ± 43.10	~0.68
		21	798.00 ± 23.12	~1.00
	200	**7**	N.D.	–
		10	83.00 ± 10.81	~0.50
		14	246.00 ± 34.95	~0.63
		21	794.00 ± 37.56	~0.94
	300	**7**	N.D.	–
		10	81.00 ± 10.11	~0.49
		14	231.00 ± 48.18	~0.59
		21	738.00 ± 81.27	~0.88

Results are expressed in absolute values and represent mean ± SEM of at least three independent experiments; *$p \leq 0.05$ relative to control at each time point. Fold changes represent noggin value divided by the control value for each time point.

N.D. = not detected.

Table 23. Concentration-dependent effects of early and limited exposure to noggin on the expression of markers of mineralization and odontoblast differentiation in primary dental pulp cultures.

		Gene	CT	ΔCT	-ΔΔCT	Fold change
Control	**Day 7**	*Gapdh*	19.56 ± 0.27	–	–	–
		Bsp	26.15 ± 0.15	6.60 ± 0.39	0	1
		Dmp1	29.59 ± 0.41	10.32 ± 0.24	0	1
		Osteocalcin	22.05 ± 0.82	2.49 ± 0.66	0	1
		Dspp	38.76 ± 0.72	19.21 ± 0.62	N.D.	N.D.
	Day 10	*Gapdh*	19.44 ± 0.31	–	–	–
		Bsp	20.32 ± 0.35	0.88 ± 0.31	5.72 ± 0.09	53.09 ± 3.20
		Dmp1	20.62 ± 0.69	1.35 ± 0.38	8.98 ± 0.15	511.84 ± 68.15
		Osteocalcin	19.90 ± 0.56	0.46 ± 0.77	2.04 ± 0.56	5.48 ± 2.22
		Dspp	29.39 ± 1.60	9.95 ± 1.57	0	1
	Day 14	*Gapdh*	19.56 ± 0.23	–	–	–
		Bsp	18.55 ± 0.11	-1.01 ± 0.21	7.60 ± 0.23	204.51 ± 31.37
		Dmp1	20.07 ± 0.46	0.75 ± 0.39	9.58 ± 0.34	824.14 ± 201.45
		Osteocalcin	18.93 ± 0.75	-0.63 ± 0.63	3.12 ± 0.08	8.76 ± 0.58
		Dspp	27.36 ± 1.28	7.80 ± 1.21	2.15 ± 0.52	5.54 ± 1.64
	Day 21	*Gapdh*	19.82 ± 0.05	–	–	–
		Bsp	18.03 ± 0.45	-1.79 ± 0.44	8.39 ± 0.08	337.29 ± 19.16
		Dmp1	19.50 ± 0.37	-0.31 ± 0.41	10.64 ± 0.16	1623.12 ± 228.69
		Osteocalcin	17.91 ± 0.64	-1.91 ± 0.63	4.41 ± 0.26	22.84 ± 5.60
		Dspp	26.41 ± 1.38	6.59 ± 1.35	3.36 ± 0.40	11.62 ± 2.32

		Gene	CT	ΔCT	-ΔΔCT	Fold change
Noggin (100 ng/ml)	**Day 7**	*Gapdh*	19.99 ± 0.46	–	–	–
		Bsp	27.26 ± 0.42	6.60 ± 0.39	-0.91 ± 0.19	0.55 ± 0.07
		Dmp1	30.86 ± 0.31	11.42 ± 0.44	-1.03 ± 0.84	0.57 ± 0.30
		Osteocalcin	23.42 ± 1.29	3.42 ± 0.98	-0.75 ± 0.10	0.60 ± 0.05
		Dspp	39.93 ± 0.05	19.94 ± 0.44	N.D.	N.D.
	Day 10	*Gapdh*	19.49 ± 0.51	–	–	–
		Bsp	20.86 ± 0.54	0.88 ± 0.31	4.99 ± 0.19	32.72 ± 4.49
		Dmp1	20.41 ± 0.03	1.54 ± 0.02	8.86 ± 0.01	480.36 ± 123.86
		Osteocalcin	21.32 ± 0.75	1.83 ± 0.18	0.85 ± 0.59	2.20 ± 0.78
		Dspp	32.07 ± 2.91	12.58 ± 2.63	-2.02 ± 0.78	0.36 ± 0.17
	Day 14	*Gapdh*	19.37 ± 0.31	–	–	–
		Bsp	19.11 ± 0.41	-1.01 ± 0.31	6.63 ± 0.30	105.72 ± 23.79
		Dmp1	19.39 ± 0.59	0.35 ± 0.26	10.05 ± 0.10	1061.86 ± 99.32

		Osteocalcin	19.60 ± 1.12	0.23 ± 0.97	2.44 ± 0.13	5.49 ± 0.57
		Dspp	28.91 ± 1.86	9.54 ± 1.90	1.02 ± 0.67	2.80 ± 1.27
	Day 21	*Gapdh*	19.61 ± 0.21	–	–	–
		Bsp	17.90 ± 0.32	-1.79 ± 0.44	8.07 ± 0.15	273.86 ± 29.98
		Dmp1	19.81 ± 0.86	0.21 ± 0.42	10.19 ± 0.02	1166.42 ± 17.50
		Osteocalcin	18.37 ± 0.99	-1.25 ± 1.13	3.92 ± 0.27	15.89 ± 3.18
		Dspp	27.53 ± 1.59	7.91 ± 1.75	2.64 ± 0.25	6.52 ± 1.11

		Gene	CT	ΔCT	-ΔΔCT	Fold change
Noggin (200 ng/ml)	**Day 7**	*Gapdh*	19.56 ± 0.28	–	–	–
		Bsp	28.24 ± 0.66	8.30 ± 0.51	-1.94 ± 0.42	0.30 ± 0.08
		Dmp1	31.95 ± 0.48	12.52 ± 0.51	-2.12 ± 0.91	0.29 ± 0.15
		Osteocalcin	23.68 ± 1.87	3.74 ± 0.92	-1.07 ± 0.18	0.49 ± 0.07
		Dspp	40.00 ± 0.00	20.06 ± 0.41	N.D.	N.D.
	Day 10	*Gapdh*	19.04 ± 0.16	–	–	–
		Bsp	20.63 ± 0.25	1.59 ± 0.39	4.77 ± 0.39	30.21 ± 6.97
		Dmp1	19.85 ± 0.73	0.86 ± 0.39	9.54 ± 0.19	743.19 ± 4.19
		Osteocalcin	20.49 ± 0.11	1.44 ± 0.31	1.23 ± 0.58	2.91 ± 1.20
		Dspp	32.14 ± 2.78	13.10 ± 2.93	-2.48 ± 1.05	0.31 ± 0.12
	Day 14	*Gapdh*	19.00 ± 0.31	–	–	–
		Bsp	18.73 ± 0.25	-0.28 ± 0.33	6.64 ± 0.16	101.48 ± 10.52
		Dmp1	19.58 ± 0.46	0.96 ± 0.31	9.44 ± 0.07	695.28 ± 45.04
		Osteocalcin	18.72 ± 0.87	-0.28 ± 0.71	2.96 ± 0.20	8.00 ± 1.37
		Dspp	29.32 ± 2.04	10.32 ± 2.02	0.24 ± 0.84	1.87 ± 0.94
	Day 21	*Gapdh*	19.37 ± 0.09	–	–	–
		Bsp	17.73 ± 0.26	-1.64 ± 0.25	8.01 ± 0.23	267.00 ± 40.15
		Dmp1	20.03 ± 0.77	0.57 ± 0.67	9.82 ± 0.19	922.92 ± 174.00
		Osteocalcin	18.92 ± 1.11	-0.45 ± 1.21	3.12 ± 0.25	9.15 ± 2.09
		Dspp	27.42 ± 1.61	8.05 ± 1.68	2.50 ± 0.29	5.97 ± 1.04

		Gene	CT	ΔCT	-ΔΔCT	Fold change
Noggin (300 ng/ml)	**Day 7**	*Gapdh*	20.44 ± 0.76	–	–	–
		Bsp	28.24 ± 0.66	8.28 ± 0.83	-1.91 ± 0.40	0.29 ± 0.07
		Dmp1	31.96 ± 0.56	12.44 ± 0.47	-2.05 ± 0.87	0.29 ± 0.15
		Osteocalcin	24.95 ± 1.30	4.51 ± 0.62	-1.83 ± 0.29	0.30 ± 0.07
		Dspp	40.00 ± 0.00	19.56 ± 0.76	N.D.	N.D.
	Day 10	*Gapdh*	19.31 ± 0.25	–	–	–
		Bsp	20.78 ± 0.25	1.47 ± 0.32	4.89 ± 0.32	31.84 ± 6.02
		Dmp1	19.90 ± 0.45	0.83 ± 0.13	9.56 ± 0.08	769.00 ± 142.59
		Osteocalcin	21.04 ± 0.18	1.73 ± 0.13	0.95 ± 0.71	2.57 ± 1.09

		Dspp	32.33 ± 2.37	13.03 ± 2.46	-2.48 ± 1.05	0.31 ± 0.12
	Day 14	*Gapdh*	19.48 ± 0.18	–	–	–
		Bsp	18.77 ± 0.42	-0.71 ± 0.29	7.07 ± 0.60	166.94 ± 52.41
		Dmp1	19.40 ± 0.05	0.12 ± 0.08	10.28 ± 0.34	1308.15 ± 418.91
		Osteocalcin	19.16 ± 0.95	-0.32 ± 0.83	3.00 ± 0.19	8.21 ± 1.43
		Dspp	30.00 ± 2.49	10.51 ± 2.46	0.04 ± 0.53	1.27 ± 0.47
	Day 21	*Gapdh*	19.50 ± 0.17	–	–	–
		Bsp	18.10 ± 0.33	-1.40 ± 0.20	7.76 ± 0.44	244.44 ± 60.60
		Dmp1	19.84 ± 0.52	0.22 ± 0.81	10.18 ± 0.29	1204.21 ± 335.42
		Osteocalcin	19.05 ± 0.94	-0.46 ± 0.86	3.13 ± 0.08	8.79 ± 0.62
		Dspp	27.53 ± 1.43	8.03 ± 1.37	2.52 ± 0.60	7.10 ± 2.11

Results represent mean ± SEM of at least three independent experiments; *$p \leq 0.05$ relative to the control at each time point.

All fold changes are relative to VH at day 7, except *Dspp*, which is relative to VH at day 10.

N.D. = not detected.

Table 24. Concentration-dependent effects of early and limited exposure to noggin on intensity of BSP-GFP transgene in primary dental pulp cultures.

Treatment		Day of culture	Epifluorescence intensity	Fold change
Control		**7**	747.00 ± 28.84	1
		10	1029.00 ± 27.05	1
		14	1334.00 ± 40.70	1
		21	2870.00 ± 125.90	1
BMP2 (ng/ml)	10	**7**	442.00 ± 66.84	~0.59
		10	1029.00 ± 19.46	1
		14	1182.00 ± 42.43	~0.89
		21	2612.00 ± 44.88	~0.91
	20	**7**	375.00 ± 40.03	~0.50
		10	995.00 ± 11.84	~0.97
		14	1171.00 ± 62.70	~0.88
		21	2533.00 ± 94.41	~0.88
	50	**7**	274.00 ± 0.73	~0.37
		10	999.00 ± 12.25	~0.97
		14	1209.00 ± 89.00	~0.91
		21	2539.00 ± 39.98	~0.88

Results are expressed in absolute values and represent mean ± SEM of at least three independent experiments; $*p \leq 0.05$ relative to control at each time point. Fold changes represent BMP2 value divided by the control value for each respective time point.

Table 25. Concentration-dependent effects of early and limited exposure to noggin on intensity of DMP1-mCherry transgene in primary dental pulp cultures.

Treatment		Day of culture	Epifluorescence intensity	Fold change
Control		**7**	88.00 ± 12.09	1
		10	210.00 ± 21.30	1
		14	1580.00 ± 137.08	1
		21	2353.00 ± 201.53	1
BMP2 (ng/ml)	10	**7**	52.00 ± 8.06	~0.59
		10	276.00 ± 19.38	~1.31
		14	1737.00 ± 202.15	~1.10
		21	2272.00 ± 83.39	~0.97
	20	**7**	57.00 ± 6.62	~0.64
		10	219.00 ± 27.57	~1.05
		14	1543.00 ± 183.03	~0.98
		21	2200.00 ± 134.53	~0.94
	50	**7**	56.00 ± 6.84	~0.63
		10	238.00 ± 1.52	~1.13
		14	1657.00 ± 154.40	~1.05
		21	2196.00 ± 7.22	~0.93

Results are expressed in absolute values and represent mean ± SEM of at least three independent experiments; $*p \leq 0.05$ relative to control at each time point. Fold changes represent BMP2 value divided by the control value for each respective time point.

Table 26. Concentration-dependent effects of early and limited exposure to noggin in combination with FGF2 on intensity of Xylenol Orange staining in primary dental pulp cultures.

Treatment		Day of culture	Epifluorescence intensity	Fold change
Control		**7**	N.D.	–
		10	165.00 ± 16.04	1
		14	390.00 ± 38.69	1
		21	841.00 ± 35.34	1
FGF2		**7**	N.D.	–
		10	178.00 ± 15.23	~1.08
		14	395.00 ± 22.42	~1.01
		21	1264.00 ± 115.00	~1.52
FGF2 + Noggin (ng/ml)	**100**	**7**	N.D.	–
		10	178.00 ± 18.18	~1.08
		14	404.00 ± 34.31	~1.04
		21	922.00 ± 25.20	~1.10
	200	**7**	N.D.	–
		10	168.00 ± 20.57	~1.02
		14	387.00 ± 31.24	~0.99
		21	780.00 ± 8.82	~0.93
	300	**7**	N.D.	–
		10	117.00 ± 22.76	~0.71
		14	274.00 ± 49.85	~0.70
		21	684.00 ± 17.61	~0.81

Results are expressed in absolute values and represent mean ± SEM of at least three independent experiments; *$p \leq 0.05$ relative to control at each time point. Fold changes represent value obtained in a treated culture divided by control value for each time point. N.D. = not detected.

Table 27. Concentration-dependent effects of early and limited exposure to noggin in combination with FGF2 on expression of markers of mineralization and odontoblast differentiation in primary dental pulp cultures.

		Gene	CT	ΔCT	-ΔΔCT	Fold change
Control	**Day 7**	*Gapdh*	19.56 ± 0.27	–	–	–
		Bsp	26.15 ± 0.15	6.60 ± 0.39	0	1
		Dmp1	29.59 ± 0.41	10.32 ± 0.24	0	1
		Osteocalcin	22.05 ± 0.82	2.49 ± 0.66	0	1
		Dspp	38.76 ± 0.72	19.21 ± 0.62	N.D.	N.D.
	Day 10	*Gapdh*	19.44 ± 0.31	–	–	–
		Bsp	20.32 ± 0.35	0.88 ± 0.31	5.72 ± 0.09	53.09 ± 3.20
		Dmp1	20.62 ± 0.69	1.35 ± 0.38	8.98 ± 0.15	511.84 ± 68.15
		Osteocalcin	19.90 ± 0.56	0.46 ± 0.77	2.04 ± 0.56	5.48 ± 2.22
		Dspp	29.39 ± 1.60	9.95 ± 1.57	0	1
	Day 14	*Gapdh*	19.56 ± 0.23	–	–	–
		Bsp	18.55 ± 0.11	-1.01 ± 0.21	7.60 ± 0.23	204.51 ± 31.37
		Dmp1	20.07 ± 0.46	0.75 ± 0.39	9.58 ± 0.34	824.14 ± 201.45
		Osteocalcin	18.93 ± 0.75	-0.63 ± 0.63	3.12 ± 0.08	8.76 ± 0.58
		Dspp	27.36 ± 1.28	7.80 ± 1.21	2.15 ± 0.52	5.54 ± 1.64
	Day 21	*Gapdh*	19.82 ± 0.05	–	–	–
		Bsp	18.03 ± 0.45	-1.79 ± 0.44	8.39 ± 0.08	337.29 ± 19.16
		Dmp1	19.50 ± 0.37	-0.31 ± 0.41	10.64 ± 0.16	1623.12 ± 228.69
		Osteocalcin	17.91 ± 0.64	-1.91 ± 0.63	4.41 ± 0.26	22.84 ± 5.60
		Dspp	26.41 ± 1.38	6.59 ± 1.35	3.36 ± 0.40	11.62 ± 2.32

		Gene	CT	ΔCT	-ΔΔCT	Fold change
FGF2 (20 ng/ml)	**Day 7**	*Gapdh*	20.44 ± 0.76	–	–	–
		Bsp	25.86 ± 0.43	5.87 ± 1.00	0.87 ± 0.51	2.23 ± 0.84
		Dmp1	21.85 ± 1.51	2.59 ± 0.96	7.50 ± 0.86	214.44 ± 115.09
		Osteocalcin	20.86 ± 0.90	0.87 ± 0.29	2.16 ± 0.21	4.61 ± 0.74
		Dspp	29.63 ± 1.80	9.64 ± 1.61	1.71 ± 0.41	3.64 ± 0.89
	Day 10	*Gapdh*	19.23 ± 0.22	–	–	–
		Bsp	20.15 ± 0.06	0.92 ± 0.23	5.82 ± 0.48	66.17 ± 19.80
		Dmp1	20.00 ± 0.38	0.90 ± 0.02	9.19 ± 0.38	585.63 ± 47.26
		Osteocalcin	19.23 ± 0.67	-0.004 ± 0.71	3.04 ± 0.14	8.34 ± 1.01
		Dspp	27.85 ± 1.30	8.62 ± 1.33	2.73 ± 0.13	6.70 ± 0.61
	Day 14	*Gapdh*	19.53 ± 0.39	–	–	–
		Bsp	18.01 ± 0.12	-1.53 ± 0.88	8.27 ± 0.41	352.14 ± 109.20
		Dmp1	18.86 ± 1.46	-0.41 ± 0.76	10.50 ± 0.47	1605.66 ± 692.87

		Osteocalcin	19.39 ± 0.88	-0.14 ± 0.80	3.18 ± 0.24	9.41 ± 1.76
		Dspp	28.20 ± 1.16	6.67 ± 1.41	4.68 ± 0.72	35.67 ± 15.23
	Day 21	*Gapdh*	20.07 ± 0.02	–	–	–
		Bsp	18.53 ± 0.66	-1.54 ± 0.67	8.28 ± 0.35	337.87 ± 73.42
		Dmp1	19.32 ± 0.001	-0.75 ± 0.49	10.84 ± 0.10	1842.05 ± 176.72
		Osteocalcin	18.29 ± 0.65	-1.77 ± 0.66	4.81 ± 0.11	28.28 ± 2.59
		Dspp	25.20 ± 1.24	5.13 ± 1.25	6.22 ± 0.17	76.07 ± 9.60

		Gene	CT	ΔCT	-ΔΔCT	Fold change
FGF2 + noggin (100 ng/ml)	**Day 7**	*Gapdh*	19.74 ± 0.29	–	–	–
		Bsp	26.05 ± 0.37	6.31 ± 0.55	0.43 ± 0.06	1.35 ± 0.06
		Dmp1	26.65 ± 2.09	7.19 ± 1.70	2.90 ± 1.60	12.52 ± 10.08
		Osteocalcin	20.86 ± 0.66	1.18 ± 0.32	1.85 ± 0.40	4.03 ± 1.30
		Dspp	36.70 ± 2.69	16.97 ± 2.75	-5.62 ± 1.53	0.08 ± 0.06
	Day 10	*Gapdh*	20.14 ± 1.79	–	–	–
		Bsp	22.20 ± 1.24	2.06 ± 0.07	4.69 ± 0.56	30.98 ± 8.59
		Dmp1	20.01 ± 1.14	1.28 ± 0.63	8.81 ± 0.14	478.66 ± 168.47
		Osteocalcin	20.30 ± 2.17	0.16 ± 1.14	2.88 ± 0.49	8.86 ± 4.01
		Dspp	28.89 ± 2.20	8.75 ± 1.33	2.60 ± 0.07	6.08 ± 0.31
	Day 14	*Gapdh*	19.22 ± 0.24	–	–	–
		Bsp	19.52 ± 1.27	0.30 ± 0.17	6.44 ± 1.59	243.16 ± 121.62
		Dmp1	19.66 ± 1.10	0.67 ± 0.80	9.42 ± 0.50	766.51 ± 345.54
		Osteocalcin	20.74 ± 1.61	1.52 ± 1.44	1.51 ± 0.82	4.08 ± 1.74
		Dspp	27.11 ± 1.35	7.89 ± 1.41	3.46 ± 0.51	13.01 ± 3.96
	Day 21	*Gapdh*	19.83 ± 0.21	–	–	–
		Bsp	18.68 ± 0.17	-1.15 ± 0.37	7.90 ± 0.50	287.95 ± 103.11
		Dmp1	19.04 ± 0.68	-0.66 ± 0.30	10.75 ± 0.15	1742.81 ± 248.18
		Osteocalcin	20.15 ± 1.14	0.32 ± 1.15	2.72 ± 0.52	7.89 ± 3.17
		Dspp	26.87 ± 1.77	7.04 ± 1.86	4.31 ± 0.61	25.84 ± 10.52

		Gene	CT	ΔCT	-ΔΔCT	Fold change
FGF2 + noggin (200 ng/ml)	**Day 7**	*Gapdh*	19.77 ± 0.44	–	–	–
		Bsp	26.25 ± 0.32	6.47 ± 0.61	0.27 ± 0.12	1.22 ± 0.11
		Dmp1	27.69 ± 1.08	8.38 ± 0.62	-1.70 ± 0.52	3.47 ± 1.21
		Osteocalcin	22.79 ± 1.08	2.01 ± 0.63	-1.02 ± 0.30	2.16 ± 0.57
		Dspp	37.18 ± 2.29	17.40 ± 2.33	6.05 ± 1.20	0.03 ± 0.01
	Day 10	*Gapdh*	19.46 ± 0.59	–	–	–
		Bsp	22.02 ± 0.97	2.55 ± 0.63	4.18 ± 1.09	33.21 ± 15.05
		Dmp1	20.04 ± 1.22	1.06 ± 0.28	9.02 ± 0.13	524.71 ± 70.06
		Osteocalcin	19.90 ± 1.12	0.44 ± 1.05	2.59 ± 0.41	6.91 ± 2.69

		Dspp	30.97 ± 2.66	11.50 ± 2.42	0.16 ± 1.33	1.87 ± 0.72
	Day 14	*Gapdh*	19.59 ± 0.39	–	–	–
		Bsp	20.94 ± 1.44	1.35 ± 0.77	5.39 ± 1.59	112.67 ± 65.05
		Dmp1	19.80 ± 0.65	0.65 ± 0.34	9.43 ± 0.17	700.91 ± 118.49
		Osteocalcin	21.84 ± 1.76	2.24 ± 1.40	0.78 ± 0.77	2.40 ± 1.06
		Dspp	28.24 ± 1.95	8.64 ± 1.78	2.70 ± 0.45	7.61 ± 2.53
	Day 21	*Gapdh*	19.77 ± 0.23	–	–	–
		Bsp	18.76 ± 0.12	-1.01 ± 0.35	7.76 ± 0.46	254.28 ± 84.10
		Dmp1	19.21 ± 0.61	-0.44 ± 0.16	10.53 ± 0.04	1488.25 ± 66.32
		Osteocalcin	20.15 ± 1.09	0.37 ± 1.11	2.66 ± 0.50	7.45 ± 2.69
		Dspp	27.86 ± 2.19	8.08 ± 2.28	3.26 ± 0.95	18.88 ± 11.87

		Gene	CT	ΔCT	-ΔΔCT	Fold change
FGF2 + noggin (300 ng/ml)	**Day 7**	*Gapdh*	19.97 ± 0.60	–	–	–
		Bsp	26.35 ± 0.38	6.38 ± 0.65	-0.36 ± 0.25	1.34 ± 0.21
		Dmp1	28.42 ± 0.62	8.87 ± 0.49	1.21 ± 0.40	2.41 ± 0.65
		Osteocalcin	22.27 ± 0.98	2.30. ± 0.61	0.72 ± 0.19	1.70 ± 0.27
		Dspp	37.44 ± 2.08	17.47 ± 1.99	6.12 ± 0.87	0.02 ± 0.00
	Day 10	*Gapdh*	19.36 ± 0.55	–	–	–
		Bsp	20.76 ± 0.27	1.39 ± 0.20	5.34 ± 0.28	42.9 ± 7.34
		Dmp1	19.83 ± 1.41	0.71 ± 0.87	9.37 ± 0.54	763.88 ± 376.01
		Osteocalcin	19.72 ± 1.19	0.35 ± 1.19	2.67 ± 0.53	7.93 ± 3.74
		Dspp	32.40 ± 3.23	13.04 ± 3.13	-1.69 ± 2.03	1.19 ± 0.52
	Day 14	*Gapdh*	19.72 ± 0.18	–	–	–
		Bsp	19.43 ± 0.34	-0.29 ± 0.16	7.03 ± 0.17	134.37 ± 16.46
		Dmp1	20.25 ± 0.52	0.71 ± 0.73	9.37 ± 0.45	730.74 ± 306.07
		Osteocalcin	19.99 ± 0.32	0.27 ± 0.24	2.76 ± 0.43	7.75 ± 2.80
		Dspp	30.21 ± 2.82	10.48 ± 2.64	0.86 ± 1.41	4.68 ± 2.63
	Day 21	*Gapdh*	19.62 ± 0.09	–	–	–
		Bsp	18.94 ± 0.26	-0.67 ± 0.24	7.42 ± 0.53	207.22 ± 67.49
		Dmp1	19.16 ± 0.74	-0.35 ± 0.76	10.44 ± 0.47	1540.75 ± 666.16
		Osteocalcin	20.85 ± 1.23	1.23 ± 1.13	1.79 ± 0.49	4.14 ± 1.68
		Dspp	28.09 ± 2.21	8.47 ± 2.14	2.87 ± 0.81	11.67 ± 6.12

Results represent mean ± SEM of at least three independent experiments; *$p \leq 0.05$ relative to the control at each time point.

All fold changes are relative to VH at day 7, except *Dspp*, which is relative to VH at day 10. N.D. = not detected.

Table 28. Concentration-dependent effects of early and limited exposure to noggin in combination with FGF2 on intensity of BSP-GFP transgene in primary dental pulp cultures.

Treatment		Day of culture	Epifluorescence intensity	Fold change
Control		7	747.00 ± 28.84	1
		10	1029.00 ± 27.05	1
		14	1334.00 ± 40.70	1
		21	2870.00 ± 125.90	1
FGF2		7	919.00 ± 11.02	~1.23
		10	1039.00 ± 55.00	~1.01
		14	1296.00 ± 71.29	~0.97
		21	2805.00 ± 39.98	~0.98
FGF2 + Noggin (ng/ml)	100	7	835.00 ± 36.56	~1.12
		10	939.00 ± 62.52	~0.91
		14	1305.00 ± 41.69	~0.98
		21	2538.00 ± 188.05	~0.88
	200	7	823.00 ± 30.98	~1.08
		10	916.00 ± 51.44	~0.89
		14	1233.00 ± 59.76	~0.92
		21	2490.00 ± 185.69	~0.87
	300	7	792.00 ± 28.90	~1.06
		10	867.00 ± 68.48	~0.84
		14	1111.00 ± 73.94	~0.83
		21	26432.00 ± 145.8	~0.92

Results are expressed in absolute values and represent mean ± SEM of at least three independent experiments; *$p \leq 0.05$ relative to control at each time point. Fold changes represent value obtained in a treated culture divided by the control value for each time point.

Table 29. Concentration-dependent effects of early and limited exposure to noggin in combination with FGF2 on intensity of DMP1-mCherry transgene in primary dental pulp cultures.

Treatment		Day of culture	Epifluorescence intensity	Fold change
Control		7	88.00 ± 12.09	1
		10	210.00 ± 21.30	1
		14	1580.00 ± 137.08	1
		21	2353.00 ± 201.53	1
FGF2		7	212.00 ± 22.62	~2.41
		10	427.00 ± 108.21	~2.04
		14	1599.00 ± 37.87	~1.01
		21	2636.00 ± 158.17	~1.12
FGF2 + Noggin (ng/ml)	100	7	93.00 ± 1.05	~1.05
		10	355.00 ± 68.74	~1.69
		14	1438.00 ± 91.22	~0.91
		21	2647.00 ± 113.05	~1.12
	200	7	74.00 ± 0.78	~0.83
		10	305.00 ± 48.87	~1.46
		14	1397.00 ± 87.51	~0.88
		21	2448.00 ± 209.22	~1.04
	300	7	61.00 ± 1.44	~0.69
		10	330.00 ± 63.03	~1.57
		14	1497.00 ± 101.17	~0.95
		21	2304.00 ± 352.15	~0.98

Results are expressed in absolute values and represent mean ± SEM of at least three independent experiments; *$p \leq 0.05$ relative to control at each time point. Fold changes represent value obtained in a treated culture divided by the control value for each time point.

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