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The Canonical BMP Signaling Pathway Plays a Crucial Part in Stimulation of Dentin Sialophosphoprotein Expression by BMP-2*

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Dentin sialophosphoprotein (DSPP), a typical dentin-specific protein, is mainly expressed in the dentin extracellular matrix and plays a role in dentin mineralization. BMP-2 provides a strong signal for differentiation and mineralization of odontoblasts and osteoblasts. Previously, BMP-2 treatment is reported to stimulate *Dspp* expression in the MD10-F2 pre-odontoblast cells through activation of the heterotrimeric transcription factor Y (NF-Y). The canonical BMP signaling pathway is known to contribute greatly to biomineralization, however, it is not known whether it is involved in *Dspp* expression. Here, we investigated this question. Activation of the canonical BMP-2 signaling pathway in MDPC-23, preodontoblast cell, by overexpression of constitutively active Smad1/5 or downstream transcription factors Dlx5 and Runx2 stimulated *Dspp* expression. Conversely, knockdown of each element with siRNA significantly blocked the BMP-2-induced *Dspp* expression. To test whether these transcription factors downstream of BMP-2 are directly involved in regulating *Dspp*, we analyzed the mouse *Dspp* promoter. There are 5 well conserved homeodomain binding elements, H1 to H5, in *Dspp* proximal promoter regions (−791 to +54). A serial deletion of H1 and H2 greatly changed basal promoter activity and responsiveness to Dlx5 or Msx2. However, further deletions did not change the responsiveness to Dlx5 or Msx2. H1 and H2 sites can be suggested as specific response elements of Dlx5 and Msx2, respectively, based on their promoter activity modulation. Thus, the canonical BMP-2 signaling pathway plays a crucial part in the regulation of *Dspp* expression through the action of Smads, Dlx5, Runx2, and Msx2.

Dentin sialophosphoprotein (DSPP)[2] is a major non-collagenous dentin matrix protein and is mainly expressed by odontoblasts (1). DSPP is synthesized as a single polypeptide and then cleaved into three peptides, dentin sialoprotein (DSP), dentin glycoprotein (DGP), and dentin phosphoprotein (DPP) (2, 3). Evidence from human and mouse genetic studies indicates that DSPP is important for dentin mineralization (4); mutations of the *DSPP* gene have been identified in human families with dentinogenesis imperfecta II and III (5, 6), in whom dentin mineralization is defective. Moreover, *Dspp*-null mice show dentin mineralization defects that are very similar to human dentinogenesis imperfecta III.

DSPP is a member of the small integrin-binding ligand *N*-linked glycoproteins (SIBLINGS) family of proteins, which also includes bone sialoprotein, osteopontin, dentin matrix protein 1, and matrix extracellular phosphoglycoprotein. The genes encoding these proteins are clustered on chromosome 4q21 in humans and 5q in mouse, and the proteins are commonly involved in dentin and bone mineralization and share an acidic serine- and aspartate-rich motif (7).

The cleavage of DSPP into smaller proteins has been proposed to be an activation step (5). The failure of the cleavage process is a critical cause of defects in developmental dentin formation (3). DSP is in the N-terminal portion of DSPP; it is a 95-kDa glycoprotein that was first identified within the extracellular matrix of dentin, but its biological function is not known (2). DGP was identified recently as an 81-amino acid segment between DSP and DPP (3). DPP is a major constituent of non-collagenous dentin matrix proteins in the extracellular matrix of dentin that is rich in aspartic acid and phosphoserine and binds to calcium. Therefore, DPP is strongly associated with the mineral phase of dentin, acting as an important initiator and modulator of dentin apatite crystal formation (2, 8).

The odontogenic process is composed of tooth initiation, morphogenesis, epithelial histogenesis, and cytodifferentiation (9). The fibroblast growth factor, bone morphogenetic protein (BMP), sonic hedgehog, and wingless (Wnt) families are components of signaling pathways for tooth development stimulated by epithelial-mesenchymal interactions (10). During tooth development, BMPs can induce apoptosis, or cell proliferation or differentiation (11). BMPs are the largest subgroup in the transforming growth factor-β superfamily and were identified from demineralized bone matrix as factors that induce ectopic bone formation (12). BMP-2 is one of the strongest signals stimulating biomineralization. BMP-2 signaling is transduced into cells through a heterotetrameric complex of BMP receptors, types I and II, which have serine/threonine kinase activity. The binding of BMP ligands to their receptors phosphorylates and subsequently activates Smads (the canonical BMP pathway) or acts through other mechanisms (non-canonical BMP pathway) (13). BMP-2 stimulates osteoblast differentiation and bone mineralization via the canonical BMP

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[2] The abbreviations used are: DSPP, dentin sialophosphoprotein; DSP, dentin sialoprotein; DGP, dentin glycoprotein; DPP, dentin phosphoprotein.

pathway; activated BMP R-Smads stimulate *Dlx5* expression in the osteoblasts, which in turn stimulates *Alp* (14), *Runx2* (15), *Osx* (16), and *Mepe* (7) expression.

Because BMP-2 is a potent factor in tooth development and DSPP is an extracellular matrix protein almost exclusively expressed in dentin, BMP-2 signaling might also regulate *DSPP* expression during tooth development. In this study, we proved the relationship between canonical BMP-2 signaling and *DSPP* expression using mouse cells as a model for the human process. Many data proved the relationship between BMP-2 and *DSPP* expression. *Bmp-2* is expressed in the bell stage during which dentin mineralization progresses very actively, and *Dspp* expression becomes detectable from the subsequent secretory stage, just following *Bmp-2* expression. Previously, Nakashima (18) reported that the expression of BMP-2 is increased during terminal differentiation of odontoblast, and Iohara *et al.* (17) indicated that BMP-2 induces *Dspp* mRNA expression. Chen *et al.* (19) also proved that *Dspp* mRNA expression is followed by additional *Bmp2* expression, through an *in situ* hybridization experiment. *Dspp* expression is suppressed by TGF-β1 treatment or activation of Smad2 and Smad3 (20, 21). *Nuclear factor I-C* null mice show shortened molar roots and disorganized odontoblasts in which *Dspp* gene expression is strongly down-regulated (22). *Dspp* expression is regulated by BMP-2 through the activation of the heterotrimeric transcription factor NF-Y (19), but these authors did not investigate whether Smad-mediated canonical BMP signaling is involved in *Dspp* expression. We have been studying the role of this pathway in osteoblast differentiation and biomineralization (7, 14–16) and have found that BMP R-Smads stimulate *de novo* expression of Dlx5, which stimulates bone marker genes directly or indirectly through the activation of other osteogenic transcription factors such as Runx2 (15) and Osx (16). Thus, Dlx5 may be a central regulator of the osteogenic BMP signaling pathway. On the other hand, Dlx5 seems to be an immediate early target of TGF-β-induced suppression of osteoblast differentiation; the treatment of C2C12 cells with TGF-β or its downstream Smad2 or Smad3 activation suppresses *Dlx5* expression and bone marker gene expression (15). Taking together the fact that *Dspp* expression is suppressed by TGF-β1 treatment or Smad2 and Smad3 activation (20), we hypothesized that the canonical BMP signal and its downstream transcription factor, Dlx5, can regulate *Dspp* expression. This study was designed to illuminate the regulatory relationship between the canonical BMP-2 signaling pathway and *Dspp* transcriptional regulation.

EXPERIMENTAL PROCEDURES

Cell Culture—C2C12 mouse myogenic cells and MDPC-23 mouse pre-odontoblast cells were maintained in Dulbecco's modified Eagle's medium (Hyclone, Logan, UT) supplemented with 10% fetal bovine serum (FBS).

Antibodies—The anti-hemagglutinin (HA) (HA11.3) antibody was purchased from Covance (Princeton, NJ). Horseradish peroxidase-conjugated anti-FLAG (M2) (anti-FLAG-horseradish peroxidase) was purchased from Sigma. The anti-Dlx5 antibody was purchased from Takara (Takara Shuzo, Shiga, Japan). Anti-β-actin and -Smad1/5/8 antibodies were purchased from Abcam (Cambridge, MA), and HRP-conjugated anti-mouse and anti-rabbit secondary antibodies were purchased from Pierce. The anti-Runx2 antibody was purchased from MBL International (Woburn, MA).

Materials—Bioactive recombinant human BMP-2 protein was purchased from R&D Systems (Minneapolis, MN).

DNA Construction—The construction of the Dlx5 (pcDNA3.1-Dlx5), Msx2, and Runx2 expression vectors has been described previously (15, 24). Smad1 and Smad5 full-length cDNAs were generated by PCR and subcloned, respectively, into pcDNA3.1 to create HA-epitope fusion proteins and pcDNA3 to create FLAG (M2)-epitope fusion proteins. The mouse Yy1 expression vector is based on NCBI reference sequence NM_009537. Yy1 full-length cDNA was generated by PCR and subcloned into pcDNA3.1 to create HA-epitope fusion proteins. All fusion proteins had N-terminal tags and were confirmed by Western blot analysis as described previously (25). The *Dspp* promoter construct, D-791 (pGL3LUC bp −791 to +54) was provided by Dr. J.-C. Park (Department of Oral Histology-Developmental Biology, School of Dentistry and Dental Research Institute, Seoul National University, Seoul, Korea) (22). *Dspp* promoter deletion constructs were generated by serial deletion from the 5′-end of the promoter with PCR, and the fragments were ligated into the XhoI and HindIII sites, respectively. The *Dspp* promoter deletion constructs, D-610 (−610 to +54 bp), D-426 (−426 to +54 bp), D-249 (−249 to +54 bp), D-216 (−216 to +54 bp), D-94 (−94 to +54 bp), and D-15 (−15 to +54 bp) were subcloned into the pGL3-enhancer vector (Promega, Madison, WI) for the luciferase reporter assay. The forward and reverse primers for the amplification of *Dspp* promoter deletion constructs are listed in Table 1.

Site-directed Mutagenesis of Homeodomain Binding Sites—To produce constructs bearing mutations in the putative homeodomain binding sites, a site-directed mutagenic PCR was performed with the −433 Mut and −415 Mut primers and universal RVprimer3 (RV3) and GLprimer2 (GL2) listed in Table 1 establishing mutant promoters M-433 and M-415 (see Table 1 for the primer sequence). The PCR products of the mutated promoters were digested with XhoI and HindIII and used to replace the wild-type counterpart of the reporter vector.

Reverse Transcription-PCR and Quantitative Real Time PCR—The SuperScript™ first-strand synthesis system for reverse transcription was purchased from Invitrogen. Quantitative real time-PCR was performed using Takara SYBR Premix Ex Taq (Takara, Japan) on an Applied Biosystems 7500 Real Time PCR system (Foster City, CA). PCR primers were synthesized by Integrated DNA Technology (Coralville, IA). All samples were run in duplicate, and the relative levels of *Dspp* mRNA expression were normalized to those of glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*). The primer sets for real time PCR are listed in Table 2.

Knockdown Assays with siRNA—siRNAs were used to knock down Smad1/5, Dlx5, and Runx2 expression. siRNAs against Dlx5 and Runx2 (siGENOME SMART pool) were purchased from Dharmacon (Lafayette, CO). The siRNA against Smad1/5 (Stealth™/siRNA duplex oligoribonucleotides) was purchased from Invitrogen. siGENOME Non-Targeting siRNA #2 was used as a control (scrambled siRNA). Cells were seeded into 12-well plates and, after overnight culture, transfected with 60

TABLE 1

Primer sequences for construction of *Dspp* promoter deletion and mutant constructs

The boldfaced letters correspond to restriction enzyme sites for XhoI (forward primers) or HindIII (reverse primer). The lowercase letters designate the substitution of nucleotide for site-directed mutagenesis.

Name	Oligonucleotide sequence	Sequence location
		bp
−610 Del (forward)	5′-AAA**CTCGAG**CCTTTGGAACAGCTATTT-3′	−610 to −592
−426 Del (forward)	AAA**CTCGAG**CGCACCAACTTTAATATG	−426 to −408
−249 Del (forward)	AAA**CTCGAG**ACACAAAACAGTCTTCCAG	−249 to −230
−216 Del (forward)	AAA**CTCGAG**TGAAAGTAAGTCTAGTCCTTTTG	−216 to −194
−94 Del (forward)	AAA**CTCGAG**GATCCTAAGCAGTGATTG	−94 to −77
−15 Del (forward)	AAA**CTCGAG**CCCAGGACAGTGTG	−15 to −2
+54 Del (reverse)	ACA**AAGCTT**CGAGGGGACTTTGAA	+40 to +54
−433 Mut (forward)	ACAGCCGCTTGgcgggTAGCGCACCAA	−445 to −419
−433 Mut (reverse)	TTGGTGCGCTAcccgcCAAGCGGCTGT	−445 to −419
−415 Mut (forward)	GCGCACCAACTTgcggATGTACCTCAGG	−427 to −400
−415 Mut (reverse)	CCTGAGGTACATccgcAAGTTGGTGCGC	−427 to −400
RV3 (forward)	CTAGCAAAATAGGCTGTCCCCAGTGCAAGTGCA	
GL2 (reverse)	CTTTATGTTTTTGGCGTCTTCCATGGTGGCTTTACC	

TABLE 2

Primer sequences for quantitative real time PCR

Name	Oligonucleotide sequence
DSPP (forward)	5′-ATTCCGGTTCCCCAGTTAGTA-3′
DSPP (reverse)	5′-CTGTTGCTAGTGGTGCTGTT-3′
GAPDH (forward)	5′-GGCCTCACCCCATTTGATGT-3′
GAPDH (reverse)	5′-CATGTTCCAGTATGACTCCACTC-3′

or 40 pmol of siRNA using Lipofectamine™ 2000 (Invitrogen) in accordance with the manufacturer's instructions.

Transient Transfection—C2C12 cells were seeded in 100-mm plates and cultured to 90% confluence. The cells were then trypsinized and transfected with the desired expression vectors by electroporation using a Microporator (Invitrogen) with a 10-μl gold tip in accordance with the manufacturer's instructions. MDPC-23 cells were seeded in a 96-well assay plate, and after overnight culture the cells were transfected using Lipofectamine 2000 (Invitrogen) or Genefectine (Genetrone Biotech, Korea) transfection reagents in accordance with the manufacturer's instructions. Transfections were performed with 0.5 μg of Dlx5 or Msx2 or Yy1 expression vectors, or the pcDNA3.1 empty vector as a control, and 0.15 μg of the *Dspp* promoter reporter vectors (D-791 or deletion/mutant constructs). All plasmid DNAs were prepared using a DNA Maxi-prep kit (GENOMED, Loehne, Germany). The Dlx5, Msx2, Runx2, and Yy1 expression vectors were previously described and confirmed by Western blot analysis (15, 25, 26).

Luciferase Reporter Assay—After the cells were lysed with passive lysis buffer (Promega, Madison, WI), luciferase activity was determined by using a Bright-Glo™ Luciferase assay system (Promega) with a GloMax-Multi Detection System machine (Promega).

Electrophoretic Mobility Shift Assay (EMSA)—The sequences of the wild-type oligonucleotides are shown in Fig. 3*C*. These double-stranded DNA probes were end-labeled with [γ-^{32}P]ATP (PerkinElmer Life Science) using a DNA 5′ End Labeling System (Promega). 5′-DNA end labeling and EMSA were performed in accordance with the manufacturer's instructions and our previous papers (7, 25). The Dlx5 and Msx2 proteins were produced by *in vitro* transcription and translation using TNT-coupled Reticulocyte Lysate (Promega). The Dlx5 protein was incubated with the labeled, double-stranded DNA probes in the presence or absence of a 10-, 50-, or 100-fold molar excess of the unlabeled competitor for 30 min at room temperature. For the supershift assay, the Dlx5 proteins were preincubated with an anti-HA antibody for 30 min at room temperature. The protein-DNA complexes were then separated at room temperature in a 5% polyacrylamide gel containing 0.5× TBE buffer.

In Silico Promoter Analysis—We used the "Transcription Element Search System (TESS)," which is served by the University of Pennsylvania, and searched transcription factors that bind to the *Dspp* (NCBI gene number 666279) promoter region (from −791 to +54). Then, we performed genomic alignments among human, mouse, and rat *DSPP* promoter region with the "Ensembl" program.

RESULTS

Dspp Expression Is Stimulated by the Canonical BMP-2 Signaling Pathway—Treatment of MDPC-23 pre-odontoblast cells with BMP-2 (100 ng/ml) stimulated *Dspp* expression (Fig. 1*A*). Similarly, overexpression of constitutively active Smad1/5 (Fig. 1*B*), Dlx5 (Fig. 1*C*), or Runx2 (Fig. 1*D*), which are downstream factors of the canonical BMP-2 signaling pathway, stimulated *Dspp* mRNA expression even in the absence of BMP-2 treatment. In contrast, knockdown of Smad1/5 (Fig. 1*E*), Dlx5 (Fig. 1*F*), or Runx2 (Fig. 1*G*) suppressed the stimulation of *Dspp* mRNA expression by BMP-2.

Dlx5 Specifically Induces Dspp Gene Expression—We performed an *in silico* analysis of the mouse *Dspp* proximal promoter region between nucleotide −791 and the putative transcription start site (+1) and found five putative homeodomain binding sequences (H1 to H5) well conserved in human, mouse, and rat (Fig. 2*A*). To check the binding affinity of Dlx5 to the homeodomain binding sequences, we prepared 5′-serial deletion constructs of the *Dspp* promoter, D-791, designated D-426, D-249, D-216, D-94, and D-15 (Fig. 2*B*). A luciferase reporter assay with these deletion constructs showed strong changes in basal promoter activity and Dlx5/Msx2 responsiveness in D-426 and D-249 (Fig. 2*C*); therefore, we selected H1 and H2 as candidates of strong homeodomain protein response elements. Even though the other deletion constructs (D-216, D-94, and D-15) showed some changes in basal promoter activity, however, they did not show a significant change in Dlx5/

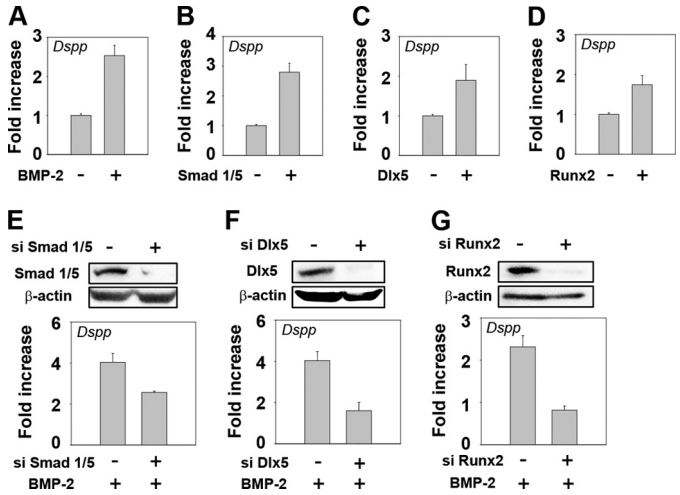

FIGURE 1. ***Dspp* expression is stimulated by the canonical BMP-2 signaling pathway.** *A–D, Dspp* mRNA expression was determined by quantitative real time PCR. MDPC-23 cells were treated with 100 ng/ml of BMP-2 (*A*) or transfected with a Smad1/Smad5 expression vector (*B*), Dlx5 expression vector (*C*), or Runx2 expression vector (*D*). After 24 h of treatment, total cellular RNA was harvested. *E–G,* MDPC-23 cells were transfected with 60 pmol of siRNA against Smad1/5 (*E*), Dlx5 (*F*), or Runx2 (*G*). Twenty-four hours after the siRNA transfection, cells were treated with 100 ng/ml of BMP-2 for 24 h. The protein levels of Smad1/5, Dlx5, Runx2, and β-actin were detected by immunoblotting (*upper panels*), and *Dspp* mRNA expression was determined by quantitative real-time PCR normalized to *Gapdh* (*lower panels*). Fold increase value is expressed as the mean ± S.D. of triplicate experiments.

Msx2 responsiveness by the serial deletions (Fig. 2*C*) so we ruled out H3, H4, and H5 for further study.

This *Dspp* promoter deletion analysis showed that deleting the region from −791 to −427 (Fig. 2*C*) strongly increased basal promoter activity but decreased the promoter activity stimulated by Dlx5 overexpression. These results indicate that there might be a strong negative regulatory element between bp −794 and −427, and that H1 is a good candidate region for a Dlx5 response element. The computerized promoter analysis showed that there is a possible response element for Yy1, a negative transcription factor, at position −622. A luciferase reporter assay with an Yy1 binding site deletion construct (D-610, Fig. 3*A*) showed that basal promoter activity increased after the Yy1 binding site was deleted. The overexpression of Yy1 strongly suppressed reporter activity in D-791, whereas suppression by Yy1 was not as strong after the Yy1 binding site was deleted (D-610, Fig. 3*A*). Based on this finding, we used D-610 to eliminate the strong Yy1 effect to the basal promoter activity with deletion constructs (Fig. 3*B*). To check the Dlx5 binding affinity for the putative homeodomain response elements, we made WT probes for H1 and H2, which we named h1 and h2 (Fig. 3*C*), and performed an EMSA with these probes. When we incubated the radiolabeled h1 and h2 WT probes with *in vitro* transcribed and translated Dlx5 protein, the Dlx5 protein made a complex with the radiolabeled probes (Fig. 3*D*, *lanes 2* and *9*). We added a molar excess of cold h1 or h2 to compete for the binding of Dlx5 to the radiolabeled h1 probe (Fig. 3*D*, *lanes 3–6*) or the radiolabeled h2 probe (Fig. 3*D*, *lanes 10–13*). The HA-tagged Dlx5-DNA probe complex was confirmed by a supershift after treatment with an anti-HA antibody (Fig. 3*D, lanes 7* and *14*). Our EMSA data indicated that the binding affinity of Dlx5 to H1 and H2 was similar. However, in the luciferase reporter assay with *Dspp* promoter mutant constructs, both the basal promoter activity (Mock) and induction by Dlx5 overexpression were lower from the M-433 mutant than from the M-415 mutant in C2C12 and MDPC-23 cells, showing that H1 is more important than H2 in the stimulation by Dlx5 (Fig. 3, *E–G*).

Msx2 Antagonizes Dlx5 by Inhibiting Its Binding to the Dspp Promoter Region—Our luciferase assay result with *Dspp* promoter deletion constructs showed different responsiveness to Dlx5 and Msx2 at H1 and H2 (Fig. 2*C*). We previously reported that Dlx5 stimulates *Alp* and *Mepe* expression, but Msx2 antagonizes the stimulatory effect of Dlx5 by competing for binding to the same response elements in promoters (7, 14). On the basis of these results, we hypothesized that Msx2 also suppresses *Dspp* expression. Msx2 overexpression in MDPC-23 cells suppressed *Dspp* expression with or without BMP-2 treatment (Fig. 4*A*). To investigate the antagonizing effect of Msx2 on *Dspp* expression, we transfected the Dlx5 and/or Msx2 expression vectors and a *Dspp* promoter reporter vector (D-791) into C2C12 cells (Fig. 4*B*). *Dspp* promoter reporter activity was increased by Dlx5 overexpression, but decreased by Msx2 overexpression, and Dlx5-stimulated promoter reporter activity was completely suppressed by Msx2 overexpression (Fig. 4*B*). A luciferase reporter assay with *Dspp* promoter deletion constructs showed that Msx2 suppresses reporter activity more at H2 than H1 (Fig. 4*C*). To examine the Msx2 binding affinity in the *Dspp* promoter region, we performed an EMSA with radiolabeled h1 and h2 probes (Fig. 4*D*). We incubated the h1 and h2 WT probes with a constant amount of Dlx5 protein (Fig. 4*D*, *lanes 3* and *10*) and increasing amounts (1×, 2×, and 4×) of Msx2 protein (Fig. 4*D*, *lane 4-6* and *11–13*). There was little change in the amount of h1 probe-Dlx5 complex (Fig. 4*D*, *lane 4-6*), but the h2 probe-Dlx5 complex decreased with increase of the Msx2 protein (Fig. 4*D*, *lanes 11–13*). The presence of HA-tagged Dlx5-DNA probe complex was confirmed by supershift with an anti-HA antibody (Fig. 4*D*, *lanes 7* and *14*, *asterisk*). Contrary to the results with the *Alp* and *Mepe* promoters (7, 14), Msx2 did not form a complex with the probes when Msx2 protein was increased, although it did inhibit Dlx5 binding to h2 (Fig. 4*D*). A luciferase reporter assay with *Dspp* promoter mutant constructs for H1 (M-433) and H2 (M-415) (Fig. 4*E*) showed that suppression by Msx2 overexpression was similar to D-791 in M-433-transfected cells, but Msx2-induced suppression of Dspp promoter activity was almost abrogated in M-415 (H2 site mutant)-transfected C2C12 and MDPC-23 cells (Fig. 4, *F* and *G*). These data indicate that Msx2 antagonizes Dlx5 binding more at H2 than H1.

DISCUSSION

Tooth development is regulated by various cytokines and transcription factors. Among them, BMP-2 is one of the most crucial for regulating epithelial-mesenchymal interactions and is expressed in the primary enamel knot, which is an epithelial signaling center and plays a central role in tooth morphogenesis (9, 27). In tooth development, DSPP is a marker of odontoblast differentiation and is associated with dentin mineralization. The transcriptional regulators of *Dspp* gene expression have been identified: Runx2 and CCAAT-binding factor (NF-Y) are

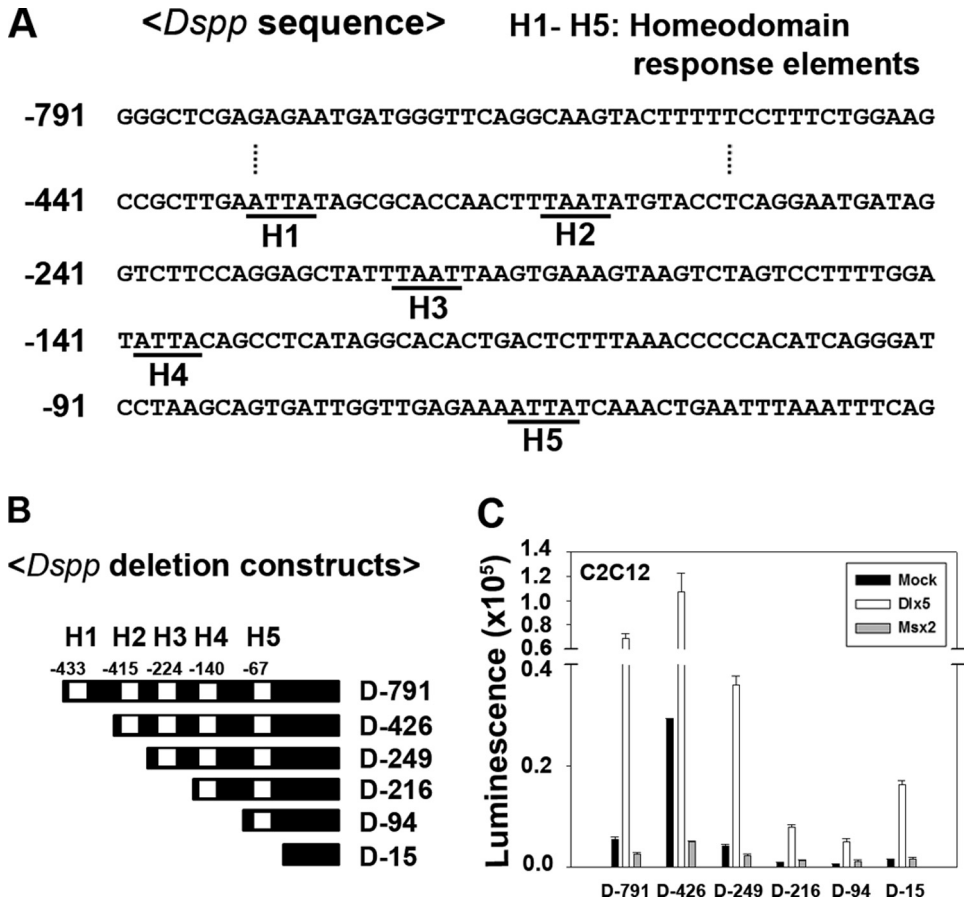

FIGURE 2. **The *Dspp* promoter has homeodomain response elements.** *A,* the mouse *Dspp* promoter region has homeodomain binding sites between −791 and +54 bp that are conserved among vertebrate species (human, mouse, and rat). These response elements were designated H1, H2, H3, H4, and H5 (*H1*, −433 to −430; *H2*, −415 to −412; *H3*, −224 to −221; *H4*, −140 to 137; *H5*, −67 to −64). *B,* serial deletion constructs of the *Dspp* promoter are illustrated. *C,* C2C12 cells were transfected with reporter vectors including the *Dspp* promoter region (D-791 and 5′-serial deletion constructs D-426, D-249, D-216, D-94, and D-15) and a pcDNA3.1 empty vector to determine the basal promoter activity and Dlx5 or Msx2 expression vectors were transfected along with the promoter reporter vector. Luciferase activities are normalized by total protein level and are expressed as the mean ± S.D. of triplicate experiments. Between 0.4 and 0.6 ($\times 10^5$) in the *y* axis was deleted to express the *bar* in the bottom of graph.

transcriptional activators (19, 28), and Twist1 is a novel transcriptional repressor (29). Here, we have added the components of the canonical BMP-2 signaling pathway to the transcriptional regulation of *Dspp* gene expression in MDPC-23 mouse preodontoblast cells.

Dspp Expression Is Regulated by the Canonical BMP-2 Signaling Pathway—BMP-2 induces osteoblast differentiation by activating transcription factors Dlx3, Dlx5, and Runx2 (13, 26). Previously, Chen *et al.* (19) showed that BMP-2 treatment of the MD10-F2 mouse pre-odontoblast cell line increased *Dspp* mRNA expression. They reported that BMP-2 induces *Dspp* expression through NF-Y, but they did not address the relationship between canonical BMP-2 signaling and *Dspp* expression. We have investigated the role of the canonical BMP-2 signaling pathway in expression of various bone marker genes and have identified *Mepe* as one gene whose expression is regulated by this pathway (7). Because BMP-2 is also important in odontoblast differentiation, and DSPP is involved in dentin mineralization as matrix extracellular phosphoglycoprotein is in bone mineralization, we hypothesized that *Dspp* expression is also regulated by the canonical BMP-2 signaling pathway. We confirmed here that *Dspp* expression is stimulated by BMP-2 treatment (Fig. 1*A*). *Dspp* expression was increased by treatment with BMP-2 or overexpression of downstream factors in the canonical signaling pathway, Smad1/5, Dlx5, and Runx2. Up-regulation of the *Dspp* mRNA expression level by overexpression of BMP R-Smads, Dlx5, and Runx2 showed an additive effect over the BMP-2 treatment (data not shown). Conversely, siRNA knockdown of Smad1/5, Dlx5, and Runx2 suppressed the expression of *Dspp* mRNA. Previously, Chen *et al.* (28) demonstrated that Runx2 regulates *Dspp* expression by direct binding to the highly conserved Runx2 response element in the *Dspp* promoter. Our data showing changes in *Dspp* mRNA expression by Runx2 overexpression or knockdown with siRNA in MDPC-23 pre-odontoblast cells were very consistent with their finding. We did not find a Runx2 response element in our *Dspp* proximal promoter regions downstream of −791, but a previous report indicated that the Runx2 response element is more than 1 kb upstream of the transcription start site (28). Therefore, in this study, we focused on the homeodomain protein (Dlx/Msx) response elements in the *Dspp* promoter. Our results suggest that the canonical BMP-2 signaling pathway through BMP R-Smads, Dlx5, and Msx2 is also important for regulation of *Dspp* expression, in concert with other known factors.

The Possibility of Complementary Cooperation with Other Transcription Factors—Knockdown of Smad1/5, Dlx5, and Runx2, factors downstream of canonical BMP-2 signaling with siRNA was not enough to block *Dspp* expression due to BMP-2 treatment; we therefore tried to identify another pathway that modulates *Dspp* expression. One possible pathway is that of NF-Y, which interacts with the inverted CCAAT box binding site and is controlled by BMP-2 (19). NF-Y binds to a BMP-2 response element in the mouse *Dspp* promoter, especially between nucleotides −97 and −72 (19). Therefore, we could suppose NF-Y to be another factor that stimulates *Dspp* expression in response to BMP-2 treatment, but is independent of the canonical BMP signaling pathway.

In addition to the BMP signaling, canonical Wnt signaling is another possible strong regulator of biomineralization. The LEF/TCF-β-catenin complex can be stabilized by glycogen synthase kinase-3β phosphorylation, and glycogen synthase kinase-3β activity can be controlled by BMP-2 treatment (30).

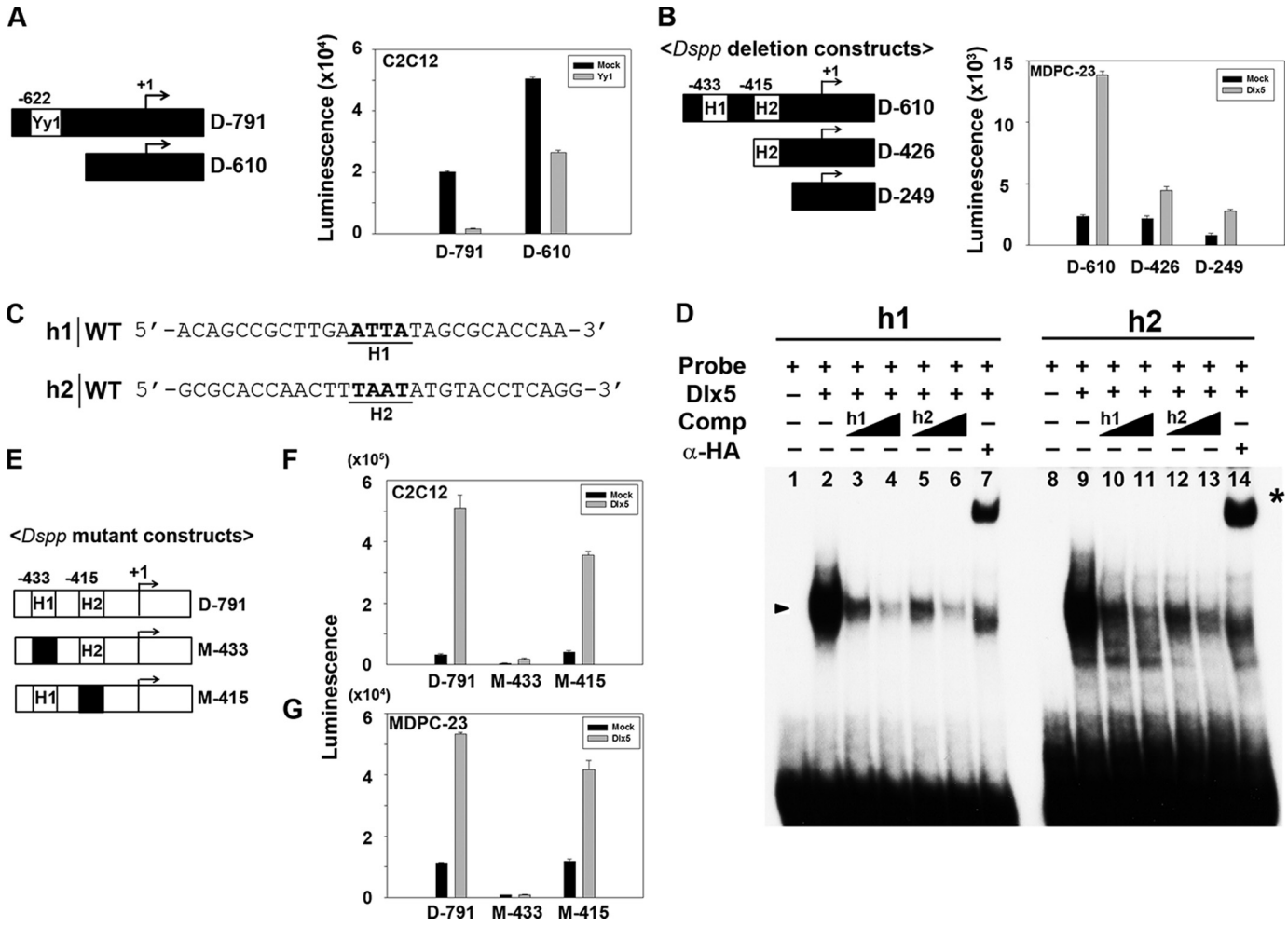

FIGURE 3. **Putative homeodomain response elements in the *Dspp* promoter specifically bind to Dlx5.** *A,* luciferase reporter assay. C2C12 cells were transfected with *Dspp* promoter constructs D-791 and D-610, along with the pcDNA 3.1 empty vector or Yy1 expression vector. *B,* luciferase reporter assay. MDPC-23 pre-odontoblastic cells were transfected with reporter vectors including the *Dspp* promoter region (D-610 and 5′-serial deletion constructs D-426 and D-249) and with a pcDNA3.1 empty vector to determine the basal level (*Mock*), and with the Dlx5 expression vector to determine the fold-induction level. Luciferase activities are expressed as the mean ± S.D. of triplicate experiments. *C,* two probes for EMSA (probes *h1* and *h2*) were constructed from the *Dspp* promoter construct bearing two representative homeodomain response elements, *H1* and *H2*, between −791 and +54 bp. *D,* ^{32}P-labeled h1 and h2 probes were incubated with *in vitro* transcribed and translated HA-Dlx5 protein. *Lanes 1* and *8*, free probe; *lanes 2–7* and *9–14*, Dlx5 protein incubated separately with the labeled h1 and h2 probes alone (*lanes 2* and *9*) or in the presence of a 50- or 100-fold molar excess of unlabeled h1 or h2 oligonucleotides (*lanes 3, 4, 10*, and *11* for h1 probe, and *5*, *6*, *12*, and *13* for the h2 probe); *lanes 7* and *14*, binding of HA-Dlx5 protein to radiolabeled probes h1 and h2 was confirmed by supershift assays with an anti-HA antibody. The *arrowhead* indicates binding of Dlx5 to each probe, and the *asterisk* indicates the supershift by the anti-HA antibody. *E, Dspp* promoter mutant constructs are illustrated. *F* and *G*, luciferase reporter assay. C2C12 (*F*) and MDPC-23 (*G*) cells were transfected with a reporter vector containing the *Dspp* promoter region D-791 or mutant constructs M-433 and M-415 missing the H1 and H2 region, respectively, and the pcDNA3.1 empty vector or Dlx5 expression vector. Luciferase activities are expressed as the mean ± S.D. of triplicate experiments.

Because we found several highly conserved LEF/TCF binding sites in the *Dspp* proximal promoter region (data not shown), it is highly probable that *Dspp* is a direct Wnt target gene. In addition, considering the cross-talk between the BMP-2 and Wnt signaling pathways, BMP-2 may stimulate *Dspp* expression indirectly by regulating glycogen synthase kinase-3β action, which would subsequently stabilize β-catenin and LEF/TCF action.

Yy1 Is a Negative Regulator of Dspp Expression—Interestingly, the basal level of reporter activity strongly increased when the promoter region −791 to −427 was deleted (Fig. 2*C*, from D-791 to D-426). We therefore hypothesized that this region includes a suppressor binding region and investigated candidates. Twist1, which is a novel repressor of *Dspp* expression, was a possible candidate (29), but there were no Twist1 binding elements in the *Dspp* proximal promoter region between −791 and −427. Instead, we found an Yy1 binding element at −622. Yy1 is a ubiquitously distributed transcription factor belonging to the GLI-Kruppel class of zinc finger proteins and is a bifunctional protein that acts as an activator or repressor of many promoters (31). To check the effect of Yy1 on the *Dspp* promoter, we made a Yy1 expression vector and a luciferase reporter vector construct (D-610) in which the Yy1 binding region is deleted. The luciferase reporter assay indicated that Yy1 overexpression suppressed the reporter activity, and suppression almost disappeared when the Yy1 binding region was deleted (Fig. 3*A*). These results suggest that Yy1 suppresses *Dspp* expression by directly binding to the response element at position −622 of the promoter.

Msx2 Antagonizes Dlx5, Inhibiting the Binding of Dlx5 to the Dspp Promoter—Dlx/Msx homeoproteins are critical determinants of early tooth development and are downstream mole-

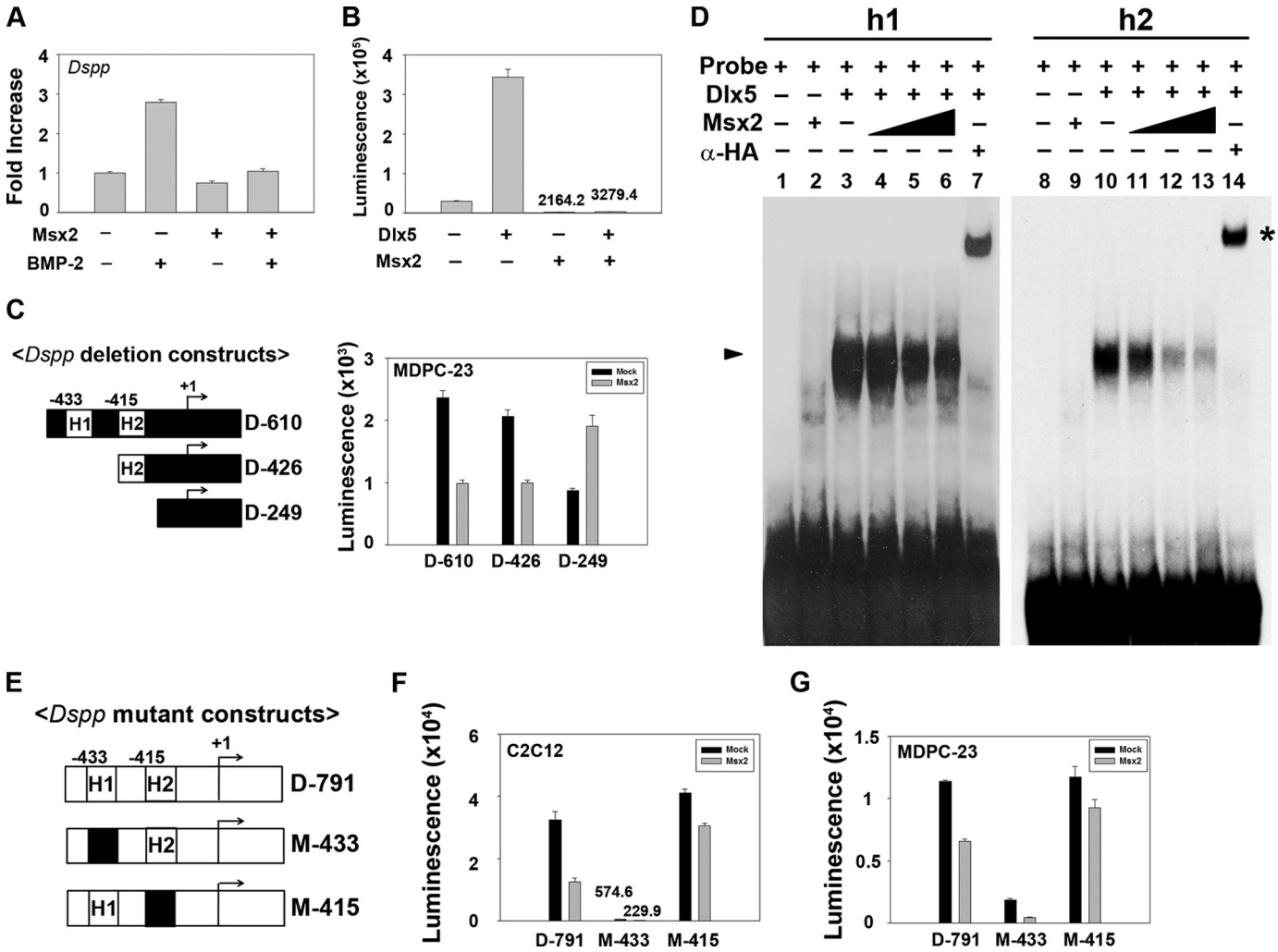

FIGURE 4. ***Dlx5* and *Msx2* reciprocally antagonize binding to the DSPP promoter region to each other.** *A,* MDPC-23 cells were transfected with pcDNA3.1 empty vector or Msx2 expression vector. After the culture reached visual confluence, the cells were treated with BMP-2 (100 ng/ml) for an additional 24 h, and total cellular RNA was extracted. *Dspp* mRNA expression was determined by quantitative real time PCR. The relative level of *Dspp* mRNA was normalized to the *Gapdh* level. *B,* C2C12 cells were transiently transfected with a *Dspp* promoter reporter vector (D-791) and the Dlx5 and/or Msx2 expression vector, and a luciferase assay was performed. *C,* MDPC-23 cells were transfected with reporter vectors containing the *Dspp* promoter region (D-610 and 5′-serial deletion constructs D-426 and D-249) along with a pcDNA3.1 empty vector to determine the basal level or an Msx2 expression vector to determine the fold-induction level. Luciferase activities were determined based on triplicates in each experiment and three independent experiments, and normalized to the basal level with empty vector expression. *D,* radiolabeled h1 and h2 probes were used to compete with the Msx2 protein for binding to the Dlx5 protein. Each protein was transcribed and translated *in vitro*. The h1 and h2 probes were separately incubated with a fixed amount of Dlx5 protein. *Lanes 1* and *8*, free probe; *lanes 2* and *9*, Msx2 protein incubated with the labeled h1 or h2 probe. *Lanes 3–7* and *10–14*, Dlx5 protein incubated with the labeled h1 or h2. *Lanes 4–6*, the binding complex with Dlx5 (*arrowhead*) and h1 probe did not decrease after the addition of increasing amounts of Msx2 (no specific bands). *Lanes 11–13*, the binding complex with Dlx5 (*arrowhead*) and h2 probe gradually decreased after the addition of increasing amounts of Msx2 (no specific bands). *Lanes 7* and *14*, *asterisk* indicates a supershift created by the anti-HA antibody against HA-Dlx5. *E, Dspp* promoter mutant constructs lacking H1 (*M-433*) or H2 (*M-415*) are illustrated. *F* and *G,* luciferase reporter assay. C2C12 (*F*) and MDPC-23 (*G*) cells were transfected with reporter vectors containing the *Dspp* promoter region (D-791 or mutant constructs M-433 and M-415) and the pcDNA3.1 empty vector (*Mock*) or Msx2 expression vector. Luciferase activities are expressed as the mean ± S.D. of triplicate experiments.

cules of the canonical BMP-2 signaling pathway (13, 32, 33). Dspp is expressed in odontoblasts and ameloblasts, and Dlx and Msx are also commonly expressed in these cells, giving them the potential to regulate *Dspp* gene expression (34). We found conserved Dlx/Msx homeodomain binding elements in the *Dspp* promoter region. Msx2 antagonizes Dlx5 activity by acting as a repressor at the promoters of osteocalcin (35), bone sialoprotein (36), α1(I) collagen (23), *Alp* (14), and *Mepe* (7). Two mechanisms have been proposed to explain how Msx2 counteracts Dlx5. One is that the two factors compete for the same binding sites in the target promoters, and the other is that protein interactions occur between the Msx2 and Dlx5 homeodomains, which may inhibit the binding of Dlx5 to the target promoters (7). Our EMSA data support the latter idea for *Dspp* gene regulation because Msx2 did not bind directly to the response region in the *Dspp* promoter (Fig. 4*D*). It is clear that Msx2 antagonizes the Dlx5 stimulation of *Dspp* expression, but the mechanism is different from its action in *Alp* (14) and *Mepe* (7) promoters, in which Msx2 competes with Dlx5 for binding to a common response element.

In conclusion, we report evidence here that the canonical BMP-2 signaling pathway regulates *Dspp* expression via BMP-R Smads, Runx2, and Dlx5, and is antagonized by Msx2 in MDPC-23 mouse pre-odontoblast cells. These downstream

factors of canonical BMP signaling, together with NF-Y, act independently or work together to stimulate *Dspp* expression at the transcriptional level. Other factors, such as Msx2, Twist1, and Yy1, act as negative regulators, and the positive and negative factors work together to determine the transcriptional level of the *Dspp* expression. As previous studies strongly indicated that Dspp played a critical role in dentinogenesis and dentin mineralization (4–6), the up-regulation of Dspp expression would be quite important in secondary dentin formation after exposure of odontoblast processes by dental cavity preparation (17). In this context, we can suggest that a direct application of BMP2 or activators of its downstream transcription factors in dental cavities would be a possibility to enhance secondary dentin bridge formation through up-regulation of *Dspp* expression.

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