

ORIGINAL

BMP2-induced gene profiling in dental epithelial cell line

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Abstract : Tooth development is regulated by epithelial-mesenchymal interactions and their reciprocal molecular signaling. Bone morphogenetic protein 2 (BMP2) is known as one of the inducers for tooth development. To analyze the molecular mechanisms of BMP2 on ameloblast differentiation (amelogenesis), we performed microarray analyses using rat dental epithelial cell line, HAT-7. After confirming that BMP2 could activate the canonical BMP-Smads signaling in HAT-7 cells, we analyzed the effects of BMP2 on 14,815 gene expressions and profiled them. Seventy-three genes were up-regulated and 28 genes were down-regulated by BMP2 treatment for 24 hours in HAT-7 cells. Functional classification revealed that 18% of up-regulated genes were ECM/adhesion molecules present in the enamel organ. Furthermore, we examined the expression of several differentiation markers in dental epithelial four cell-lineages including inner enamel epithelium (ameloblasts), stratum intermedium, stratum reticulum, and outer enamel epithelium. The results indicated that BMP2 might induce at least two different cell-lineage markers including a BMP antagonist expressed in HAT-7 cells, suggesting that BMP2 could accelerate amelogenesis via BMP signaling. *J. Med. Invest.* 55 : 216-226, August, 2008

Keywords : dental epithelium, BMP2, microarray, gene profiling

INTRODUCTION

Tooth development is initiated in dental epithelial cells derived from ectodermal cells, and underlying ectomesenchymal cells that are subsequently activated to promote the developmental cascade. The reciprocal interactions between epithelial and mesenchymal cells are strictly regulated during tooth development not only by growth factors and cytokines such as bone morphogenetic proteins (BMPs), fibroblast growth factors, Wnts, and tu-

mor necrosis factors but also by extracellular matrices (1).

BMPs are members of the TGF- β superfamily, and their production and receptors have been detected in both epithelial and mesenchymal cells during tooth development (2, 3). Previous studies using the organ culture system and genetic mouse models have demonstrated the critical role of BMPs in tooth development and its morphogenesis (4, 5). Moreover, two BMP antagonists, follistatin and ectodin (USAG-1, Wise), have recently been reported to control tooth morphogenesis by fine-tuning of BMP signals (6-9). In follistatin transgenic mice, follistatin negatively regulated ameloblast differentiation and resulted in no enamel formation in the incisors (6). Conversely, follistatin knockout mice ectopically produced enamel in the enamel free area

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(the lingual side of incisors in rodents) (7). Ectodin has been shown to control the activity of the enamel knot, a signal center, in tooth germ (10). Consistently, loss of function analysis using ectodin knockout mice revealed both extra-tooth formation and altered cusp patterning (8, 9, 11, 12). On the other hand, the application of BMP4 to the ectodin-deficient tooth germ culture accelerated the cytodifferentiation of ameloblasts and odontoblasts (9). In addition, BMP2 has been also reported as one of the potent inducers of ameloblast differentiation (amelogenesis) in combination with the apatite *in vitro* system (13). These results indicated that BMPs play an essential role in tooth development. However, the downstream molecular events and critical target genes of BMPs during amelogenesis remain still unclear.

HAT-7 cells are dental epithelial cells derived from the apical loop of rat incisors where is the dental stem cell niche (14). In addition, HAT-7 cells have a potential to differentiate into ameloblasts by cell-cell contact and overexpression of Notch signaling *in vitro* (15, 16).

In this study, we examined the regulatory roles of BMP2 on gene expression in HAT-7 cells using microarray and RT-PCR analysis.

MATERIALS & METHODS

Materials

Dulbecco's Modified Eagle Medium (DMEM) and Ham's F-12 Medium (F12) were purchased from Nissui Pharmaceutical (Tokyo, Japan). Fetal Bovine Serum (FBS) was purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA). BMP2 was provided by Yamanouchi Pharmaceutical (Tokyo, Japan). TRI Reagent was purchased from Molecular Research Center, Inc. (Cincinnati, OH, USA). RNA-PCR kit was from Takara Bio Inc. (Otsu, Japan). Go Taq DNA polymerase was purchased from Promega (Madison, WI, USA) and SYBR Green PCR Master Mix was from Applied Biosystems (Foster City, CA, USA).

Cells and cell culture

HAT-7 cells (14) were maintained in DMEM/F12 medium with 10% FBS under 5% CO₂ in air at 37°C. BMP2 was added when the cell growth was observed at 80% confluence. After the cells were washed twice with phosphate-buffered saline (PBS) without calcium and magnesium [PBS (-) : 137 mM

NaCl, 8.1 mM Na₂HPO₄ · 12H₂O, 2.68 mM KCl, and 1.47 mM KH₂PO₄, pH 7.4], they were cultured with or without 300 ng/ml of BMP2.

Western blot analysis

HAT-7 cells were lysed in a buffer containing 1% (v/v) Nonidet P-40, 150 mM NaCl, 0.1% SDS, 1 mM EDTA, 10% glycerol, 10 mM NaF, 2 mM phenylmethanesulfonyl fluoride, 0.1 mM leupeptin, and 20 mg/ml aprotinin in 50 mM Tris-HCl (pH 8.0) and rotated for 1 hr at 4°C. Lysates were separated by centrifugation at 20,000 × g for 15 min and supernatants were collected in a microtube. Measurement of protein contents and Western blot analysis were carried out as described previously (17). Activated Smad1/5/8 was detected using anti-phospho-Smad1/5/8 (1 : 1,000 ; #9511, Cell Signaling, Danvers, MA, USA).

cDNA microarray analysis

HAT-7 cells were cultured in DMEM/F12 medium with 10% FBS until 80% confluence was observed. The medium was then replaced by serum-free medium with or without 300 ng/ml of BMP2. After 24 hrs, the cells were harvested and total RNA was isolated using TRI Reagent followed by two rounds of ethanol precipitation. Each 20 µg of total RNA was reverse transcribed and labeled with Cy3 or Cy5. The labeled probes were hybridized with a cDNA microarray that printed 14,815 cDNA fragments (Agilent, Palo Alto, Cal., USA) on the slide. The arrays were scanned and analyzed. Differential expression of the genes was determined as previously reported (17).

Quantitative RT-PCR

Total RNA was treated with DNase I (Invitrogen, Carlsbad, CA) to eliminate genomic DNA contamination. One µg of total RNA was reverse-transcribed to cDNA using RNA-PCR kit following the manufacturer's instruction. A part of this reaction was used as a template in the polymerase chain reaction (PCR) with SYBR Green PCR Master mix (Applied Biosystems) or FastStart Universal SYBR Green Master (ROX) (Roche Diagnostics, Mannheim, Germany). PCR amplification was performed on the following genes ; *Cyp26b*, *CRP2*, *β-igh3*, *FN1*, *Smad7*, *OBF-1*, *TIEG*, *BMP2*, *BMP4*, *BMP7*, *follistatin*, *ectodin* and *18S rRNA*. The primer sequences are listed in Table 1. The ABI PRISM 7000 (Applied Biosystems) was used for Quantitative RT-PCR and expression ratios were cal-

Table 1 Primers for Quantative RT-PCR

Gene	Primers
<i>18S</i>	5'-TACCTGGTTGATCCTGCCAGTAGCAT-3' 5'-CCCGTCGGCATGTATTAGCTCTAGAA-3'
<i>Amelogenin</i>	5'-CAAGAAATGGGGACCTGGATCTTGTTT-3' 5'-GCTGCCTTATCATGCTCTGG-3'
<i>Cyp26b</i>	5'-AGGGCCTAGAGAAGGCTGTC-3' 5'-GTTCCCTCCCGTAGCTTTTCC-3'
<i>Crp2</i>	5'-GATCATTGGAGCTGGAAAGC-3' 5'-TCCTTGACCATAGCCGAATC-3'
<i>β-igh3</i>	5'-CATTGACGGCCAAATGAAGAC-3' 5'-TGGTGAACAGGGTCCCATAC-3'
<i>Fibronectin</i>	5'-GAAAGGCAACCAGCAGAGTC-3' 5'-CTGGAGTCAAGCCAGACAC-3'
<i>Smad7</i>	5'-TTGGAGTCTTTTCTCTCTC-3' 5'-GGCTCAATGAGCATGCTCAC-3'
<i>OBF-1</i>	5'-CTGTCAGCTGCCCTTACTCC-3' 5'-GGCCACGGGAAATAGGTAAG-3'
<i>TIEG</i>	5'-ATCTGTAGCCACCCAGGATG-3' 5'-GGGACAGGCAAATTTCTTCTC-3'
<i>BMP2</i>	5'-TGAACACAGCTGGTCTCAGG-3' 5'-GCTAAGCTCAGTGGGGACAC-3'
<i>BMP4</i>	5'-CAGAGCCAACACTGTGAGG-3' 5'-TCCACTCCCTTGAGGTAACG-3'
<i>BMP7</i>	5'-AGACGCCAAAGAACCAAGAG-3' 5'-GCTGTCGTCGAAGTAGAGGA-3'
<i>Follistatin</i>	5'-CGGAACCTCATCTTCAGAG-3' 5'-GCCAACCTTGAAATCCCATA-3'
<i>Ectodin</i>	5'-GAGGCAGGCATTTTCAGTAGC-3' 5'-CATAGCCTCCTCCGATCCAG-3'

culated from the threshold cycles, normalized by *18S rRNA* results. All data were confirmed by triplicate experiments.

RT-PCR analysis

Total RNA was treated with DNase I (Invitrogen, Carlsbad, CA) to eliminate genomic DNA contamination. One μg of the treated RNA was converted to cDNA in a 20 μl using RNA-PCR kit. One μl of this reaction was used as a template in the PCR with Go Taq DNA polymerase. The primer sequences are listed in Table 1 and 2. The results

Table 2 Primers for RT-PCR

Gene	Primers
<i>GAPDH</i>	5'-CATTGACCTCAACTACATGG-3' 5'-CTCAGTGTAGCCCAGGATGC-3'
<i>P75Ngfr</i>	5'-GAGGGCACATACTCAGACGAAGCC-3' 5'-GTCTATATGTTTCAGGCTGGTAACC-3'
<i>Jagged1</i>	5'-GTCCACGGCACCTGCAATG-3' 5'-ACAAGGCTTGGCCCTCGCAC-3'
<i>Amelogenin</i>	5'-CAAGAAATGGGGACCTGGATC-3' 5'-GCTGCCTTATCATGCTCTGG-3'
<i>Notch1</i>	5'-CTACAATCAGGGCACCTGTG-3' 5'-GCCATCACTGAAGTGGTCC-3'
<i>Alkaline phosphatase</i>	5'-CTGGACCTCATCAGCATTG-3' 5'-GCTGTGAAGGGCTTCTTGTC-3'
<i>Hes1</i>	5'-CACGCTCGGGTCTGTGCTGAGAGC-3' 5'-ATGCCAGCTGATATAATGGAG-3'
<i>Notch2</i>	5'-GAGTGTCTGAAGGGCTACGC-3' 5'-TGCAGATGCAGGTGTAGGAG-3'

were captured using Chemi-Doc XRS (BIO-RAD, Tokyo, Japan) and expression levels were estimated using Quantity One software (BIO-RAD).

RESULTS

BMP2-Smads signaling is functional in the dental epithelial cell line, HAT-7

Previously, Coin, *et al.* reported that BMP2 combined with apatite could induce both morphological and functional differentiation of ameloblasts using the *in vitro* culture system (13). However, the precise molecular mechanism of ameloblast differentiation remains unclear. To analyze the roles of BMP2 on ameloblast differentiation, we first examined whether the BMP2-Smads pathway is functional in HAT-7 cells. Western blot analysis using the antibody against the phosphorylated Smad1/5/8 demonstrated that BMP2 specifically activated Smad1/5/8 within 15 min and maintained their phosphorylated state up to 24 hrs in HAT-7 cells as shown in Fig. 1.

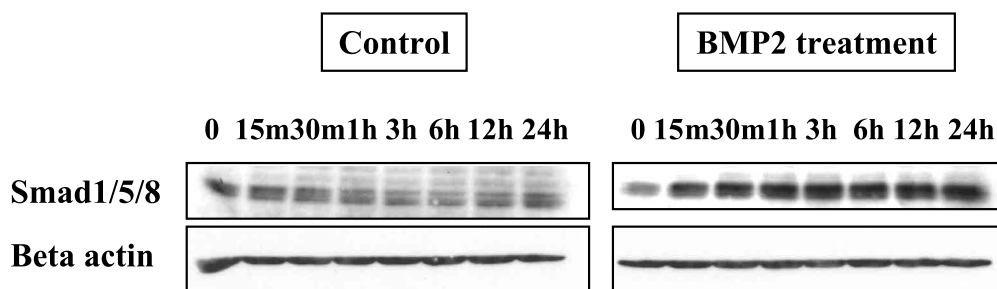


Fig. 1 Smad1/5/8 activation by BMP2 in HAT-7 cells.

HAT-7 cells were treated with 300ng/ml of BMP2 during the indicated time. Forty μg of total protein was applied in each lane. Phospho-Smad1/5/8 was detected. Beta-actin was used as the internal control.

Gene profiling in BMP2-treated HAT-7 cells

To examine the effects of BMP2 on gene expression patterns in HAT-7 cells, we performed cDNA

Table 3 Up-regulated genes by BMP2 in HAT-7

Gene Name	Fold
Rat cytochrome P450RAI-2 (Cyp26b1)	14.31
Rat cysteine and glycine-rich protein 2	6.68
Rat insulin-like growth factor binding protein 5	6.44
Rat rho GTPase activating protein 7	6.14
Rat beta ig-h3	5.87
Rat synaptotagmin-like 2	5.24
Rat DOC-2 p82	4.88
Rat carbonic anhydrase III (CA3)	4.85
Mouse capping protein alpha 1	4.19
Rat mRNA for fibronectin	4.06
Mouse NCAM-140 and NCAM-180 isoforms.	3.91
Mouse putative membrane-associated guanylate kinase 1 (Magi-1)	3.75
Mouse cytohesin binding protein (Cbp)	3.72
Rat lysyl oxidase	3.64
Rat Smad7 protein	3.50
Rat secernin 1	3.41
Rat RDC-1 protein.	3.16
Rat protein kinase C epsilon subspecies.	3.13
Mouse pre B-cell leukemia transcription factor 1	3.11
Rat New England Deaconess transcription factor	3.08
Rat G-protein beta 5 subunit	2.86
Mouse Id4 dominant negative helix-loop-helix gene.	2.74
Rat mRNA for gal beta 1,3 galNAc alpha 2,3-sialyltransferase.	2.73
Connective tissue growth factor precursor	2.70
Rat podocalyxin	2.69
Rat adenylyl cyclase	2.68
Plakophilin 2a	2.66
Periostin, osteoblast specific factor	2.63
Mouse nicotinamide nucleotide transhydrogenase (Nnt)	2.62
Mouse TGF-beta-inducible protein (TSC-36)	2.61
Rat low voltage-activated, T-type calcium channel alpha subunit (CACNA1G)	2.56
Rat PGES mRNA for prostaglandin E synthase	2.56
Rat activin type I receptor	2.55
Rat tensin1 (Tns)	2.53
Mouse hereditary multiple exostoses (Ext1)	2.43
Rat peroxisome proliferator-activated receptor gamma 2 (PPARgamma2)	2.42
Rat mindin precursor,	2.42
Rat developmentally regulated intestinal protein (OCI-5)	2.39
Rat mRNA for multidrug resistance protein (MRP5)	2.33
Rat alpha 1 type V collagen	2.29
Rat follistatin-related protein precursor	2.28
Actin binding LIM protein family, member 3	2.25
Mouse homeobox gene Prx2 mRNA.	2.25
Mouse HIC-5	2.18
Rat lung beta-galactoside-binding lectin	2.15
Rat alpha-tropomyosin	2.05
Mouse Ulip protein.	2.03
Mouse sialic acid binding Ig-like lectin 10	2.03

microarray analysis. Before the experiment, we checked the effects of BMP2 on the *amelogenin* gene expression and found that BMP2 could induce *amelogenin* expression transiently in dose- and time-dependent manner (data not shown). Based on the results, HAT-7 cells were treated with or without 300 ng/ml of BMP2 for 24 hrs, and total RNA was purified as mentioned in MATERIALS & METHODS. We found that 101 genes were regulated in response to BMP2 treatment (Tables 3 and 4). Seventy-three genes were up-regulated including 25 unknown genes and 28 genes were down-regulated including 8 unknown genes (Fig. 2A). The results were summarized based on the gene function in Fig. 2B. Interestingly, 18 % of BMP2-induced genes are ECM/adhesion molecules and signaling molecules. Gene expressions of this category including *fibronectin* (18), *NCAM* (19), and *periostin* (20, 21) have been reported in both ectomesenchyme and dental epithelia during tooth development. About 10% of BMP2-induced genes are transcriptional regulators including *Prx2*, *Id4*, *PBX*, and *CRP2*. However, their roles in tooth development have not been described in detail.

Confirmation of gene expression by Quantitative RT-PCR

To confirm the microarray data, we further ana-

Table 4 Down-regulated genes by BMP2 in HAT-7

Gene Name	Fold
Mouse C57Bl/6J clone L45 odorant receptor	0.48
Rat transcription factor Maf1	0.48
Kruppel-like factor 10, (TIEG)	0.47
Rat cDNA for glutamate receptor subunit (GluR6), kainate subtype.	0.46
Extensin-like protein	0.46
Rat mRNA for carbonic anhydrase II.	0.45
Rat brain glucose-transporter protein	0.45
Mouse Mort1 Fas-associated protein	0.42
Mouse hybrid receptor gp250 precursor	0.41
Mouse cytoplasmic protein Ndr1 (Ndr1)	0.41
Rat high affinity glutamate transporter EAAC1	0.39
Mouse neural precursor cell expressed developmentally downregulated Nedd9 (Nedd9)	0.39
Mouse Oct binding factor 1 (OBF-1)	0.39
Rat thrombomodulin	0.38
Incyte EST/inositol 1,4,5-trisphosphate 3-kinase C	0.37
Rat GADD153	0.35
Rat galectin-7	0.31
Rat PFK-L mRNA for liver phosphofructokinase.	0.27
Rat aldehyde dehydrogenase	0.25
Rat interferon regulatory factor 1 (IRF-1)	0.21

A

	Known Gene	Unknown Gene	Total
Up	48	25	73
Down	20	8	28
Total	68	33	101

(more than 2-fold, less than 0.5-fold.)

B

[UP]	
Secretion :	Growth factor (1) Secreting machinery (2)
Cell-cell interaction :	ECM/Adhesion (13)
Cell surface :	Receptor (membrane) (2) Transporter/Channel (2) Membrane protein (1)
Cytoskeleton :	Cytoskeleton (2)
Cytosol :	Kinase (2) Enzyme (w/o kinase) (7) Signaling (8)
Nuclear :	Transcriptional regulator (8)
[DOWN]	
Apoptosis :	Apoptosis (2)
Cell-cell interaction :	Adhesion/Glycoprotein (3)
Cell surface :	Receptor (membrane) (3) Transporter (2)
Cytoskeleton :	Cytoskeleton (1)
Cytosol :	Kinase (3) Enzyme (w/o kinase) (2) Signaling (1)
Nuclear :	Transcriptional regulator (3)

Fig. 2 Summary of microarray analysis. (A) Overview of gene expression change. "Up" and "Down" indicate that the expression level is 2-fold higher or less than 0.5-fold by BMP2 treatment. (B) Functional classification of up- and down-regulated genes. The number in parenthesis indicates the gene numbers.

lyzed the following 7 genes involved in developmental events: *Cytochrome P450 family26 subfamily b (Cyp26b)* (22), *cysteine and glycine-rich protein 2 (CRP2)* (23, 24), *TGF β -induced gene H3 (β ig-h3)* (25), *Fibronectin 1 (FNI)* (18, 26), *Smad7* (27), *Oct binding factor-1 (OBF)* (28, 29), and *TGF β -inducible early transcription factor gene (TIEG/KLF10)* (30) (Fig. 3A). *In silico* analysis of the potential BMP-inducible Smads-binding sites located within the 3.0 kb upstream region from each transcription start site identified that the numbers of binding sites in *Cyp26b*, *CRP2*, *β ig-h3*, *FNI*, *Smad7*, *OBF*, and *TIEG* were 79, 69, 59, 55, 92, 54, and 79, respectively. Subsequently, we performed the quantitative RT-PCR to validate the microarray results as shown in Fig. 3B. These results indicated that the microarray data are reproducible. To further examine these responses, we also performed RT-PCR analysis using additional two other dental epithelial cell lines treated with BMP2. The result shows similar responses to BMP2 treatment as HAT-7 cells (data

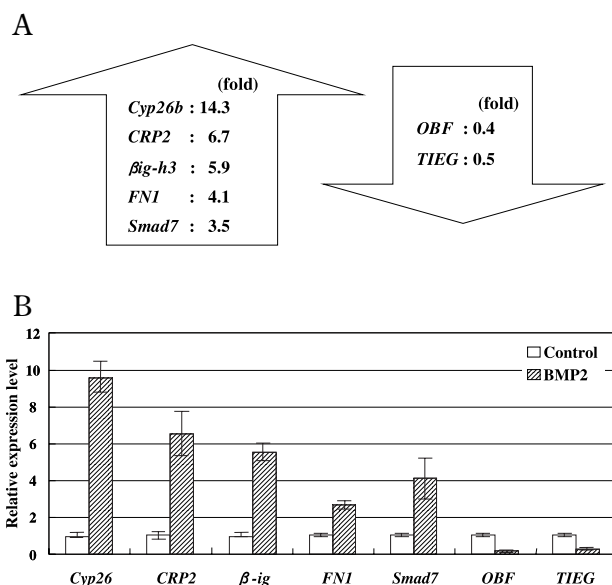


Fig. 3 Confirmation of microarray data by Quantitative RT-PCR. (A) The changes in the expression levels of genes related to differentiation. Up- and down-regulated genes are indicated by up- and down-arrows, respectively. (B) Confirmation of microarray data by Quantitative RT-PCR. Expression levels of each gene are normalized to those of *18S rRNA*. Relative level is calculated as BMP2 treatment sample divided by untreated sample. White bar : untreated HAT-7 as a control, striped bar : 300 ng/ml of BMP2 treated HAT-7.

not shown).

BMP2 enhanced gene expression of ameloblast markers in HAT-7 cells

Since the number of cDNA that can be printed on a microarray is limited, we could not detect any well-known amelogenesis-related genes. Dental epithelial cells differentiate into four cell-lineages during tooth development: inner enamel epithelium (IEE), stratum intermedium (SI), stratum reticulum (SR), and outer enamel epithelium (OEE). Ameloblasts are derived from IEE by several growth factors, cytokines, and cell-cell interactions. To assess whether BMP2 can promote *in vitro* differentiation of undifferentiated HAT-7 cells into inner enamel epithelial-lineage cells or ameloblasts, we analyzed mRNA induction of marker genes following BMP2 treatment.

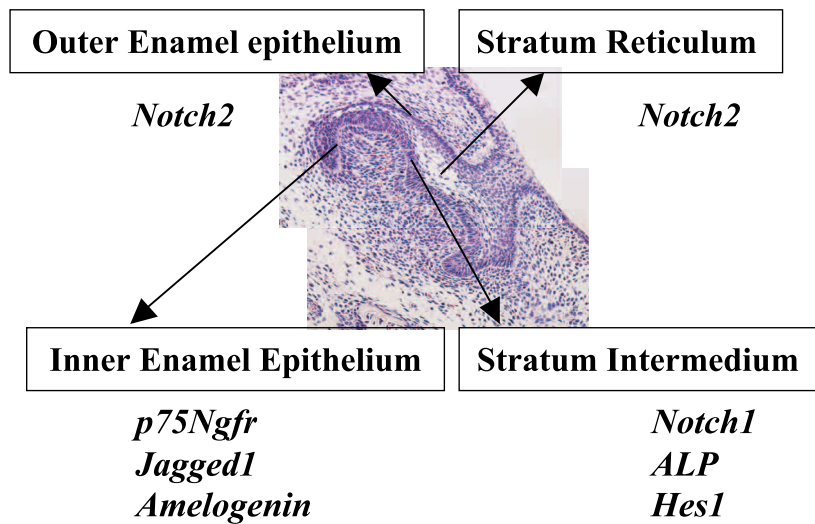
Fig. 4A depicts well-known differentiation markers related to the dental epithelial cell-lineages (16). As shown in Fig. 4B, BMP2 enhanced the gene expressions of *p75Ngfr* and *amelogenin* (IEE and ameloblast markers) whereas that of *Hes1* (SI marker) was slightly reduced. Moreover, the gene expression of *Notch2* (SR or OEE marker) was also enhanced by BMP2 treatment. The other marker genes for IEE and SI, *Notch1* and *Jagged1*,

were unaffected. These results indicated that BMP2 could promote ameloblastic marker genes induction.

Furthermore, we analyzed the effects of BMP2 on the gene expression of other BMP families and their antagonists that are closely related to amelo-

genesis. BMP2 treatment enhanced the expression level of *BMP4* (Fig. 5A, 5B), and significantly induced that of *ectodin* in HAT-7 cells (Fig. 5A). Conversely, the expression levels of *BMP2*, *BMP7*, and *follistatin* were unaffected by BMP2 treatment (Fig. 5A, 5B).

A



B

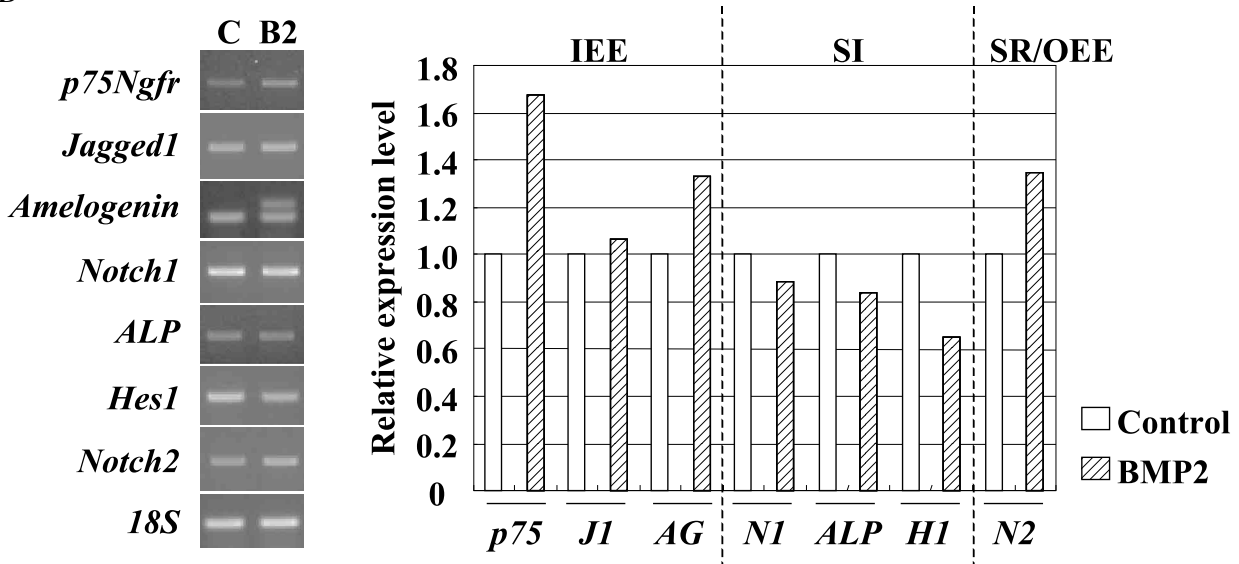


Fig. 4 Effects of BMP2 on marker gene expression of four dental epithelial cell-lineages in HAT-7 cells.

(A) The localization of four dental epithelial cell-lineages at bell stage of tooth germ. The marker genes of each cell-lineage are indicated under the name of the cell-lineage.

IEE : inner enamel epithelium, SI : stratum intermedium, SR : stratum reticulum, and OEE : outer enamel epithelium. (B) Effects of BMP2 on 4-cell-lineage marker genes in HAT-7 cells. Left panel : RT-PCR analysis was performed and PCR products were stained and captured using Chemi-Doc XRS. C : no treatment, B2 : 300ng/ml BMP2 was treated for 24 hrs. Right panel : relative ratio of gene expression levels. The expression levels were estimated using Quantity One software, and normalized by each *18S rRNA* level. Relative level was calculated as BMP2 treatment sample divided by untreated sample. IEE : inner enamel epithelium, SI : stratum intermedium, SR : stratum reticulum, OEE : outer enamel epithelium, *p75* : *p75Ngfr*, *J1* : *Jagged1*, *AG* : *amelogenin*, *N1* : *Notch1*, *ALP* : *alkaline phosphatase*, *H1* : *Hes1*, *N2* : *Notch2*. White bar : untreated HAT-7 as a control, striped bar : 300 ng/ml of BMP2 treated HAT-7. These are representative data among three repeated experiments.

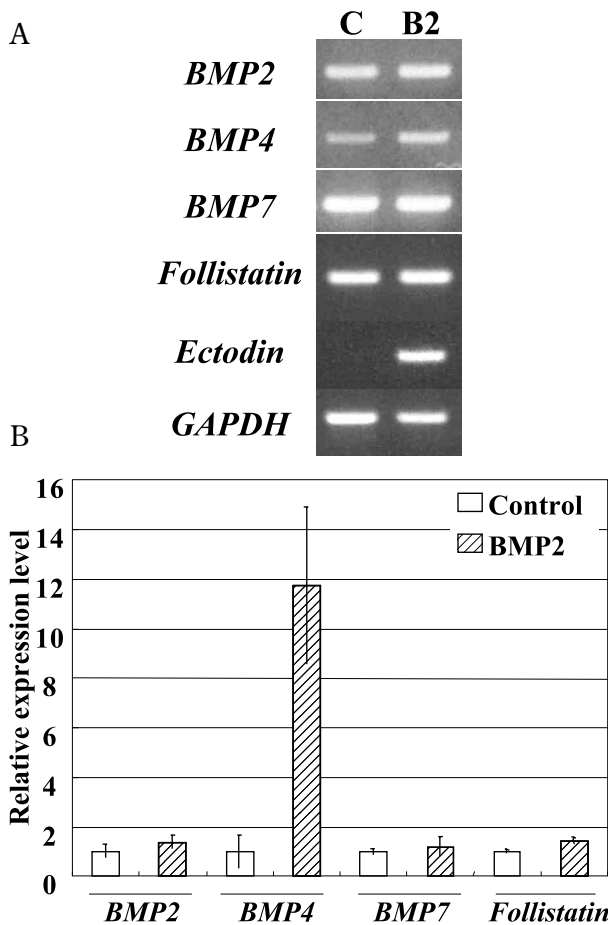


Fig. 5 Effects of BMP2 on *BMP2*, 4, 7 and BMP antagonists, *ectodin*, *follistatin* mRNAs in HAT-7 cells.

(A) RT-PCR analysis was performed. The *ectodin* mRNA could not be detected initially, but it was induced by BMP2 in HAT-7 cells. C : untreated, B2 : BMP2 treated with 300ng/ml for 24 hrs. (B) The relative level of each gene expression in HAT-7 cells. Quantitative RT-PCR was performed on *BMP2*, 4, 7 and BMP antagonists, *ectodin*, *follistatin* mRNAs in HAT-7 cells. Each expression level was normalized by the *18S rRNA* level. Relative level was calculated as BMP2 treated sample divided by untreated sample. White bar : untreated HAT-7 as a control, striped bar : 300 ng/ml of BMP2 treated HAT-7 cells. PCR products of *Ectodin* were not detected in untreated HAT-7 cells. Therefore, the relative ratio could not be calculated.

DISCUSSION

Here we reported that canonical BMP-Smad signaling pathway in HAT-7 cells could be activated in response to BMP2 stimulation (Fig. 1). Moreover, BMP2 was able to enhance the expression levels of *p75Ngfr* and *amelogenin*, the well-known ameloblast differentiation markers, whereas that of *Hes1*, a marker for stratum intermedium, was reduced (Fig. 4). These findings suggest that the gene expression pattern in HAT-7 cells was shifted from the transient amplifying stage of IEE (15) to the ameloblast-lineage by BMP2 treatment which is consistent with the results of the previous study sug-

gesting that BMP may play a role in amelogenesis (13). The other marker genes for IEE and SI, *Jagged1*, *Notch1*, and *ALP* were unaffected. We need further investigation for these genes but it is possible that the effects of BMP2 are not so significant on these gene expressions at this time point. Recently, it has been reported that *Jagged1* is one of the target genes of Shh signaling (31), and *Notch1* is regulated via IKK signaling (32). Furthermore, the induction of *ALP* gene expression is mediated through MAPK activity (33), and Wnt signaling suppresses BMP2-induced *ALP* expression in osteoblasts (34). These data suggested that other signaling molecules might be involved in the regulation of their gene expression.

Taken together, we conclude that HAT-7 cells are a suitable *in vitro* assay system to analyze BMP2-mediated gene regulation.

We performed microarray analysis using total RNA from BMP2-treated and untreated HAT-7 cells. The results clearly demonstrated that BMP2 regulated multiple gene expression positively and negatively in HAT-7 cells as listed in Fig. 2B. Up-regulated genes are listed in Table 3 and include several tooth-related genes. *Connective tissue growth factor precursor* was up-regulated 2.7-fold by BMP2 in HAT-7 cell, and the expression of *connective tissue growth factor (CTGF)* has been reported in IEE, preameloblast, and OEE (35). *Periostin* was up-regulated 2.6-fold by BMP2, and it has been detected in SI cells (21). Notably, periostin null mice showed abnormal ameloblast differentiation (20).

To further analyze the microarray data, we focused on 7 genes that are reported to play a role in cellular differentiation. The highest induction was observed with *Cyp26b1* gene expression in BMP2 treated HAT-7 cells as shown in Table 3, *Cyp26b1*, belonging to the Cyp26 family are a group of P450 enzymes that metabolize retinoic acid to inactive forms (36). *Cyp26b1* is highly expressed in the restricted regions of the developing limb, and a knock-out mouse model showed that *Cyp26b1* prevents apoptosis and promotes chondrocyte maturation in limb development (37). The *Cyp26* gene has two additional isoforms, *Cyp26a1* and *Cyp26c1*, that have been reported to be expressed in mouse dental epithelium (22, 38, 39); *Cyp26b1* has been detected in the craniofacial mesenchymal cells neighboring *Cyp26a1*- and *Cyp26c1*-expressing cells (22, 39). *Fibronectin (FN)*, an extracellular matrix protein, is also induced by BMP2. In tooth development, *FN* is expressed not only in mesenchymal

cells but also in the stellate reticulum and is deposited in basement membrane (18, 40). FN has also been shown to appear in the epithelium during submandibular gland development and is suggested to induce the EMT event in development of the cleft of alveoli (26). Since HAT-7 cells are derived from an apical loop containing stem cell fraction, it is possible that some population of HAT-7 cells could differentiate into SR cells. *Smad7*, an inhibitory Smad, was increased 3.5-4-fold in BMP2 treated HAT-7. *Smad7* has been shown to inhibit TGF- β signaling to a greater degree than BMP signaling (41, 42). It has also been reported that keratin 5 promoter-driven *Smad7* transgenic mice caused defects in amelogenesis (27). These results suggested that BMP2 may define the spatio-temporal domain of BMP target field by balancing between TGF- β and BMP signaling through regulating *Smad7* gene expression. OBF is a B-cell specific transcriptional co-activator that functions with octamer binding transcription factors to regulate B cell differentiation (28). *TIEG* (KLF10) was identified as a TGF- β inducible gene and it was also reported that BMP2 could induce *TIEG* mRNA and regulate cerebellar granule cell differentiation (43, 44). However, *OBF* and *TIEG* were down-regulated in BMP2-treated HAT-7 and this may be due to the difference in cell types (Table 4 and Fig. 3).

Moreover, we discovered two interesting genes that have never been discussed in amelogenesis study. One is CRP2, a member of the LIM family of proteins, which contains the LIM motif and double zinc finger domains (24). In general, LIM proteins are important for both cellular differentiation and function (45). LIM family proteins, such as *Islet1*, *Lhx6*, *Lhx7*, and *Lhx8*, have shown to be important regulators in tooth formation and patterning (46). *Islet1* is expressed in dental epithelium and activates BMP4 expression. *Lhx 6, 7, and 8* are expressed in dental mesenchyme (47). CRP2 has been reported to have restricted expression in arteries, cardiac myocytes, and fibroblasts (24, 48); however, there are no reports of its expression in tooth development. Our microarray data indicated that BMP2 strongly induces CRP2 mRNA in the dental epithelial cell line (6.7-fold compare to untreated HAT-7 cells). Western blot analysis could also detect enhanced CRP2 protein expression in BMP2-treated HAT-7 cells (unpublished results). However, we could not detect specific CRP2 reactivity in the rat tooth during amelogenesis by immunohistochemistry (data not shown). Therefore,

further investigation is required to determine the expression of CRP2 in tooth structures and to analyze its role in amelogenesis.

Another gene identified in this study is *β ig-h3*, originally recognized as a TGF- β inducible gene, whose mRNA was increased 5.9-fold by BMP2 treatment. *β ig-h3* is a secretory protein and a member of the periostin family (49) that contains both an RGD sequence and four fasciclin-1 domains. It is expressed in several tissues, including lung, kidney, skin, bone, and cartilage (25, 50, 51). *β ig-h3* has been shown to bind not only to ECM proteins such as FN, laminin, and collagens, but also to cell surface molecules such as $\alpha\beta$ 5 integrin, decorin, and biglycan (52, 53). To confirm *β ig-h3* expression, we performed immunohistochemical analysis using a commercially available antibody. Unfortunately, we could not detect any specific signals in tooth sections, possibly due to the inability of the antibody to detect the antigen on paraffin-embedded samples (data not shown). To understand the roles of CRP2 and *β ig-h3* in tooth development *in vivo*, it will be necessary to generate new antibodies against them and the gene knock-out mice in the next study.

Microarray data did not include other tooth-related genes such as BMP family members (*BMP-2*, *BMP-4*, and *BMP-7*) and BMP antagonists (*ectodin*, *follistatin*) since the gene numbers on the microarray chip were limited. Therefore, we further performed RT-PCR analysis to examine their expression as shown in Fig. 5. BMP2 treatment significantly enhanced the level of *BMP4* mRNA. This finding is novel and important in the dental epithelial cells since BMP4 plays a distinct role in early tooth development (5). Moreover, RT-PCR analysis demonstrated that BMP2 regulated the expression of its own antagonists. BMP2 induced *ectodin* mRNA expression and slightly enhanced *follistatin* mRNA expression (Fig. 5B). Since *ectodin* is also an inhibitor of Wnt via competitive binding to LRP 6 (54), both BMP and Wnt might form a regulatory loop *in vivo*. Previous studies demonstrated that both *ectodin* and *follistatin* are expressed in IEE and OEE, and play important roles in tooth morphogenesis (6-9). Since overexpression of *Wnt3* caused defects of ameloblast differentiation in the lower incisor (55), it is suggested that the induction of *ectodin* may form a negative feedback loop serving to control ameloblast differentiation in HAT-7 cells.

Taken together, our findings suggest that BMP2

might accelerate or support amelogenesis at the molecular level. Therefore, our gene profiling data will facilitate identification of a new approach for the molecular analysis of amelogenesis and tooth regeneration.

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