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

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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 tumor 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 controls 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/F 12 medium with 10% FBS under 5% CO_2 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 $Na_2HPO_4 \cdot 12H_2O$, 2.68 mM KCl, and 1.47 mM KH_2PO_4, 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 Quantative RT-PCR and expression ratios were cal-

Table 1 Primers for Quantative RT-PCR

Gene	Primers
18S	5'-TACCTGGTTGATCCTGCCAGTAGCAT-3'
	5'-CCCGTCGGCATGTATTAGCTCTAGAA-3'
Amelogenin	5'-CAAGAAATGGGGACCTGGATCTTGTTT-3'
	5'-GCTGCCTTATCATGCTCTGG-3'
Cyp26b	5'-AGGGCCTAGAGAAGGCTGTC-3'
	5'-GTTCCTCCCGTAGCTTTTCC-3'
Crp2	5'-GATCATTGGAGCTGGAAAGC-3'
	5'-TCCTTGACCATAGCCGAATC-3'
β-igh3	5'-CATTGACGGCCAAATGAAGAC-3'
	5'-TGGTGAACAGGGTCCCATAC-3'
Fibronectin	5'-GAAAGGCAACCAGCAGAGTC-3'
	5'-CTGGAGTCAAGCCAGACAC-3'
Smad7	5'-TTGGAGTCCTTTCCTCTCTC-3'
	5'-GGCTCAATGAGCATGCTCAC-3'
OBF-1	5'-CTGTCAGCTGCCCTTACTCC-3'
	5'-GGCCACGGGAAATAGGTAAG-3'
TIEG	5'-ATCTGTAGCCACCCAGGATG-3'
	5'-GGGACAGGCAAATTTCTTCTC-3'
BMP2	5'-TGAACACAGCTGGTCTCAGG-3'
	5'-GCTAAGCTCAGTGGGGACAC-3'
BMP4	5'-CAGAGCCAACACTGTGAGG-3'
	5'-TCCACTCCCTTGAGGTAACG-3'
BMP7	5'-AGACGCCAAAGAACCAAGAG-3'
	5'-GCTGTCGTCGAAGTAGAGGA-3'
Follistatin	5'-CGGAACCCTCATCTTCAGAG-3'
	5'-GCCAACCTTGAAATCCCATA-3'
Ectodin	5'-GAGGCAGGCATTTCAGTAGC-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
GAPDH	5'-CATTGACCTCAACTACATGG-3'
	5'-CTCAGTGTAGCCCAGGATGC-3'
P75Ngfr	5'-GAGGGCACATACTCAGACGAAGCC-3'
	5'-GTCTATATGTTCAGGCTGGTAACC-3'
Jagged1	5'-GTCCACGGCACCTGCAATG-3'
	5'-ACAAGGCTTGGCCTCGCAC-3'
Amelogenin	5'-CAAGAAATGGGGACCTGGATC-3'
	5'-GCTGCCTTATCATGCTCTGG-3'
Notch1	5'-CTACAATCAGGGCACCTGTG-3'
	5'-GCCATCACTGAAGTGGTCC-3'
Alkaline phosphatase	5'-CTGGACCTCATCAGCATTTG-3'
	5'-GCTGTGAAGGGCTTCTTGTC-3'
Hes1	5'-CACGCTCGGGTCTGTGCTGAGAGC-3'
	5'-ATGCCAGCTGATATAATGGAG-3'
Notch2	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.

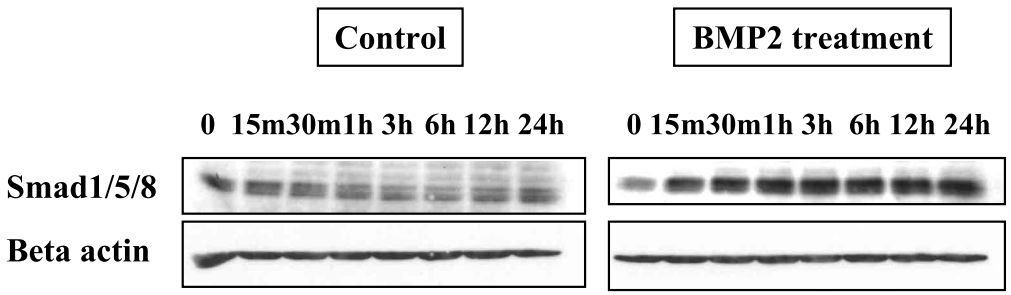

Fig. 1 Smad1/5/8 activation by BMP2 in HAT-7 cells.
HAT-7 cells were treated with 300ng/ml of BMP2 during the indicated time. Forty μg of total protein was applied in each lane. Phospho-Smad1/5/8 was detected. Beta-actin was used as the internal control.

Gene profiling in BMP2-treated HAT-7cells

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

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

Confirmation of gene expression by Quantative 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.

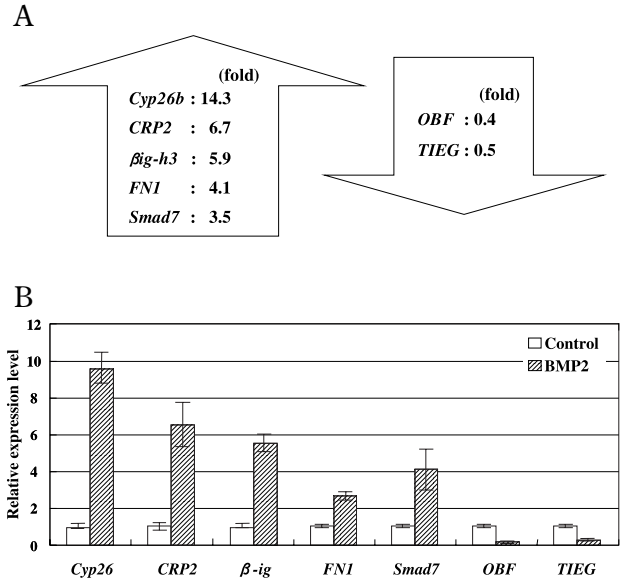

Fig. 3 Confirmation of microarray data by Quantative RT-PCR.
(A) The changes in the expression levels of genes related to differentiation. Up- and down-regulated genes are indicated by up- and down-arrows, respectively. (B) Confirmation of microarray data by Quantative RT-PCR. Expression levels of each 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.

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), *TGFβ-induced gene H3* (*βig-h3*) (25), *Fibronectin 1* (*FN1*) (18, 26), *Smad7* (27), *Oct binding factor-1* (*OBF*) (28, 29), *and TGFβ-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*, *βig-h3*, *FN1*, *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 not shown).

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

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 *Jagged* 1,

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 amelogenesis. 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).

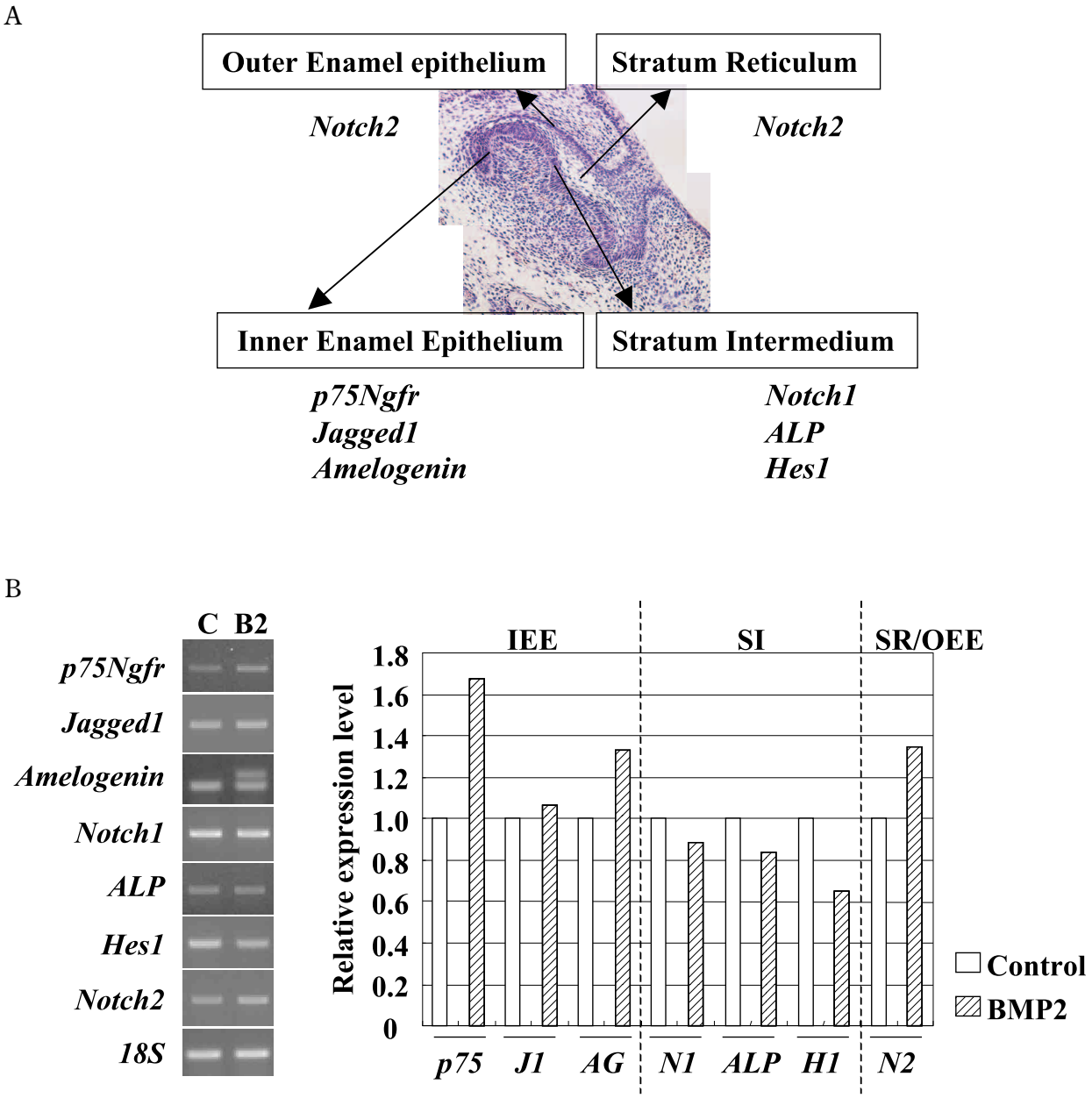

Fig. 4 Effects of BMP2 on marker gene expression of four dental epithelial cell-lineages in HAT-7 cells.
(A) The localization of four dental epithelial cell-lineages at bell stage of tooth germ. The marker genes of each cell-lineage are indicated under the name of the cell-lineage.
IEE : inner enamel 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.

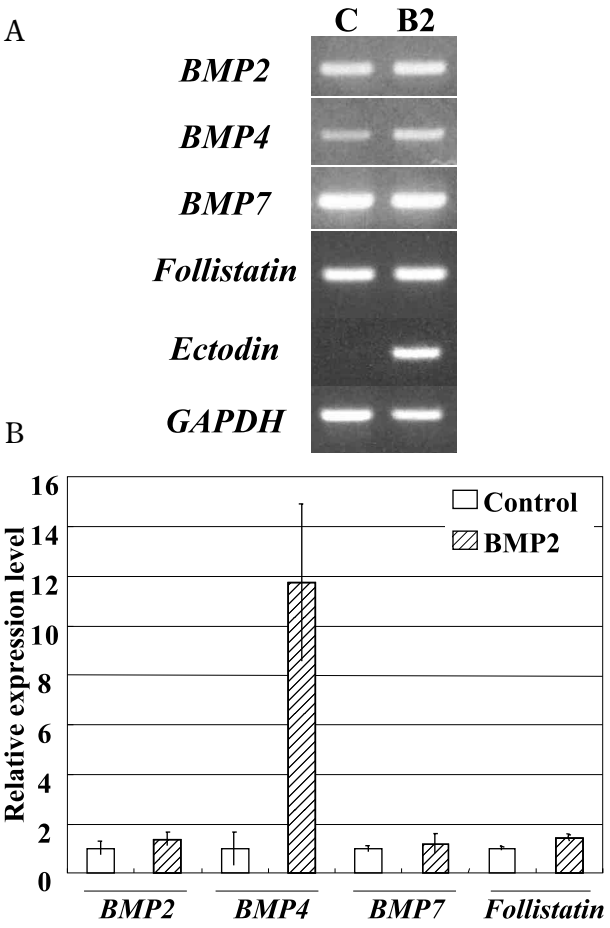

Fig. 5 Effects of BMP2 on *BMP2, 4, 7* and BMP antagonists, *ectodin*, *follistatin* mRNAs in HAT-7 cells.
(A) RT-PCR analysis was performed. The *ectodin* mRNA could not be detected initially, but it was induced by BMP2 in HAT-7 cells. C : untreated, B2 : BMP2 treated with 300ng/ml for 24 hrs. (B) The 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 suggesting 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 knockout 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 *βig-h3*, originally recognized as a TGF-β inducible gene, whose mRNA was increased 5.9-fold by BMP2 treatment. βig-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). βig-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 αvβ5 integrin, decorin, and biglycan (52, 53). To confirm βig-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 βig-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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REFERENCES

1. Thesleff I : Epithelial-mesenchymal signalling regulating tooth morphogenesis. J Cell Sci 116 : 1647-8, 2003
2. Aberg T, Wozney J, Thesleff I : Expression patterns of bone morphogenetic proteins (Bmps) in the developing mouse tooth suggest roles in morphogenesis and cell differentiation. Dev Dyn 210 : 383-96, 1997
3. Nadiri A, Kuchler-Bopp S, Perrin-Schmitt F, Lesot H : Expression patterns of BMPRs in the developing mouse molar. Cell Tissue Res 324 : 33-40, 2006
4. Andl T, Ahn K, Kairo A, Chu EY, Wine-Lee L, Reddy ST, Croft NJ, Cebra-Thomas JA, Metzger D, Chambon P, Lyons KM, Mishina Y, Seykora JT, Crenshaw EB, 3rd, Millar SE : Epithelial Bmpr1a regulates differentiation and proliferation in postnatal hair follicles and is essential for tooth development. Development 131 : 2257-68, 2004
5. Bei M, Kratochwil K, Maas RL : BMP4 rescues a non-cell-autonomous function of Msx1 in tooth development. Development 127 : 4711-8, 2000
6. Wang XP, Suomalainen M, Jorgez CJ, Matzuk MM, Werner S, Thesleff I : Follistatin regulates enamel patterning in mouse incisors by asymmetrically inhibiting BMP signaling and ameloblast differentiation. Dev Cell 7 : 719-30, 2004
7. Wang XP, Suomalainen M, Jorgez CJ, Matzuk MM, Wankell M, Werner S, Thesleff I : Modulation of activin/bone morphogenetic protein signaling by follistatin is required for the morphogenesis of mouse molar teeth. Dev Dyn 231 : 98-108, 2004
8. Murashima-Suginami A, Takahashi K, Kawabata T, Sakata T, Tsukamoto H, Sugai M, Yanagita M, Shimizu A, Sakurai T, Slavkin HC, Bessho K : Rudiment incisors survive and erupt as supernumerary teeth as a result of USAG-1 abrogation. Biochem Biophys Res Commun 359 : 549-55, 2007
9. Kassai Y, Munne P, Hotta Y, Penttila E, Kavanagh K, Ohbayashi N, Takada S, Thesleff I, Jernvall J, Itoh N : Regulation of mammalian tooth cusp patterning by ectodin. Science 309 : 2067-70, 2005
10. Laurikkala J, Kassai Y, Pakkasjarvi L, Thesleff I, Itoh N : Identification of a secreted BMP antagonist, ectodin, integrating BMP, FGF, and SHH signals from the tooth enamel knot. Dev Biol 264 : 91-105, 2003
11. Yanagita M, Oka M, Watabe T, Iguchi H, Niida A, Takahashi S, Akiyama T, Miyazono K, Yanagisawa M, Sakurai T : USAG-1 : a bone morphogenetic protein antagonist abundantly expressed in the kidney. Biochem Biophys Res Commun 316 : 490-500, 2004
12. Yanagita M, Okuda T, Endo S, Tanaka M, Takahashi K, Sugiyama F, Kunita S, Takahashi S, Fukatsu A, Yanagisawa M, Kita T, Sakurai T : Uterine sensitization-associated gene-1 (USAG-1), a novel BMP antagonist expressed in the kidney, accelerates tubular injury. J Clin Invest 116 : 70-9, 2006
13. Coin R, Haikel Y, Ruch JV : Effects of apatite, transforming growth factor beta-1, bone morphogenetic protein-2 and interleukin-7 on ameloblast differentiation *in vitro*. Eur J Oral Sci 107 : 487-95, 1999
14. Kawano S, Morotomi T, Toyono T, Nakamura N, Uchida T, Ohishi M, Toyoshima K, Harada H : Establishment of dental epithelial cell line (HAT-7) and the cell differentiation dependent on Notch signaling pathway. Connect Tissue Res 43 : 409-12, 2002
15. Harada H, Ichimori Y, Yokohama-Tamaki T, Ohshima H, Kawano S, Katsube K, Wakisaka S : Stratum intermedium lineage diverges from ameloblast lineage via Notch signaling. Biochem Biophys Res Commun 340 : 611-6, 2006
16. Kawano S, Saito M, Handa K, Morotomi T, Toyono T, Seta Y, Nakamura N, Uchida T, Toyoshima K, Ohishi M, Harada H : Characterization of dental epithelial progenitor cells

derived from cervical-loop epithelium in a rat lower incisor. J Dent Res 83 : 129-33, 2004
17. Ruspita I, Miyoshi K, Muto T, Abe K, Horiguchi T, Noma T : Sp6 downregulation of follistatin gene expression in ameloblasts. J Med Invest 55 : 87-98, 2008
18. Thesleff I, Barrach HJ, Foidart JM, Vaheri A, Pratt RM, Martin GR : Changes in the distribution of type IV collagen, laminin, proteoglycan, and fibronectin during mouse tooth development. Dev Biol 81 : 182-92, 1981
19. Obara N, Suzuki Y, Nagai Y, Nishiyama H, Mizoguchi I, Takeda M : Expression of neural cell-adhesion molecule mRNA during mouse molar tooth development. Arch Oral Biol 47 : 805-13, 2002
20. Kii I, Amizuka N, Minqi L, Kitajima S, Saga Y, Kudo A : Periostin is an extracellular matrix protein required for eruption of incisors in mice. Biochem Biophys Res Commun 342 : 766-72, 2006
21. Suzuki H, Amizuka N, Kii I, Kawano Y, Nozawa-Inoue K, Suzuki A, Yoshie H, Kudo A, Maeda T : Immunohistochemical localization of periostin in tooth and its surrounding tissues in mouse mandibles during development. Anat Rec A Discov Mol Cell Evol Biol 281 : 1264-75, 2004
22. MacLean G, Abu-Abed S, Dolle P, Tahayato A, Chambon P, Petkovich M : Cloning of a novel retinoic-acid metabolizing cytochrome P450, Cyp26B1, and comparative expression analysis with Cyp26A1 during early murine development. Mech Dev 107 : 195-201, 2001
23. Herrmann J, Borkham-Kamphorst E, Haas U, Van de Leur E, Fraga MF, Esteller M, Gressner AM, Weiskirchen R : The expression of CSRP2 encoding the LIM domain protein CRP2 is mediated by TGF-beta in smooth muscle and hepatic stellate cells. Biochem Biophys Res Commun 345 : 1526-35, 2006
24. Weiskirchen R, Gunther K : The CRP/MLP/TLP family of LIM domain proteins : acting by connecting. Bioessays 25 : 152-62, 2003
25. Ferguson JW, Mikesh MF, Wheeler EF, LeBaron RG : Developmental expression patterns of Beta-ig (betaIG-H3) and its function as a cell adhesion protein. Mech Dev 120 : 851-64, 2003
26. Sakai T, Larsen M, Yamada KM : Fibronectin requirement in branching morphogenesis. Nature 423 : 876-81, 2003
27. Klopcic B, Maass T, Meyer E, Lehr HA, Metzger D, Chambon P, Mann A, Blessing M : TGF-beta superfamily signaling is essential for tooth and hair morphogenesis and differentiation. Eur J Cell Biol 86 : 781-99, 2007
28. Corcoran LM, Hasbold J, Dietrich W, Hawkins E, Kallies A, Nutt SL, Tarlinton DM, Matthias P, Hodgkin PD : Differential requirement for OBF-1 during antibody-secreting cell differentiation. J Exp Med 201 : 1385-96, 2005
29. Hess J, Nielsen PJ, Fischer KD, Bujard H, Wirth T : The B lymphocyte-specific coactivator BOB.1/OBF.1 is required at multiple stages of B-cell development. Mol Cell Biol 21 : 1531-9, 2001
30. Subramaniam M, Gorny G, Johnsen SA, Monroe DG, Evans GL, Fraser DG, Rickard DJ, Rasmussen K, van Deursen JM, Turner RT, Oursler MJ, Spelsberg TC : TIEG1 null mouse-derived osteoblasts are defective in mineralization and in support of osteoclast differentiation *in vitro*. Mol Cell Biol 25 : 1191-9, 2005
31. McGlinn E, van Bueren KL, Fiorenza S, Mo R, Poh AM, Forrest A, Soares MB, Bonaldo Mde F, Grimmond S, Hui CC, Wainwright B, Wicking C : Pax9 and Jagged1 act downstream of Gli3 in vertebrate limb development. Mech Dev 122 : 1218-33, 2005
32. Ohazama A, Hu Y, Schmidt-Ullrich R, Cao Y, Scheidereit C, Karin M, Sharpe PT : A dual role for Ikk alpha in tooth development. Dev Cell 6 : 219-27, 2004
33. Jadlowiec J, Koch H, Zhang X, Campbell PG, Seyedain M, Sfeir C : Phosphophoryn regulates the gene expression and differentiation of NIH3T3, MC3T3-E1, and human mesenchymal stem cells via the integrin/MAPK signaling pathway. J Biol Chem 279 : 53323-30, 2004
34. Fujita K, Janz S : Attenuation of WNT signaling by DKK-1 and -2 regulates BMP2-induced osteoblast differentiation and expression of OPG, RANKL and M-CSF. Mol Cancer 6 : 71, 2007
35. Shimo T, Wu C, Billings PC, Piddington R, Rosenbloom J, Pacifici M, Koyama E : Expression, gene regulation, and roles of Fisp12/CTGF in developing tooth germs. Dev Dyn 224 : 267-78, 2002
36. Fujii H, Sato T, Kaneko S, Gotoh O, Fujii-Kuriyama Y, Osawa K, Kato S, Hamada H : Metabolic inactivation of retinoic acid by a

novel P450 differentially expressed in developing mouse embryos. Embo J 16 : 4163-73, 1997
37. Yashiro K, Zhao X, Uehara M, Yamashita K, Nishijima M, Nishino J, Saijoh Y, Sakai Y, Hamada H : Regulation of retinoic acid distribution is required for proximodistal patterning and outgrowth of the developing mouse limb. Dev Cell 6 : 411-22, 2004
38. Tahayato A, Dolle P, Petkovich M : Cyp26C1 encodes a novel retinoic acid-metabolizing enzyme expressed in the hindbrain, inner ear, first branchial arch and tooth buds during murine development. Gene Expr Patterns 3 : 449-54, 2003
39. Abu-Abed S, MacLean G, Fraulob V, Chambon P, Petkovich M, Dolle P : Differential expression of the retinoic acid-metabolizing enzymes CYP26A1 and CYP26B1 during murine organogenesis. Mech Dev 110 : 173-7, 2002
40. Jowett AK, Kimber SJ, Ferguson MW : Immunofluorescent lectin binding patterns and glycoprotein co-localization in the developing murine molar tooth. Arch Oral Biol 37 : 303-14, 1992
41. Nakao A, Afrakhte M, Moren A, Nakayama T, Christian JL, Heuchel R, Itoh S, Kawabata M, Heldin NE, Heldin CH, ten Dijke P : Identification of Smad7, a TGFbeta-inducible antagonist of TGF-beta signalling. Nature 389 : 631-5, 1997
42. Nagarajan RP, Zhang J, Li W, Chen Y : Regulation of Smad7 promoter by direct association with Smad3 and Smad4. J Biol Chem 274 : 33412-8, 1999
43. Hefferan TE, Subramaniam M, Khosla S, Riggs BL, Spelsberg TC : Cytokine-specific induction of the TGF-beta inducible early gene (TIEG) : regulation by specific members of the TGF-beta family. J Cell Biochem 78 : 380-90, 2000
44. Alvarez-Rodriguez R, Barzi M, Berenguer J, Pons S : Bone Morphogenetic Protein 2 Opposes Shh-mediated Proliferation in Cerebellar Granule Cells through a TIEG-1-based Regulation of Nmyc. J Biol Chem 282 : 37170-80, 2007
45. Kadrmas JL, Beckerle MC : The LIM domain : from the cytoskeleton to the nucleus. Nat Rev Mol Cell Biol 5 : 920-31, 2004
46. Tucker A, Sharpe P : The cutting-edge of mammalian development ; how the embryo makes teeth. Nat Rev Genet 5 : 499-508, 2004
47. Mitsiadis TA, Angeli I, James C, Lendahl U, Sharpe PT : Role of Islet1 in the patterning of murine dentition. Development 130 : 4451-60, 2003
48. Chang DF, Belaguli NS, Chang J, Schwartz RJ : LIM-only protein, CRP2, switched on smooth muscle gene activity in adult cardiac myocytes. Proc Natl Acad Sci USA 104 : 157-62, 2007
49. Litvin J, Zhu S, Norris R, Markwald R : Periostin family of proteins : therapeutic targets for heart disease. Anat Rec A Discov Mol Cell Evol Biol 287 : 1205-12, 2005
50. Skonier J, Neubauer M, Madisen L, Bennett K, Plowman GD, Purchio AF : cDNA cloning and sequence analysis of beta ig-h3, a novel gene induced in a human adenocarcinoma cell line after treatment with transforming growth factor-beta. DNA Cell Biol 11 : 511-22, 1992
51. Skonier J, Bennett K, Rothwell V, Kosowski S, Plowman G, Wallace P, Edelhoff S, Disteche C, Neubauer M, Marquardt H, Rodgers J, Purchio AF : beta ig-h3 : a transforming growth factor-beta-responsive gene encoding a secreted protein that inhibits cell attachment *in vitro* and suppresses the growth of CHO cells in nude mice. DNA Cell Biol 13 : 571-84, 1994
52. Kim JE, Jeong HW, Nam JO, Lee BH, Choi JY, Park RW, Park JY, Kim IS : Identification of motifs in the fasciclin domains of the transforming growth factor-beta-induced matrix protein betaig-h3 that interact with the alphavbeta5 integrin. J Biol Chem 277 : 46159-65, 2002
53. Reinboth B, Thomas J, Hanssen E, Gibson MA : Beta ig-h3 interacts directly with biglycan and decorin, promotes collagen VI aggregation, and participates in ternary complexing with these macromolecules. J Biol Chem 281 : 7816-24, 2006
54. Itasaki N, Jones CM, Mercurio S, Rowe A, Domingos PM, Smith JC, Krumlauf R : Wise, a context-dependent activator and inhibitor of Wnt signalling. Development 130 : 4295-305, 2003
55. Millar SE, Koyama E, Reddy ST, Andl T, Gaddapara T, Piddington R, Gibson CW : Over- and ectopic expression of Wnt3 causes progressive loss of ameloblasts in postnatal mouse incisor teeth. Connect Tissue Res 44 (Suppl 1) : 124-9, 2003