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상아질모세포에서 Thymosin β4에 의한 BSP(bone sialoprotein)와 DSPP(dentin sialophosphoprotein) 발현조절

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ABSTRACT

Thymosin β4 regulates the expression of bone sialoprotein (BSP) and dentin sialophosphoprotein (DSPP) in odontoblast

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Thymosin β4 (Tβ4) is involved in wound healing, angiogenesis, proliferation, migration and differentiation in mammalians. Tβ4-overexpressed transgenic mice present enamel hyperplasia and the knockdown of Tβ4 inhibits the growth of tooth germs. In addition, Tβ4 is involved in the proliferation of oral epithelial cells, and regulates the expression and secretion of the proteins related to differentiation and mineralization. Based on the previous results, Tβ4 regulates the expression of the factors related with dentinogenesis, but the signal transduction pathway of Tβ4 is insufficient in relation to the expression of these factors. Therefore, this study investigates the signal transduction pathway of Tβ4 associated with the expression of bone sialoprotein (BSP) and dentin sialophosphoprotein (DSPP), among the regulating factors to dentinogenesis, in odontoblastic cell lines, MDPC-23 cells.

As a result of membrane hybridization, a synthetic antisense Tβ4 probe bound to specifically with a sense Tβ4 probe and identified the hybridization between antisense Tβ4 probe and the intracellular Tβ4 mRNA in odontoblasts. In *in situ* hybridization, the Tβ4 mRNA was increased rapidly in the odontoblasts of molar tissue at postnatal day 5 (PN5) of the advanced bell stage. In Tβ4-treated MDPC-23 cells (MDPC-23/Tβ4), the expression of BSP was increased, but DSP

expression was decreased compared to the control. In addition, in immunofluorescence, the expression of cytoplasmic BSP was increased in MDPC-23/Tβ4, but DSP expression was decreased. The phosphorylation of ERK1/2, cytoplasmic or nuclear Runx2 and BSP expression were increased MDPC-23/Tβ4 compared to that of the control, but DSP expression was decreased. In PD98059-treated MDPC-23 cells (MDPC-23/PD98059), the phosphorylation of ERK1/2, cytoplasmic or nuclear Runx2, and BSP expression were decreased, but the reduced expression pattern of DSP was similar to that of MDPC-23/Tβ4. In MDPC-23/Tβ4, the phosphorylation of β-catenin was increased, and the nuclear translocation of β-catenin was induced with the reduction of cytoplasmic β-catenin compared to that of control, and the expression of BSP was increased, but DSP expression decreased. In addition, in MDPC-23/PD98059, the phosphorylation of β-catenin was decreased and cytoplasmic β-catenin was increased with the induction of β-catenin nuclear translocation. In MDPC-23/Tβ4, phosphorylated Smad3 translocated into the nucleus and the pattern of the phosphorylation of β-catenin, cytoplasmic β-catenin, Runx2, and BSP expression were similar to that of ERK1/2 pathway. In SIS3-treated MDPC-23 cells (MDPC-23/SIS3), the phosphorylation of Smad3 was decreased compared to that of MDPC-23/Tβ4, and the pattern of phosphorylation of β-catenin, β-catenin, Runx2, BSP, and DSP expression were similar to MDPC-23/PD98059. In MDPC-23/Tβ4, the BSP promoter activity was increased significantly compared to the negative control (pGL3-Luc), but it was decreased in pGL3-BSP/PD98059 and pGL3-BSP/SIS3 compared to that of pGL3-BSP-Luc. In addition, the promoter activity of pGL3-BSP-Luc/SIS3 was more decreased than pGL3-BSP-Luc/PD98059.

In conclusion, the increase of BSP expression was induced through ERK1/2/Runx2, ERK1/2/β-catenin, Smad3, Smad3/Runx2, or Smad3/β-catenin signaling pathways, but decreased DSP expression was not affected by MEK and pSmad3 inhibitors, indicating that DSP expression by Tβ4 is not related with ERK or Smad3 signaling pathways in MDPC-23 cells. This result suggests that Tβ4 may regulate dentin matrix formation with the increase of BSP through ERK1/2 or Smad3 signaling pathways in odontoblasts.

I. INTRODUCTION

Odontoblasts are formed by the differentiation of dental papilla cells derived from the assembling of ectomesenchymal cells during tooth development (Maas and Bei, 1997; Theslef and Sharpe, 1997). The differentiation of odontoblasts is most active at the early bell stage, and when this cell initiates making the dentin matrix at the late bell stage, this process is called dentinogenesis (Butler, 1995). Odontoblasts regulate a series of complex processes that determine the mineralization of dentin and secretion of collagen type I (Col I), collagen type III (Col III), and membrane-bound matrix vesicles in the dentin matrix. In addition, odontoblasts synthesize and secrete non-collagenous proteins such as dentin sialophosphoprotein (DSPP), dentin phosphoprotein (DPP), and dentin sialoprotein (DSP) through the proteolytic cleavage of DSPP, dentin matrix proteins-1, -2, and -3 (DMP-1, DMP-2, and DMP-3) to regulate the mineralization of dentin. In addition, odontoblasts not only secrete the non-collagenous proteins that abundantly exist in bone such as osteopontin (OPN), bone sialoprotein (BSP), osteonectin (ON), and osteocalcin (OCN), but also secretory leukocyte protease inhibitor (SLPI) (Butler and Ritchie, 1995; Papagerakis et al., 2002; He et al., 2003; Choi et al., 2009).

BSP was first extracted from the compact bone of bovine, and is one of the proteins in mineralized tissues such as bone, dentin, cementum, and calcified cartilage (MacNeill et al., 1994). BSP is a phosphorylated or sulfurized glycoprotein with a high level of a sialic acid such as OPN, and tends to bind with hydroxyapatite (Oldberg et al., 1988; Stubbs et al., 1997). BSP acts as the nucleator of primary apatite crystals, and it regulates the growth direction of crystals of ribbon-like apatite, based on collagen during the mineralization process (Hunter and Goldberg, 1994). In addition, BSP contributes to the activation of osteoclasts by interacting with the integrin on cell surfaces (Razzouk et al., 2002). DSPP in its inactive form is changed to DSP and DPP in an active form through hydrolysis to promote the mineralization of predentin (George et al., 1996). DPP is the most abundant non-collagenous protein in dentin extracellular matrix, which

activates the initiation of hydroxyapatite formation during dentinogenesis (Saito et al., 1997). DPP rapidly moves to the mineralization front after synthesis, and then binds to the collagen in dentinogenesis (Butler, 1995; Butler and Ritchie, 1995). DSP is mainly expressed in odontoblasts, and preameloblasts are poorly involved or restricted in the formation of hydroxyapatite and the growth of mineral crystals, but it regulates the suppression or control of DPP activity during dentinogenesis (Butler et al., 1992; Bègue-Kirn et al., 1998; Boskey et al., 2000; Butler et al., 2003). Recently, it has been reported that DSP regulates the initiation of the mineralization of dentin, and DPP is involved in the maturation of mineralized dentin (Suzuki et al., 2012).

The Wnt signal transduction pathways contribute to the differentiation of C3H10T1/2 and C2C12, pluripotent mesenchymal cell line, and MC3T3-E1 osteoblastic cells by the induction of alkaline phosphatase (ALP) and Runx2 gene expression (Bain et al., 2003; Rawadi et al., 2003; Gaur et al., 2005). Runt-related transcription factor 2 (Runx2), one of the transcription factors, is increased by general factors for osteoblastogenesis such as transforming growth factor-β1 (TGF-β 1) or bone morphogenic protein-2 (BMP-2), and it regulates the gene expressions related with bone development, maturation, and maintenance (Lee et al., 2002; Huang et al., 2007; Deng et al., 2008). Runx2 regulates the expression of Col I, OCN and DSP (Chen et al., 2002). In Runx2 overexpressed transgenic mice, the expression of BSP and DSPP are increased at postnatal day three (PN3), but these expressions decrease after one month (Li et al., 2011). A number of Runx2 binding sites are in the promoter region of the DSPP gene, and Runx2 increases the DSPP expression in immature odontoblasts, but decreases in mature odontoblasts, according to the differentiation stage of the odontoblast in vitro (Bronckers et al., 2001; Yamashiro et al., 2002; Chen et al., 2005). It is suggested that several bioactive molecules are involved in the signal transduction pathways related with the expression of BSP and DSPP in odontoblasts and osteoblasts. BMP-2 increases the expression of Runx2 and DSPP through the Smad1/5/4 signal transduction pathway in MDPC-23 cells, and protamine increases the expression of Runx2 and BSP through ERK1/2 pathways in osteoblasts (Cho et al., 2010; Zhou et al., 2013).

TGF-β1 decreases the expression of DMP-1 and DSPP through Smad2/3 signal transduction pathways in odontoblasts (Unterbrink et al., 2002; He et al., 2004). BMP-7 promotes osteoblast differentiation by increasing the Runx2 and BSP expression through ERK1/2 signaling, and TGF-β1 decreases the BSP expression through Smad3 and β-catenin signaling (Xiao et al., 2002; Zhou, 2011).

Thymosin β4(Tβ4), composed of 43 amino acids and a 4.9 kDa small peptide, is first extracted from the thymus, and is most abundant type of thymosin in mammals (Low et al., 1981). Tβ4 is involved in intracellular G-actin sequestering and interferes with actin polymerization with binding ATP-G- actin at a 1:1 ratio (Safer et al., 1991; 1997). Tβ4 participates in the regulation of cell proliferation, differentiation and motility through G-actin sequestering (Goldstein et al., 2005). Tβ 4 is functionally involved in angiogenesis in embryo development, and is expressed during the development of the nervous system and the differentiation of embryonic cells into cardiac cells in mice embryos, and the development of the rat brain (Gómez-Márquez et al., 1993; Grant et al., 1995; Gómez-Márquez et al., 1996; Grant et al., 1999; Anadón et al., 2001). Tβ4 is expressed in the developing mandible of mouse, and it is reported that Tβ4 is involved in the initiation, formation, and differentiation of tooth germ during the development of the molars (Yamaza et al., 2001; Akhter et al., 2005). Tβ4-overexpressed transgenic mice presented abnormal tooth development similar to enamel hyperplasia, and Tβ4 deficiency mice show the suppression of the growth of tooth germ, secretion of MMP-2 and the migratory ability of oral epithelial cells (Cha et al., 2010; Ookuma et al., 2013). In addition, the inhibition of Tβ4 mRNA synthesis largely decreases the mRNA expression of BSP, DSPP, OCN, ON and Col I compared to that of control in the differentiation of MDPC-23 cells (Choi et al., 2012).

In recent research, Tβ4 accelerates the migration of gastric cancer cells by a reduction of E-cadherin expression with the degradation of phosphorylated β-catenin through ERK1/2 signaling (Ryu et al., 2012). In Tβ4 knockdown mice, Runx2 expression is decreased in tooth germ and the mandibular tissue of the embryonic mice (Ookuma et al., 2013). Tβ4 decreases the osteoblast differentiation through the reduced expression of OCN, Col I, ON, OPN, and ALP in human mesenchymal

stem cells (Ho et al., 2010). In addition, recent studies have reported that Tβ4 promotes odontoblast differentiation due to the increase of ALP, OPN, OCN, DMP-1, DSPP expression, and mineralization in human dental pulp cell through p38, JNK, ERK, BMP, and integrin signaling pathways (Lee et al., 2013). From previous studies, Tβ4 expects to regulate the expression of factors involved with the formation of dentin matrix in odontoblasts, but the study is insufficient to determine the signal transduction pathway of Tβ4, related with the secreted odontoblast molecules. Therefore, this study tried to investigate the signal transduction pathway of Tβ4 associated with the expression of BSP and DSPP in the odontoblastic cell line, MDPC-23 cells.

Ⅱ. MATERIALS AND METHODS

Ⅱ-1. Preparation of tissues

All animal studies were approved by the "Institutional Animal Care and Use Committees" at Chosun University, and animal care was carried out using the SPF level systems according to "Guide for the Care and Use of Laboratory Animals." ICR outbred mice at postnatal day 1 (PN1), PN3, PN5, PN15, and PN21 were used in this study. The heads, dissected from PN1, PN3, and PN5 mice, were fixed in 4% paraformaldehyde (PFA) in diethylpyrocarbonate-treated phosphate-buffer saline (DEPC-PBS, pH 7.4). The PN15 and PN21 mice were anesthetized with ketamine (Yuhan, KOR), and then DEPC-PBS was circulated by intracardiac perfusion for the removal of blood, and was fixed by the intracardiac perfusion of 4% PFA. The heads of the PN15 and PN21 mice were dissected out, and were postfixed for an additional 18 h in fresh 4% PFA, and then decalcified in solution of 10% EDTA supplement with 1% PFA for four weeks. The heads were dehydrated by sequential washes in 70%, 80%, 90%, 100% I, 100% II, 100% III and finally 100% IV ethanol after decalcification. The clearing process was performed using xylene, and paraffin was embedded in the samples. The paraffin-embedded tissue was made a section of 6 μm thickness using the Histocut 820 (Leica, GER) and dried on a 37 °C slide warmer overnight after being placed onto a glass slide coated by 3-(Trimethoxysilyl) propyl methacrylate (Sigma-Aldrich, USA).

Ⅱ-2. Synthesis of Tβ4 cRNA probes and peptide

Gene specific probes for 405 bp Tβ4 cDNA were designed according to the previous study (Akhter et al; 2005). pGEM-3Z vector (Promega, USA) inserting T β4 cDNA was linearized by digestion with restriction enzymes (EcoR I or Hind III) for the synthesis of Tβ4 sense and antisense cRNA probes using an in vitro

transcription kit (Roche molecular biochemical, GER). The probes were labeled with digoxigenin (DIG)-11-UTP using the SP6/T7 DIG RNA Labeling Kit (Roche molecular biochemicals). The full amino acid sequence of mouse Tβ4 peptide, Ac-SDKPDMAEIEKFDKSKLKKTETQEKNPLPSKETIEQEKQAGES, was synthesized by solution phase peptide synthesis (A&PEP, KOR).

Ⅱ-3. Membrane hybridization

An unlabeled Tβ4 sense RNA probe (unlabeled S, 10 ~ 0.01 ng/μl) and an unlabeled Tβ4 anti-sense RNA probe (unlabeled AS, 10 ~ 0.01 ng/μl) were anchored to the nucleic acid transfer membranes (GE Healthcare, UK) using a UV crosslinker (STRATAGENE, USA) before hybridization with the DIG-labeled antisense Tβ4 probe (DIG-labeled AS). In addition, the membrane-anchored unlabeled S was incubated with the DIG-labeled AS mixed with unlabeled S or unlabeled AS (1 - 10 ng/μl, respectively) in the hybridization solution (50% deionized formamide, 5X SSC, 50 μg/ml yeast tRNA, 1% SDS, 50 μg/ml heparin) at 55 °C for 16 h. The DIG-labeled AS was reacted with alkaline phosphatase (AP)-conjugated DIG antibodies for 1 h at room temperature, and then detected using a DIG nucleic acid detection kit (Roche molecular biochemical).

Ⅱ-4. *In situ* hybridization

After deparaffinization and hydration, the section was incubated with proteinase K and then treated with 4% PFA, treated at room temperature. Subsequently, the tissue was immersed in 0.1M Triethanolamine-HCl (TEA-HCl) and 0.1M TEA-0.25% acetic anhydride for the removal of background signals. Hybridization was carried with the hybridization mixture (hybridization solution, 1 ng/μl Dig-labeled S or Dig-labeled AS) in a humidified chamber at 55 °C for 16 h. The sections were washed twice in 2X and 0.2X SSC containing 50% formamide, and then incubated with a 1:500 dilution of AP-conjugated DIG antibodies for 1 h at

room temperature. Dig-labeled S or Dig-labeled AS was detected using the DIG nucleic acid detection kit (Roche molecular biochemical) and the section was counterstained with methyl green for 20 sec. The expression of Tβ4 mRNA in the tissue was analyzed by Axiovision LE release 4.6 software (Carl Zeiss, GER).

Ⅱ-5. Cell culture

MDPC-23 cells, the odontoblastic cell line derived from the dental papilla of fetal mouse molars, were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL, USA) containing 10% fetal bovine serum (WelGENE, KOR) and 1% antibiotic-antimycotic solution (WelGENE). Cells were incubated in a humidified chamber maintained with 5% CO_2 and at 37 ºC.

Ⅱ-6. Extraction of total RNA and reverse transcription and polymerase chain reaction

MDPC-23 cells were grown to 70% confluence in 60 mm cell culture dish and then proceeded with serum starvation for 16 h. After serum starvation, 2 μg/ml of Tβ4 was treated to MDPC-23 cells (MDPC-23/Tβ4) for 24 h. Total RNA was extracted from the cells using Tri reagent (MRC Inc, USA), and a PCR reaction was carried out according to the manufacturer's instructions (GeneAll, KOR). The following primers were synthesized (Bioneer, KOR) for RT-PCR analysis: 1) Bone sialoprotein (BSP) forward 5'-ACC GGC CAC GCT ACT TTC TTT AT-3', reverse 5'-TCC TCG TCG CTT TCC TTC ACT TT-3'; 2) dentin sialophosphoprotein (DSPP) forward 5'-CGA CCC TTG TCC AGG A-3', reverse 5'-CAT GGA CTC GTC ATC GAA-3' and 3) glyceraldehyde 3-phosphate dehydrogenase (GAPDH) forward 5'-CCA TGG AGA AGG CTG GG-3', reverse 5'-CAA AGT TGT CAT GGA TGA CC-3'. GAPDH was used as the internal control for RT-PCR. The annealing temperature for each primer and number of cycles were as follows: 1) BSP 60 °C (30 cycles); 2) DSPP 56 °C (35 cycles) and

3) GAPDH 56 °C (30 cycles). The products were electrophoresed on 1.5% agarose gel buffered with 0.5X Tris-Borate-EDTA, and stained with ethidium bromide after amplification. The staining bands were visualized by Gel-Doc (BioRad Laboratories, USA). These primer sets only recognized the genes of interest, as indicated by the amplification of single bands of expected sizes (358 bp for BSP; 824 bp for DSPP, and 199 bp for GAPDH) according to the nucleotide sequences of the BSP (Genbank # L20232), DSPP (Genbank # NM__010080), and GAPDH (Genbank # M33197). The intensities of the bands were measured using a Science Lab Image Gauge (FUJI FILM, JPN).

Ⅱ-7. Extraction of proteins and western blotting

MDPC-23 cells were grown to 70% confluence in a 60 mm cell culture dish, and then serum starvation was carried out for 16 h. After serum starvation, 2 μg/ml of Tβ4 was treated to MDPC-23 cells (MDPC-23/Tβ4) for 5, 10, 15, or 30 min, or 6, 12, or 24 h. A 10 ng/ml transforming growth factor-β1 (TGF-β1; R&D Systems, USA) was also treated to MDPC-23 cells (MDPC-23/TGF-β1) for 30 min. Either a 5 μM MEK inhibitor PD98059 (Sigma-Aldrich) or a 5 μM Smad3 phosphorylation inhibitor SIS3 (Sigma-Aldrich) was treated to the MDPC-23 cells (MDPC-23/PD98059 or MDPC-23/SIS3) for 1 h. In addition, PD98059 or SIS3 was pretreated to the MDPC-23 cells for 1 h before the Tβ4 treatment (MDPC-23/PD98059/Tβ4 or MDPC-23/SIS3/Tβ4). The cytosolic protein was extracted using an NP-40 lysis buffer (150 mM NaCl, 1% NP-40, 50 mM Tris-Cl (pH 7.4), 2 mM Na_3VO_4, 2 mM $Na_4P_2O_7$, 50 mM NaF, 2 mM EDTA (pH7.4), 0.1 μg/ml leupeptin and 1 μg/ml aprotinin). Cell lysates were incubated on ice for 30 min and centrifuged at 13,000 rpm and 4 °C. The nuclear protein was extracted from the cells using a Cell Fractionation kit (BioVision, USA) according to the manufacturer's instructions. The concentration of extracted protein was determined using a *DC* protein assay kit (BioRad Laboratories). The proteins (30 μg) were denatured and then electrophoresed in 7.5% SDS-polyacrylamide gel. After electrophoresis, the protein was transferred to

PVDF membranes and blocked with either 5% non-fat dry milk or 5% bovine serum albumin (Bioshop, USA) for 1 h at room temperature. The membranes were blotted with 1:2,500 of anti-rabbit BSP (Millipore, USA), 1:1,000 of anti-rabbit DSP (Santa Cruz Biotechnology, USA), 1:2,000 of anti-rabbit Runx2 (Santa Cruz Biotechnology), 1:2,500 of anti-rabbit pERK1/2 (Cell signaling, USA), 1:2,500 of anti-rabbit ERK1/2 (Upstate, USA), 1:1,000 of anti-rabbit pβ-catenin (Cell signaling), 1:1,000 of anti-rabbit β-catenin (Cell signaling), 1:1,000 of anti-rabbit pSmad2 (Cell signaling), 1:1,000 of anti-rabbit pSmad3 (Cell signaling), 1:1,000 of anti-rabbit Smad2/3 (Cell signaling), and 1:2,500 of anti-mouse β-actin (Santa Cruz Biotechnology) at 4 ℃ overnight . After washing, the membrane was blotted with 1:5,000 - 1:10,000 of HRP-conjugated goat anti-rabbit or mouse-IgG (SantaCruz Biotechnology, Inc) at room temperature for 1 h. The developing was performed using X-ray film (FUJI FILM) after treatment with an ECL solution (Millipore). The molecular weights of BSP, DSP, Runx2, pERK1/2, pβ-catenin, pSmad2, pSmad3, Smad2/3, and β-actin on the representative bands indicated 39 kDa, 70 kDa, 55 kDa, 44/42 kDa, 92 kDa, 60 kDa, 52 kDa, 52/60 kDa and 42 kDa, respectively. The density of the expressed bands was measured using a Science Lab Image Gauge.

Ⅱ-8. Immunofluorescence

MDPC-23 cells were placed on coverslips in six-well culture plates. Subsequently, the cells were treated with 2 μg/ml Tβ4 (MDPC-23/Tβ4), 5 μM PD98059 (MDPC-23/PD98059) or 5 μM SIS3 (MDPC-23/SIS3) for 24 h, after serum starvation for 16 h. Cells were fixed with 4% formaldehyde for 15 min and treated with 0.1 M glycine for 5 min to quench the excessive aldehyde. The cells were permeabilized with 0.2% Triton X-100 for 5 min and then blocked with 5% normal goat serum for 1 h. The cells were reacted with 1:100 of anti-rabbit BSP, anti-rabbit DSP, anti-rabbit pSmad3, anti-rabbit β-catenin, or anti-rabbit Runx2 was treated at 4 ℃ for overnight after blocking. After washing, 1:1,000 of

goat-anti-rabbit IgG conjugated with FITC (SantaCruz Biotechnology, Inc) as a secondary antibody was reacted for 2 h at room temperature in a dark condition. Cells were mounted with DAPI (Vector Lab, USA) for nucleus staining and fluorescence cells were visualized and photographed by fluorescent microscopy (Carl Zeiss). The fluorescence signal intensity in cells was analyzed by Axiovision LE release 4.6 software.

Ⅱ-9. Plasmid construction and transfection

The 2.5 kb of the mouse BSP gene promoter region from −2472 to +41 was artificially synthesized (Bioneer) and then cloned into the 5' to 3' Nhe I-Xho I site of pGL3-basic vector (pGL3-BSP-Luc). All corrective constructs were verified by DNA sequencing and restriction enzyme digestion. The MDPC-23 cells were subcultured in six-well culture dishes (1×10^5 cells/ml) and then the transient was transfected with 2 μg/ml pGL3-basic vector (pGL3-Luc) or 2 μg/ml pGL3-BSP-Luc for 48 h using WellFect-EX^{TM} (WelGENE) according to the manufacturer's instructions.

Ⅱ-10. Luciferase assay

The pGL3-BSP-Luc-transfected MDPC-23 cells were treated with 2 μg/ml Tβ4 (pGL3-BSP-Luc/Tβ4), 5 μM PD098509 (pGL3-BSP-Luc/PD98059), 5 μM SIS3 (pGL3-BSP-Luc/PD98059) for 24 h after serum starvation. In addition, the pGL3-BSP-Luc-transfected MDPC-23 cells were pretreated with PD98059 or SIS3 for 1 h before Tβ4 treatment (pGL3-BSP-Luc/PD98059/Tβ4 or pGL3-BSP-Luc/SIS3/Tβ4). The extraction of proteins and reaction with luciferase substrates were processed using a luciferase assay kit according to the manufacturer's instructions (Promega). The luciferase activity was measured using luminometer (Thermo Scientific, USA).

Ⅱ-11. Statistical analysis

All experiments were performed at least in triplicate. All data was reported as the mean and standard deviation using Excel 2010 statistical software (Microsoft, USA). The significant differences (*$p<0.05$, **$p<0.001$) were determined using a Student's *t*-test.

Ⅲ. RESULTS

Ⅲ-1. Specificity and binding activity of the *in situ* probes

For *in situ* hybridization, membrane hybridization was performed to confirm the specificity and binding activity of the Tβ4 cRNA probe. Hybridization was performed to take measurements of the DIG-labeled antisense Tβ4 probe (DIG-labeled AS) specificity. Unlabeled Tβ4 sense RNA (unlabeled S) probe and unlabeled Tβ4 anti-sense RNA probe (unlabeled AS) were fixed in nucleic acid transfer membrane in concentration sequence 10, 1, 0.1, 0.01 and 0.001 ng/μl before being hybridized with the same concentration of DIG-labeled AS Hybridization signal between DIG-labeled AS and unlabeled S was decreased in 10 ng/μl and 1 ng/μl by concentration sequence, and was not detected below 0.1 ng/μl. The hybridization signal between DIG-labeled AS and unlabeled AS was not detected in all concentrations. The appropriate concentration of DIG-labeled AS was determined to be 1 ng/μl using *in situ* hybridization (Fig. 1A).

Furthermore, to examine DIG-labeled AS binding activity, hybridization was performed on previously fixed unlabeled S (1 ng/μl) on a membrane with a probe mixture of DIG-labeled AS (1 ng/μl) adding 1, 5, 10 ng/μl of unlabeled S or unlabeled AS. The hybridization signal between the mixture, DIG-labeled AS and unlabeled S, and the membrane-fixed unlabeled S was detected in 1 ng/μl of the unlabeled S of mixture, and was not detected in the 5 ng/μl or 10 ng/μl. In addition, the hybridization signal between the mixture, DIG-labeled AS, and unlabeled AS, and membrane fixed unlabeled S was strongest in the 1 ng/μl unlabeled AS of mixture, and was gradually decreased in both the 5 ng/μl and 10 ng/μl (Fig. 1B). The expression of Tβ4 mRNA was not detected in *in situ* hybridization using no probe (negative control) or DIG-labeled S (Fig. 1Ca and b), but was detected in odontoblast using DIG-labeled AS (Fig. 1Cc).

Ⅲ-2. *In situ* hybridization of Tβ4 mRNA in odontoblasts during dentinogenesis

The developing molar between the early bell stage and functional stage was used to investigate the expression pattern of Tβ4 mRNA in odontoblasts. The deposition of dentin was observed at postnatal day 1 (PN1) of early bell stage (Fig. 2A), and was more thickened with the increase of enamel formation at PN3 and PN5 of the advanced bell stage (Fig 2B and C). Dentin was more thickened at PN15 as a crown stage, and tooth eruption was observed at PN21 of the functional stage (Fig 2D and E). Tβ4 mRNA increased rapidly in odontoblasts at PN5 of the advanced bell stage, and was observed in the odontoblasts of the crown and functional stages with a similar pattern to the early bell stage (Fig. 2a - e and F, $p<0.001$).

Ⅲ-3. The mRNA and protein expression of BSP and DSP after Tβ4 treatment in MDPC-23 cells

In MDPC-23 cells treated with Tβ4 for 24 h (MDPC-23/Tβ4), the expression of BSP mRNA was increased 3.3 times compared to that of the control, but DSPP was decreased 0.7 times. The expression of BSP protein was increased 2.5 times compared to that of the control, but DSPP was decreased 0.6 times (Fig. 3A and B). In immunofluorescence, the expression pattern of BSP and DSP proteins were respectively diffuse and punctated in the cytoplasm of the MDPC-23 cells. The expression of BSP protein was 2 times higher in MDPC-23/Tβ4 compared to the control, but the expression of DSP was decreased to 0.6 of that of the control and the punctated form of the DSP protein was changed to a diffuse form in the cytoplasm (Fig. 3C and D).

Ⅲ-4. BSP and DSP expression through the ERK1/2, β-catenin, and Runx2 signaling after Tβ4 treatment in MDPC-23 cells

The phosphorylation of ERK1/2 was most increased (8.5 times) at 10 min in MDPC-23/Tβ4 compared to that of the control, and it was increased by 5.3, 4.8, and 2.5 times at 5, 15, and 30 min, respectively. The phosphorylation of β-catenin was increased 1.7 times at 10 min compared to that of the control, and was increased 1.3, 2.1, 2.4 times at 5, 15, and 30 min, respectively ($p<0.001$). The expression of β-catenin was respectively decreased by 0.1 and 0.14 times at 5 and 10 min, and by 0.2 times at both 15 and 30 min compared to the control (Fig 4A, $p<0.05$).

In MDPC-23 cells treated with Tβ4 for 10 min (MDPC-23/Tβ4), the phosphorylation of ERK1/2 was increased 3.2 times at 10 min compared to that of the control. In MDPC-23 cells treated with PD98059 for 1 h (MDPC-23/PD98059), the phosphorylation of ERK1/2 was decreased 0.7 times compared to that of MDPC-23/Tβ4. The phosphorylation of ERK1/2 was decreased 0.7 times in Tβ4 treated MDPC-23/PD98059 cells compared to that of MDPC-23/Tβ4. The phosphorylation of β-catenin was increased 2.1 times in MDPC-23/Tβ4 compared to that of the control but it was decreased 0.5 times in MDPC-23/PD98059 and MDPC-23/PD98059/Tβ4, respectively compared to that of MDPC-23/Tβ4. The expression of β-catenin protein was decreased 0.5 times in MDPC-23/Tβ4 compared to that of the control, but it was increased 1.7 times in MDPC-23/PD98059 and MDPC-23/PD98059/Tβ4, compared to that of MDPC-23/Tβ4. The expression of Runx2 protein was increased 1.3 times in MDPC-23/Tβ4 compared to that of the control, but it was decreased 0.6 and 0.7 times in MDPC-23/PD98059 and MDPC-23/PD98059/Tβ4, respectively, compared to that of MDPC-23/Tβ4. The expression of BSP protein was increased 1.3 times in MDPC-23/Tβ4 compared to that of the control ($p<0.001$), but it was decreased 0.4 and 0.5 times in MDPC-23/PD98059 and MDPC-23/PD98059/Tβ4, respectively, compared to

MDPC-23/Tβ4. The expression of DSP protein was decreased 0.4 times in MDPC-23/Tβ4 compared to that of the control ($p<0.001$), but the expression of DSP in MDPC-23/PD98059 and MDPC-23/PD98059/Tβ4 was similar to MDPC-23/Tβ4 (Fig. 4B).

The phosphorylation of β-catenin was most increased 18 times at 24 h in MDPC-23/Tβ4 compared to that of control and it was increased 13 times at 6 and 24 h, respectively. The expression of β-catenin protein in cytoplasm was no different at 6 and 12 h compared to that of the control, but it was decreased by 0.6 times at 24h. In the nucleus, β-catenin was decreased 0.2 times at 6 h, and this pattern was maintained until 24 h. The expression of Runx2 protein in cytoplasm was increased 1.4 times at 6 h compared to that of the control, and it was decreased 0.3 times at both 12 and 24 h. The expression of Runx2 protein in the nucleus was increased 1.3 times at 6 h compared to that of control, and it was similar to the control at both 12 and 24 h. The expression of BSP protein was increased 1.2, 1.7, and 2.2 times at 6, 12 and 24 h, respectively, compared to that of the control, but the expression of DSP protein was decreased 0.5, 0.4 and 0.3 times, at 6, 12 and 24 h, respectively (Fig. 5A).

In immunofluorescence, the distribution pattern of β-catenin protein was observed in its diffuse and punctated form in the cytoplasm and nucleus of MDPC-23 cells, respectively. In MDPC-23 cells treated with Tβ4 for 24 h (MDPC-23/Tβ4), the expression of cytoplasmic β-catenin protein was decreased 0.3 times compared to that of the control. In MDPC-23 cells treated with PD98059 for 24 h (MDPC-23/PD98059), the expression of cytoplasmic β-catenin protein was increased 1.9 times compared to that of MDPC-23/Tβ4. The expression of β-catenin protein in the nucleus was decreased 0.7 times in MDPC-23/Tβ4 compared to that of the control, but it was increased 1.8 times in MDPC-23/PD98059 compared to MDPC-23/Tβ4 (Fig. 5B). In the control and MDPC-23/PD98059, Runx2 protein was in its diffuse form in the cytoplasm, but this form was punctated in the cytoplasm and nucleus in MDPC-23/Tβ4. The expression of Runx2 protein in the cytoplasm of MDPC-23/Tβ4 was increased 2.7 times compared to that of the control, but it was decreased 0.8 times in MDPC-23/PD98059 compared to that of

MDPC-23/Tβ4. The expression of Runx2 protein in the nucleus of MDPC-23/Tβ4 was increased 2.6 times compared to that of the control, but it was decreased 0.7 times in MDPC-23/PD98059 compared to that of MDPC-23/Tβ4 (Fig. 5C).

Ⅲ-5. BSP and DSP expression through the Smad3, β-catenin, and Runx2 signaling after Tβ4 treatment in MDPC-23 cells

In MDPC-23 cells treated with TGF-β1 for 30 min (MDPC-23/TGF-β1), the phosphorylation of Smad2 was increased 72 times compared to that of the control, but it was not induced in MDPC-23 cells treated with Tβ4 for 30 min (MDPC-23/Tβ4). The phosphorylation of Smad3 was increased 2.3 times and 1.9 times in MDPC-23/TGF-β1 and MDPC-23/Tβ4, respectively, compared to the control (Fig. 6A). The phosphorylation of Smad2 was not induced in MDPC-23 cells treated with Tβ4 (MDPC-23/Tβ4) for 5, 10, 15, or 30 min. In MDPC-23/Tβ4, the phosphorylation of Smad3 was most increased 8.5 times at 5 min, and was increased 3.2, 3, and 2.9 times at 10, 15, and 30 min, respectively, compared to that of the control (Fig. 6B).

In MDPC-23 cells treated with Tβ4 for 10 min (MDPC-23/Tβ4), the phosphorylation of Smad3 was increased 2 times compared to that of the control. In MDPC-23 cells treated with SIS3 for 1 h (MDPC-23/SIS3), the phosphorylation of Smad3 was decreased 0.4 times compared to that of MDPC-23/Tβ4. The phosphorylation of Smad3 was decreased 0.7 times in Tβ4 treated MDPC-23/SIS3 cells compared to that of MDPC-23/Tβ4. The phosphorylation of β-catenin was increased 4.9 times in MDPC-23/Tβ4 compared to that of the control, but it was decreased 0.5 and 0.2 times in MDPC-23/SIS3 and MDPC-23/SIS3/Tβ4, respectively, compared to that of MDPC-23/Tβ4. The expression of β-catenin protein was decreased 0.4 times in MDPC-23/Tβ4 compared to that of the control, but it was increased 1.4 times in both MDPC-23/SIS3 and MDPC-23/SIS3/Tβ4, compared to that of MDPC-23/Tβ4 ($p<0.001$). The expression of Runx2 protein was

increased 1.5 times in MDPC-23/Tβ4 compared to that of the control, but it was decreased 0.6 and 0.7 times in MDPC-23/SIS3 and MDPC-23/SIS3/Tβ4, respectively, compared to that of MDPC-23/Tβ4. The expression of BSP protein was increased 4.5 times in MDPC-23/Tβ4 compared to that of the control, but it was decreased 0.4 and 0.2 times in MDPC-23/SIS3 and MDPC-23/SIS3/Tβ4, respectively, compared to that of MDPC-23 /Tβ4. The expression of DSP protein was decreased 0.4 times in MDPC-23/Tβ4 compared to that of the control, but there was no difference in MDPC-23/SIS3 and MDPC-23/SIS3/Tβ4 compared to MDPC-23 /Tβ4 (Fig. 6C).

The phosphorylation of cytoplasmic Smad3 was at most increased by 1.6 times at 6 h in MDPC-23/Tβ4 compared to that of the control, and it was increased by 1.4 times at both 12 and 24 h. The phosphorylation of nuclear Smad3 was at most increased 6.8 times at 24 h in MDPC-23/Tβ4 compared to that of the control, and it was increased 4 and 5.3 times at 6 and 12 h, respectively. The expression of BSP protein was increased 1.7 times at 6, 12 and 24 h compared to that of the control, but the expression of DSP protein was decreased 0.3, 0.5 and 0.6 times at 6,12 and 24 h, respectively (Fig. 7A).

In immunofluorescence, the distribution pattern of the phosphorylated Smad3 protein was punctated form in both the cytoplasm and nucleus of the MDPC-23 cells. In MDPC-23 cells treated with Tβ4 for 24 h (MDPC-23/Tβ4), the phosphorylation of Smad3 protein in cytoplasm was increased 1.4 times compared to that of the control. In MDPC-23 cells treated with SIS3 for 24 h (MDPC-23/SIS3), the phosphorylation of Smad3 protein in cytoplasm was decreased 0.5 times compared to that of MDPC-23/Tβ4. The phosphorylation of Smad3 proteins in the nucleus was increased 3.8 times in MDPC-23/Tβ4 compared to that of the control, but it was decreased 0.8 times in MDPC-23/SIS3 compared to MDPC-23/Tβ4 (Fig. 7B). The expression of β-catenin proteins in cytoplasm was decreased 0.6 times in MDPC-23/Tβ4 compared to that of the control, and it was increased 1.7 times in MDPC-23/SIS3 compared to MDPC-23/Tβ4. The expression of β-catenin proteins in the nucleus was decreased 0.7 times in MDPC-23/Tβ4 compared to that of the control, but it was increased 3 times in MDPC-23/SIS3 compared to MDPC-23/Tβ4 (Fig. 7C).

Ⅲ-6. BSP promoter activity through ERK1/2 and Smad3 signaling after Tβ4 treatment in MDPC-23 cells

The vector containing the Luciferase gene (pGL3-Luc) and an inserted vector with promoter region of the BSP gene (pGL3-BSP-Luc) were transfected into MDPC-23 cells, and used as negative control and positive control, respectively . The luciferase activity was measured in pGL3-BSP-Luc transfected MDPC-23 cells after treatment with Tβ4 (pGL3-BSP-Luc/Tβ4), PD98059 (pGL3-BSP-Luc/PD98059), PD98059/Tβ4 (pGL3-BSP-Luc/PD98059/Tβ4), SIS3 (pGL3-BSP-Luc/SIS3), SIS3/Tβ4 (pGL3-BSP-Luc/SIS3/Tβ4) for 24 h. The luciferase activity was increased 16 times in pGL3-BSP-Luc compared to pGL3-Luc, and it was increased 2.5 times in pGL3-BSP-Luc/Tβ4 compared to pGL3-BSP-Luc. The luciferase activity of pGL3-BSP-Luc/PD98059 and pGL3-BSP-Luc/PD98059/Tβ4 were decreased 0.4 times compared to pGL3-BSP-Luc/Tβ4. The luciferase activity of pGL3-BSP-Luc/SIS3 and pGL3-BSP-Luc/SIS3/Tβ4 were decreased 0.9 times compared to pGL3-BSP-Luc/Tβ4 (Fig. 8).

Ⅲ-7. Schematic diagram of Tβ4 signaling pathway related with BSP and DSP expression

Tβ4 increased the phosphorylation of ERK1/2 and Runx2 in cytoplasm, and induced the nuclear translocation of Runx2 in MDPC-23 cells. In addition, Tβ4 increased the phosphorylation of ERK1/2 and β-catenin, and induced the reduction of cytoplasmic β-catenin, the nuclear translocation of β-catenin, and it increased the BSP expression, but decreased the DSP expression in MDPC-23 cells. PD98059 decreased the phosphorylation of ERK1/2, Runx2, and the phosphorylation of β-catenin, but it increased cytoplasmic β-catenin and the decreased BSP and DSP expression in MDPC-23 cells (Fig. 9A).

Tβ4 increased the phosphorylation of Smad3 and cytoplasmic Runx2, and induced the nuclear translocation of these proteins in MDPC-23 cells. In addition, T

β4 increased the phosphorylation of Smad3 and β-catenin, and induced a reduction of cytoplasmic β-catenin and the nuclear translocation of β-catenin, and it increased BSP but decreased the DSP expression in MDPC-23 cells. SIS3 decreased the phosphorylation of Smad3, Runx2, and β-catenin, but it increased the cytoplasmic β-catenin and decreased the BSP and DSP expression in MDPC-23 cells (Fig. 9B).

Ⅳ. DISCUSSION

The differentiation of odontoblasts occurs actively at the early bell stage, and secretory or aged odontoblasts can be observed in the cusp and cervical region of a developing molar at advanced bell stages (Lisi et al., 2003; Zhang et al., 2009; Choi et al., 2012). Secretory odontoblast secretes non-collagenous proteins such as BSP, DSP, OCN, and ON at the advanced bell stage (Fisher et al., 1983; Bronckers et al., 1987; Fujisawa and Kuboki, 1989; D'Souza et al., 1992). Tβ4 mRNA is expressed in the mandibular tissue of an embryo, and it is expressed in the proliferative inner dental epithelium and dental lamina, but not odontoblast (Yamaza et al., 2001; Akhter et al., 2005). In a recent study, the expression of Tβ4 protein is increased in the odontoblast of the cusp and cervical region on developing molars at an advanced bell stage, compared to early bell stages (Choi et al., 2012). In addition, Tβ4-overexpressed transgenic mice present with enamel hyperplasia, and Tβ4 knockdown inhibits the growth of tooth germ (Cha et at al., 2010; Ookuma et al., 2013). Therefore, in this study, the expression of Tβ4 mRNA was confirmed in odontoblasts on developing molar tissues from the early bell stage to eruption. In membrane hybridization, an antisense Tβ4 probe bound specifically with a sense Tβ4 probe and identified the hybridization between antisense Tβ4 probe and the intracellular Tβ4 mRNA of odontoblasts. In addition, the expression of Tβ4 mRNA from early bell stage to eruption was the strongest in the odontoblast of a cusp region at an advanced bell stage with the activation of dentin matrix synthesis. Therefore, in accordance with other results, the strong expression of Tβ4 in secretory odontoblast indicates that Tβ4 may be a regulator for differentiation of odontoblasts or the formation of dentin during tooth development.

Tβ4 downregulates the expression of OCN, Col I, ON, OPN, and AP and then inhibits the differentiation of mesenchymal stem cells into osteoblast (Ho et al., 2010). In addition, Tβ4 siRNA treatment before the differentiation of MDPC-23 cells strongly decreases the expression of BSP, DSPP, OCN, ON, and Col I mRNA related with dentinogenesis and the formation of mineralized nodules (Choi et al.,

2012). In bone, similar to the properties of dentin, BSP knock-out transgenic mice form undermineralized bones, and the thickness of the cortical bone is thinner than in normal mice (Malaval et al., 2008). The mutation of DSPP induces dentinogenesis imperfecta type II and type III and dentin dysplasia type II by inhibiting the dentin mineralization compared to that of normal mice (Dong et al., 2005; Holappa et al., 2006; McKnight et al., 2008). Protamine increases the expression of BSP in osteoblasts, and BMP-2 increases the expression of DSPP and promotes mineralization in stem cells from human exfoliated deciduous teeth (SHED) (Hara et al., 2011; Zhou et al., 2013). In addition, TGF-β1 promotes BSP expression and mineralization in osteoblasts, and hepatocyte growth factor-1 (HGF-1) increases DSPP expression, proliferation, and differentiation in dental papilla cells (Zhang et al., 2005; Li et al., 2009). In this study, BSP expression was increased, but DSP expression was decreased in MDPC-23/Tβ4 compared to that of the control. In addition, immunofluorescence resulted in MDPC-23/Tβ4, the expression of cytoplasmic BSP was increased, but DSP expression was decreased. Therefore, Tβ4 may regulate the formation of dentin matrix through an increase of BSP expression and a reduction of DSP expression in odontoblasts.

ERK, p38, and JNK, as the MAPKs, are known to have a critical function in intracellular signal transduction of embryo development, immune response, and neural canal development in vertebrates (Kuan et al., 1999; Dong et al., 2002). In addition, MAPKs is identified as the central signal transducers to modulate osteogenesis and bone mass (Ge et al., 2007; Greenblatt et al., 2010; Zou et al., 2011). The transgenic mice, germline deletion of *Erk1*, or the conditional deletion of *Erk2* in limb mesenchyme, severely decreases bone mineralization (Matsushita et al., 2009). The low bone mass and hypomineralization are occurred in the clavicle and calvarium by *MEK1* mutation in the osteoblasts (Ge et al., 2007). Tβ4 increases the migration of gastric cancer cells by downregulating E-cadherin through the ERK1/2/GSK3α/β-catenin signaling pathway (Ryu et al., 2012). In the present study, the phosphorylation of ERK1/2 was increased with cytoplasmic, nuclear Runx2 and the expression of BSP in MDPC-23/Tβ4, but DSP expression was decreased compared to that of control. In addition, in MDPC-23/PD98059, the

phosphorylation of ERK1/2 was decreased with cytoplasmic, nuclear Runx2 and the expression of BSP compared to that of MDPC-23/Tβ4, but the reduction of DSP expression was maintained similar to that of MDPC-23/Tβ4. In MDPC-23/Tβ4, the phosphorylation of β-catenin was increased, cytoplasmic β-catenin was decreased with the nuclear translocation of β-catenin, and BSP expression was decreased, but DSP expression was decreased compared to that of the control. In MDPC-23/PD98059, the phosphorylation of β-catenin was decreased and cytoplasmic β-catenin was increased with the induction of β-catenin nuclear translocation. BSP expression was decreased, but the reduction of DSP expression was similar to that of MDPC-23/Tβ4. Protamine or BMP-7 increased the expression of Runx2 and BSP through the activation of ERK1/2 in osteoblasts, and Ugonin K increased the expression of Runx2, osterix, BSP, and OCN by the activation of p38 or the ERK1/2 pathway (Xiao et al., 2002; Lee et al., 2011; Zhou et al., 2013). In a recent study, Wnt3a stimulation is found to increase the nuclear translocation of β-catenin, but decrease the expression of Runx2, osterix, ALP, BSP, and OCN in cementoblasts and dental follicle cells (Nemoto et al., 2009; Silvério et al., 2012). In addition, LEF1, a transcription factor, decreases the promoter activity of Runx2 and OCN in β-catenin-overexpressed MC3T3-E1 cells compared to that of normal cells (Kahler and Westendorf, 2003). Therefore, Tβ4 increases the BSP expression through the ERK1/2/Runx2 or ERK1/2/β-catenin pathway, but these signaling pathways may not be associated with a reduction of DSP expression.

A recent study has suggested that mineral trioxide aggregate (MTA) increases the differentiation of primary human dental pulp cells into odontoblasts, and mineralization by elevation of DSPP and DMP-2 expression through the activation of ERK (Jung et al., 2014). In addition, amelogenin increases the expression of DSPP and DMP-1 by the activation of the ERK1/2 and p38 MAPK signal transduction pathway in MDPC-23 cells (Yao et al., 2011). BMP-2 increases the expression of Runx2 and DSPP through the Smad1/5 pathway, and BMP-2 increases the expression of DSPP, ALP, and OCN through the β-catenin pathway in dental papilla cells (Cho et al., 2010; Koizumi et al., 2013). Wnt1 increases the expression of DSPP, ALP, and OCN through the stimulation of the β-catenin

pathway in apical papilla stem cells (Wang et al., 2012). TGF-β1 decreases the promoter activity of DSPP through the Smas2/3 pathway in MO6-G3, odontoblastic cell line compared to the control, and that activity is decreased through the Smad3 pathway in MDPC-23 cells (Unterbrink et al., 2002; He et al., 2004). TGF-β1 inhibits the differentiation of mesenchymal stem cells into osteoblasts with the decrease of ALP and BSP expression by the induction of the elevation of β-catenin through the Smad3 pathway (Zhou, 2011). Based on previous reports, TGF-β1 was used as the control of Tβ4 to compare Smad2/3 phosphorylation in this study. MDPC-23/TGF-β1 showed the increased phosphorylation of Smad2 and Smad3, but MDPC-23/Tβ4 only presented an increase of Smad3. The phosphorylated-Smad3 was translocated into the nucleus, and the phosphorylation of β-catenin, expression of cytoplasmic β-catenin, Runx2, and BSP were of a similar pattern to that of the ERK1/2 pathway in MDPC-23/Tβ4. In MDPC-23/SIS3, the phosphorylation of Smad3 and β-catenin were decreased, and the expression of β-catenin was increased, but Runx2 and BSP expression were downregulated compared to that of MDPC-23/Tβ4. However, the reduced expression of DSP was maintained, similar to that of MDPC-23/Tβ4. In addition, the promoter activity of pGL3-BSP-Luc/Tβ4 was significantly increased compared to that of pGL3-Luc, but it was decreased by pGL3-BSP-Luc/PD98059 and pGL3-BSP-Luc/SIS3 compared to pGL3-BSP-Luc/Tβ4 in MDPC-23 cells. In addition, the BSP promoter activity of pGL3-BSP-Luc/SIS3 was more decreased than pGL3-BSP-Luc/PD98059. Therefore, Tβ4 increases the BSP expression through the Smad3, Smad3/Runx2, or Smad3/β-catenin pathway, but the signaling of these pathways may not be associated with a reduction of DSP expression. In addition, Smad3 may be a more predominant signal transduction pathway than ERK1/2 in BSP expression.

In conclusion, an increase of BSP expression was induced through ERK1/2/Runx2, ERK1/2/β-catenin, Smad3, Smad3/Runx2, or Smad3/β-catenin signaling pathways, but the decreased DSP expression was not affected by MEK and pSmad3 inhibitors, indicating that DSP expression by Tβ4 is not related to the ERK or Smad3 signaling pathways in MDPC-23 cells. This result suggests that Tβ4 may regulate dentin matrix formation with the increase of BSP through the

ERK1/2 or Smad3 signaling pathways in odontoblasts. Further study will be needed to see whether other signal transduction pathways may exist in the regulation of DSP expression for Tβ4 signaling.

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FIGURE LEGENDS

Figure 1. Specificity and binding activity analysis of the *in situ* probes through membrane hybridization and the expression of Tβ4 mRNA in odontoblasts.

(A) The hybridization signal between DIG-labeled AS and unlabeled S was presented in concentrations of 10 ng/μl and 1 ng/μl, but was not detected below 0.1 ng/μl. (B) The hybridization signal between DIG-labeled AS (1 ng/μl) and membrane-dotted unlabeled S (1 ng/μl) was decreased by increasing the concentration of unlabeled S (upper panel) or unlabeled AS (lower panel) in the mixture. (C) The expression of Tβ4 mRNA was not detected in the negative control (a) or DIG-labeled S (b), but it was detected in odontoblast PN3 using DIG-labeled AS in *in situ* hybridization (c). Scale bars: 20 μm.

Figure 2. *In situ* hybridization of Tβ4 mRNA expression in dentinogenesis.

(A - E) Low magnification images of the tooth germ of molars from PN1 to PN21. The expression of Tβ4 mRNA was expressed in odontoblasts and ameloblasts. (a - e) The expression of Tβ4 mRNA was increased a large amount in the odontoblasts (arrow) at PN5 of the advanced bell stage, and Tβ4 mRNA expression at the crown and functional stages were similar to the early bell stage. (F) The hybridization signal intensity of Tβ4 mRNA was strongest at PN5 odontoblasts. PN1, postnatal day 1; PN3, postnatal day 3; PN5, postnatal day 5; PN15, postnatal day 15; PN21, postnatal day 21. Scale bars, 100 μm (A - E), 20 μm (a - e).

Figure 3. The mRNA and protein expression of BSP and DSP in Tβ4 treated MDPC-23 cells.

(A) BSP mRNA and protein expression was increased in MDPC-23/Tβ4 compared to that of the control, and (B) DSPP mRNA and DSP protein expression was decreased compared to that of control. (C and D) In immunofluorescence, an increase of BSP protein and a reduction of DSP proteins in the cytoplasm were

detected in MDPC-23/Tβ4 compared to that of control. M, DNA marker; Con, control; Arrows, BSP or DSP protein; Scale bars, 20 μm.

Figure 4. BSP and DSP expression through the ERK1/2, β-catenin, and Runx2 signaling in Tβ4 treated MDPC-23 cells.

(A) The phosphorylation of ERK1/2 and β-catenin was increased gradually in MDPC-23/Tβ4 in a time dependent manner compared to that of the control. β-catenin expression was decreased gradually in a time dependent manner. (B) The phosphorylation of ERK1/2 and β-catenin was decreased in MDPC-23/PD98059 and MDPC-23/PD98059/Tβ4 compared to MDPC-23/Tβ4. The expression of Runx2 and BSP protein was decreased in MDPC-23/PD98059 and MDPC-23/PD98059/Tβ4 compared to MDPC-23/Tβ4. DSP protein in MDPC-23/PD98059, and MDPC-23/PD98059/Tβ4 were expressed similarly to MDPC-23/Tβ4. C, control; PD, PD98059; PD/ Tβ4, PD98059/Tβ4.

Figure 5. The cytoplasm and nuclear expression of β-catenin and Runx2 protein in Tβ4 treated MDPC-23 cells.

(A) The phosphorylation of β-catenin was increased gradually in MDPC-23/Tβ4 in a time dependent manner compared to that of the control. Cytoplasmic and nuclear β-catenin expression was decreased in MDPC-23/Tβ4 compared to that of the control. Cytoplasmic and nuclear Runx2 protein expression was increased at 6 h compared to that of the control, as was BSP protein, but DSP protein was decreased compared to that of the control. (B) In immunofluorescence, the expression of β-catenin protein in cytoplasm (arrows) and nuclear (arrowheads) were decreased in MDPC-23/Tβ4 compared to that of the control, but it was increased in MDPC-23/PD98059 compared to MDPC-23/Tβ4. (C) In immunofluorescence, the expression of Runx2 protein in cytoplasm (arrows) and the nucleus (arrowheads) were increased in MDPC-23/Tβ4 compared to that of the control but it was decreased in MDPC-23/PD98059 compared to MDPC-23/Tβ4. Con, control; Scale bars; 20 μm.

Figure 6. BSP and DSP expression through the Smad3, β-catenin and Runx2 signaling in Tβ4 treated MDPC-23 cells.

(A) The phosphorylation of Smad2 was increased in MDPC-23/TGF-β1 compared to that of the control, but it did not occur in MDPC-23/Tβ4. The phosphorylation of Smad3 was increased in MDPC-23/TGF-β1 and MDPC-23/Tβ4 compared to that of the control. (B) The phosphorylation of Smad2 was not occurred in MDPC-23/Tβ4, but the phosphorylation of Smad3 was increased in a time dependent manner compared to that of the control. (C) The phosphorylation of Smad3 and β-catenin were decreased in both MDPC-23/SIS3 and MDPC-23/SIS3/Tβ4 compared to MDPC-23/Tβ4, but the expression of β-catenin protein was increased. The expression of Runx2 and BSP protein was decreased in MDPC-23/SIS3 and MDPC-23/SIS3/Tβ4 compared to MDPC-23/Tβ4. DSP protein in MDPC-23/SIS3 and MDPC-23/SIS3/Tβ4 were expressed similarly to that of MDPC-23/Tβ4.

Figure 7. The cytoplasm and nuclear expression of β-catenin and Smad3 protein in Tβ4-treated MDPC-23 cells.

(A) The phosphorylation of Smad3 both in cytoplasm and nuclear, and BSP expression were increased in MDPC-23/Tβ4 compared to that of the control, but DSP protein expression was decreased. (B) In immunofluorescence, phosphorylated Smad3 in cytoplasm (arrows) and nucleus (arrowheads) were increased in MDPC-23/Tβ4 compared to that of the control, but it was decreased in MDPC-23/SIS3 compared to MDPC-23/Tβ4. (C) The expression of β-catenin protein in the cytoplasm (arrows) and nucleus (arrowheads) were decreased in MDPC-23/Tβ4 compared to that of the control, and it was increased in MDPC-23/SIS3 compared to MDPC-23/Tβ4. Con, control; Scale bars: 20 μm

Figure 8. BSP promoter activity through ERK1/2 and Smad3 signaling in Tβ4-treated MDPC-23 cells.

Luciferase activity was increased in pGL3-BSP-Luc and pGL3-BSP-Luc/Tβ4 compared to pGL3-Luc. The luciferase activity of pGL3-BSP-Luc/PD98059 or

pGL3-BSP-Luc/PD98059/Tβ4 was decreased compared to pGL3-BSP-Luc/Tβ4. Luciferase activity in both pGL3-BSP-Luc/SIS3 and pGL3-BSP-Luc/SIS3/Tβ4 was decreased compared to pGL3-BSP-Luc/Tβ4.

Figure 9. Schematic diagram of the Tβ4 signaling pathway related with BSP and DSP expression.

(A) An increase of BSP expression in Tβ4-treated MDPC-23 cells was induced through either the ERK1/2/Runx2 or ERK1/2/β-catenin signal transduction pathway, but was not associated with a decrease of DSP expression. (B) An increase of BSP expression in Tβ4-treated MDPC-23 cells was induced through the Smad3, Smad3/Runx2, or Smad3/β-catenin signal transduction pathways, but was not associated with a decrease of DSP expression.

FIGURES

Figure 1.

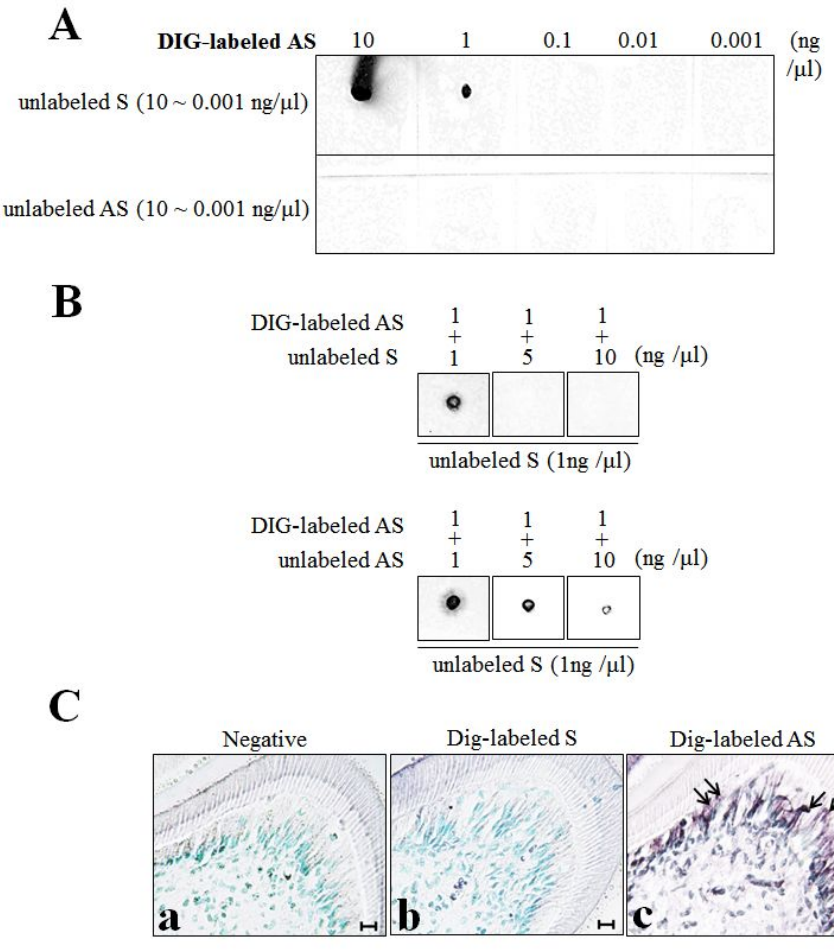
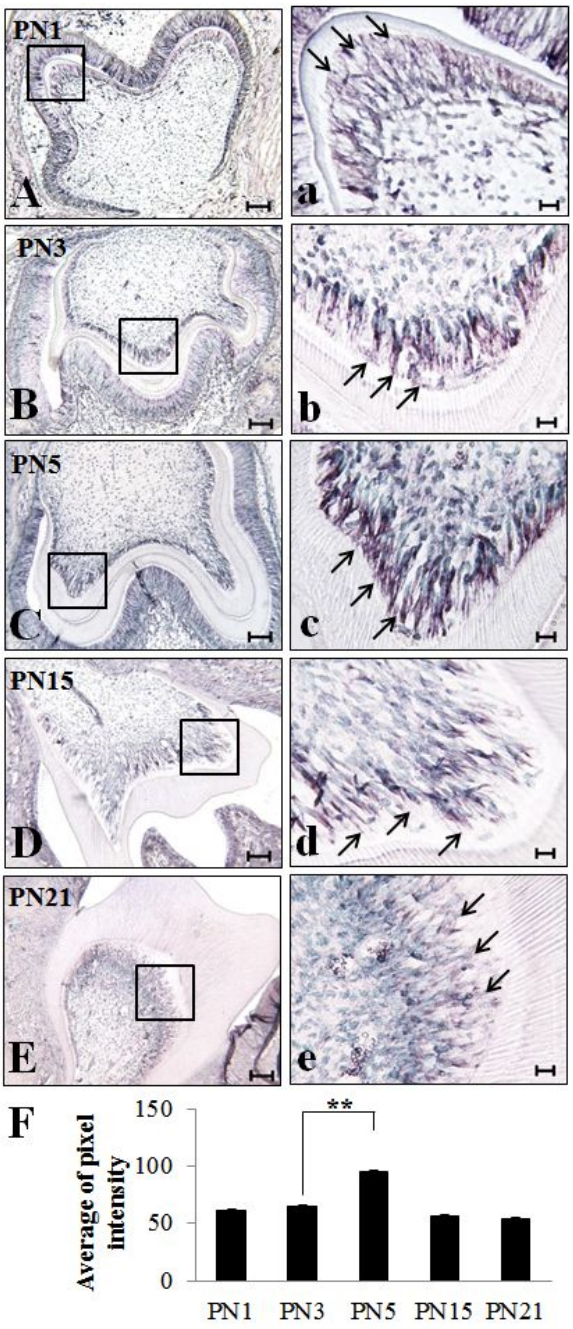


Figure 2.



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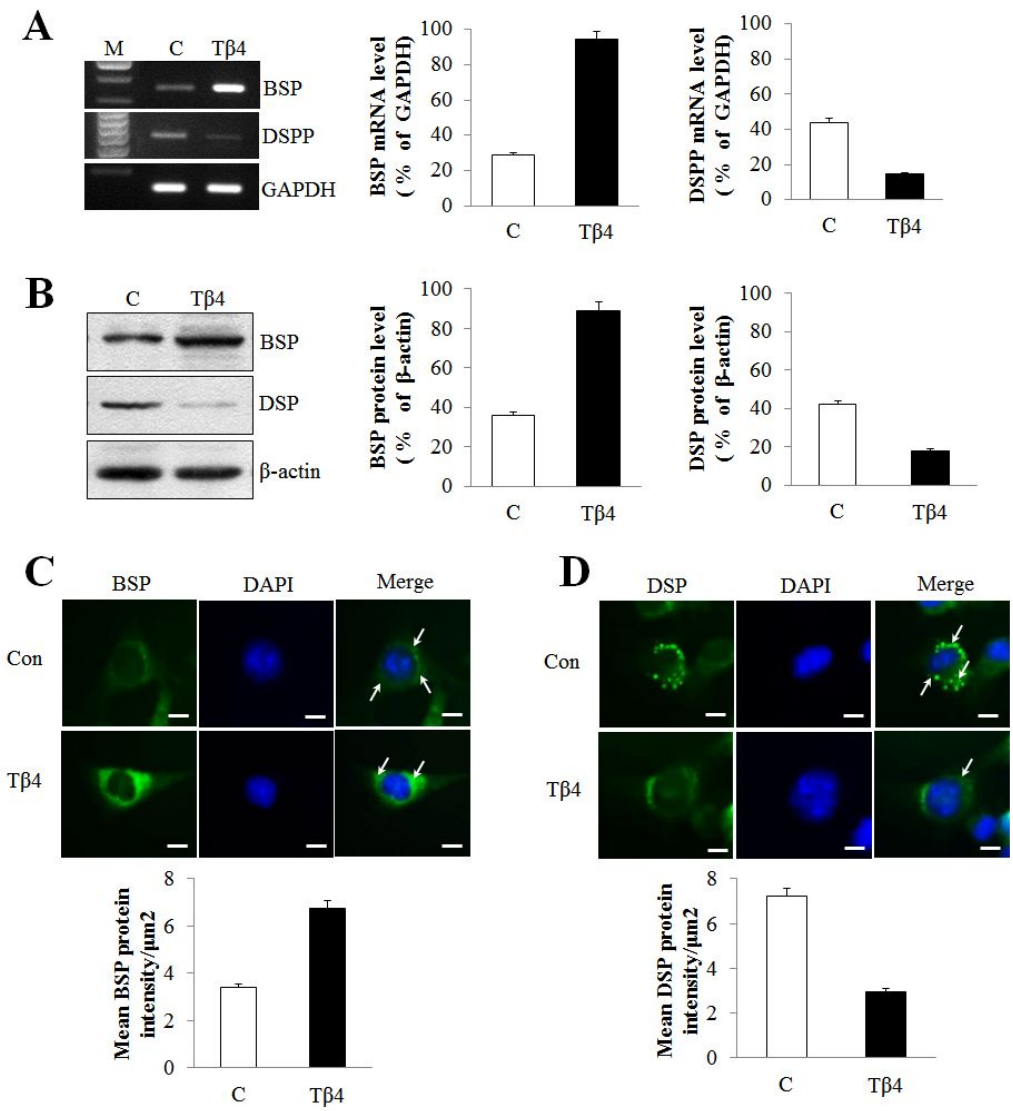

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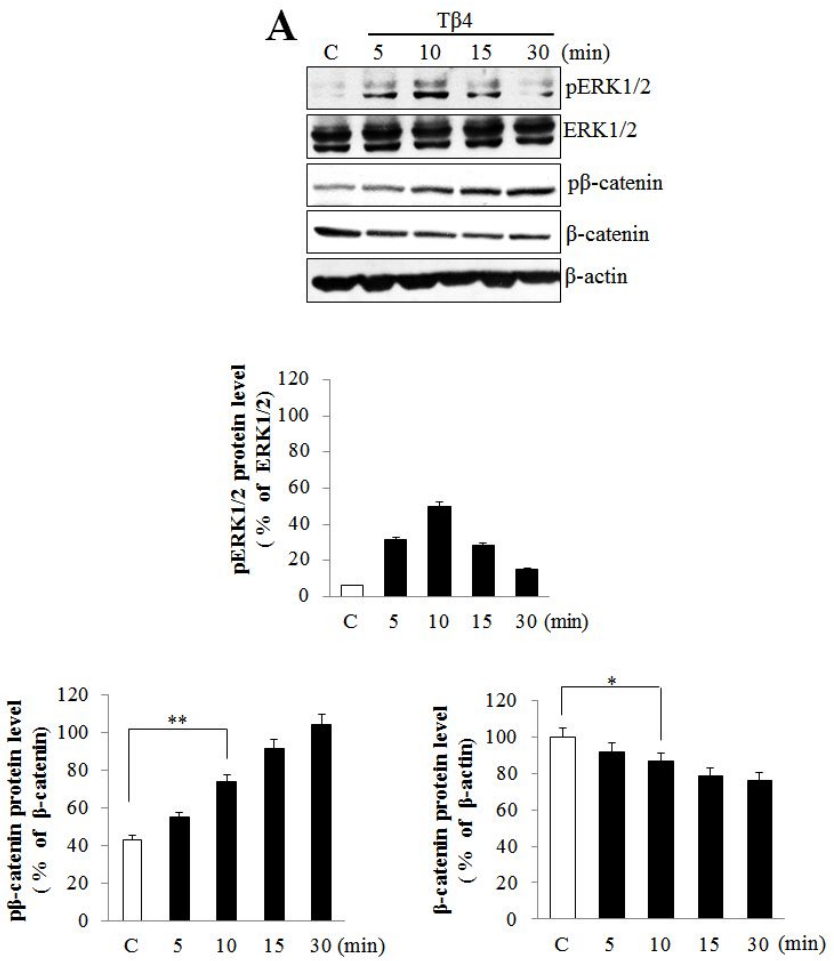

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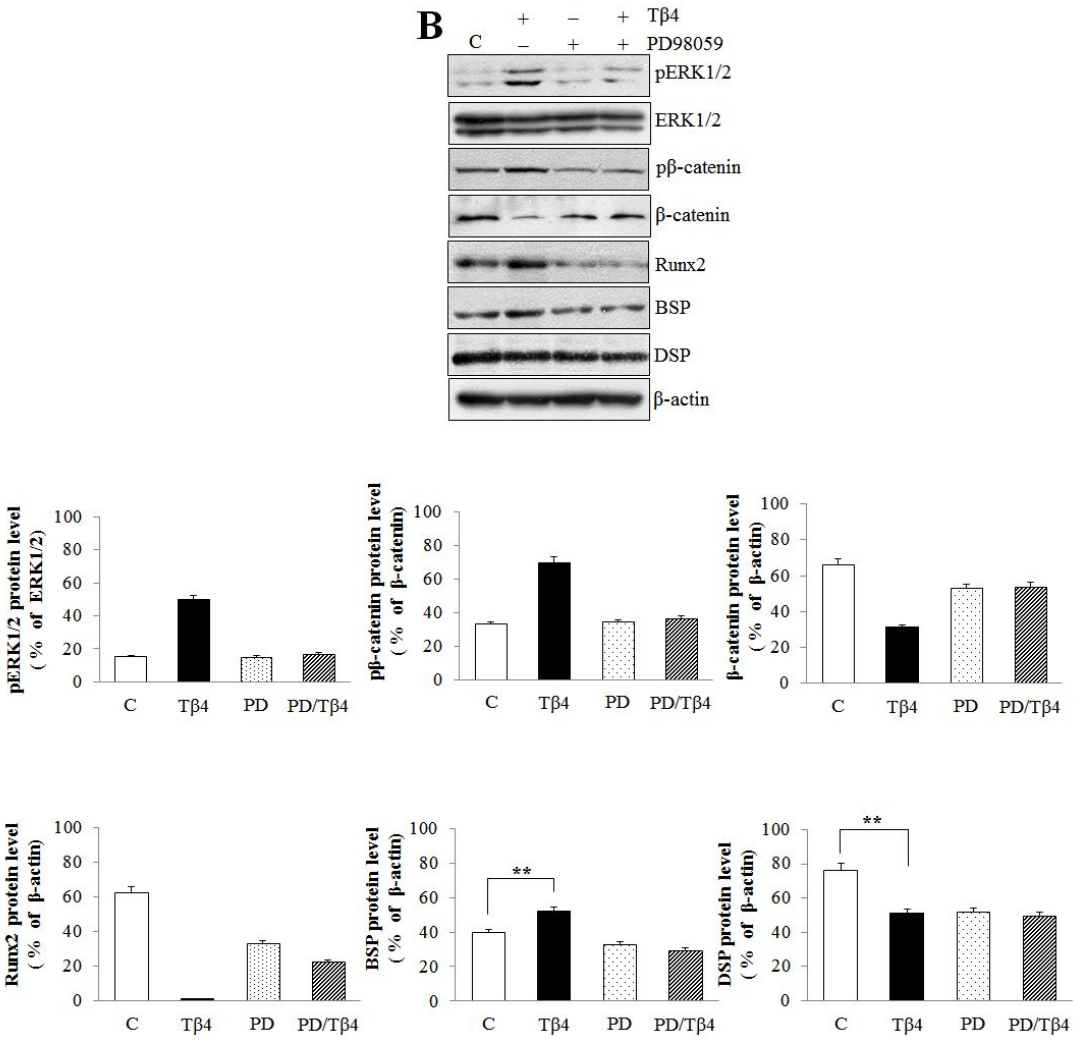

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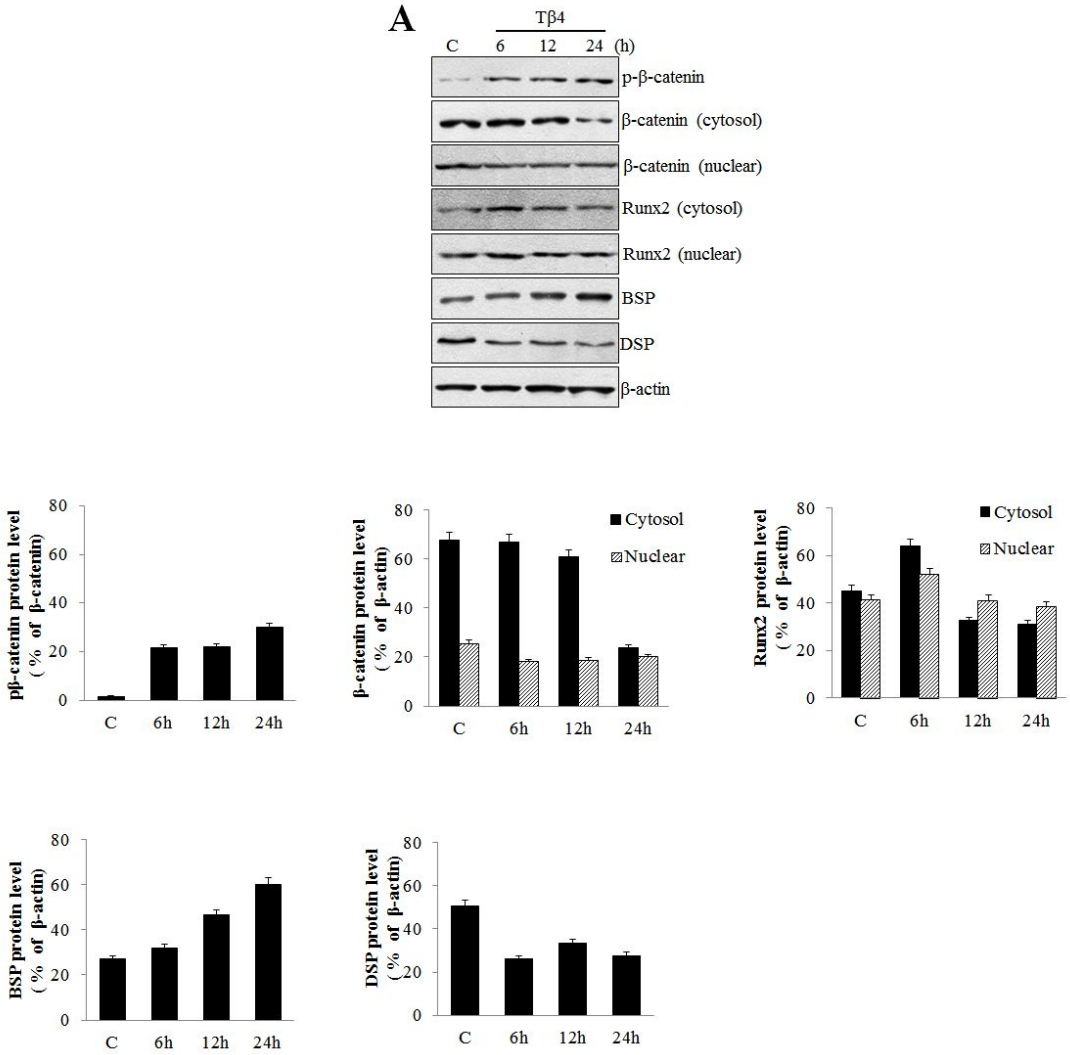

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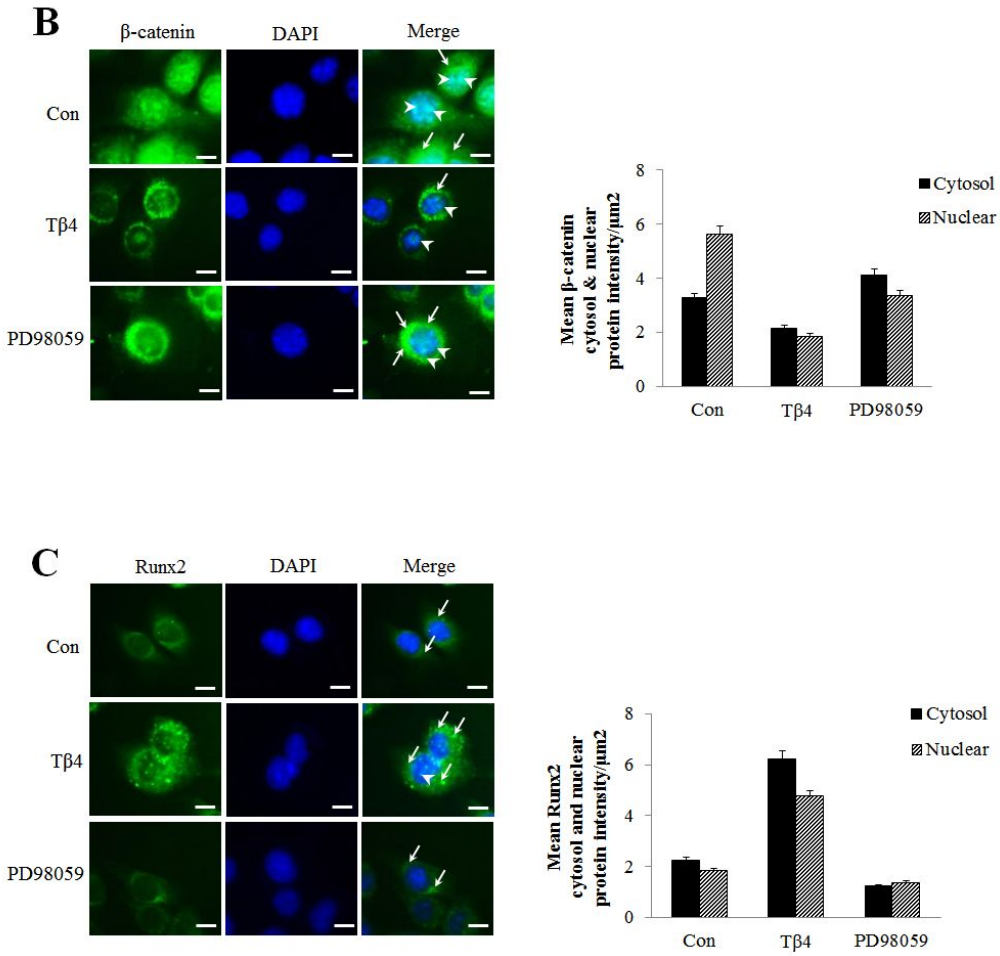

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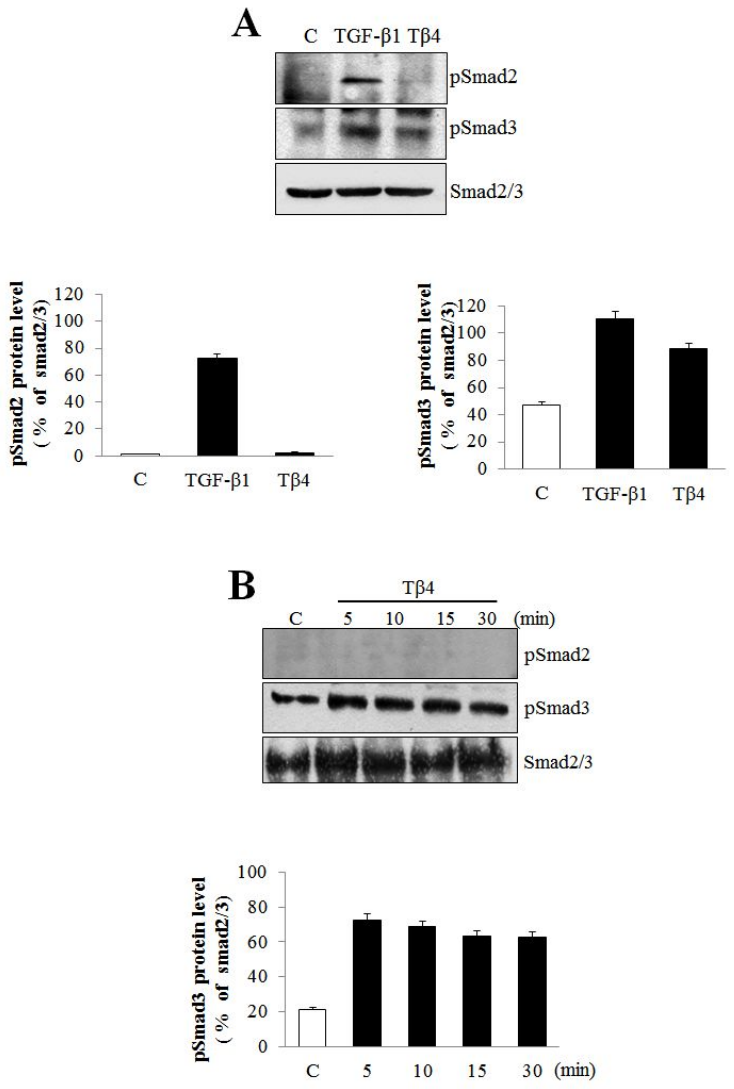

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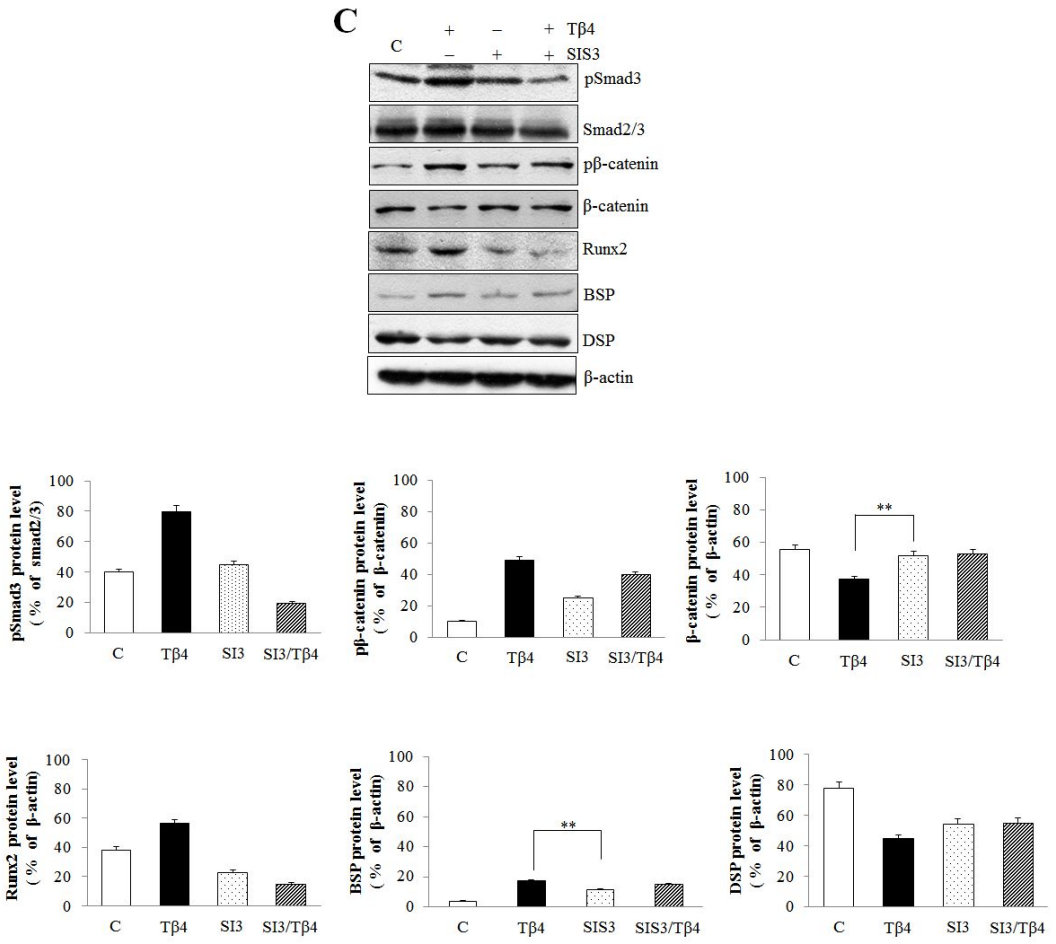

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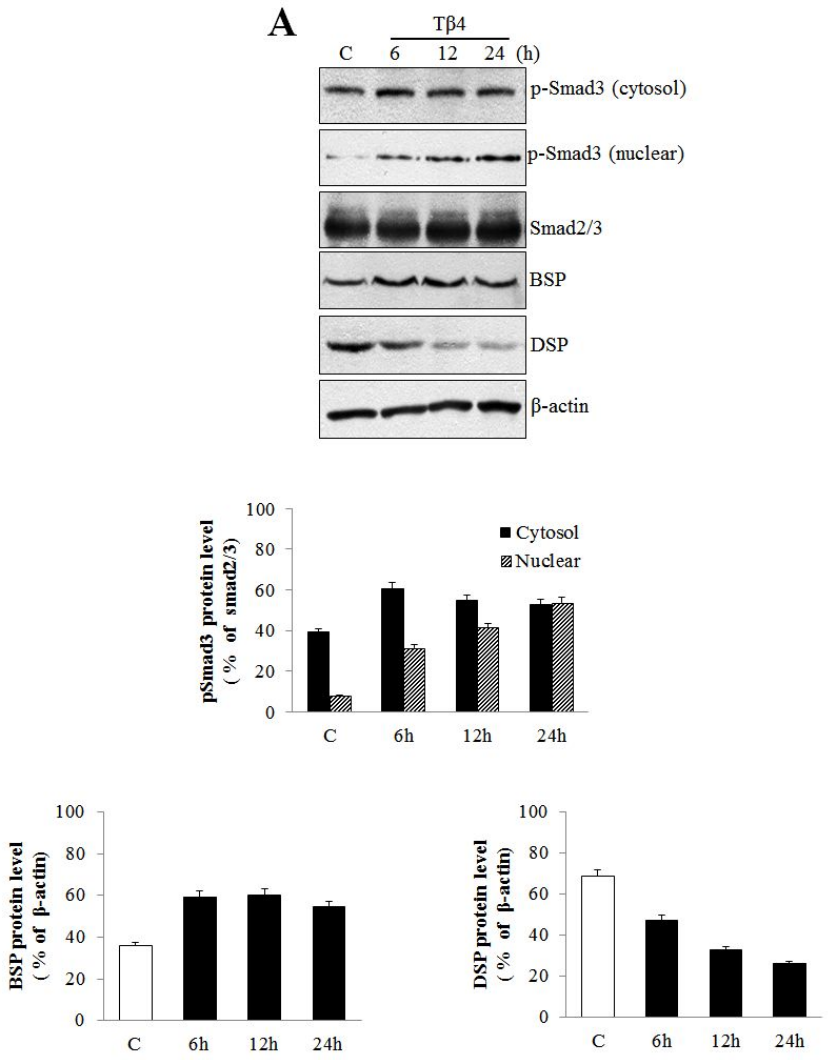

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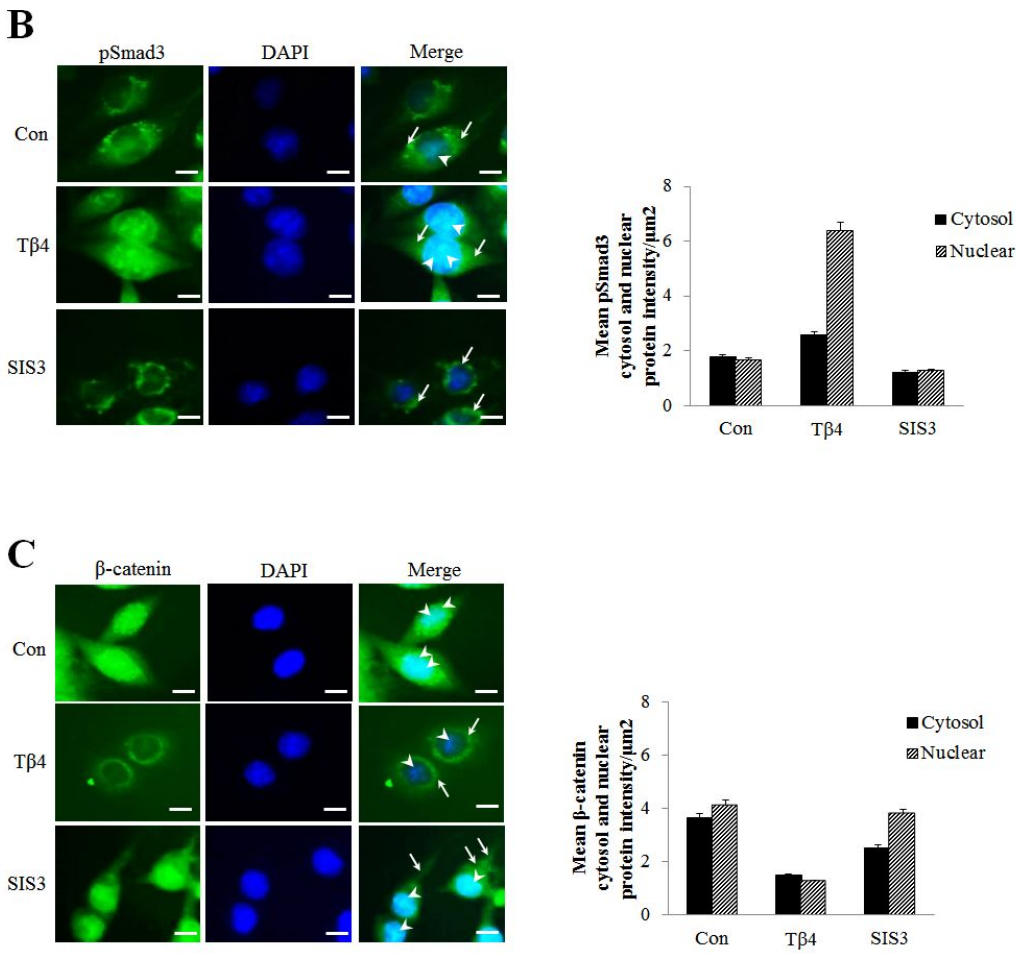

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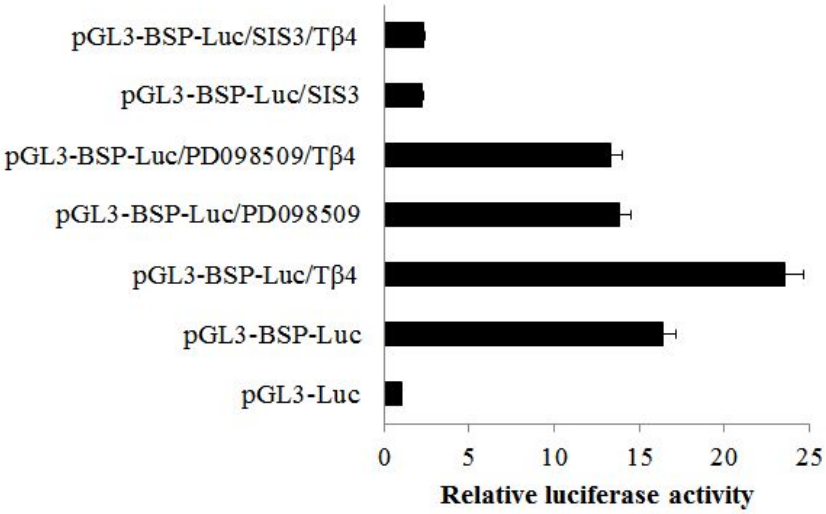

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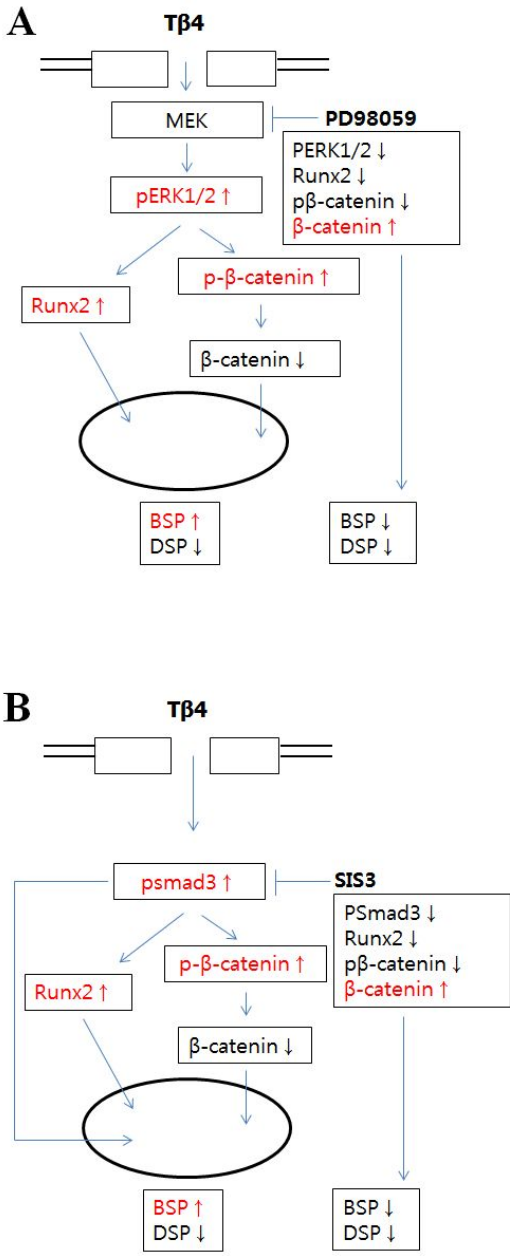

ABSTRACT IN KOREAN

상아질모세포에서 Thymosin β4에 의한 BSP(bone sialoprotein)와 DSPP(dentin sialophosphoprotein) 발현조절

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Thymosin β4(Tβ4)는 포유류 세포에서 상처치유, 혈관생성, 세포 증식, 이동, 분화에 관여한다. Tβ4가 과발현된 형질전환 생쥐는 법랑질형성부전을 보였고, Tβ4 knockdown은 치배의 성장을 억제시켰다. 또한 Tβ4는 구강상피세포 증식에 관여했고, 상아질모세포 분화와 광화에 관련된 단백질들의 발현과 분비를 조절했다. 선행된 연구결과들로 보아 Tβ4는 상아질모세포에서 상아기질 형성에 관련된 인자들의 발현을 조절할 것으로 생각되지만, 이들 인자들의 발현과 연관된 Tβ4의 신호전달체계에 관한 연구는 미흡한 실정이다. 따라서 본 연구에서는 상아질모세포주인 MDPC-23 세포에서 상아기질형성 조절인자 중 bone sialoprotein(BSP)과 dentin sialoprotein(DSP)의 발현과 관련된 Tβ4의 신호전달경로를 규명하고자 하였다.

Membrane hybridization 실험 결과, 합성된 antisense Tβ4 probe가 sense Tβ4 probe에 특이적으로 결합했고, antisense Tβ4 probe와 상아질모세포 내 Tβ4 mRNA 사이에 hybridization을 확인했다. *In situ* hybridization 방법을 통해 발생 중인 구치 조직의 상아질모세포에서 Tβ4 mRNA는 후기 종시기인 postnatal day 5(PN5)에 급격하게 증가했다. Tβ4가 처리된 MDPC-23 세포(MDPC-23/Tβ4)에서 BSP 발현은 대조군에 비해 증가했지만, DSP 발현은 감소했다. MDPC-23/Tβ4에 대한 면역형광 결과에서도 세포질 내 BSP 발현은 증가했지만, DSP는 감소했다. MDPC-23/Tβ4에서 ERK1/2 인산화, 세포질과 핵 내 Runx2 그리고 BSP 발현은 대조군에 비해 증가됐으나 DSP 발현은 감소됐다. MEK inhibitor인 PD98059가 처리된 MDPC-23 세포(MDPC-23/PD98059)에서 ERK1/2 인산화, 세포질과 핵 내 Runx2 그리고 BSP 발현은 MDPC-23/Tβ4에 비해 감소했지만, DSP의 감소된 발현 경향은 유지됐다.

MDPC-23/Tβ4에서 β-catenin 인산화는 대조군에 비해 증가했고, 세포질 내 β-catenin은 감소했지만, β-catenin 핵 이동이 일어났으며 BSP 발현은 대조군에 비해 증가됐으나, DSP 발현은 감소했다. 또한 MDPC-23/PD98059에서 β-catenin 인산화는 MDPC-23/Tβ4에 비해 감소했지만, 세포질 내 β-catenin 및 핵 이동은 증가했다. MDPC-23/Tβ4에서 인산화된 Smad3는 핵으로 이동했으며, β-catenin 인산화와 세포질 내 β-catenin, Runx2 그리고 BSP 발현 경향은 ERK1/2 경로와 유사했다. Smad3의 inhibitor인 SIS3가 처리된 MDPC-23 세포(MDPC-23/SIS3)에서 Smad3 인산화는 MDPC-23/Tβ4에 비해 감소했고 β-catenin 인산화, β-catenin, Runx2와 BSP 그리고, DSP 단백질 발현 경향은 MDPC-23/PD98059와 유사했다. MDPC-23/Tβ4 에서 BSP promoter activity를 측정한 결과 음성대조군(pGL3-Luc)에 비해 크게 증가했지만, PD98059와 SIS3를 처리하면 Tβ4 처리군에 비해 감소했다. 또한 SIS3를 처리한 군의 promoter activity는 PD98059를 처리한 군보다 더 감소했다.

결론적으로, Tβ4는 상아질모세포에서 BSP 발현 증가와 DSP 발현 감소를 통해 상아질형성을 조절하고, Tβ4에 의한 BSP 발현 증가는 ERK1/2/Runx2, ERK1/2/β-catenin, Smad3, Smad3/Runx2 또는 Smad3/β-catenin 경로를 통해 일어나지만, DSP 발현 감소는 ERK1/2 또는 Smad3가 아닌 다른 신호전달 기작이 있을 것으로 생각된다.

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