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Kaempferol Stimulates Bone Sialoprotein Gene Transcription and New Bone Formation

Li Yang,[1,2] Hideki Takai,[1,3] Tadahiko Utsunomiya,[3,4] Xinyue Li,[1,2] Zhengyang Li,[1,2] Zhitao Wang,[1,2] Shuang Wang,[1,5] Yoko Sasaki,[1] Hirotsugu Yamamoto,[3,4] and Yorimasa Ogata[1,3*]

[1]*Department of Periodontology, Nihon University School of Dentistry at Matsudo, Chiba 271-8587, Japan*
[2]*Tianjin Stomatology Hospital, Tianjin, China*
[3]*Research Institute of Oral Science, Nihon University School of Dentistry at Matsudo, Chiba 271-8587, Japan*
[4]*Department of Oral Pathology, Nihon University School of Dentistry at Matsudo, Chiba 271-8587, Japan*
[5]*Stomatology College of Tianjin Medical University, Tianjin, China*

ABSTRACT

Kaempferol is a typical flavonol-type flavonoid that is present in a variety of vegetables and fruits, and has a protective effect on postmenopausal bone loss. Bone sialoprotein (BSP) is thought to function in the initial mineralization of bone and could be crucial for osteoblast differentiation, bone matrix mineralization and tumor metastasis. In the present study we investigated the regulation of BSP transcription by kaempferol in rat osteoblast-like UMR106 cells, and the effect of kaempferol on new bone formation. Kaempferol (5 μM) increased BSP and Osterix mRNA levels at 12 h and up-regulated Runx2 mRNA expression at 6 h. Kaempferol increased luciferase activity of the construct pLUC3, which including the promoter sequence between nucleotides −116 to +60. Transcriptional stimulation by kaempferol abrogated in constructs included 2 bp mutations in the inverted CCAAT, CRE, and FRE elements. Gel shift analyses showed that kaempferol increased nuclear protein binding to CRE and FRE elements, whereas the CCAAT-protein complex did not change after kaempferol stimulation. Twelve daily injections of 5 μM kaempferol directly into the periosteum of parietal bones of newborn rats increased new bone formation. These data suggest that kaempferol increased BSP gene transcription mediated through inverted CCAAT, CRE, and FRE elements in the rat BSP gene promoter, and could induce osteoblast activities in the early stage of bone formation. J. Cell. Biochem. 110: 1342–1355, 2010.

KEY WORDS: BONE SIALOPROTEIN; KAEMPFEROL; FLAVONOID; BONE FORMATION; OSTEOBLASTS; TRANSCRIPTION; IMMUNOLOCALIZATION

Flavonoids are a group of polyphenolic compounds that are widely found in plants. As intrinsic components of fruits, vegetables and beverages such as wine and tea [Gerritsen et al., 1995; Bai et al., 1998], over 4,000 different flavonoids known to date are part of the regular human diet. They have various biological properties, such as anti-oxidant, anti-inflammation, anti-viral, anti-cancer, gastroprotective, protein kinase C inhibition, topoisomerases II inhibition, cytotoxic activities, and the prevention of age-related pathologies [Akama et al., 1996; Manna et al., 1999; Shimizu and Ogata, 2002]. Flavonoids are present mainly as glycosides, in which hydrogen is substituted by the sugar moiety [Murota et al., 2000, 2002; Kim et al., 2007]. Soybeans are rich in isoflavones, such as genistein and daidzein, and have been reported to reduce the occurrence of osteoporosis [Morabito et al., 2002; Devareddy et al., 2006]. Genistein could prevent the loss of bone mass in ovariectomized rats [Anderson and Garner, 1998]. A synthetic analogue, ipriflavone, is effective in inhibiting bone resorption in postmenopausal osteoporosis, although its action may not occur directly through estrogen receptors (ER) [Petilli et al., 1995]. Kaempferol (3,4′,5,7-tetrahydroxyflavone) and quercetin (3,3′,4′,5,7-pentahydroxyflavone) belong to the flavonol family [Comalada et al., 2006]. Flavonols, in contrast to soybean isoflavones, are the most abundant phytoestrogens in western diets, being present in apples, onions, leeks, citrus fruits, grapes, tea and red wine. Quercetin and its

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*Correspondence to: Prof. Yorimasa Ogata, Department of Periodontology, Nihon University School of Dentistry at Matsudo, Chiba 271-8587, Japan. E-mail: ogata.yorimasa@nihon-u.ac.jp

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glycoside derivative rutin reduced osteoclastic bone resorption by inhibiting the receptor activator of nuclear factor κB (NFκB) (RANK) protein and activating caspases [Rassi et al., 2005]. Kaempferol, but not quercetin, inhibited tumor necrosis factor α (TNFα)-induced production of IL-6 and monocyte chemoattractant protein-1 (MCP-1) in MC3T3-E1 osteoblast-like cells, and only kaempferol blocked TNFα-induced translocation of NFκB subunit p65 from the cytoplasm to the nucleus [Pang et al., 2006]. Kaempferol and quercetin increased alkaline phosphatase (ALP) activity in MG-63 osteoblast-like cells through extracellular regulated kinase (ERK) and the ER pathway [Prouillet et al., 2004]. The results indicate that kaempferol is a potent anti-osteoclastic agent due to its action on both osteoclasts and osteoblasts.

The major posphoproteins in bone are bone sialoprotein (BSP) and osteopontin (OPN), which contain an Arg-Gly-Asp (RGD) cell attachment sequence [Oldberg et al., 1986, 1988; Ganss et al., 1999; Ogata, 2008]. The RGD sequence is essential for the cell-binding properties of fibronection, vitronectin, fibrinogen and Small Integrin-Binding LIgand, N-Linked Glycoproteins (SIBLING) [Oldberg et al., 1988; Fisher and Fedarko, 2003]. The SIBLING family of proteins (BSP, OPN, dentin matrix protein-1, dentin sialophosphoprotein and matrix extracellular phosphoglycoprotein) have a noncoding exon 1, a leader sequence and the first two amino acids in exon2, casein kinase II phosphorylation consensus sequences in exon 3 and 5, proline-rich and often basic exon 4, and the integrin-binding RGD sequence, within one of the last two exons [Rowe et al., 2000; Chen et al., 2004]. The RGD sequence in BSP is recognized by a $\alpha_V\beta_3$ vitronectin receptor ($\alpha_V\beta_3$ integrin). Flanking the RGD sequence at the C-terminus of BSP, there is 7–12 of the sulfated tyrosine residues. BSP contains ~15% sialic acid, tyrosine sulfates and clusters of up to 10 consecutive glutamic acid residues. These negatively charged domains are presumably responsible for the strong binding of BSP to hydroxyapatite [Oldberg et al., 1986, 1988]. BSP expression is restricted in mineralized connective tissues, and first expressed at the onset of bone, cementum and dentin formation [Chen et al., 1992]. BSP has been shown to induce the formation of hydroxyapatite in a steady-state agarose gel system [Hunter and Goldberg, 1990]. Therefore, BSP might be involved in the nucleation of hydroxyapatite at the mineralization front of bone. BSP deficiency impairs bone growth and mineralization, concomitant with dramatically reduced bone formation [Malaval et al., 2008]. BSP is also expressed by breast, prostate and lung cancers and is associated with the formation of ectopic hydroxyapatite microcrystals in tumor tissues and tumor metastasis [Waltregny et al., 2000; Ogata, 2008]. Rat, mouse and human BSP genes have been cloned and partially characterized [Kerr et al., 1993; Li and Sodek, 1993; Kim et al., 1994; Benson et al., 1999; Kiyoshima et al., 2002]. These promoters have an inverted TATA box (−24 to −19) [Li et al., 1995], an inverted CCAAT box (−50 to −46) [Kim and Sodek, 1999; Shimizu and Ogata, 2002], a cAMP response element (CRE; −75 to −68) [Samoto et al., 2003; Araki et al., 2009; Mezawa et al., 2009], a fibroblast growth factor 2 response element (FRE; −92 to −85) [Shimizu-Sasaki et al., 2001; Samoto et al., 2003; Shimizu et al., 2005, 2006; Nakayama et al., 2006], a pituitary-specific transcription factor-1 (Pit-1) motif (−111 to −105) [Ogata et al., 2000; Shimizu et al., 2006], and a homeodomain protein binding site (HOX; −199 to −192) [Benson et al., 2000; Shimizu et al., 2004; Nakayama et al., 2006]. Previously, we have reported that isoflavone activated BSP transcription through an inverted CCAAT box [Shimizu and Ogata, 2002], and quercetin (3,3,4′,5,7-pentahydroxyflavone) up-regulated BSP gene expression through inverted CCAAT and FRE [Kim et al., 2007].

In this study, we analyzed the effects of kaempferol on the expression of BSP using rat osteoblast-like UMR106 cells and new bone formation of rat calvaria.

METHODS

MATERIALS

Alpha minimum essential medium (α-MEM), fetal calf serum (FCS), lipofectamine, penicillin and streptomycin, and trypsin were obtained from Invitrogen (Carlsbad, CA). The pGL3-basic, pSV-β-galactosidase (β-Gal) control vector and U0126 (MAP kinase kinase inhibitor) were purchased from Promega Co. (Madison, WI). Kaempferol and Herbimycin A (HA, tyrosine kinase inhibitor) were purchased from Wako Pure Chemical Industries (Tokyo, Japan). The EXScript RT reagent kit and SYBR Premix Ex Taq were purchased from Takara (Tokyo, Japan). H7 (protein kinase C inhibitor) and H89 (protein kinase A inhibitor) were purchased from Seikagaku Corporation (Tokyo, Japan). LY249002 (phosphatidylinositol 3-kinase inhibitor (PI3-K)) was from Calbiochem (San Diego, CA). EnVision System-HRP was from Dako (Carpinteria, CA). Block Ace was from DS Pharma Biomedical (Osaka, Japan).

CELL CULTURE

Rat osteoblast-like UMR106 cells were cultured in α-MEM containing 10% FCS. Cells were grown to confluence in 60 mm tissue culture dishes and then cultured in α-MEM without serum and incubated with or without kaempferol at doses from 0.05 to 50 μM for 12 h and time (5 μM) from 3 to 24 h. Total RNA was isolated from triplicate cultures.

NORTHERN HYBRIDIZATION

Following kaempferol treatment, total RNA was extracted from UMR 106 cells with guanidium thiocyanate. Aliquots (20 μg) of total RNA were fractionated in 1.2% agarose gels and transferred onto a Hybond-N+ membrane. Hybridizations were performed at 42°C with either ^{32}P-labeled rat BSP, osteopontin (OPN) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probes. The hybridized bands, representing the two polyadenylated forms (1.6 and 2.0 kb) of rat BSP mRNA, were scanned using a Bio-imaging analyzer (Fuji BAS2500).

REAL-TIME PCR

One microgram of total RNA was used as a template for cDNA, which was prepared using the EXScript RT reagent kit. Quantitative real-time PCR was performed using the following primer sets: Runx2 forward, 5′-CAAGTGGCCAGGTTCAACGA-3′; Runx2 reverse, 5′-TGTGAAGACCGTTATGGTCAAAGTG-3′; Osterix forward, 5′-GCAAGGCTTCGCATCTGA-3′; Osterix reverse, 5′-CTTGGAGCAGAGCAGACAGG-3′; GAPDH forward, 5′-GACAACTTTGGCATCGTGGA-3′; GAPDH reverse, 5′-ATGCAGGGATGATGTTCTGG-3′ using SYBR

Premix Ex Taq in a TP800 thermal cycler dice real-time system (Takara). The amplification reactions were performed in 25 μl of the final volume containing 2× SYBR Premix EX Taq (12.5 μl), 25 ng cDNA (2.5 μl) and 0.2 μM forward and reverse primers (0.1 μl) for BSP, Runx2, Osterix and 10 ng cDNA (1.0 μl) for GAPDH. To reduce variability between replicates, PCR premixes, which contained all reagents except for cDNA, were prepared and aliquoted into 0.2 ml Hi-8-tubes (Takara). The thermal cycling condition was 10 s at 95°C and 40 cycles of 5 s at 95°C and 30 s at 60°C. Post-PCR melting curves confirmed the specificity of single-target amplification and the expressions of BSP, Runx2 and Osterix relative to GAPDH were determined in triplicate.

WESTERN BLOT

For Western blot analyses, cell lysate from UMR106 cells were separated on 10% SDS–PAGE and transferred onto a Hybond-P membrane. The membrane was then incubated for 3 h by anti-human BSP (LF-100 provided by Dr. Larry W. Fisher), anti-Runx2 (M06, Abnova), anti-Osterix (sc-22538, Santa Cruz Biotechnology) and anti-α-tubulin (sc-5286, Santa Cruz Biotechnology) antibodies. Anti-rabbit or mouse IgG conjugated with HRP (GE Healthcare UK Ltd.) were used as the secondary antibodies. Immunoreactivities were detected by ECL Plus Western Blotting Detection Reagents.

TRANSIENT TRANSFECTION ASSAYS

Exponentially growing UMR106 cells were used for transfection assays. Twenty-four hours after plating, cells at 40–60% confluence were transfected using a lipofectamine reagent. Transfection included 1 μg of a luciferase (LUC) construct and 2 μg β-Gal vectors as an internal control. Two days post-transfection, the cells were deprived of serum for 12 h, kaempferol (5 μM) was added, and the cells were cultured for a further 12 h prior to harvesting. The luciferase assay was performed according to the supplier's protocol using a luminescence reader (Acuu Flex Lumi 400; Aloka, Tokyo, Japan) to measure luciferase activities.

GEL MOBILITY SHIFT ASSAYS

Confluent UMR 106 cells in T-75 flasks incubated for 3, 6, and 12 h with 5 μM kaempferol in α-MEM without serum were used to prepare nuclear extracts. Nuclear protein was extracted according to the method of Dignam et al. [1983] with the addition of extra proteinase inhibitors (extraction buffer was 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 1 mM dithiothreitol (DTT), 25% (v/v) glycerol, 0.5 mM phenylmethyl-sulfonyl fluoride, 2 μg/ml leupeptin, 2 μg/ml pepstatin A, 1 μg/ml aprotinin, pH 7.9). Double-standard oligonucleotides encompassing the inverted CCAAT (nt, −61 to −37, 5′-CCGTGACCGTGA**TTGG**CTGCTGAGA), CRE (nt, −84 to −59, 5′-CCCACAGCC**TGACGTCG**CACCGGCCG), FRE (nt, −98 to −79, 5′-TTTTCT**GGTGAG**AACCCACA) and Pit-1 wild (nt, −115 to −96, 5′-CGTG**TTGTAGT**TACGGATTT) in the rat BSP promoter were prepared. For gel shift analysis, double-stranded oligonucleotides were end-labeled with [γ-^{32}P] ATP and T4 polynucleotide kinase. Nuclear protein extracts (3 μg) were incubated for 20 min at room temperature with 0.1 pM radiolabeled double-strandard oligonucleotide. Following incubation, the protein-DNA complexes were resolved by electrophoresis on 5% non-denaturing acrylamide gels (38:2 acrylamide/bis acrylamide) run at 200 V at RT. After electrophoresis, the gels were dried and autoradiograms were prepared and analyzed using an image analyzer. Supershift experiments were performed using anti-Runx2 (PC-287L-100UG) (AML3; Oncogene Research Product), anti-Dlx5 (AB5728) (Chemicon International), anti-CREB1 (p-43; Rockland), anti-phospho-CREB (Ser133) (UpState), anti-NF-YA (556359) (BD Pharmingen). anti-Msx-2 (sc-35196), anti-Smad1 (sc-7965), anti-c-Fos (sc-253-G), anti-c-Jun (sc-1694), anti-JunD (sc-74) and anti-Fra2 (sc-604) antibodies were from Santa Cruz Biotechnology. Antibodies were added to the reaction mixture and incubated for 3 h at 4°C before electrophoresis, which was performed under the same conditions as described above.

HISTOLOGICAL AND IMMUNOHISTOCHEMICAL ANALYSES

Newborn Sprague–Dawley rats were given daily injections of 5 μl of the solution containing kaempferol or vehicle (DMSO) alone into the top of the periosteum of the parietal bone for 12 days. Kaempferol was injected on the right side and the vehicle on the left side. The injection sites were marked on the first day and kaempferol was injected at the same site as precisely as possible. For time-course studies, 5 μl/day kaempferol (5 μM) or vehicle alone was injected for up to 12 days, and parietal bone was harvested on days 3, 6, 12, 15, and 19. Parietal bone was dissected and fixed in 10% neutral-buffered formalin following decalcification with 5% EDTA solution. Following fixation, 5 μm-thick sections were prepared from the middle and posterior parts of paraffin-embedded decalcified parietal bone and stained with hematoxylin and eosin. The central point of the sections was determined with a microscope as the point between the sagittal suture and the attachment site of the temporal muscle. The effect of kaempferol was evaluated by measuring the quantity of new bone formation using Image-Pro® Plus (MediaCybernatics, Inc.) and the number of osteoblasts per bone surface (within 0.05 mm × 0.1 mm) at the injection point in parietal bone. New bone was defined as the area between periosteal and endosteal surfaces containing both calcified bone and osteoid. The quantity of new bone was calculated as the ratio of bone volume in the visual field over the whole area of the visual field (0.225 mm × 0.3 mm). Immunoperoxidase staining for BSP, Runx2 and Osterix in tissue sections from parietal bone harvested on day 6 was performed using the EnVision System-HRP according to the manufacturer's instructions. Tissue sections, prepared as described above, were manually dewaxed in three 5 min xylene washes and rehydrated through incubation in graded ethanol to water. Endogenous peroxidase activity was destroyed by treating the sections with 0.03% hydrogen peroxide for 10 min. Sections were then washed three times in phosphate buffered saline (PBS) for at least 5 min each before incubated in blocking solution (Block Ace) for 10 min. Anti-BSP (LF-100), anti-Runx2 (M06) and anti-Osterix (sc-22538) antibodies were applied and tissue sections were incubated for 18 h in 4°C. The sections were washed three times 5 min with PBS before incubation with labeled polymer-HRP secondary antibody for 1 h in room temperature and treated with DAB chromogen (3,3′-diaminobenzidine chromogen solution) for 2 min. Various sections were counterstained with Mayer's hematoxylin. All immunostaining

was performed at least three times on sections obtained from at least three individual rats.

STATISTICAL ANALYSIS

Triplicate or quadruplicate samples were analyzed for each experiment, and the experiments were replicated to ensure the consistency of the responses to kaempferol. Significant differences between the control and kaempferol treatments were determined using unpaired Student's *t*-test.

RESULTS

STIMULATION OF BSP mRNA EXPRESSION IN UMR106 CELLS

To study the regulation of BSP expression by kaempferol, we performed Northern hybridization analysis of total RNA extracted from osteoblastic UMR 106 cells. First, a dose–response relation for kaempferol induction of BSP was established by treating the UMR 106 cells with different concentrations of kaempferol for 12 h. Kaempferol increased BSP mRNA levels at 0.05–5 μM and had a maximal effect at 5 μM (Fig. 1A), whereas 50 μM kaempferol decreased BSP expression. This optimal level of kaempferol was used to determine the time course of BSP mRNA expression. Kaempferol up-regulated BSP mRNA accumulation at 6 and 12 h, whereas no effect on GAPDH mRNA was observed (Fig. 1B). Kaempferol increased the expression of OPN mRNA levels in either a dose- and time-dependent manner (Fig. 1A,B). The results of real-time PCR showed that the treatment of UMR106 cells with 5 μM kaempferol significantly increased Runx2 mRNA levels at 6 h. Kaempferol (5 μM) induced Osterix mRNA expression at 3 h and reached maximal at 12 and 24 h, respectively (Fig. 1C,D). Kaempferol (5 μM) induced BSP, Runx2 and Osterix protein expressions at 3 h and reached maximal at 6 h (Osterix) and 12 h (BSP and Runx2) in UMR106 cells. Tubulin was used as loading control (Fig. 2).

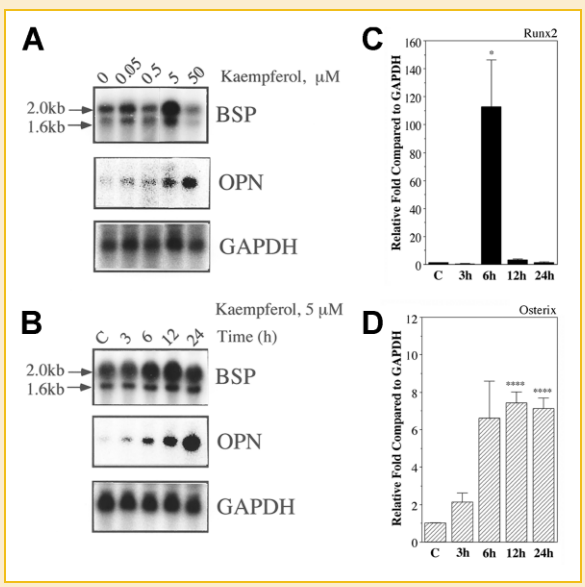


Fig. 1. Effects of kaempferol on BSP, OPN, Runx2 and Osterix mRNA levels in UMR106 cells. A: Dose–response effect of kaempferol on BSP mRNA levels in osteoblast-like UMR106 cells treated for 12 h. B: 24 h time course revealed an increase in BSP mRNA following administration of 5 μM kaempferol to UMR106 cells. Total RNA was isolated from triplicate cultures harvested after stimulation at 3, 6, 12, 24 h and used for Northern hybridization using BSP, OPN and GAPDH cDNA probes. Results of representative hybridization analysis for control and kaempferol-treated cells are shown. C,D: The expressions of Runx2, Osterix and GAPDH mRNA treated with 5 μM kaempferol for 24 h in UMR106 cells were measured by real-time PCR. The relative amounts of mRNA of Runx2 (C) and Osterix (D) to GAPDH were calculated. The experiments were performed in triplicate for each data point. Quantitative analyses of triplicate data sets are shown with standard errors. Significant differences from control: $^{*}P < 0.1$; $^{****}P < 0.01$.

TRANSIENT TRANSFECTION ANALYSIS OF RAT BSP PROMOTER CONSTRUCTS

To further determine the effects of kaempferol on the activation of BSP transcription, various-sized rat BSP promoters ligated to a luciferase reporter gene (pLUC1–pLUC6, pLUC1; −18 to +60, pLUC2; −43 to +60, pLUC3; −116 to +60, pLUC4; −425 to +60, pLUC5; −801 to +60 and pLUC6; −938 to +60) were transiently transfected into UMR106 cells. The results of luciferase assays indicated an increase in transcription after 12 h treatment with 5 μM kaempferol using pLUC3 constructs, which encompass nucleotides from −116 to +60, as well as longer constructs (pLUC4, pLUC5 and pLUC6) (Fig. 3A). In shorter constructs (pLUC1 and pLUC2), luciferase activities were not increased by kaempferol. Within the DNA sequence unique to the pLUC3 construct is an inverted CCAAT box (ATTGG; nt −50 and −46), a cAMP response element (CRE; nt −75 and −68), a putative Runx2 binding site (Runx2; nt −84 and −79), a FGF2 response element (FRE; nt −92 and −85), and a pituitary-specific transcription factor-1 motif (Pit-1; nt −111 and −105), which is the target of parathyroid hormone (Fig. 4). To determine more precisely the target sites in the BSP promoter through which the kaempferol effects were mediated, we prepared a series of 5′ deletion constructs between nt −280 and −43. The luciferase activities of promoter constructs (−60, −84, −108, −116, −280BSPLUC) were increased by kaempferol (5 μM, 12 h) (Fig. 3B). Next, we introduced 2 bp mutations in the putative response elements targeted by kaempferol within nt −116 to −43 of pLUC3 (MCCAAT, MCRE, and MFRE) (Fig. 5A). The basal transcriptional activities of MCCAAT, MCRE, and MFRE were lower than the basal

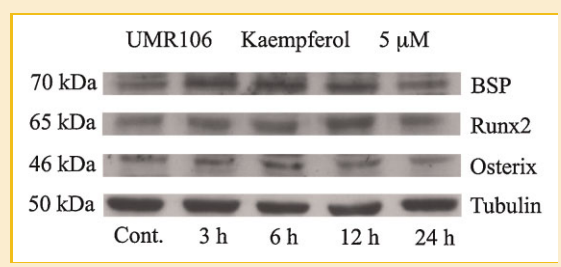


Fig. 2. Effects of kaempferol on BSP, Runx2 and Osterix protein levels in UMR106 cells. UMR106 cells were treated with or without 5 μM kaempferol for 24 h. The expressions of BSP, Runx2 and Osterix were analyzed by Western blotting using anti-BSP, Runx2 and Osterix antibodies. Anti-α-tubulin antibody was used for loading control.

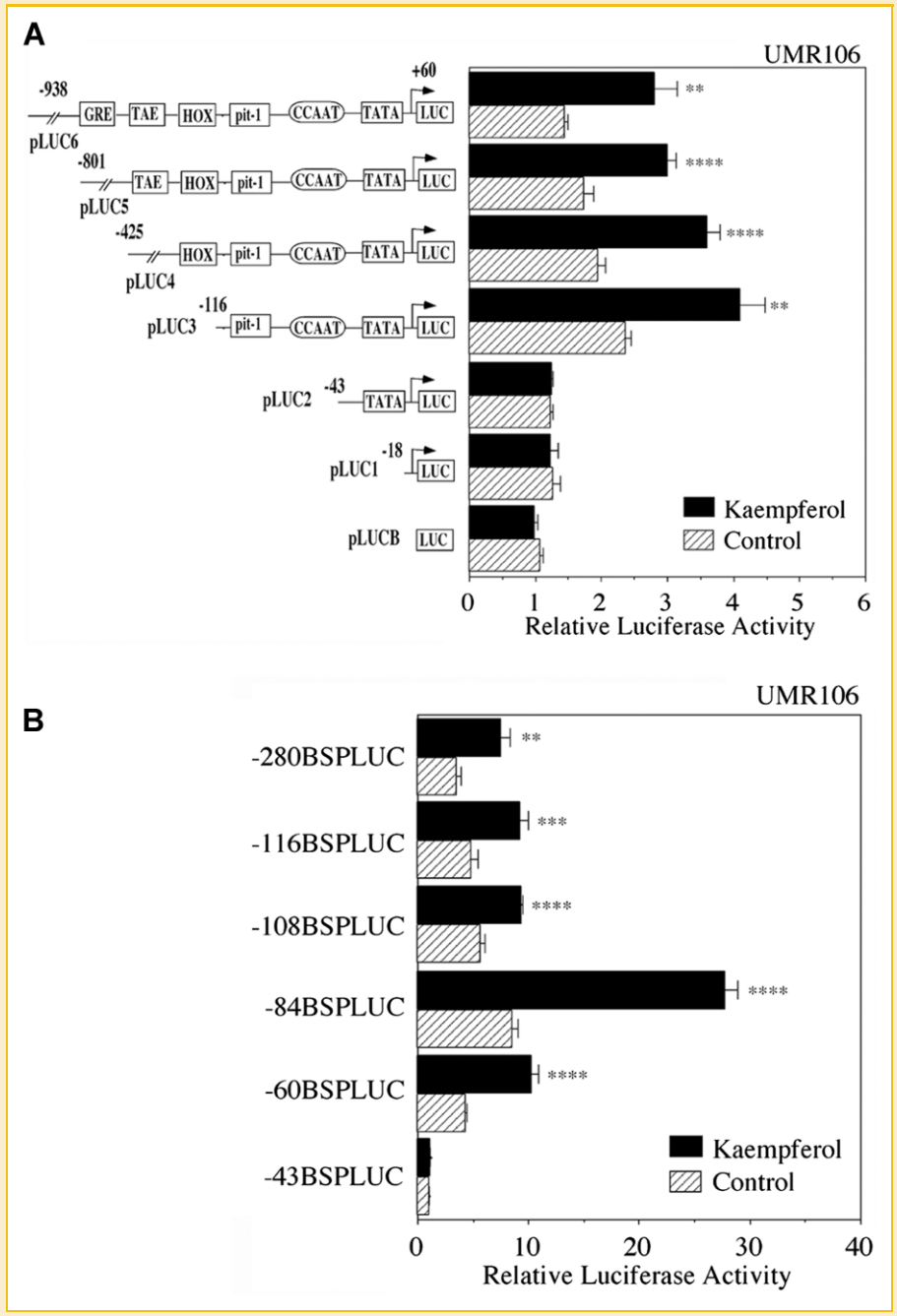


Fig. 3. Kaempferol up-regulates BSP promoter activity in UMR106 cells. A: Transient transfections of UMR106 cells, in the presence or absence of kaempferol (5 μM) for 12 h, were used to determine transcriptional activity of chimeric constructs that included various regions of the BSP promoter ligated to a luciferase reporter gene. B: Fine 5′ deletion mapping of the nt −280 to −43 elements in the BSP promoter. A series of rat BSP promoter 5′ deletion constructs was analyzed for relative promoter activity after transfection into UMR106 cells and examined for induction in the presence of kaempferol (5 μM). The results of transcriptional activity obtained from three separate transfections have been combined and the values expressed with standard errors. Significant differences from control: $^{**}P < 0.05$; $^{***}P < 0.02$; $^{****}P < 0.01$.

level of pLUC3. Transcriptional stimulation by kaempferol was partially abrogated in these three single mutation constructs (Fig. 5A). To confirm the functional elements, we also performed double and triple mutation analyses. When mutations were made in pairs of target elements (MCRE/MFRE, MCCAAT/MCRE, MCCAAT/MFRE), kaempferol-induced luciferase activities was partially abolished only with the paired mutation of the MCCAAT/MCRE construct. In triple mutation (MCCAAT/MCRE/MFRE), kaempferol-induced luciferase activities were totally abrogated (Fig. 5A). We then used six signaling pathway inhibitors; protein kinase C inhibitor (H7), cAMP-dependent protein kinase inhibitor (H89), tyrosine kinase inhibitor herbimycin A (HA), MAP kinase kinase (MEK) inhibitor (U0126), PI3-K inhibitor (LY294002) and antioxidant NAC, to determine the signaling pathway after kaempferol

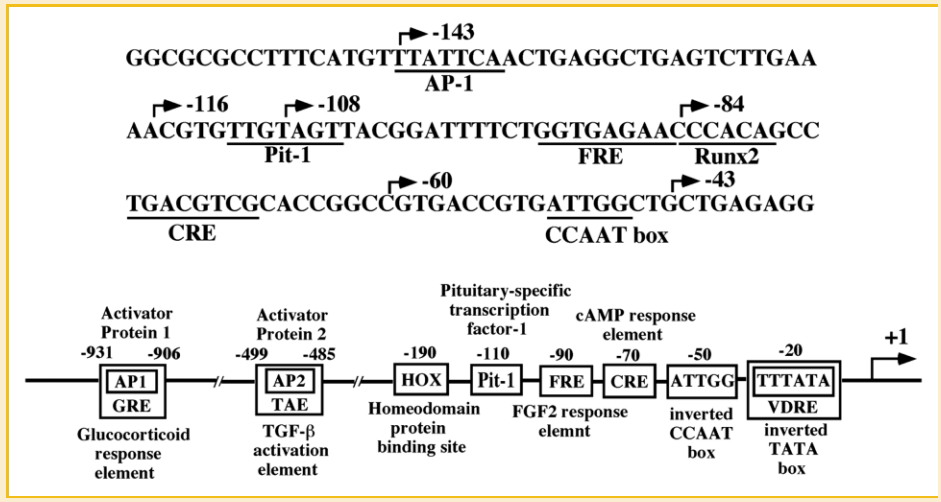


Fig. 4. Regulatory elements in the proximal rat BSP promoter. Upper panel: The nucleotide sequence of the rat BSP gene proximal promoter is shown from −159 to −35. Inverted CCAAT box, CRE, Runx2, FRE, Pit-1, and AP-1 are present. Lower panel: The position of inverted TATA and CCAAT boxes, a cAMP response element (CRE), FGF2 response element (FRE), pituitary-specific transcription factor-1 (Pit-1), and homeobox-binding site (HOX). The numbering of nucleotides is relative to the transcription start site (+1).

stimulation. Transcriptional stimulations by kaempferol were inhibited by H89, HA, U0126 and LY294002 (Fig. 5B).

GEL MOBILITY SHIFT ASSAY

To identify which nuclear proteins can bind to the promoter region of pLUC3, double-stranded oligonucleotides of CCAAT, CRE, FRE, and Pit-1 elements were end-labeled and incubated with equal amounts (3 μg) of nuclear proteins extracted from confluent UMR106 cells treated with or without kaempferol (5 μM) for 3, 6, and 12 h. When we used the inverted CCAAT as a probe, the DNA–NF-Y protein complex [Kim and Sodek, 1999; Shimizu and Ogata, 2002] did not change after kaempferol treatment (Fig. 6, lanes 1–4). With nuclear extracts from confluent control cultures of UMR106 cells, shifts of CRE-, FRE-, and Pit-1 protein complexes were evident (Fig. 6, lanes 5, 9, and 13). After kaempferol stimulation for 3 and 6 h, CRE- and FRE-protein complexes formations were increased (Fig. 6, lanes 6 and 7, 10 and 11), and decreased again at 12 h. On the other hand, Pit-1-protein complex formation did not change (Fig. 6, lanes 14–16). That the CCAAT-, CRE-, and FRE-protein complexes represent specific interactions were indicated by competition experiments in which 40-fold molar excesses of CCAAT, CRE, and FRE reduced the amount of complex formations (Fig. 7, lanes 3, 9, and 15). In contrast, CRE, FRE, and Pit-1 did not compete with CCAAT-protein complex formation (Fig. 7, lanes 4–6), FRE, CCAAT, and Pit-1 did not compete with CRE-protein complex formation (Fig. 7, lanes 10–12), CRE, CCAAT, and Pit-1 did not compete with FRE-protein complex formation (Fig. 7, lanes 16–18). Further competition experiments in which a 20- and 40-fold molar excess of CCAAT, and 40-fold molar excess of CRE and FRE double-stranded oligonucleotides reduced the amount of complexes formation (Fig. 8, lanes 3 and 4, Fig. 9, lane 3; Fig. 10, lane 3). In contrast, mutation CCAAT (Fig. 8, lanes 5 and 6), mutation CRE (Fig. 9, lane 4) and mutation FRE (Fig. 10, lane 4) did not compete with complex formation. Interestingly, FRE-protein complex formation was in competition with a by 40-fold molar excess of HOX (Fig. 10, lane 5), suggesting that FRE- and HOX-binding proteins were similar. To further characterize the nuclear proteins in complex formation we used several antibodies. For inverted CCAAT box, NFYA antibody partially blocked CCAAT-protein complex formation (Fig. 8, lanes 8–10). CRE-binding proteins were supershifted by CRE binding protein 1 (CREB) and phospho-CREB antibodies, and disrupted by c-Fos, c-Jun, JunD and Fra2 antibodies (Fig. 9, lanes 6–11). FRE-protein complexes formations were disrupted by Runx2, Dlx5 and Msx2 antibodies (Fig. 10, lanes 7–9).

STIMULATORY EFFECTS OF KAEMPFEROL ON NEWBORN RAT CALVARIA

In order to further clarify the direct effects of kaempferol on bone formation, 5 μl kaempferol (5 μM) was injected onto the parietal bone of newborn rats. Histologically, only osteoid matrix formation was seen in the control group 6 days after treatment, suggesting that calcification in the area of new bone formation was accelerated by kaempferol treatment compared to the control (Fig. 11). Immunohistochemical analyses were performed on the tissue sections of parietal bone which was harvested on day 6. Immunostaining with BSP antibody revealed staining of the bone matrix and osteoblastic cells on the superior surface of the calvariae. The increase in the thickness of mineralized bone matrix stained with BSP antibody was more pronounced in kaempferol group (Fig. 11g). Osteoblasts lining the bone surface, osteocytes, cells with fibroblastic morphology associated with the soft tissue stroma were stained with Runx2 and Osterix antibodies. These staining were accelerated by kaemoferol treatment (Fig. 11h,i). The bone area normalized to total area (0.225 mm × 0.3 mm) has been increased after kaempferol stimulation on 3, 6, 12, 15, and 19 days (Fig. 12). The number of osteoblasts per bone surface (within 0.05 mm × 0.1 mm) were significantly increased on 3, 6, 12, 15, and 19 days after kaempferol stimulation (Fig. 12). After 12, 15, and 19 days, osteoblasts remarkably showed angular-shaped cytoplasm with nuclear polarity, indicating an activated state of osteoblast differentiation. Suggesting new bone

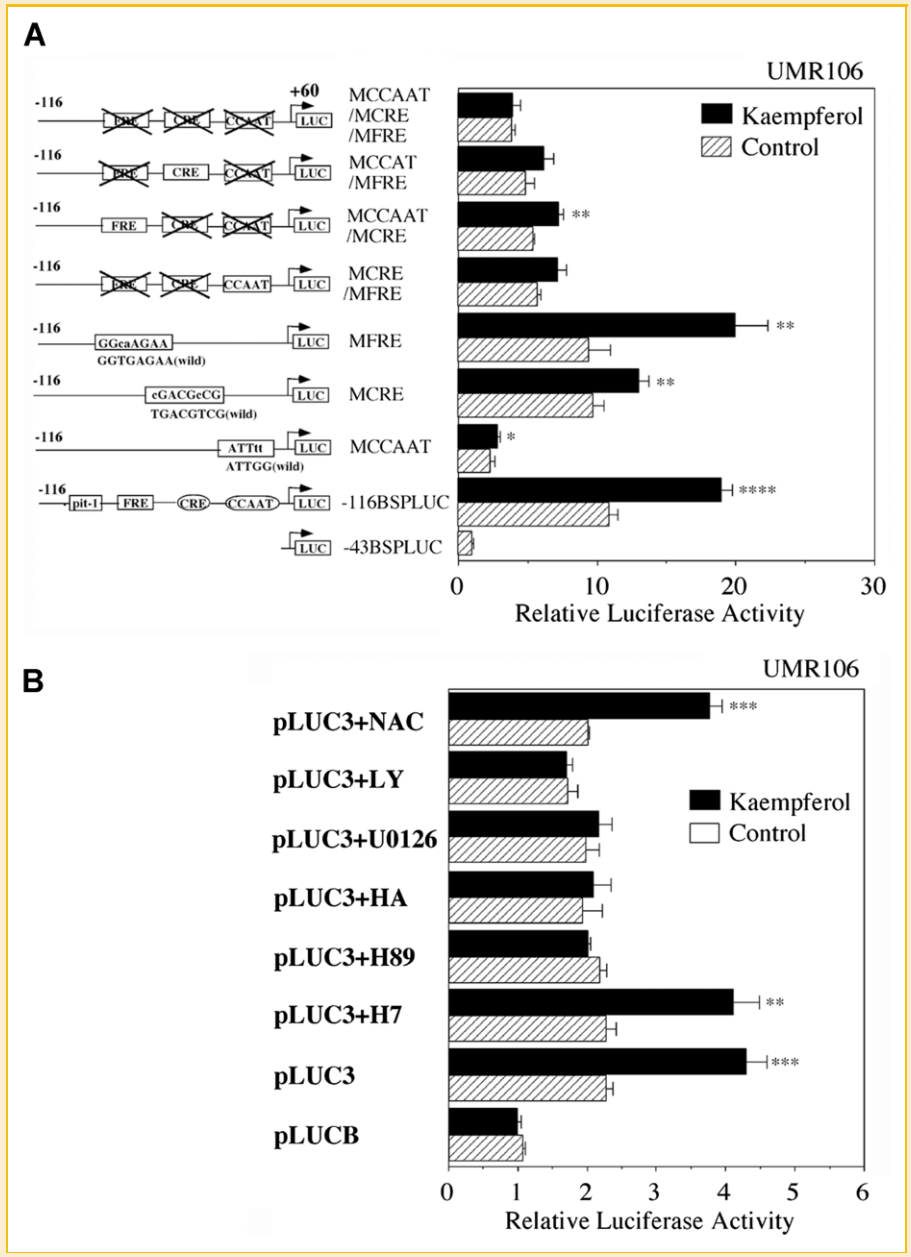


Fig. 5. A: Site mutation analysis of luciferase activities. Dinucleotide substitutions were made within the context of the homologous −116 to +60 (pLUC3) BSP promoter fragments. M-CCAAT (ATTtt), M-CRE (cGACGcCG), M-FRE (GGcaAGAA), double and triple-mutated constructs were analyzed for relative promoter activity after transfection into UMR106 cells and examined for induction after treatment with kaempferol (5 μM) for 12 h. B: Effect of kinase inhibitors on transcriptional activation by kaempferol. Transient transfection analysis of pLUC3 treated with kaempferol (5 μM) for 12 h in UMR106 cells is shown together with the effects of a PKC inhibitor (H7, 5 μM), PKA inhibitor (H89, 5 μM), tyrosine kinase inhibitor (herbimycin A; HA, 1 μM), MEK inhibitor (U0126, 5 μM), PI3-K inhibitor (LY294002, 10 μM), and antioxidant (*N*-acetylcysteine; NAC, 20 mM). The results of transcriptional activity obtained from three separate transfections with constructs were combined and the values expressed with standard errors. Significant differences from control: $^{*}P<0.1$; $^{**}P<0.05$; $^{***}P<0.02$, $^{****}P<0.01$.

formations were more active by kaempferol treatment (Figs. 11 and 12).

DISCUSSION

Flavonoids are widely found in vegetables and fruits [Gerritsen et al., 1995; Bai et al., 1998], and four of the major subclasses are flavones, flavonols, isoflavones and flavanones [Havsteen, 1983], which have multiple biological and pharmacological activities owing to their anti-oxidant [Suh et al., 2009], anti-inflammatory [Hämäläinen et al., 2007] and estrogenic effects [Dang and Lowik, 2005]. Quercetin exhibits anti-tumor properties, possibly through its ability to stimulate the immune system, induce apoptosis, alter the mitotic cycle, scavenge free radicals, modify gene expression, and block angiogenesis [Hayashi et al., 2000]. We previously reported that genistein increased BSP transcription through an inverted CCAAT

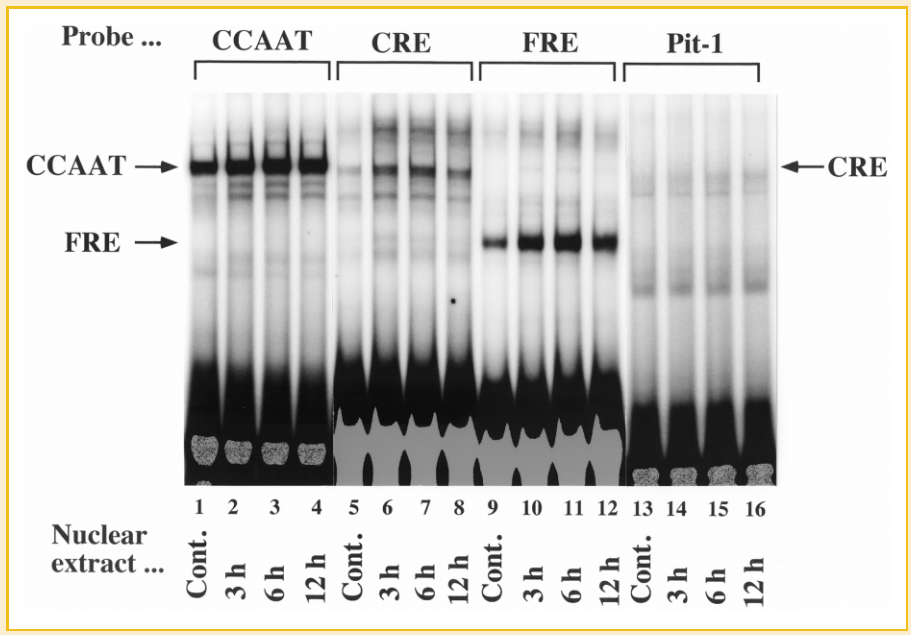


Fig. 6. Gel mobility shift analysis of inverted CCAAT, CRE, FRE, and Pit-1 oligonucleotides. Radiolabeled double-stranded CCAAT (−61 CCGTGACCGTGA**ATTGG**CTGCTGAGA −37; lanes 1–4), CRE (−84 CCCACAGCC**TGACGTCG**CACCGGCCG −59; lanes 5–8), FRE (−98 TTTTCT**GGTGAGAA**CCCACA −79; lanes 9–12), and Pit-1 (−115 CGTG**TTGTAGT**TACGGATTT −96; lanes 13–16) were incubated with nuclear protein extracts (3 μg) obtained from UMR106 cells stimulated without (lanes 1, 5, 9, and 13) or with kaempferol (5 μM) for 3 h (lanes 2, 6, 10, and 14), 6 h (lanes 3, 7, 11, and 15), and 12 h (lanes 4, 8, 12, and 16). DNA–protein complexes were separated on 5% polyacrylamide gel in low-ionic-strength Tris-borate buffer, dried under a vacuum and exposed to an imaging plate for quantitation using image analysis.

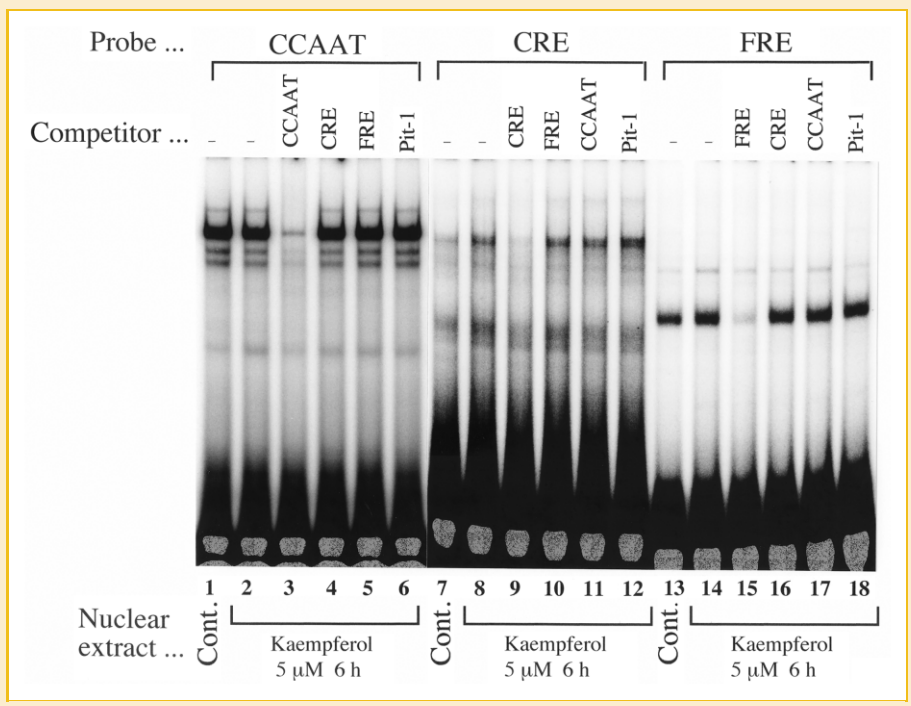


Fig. 7. Specific binding of nuclear proteins to inverted CCAAT, CRE and FRE. Radiolabeled double-stranded CCAAT (−61 CCGTGACCGTGA**ATTGG**CTGCTGAGA −37), CRE (−84 CCCACAAGCC**TGACGTCG**CACCGGCCG −59) and FRE (−98 TTTTCT**GGTGAGAA**CCCACA −79) oligonucleotides were incubated with nuclear protein extracts (3 μg) obtained from UMR106 cells treated without (lanes 1, 7, and 13) or with kaempferol (5 μM) for 6 h (lanes 2–6, 8–12, and 14–18). Competition reactions were performed using a 40-fold molar excess of unlabeled CCAAT (lanes 3, 11, and 17), CRE (lanes 4, 9, and 16), FRE (lanes 5, 10, and 15), and Pit-1 (lanes 6, 12, and 18) oligonucleotides.

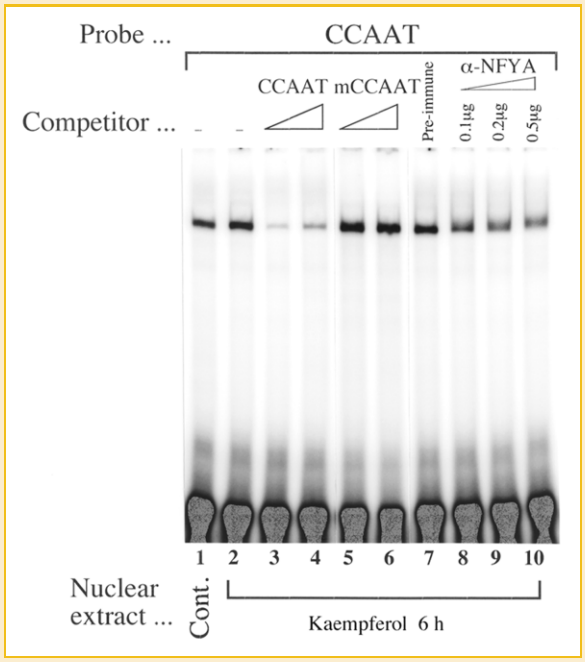
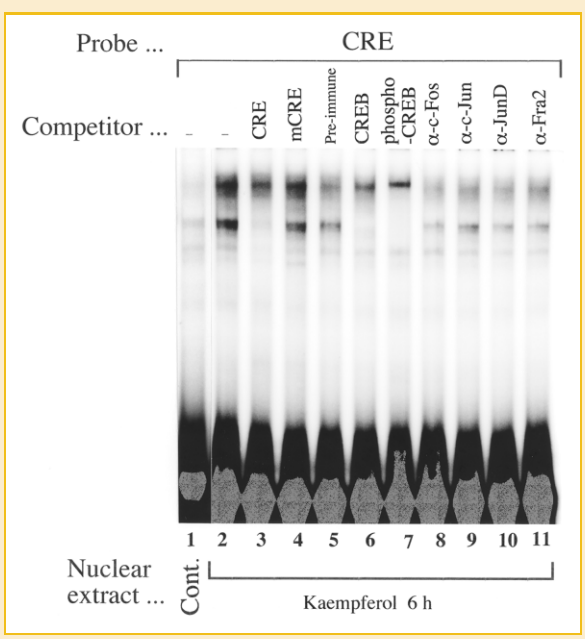


Fig. 8. Specific binding of nuclear protein to the inverted CCAAT. Radiolabeled double-stranded CCAAT (−61 CCGTGACCGTG**ATTGG**CTGCTGAGA −37) was incubated with nuclear protein extracts (3 μg) obtained from UMR106 cells treated without (lane 1) or with kaempferol (5 μM) for 6 h (lanes 2–10). Competition reactions were performed using a 20- and 40-fold molar excess of unlabeled CCAAT (lanes 3 and 4) and mutation CCAAT (CCGTGACCGT-G**ATTtt**CTGCTGAGA; (lanes 5 and 6). Supershift experiments were performed with 0.1, 0.2, and 0.5 μg anti-NF-YA monoclonal antibody, which recognizes the A subunit of NF-Y (lanes 8–10).



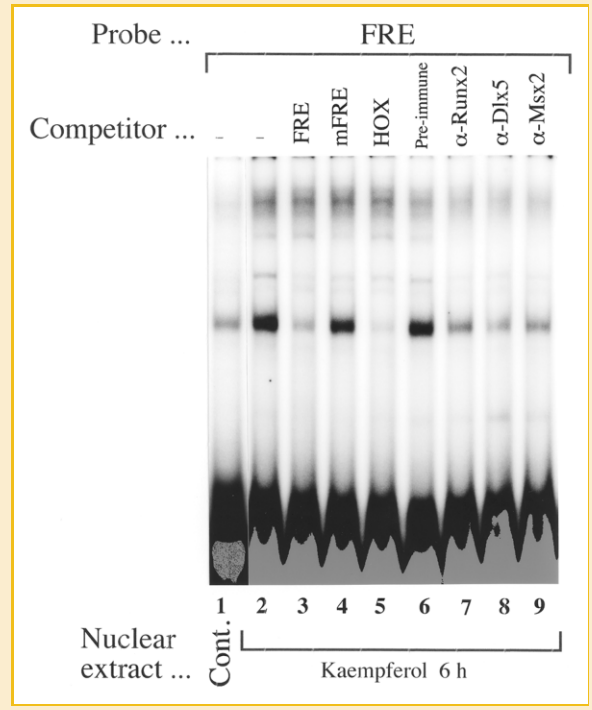


Fig. 10. Specific binding of nuclear protein to the FRE. Radiolabeled double-stranded FRE (−98 TTTTCT**GGTGAGAA**CCCACA −79) was incubated with nuclear protein extracts (3 μg) obtained from UMR106 cells treated without (lane 1) or with kaempferol (5 μM) for 6 h (lane 2). Competition reactions were performed using a unlabeled FRE (lane 3), mutation FRE (TTTTCT**GGcaA-GAA**CCCACA; lane 4) and HOX (TCCTCAGCCT**TCAATTAAA**TCCCACA; line 5). Supershift experiments were performed with antibodies against Runx2 (lane 7), Dlx5 (lane 8) and Msx2 (lane 9) added separately to each gel shift reaction.

box [Shimizu and Ogata, 2002], and quercetin up-regulated BSP expression via inverted CCAAT and FRE elements [Kim et al., 2007]. These results suggested that the effects of genistein (isoflavone) and quercetin (flavonol) were different. Quercetin inhibited RANK ligand (RANKL)-induced NFκB and activator protein 1 (AP1) activation, and decreased osteoclastic differentiation [Wattel et al., 2004]. Kaempferol belongs to the flavonol family, and could have more potent antiosteoclastic activities than quercetin [Pang et al., 2006]. In addition, kaempferol decreased osteoclastic bone resorption by directly targeting the osteoclasts by a mechanism at least partially involving the ER [Rassi et al., 2005; Tang et al., 2008]. Kaempferol increased ALP activity, and the effect was reduced by inhibitor of extracellular regulated kinase (ERK) and by an antagonist of ER

Fig. 9. Specific binding of nuclear protein to the CRE. Radiolabeled double-stranded CRE (−84 CCCACAGCC**TGACGTCG**CACCGGCCG −59) was incubated with nuclear protein extracts (3 μg) obtained from UMR106 cells treated without (lane 1) or with kaempferol (5 μM) for 6 h (lane 2). Competition reactions were performed using a unlabeled CRE (lane 3) and mutation CRE (CCCACAGCC**cGACGcCG**CACCGGCCG; lane 4). Supershift experiments were performed using antibodies against CREB (lane 6), phospho-CREB (lane 7), c-Fos (lane 8), c-Jun (lane 9), JunD (lane 10), and Fra2 (lane 11) added separately to each gel shift reaction.

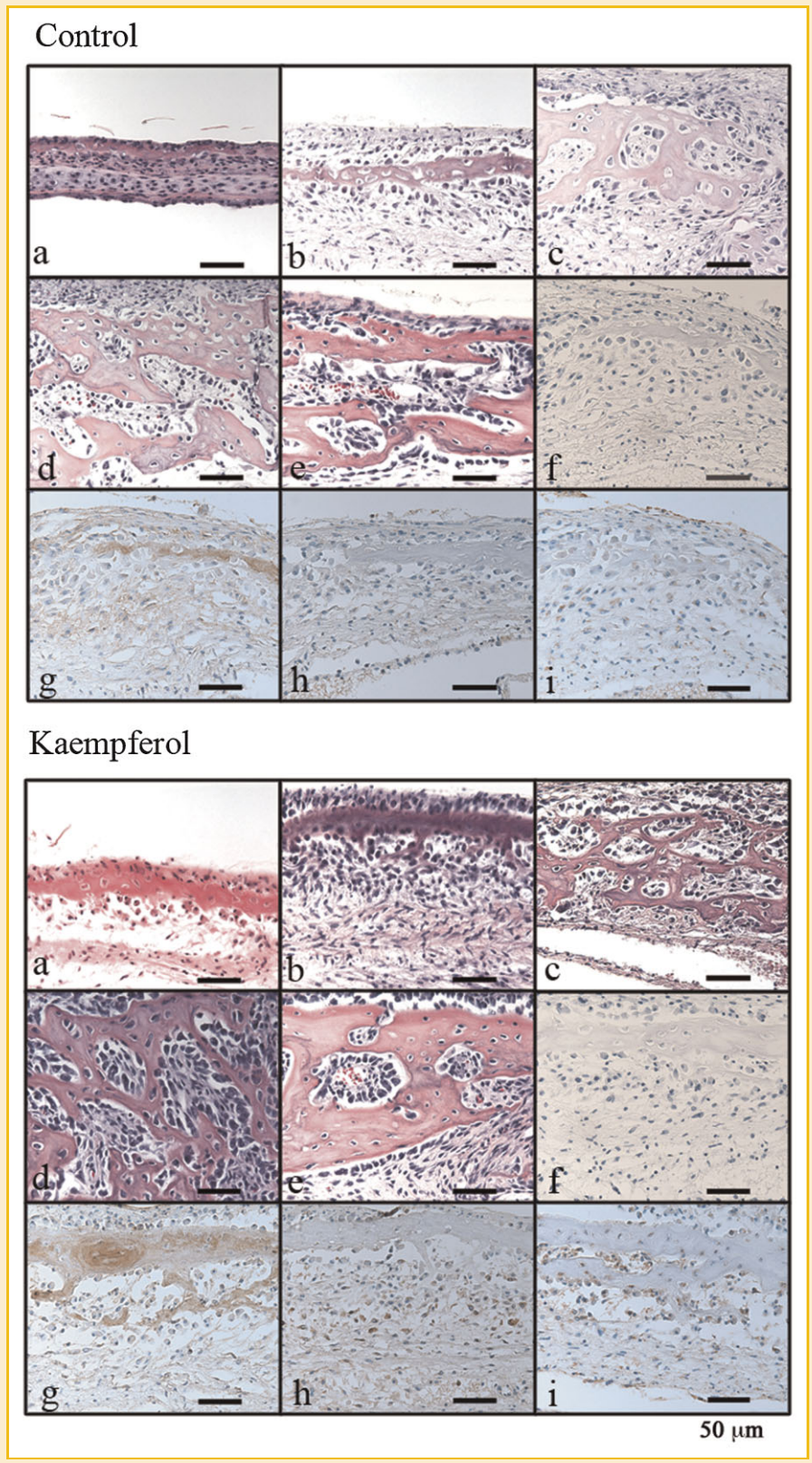


Fig. 11. Histological and immunohistochemical analyses of newborn rats treated with kaempferol. Five microliter per day kaempferol (5 μM) or control (DMSO) alone were injected for up to 12 days, and parietal bone was harvested on days 3 (a), 6 (b), 12 (c), 15 (d), and 19 (e). Parietal bones were dissected and fixed in 10% neutral-buffered formalin following decalcification with 5% EDTA solution. The sections were stained with hematoxylin and eosin. The central point of the sections was determined with a microscope as the point between the sagittal suture and the attachment site of the temporal muscle. Immunostaining of BSP (g), Runx2 (h) and Osterix (i) in parietal bone which was harvested on day 6. Sections immunostained without primary antibody as negative controls (f). All sections were counterstained with Mayer's hematoxylin. Bars = 50 μm.

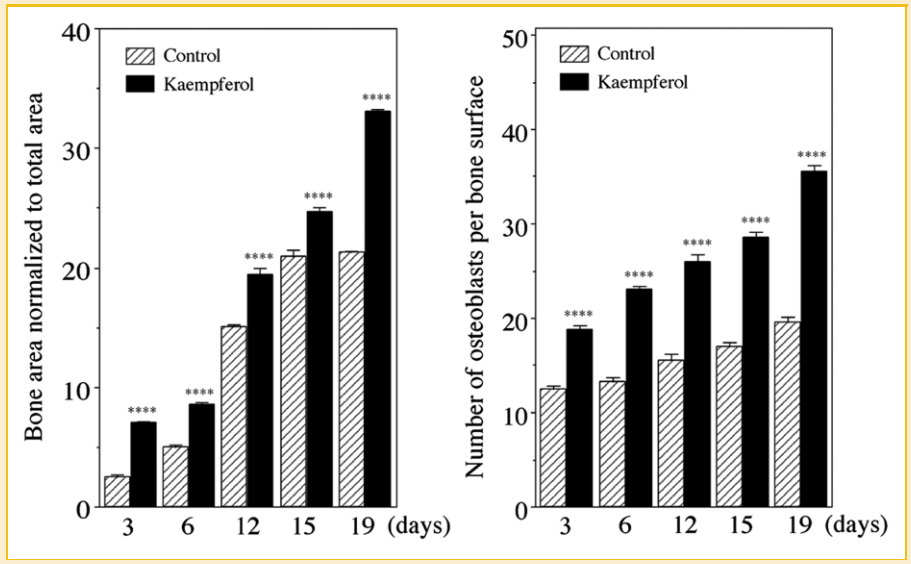


Fig. 12. The effect of kaempferol was evaluated by measuring the quantity of new bone formation using Image-Pro® Plus and the number of osteoblasts per bone surface (within 0.05 mm × 0.1 mm) at the injection point in parietal bone. New bone was defined as the area between periosteal and endosteal surfaces containing both calcified bone and osteoid. The quantity of new bone was calculated as the ratio of bone volume in the visual field over the whole area of the visual field (0.225 mm × 0.3 mm).

[Prouillet et al., 2004]. Sex steroid hormones have major beneficial effects on the development and maintenance of the skeleton [Syed and Khosla, 2005]. 17β-estradiol does not upregulate BSP transcription (data not shown) and there is no putative estrogen response element in the proximal promoter of the rat BSP gene (Fig. 4), suggesting that the regulation of BSP gene expression by kaempferol is independent of its estrogenic activity.

In this study we have shown that kaempferol increased BSP transcription in osteoblast-like UMR106 cells through an inverted CCAAT box, CRE, and FRE in the proximal promoter of the BSP gene. Kaempferol increased BSP mRNA levels at 12 h in UMR106 cells (Fig. 1A,B). Additionally, kaempferol up-regulated the mRNA expression of Runx2 and Osterix at 6 and 12 h (Fig. 1C,D). Runx2 and Osterix play an essential role in osteoblast differentiation. Runx2 and Osterix deficient mice display absence of bone due to arrested osteoblast differentiation [Komori et al., 1997; Nakashima et al., 2002]. Runx2 might involve the FRE binding transcription factor since a putative Runx2 binding site (CCCACA) is juxtaposed to FRE (GGTGAGAA) (Fig. 4) and FRE-protein complex formations were disrupted by Runx2, Dlx5 and Msx2 antibodies. Dlx5 are activated after BMP2 addition to the mouse 2T3 osteoblast and primary fetal rat calvarial osteoblasts [Harris et al., 2003]. BMP2-induced Runx2 expression is mediated by Dlx5 [Lee et al., 2003]. Further, Dlx5 reverses Msx2 inhibition of osteocalcin promoter activation by FGF2/forskolin [Newberry et al., 1998]. Msx2 suppresses BSP transcription and the effect can be de-repressed by increasing Dlx5 levels [Barnes et al., 2003].

From transient transfection assays using a series of 5′ deletion constructs between nt −280 and −43, the luciferase activities of −60 to −280BSPLUC were increased by kaempferol (5 μM, 12 h), and had a maximal effect on -84BSPLUC (Fig. 3B). CRE and Runx2 binding sites are specific sequences in −84BSPLUC, suggesting that CRE and Runx2 binding transcription factors are crucial for kaempferol-induced BSP transcription. CRE-protein complexes were supershifted by CREB and phospho-CREB antibodies and disrupted by c-Fos, c-Jun, JunD and Fra2 antibodies (Fig. 9). CREB and AP1 transcription factors JunD and Fra2 regulate BSP gene expression in breast cancer cells [Detry et al., 2008]. The results indicate that CREB, phospho-CREB, c-Fos, c-Jun, JunD and Fra2 might interact with CRE.

Transcription of the BSP gene is stimulated by v-Src through an inverted CCAAT box [Kim and Sodek, 1999], which is bound with high affinity by an ubiquitous trimeric complex, NF-Y transcription factor. This complex is composed of three conserved subunits, NF-YA, NF-YB, and NF-YC [Li et al., 1992; Caretti et al., 1999], all of which are required for DNA binding. It is essential for expression of the class II genes of the major histocompatibility complex (MHC) and is likely involved in the regulation of albumin, α1(I) and α2(I)collagen genes [Maity et al., 1992; Caretti et al., 1999]. NF-Y is also required for cyclin B1 transcription and that the switch-off of cyclin B1 expression in terminally differentiated skeletal muscle cells depends upon the loss of a functional NF-Y complex [Manni et al., 2001]. That the promoters of several cell cycle regulatory genes such as cyclin A, cyclin B1, cyclin B2, cdk1 and cdc25C contain CCAAT boxes suggests NF-Y is a key regulator of cell cycle [Manni et al., 2001]. However, the mechanism of NF-Y mediated gene transcriptional regulation is not well understood. In gel shift assays, we could not see any differences in the DNA–NF-Y protein complex formed with the inverted CCAAT sequence obtained from nuclear extracts of cells treated or not with kaempferol (Fig. 8). Therefore, it is likely that kaempferol treatment results in modifications of NF-Y that influence its transactivation properties, but these changes do not affect the binding of the DNA–NF-Y protein complex to the CCAAT element.

In transient transfection assays, H89, HA, U0126, and LY294002 abrogated the transcriptional activity induced by kaempferol (Fig. 5B). H89 could suppress the increased binding of CRE-protein complexes, and HA and U0126 inhibited the increased binding of FRE-protein complexes. LY294002 is an inhibitor of PI3-K. PI3-K signaling is one of critical pathways for the differentiation of skeletal component cells, including chondrocytes, osteoblasts, myoblasts and adypocytes [Kaliman et al., 1996; Sakaue et al., 1998; Ghosh et al. 2002], and bone development is delayed in mice lacking Akt1 and Akt2 [Peng et al., 2003]. Further, Runx2-induced osteoblast differentiation is inhibited by the PI3-K inhibitor LY294002 and a dominant-negative Akt [Fujita et al., 2004], suggesting that Runx2 and PI3-K may be crucial signaling molecules for kaempferol-induced BSP transcription.

Twelve daily injections of 5 μM kaempferol directly onto the periosteum of parietal bones of newborn rats increased the number of osteoblasts and new bone formation. And also enhanced BSP, Runx2 and Osterix protein expressions in parietal bone and osteoblasts lining the bone surface after kaempferol injection for 6 days. Therefore kaempferol induces osteoblast activities at early stage of bone formation.

In conclusion, we have shown inverted CCAAT, CRE, and FRE elements in the rat BSP proximal promoter through which the stimulatory effects of kaempferol on BSP gene transcription are mediated. Kaempferol stimulated new bone formation and increased BSP gene expression. Moreover, CREB, phospho-CREB, c-Fos, c-Jun, JunD, Fra2, Runx2, Dlx5, and Msx2 transcription factors appear to be key regulators of the kaempferol effects on BSP transcription and bone formation.

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