

Stimulation of Bone Formation in Cortical Bone of Mice Treated with a Receptor Activator of Nuclear Factor- κ B Ligand (RANKL)-binding Peptide That Possesses Osteoclastogenesis Inhibitory Activity

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Background: A RANKL-binding peptide WP9QY (W9) is known to inhibit osteoclastogenesis.

Results: W9 showed an anabolic effect on cortical bone in mice. W9 bound RANKL and differentiated osteoblasts with production of autocrine factors like BMP-4.

Conclusion: Signaling through RANKL is involved in part in the W9-induced osteoblast differentiation.

Significance: The RANKL pathway could be a novel mechanism in osteoblast differentiation.

To date, parathyroid hormone is the only clinically available bone anabolic drug. The major difficulty in the development of such drugs is the lack of clarification of the mechanisms regulating osteoblast differentiation and bone formation. Here, we report a peptide (W9) known to abrogate osteoclast differentiation *in vivo* via blocking receptor activator of nuclear factor- κ B ligand (RANKL)-RANK signaling that we surprisingly found exhibits a bone anabolic effect *in vivo*. Subcutaneous administration of W9 three times/day for 5 days significantly augmented bone mineral density in mouse cortical bone. Histomorphometric analysis showed a decrease in osteoclastogenesis in the distal femoral metaphysis and a significant increase in bone formation in the femoral diaphysis. Our findings suggest that W9 exerts bone anabolic activity. To clarify the mechanisms involved in this activity, we investigated the effects of W9 on osteoblast differentiation/mineralization in MC3T3-E1 (E1) cells. W9 markedly increased alkaline phosphatase (a marker enzyme of osteoblasts) activity and mineralization as shown by alizarin red staining. Gene expression of several osteogenesis-related factors was increased in W9-treated E1 cells. Addition of W9 activated p38 MAPK and Smad1/5/8 in E1 cells, and W9 showed osteogenesis stimulatory activity synergistically with BMP-2 *in vitro* and ectopic bone formation. Knockdown of RANKL expression in E1 cells reduced the effect of W9. Furthermore, W9 showed a weak effect on RANKL-deficient osteoblasts in alkaline phosphatase assay. Taken together, our findings suggest that this peptide may be useful for the treatment of bone diseases, and W9 achieves its bone anabolic activity through

RANKL on osteoblasts accompanied by production of several autocrine factors.

The morphogenesis and remodeling of bone depend on the integrated activity of osteoblasts that form bone and osteoclasts that resorb bone. Receptor activator of nuclear factor- κ B ligand (RANKL)³ is a member of the tumor necrosis factor (TNF) superfamily and is produced on osteoblasts and osteocytes (1–4). It binds the receptor RANK, which is produced on osteoclasts and their progenitors (1, 2, 5, 6). The interaction of RANK with RANKL is required for osteoclast formation, differentiation, activation, and survival. Osteoprotegerin (OPG) is a natural decoy receptor for RANKL (1, 2, 7, 8). Bone resorption is mediated by RANKL-RANK signaling, and excessive osteoclastic bone resorption plays a central role in the pathogenesis of age-related bone loss and microstructural deterioration, leading to fragility fractures (9–11).

Inhibition of the RANKL/RANK signal in bone can increase bone mass and is useful for treatment of osteoporosis. RANKL-deficient mice and RANK-deficient mice exhibited severe osteopetrosis with lack of osteoclasts (12, 13). OPG and soluble RANK have been developed as pharmaceutical candidates, and anti-human RANKL-neutralizing antibody (denosumab) has

³ The abbreviations used are: RANKL, receptor activator of nuclear factor- κ B ligand; RANK, receptor activator of nuclear factor- κ B; ALP, alkaline phosphatase; OPG, osteoprotegerin; TRAP, tartrate-resistant acid phosphatase; BGP, β -glycerophosphate; Dex, dexamethasone; BMD, bone mineral density; BMP, bone morphogenetic protein; PTH, parathyroid hormone; sRANKL, soluble human RANKL; Oc, osteoclast; Ob, osteoblast; BS, bone surface; hMSC, human mesenchymal stem cell; ANOVA, analysis of variance; pNPP, *p*-nitrophenyl phosphate; Wnt, Wingless-type; IGF, insulin-like growth factor, M-CSF, macrophage colony-stimulating factor.

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been clinically used for osteoporosis and cancer-related bone disorders (14–16).

WP9QY (W9) is a peptide designed to be structurally similar to one of the cysteine-rich domains in TNF receptor type I, and it is known to bind and block the activity of TNF- α (17). The peptide also binds RANKL and inhibits RANKL-induced osteoclastogenesis *in vitro* (18). Furthermore, this peptide increases bone mineral density (BMD) via inhibition of osteoclastogenesis in the bone-loss model of wild-type and TNF receptor-deficient mice that underwent ovariectomy or low calcium diet (18).

Bone formation is regulated by cytokines and factors inducing differentiation and mineralization of osteoblasts such as bone morphogenetic proteins (BMPs) (19), parathyroid hormone (PTH) (20), statins (21), Wingless-type (Wnt) (22), growth hormone (23), and insulin-like growth factor (IGF)-1 (24). These factors are candidates for pharmaceuticals with bone anabolic activity, but PTH is the only one clinically available.

This study explored the effects of the W9 peptide that has osteoclastogenesis inhibitory activity on bone. We surprisingly found that this peptide stimulated bone formation *in vivo* probably through a novel mechanism of osteoblast differentiation and activation.

EXPERIMENTAL PROCEDURES

Reagents—Recombinant soluble human RANKL (sRANKL) was prepared by Oriental Yeast Co., Ltd., as described previously (25). Recombinant human TNF- α , recombinant human BMP-2, anti-BMP-2/4-neutralizing polyclonal antibody, recombinant human soluble BMP receptor type 1A (sBMPR-1A), recombinant human TNF- α , recombinant human Wnt5a, recombinant human Wnt10b, and recombinant mouse Dickkopf-related protein (Dkk-1) were purchased from R&D Systems (Minneapolis, MN). SB203580, p38 inhibitor, was purchased from Merck. Recombinant human BMP-2 for ectopic bone formation was purchased from Osteogenetics GmbH (Wuerzburg, Germany). β -Glycerophosphate (BGP), ascorbic acid phosphate, actinomycin-D, dexamethasone (Dex), and calcein were purchased from Sigma. Recombinant human macrophage colony-stimulating factor (M-CSF, Leukoprol) was obtained from Kyowa Hakko (Tokyo, Japan). *p*-Nitrophenyl phosphate and other reagents were purchased from Nacal Tesque (Tokyo, Japan).

Peptides—Peptides (WP9QY (W9); YCWSQYLCY, Y6N (Y6); YCWSQNLCY, and WP5JY (W5), YCASENHICY) were synthesized by the Fluorenylmethoxycarbonyl method and formed a disulfide link. These peptide solutions were prepared by Oriental Yeast Co., Ltd.

Bone Analysis in Normal Mice Treated with W9—Five-week-old female C57BL/6N mice were purchased from Charles River, Inc., and acclimated for 1 week under standard laboratory conditions at $24 \pm 2^\circ\text{C}$ and 40–70% humidity. W9 and vehicle (PBS) were administered subcutaneously three times/day to 6-week-old mice ($n = 8$) for 5 days. Calcein is a fluorescent dye, and it was injected subcutaneously for labeling of bones in mice on days 1 and 4. After 12 h from the last administration, both femurs were extirpated from the examined mice and fixed with

70% ethanol. BMD of fixed right femurs was determined by peripheral quantitative computed tomography (XCT Research SA+, Stratec Medizintechnik, Pforzheim, Germany) using the voxel size of $0.08 \times 0.08 \times 0.46$ mm. Image analysis was carried out using integrated XCT 2000 software version 6.00f. Trabecular BMD was defined under 395 mg/cm^3 at 1.0 mm from growth plate, and cortical BMD was defined over 690 mg/cm^3 at 5.0 mm from the end of epiphysis.

Histomorphometry—Fixed and undecalcified femurs were embedded in glycol methacrylate or methylmethacrylate, and $3\text{-}\mu\text{m}$ sections in the distal femoral metaphysis and femoral diaphysis region were stained with toluidine blue O. Histomorphometry was performed with an image analyzing system (Osteoplan II, Carl Zeiss, Thornwood, NY) linked to a light microscope. Histomorphometric measurement was made at $\times 400$ magnification at 1.05–1.2 mm in the secondary spongiosa area from the growth plate and at 4.0–5.0 mm from the end of metaphysis. Oc surface/BS (%), Ob surface/BS (%), mineral apposition rate ($\mu\text{m/day}$), and bone formation rate ($\text{mm}^3/\text{mm}^2/\text{year}$) were calculated and expressed according to standard formulas and nomenclatures (26).

ALP Assay—Mouse preosteoblastic MC3T3-E1 (E1) cells obtained from RIKEN Cell Bank (Tsukuba, Japan) were seeded at 2×10^4 cells/well in a 96-well plate. The cells were cultured with 50–200 μM W9 peptide, 200 μM negative control peptide, or 10 ng/ml BMP-2 for 5 days and fixed by acetone/ethanol (1:1). ALP solution, including 20 nM *p*-nitrophenyl phosphate (pNPP) and 80 mM bicarbonate, was added to each fixed well and incubated for 1–2 h at 37°C . ALP activity in the well was measured at 405 nm with a microplate reader.

Human mesenchymal stem cells (hMSCs) were purchased from Lonza (Basel, Switzerland). The cells were seeded at 2×10^3 cells/well in a 96-well plate and cultured in the presence of 100 nM Dex, 50 $\mu\text{g/ml}$ ascorbic acid phosphate, and 10 mM BGP with 100–200 μM W9 or 200 μM W5 for 4 days. They were fixed, and the ALP activity was measured as described above. Primary calvarial cells, including osteoblasts, were prepared from newborn RANKL-deficient mice and wild-type littermates, inoculated at 1×10^4 cells/well, and cultured with 100 μM W9 or 40 ng/ml BMP-2 for 5 days. They were fixed, and the ALP activity was measured as described above.

Alizarin Red Staining—E1 cells were seeded at 2×10^4 cells/well in a 48-well plate and cultured for 3 days. Culture medium was changed to differential medium containing 100 nM Dex, 50 $\mu\text{g/ml}$ ascorbic acid phosphate, and 10 mM BGP with 200 μM W9 or W5 and replaced with new differential medium every 3 or 4 days until day 21. hMSCs were seeded at 4×10^3 cells/well in a 48-well plate and cultured in the presence of 100 nM Dex, 50 $\mu\text{g/ml}$ ascorbic acid phosphate, and 10 mM BGP with 200 μM W9; and the culture medium was replaced with new medium every 3 or 4 days until day 21. The cultured cells were fixed by 10% neutral buffered formalin and stained with 1% alizarin red S solution for 10 min at room temperature. To evaluate the synergistic effect of W9 and BMP-2, E1 cells were cultured in another osteoblastic differentiation medium (including 50 $\mu\text{g/ml}$ ascorbic acid phosphate, 5 mM BGP, and 10 nM Dex), and the culture medium was replaced with new medium every 3 days until day 10.

W9 Peptide Has Anabolic Effect on Osteoblasts through RANKL

TRAP Assay—RAW264 cells (mouse macrophage cell line) obtained from RIKEN Cell Bank were seeded at 2×10^3 cells/well in a 96-well plate. The cells were cultured in the presence of 5 nM sRANKL with 25–200 μ M W9 or 200 μ M W5 for 4 days and fixed by acetone/ethanol (1:1). TRAP is a marker enzyme of osteoclasts. TRAP activity was measured as described previously (25). For TRAP staining, RAW264 cells were seeded at 2×10^3 cells/well in a 48-well plate and cultured with 10 nM sRANKL in the presence or absence of W9 for 5 days. The cells were fixed with neutral buffered formalin and acetone/ethanol (1:1) and stained for TRAP.

Formation of Osteoclasts in Bone Marrow Cell Cultures—Seven-week-old male mice of the ddY strain were obtained from Japan SLC (Shizuoka, Japan). Mouse bone marrow cells (2.5×10^5 cells/well) were prepared as osteoclast precursors. Bone marrow cells were cultured in α -minimal essential medium containing 10% FBS in 48-well plates in the presence of sRANKL (100 ng/ml) and M-CSF (50 ng/ml) with or without 100 μ M W9 peptide. After 7 days, cells were fixed and stained for TRAP and ALP as described (27).

Gene Chip Assay—E1 cells (1×10^6 cells) were incubated with or without 200 μ M W9 for 12 h. After stimulation, total RNA of the cells was isolated using TRIzol (Invitrogen). Gene chip analysis was carried out using Gene Chip Mouse Genome 430 2.0 (Kurabo, Japan).

Western Blot Analysis—E1 cells were stimulated with 100 μ M W9 for 10 min to 18 h in α -minimal essential medium, including 0.1% FBS. After stimulation, whole cell lysates were prepared with modified RIPA buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 1 mM EDTA supplemented with 1 mM NaF, 1 mM Na_3VO_4 , 10 μ g/ml leupeptin, 10 μ g/ml aprotinin, 10 μ g/ml pepstatin, and 1 mM phenylmethylsulfonyl fluoride). Proteins in the lysates (10 μ g) were analyzed by SDS-PAGE and immunoblotting with the following antibodies according to the manufacturer's instructions. Antibodies for phospho-p38, phospho-Smad1/5/8, Smad1, and β -catenin were purchased from Cell Signaling (Danvers, MA). Antibody for p38 was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and antibody for β -actin was from Imgenex (San Diego). Immune complex were detected by ECL-Plus (GE Healthcare).

Ectopic Bone Formation—Five-week-old male C57BL/6 mice were purchased from NIHON CLEAR Inc. (Tokyo, Japan) and acclimated for 1 week under standard laboratory conditions at $24 \pm 2^\circ\text{C}$ and 40–70% humidity. Before surgery, mice were anesthetized by subcutaneous injection of medetomidine hydrochloride (Domitor, Meijiseika, Tokyo, Japan) and ketamine hydrochloride (Sankyo, Tokyo, Japan). Helistat absorbable collagen sponge (Integra Life Science Corp., Plainsboro, NJ) was used as a carrier of BMP-2. Biopsy punch (Kai Industries Co., Ltd., Gifu, Japan) was used to make the collagen disc (3.5 mm diameter, 3 mm thickness). These discs were then freeze-dried with 2 μ g of BMP-2, and they were surgically implanted into dorsal muscle pouches. After a week, 5 and 10 mg/kg W9 peptides were administered to the implanted mice three times/day for 5 days subcutaneously. All the mice were sacrificed on day 12 after implantation. The formed ectopic bones were collected and fixed in PBS-buffered glutaraldehyde (0.25%), formalin (4%) fixative, pH 7.4, for 3 days at 4°C and

washed with PBS. Soft x-ray photographs of ectopic bones were taken by using a cabinet x-ray apparatus (type SRO-M50; Sofron, Tokyo, Japan). The bone mineral content of ectopic bones was measured by dual-energy x-ray absorptiometry (DCS-600R, Aloka, Tokyo, Japan). Three-dimensional reconstruction images of ectopic bones were obtained by micro-focal computed tomography (Scan Xmate-E090; Comscan, Kanagawa, Japan).

Stealth RNAi—Stealth RNAs targeting the 25-nucleotide sequence of RANKL and TNF- α and the control were obtained from Invitrogen. E1 cells were transfected with 10 nM stealth RNAs using LipofectamineTM (Invitrogen). After 48 h of culture, total RNA of the cells was isolated using TRIzol. To carry out RT-PCR, cDNAs were synthesized with 500 ng of the RNA and random hexamer using ThermoScriptTM RT (Invitrogen). PCRs were performed using the following primer sets for semi-quantitative analysis of RANKL, TNF- α , and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expressions: RANKL forward, GGCAAGCCTGAGGCCAGCCATTT, and reverse, GTCTCAGTCTATGTCCTGAACTTT; TNF- α forward, AGCCACGTAGCAAACCACCAA, and reverse, ACACCCATTCCCTTCACAGAGCAAT; and GAPDH forward, CACCATGGAGAAGGCCGGGG, and reverse, GACGGACACATTGGGGGTAG. The PCR conditions were as follows: 94°C (20 s), 65°C (20 s), and 72°C (40 s) for RANKL (35 cycles); 94°C (20 s), 60°C (20 s), and 72°C (40 s) for TNF- α (35 cycles); and 95°C (10 s), 60°C (15 s), and 68°C (1 min) for GAPDH (20 cycles). The transfected cells with stealth RNA also were seeded at 5×10^3 cells/well in a 96-well plate. After 48 h of culture, the cells were further cultured with 100 μ M W9 and 10 ng/ml BMP-2 for 5 days, and ALP activity of the well was measured as described above.

TNF- α Apoptosis Assay—L929 cells obtained from RIKEN Cell Bank were seeded at 1×10^4 cells/well in a 96-well plate. The cells were cultured with 1 mg/ml (microgram/ml) actinomycin D in the presence or absence of 25 pg/ml recombinant human TNF- α and 25 and 100 μ M W9 and W5 for 24 h. The viable cells were detected with the WST-1 assay kit (Roche Applied Science).

Statistical Analysis—The results were expressed as means \pm S.D. for all data. Significant difference was determined using ANOVA with Dunnett's test or Student's *t* test.

RESULTS

Identification of Bone Anabolic Effect of W9—To characterize the effects of W9 on bone in detail, we administered 10 mg/kg W9 to normal mice three times/day for 5 days. In W9-treated mice, soft x-ray images unexpectedly showed an increase in the density of cortical bone (Fig. 1A). Peripheral quantitative computed tomography showed a significant increase in BMD in cortical bone but not in trabecular bone (Table 1). Calcein labeling revealed an increase in bone formation in the cortical bone in histological analysis (Fig. 1B). Histomorphometric analysis also revealed that the increase in cortical BMD was due to significant acceleration of the mineral apposition rate and bone formation rate (Table 1). Administration of W9 showed decreased tendencies in the number of osteoclasts/bone perimeter ($p = 0.051$), osteoclast surface/BS ($p = 0.055$), and eroded surface/BS in trabecular areas, but there was no significance

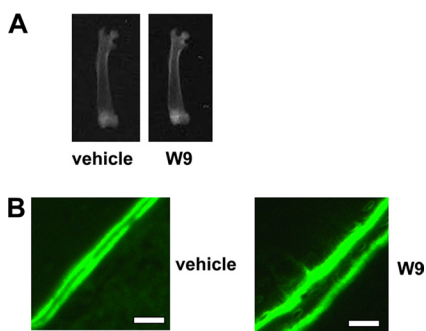


FIGURE 1. Anabolic effect of W9 peptide on cortical bone in normal mice. W9 (10 mg/kg) or vehicle (PBS) was administered subcutaneously to 6-week-old female mice for 5 days ($n = 8$). *A*, soft x-ray images of femurs of treated mice. *B*, calcein labeling in mice treated with W9 or vehicle was carried out on days 1 and 4. After 12 h from the last administration of W9 on day 5, both femurs were extirpated and fixed with 70% ethanol. The inner and outer lines show the labeled bones on days 1 and 4, respectively. *Bar*, 10 μm .

(Table 1). In trabecular bone, W9 showed no effect on osteoblast surface/BS.

In Vitro Analysis of Bone Anabolic Effect of W9—We next evaluated the bone anabolic effect of W9 *in vitro*. E1 cells were cultured with 50–200 μM W9 for 5 days to measure the ALP activity and then with 200 μM W9 for 3 weeks to detect mineralization activity by alizarin red staining. The peptide increased the ALP activity in a dose-dependent manner and strongly stimulated mineralization in preosteoblastic cells compared with the negative control peptide (W5) containing four amino acid mutations in W9 (Fig. 2A). W5 did not enhance mineralization in the cells but weakly increased ALP activity (Fig. 2A).

We then treated hMSCs with 100 or 200 μM W9 in osteoblastic differentiation medium. W9 markedly enhanced ALP activity and mineralization in hMSCs, although W5 did not increase ALP activity (Fig. 2B). We next examined the effects of W9 with mouse bone marrow cells, including osteoblastic stromal cells and osteoclast progenitors in the presence of sRANKL and M-CSF. Addition of W9 to the culture simultaneously inhibited osteoclast differentiation and stimulated osteoblast differentiation in the identical culture plate (Fig. 2C). To further explore the role of W9 in osteoclastogenesis, RAW264 cells were cultured in the presence of sRANKL. Addition of the peptide strongly suppressed osteoclast differentiation (Fig. 2D) and inhibited osteoclastogenesis in a dose-dependent manner as shown by TRAP activity; W5 showed no effect (Fig. 2E).

Gene chip analysis using E1 cells treated with 100 μM W9 demonstrated up-regulation of several genes associated with osteogenesis (Table 2). In particular, expression of bone γ -carboxyglutamate protein 1 (*Bglap1*), fibromodulin, *Igf2*, *Bmp4*, and connective tissue growth factor (*Ctgf*) was strongly up-regulated and that of fibroblast growth factor receptor 2 (*Fgfr2*), insulin receptor substrate 1 (*Irs1*), *Igf1*, *Alpl*, and collagen type I $\alpha 1$ and $\alpha 2$ was weak.

Analysis of Signaling Pathways of W9 Stimulation in E1 Cells—To understand its mechanism, we analyzed signaling pathways of W9-induced osteoblastogenesis. Activation of p38 MAPK is known to be required for osteoblast differentiation (28). The p38 MAPK inhibitor, SB203580, significantly diminished the ALP activity that was increased by W9 in E1 cells in a dose-dependent manner (Fig. 3A). The inhibitor also suppressed the

ALP activity stimulated by BMP-2. Addition of W9 to the cell culture markedly enhanced phosphorylation of p38 after 30 min and the phosphorylation was maintained for 6 h (Fig. 3B).

We also explored factors upstream of the p38 signaling pathway. Among Wnt family members, Wnt3a is known to enhance ALP activity in mesenchymal stem cells and osteoblasts (29). Wnt3a increased the ALP activity and stimulated accumulation of β -catenin in E1 cells (Fig. 3, C and D). Although Wnt3a induced accumulation of β -catenin after a 6-h stimulation, W9 showed no accumulation (Fig. 3D). Moreover, recombinant Dkk-1 did not suppress enhancement of ALP activity by W9 (Fig. 3E). Wnt5a and Wnt10b are known to exert their activities via a noncanonical Wnt signaling pathway, but neither of these noncanonical Wnt proteins induced ALP activity like W9 in E1 cells (Fig. 3, F and G).

Next, we examined the involvement of BMP signaling in W9-induced osteoblastogenesis. sBMPR-1A is recombinant extracellular domain of BMPR-1A that binds BMPs like BMP-2/4/6 and inhibits their activities. Addition of sBMPR-1A significantly, but not completely, suppressed the ALP activity increased by W9 in E1 cells in a dose-dependent manner (Fig. 4A). Smad1/5/8 are known to be involved in BMP-2 signaling in osteoblast differentiation (30). Addition of W9 to the cell culture enhanced phosphorylation of Smad1/5/8 after 30 min (Fig. 4B). Moreover, addition of anti-BMP-2/4-neutralizing polyclonal antibody significantly decreased the ALP activity increased by W9 in E1 cells but only partially (Fig. 4C).

W9 Synergistically Exerts Its Effect with BMP-2 in Vitro and in Vivo—To investigate whether BMP-2 shows a synergistic effect with W9, we added various doses of BMP-2 to E1 cell cultures in the presence of W9. BMP-2 increased ALP activity in a dose-dependent manner in the presence or absence of W9 (Fig. 5A). Moreover, W9 induced mineralization in E1 cell culture on day 10 in a dose-dependent manner in the presence of BMP-2 (Fig. 5B). As the mineralization in E1 cell culture was usually carried out for 21 days, W9 did not induce mineralization on day 10 in the absence of BMP-2. The synergistic effect of W9 with BMP-2 found in cell culture was examined using an ectopic bone formation model (Fig. 5C). Collagen sponges soaked with 2 μg of BMP-2 were implanted in the dorsal fascia of normal mice. Subcutaneous administration of W9 to the mice for 5 days increased bone mineral content of collagen sponges, including BMP-2 in a dose-dependent manner (Fig. 5D). The negative control peptide, W5, showed no effect. The shape of the collagen sponges in the mice treated with 10 mg/kg W9 was markedly bigger than those with vehicle or W5 (Fig. 5E).

Investigation of the Mechanism of W9-induced Osteoblastogenesis—Y6 containing one amino acid mutation in W9 has been found to weakly bind RANKL (18). We found that both W9 and Y6 increased ALP activity in E1 cells in a dose-dependent manner, but the effect of Y6 was weaker than that of W9 (Fig. 6A). As TNF- α and RANKL were the only W9-binding proteins described in previous reports (17, 18), we investigated whether the interaction of W9 with membrane-bound RANKL or TNF- α on osteoblasts leads to osteoblastogenesis. We knocked down RANKL and/or TNF- α expression in E1 cells by RNAi. The cells transfected with StealthTM RNAi oligonucleo-

W9 Peptide Has Anabolic Effect on Osteoblasts through RANKL

TABLE 1

Anabolic effect of W9 peptide on cortical bone in normal mice

Cortical (upper) and trabecular (lower) indices of the fixed femurs were determined by peripheral quantitative computed tomography and histomorphometric analyses. The following abbreviations are used: MAR, mineral apposition rate; BFR, bone formation rate; N.Oc, number of Oc; Oc.S, Oc surface; ES, eroded surface.

	Vehicle	W9
Cortical BMD	837.10 ± 14.75 mg/cm ³	851.62 ± 9.65 mg/cm ^{3a}
MAR	2.75 ± 0.34 μm/day	3.60 ± 0.40 μm/day ^b
BFR/BS	0.80 ± 0.18 mm ³ /mm ² /year	1.13 ± 0.16 mg/cm ^{3c}
Trabecular BMD	275.71 ± 12.31 mg/cm ³	270.60 ± 17.16 mg/cm ³
N.Oc/bone perimeter (1/100 mm)	539.32 ± 154.87	395.68 ± 112.19 ^d
Oc.S/BS	6.97 ± 1.98%	5.06 ± 1.68% ^e
ES/BS	10.12 ± 2.76%	7.74 ± 2.28%
Ob/BS	25.80 ± 8.24%	20.82 ± 3.61%

^a *p* < 0.05 versus vehicle (Student's *t* test).

^b *p* < 0.001 versus vehicle (Student's *t* test).

^c *p* < 0.01 versus vehicle (Student's *t* test).

^d *p* = 0.051 versus vehicle (Student's *t* test).

^e *p* = 0.055 versus vehicle (Student's *t* test).

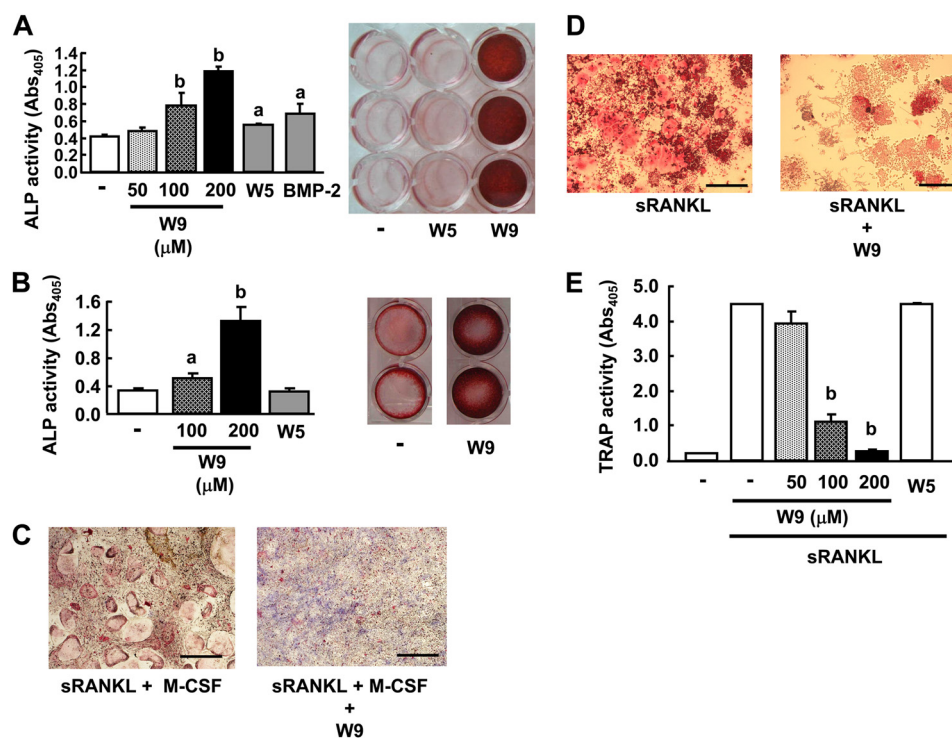


FIGURE 2. Effects of W9 on ALP activity and mineralization in mouse preosteoblastic cells and human mesenchymal stem cells. *A*, E1 cells were cultured with 50–200 μM W9, 200 μM W5 (negative control peptide), or 10 ng/ml BMP-2 for 5 days. ALP activity was measured by the pNPP assay and expressed as the mean ± S.D. *a*, *p* < 0.05; *b*, *p* < 0.01 versus control. Significant difference was determined using ANOVA with Dunnett's test. Mineralization of E1 cells was evaluated by alizarin red staining. They were cultured in osteoblastic differential media with 200 μM W9 or W5 for 21 days. *B*, hMSCs were cultured in the osteoblastic differential media with 100 and 200 μM W9 or 200 μM W5 for 4 days. Data were measured as described above and expressed as the mean ± S.D. *a*, *p* < 0.05; *b*, *p* < 0.01 versus control. Significant difference was determined using ANOVA with Dunnett's test. Mineralization of hMSCs was evaluated by alizarin red staining. They were cultured in osteoblastic differential media with 200 μM W9 for 21 days. *C*, mouse bone marrow cells were cultured in α-minimal essential medium containing 10% FBS in the presence of 10 nM sRANKL and 50 ng/ml M-CSF with or without 100 μM W9. After 7 days, cells were fixed and stained for TRAP and ALP. Bar, 500 μm. *D*, RAW264 cells were cultured in the presence of 10 nM sRANKL with or without 100 μM W9 for 5 days, and the cells were fixed and stained for TRAP. Bar, 500 μm. *E*, suppressive effect of W9 on osteoclastogenesis was examined in RAW264 cells cultured in the presence of 10 nM sRANKL with 0–200 μM W9 and 200 μM W5 for 4 days. TRAP activity was detected by TRAP solution assay. *b*, *p* < 0.01 versus sRANKL. Significant difference was determined using ANOVA with Dunnett's test.

tide of RANKL or TNF-α for 48 h showed strong down-regulation of these gene expressions (Fig. 6B). RANKL knockdown reduced, but not completely, ALP activity in cells treated with 100 μM W9 (Fig. 6B). TNF-α knockdown reduced the activity slightly. Double knockdown did not further reduce the ALP activity decreased by RANKL knockdown, whereas knockdown of either RANKL or TNF-α showed no decrease but rather an increase in ALP activity in the cells treated with BMP-2 (Fig. 6B). We also examined the effect of W9 on TNF-α activity. Although TNF-α induced apoptosis of L929 cells, addition of

various concentrations of W9 showed no effect on the apoptosis (Fig. 6C). To investigate the involvement of RANKL in W9-induced osteoblastogenesis, we examined the effects of W9 and BMP-2 on RANKL-deficient osteoblasts in ALP assay. Although BMP-2 increased ALP activity similarly in RANKL-deficient and wild-type osteoblasts, W9 showed a strong effect on wild-type cells and a weak effect on the deficient cells (Fig. 6D). The relative ratio of BMP-2 to control in the increase in ALP activity is about 1.4 in both wild-type and RANKL-deficient cells but that of W9 to control is

TABLE 2
Up-regulated expressions of osteogenesis-related factors in gene chip analysis

Gene name	Representative public identification	W9 signal	Control signal	W9/control	Change <i>p</i> value
<i>Alpl</i>	AW319615	179	105.3	1.702	0.00225
<i>Eglap1</i>	NM_007541	1775	213.5	8.314	0.00002
<i>Bmp4</i>	NM_007554	503	128.5	3.921	0.00002
Collagen I α 1	U08020	34,766	25,828	1.346	0.000167
Collagen I α 2	BF227507	33,013	21,834	1.511	0.00002
<i>Ctgf</i>	NM_010217	841	220	3.821	0.00002
Fibromodulin	NM_021355	13,977	2140	6.528	0.00002
<i>Igf1</i>	BG075165	11,353	6302	1.801	0.000438
<i>Igf2</i>	NM_010514	4530	887	5.107	0.00002
<i>Irs1</i>	BB345784	11,154	5644	1.976	0.00002
<i>Egfr2</i>	BG873440	2422	1190	2.0347	0.000966

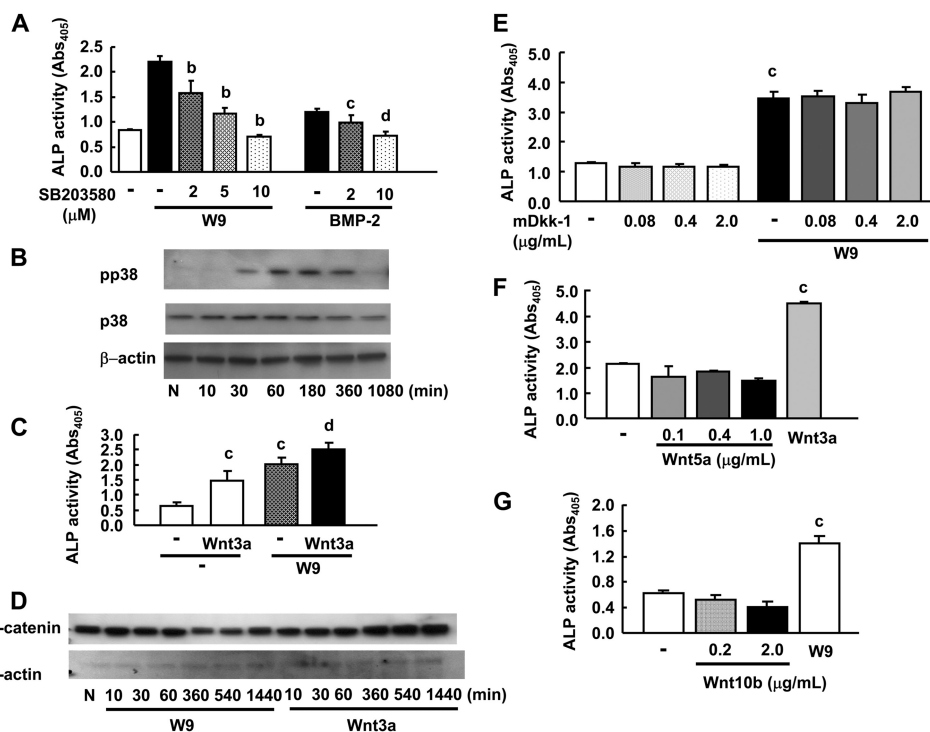


FIGURE 3. Signaling pathways involved in W9 stimulation in E1 cells. *A*, E1 cells were cultured in the presence of 100 μ M W9 or 5 ng/ml BMP-2 with or without 2–10 μ M p38 inhibitor (SB203580) for 5 days. ALP activity was measured by the pNPP method and expressed as the mean \pm S.D. *b*, $p < 0.01$ versus W9; *c*, $p < 0.05$; *d*, $p < 0.01$ versus BMP-2. Significant difference was determined using ANOVA with Dunnett's test. *B*, postconfluent E1 cells were stimulated with 100 μ M W9 peptide for 10–1080 min. Each whole cell lysate was prepared from the cells using RIPA buffer. Phosphorylated p38, total p38, and β -actin were detected by Western blotting. *N*, no treatment. *C*, E1 cells were cultured in the presence or absence of 50 ng/ml Wnt3a and 100 μ M W9 for 5 days. Data were measured as described above and expressed as the mean \pm S.D. *c*, $p < 0.001$ versus control; *d*, $p < 0.01$ versus W9. Significant difference was determined using Student's *t* test. *D*, postconfluent cells were stimulated with 100 μ M W9 or 100 ng/ml Wnt3a for 10–1440 min, and each whole cell lysate was prepared as described above. β -Catenin and β -actin were detected by Western blotting. *E*, E1 cells were cultured in the presence or absence of 100 μ M W9 with or without 0.08–2.0 μ g/ml Dkk-1 for 5 days. *F* and *G*, E1 cells were cultured with 0.1–1.0 μ g/ml Wnt5a or 0.2 and 2.0 μ g/ml Wnt10b for 5 days. Wnt3a (50 ng/ml) and W9 (50 μ M) were used as positive controls. Data were measured as described above and expressed as the mean \pm S.D. *c*, $p < 0.001$ versus control. Significant difference was determined using Student's *t* test.

about 1.5 in wild-type cells and about 1.2 in RANKL-deficient cells. W9 showed weaker activity in RANKL-deficient cells than wild-type cells, but BMP-2 did the similar activity irrespective of genotype.

DISCUSSION

In this study, we demonstrated for the first time that the osteoclastogenesis-inhibiting peptide W9 stimulated bone formation in cortical areas *in vivo*. Histomorphometry revealed that the rapid increase of BMD in cortical bone in the femoral diaphysis due to the addition of W9 leads to bone anabolic activity of the peptide. Consistent with a previous report (18), the number of Oc/bone perimeter and Oc surface/BS in trabe-

cular bone showed decreased tendencies with the addition of W9 due to inhibition of osteoclastogenesis, but the trabecular BMD did not change. In some experiments, administration of W9 significantly increased trabecular BMD, but W9 always increased cortical BMD in the experimental conditions (data not shown). W9 stimulated differentiation and mineralization of osteoblastic cells and inhibited osteoclast formation independently *in vitro*, indicating the dual function of the single peptide, up-regulation of osteoblastogenesis and down-regulation of osteoclastogenesis. Taken together, these results suggested that W9 exerted a dominant effect on stimulation of bone formation and a subdominant effect on inhibition of bone resorption for increase of bone mass in mice. The reasons why

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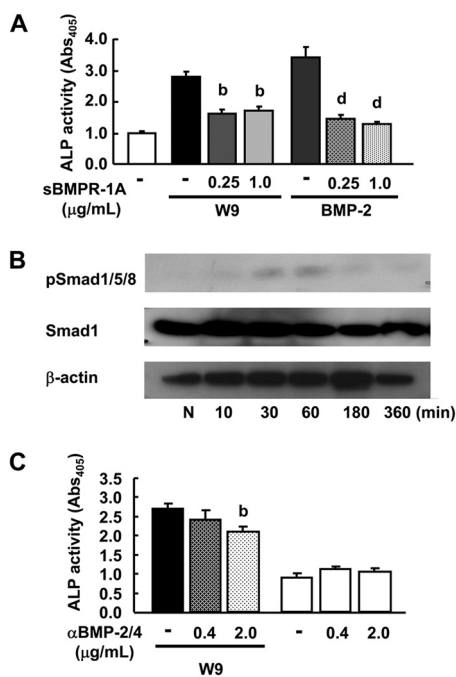


FIGURE 4. Participation of BMPs in W9 stimulation in E1 cells. *A*, E1 cells were cultured in the presence of 50 μM W9 or 5 ng/ml BMP-2 with or without 0.25 and 1.0 μg/ml sBMPR-1A for 5 days. ALP activity was measured by the pNPP method and expressed as the mean ± S.D. *b*, $p < 0.01$ versus W9; *d*, $p < 0.01$ versus BMP-2. Significant difference was determined using ANOVA with Dunnett's test. *B*, postconfluent E1 cells were stimulated with 100 μM W9 for 10–360 min, and each whole cell lysate was prepared from the cells using RIPA buffer. Phosphorylated Smad1/5/8, total Smad1, and β-actin were detected by Western blotting. *C*, E1 cells were cultured in the presence or absence of 100 μM W9 with or without 0.4 and 2.0 μg/ml anti-BMP-2/4 neutralizing antibody (αBMP-2/4) for 5 days. Data were measured as described above and expressed as the mean ± S.D. *b*, $p < 0.01$ versus W9. Significant difference was determined using ANOVA with Dunnett's test.

W9 acted on osteoblasts dominantly *in vivo* remain to be elucidated, although endogenous BMPs may enhance the effect of W9 on osteoblasts *in vivo*.

Wnt3a and BMP-2 are known to stimulate osteoblast differentiation (19, 29). W9 worked with Wnt3a synergistically in osteoblast differentiation, but β-catenin accumulation did not occur as seen for osteoblast differentiation by Wnt3a. These results indicate that W9 does not play a role in Wnt signaling. On the contrary, the effect of W9 was partially reduced by the addition of sBMPR-1A- or anti-BMP-2/4-neutralizing antibody in mouse osteoblastic cells. Furthermore, W9 showed a synergistic effect with BMP-2 in osteoblast differentiation, especially in the presence of endogenous BMP-2/4. W9 also activated Smad1/5/8 as shown previously in osteoblast differentiation by BMP-2 (30). W9 was further confirmed to enhance differentiation and mineralization of osteoblastic cells synergistically with exogenous BMP-2 and to induce ectopic bone formation using collagen gels containing BMP-2. Taken together, these results suggest an involvement of BMPs in W9 signaling in osteoblasts.

In this study, we administered W9 to mice three times per day for 5 days to achieve an increase of cortical BMD in the femur. Although daily subcutaneous injection of PTH to mice for 2 weeks increased cortical BMD in a previous study (31), the effect of PTH seems to be comparable with that of W9 in the experimental conditions. Because PTH is the only bone ana-

bolic drug that is clinically used for treatment of osteoporosis, the effect of W9 is not relatively weak. Meanwhile, local administration of W9 may be useful for the treatment of the osseous defects, as it can induce ectopic bone formation with BMP-2. To clarify this, further examination of ectopic bone formation using collagen gels, including W9 and BMP-2, is now ongoing.

W9 is known to bind TNF-α and RANKL to inhibit their activities (17, 18). The presence of four amino acid mutations in the W9 peptide (*i.e.* W5) almost completely reduced its activity, indicating the importance of the amino acid sequence, WSQYL, located between two cysteines for its activity. One amino acid mutation in the W9 peptide (Y6), which has been shown to bind RANKL with one-third affinity compared with W9 (18), reduced osteoblastogenesis stimulatory activity of W9.

To examine whether W9 exerts its activity by binding TNF-α or RANKL, we decreased TNF-α and/or RANKL expressions in E1 cells using RNAi and found the activity of W9 was partially blocked in the RANKL-knockdown cells. In contrast, knockdown of TNF-α showed little effect on the activity of W9, and this peptide did not inhibit apoptosis of L929 cells by TNF-α. Thus, it is unlikely that TNF-α is the target of W9. To confirm that W9 depends on RANKL in its osteoblast differentiation activity, we used osteoblasts prepared from RANKL-deficient mice. Consistent with the RANKL knockdown experiments, W9 showed a very weak effect on the deficient cells compared with the cells from wild-type littermates. Because BMP-2 was affected similarly on both type of the cells, BMP signaling seemed normal in RANKL-deficient cells. The difference in the reactivity of W9 between the deficient and wild-type cells showed that RANKL was involved at least partially in W9-induced osteoblastogenesis.

Expression of several growth factors and their receptors, including *Bmp4*, *Ctgf*, *Igf1*, *Igf2*, and *Fgfr2*, was increased in mouse osteoblastic cells by W9. The results suggest that W9 binds RANKL on osteoblasts and stimulates signaling pathways for the expression of several autocrine factors in the cells. Because addition of W9 activated p38 and Smad1/5/8 in osteoblastic cells after 30 min, its signal is directly transmitted through RANKL on the cells.

W9 was found to exert its osteoclastogenesis inhibitory activity via directly binding RANKL (18). It is well known that RANKL transmits the osteoclast differentiation signal through RANK. As W9 is an artificial synthetic peptide that can bind RANKL, we hypothesized that an endogenous ligand for RANKL is RANK. The binding of RANKL and RANK may transmit a bidirectional signal to activate osteoclasts and osteoblasts through a forward and reverse signal, respectively (Fig. 7).

Reverse signaling occurs among the members of the TNF family, including TNF-α, CD40L, FasL, and RANKL, and a membrane-bound ligand transmitting its signal as a receptor (32–34). Consistent with a previous report that activation of p38 is involved in a signaling through RANKL in T cells (34), we demonstrated that W9 activates p38 in osteoblastic cells via a signaling through RANKL. However, we cannot exclude the possibility that W9 transmits its signal through other receptors.

OPG and RANK are known to bind RANKL. The enhanced osteoclastogenesis and osteoblastogenesis found in OPG-defi-

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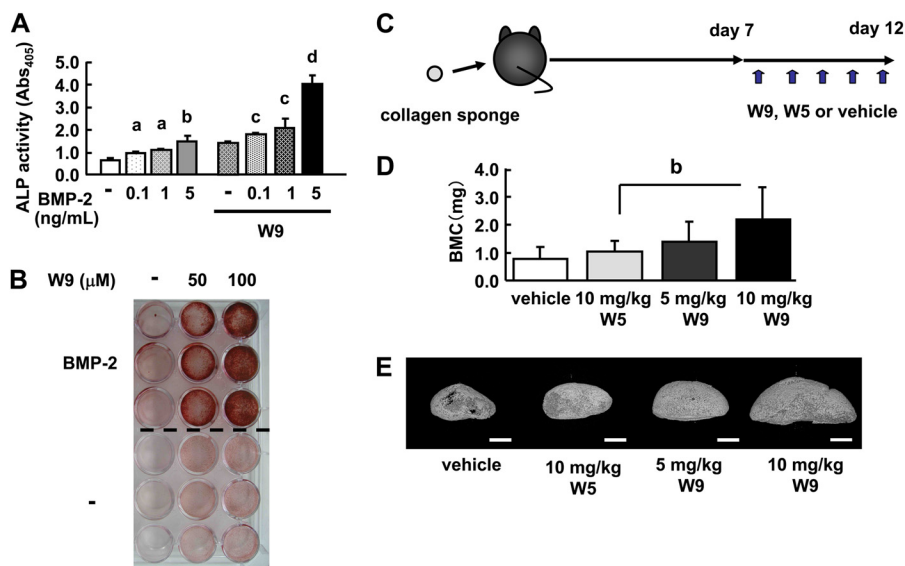


FIGURE 5. Synergistic effect of W9 with BMP-2 on osteoblast differentiation/mineralization and ectopic bone formation. *A*, E1 cells were cultured in the presence or absence of 25 μM W9 with or without 0.1–5 ng/ml BMP-2 for 5 days. Data were expressed as the mean ± S.D. *a*, $p < 0.05$; *b*, $p < 0.01$ versus control; *c*, $p < 0.05$; *d*, $p < 0.01$ versus W9. Significant difference was determined using ANOVA with Dunnett's test. *B*, mineralization of E1 cells cultured in the presence or absence of 10 ng/ml BMP-2 with or without 50 and 100 μM W9 for 10 days was evaluated by alizarin red staining. *C*, experimental protocol. Collagen sponge disc was used as a carrier of BMP-2. These collagen discs were freeze-dried with BMP-2 and then surgically implanted into dorsal muscle pouches. After a week, 5 or 10 mg/kg W9, 10 mg/kg W5, or vehicle (PBS) was administered subcutaneously to the implanted mice three times/day for 5 days. *D*, bone mineral content (BMC) of ectopic bone was measured by dual-energy x-ray absorptiometry and expressed as the mean ± S.D. *b*, $p < 0.01$ versus W5. Significant difference was determined using ANOVA with Dunnett's test. *E*, three-dimensional reconstruction images of ectopic bones were obtained by micro-focal computed tomography. Bar, 1 mm.

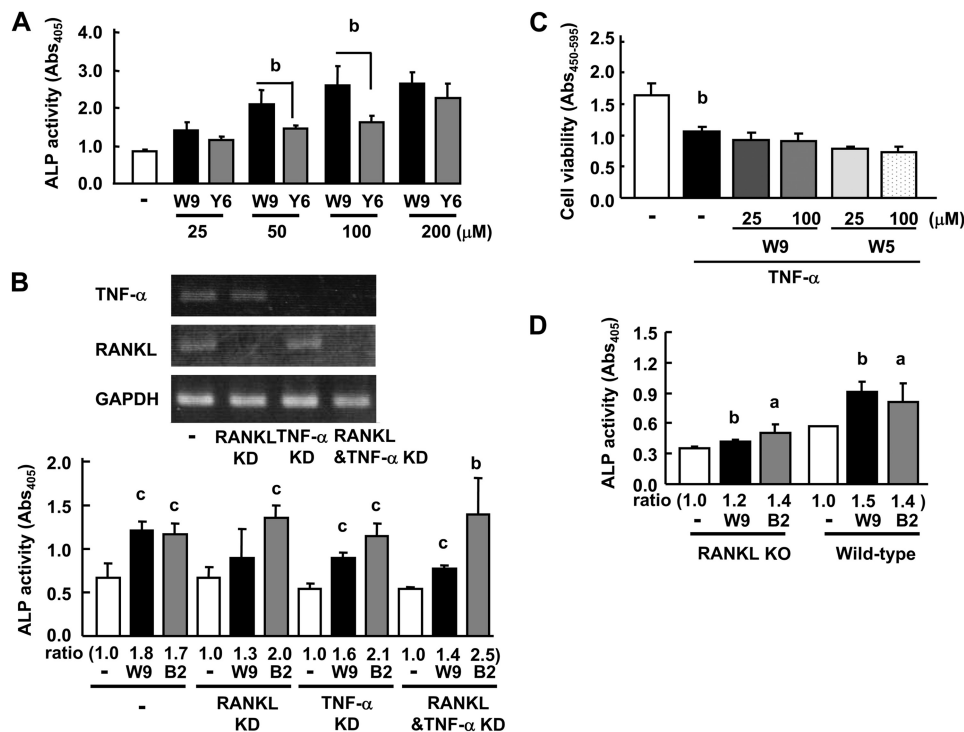


FIGURE 6. A signaling through RANKL is involved in part in W9-induced osteoblast differentiation. *A*, E1 cells were cultured in the presence of 25–200 μM W9 or Y6 for 5 days. ALP activity was measured by the pNPP method. Data were expressed as the mean ± S.D. *b*, $p < 0.01$ versus each concentration of Y6. Significant difference was determined using Student's *t* test. *B*, RANKL and TNF-α gene expression was suppressed by siRNA-mediated knockdown with 10 nm stealth RNAi in E1 cells. The cells were transfected with stealth RNAi for 48 h. *Upper panel*, expression of RANKL, TNF-α, and GAPDH was measured by RT-PCR. *Lower panel*, treated cells were further cultured with 100 μM W9 or 10 ng/ml BMP-2 (B2) for 5 days. Data were measured as described above and expressed as the mean ± S.D. The ratios of A_{405} /each control are shown in parentheses. *c*, $p < 0.001$ versus each control. *C*, L929 cells were cultured with 1 μg/ml actinomycin D in the presence or absence of 25 pg/ml TNF-α with or without 25 and 100 μM W9 and W5 for 24 h. The viable cells were detected with WST-1 assay kit. *b*, $p < 0.01$ versus control. Significant difference was determined using Student's *t* test. *D*, osteoblasts from RANKL-deficient (KO) mice and wild-type mice were cultured in the presence or absence of 100 μM W9 or 40 ng/ml BMP-2 (B2) for 5 days. Data were measured as described above and expressed as the mean ± S.D. The ratios of A_{405} /each control are shown in parentheses. *a*, $p < 0.05$; *b*, $p < 0.01$ versus each control.

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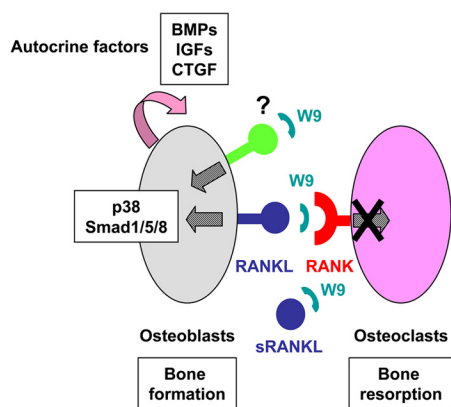


FIGURE 7. A model illustrating a mechanism by which W9 differentiates osteoblasts; a hypothesis that a bidirectional signaling through RANKL and RANK regulates osteoblastogenesis and osteoclastogenesis. W9 binds RANKL on osteoblasts and stimulates production of autocrine factors like BMPs, IGFs, and connective tissue growth factor (CTGF) for osteoblast differentiation. The p38 and Smad1/5/8 signaling pathways are involved in W9-induced osteoblastogenesis. W9 may bind other receptors on osteoblasts to exert its activity in part. In contrast, W9 binds RANKL and sRANKL to inhibit osteoclastogenesis. In the hypothesis RANK is an endogenous ligand for RANKL. The bidirectional signaling between RANKL and RANK could regulate the coupling between bone formation and resorption. RANKL and RANK could be pivotal coupling factors.

cient mice (35–37) suggest enhanced the bidirectional signaling without OPG, as OPG is a decoy receptor for RANKL. The observation of similar phenotypes of RANKL- and RANK-deficient mice also supports our hypothesis (12, 13).

Once the signaling through RANKL is activated in osteoblasts, the cells produce many autocrine growth factors and their receptors to activate their differentiation in the particular model. These growth factors of the RANKL signaling may subsequently affect neighboring cells to further activate their differentiation in a paracrine manner. The reverse signal from RANK on osteoclasts to RANKL on osteoblasts, and the forward signal from RANKL to RANK, could play an important role in the coupling between bone formation and resorption.

Our findings that W9 has a bone anabolic effect emphasized its potential as a treatment of bone diseases, and it may shed light on the identification of the mechanism regulating the coupling between bone formation and resorption.

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