Influence of Water-soluble Extracts of Long-term Fermented *Doenjang* on Bone Metabolism Bioactivity and Breast Cancer Suppression

Ja young Seol¹, Yu Na Youn¹, Minseon Koo^{2,3}, Hyun Jung Kim^{2,3}, and Soon Young Choi*

¹Division of Biological Science, Sookmyung Women's University, Seoul 04310, Korea

²Korea Food Research Institute, Seongnam, Gyeonggi 13539, Korea

³Department of Food Biotechnology, Korea University of Science and Technology, Daejeon 34113, Korea

Received June 28, 2015
Revised November 10, 2015
Accepted December 10, 2015
Published online April 30, 2016

*Corresponding Author
Tel: +82-2-710-9510
Fax: +82-2-2077-7322
E-mail: sychoi@sookmyung.ac.kr

pISSN 1226-7708
eISSN 2092-6456

© KoSFoST and Springer 2016

Abstract Anti-cancer effects of *doenjang* extracts are usually studied based on methanol extraction focusing on isoflavones, the principal extracted components. In this study, effects of water-soluble extracts of long-term fermented *doenjang* containing water-soluble low Mw peptides produced by microorganisms were studied. *Doenjang* extracts had effects which arrested the cell cycle, inhibited proliferation, and caused consequential apoptosis in breast cancer cells. *Doenjang* water-soluble extracts increased bone density via activation of osteoblast differentiation and suppression of osteoclast differentiation. Effects increased as the fermentation period extended because the *doenjang* fermentation period influenced extract contents. The value and utility of *doenjang* as a functional food should be considered.

Keywords: *doenjang*, anti-cancer, osteoblast, osteoclast

Introduction

Doenjang is an important and representative fermented soybean product in Korea. The functional superiority of *doenjang* over natural soybeans is due to an abundance of useful bioactive components of diverse kinds of peptides created via soybean protein decomposition by hydrolase that is produced by microorganisms during fermentation, and saponin, phytic acid, oligosaccharide, and isoflavones, which originate from soybeans (1).

Anti-cancer effects of *doenjang* extracts against hormone-induced colon, prostate, and breast cancers have been reported (2-5). Suppression of osteoporosis, a hormone-induced disease, are documented as well (6,7). Anti-mutagenesis (8), fibrinolytic activity (9), immune system improvement (10), angiotensin-converting enzyme (ACE) inhibitory activity (11,12), hypoglycemic effects (13), antidiabetic (14), and anti-oxidative effects (15,16) have also been identified. Studies about these effects of *doenjang* have used methanol extraction generally. Isoflavones, fatty acids, and linoleic acids have been isolated from methanol extracts for study of cancer cell growth inhibition (17,18). Kwon *et al.* (19) compared bio-activities between extracts obtained from *meju* using both water and methanol extraction and explored the effects of 2 *doenjang* extracts on the insulin sensitivity of 3T3-L1 fibroblasts (19). Park *et al.* (20,21) reported effects of synthetic peptides in contrast with peptides

separated directly from soybeans. Synthetic peptides decreased amounts of body fat, decreased blood pressure, and decreased blood glucose levels.

Useful bio-active components can be created via fermentation and some studies have recently reported a close relationship between the effects of bio-activity and the fermentation period of *doenjang* (19,22). Improved anti-oxidative effects with an extended fermentation period were also reported (23,24). In this study, water-soluble extracts of *doenjang* containing several low Mw peptides created by microorganisms via fermentation were examined for anti-cancer and bone metabolism effects, and compared with methanol extracts and with raw soybeans.

Materials and Methods

Preparation of *doenjang* water extract Five *doenjang* samples were collected from Gangwon, Gyeonggi, Jeolla, and Kyongsang Provinces, and from Jeju Island in March of 2013. Five g of each *doenjang* sample was extracted with distilled water 5x for 20 min at room temperature by Pulsifier (PULSIFIER®; Microgen, Camberley, UK). Extracts were then centrifuged (High Speed Refrigerated Centrifuge; Labogene, Seoul, Korea) at 10,000×g for 10 min and supernatants were separated between Mw=3 to 10 kDa using Microcon YM-3 and

YM-10 (Millipore, Billerica, MA, USA) for removal of high Mw substances and salt leaving low Mw peptides of 3 to 10 kDa, which were used for biological assays. The salinity of *doenjang* extracts was nearly 10%, but 2% NaCl solution is used as a control because *doenjang* extracts were cut off by microcon.

ABTS radical scavenging activity assay ABTS radical scavenging activity of water-soluble *doenjang* extracts was measured by the ABTS cation decolorization assay. ABTS (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in deionized water to 7.4 mM concentration, and potassium persulphate added to a concentration of 2.45 mM. The reaction mixture was left to stand in the dark at room temperature for 16 h before use. The ABTS radical cation (ABTS^{•+}) was produced by reaction of 7.4 mM ABTS with 2.45 mM potassium persulfate. The resultant ABTS^{•+} radical cation was diluted with 5 mM PBS (pH 7.4) to an absorbance of 0.7 (± 0.02) at 734 nm. Ten microliters of the water-soluble *doenjang* extracts was added to 990 μ L of diluted ABTS solution. Absorbance was measured at time intervals of 10 min after addition of the water-soluble *doenjang* extracts at 734 nm using Enzyme-Linked Immunosorbent Assay Reader (ELISA Reader SpectraMax M5^e; Molecular Devices, Sunnyvale, CA, USA). Measured values were calculated in mg of vitamin C equivalents/mL using vitamin C as a standard based on comparison with a standard curve of vitamin C (Sigma-Aldrich). Anti-oxidant activity was indicated by fading blue and green colors of ABTS due to loss of electrons to potassium persulfate.

Cell culture The human breast cancer cell lines MCF-7 and SK-BR-3, and human embryonic kidney HEK293 cells, were obtained from the Korea Cell Line Bank (KCLB, Seoul, Korea). MCF-7, SK-BR-3, and HEK293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Welgene Inc., Daegu, Korea) supplemented with 10% fetal bovine serum (FBS, Gibco; Invitrogen, Waltham, MA, USA) and 1% penicillin/streptomycin (Welgene). The osteoblast MC3T3-E1 mouse cell line (subclon 4; ATCC, Manassas, VA, USA) was cultured in α -minimal essential medium without ascorbic acid (α -MEM; Welgene). Primary bone marrow macrophage (BMM) cells separated from thigh bones of 4-week-old male C57/BL6 mice were cultured in DMEM (Welgene).

Osteocyte differentiation MC3T3-E1 osteoblast cells were cultured α -MEM for 24 h, then treated with 50 μ g/mL ascorbic acid (AA) (Sigma-Aldrich) and 10 mM β -glycerophosphate (β -GP) (Sigma-Aldrich) to induce differentiation. Cells were then transferred to fresh culture medium, including AA and β -GP, every 2 to 3 days. Primary bone marrow macrophage cells were differentiated in DMEM using 20 ng/mL macrophage colony stimulating factor (M-CSF; Sigma-Aldrich), 200 ng/mL TNF-related activation-induced cytokine (TRANC; Sigma-Aldrich), and 10% FBS.

Cell proliferation assay Absorbance values were measured at 450

Table 1. Primer sequences of differentiation-related genes of osteoblasts and osteoclasts

Target gene		Primer Sequence
Cathepsin K	Forward	5'-GGCCAACCTCAAGAAGAAAC-3'
	Reverse	5'-GTGCTTGCTTCCCTTCTGG-3'
MMP-9	Forward	5'-AGTTTGGTGTGCGGAGCAC-3'
	Reverse	5'-TACATGAGCGCTTCCGGCAC-3'
TRAP	Forward	5'-GACAAGAGGTTCCA-3'
	Reverse	5'-AGCCAGGACAGCTGAGTG-3'
OPN	Forward	5'-ATGAGATTGGCAGTGATTGCTTTT-3'
	Reverse	5'-ATGTGGCTATAGGATCTGGGTGCA-3'
RhoC	Forward	5'-ATGGCTGCGATCCGAAAGAGCT-3'
	Reverse	5'-CTTGCCATCCACTTCGATGT-3'
V-ATPase	Forward	5'-ATGGCGCTCAGTGATGAGATGTACA-3'
	Reverse	5'-TTGCAACAAGGCACCTTCT-3'
ALP	Forward	5'-ATGATCTCACCTTTTAGTACTGG-3'
	Reverse	5'-TACATTGGTGTGAGCTTTTGGAGT-3'
BSP	Forward	5'-ATGAAGACTGCTTAATTTTGCTC-3'
	Reverse	5'-GTAGGCATGCTTATAAGAAAGTA-3'
OCN	Forward	5'-ATGAGGACCATCTTCTGCTCACT-3'
	Reverse	5'-CCGGAGTATGTTCACTACCTTATT-3'
VEGF	Forward	5'-ATGAACCTTCTGCTCTTGGGTGCA-3'
	Reverse	5'-GTAGCTTCGCTGGTAGACATCCATGAA-3'
GAPDH	Forward	5'-CCTGTCCAGAGACGCCGCATCTT-3'
	Reverse	5'-TGGCAACAATCTCCACTTGGCCA-3'

nm using a WST-1 proliferation assay (Roche Diagnostics, GmbH Mannheim, Germany) and an ELISA Reader (SpectraMax M5^e; Molecular Devices).

Reverse transcriptase (RT)-PCR and quantitative Real-Time PCR

Total RNA was extracted using TRIzol reagent (Invitrogen) and cDNA was synthesized using the ImProm-IITM Reverse Transcription System (Promega, Madison, WI, USA) using gene specific primers (Table 1) for amplification of cDNA. RT-PCR was carried out using a QuantiTectTM SYBR[®] Green PCR kit (Promega) and results were analyzed using a Corbett Research Rotor-Gene RG-3000A (Qiagen, Valencia, CA, USA).

Western blotting

Total protein was extracted from cells using a lysis buffer (RIPA; Thermo Fisher Scientific, Waltham, MA, USA). After SDS-PAGE by western Kit (Bio-Rad, Hercules, CA, USA), proteins were transferred to PVDF membranes (Bio-Rad) using a TE 70 semi-dry transfer unit (Amersham Biosciences, Piscataway, NJ, USA). Membranes were blotted using a primary antibody at 4°C for 12 h. Changes in protein expressions were confirmed using an LAS-3000 apparatus (Fujifilm Co., Tokyo, Japan). Antibodies used were p27, cyclin D1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), AKT (ABR, Seoul, Korea), BID and Bcl-x_i (Cell Signaling Technology Inc., Danvers, MA, USA), p85 (Upstate Biotechnology Inc., Lake Placid, NY, USA), p53 (Oncogene Science, Inc., Cambridge, MA, USA), actin (Sigma-Aldrich), and phospho-AKT, phospho-CyclinD1, and phospho-ERK (Cell Signaling Technology).

Annexin V apoptosis assay Breast cancer cells were treated with *doenjang* extracts and stained with 5 μ L of annexin V-FITC and 5 μ L of propidium iodide (PI) using an Annexin V-FITC Apoptosis Kit (BioVision, Inc., Mountain View, CA, USA). Flow cytometric analysis was carried out using a fluorescence-activated cell sorting (FACS) Flow Cytometer (Canto™II Flow Cytometer; BD Biosciences, San Jose, CA, USA).

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay Breast cancer cells were fixed in 3% formaldehyde (Sigma-Aldrich) and added with 0.1% Triton X-100 (Sigma-Aldrich) then placed on ice for 2 min. Next, a TUNEL reaction mixture from a Cell Death Detection Kit (Roche Diagnostics) was applied. Fluorescein (Roche Diagnostics) was added to cells for 2 h. A Hoechst 33258 staining solution (Sigma-Aldrich) at a concentration of 20 μ g/mL was mixed with cells for 5 min in the dark. Finally, cells were examined under a fluorescent microscope (Axioskop 2 plus; Carl Zeiss, Thornwood, NY, USA).

Alkaline phosphatase (ALP) staining and activity MC3T3-E1 cells were differentiated for 6 days and fixed with a citrate-acetone-formaldehyde liquid (Sigma-Aldrich) for 30 s, stained with an alkaline-dye mixture (Sigma-Aldrich) for 15 min at room temperature, then counterstained with a neutral red solution (Sigma-Aldrich) for 2 min, and finally examined under an optical microscope ($\times 200$) (Optical Microscope IX-71; Olympus, Fukuoka, Japan). Cells were lysed using a Bioruptor (Cosmo bio Co. Ltd., Tokyo, Japan), and centrifuged (Heraeus™ Pico™ and Fresco™ Microcentrifuges; Thermo Fisher Scientific) for 20 min at 4°C and 12,000 $\times g$. The resulting supernatant was used for ALP-activity measurement with 180 μ L of a p-nitrophenyl phosphate liquid substrate (Sigma-Aldrich) added to each well of a 96-well plate (SPL Life Sciences, Seoul, Korea) containing 20 μ L of a cell-lysis liquid for 30 min at 37°C and 405 nm using a SpectraMax M5[®] ELISA Reader (Molecular Devices). Alkaline phosphatase is a glycoprotein that indicates bone formation (25). The ALP concentration in this study was indirectly measured based on the amount of p-nitrophenol, which is an ALP catalyst for the hydrolysis reaction of p-Nitrophenyl phosphate (p-NPP).

Von Kossa staining Osteoblastic cell strains (MC3T3-E1) were first fixed using 3.7% formaldehyde for 10 min, then were reacted with a 3% silver nitrate solution (Sigma-Aldrich) under UV light for 60 min. Cells were then observed under an optical microscope ($\times 200$) (Olympus).

Tartrate-resistant acid phosphate (TRAP) staining and activity Primary BMM cells were fixed in 3% formaldehyde (Sigma-Aldrich), then were added with a mixture consisting of a 2% TRAP Fast Garnet GBC base solution, a sodium nitrite solution, 5% naphthol AS-BI phosphoric acid (Sigma-Aldrich), a 4% acetate solution (Sigma-Aldrich), and a 2% tartrate solution (Sigma-Aldrich) for 30 min. The

Fig. 1. ABTS radical scavenging activity. Antioxidant activity of water-soluble *doenjang* extracts were measured according to different regions and different formation periods. The measured values were calculated in mg vit C eq/mL compared with the standard curve of vitamin C.

cell lysis mixture was added with a 50 mM citrate buffer (Sigma-Aldrich) at pH 4.6, 10 mM sodium tartrate (Sigma-Aldrich), and 3.7 mM p-nitrophenyl phosphate (Sigma-Aldrich) for 10 min at 37°C. The activity was measured at 405 nm using an ELISA Reader (Molecular Devices).

Statistical analysis All results were expressed as a mean $\pm$ standard deviation (SD) of 3 independent experiments. Statistical analysis was performed using SAS 9.1 (SAS Institute, Cary, NC, USA) at $p < 0.05$.

Results and Discussion

Anti-oxidative activities of water-soluble *doenjang* extracts Anti-oxidants attack polyhydric unsaturated fatty acids and eliminate oxygen and hydroxy radicals that cause oxidation (26). Therefore, it is important to examine increasing ABTS radical scavenging activities of *doenjang* extracts based on the fermentation period.

Anti-oxidant activities were examined to determine extract biological activities. Anti-oxidant activities, based on measurement of the ABTS radical scavenging activity, tended to increase in *doenjang* extracts fermented from 1 to 3 years. However, there was not ($p > 0.05$) differences in ABTS radical scavenging activities of *doenjang* from the 5 sampling regions. *Doenjang* extracts from all regions had anti-oxidant activities (Fig. 1). Anti-oxidant activities might have been caused by peptide effects based on fermentation products in water-soluble *doenjang* extracts. Thus, water-soluble *doenjang* extracts exhibited anti-oxidant activities.

Water-soluble *doenjang* extracts inhibited breast cancer cell proliferation *Doenjang* extracts were investigated for anti-cancer effects based on proliferation of breast cancer cells in extracts of different fermentation periods from 1 to 5 years. Extracts from the 5 different sampling regions exhibited anti-oxidant activities. *Doenjang*

Fig. 2. Suppression of breast cancer cell proliferation by water-soluble *doenjang* extracts. Breast cancer cells were treated with water-soluble *doenjang* extracts at 50 μ L/mL for 72 h. (A) Morphological changes in the nucleus were observed under an optical microscope ($\times 200$) after cells were stained with Hoechst 33258. (B) WST-1 Proliferation assay. Results are presented as mean $\pm$ standard deviation for representative experiments using each cell line ($p < 0.05$). (C) Western blotting. Suppression of the EGFR signaling pathway at the protein level.

extracts from *Gangwon* were used for testing water-soluble *doenjang* extracts for anti-cancer effects. MCF7 and SKBR3 breast cancer cells were treated with extracts for 72 h. Microscopic observations revealed that extract treatments effectively induced ($p < 0.05$) decrease in cell number and cell size, compared with controls (Fig. 2A). Cell proliferation inhibition by extracts was first quantified using the WST-1 reagent following manufacturer instructions. Absorbance values were markedly ($p < 0.05$) decreased in *doenjang* extract-treated cancer cells, compared with controls (Fig. 2B).

Expression changes of the proliferation-related genes ERK, p85, and AKT (27) were investigated at the protein level. Treatment of breast cancer cells with extracts slightly decreased levels of ERK1/2 and p85. But, levels of AKT phosphorylation was distinctly ($p < 0.05$) decreased compared with controls. No differences ($p > 0.05$) were observed in HEK-293 normal cells, compared with controls (Fig. 2C). *Doenjang* extracts inhibited proliferation of breast cancer cells, at least in part, via suppression of ERK, p85, and AKT activation. Furthermore, inhibition of breast cancer cell proliferation was slightly effective as the fermentation period of *doenjang* extracts increased.

Water-soluble *doenjang* extracts induced breast cancer cell apoptosis After treatment with *doenjang* extracts, proliferation inhibition of breast cancer cells were examined. In addition, cell death was also detected using a microscope. Effects of *doenjang* extracts on apoptosis of breast cancer cells were examined using annexin V - FITC and TUNEL assays. Percentages of apoptotic cells for control and 2% NaCl treated groups were 1.1 and 0.9%, respectively, in SK-BR-3 cells. However, the proportion of apoptotic cells reached 14.1% for SK-BR-3 cells treated with extracts (Fig. 3A). A distinct ($p > 0.05$) difference was not observed in normal HEK-293 cells, compared with controls, despite treatment with extracts (data not shown). Apoptosis was confirmed using the TUNEL assay (Fig. 3B).

After treatment of breast cancer cells with extracts for 48 h, 50% of cells were stained. All cells were stained after 96 h (Fig. 3B). Therefore, *doenjang* extracts caused apoptosis in breast cancer cells.

Gene expression changes related to cell death were examined. After 96 h of extract treatment, expressions of apoptosis and cell cycle regulator genes were examined at the protein level (Fig. 3C). The cell cycle arrest genes p53 and p27 and the pro-apoptotic gene Bid exhibited increased expressions. Furthermore, CyclinD1, which functions in the cell cycle progression, and Bcl-X_L, an anti-apoptotic gene product, exhibited decreased expressions. Expression of pCyclinD1 which lead to proteasomal degradation of CyclinD1, increased (Fig. 3C). However, changes in gene expressions did not occur in HEK-293 cells. Therefore, *doenjang* extracts controlled expression of genes related to cell death and cell cycle control and caused apoptosis of breast cancer cells via controlling apoptosis related genes with no effects in normal cells.

Water-soluble *doenjang* extracts promoted osteoblast differentiation

Doenjang extract effects on bone metabolism were investigated. Metabolic bone diseases, such as osteosclerosis and osteoporosis, can be caused by an imbalance in bone formation and resorption in bone remodeling (28). MC3T3-E1 osteoblast cells were treated with 10 μ L/mL *doenjang* extracts from *Gangwon* fermented for 1 to 5 years. Cells were differentiated for 6 days and the level of alkaline phosphatase was measured (Fig. 4A). Treatment with the extracts markedly ($p < 0.05$) increased the concentration of ALP, compared with controls. ALP activity increased with longer fermentation times and peaked in 3-year fermented *doenjang*. ALP activity began to decline when MC3T3-E1 cells were cultured with extracts fermented for more than 3 years. Therefore, extracts increased osteoblast differentiation and the efficacy increased with longer fermentation times up to 3 years, then decreased after 3 years. Osteoblast

Fig. 3. Induction of apoptosis in breast cancer cells by water-soluble *doenjang* extracts. Breast cancer cells were incubated with water-soluble *doenjang* extracts at 50 μ L/mL for 72 h. (A) Annexin V-FITC apoptosis assay. Breast cancer cells were stained with Annexin V and propidium iodide and analyzed using flow cytometry. (B) TUNEL assay. Top panel: DNA stained with Hoechst 33258; bottom panel: apoptotic cells stained with FITC ($\times 200$). (C) Western blotting. Regulation of apoptosis and cell cycle-related gene expression at the protein level by water-soluble *doenjang* extracts in breast cancer cells.

differentiation was investigated using 3-year fermented *doenjang* extracts, which were confirmed to have the largest differentiation effects.

The influence of extracts on differentiation of osteoblasts was confirmed based on changes in ALP expression using ALP staining for 6 days during MC3T3-E1 cell differentiation (Fig. 4B).

Osteoblasts remodel bones through mineralization after differentiation. In order to determine the impact of extracts on mineralization of osteoblasts, Von Kossa staining was carried out after 3 weeks of MC3T3-E1 cell differentiation and osteoblast calcific nodules were visualized using a dye. Mineralization was increased in osteoblasts cultured with extracts, compared with controls (Fig. 4C). Water-soluble *doenjang* extracts promoted osteoblast differentiation and increased osteoblast mineralization.

Osteoblast differentiation at the gene level was determined and the influence of 3-year fermented *doenjang* extracts on genes related to osteoblast differentiation was determined. MC3T3-E1 cells were cultured for 12 days with and without extracts. Cellular mRNA was extracted on days 0, 2, 4, 6, 8, and 10 and expression changes in differentiation-related genes were confirmed based on quantitative real-time PCR (Fig. 4D). Widely recognized genes related to osteoblast differentiation include ALP (alkaline phosphatase), BSP (bone

sialoprotein), *Colla2* (collagen 1a2), *OCN* (osteocalcin), *OPN* (osteopontin), and *VEGF* (vascular endothelial growth factor) (29-31). MC3T3-E1 cells differentiated for 12 days exhibited increased levels of ALP expression comparison with controls. BSP expression gradually increased in expression and was doubled on day 10. Expression of *OCN* (29), which is generally expressed during the terminal period of the osteoblast differentiation process, dramatically ($p < 0.05$) quadrupled, compared with controls, by day 12. *VEGF* expression gradually increased from day 0 to day 12 in extract treatment and extract no treatment. But increased expression of *VEGF* was higher ($p < 0.05$) in extract treatment compared with controls.

The ALP, BSP, OCN, and VEGF genes, expressed in osteoblast differentiation, showed different expression levels depending on the differentiation period of the middle and late stages of the osteoblast differentiation process (29-31). With water-soluble *doenjang* extract treatments, increases in gene expression were observed at almost every stage. Thus, osteoblast differentiation was promoted via increased expression of genes induced by extracts.

Water-soluble *doenjang* extracts inhibited osteoclast differentiation

Effects on osteoclast differentiation were confirmed via measurement of TRAP activities, which are indicators of differentiated osteoclast

Fig. 4. Effects of water-soluble *doenjang* extracts on osteoblast differentiation. (A) Alkaline phosphatase activity of MC3T3-E1 cells treated with and without water-soluble *doenjang* extracts at 10 μL/mL for 6 days ($p < 0.05$). (B) ALP staining (×200). (C) Von Kossa staining (×200). MC3T3-E1 cells were treated with AA and β-GP or AA, β-GP, and water-soluble *doenjang* extracts at 10 μL/mL. (D) Quantitative real-time PCR analysis of osteoblast differentiation markers in MC3T3-E1 cells. Cells were cultured with and without water-soluble *doenjang* extracts at 10 μL/mL for 12 days ($p < 0.05$).

expression (32). TRAP activation was measured after treatment of osteoclast precursor cells with 10 μL/mL water-soluble *doenjang* extracts from *Gangwon* with different fermentation periods from 1 to 5 years. After primary bone marrow macrophages differentiated, TRAP activities of cells treated with extracts distinctly ($p < 0.05$) decreased, compared with controls (Fig. 5A). There was not difference in osteoclast differentiation among fermentation periods of extracts ($p > 0.05$). But, the inhibition of osteoclast differentiation was slightly higher in 3-year fermented *doenjang*. The degree of osteoclast differentiation was confirmed based on TRAP staining (Fig. 5B). Differentiation-induced multinuclear cells were observed when osteoclast differentiation was induced using TRANCE at 200 ng/mL. However, for differentiation induction after *doenjang* extract stimulation along with TRANCE, the number of multinuclear cells stained with TRAP decreased by 30%, compared with controls treated with only TRANCE (Fig. 5B and 5C). Therefore, *doenjang* extracts inhibited osteoclast differentiation. TRAP positive multinucleated

cells containing 3 or more nuclei were numbered

Changes in gene expression related to osteoclast differentiation were examined to determine effects on osteoclast differentiation inhibition. RNA expression changes were confirmed based on induction of osteoclast differentiation for 10 days under extract stimulation (Fig. 5D). Genes related to osteoclast differentiation are *CTSK* (Cathepsin K), *MMP-9* (Matrix metalloproteinase 9), *mTRAP* (tartrate-resistant acid phosphatase), *OPN* (Osteopontin), *rhoC*, and *V-ATPase* (30–32). Expressions of *MMP-9*, *RhoC*, and *mTRAP* were reduced in osteoclasts cultured with extracts, compared with controls. However, there were no significant ($p > 0.05$) differences in *Cathepsin K* and *V-ATPase* expressions, compared with controls, probably due to different gene expressions in different differentiation stages.

In conclusion, water-soluble extracts of *doenjang* exhibited anti-cancer effects of induction of apoptosis and inhibition of proliferation-related genes in breast cancer cells. However, *doenjang* extracts did

Fig. 5. Effects of water-soluble *doenjang* extracts on osteoclast differentiation. (A) TRAP activity ($p < 0.05$). (B) TRAP staining ($\times 200$). Primary bone marrow macrophage cells were treated with M-SCF and TRAN-CE (RANKL), or with M-SCF, TRAN-CE (RANKL), and water-soluble *doenjang* extracts at 10 $\mu\text{L/mL}$ for 7 days. (C) TRAP positive multinucleated cells containing 3 or more nuclei were numbered in mouse BMM cells ($p < 0.05$). (D) RT-PCR. mRNA expression of osteoclast differentiation markers. Primary bone marrow cells were differentiated to mature osteoclasts cultured with and without water-soluble *doenjang* extracts at 10 $\mu\text{L/mL}$ for 10 days.

not affect apoptosis and proliferation in normal HEK 293 cells. In addition, extracts controlled genes associated with osteoblastic and osteoclastic differentiation in which bone-forming osteoblastic differentiation was facilitated while bone resorbing osteoclastic differentiation was inhibited (29-32). Prior studies of *doenjang* have mainly focused on the effects of soybean components and isoflavones isolated from *doenjang* methanol extracts. There are, however, no known studies about products of hydrolytic enzymes formed by microorganisms through fermentation.

Herein, for the first time, bioactive effects of water-soluble peptides against breast cancer proliferation inhibition and bone metabolism activation were investigated. Bioactivities of water-soluble *doenjang* products fermented by microorganisms in breast cancer cells and osteocytes were confirmed. Genetic regulation studies were also performed for investigation of the effects of water-soluble *doenjang* extracts. However, genetic regulation in EGFR/JAK2 protein expression was not demonstrated (data not shown). Water-soluble *doenjang* extracts decreased amounts of non-phosphorylated forms of EGFR/JAK2 proteins, perhaps due to the effect of protein degradation, a topic for future research. Nonetheless, genes related to proliferation and apoptosis were affected by *doenjang* extracts, except EGFR/JAK2.

Extracts were proteolytic fermentation products of microorganisms, and the fermentation period of *doenjang* was influential for *doenjang* extract effects. Therefore, extract effects for fermentation from 1 to 5 years on cells were also identified. Extract effects slightly increased proportionally in relation to the fermentation period. Conflicting results suggesting that breast cancer and osteoporosis are associated with an excess or deficit of estrogen hinder development of a cure (33-34). Water-soluble extracts of *doenjang* showed anti-cancer effects against breast cancer and bone metabolism bioactivities and can serve as an alternative to conventional therapies. Further studies of microorganism fermentation peptide products are required for

scientific analysis of water-soluble extracts of *doenjang*. Also, comparative studies regarding anticancer effects of methanol and water-soluble extracts of *doenjang* are required for assessment of the effective value of *doenjang*.

Acknowledgments This research was supported by the Advanced Production Technology Development Program, Ministry of Agriculture, Food and Rural Affairs, Republic of Korea, and by the SRC Research Center for Women's Diseases of Sookmyung Women's University, Republic of Korea.

Disclosure The authors declare no conflict of interest.

References

1. Lee YW, Kim JD, Zheng J, Row KH. Comparisons of isoflavones from Korean and Chinese soybean and processed products. *Biochem. Eng. J.* 36: 49-53 (2007)
2. Lim SY, Rhee SH, Park KY. Inhibitory effect of methanol extracts of *doenjang* on growth and DNA synthesis of human cancer cells. *J. Korean Soc. Food Sci. Nutr.* 33: 936-940 (2004)
3. Cui CB, Lee EY, Lee DS, Ham SS. Antimutagenic and anticancer effects of ethanol extracts from Korean traditional *doenjang* added sea tangle. *J. Korean Soc. Food Sci. Nutr.* 31: 322-328 (2002)
4. Chung NH. Studies on inhibitory effect of human breast cancer cells and protection against endocrine disruptors of fermented soy extracts. MS thesis, Catholic University of Korea, Bucheon, Korea (2002)
5. Lee SJ. Studies on the enhancement of cancer preventive effects of *doenjang*. PhD Thesis, Pusan National University, Pusan, Korea (2002)
6. Woo SS, Kim SH. Relationships between intakes of soybean food, dietary isoflavone and osteoporosis incidence among postmenopausal women in Daejeon city. *J. Korean Soc. Food Cult.* 24: 588-596 (2009)
7. Lee CH, Song GS, Kim YS. Effects of *cheonggukjang* and *doenjang* on bone loss in ovariectomized rats. *Food Sci. Biotechnol.* 17: 553-557 (2008)
8. Park KY, Moon SH, Baik HS, Cheigh HS. Antimutagenic effect of *doenjang* (Korean fermented soy paste) toward aflatoxin B1. *J. Korean Soc. Food Sci. Nutr.* 19: 158-162 (1990)
9. Ko JA, Koo SY, Park HJ. Effects of alginate microencapsulation on the fibrinolytic activity of fermented soybean paste (*cheonggukjang*) extract. *Food Chem.* 111: 921-924 (2008)
10. Park JW, Lee NK, Kim BY, Kim HK, Kwon KO, Hahm YT. Characterization of

- traditionally fermented Korean soybean paste, *eoyukjang*, and isolation of its microorganisms. Food Sci. Biotechnol. 19: 425-430 (2010)
11. Kim TW, Kim YH, Jung HJ, Park CS, Kim HY. Screening of strains with fibrinolytic activity and angiotensin-converting enzyme inhibitory activity from *doenjang*. Food Sci. Biotechnol. 21: 581-585 (2012)
 12. Ahn SW, Kim KM, Yu KW, Noh DO, Suh NH. Isolation of angiotensin I converting enzyme inhibitory peptide from soybean hydrolysate. Food Sci. Biotechnol. 9: 378-381 (2000)
 13. Yang BK, Jeong SC, Hur NJ, Ha SO, Kim KY, Kym KH, Yun JW, Song CH. Hypoglycemic effects of extracts of soybean paste containing mycelia of mushrooms in streptozotocin induced diabetic rats. Korean J. Mycol. 28: 126-129 (2000)
 14. Lee SY, Kim IS, Park SL, Lim SI, Choi HS, Choi SY. Antidiabetic activity and enzymatic activity of commercial *doenjang* certified for traditional foods. KSBB J. 27: 361-366 (2012)
 15. Kwon SH, Shon MY. Antioxidant and anticarcinogenic effects of traditional *doenjang* during maturation periods. Korean J. Food Preserv. 11: 461-467 (2004)
 16. Oh HJ, Kim CS. Antioxidant and nitrite scavenging ability of fermented soybean foods (*chungkukjang*, *doenjang*). J. Korean Soc. Food Sci. Nutr. 36: 1503-1510 (2007)
 17. Park KY, Lee JM, Moon SH, Jung KO. Inhibitory effect of *doenjang* (fermented korea soy paste) extracts and linoleic acid on the growth of human cancer cell lines. J. Food Sci. Nutr. 5: 114-118 (2000)
 18. Shin JY, Kim TH, Kim AK. Anticancer effects of the isoflavone extracts from *chungkukjang* via cell cycle arrest and apoptosis in mda-mb-453 cells. Yakhak Hoeji 58: 33-39 (2014)
 19. Kwon DY, Hong SM, Ahn IS, Kim MJ. Isoflavonoids and peptides from *meju*, long-term fermented soybeans, increase insulin sensitivity and exert insulinotropic effects *in vitro*. Nutrition 27: 244-252 (2011)
 20. Park VV, Koo MS, Kasymova TD, Kwon DY. Isolation and identification of peptides from soy 11s-globulin with hypocholesterolemic activity. Chem. Nat. Compd. 41: 710-714 (2005)
 21. Park VV, Koo MS, Lee NR, Oh SK, Kim MS, Lee JS, Kwon DY. Hypocholesterolemic soybean peptide (IAVP) inhibits HMG-CoA reductase in a competitive manner. Food Sci. Biotechnol. 14: 727-731 (2005)
 22. Jung KO, Park SY, Park KY. Longer aging time increases the anticancer and antimetastatic properties of *doenjang*. Nutrition 22: 539-545 (2006)
 23. Kim HJ, Sohn KH, Chae SH, Kwak TK, Yim SK. Brown color characteristics and antioxidizing activity of *doenjang* extracts. Korean J. Food Cook. Sci. 18: 644-654 (2002)
 24. Ku KH, Park KM, Kim HJ, Kim YS, Koo MS. Quality Characteristics of *doenjang* by aging period. J. Korean Soc. Food Sci. Nutr. 43: 720-728 (2014)
 25. Teitelbaum SL. Bone resorption by osteoclasts. Science 289: 1504-1508 (2000)
 26. Kang JS, Kim SY, Kim HS, Cho HL. The effects of vitamin C on lipid contents and fatty acid compositions of serum and liver in rats treated with radiation or aflatoxin B. J. Korean Soc. Food Sci. Nutr. 36: 163-173 (2007)
 27. Jorissen RN, Walker F, Pouliot N, Garrett TP, Ward CW, Burgess AW. Epidermal growth factor receptor: Mechanisms of activation and signalling. Exp. Cell Res. 284: 31-53 (2003)
 28. Manolagas SC. Birth and death of bone cells: Basic regulatory mechanisms and implications for the pathogenesis and treatment of osteoporosis. Endocr. Rev. 21: 115-137 (2000)
 29. Choi JY, Lee BH, Song KB, Park RW, Kim IS, Sohn KY, Jo JS, Ryoo HM. Expression patterns of bone-related proteins during osteoblastic differentiation in MC3T3-E1 cells. J. Cell. Biochem. 61: 609-618 (1996)
 30. Seibel MJ. Biochemical markers of bone turnover: Part I: Biochemistry and variability. Clin. Biochem. 26: 97-122 (2005)
 31. Owen TA, Aronow M, Shalhoub V, Barone LM, Wilming L, Tassinari MS, Kennedy MB, Pockwinse S, Lian JB, Stein GS. Progressive development of the rat osteoblast phenotype *in vitro*: Reciprocal relationships in expression of genes associated with osteoblast proliferation and differentiation during formation of the bone extracellular matrix. J. Cell. Physiol. 143: 420-430 (1990)
 32. Suda T, Takahashi N, Martin TJ. Modulation of osteoclast differentiation. Endocr. Rev. 13: 66-80 (1992)
 33. Robert WB, John CH, Edgar SD. Aromatase inhibitors in the treatment of breast cancer. Endocr. Rev. 26: 331-345 (2005)
 34. Renqing Z. Immune regulation of bone loss by Th17 cells in estrogen-deficient osteoporosis. Eur. J. Clin. Invest. 43: 1195-1202 (2013)