Thesis for the Degree of Master of Science

An Anti-inflammatory Peptide Isolated from Seahorse *Hippocampus kuda bleeler* inhibits the Invasive Potential of MG-63 Osteosarcoma Cells

by

Yun-Ji Yang

Department of Chemistry

The Graduate School

Pukyong National University

August 2013

An Anti-inflammatory Peptide Isolated from Seahorse *Hippocampus kuda bleeler* inhibits the Invasive Potential of MG-63 Osteosarcoma Cells

Hippocampus kuda bleeler 해마로부터 분리 정제된 항염증 peptide의 MG-63 골육종 세포 전이 억제효과

Advisor: Prof. Sun-Joo Park

by

Yun-Ji Yang

A thesis submitted in partial fulfillment of the requirements

for the degree of Master of science

in Department of Chemistry, The Graduate School

Pukyong National University

August 2013

An Anti-inflammatory Peptide Isolated from Seahorse *Hippocampus kuda bleeler* inhibits the Invasive Potential of MG-63 Osteosarcoma Cells

A dissertation

by

Yun-Ji Yang

Approved as to style and content by:

Prof. Se-Kwon Kim (Chairman)

Prof. Sun-Joo Park (member)

Prof. Hyun-Kwan Shim(member)

August, 2013

An Anti-inflammatory Peptide isolated from Seahorse *Hippocampus kuda bleeler* inhibits the Invasive Potential of MG-63 Osteosarcoma Cells

Yun-Ji Yang

Department of Chemistry, Graduate School,
Pukyong National University

Abstract

Osteosarcoma is the most common primary malignancy of bone, and patients often develop pulmonary metastasis. The mechanisms underlying osteosarcoma metastasis remain to be elucidated. Recently, anti-inflammatory agents were shown to be useful in the treatment of tumor progression. We previously isolated a natural anti-inflammatory peptide from the seahorse Hippocampus kuda bleeler. Here, we examined the antitumor metastasis activity of this peptide and investigated its mechanism. The peptide significantly inhibited 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced invasive migration was associated with reduced expression of matrix metalloproteinases (MMP1 and MMP2). In addition, Rac1 is a member of the Rho family of small GTPases involved in signal transduction pathways that control proliferation, adhesion, and migration of cells during embryonic development and invasiveness of tumor cells. So, we confirmed that TPA stimulation increased intracellular reactive oxygen species (ROS) generation and small GTPase Rac 1 expression, whereas the peptide decreased ROS generation and Rac 1 activation.Taken together, these results suggest that the peptide inhibits invasive migration of MG-63 osteosarcoma cells by inhibiting MMP1 and MMP2 expression through downregulation of Rac 1-ROS signaling.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

TPA	12-*O*-tetradecanoylphorbol-13-acetate
ROS	Reactive oxygen species
ECM	Extracellular matrix
MMPs	Matrix metalloproteinases
iNOS	inducible nitric oxide synthase
DMEM	Dulbecco's modified Eagle's medium
FBS	Fetal bovine serum
MTT	3-(4,5-dimethylthiazol-2-y-l)-2,5-diphenyltetrazolium
GC/MS	Gas chromatography/mass spectroscopy
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
PBS	Phosphate buffered saline
COX-2	cycloxygenase - 2
DTT	dithiothreitol
PMSF	Phenylmethanesulfonyl fluoride
SDS	Sodium dodecyl sulfate
RT-PCR	Reverse transcription-polymerization chain reaction
PKC	Protein kinase C

1. Introduction

Osteosarcoma is the most common malignant bone cancer in children and adolescents (Thompson et al., 2002; Hauben et al., 2003.) Over 80% of patients with osteosarcoma have metastatic or micrometastatic disease at diagnosis (Mehta et al,. 1986; kaste et al., 1999). For patients presenting with metastatic diseases or tumor recurrence, outcomes are far worse, with survival rates below 30% (Zhang et al., 2008). Current treatment for osteosarcoma includes chemotheraphy and surgery. Unfortunately, major problems associated with chemotheraphy persist, in particular, the cytotoxic effects of chemotherapy on normal tissues and organs. Thus, research has focused on the discovery of new agents and strategies to prevent the progression of osteosarcoma.

Our knowledge of the mechanistic control of invasion and metastasis in osteosarcoma is limited. Tumor growth, invasion, and metastasis require tumor cell proliferation, proteolytic digestion of the extracellular matrix (ECM), cell migration through basement membranes into the circulatory system, and extravasation and growth at the metastatic sites (Liotta and Kohn, 2001). Matrix metalloproteinases (MMPs) have been known to contribute to this metalloproteinases (MMPs) have been known to contribute to this metastatic process by degrading basement membranes (Arii et al., 1996; Klein et al., 2004). MMPs and their endogenous inhibitors

participate in the invasive process of human osteosarcoma (Kähäri and Saarialho Kere, 1999; Kimura et al., 2011). MMPs can also promote tumor growth by increasing the bioavailability of growth factors in the ECM (Chambers and Matrisian, 1997). Recent studies have also suggested that anti-inflammatory agents exert substantial protective effects on tumor promotion and metastasis by blocking the expression of MMPs (Kim et al., 2009). Thus, MMPs play a critical role in cancer and inflammation. In addition, among the molecules involved in inflammation, reactcive oxygen species (ROS), such as superoxide and hydrogen peroxide, likely play a role in linking inflammation to carcinogenesis. ROS are well known as key inducers of MMPs and subsequent tumor progression (Mori et al., 2004; Nelson and Melendez, 2004).These findings suggest that downregulation of ROS signals may be an effective tool for the treatment of osteosarcoma. However, the importance of ROS signals on the metastatic property of osteosarcoma cells has not been studied.

A natural peptide from the enzymatic hydrolysates of the seahorse *Hippocampus kuda Bleeler* was found to inhibit collagen release via its anti-inflammatory effect (Ryu et al., 2010). The peptide suppressed nitric oxide production via downregulation of inducible nitric oxide synthase (iNOS), a well known inflammatory factor. In the present study, we examined the effect of this peptide on the metastatic potential of MG-63 osteosarcoma cells. The peptide was found to suppress invasive migration of MG-63 osteosarcoma cells by inhibiting the expression of MMPs via downregulation

of Rac1-ROS signaling.

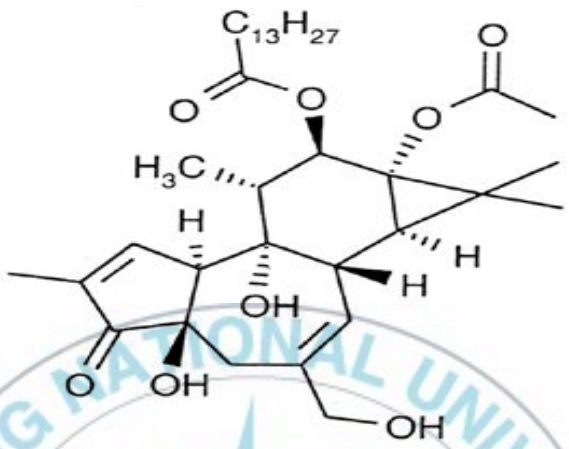

Fig 1. Molecular structure of TPA ($C_{36}H_{56}O_8$, 12-O-tetradecanoylphorbol-13-acetate)

2. Material and methods

2.1. Materials and chemicals

The anti-inflammatory seahorse peptide was prepared as reported by Ryu et al. (2010). Human osteosarcoma cells (MG-63) was obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured as monolayer in 10 cm^2 dish. Cell culture media Dulnecco's Modified Eagle Medium (DMEM), penicillin/streptomycin, fetal bovine serum (FBS) were purchased from Gibco BRL, Life technology. (Grand Island, NY, USA). (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium btomide (MTT) reagent was purchased from Sigma Chemical Co. (St. Louis, Mo, USA). For the cell invasion assay, Matrigel Invasion Chambers was purchased from BD Bioscience (BD Biosciences, SanJose, CA, USA). Antibodies against MMP1, MMP2, Rac1, and actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA), respectively. Dichlorofluorescein diacetate (DCF-DA) was acquired from Molecular Probes (Eugene, OR, USA). Chemicals and reagents were purchange from Sigma, if not otherwise noted.

2.2. Preparation of the anti-inflammatory peptide

The anti-inflammatory seahorse peptide was prepared as reported by Ryu et al. (2010). To prepare the peptide from seahorse protein, enzymatic hydrolysis using

several commercial enzymes (alcalase, neutrase, papain, pepsin, pronase E, and trypsin) was performed. At an enzyme/substrate ratio of 1/100 (w/w), the reaction mixture was incubated for 8 h with strring and then heated in a boiling water bath (100℃) for 10 min to inactivate the enzyme. Pronase E-derived seahorse hydrolysates exhibiting the greatest downregulation of collagen relase and iNOS were further purified by Hiprep 16/10 DEAE FF anion-exchange chromatography (GE Healthcare, Piscataway, NJ, USA) and reverse-phase high-performance liquid chromatography using a Primesphere $10C_{18}$ (20 x 250 mm) column. The purity of the peptide was over 99% according to assessment by reverse phase-high performance liquid chromatography and N-terminal sequence analysis. The amino acid sequence of the purified peptide was determined to be LEDPFDKDDWDNWK by electrospray ionization mass spectrometry spectroscopy.

2.3. Cell culture

MG-63 human osteosarcoma cells from the American Type Culture Collection (ATCC, Manassas, VA, USA) were routinely grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco-BRL, Life Technologies, Grand Island, NY, USA), 100 U/mL penicillin, and 100 mg/mL, streptomycin in 5% CO_2-containing air at 37℃. For experiments, cells were passaged at least three times and detached with Trypsin-EDTA.

2.4. Cell viability assay

Cytotoxic levels of the enzymatically hydrolysis cultured cells were measured using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay (Denizot and Lang, 1986).

MG-63 cells were seeded in 96-well plates at a density of 1 x 10^3 cells/well in DMEM containing 10% FBS. Twenty-four hours after seeding, the medium was changed to serum-free DMEM, and the cells were incubated with various concentrations (0-0.5 mg/mL) of peptide for 24 h. Thereafter, the medium was carefully removed, and 100μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; 1mg/mL final concentration) solution was added to each well prior to incubation for another 3 h at 37℃ in 5% CO_2. Finally, 100μL of DMSO was added to solubilize the formed formazan crystals and the amount of formazan crystal was determined by measuring the absorbance at 540 nm using a microplate reader (iMark; Bio-Rad, Hercules, CA, USA). Relative cell viability was determined by the amount of MTT converted into formazan crystal.

2.5. Cell invasion assay

For the cell invasion assay, the undersurface of the porous membranes in the Matrigel Invasion Chambers (BD Biosciences) were coated with fibronectin (25 μg/mL) at room temperature for 1 h and washed three times in DMEM. DMEM was added to the lower compartment of the chamber. Cells were cultured in DMEM without FBS overnight and treated with or without 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (10 ng/mL) for 24 h in the presence or absence of 0.1 mg.mL peptide, trypsinized, and collected. Subsequently, 200 μL of each cell suspension (2 x 10^5 cells/well in

DMEM) was added to the upper compartment of the chamber and incubated at 37℃ in a humidified atmosphere with 5% CO_2 for 24h. Cells on the upper surface of the membrane were fixed with 3.7% formaldehyde in phosphate-buffered saline (PBS), stained with crystal violet (0.4% dissolved in 10% ethanol) for 15 min, washed twice with PBS, and counted under a phase-contrast microscope with a 10x objective lens. The numbers of cells in nine randomly selected fields from triplicate chambers were counted in each experiment.

2.6. Measurement of ROS

DCF-DA was used to evaluate the generation of intracellular ROS. Cells (3.3×10^4 cells/well) in 24-well plates were first incubated with TPA for 24 h in the presence or absence of peptide. The cells were then washed with PBS and incubated with 10 μM DCF-DA for 30 min at room temperature. Fluorescence was measured using a fluorescence plate reader.

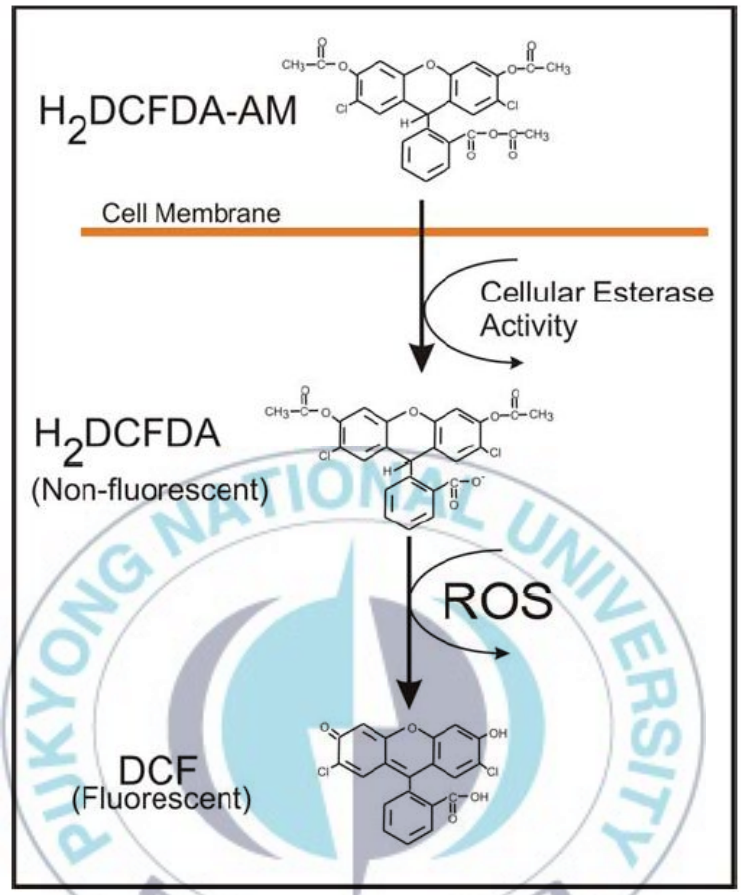

Fig 2. TPA-induced formation of ROS.

2.7. Small GTPase Rac1 activity assay

GST-PAK-CRIB fusion protein was expressed as described previously (Miki et al., 2000) and immobilized on glutathione-seahorse beads (Amersham Biosciences, Piscataway, NJ, USA). Cells were lysed in lysis buffer containing 50 mM Tris-HCl (PH 7.5), 10 mM $MgCl_2$, 1% NP-40, 10% glycerol, 200 mM NaCl, 1 μg/mL aprotinin, 1 μg/mL leupeptin, 1 mM dithiothreitol (DTT), and 1mM phenylmethanesulfonyl fluoride (PMSF). An aliquot of 5-10 μL of cell lysate was mixed with 50 μL (bed volume) of GST-PAK CRIB beads and rotated at 4℃ for 40min. The beads were washed three times with cold wash buffer containing 25 mM Tris-HCl (pH 7.5), 30 mM $MgCl_2$, 40 mM NaCl, 1% NP-40, 1 mM DTT, 1 μg/mL aprotinin, 1 μg/mL leupeptin, 1 mM PMSF, and 20 μL of sodium dodecyl sulfate (SDS) sample buffer containing 50 mM Tris-HCl (pH 6.8), 2% SDS, 6% 2-mercaptoethanol, 10% glycerol, and 0.5 mg/mL bromophenol blue. Samples were separated by SDS-gel electrophoresis, and Rac1-GTP was detected by Western blotting.

2.8. Reverse transcription-polymerization chain reaction (RT-PCR)

MG-63 cells treated with TPA and/or peptide for 24 h were washed with ice-cold PBS twice. Totla RNA was extracted using a commercial kit (RNeasy Mini Kit; Qiagen, Valencia, CA, USA) according to the manufacture's instructions. cDNA synthesis was carried out using 3 μg of total RNA. The following primers were used

to determine target gene levels. MMP1, MMP2, ALP, COX-2 and GAPDH were showed in Table 3. Amplication of target cDNA was carried out at 95 ℃ for 60 sec, 55 ℃ for 30 sec and 72 ℃ for 1 min for 35 cycle. After amplification step, the extension process proceeded consecutively at 72℃ for 10 min. PCR products were separated on 1% agarose gel for 15 min at 100 V by electrophoresis.

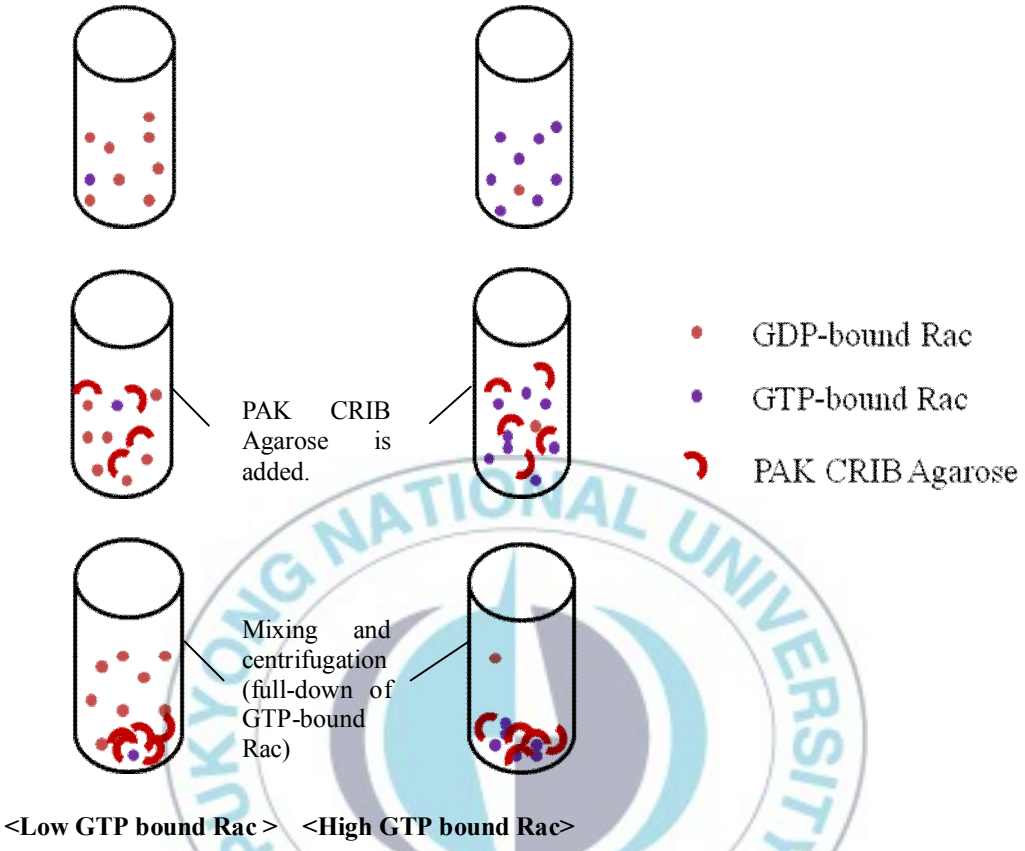

Fig 3. GST-PAK-CRIB fusion protein was expressed as described previously and immobilized on glutathione-sepharose beeds. The PAK-PBD is in the form of a GST fusion protein, which allows one to "pull-down" the PAK-PBD/GST-Rac complex with glutathione affinity beads. The assay therefore provides a simple means of quantitating Rac activation in cells. The amount of activated Rac is determined by a Western blot using a Rac-specific antibody.

Table 1. Chemicals used for PCR reaction.

PCR chemical	Used
10 X Taq buffer with KCl	5 µl
25 mM $MgCl_2$	3 µl
2.5 mM dNTP mix	4 µl
Forward Primer (1.5µM)	3.75 µl
Reverse Primer (1.5µM)	3.75 µl
Template DNA	3 µg
Taq polymerase	1 µl
Nuclease-free water to 28 µl	

Table 2. PCR conditions.

Step	**Temperature**	**Time**	**Number of Cycles**
Initial Danaturation	95 ℃	5 min	1 cycle
Denaturation	95 ℃	30 sec	35 cycles
Annealing	55 ℃	30 sec	
Eaxtension	72 ℃	1 min	
Final Extension	72 ℃	10 min	1 cycle
Soak	4 ℃	Indefinite	1 cycle

Table 3. Gene-specific primers Used for the RT-PCR

primer	sequence	
MMP1	Forward	5′-GGT-CTC-TGA-GGG-TCA-AGC-AG-3′
	Reverse	5′-AGT-TCA-TGA-GCT-GCA-ACA-CG-3′
MMP2	Forward	5′-ATG-ACA-GCT-GCA-CCA-CTC-AG-3′
	Reverse	5′-ATT-TGT-TGC-CCA-GGA-AAG-TG-3′
ALP1	Forward	5`-CCA-CGT-CTT-CAC-ATT-TGG-TG-3`
	Reverse	5`-AGA-CTG-CGC-CTG-GTA-GTT-GT-3`
COX2	Forward	5′-TGA-GCA-TCT-ACG-GTT-TGC-TG-3′
	Reverse	5′-TGC-TTG-TCT-GGA-ACA-ACT-GC-3′
Collagen Ⅰ	Forward	5`-GTG-CTA-AAG-GTG-CCA-ATG-GT-3`
	Reverse	5`-CTC-CTC-GCT-TTC-CTT-CCT-CT-3`
GAPDH	Forward	5′-GAG-TCA-ACG-GAT-TTG-GTC-GT-3′
	Reverse	5′-TTG-ATT-TTG-GAG-GGA-TCT-CG-3′

3. Result and Discussion

3.1 An anti-inflammatory peptide from the seahorse does not affect the viability of MG-63 osteosarcoma cells

We first examined peptide cytotoxicity to human osteosarcoma MG-63 cells and determined the proper peptide concentration showing anti-inflammatory effects in MG-63 cells. MG-63 cells were treated with 0-0.5 mg/mL peptide for 24 h. Peptide treatment at these concentrations did not significantly inhibit cell growth (Fig. 4). We confirmed that the cell stimulation with 10 ng/mL of the pro-inflammatory agent TPA inducs decreased expression of alkaline phosphatase (ALP), a phenotype marker of osteoblast differentiation, whereas 0.1 mg/mL peptide treatment effectively increased TPA-induced ALP expression. (Fig. 5) In addition, Fig. 6 shows that cell stimulation with 10 ng/mL of the pro-inflammatory agent TPA induces increased expression of cyclooxygenase-2 (COX-2), a key enzyme that stimulates prostaglandin production, whereas 0.1 mg/mL peptide treatment effectively suppresses TPA-induced COX-2 expression. This inhibitory effect of peptide on TPA-induced COX-2 expression was not significantly altered at increased peptide concentrations (data not shown). Thus, we performed all subsequent experiments using a peptide concentration of 0.1 mg/mL.

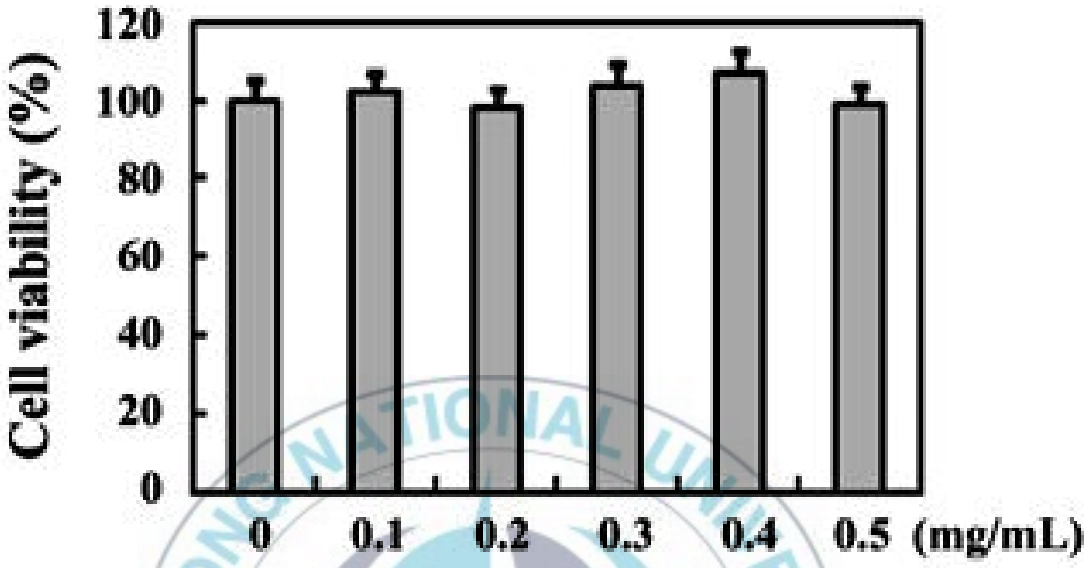

Figure 4. Effect of anti-inflammatory peptide on the cell viability of MG-63 osteosarcoma cells. MG-63 cells were treated with 0-0.5 mg/mL peptide for 24 h under serum-starved conditions, and their viability was determined by MTT assay. The average of the results of 3 independent experiments was used. Phosphate buffered saline-treated cells were used as a control.

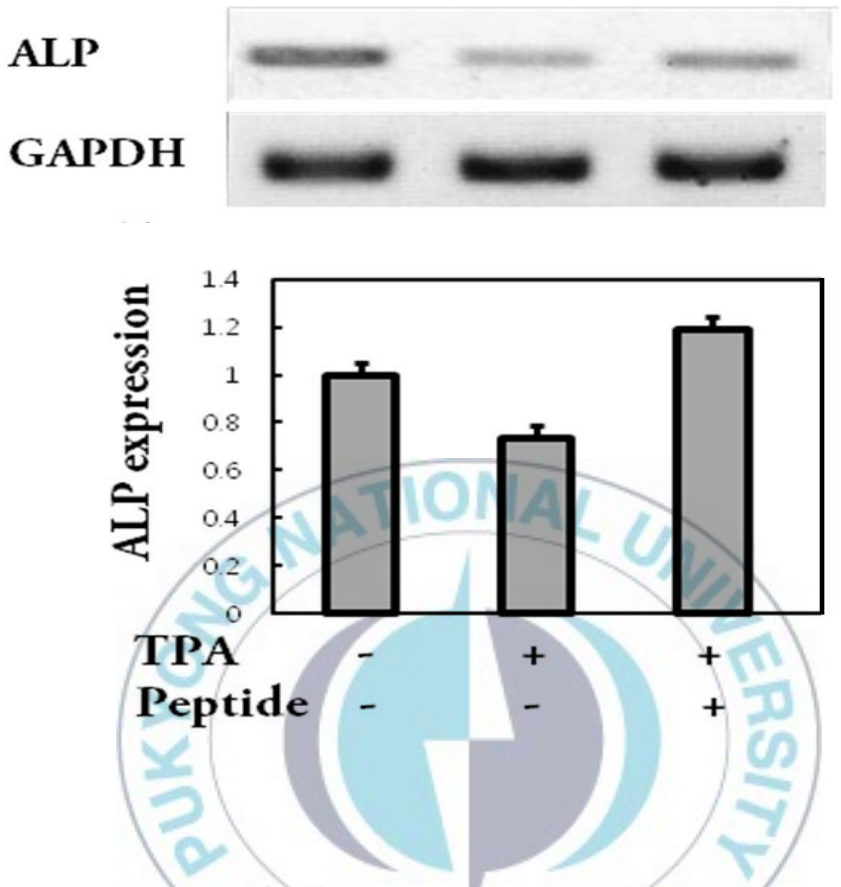

Fig 5. **Cells were incubated with 12-*O*-tetradecanoylphorbol-13 acetate(TPA, 10 ng/mL) in the presence or absence of peptide (0.1 mg/mL) for 24 h. Equal amounts of cell lysates were examined for Alkaline phosphatases (ALP) expression. Representative images are shown. Results of 3 independent experiments were averaged.**

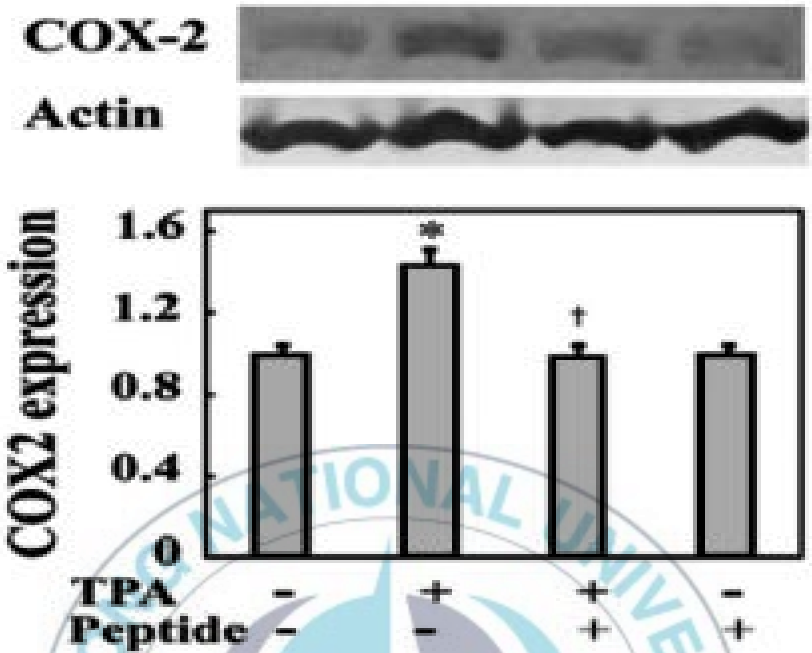

Figure 6. Cells were incubated with 12-*O*-tetradecanoylphorbol-13-acetate(TPA, 10 ng/mL) in the presence or absence of peptide (0.1 mg/mL) for 24 h. Equal amounts of cell lysates were examined for cyclooxygenase 2 (COX-2) expression. Representative images are shown. Results of 3 independent experiments were averaged.

3.2 Peptide inhibits TPA-induced invasive migration of MG-63 osteosarcoma cells.

Although many recent studies have shown that anti-inflammatory agents are capable of exerting antitumor activity in various tumor cells, their actual effects on the metastatic properties of osteosarcoma cells have not yet been described. Therefore, in this study, we investigated whether the anti-inflammatory peptide influenced the metastatic phenotype of osteosarcoma cells, such as invasive migration. As shown in (Fig. 7, Fig. 8) TPA treatment increased the invasive migration of MG-63 cells into Matrigel by approximately 7.5-fold compared to the controls, which were not treated with TPA. Treatment of MG-63 cells with 0.1 mg/mL peptide significantly inhibited TPA-induced cell invasion to approximately one-quarter of that of TPA treatment. Therefore, these results suggest that this peptide regulates an intracellular signaling cascade involved in the invasive migration of MG-63 cells.

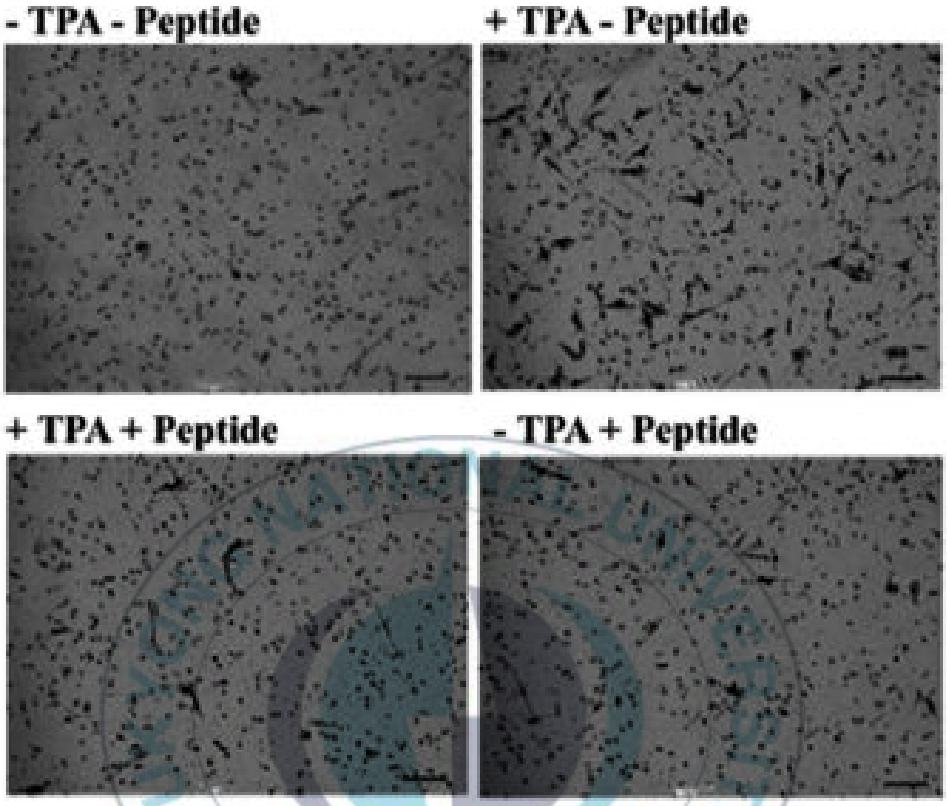

Figure 7. **Matrigel invasion assays were performed with MG-63 cells incubated with TPA (10 ng/mL) in the presence or absence of peptide (0.1 mg/mL) for 24 h. Representative images are shown.**

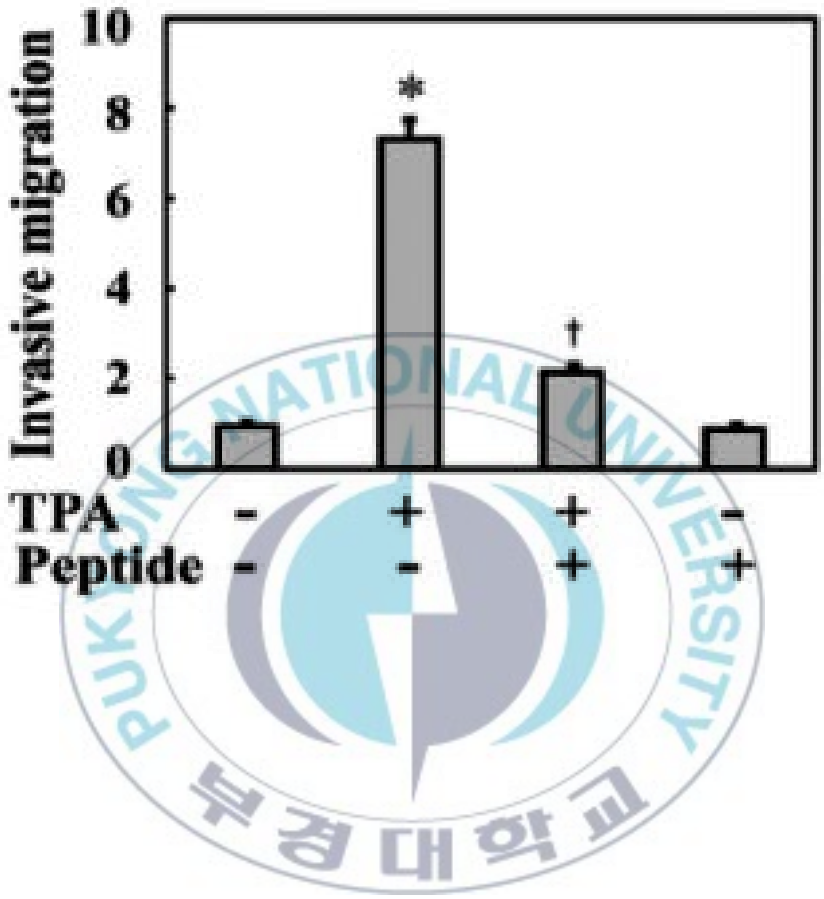

Figure 8. Anti-inflammatory peptide from seahorse inhibits 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced invasive migration of MG-63 osteosarcoma cell. The average of the results of 3 independent experiments was used. Phosphate buffered saline-treated cells were used as a control.

3.3. Peptide decreases TPA-induced intracellular ROS generation and Rac1 activation in MG-63 cells

Reactive oxygen species (ROS) have been reported to be involved in over 150 humna disorders. (B. Halliwell, 1992) ROS are continuously produced in aerobic organisms as byproducts of normal energy metabolism. Much evidence indicates that ROS play a central role in tumor cell migration, invasion, and metastasis (Radisky et al., 2005). ROS are also well known to link inflammation to tumor promotion and metastasis. Thus, ROS generation might also be induced by TPA, which is also a well-known tumor promoter in MG-63 cells. We examined the intracellular ROS levels in MG-63 cells in the presence or absence of TPA stimulation. MG-63 cells were incubated with TPA for 24 h in the presence or absence of peptide. As shown in Fig. 9 and Fig. 10, TPA treatment increased ROS levels to approximately 1.5-fold of those seen in the controls. Comparatively, peptide treatment successfully inhibited TPA-induced ROS generation in cells.

The changes in invasive cell migration under oxidative stress also suggest the involvement of the small GTPase Rac1, which is an upstream regulator critical for actin reorganization and invasive cell migration. Rac 1 is also an upstream enzyme in NADPH oxidase-dependent ROS generation (Bokoch and Knaus, 2003). Generation of ROS under a variety of physiological conditions is associated with Rac1 activation

(Werner and Werb, 2002; Nimnual et al., 2003). Therefore, we tested endogenous Rac1 activation in the presence or absence of TPA and peptide treatment via a GST-PAK binding assay using cell lysates. TPA treatment induced Rac1 activation (Fig. 11 , Fig. 12). These data suggest that the TPA-induced Rac1-ROS cascade is involved in the invasive migration of these MG-63 cells.

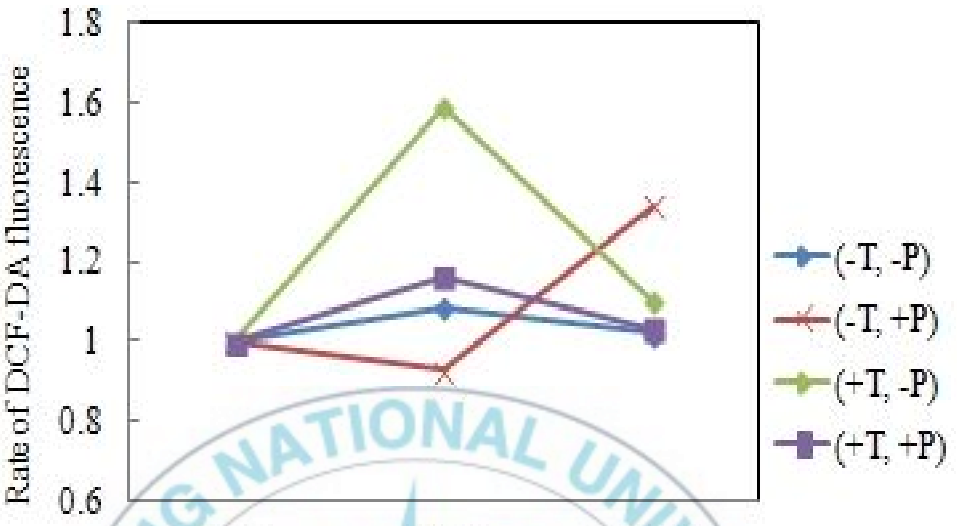

Fig 9. In this data indicated effect of peptide on production of intracellular ROS in TPA-induced MG-63cell. When MG-63 cells were stimulated TPA for 24 h, the relative amounts of ROS increased compared to the control. However, when MG-63 cells were treated with peptide in the presence of TPA, the ROS production was reduced, compared to production after TPA stimulated alone. Especially, DCF-DA treated for 1 h, the ROS peak was very highly.

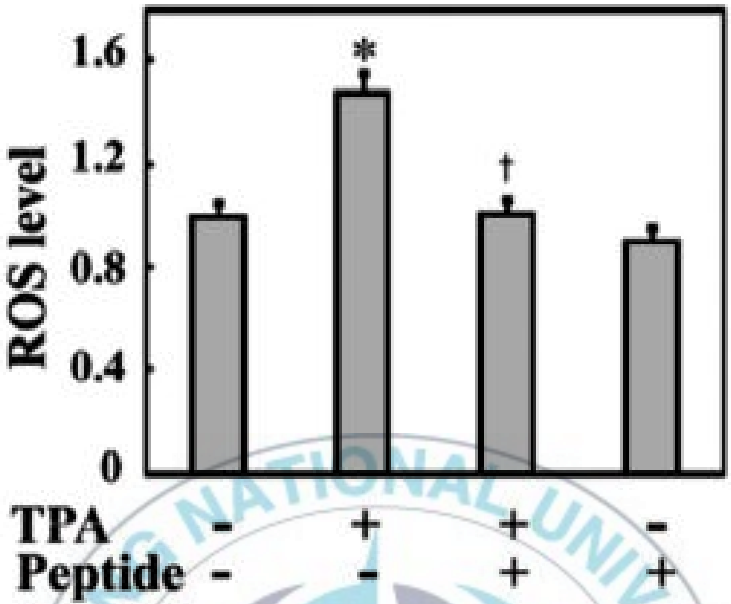

Figure 10. Anti-inflammatory peptide attenuates 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced intracellular reactive oxygen species (ROS) generation and Rac1 activity. Cells were incubated with TPA (10ng/mL) in the presence or absence of peptide (0.1 mg/mL) for 24 h. Cellular ROS levels were assessed by dichloroglucoscein diacetate.

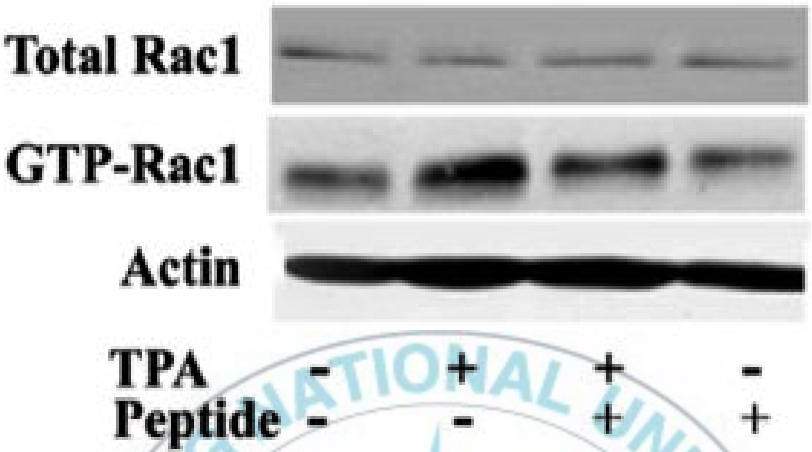

Figure 11. MG-63 cells were incubated with TPA (10 ng/mL) in the presence or absence of peptide (0.1 mg/mL) for 24 h. The purified GST-PAK-PBD fusion protein was incubated with MG-63 cells lysates. The bound proteins were collected, and the GTP-bound Rac1 was detected via western blotting with anti-Rac1 antibody.

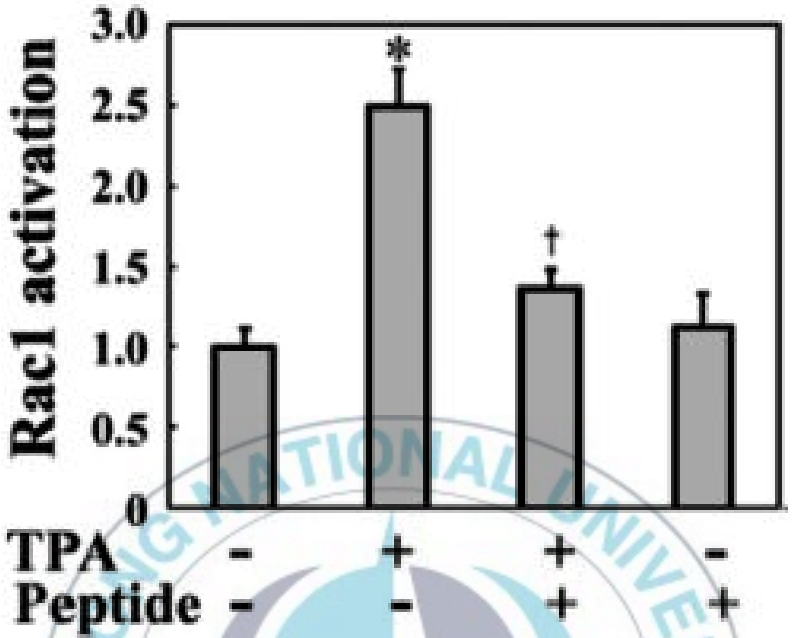

Figure 12. The figure is representative of the results of 3 independent experiments. The average of the results of 3 independent experiments was used. Phosphate buffered saline-treated cells were used as a control.

3.4. Peptide reduces TPA-induced expression of MMPs in MG-63 cells

Matrix metalloproteinases (MMPs) principally participate in the process of matrix degradation and have been regarded as major critical molecules assisting tumor cells during invasion and metastasis. MMPs are responsible for invasive cell migration via their ECM-degrading activity. The Rac1-ROS pathway induces the expression of MMPs in a variety of cell types (Nelson and Melendez, 2004; Radisky et al., 2005). Therefore, we examined whether TPA and peptide treatment affected the expression of MMPs. MG-63 cells were stimulated with TPA for 24 h in the presence or absence of peptides, and then MMP expression was examined by mRNA quantification via RT-PCR. As shown in Fig. 13, TPA stimulation increased the expression levels of MMP1 and MMP2 mRNA in MG-63 cells, while peptide treatment inhibited the TPA-induced expression of these MMPs. In particular, MMP1 mRNA levels increased over 6.2-fold after TPA treatment and were significantly inhinited by peptide treatment. These findings suggest that the peptide suppresses invasive migration of MG-63 cells by inhibiting MMP expression through downregulating intracellular Rac1-ROS signaling. (Fig. 14)

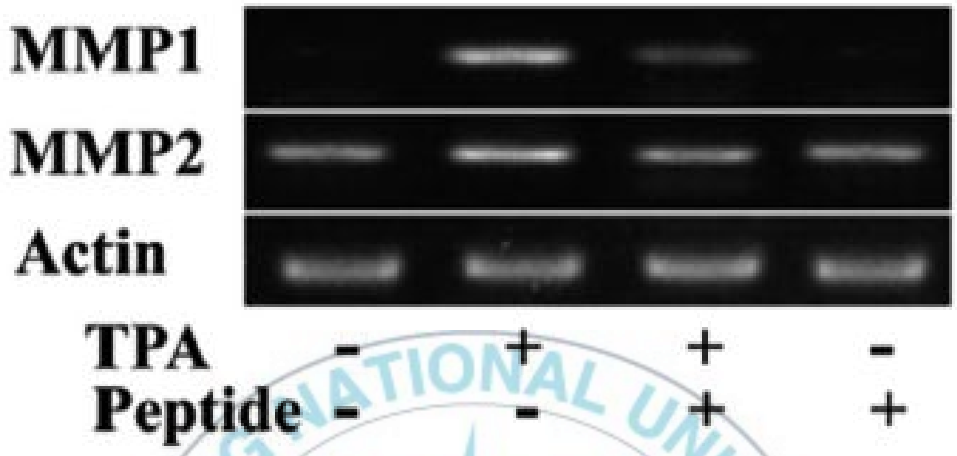

Figure 13. Anti-inflammatory peptide attenuates mRNA expression of matrix metalloproteinase (MMP) 1 and MMP2. MG-63 cells were incubated with 12-*O*-tetradecanoylphorbol-13-acetate (TPA, 10 ng/mL) in the presence or absence of peptide (0.1 mg/mL) for 24 h. Expression of MMP1 and MMP2 was determined by real-time PCR.

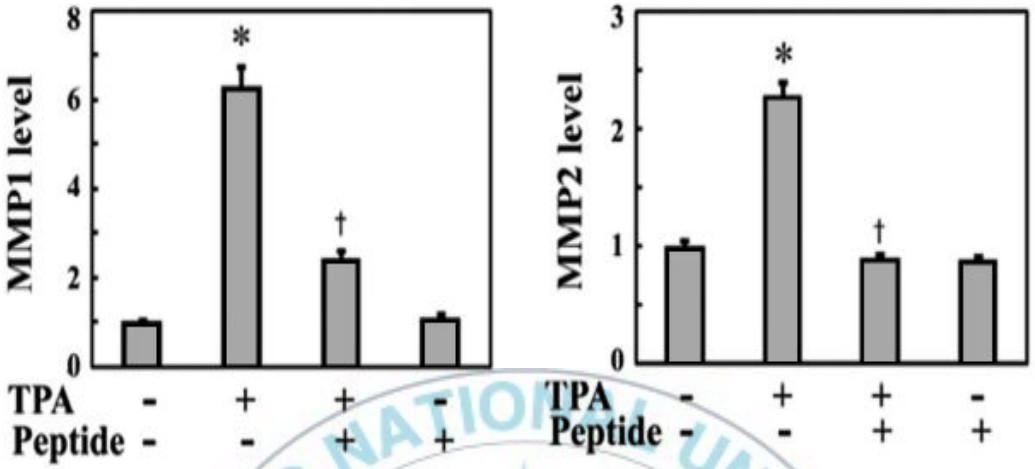

Figure 14. The average of the results of 3 independent experiments was used. PCR products after 35 cycle PCR reaction were also analyzed in 1% agarose gel.

3.5. Peptide increases TPA-induced expression of collagen Ⅰ in MG-63 cells

In the present study, we examined TPA and peptide treatment affected the expression of MMP1 and MMP2. Especially, TPA stimulation increased the over-expression levels of MMP1 mRNA in MG-63 cells, while peptide treatment inhibited the TPA-induced expression of these MMP1. MMP1 was generally known to collagenases cleave native fibrillar collagen types 1,2 and 3. Therefore, we examined whether TPA and peptide treatment affected the expression of collagen Ⅰ. MG-63 cells were stimulated with TPA for 24 h in the presence or absence of peptides, and then collagen expression was examined by mRNA quantification via RT-PCR. As shown Fig. 15, TPA stimulation decreased the expression levels Collagen1 mRNA in MG-63 cells, while peptide treatment enhanced the TPA-induced expression of Collagen Ⅰ.

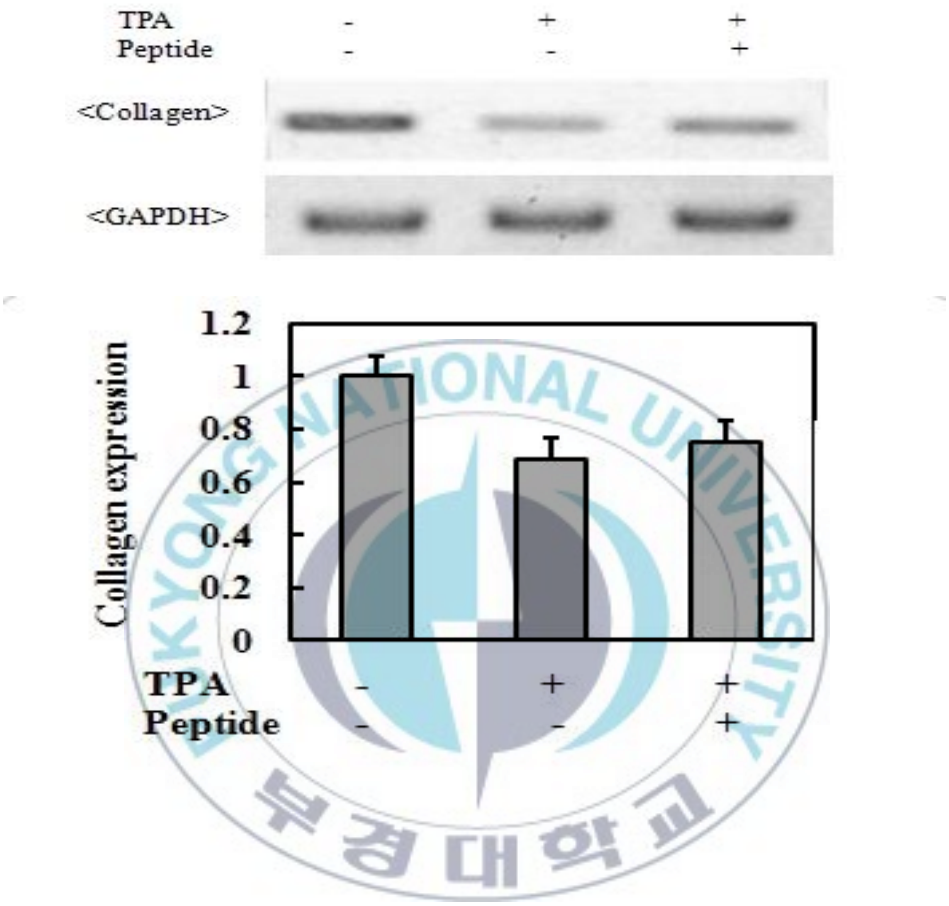

Figure 15. Cells were incubated with 12-*O*-tetradecanoylphorbol-13-acetate(TPA, 10 ng/mL) in the presence or absence of peptide (0.1 mg/mL) for 24 h. Equal amounts of cell lysates were examined for Collagen Ⅰ expression. Representative images are shown. Results of 3 independent experiments were averaged.

4. Discussion

Osteosarcoma is an aggressive malignant bone disorder with a high potential to invade and metastasize. In the present study, we examined the effects of an anti-inflammatory and anti-oxidative agent on the metastatic invasive potential of MG-63 osteosarcoma cells. TPA stimulation increases the invasive migration of MG-63 cells, which correlates with Rac1 activation and increased intracellular ROS levels as well as increased MMP1 and MMP2 expression. In contrast, an anti-inflammatory peptide isolated from seahorse enzyme extracts arrenuated TPA-induced intracellular ROS levels and Rac1 activation. The peptide also decreased the expression of MMP1 and MMP2. Therefore, these results suggest that the TPA-induced Rac1-ROS-linked cascade enhances invasive migration of MG-63 cells by inducing MMPs, whereas the peptide decreases invasive cell migration through downregulation of MMP expression by suppressing intracellular Rac1-ROS signals. Also, anti-inflammatory and anti-oxidative agents are able to exert substantial protective effects against osteosarcoma promotion by blocking MMP expression.

MMPs have been implicated in primary and metastatic tumor growth and angiogenesis (Nelson et al., 2000; Coussens and Werb, 2002). Selected inhibition of MMPs represents an important strategy for prevention or treatment of cancer. Some commercially available synthetic drugs such as Batimastat (BB-94) and Marimastat

(BB-516) have been successfully used to lower MMP expression. However, improper metabolism, low oral bioavailability, poor solubility, side effects, and the risk of increased toxicity of synthetic drugs remain substantial challenges (Thomas and Kim, 2010). Recently, great interest has been expressed in identifying a powerful, nontoxic, and natural anti-inflammatory and anti-oxidative therapeutic agent from natural resources to prevent oxidative stress and reduce inflammatory responses and cancer metastasis. Marine resources are varied and vest, and have unique bioactivities because of the diverse environment. Thus, a marine resource-derived active peptide may be a promising, cost-effective, and safe approach to the prevention of tumor progression. Here, we successfully showed that a natural peptide from seahorse suppresses the MMP signaling response involved in invasive migration of MG-63 cells through the reduction of Rac1-mediated ROS.

The relationship between inflammation and tumor progression has been well documented, for example, recent studies showing that ROS contribute to cell migration and invasion in tumor cells. Classically, ROS were regarded as the host defence molecules released by neutrophils for the destruction of exogenous pathogens. Aberrant increase in intracellular ROS results in physiological and pathological changes, such as cell cycle arrest, apoptosis, and ischemia/reperfusion injury (Boonstra and Post, 2004; Otani, 2004; Gourlay and Ayscough, 2005). In fact, some compounds have been found to exert antitumor effects against osteosarcoma cells by

including excessive ROS generation, cell growth defects, and apoptosis (Chen et al., 2012; Tian et al., 2012). However, recently, much evidence indicates that ROS generated at low concentrations play central roles in intracellular signal transduction pathways for a variety of cellular processes (Chiarugi, 2003; Aslan and Ozben, 2004; Poli et al., 2004). In particular, elevated oxidative stress has been detected in many types of cancer cells (Klaunig et al., 1998; Ambrosone, 2000), and the involvement of ROS signaling in tumor promotion and metastasis has been highlighted (Radisky et al., 2005). Consistent with these reports, we have shown that activated Rac1-ROS signaling in response to TPA stimulation successfully increases MMP expression and results in invasive migration of osteosarcoma MG-63 cells. This is the first report to show a possible mechanism of the antitumor effect of an anti-oxidative agent against the metastatic property of osteosarcoma cells.

We also found that treatment of MG-63 cells with Go6983, a selective protein kinase C (PKC) inhibitor, reduces expression of MMP1 and invasive migration of MG-63 cells (data not shown). The cellular receptor of TPA, relevant to tumor progression and metastasis, was found to be PKC (Debidda et al., 2003; Woo et al., 2004). ROS can be generated by TPA in a PKC-dependent manner (Detta et al., 2000). Therefore, the TPA/PKC-mediated signaling cascade likely cross talks with the MMP pathway to mediate TPA-induced invasive migration of MG-63 cells via ROS generation.

In addition, when we compared the seahorse peptide sequences with known sequences in the translated GenBank database, the peptide sequence was found to have a high identity (>90%) with the corresponding regions of α-enolase from various sources. α-Enolase is a surface receptor for plasminogen. Binding of plasminogen to cell surface and its activation to plasmin by plasminogen activators plays crucial roles in the intravascular and pericellular fibrinolytic systems and several ECM components including laminin and fibronectin. α-Enolase bound plasminogen promotes tumor cell invasion and cancer metastasis through conversion to plasmin and consequent ECM degradation (Pancholi and Fichetti, 1998; Vanegas et al., 2007). α-Enolase in the cytoplasm of tumor cells may be associated with cytoskeletion and other glycolytic enzymes to enhance cell migration. Surface and cytoplasmin α-Enolase may work in concert to promote tumor metastasis. ROS-generating agents have been proposed to upregulate plasminogen activator (Kiguchi et al., 2001). Plasmin also induces matrix degradation via activation of MMPs. Therefore, some other invasion-related genes, such as α-enolase and plasminogen, may also be coupled with the TPA-induced Rac1-ROS cascade to regulate MMP expression and mediate invasive migration of MG-63 cells. Additionally, the seahorse peptide is an aspartic acid-rich oligopeptide. Acidic residues are able to form ionic bonds with target molecules and also function as hydrogen bond acceptors. Many proteins that bind metal ions for structural or functional purposes possess aspartate or

glutamate side chains as metal-binding sites. Therefore, identification of the direct binding targets of the peptide in MG-63 cells may help to precisely understand how the peptide exerts its antitumor effect in MG-63 cells and is an important subject for further research.

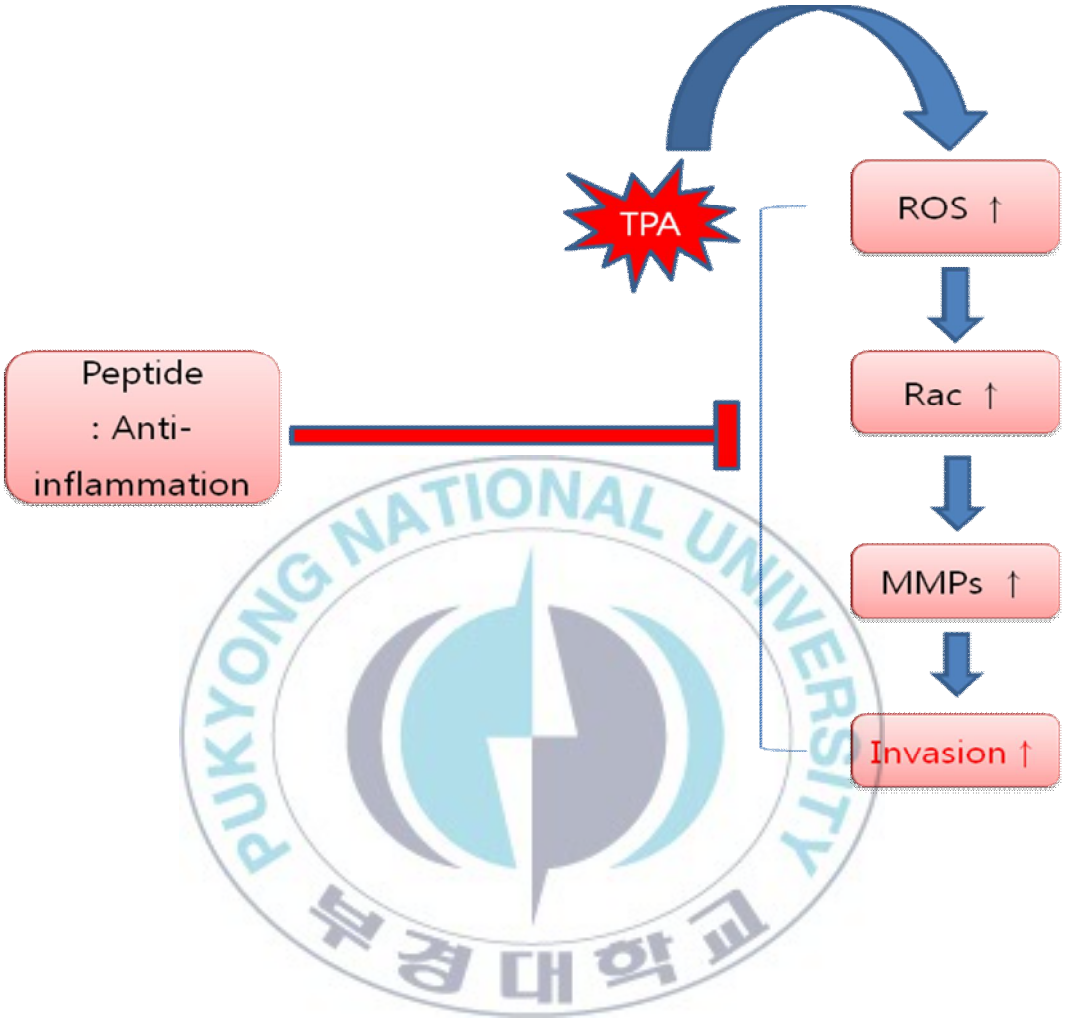

Fig 16. The stimulation of TPA, pro-inflammatory cytokines, leads to an incrase of ROS expression, and then Rac1 to control the increase of tumor cells movement. It leads to an increase in the mediators of inflammation and MMPs, finally tumor invasion induced. But the inhibition of these expression by the peptide can modulate differentiation and the TPA-induced inflammatory response.

5. Reference

Ambrosone CB. 2000. Oxidants and antioxidants in breast cancer. Anti-oxid Redox Signal 2, 903-917.

Arii S, Mise M, Harada T, Furutani M, Ishigami S, Niwano M, Mizumoto M, Fukumoto M and Imamura M. 1996. Overexpression of matrix metalloproteinase 9 gene in hepatocellular carcinoma with invasive potential. Hepatology 24, 316-322.

Aslan M and Ozben T. 2004. Reactive oxygen and nitrogen species in Alzheimer's disease. Curr Alzheimer Res 1, 111-119.

B. Halliwell, J.M. Gutteridge, C.E. Cross, Free radicals, antioxidants, and human disease: where are we now?, J Lab. Clin. Med. 119 (1992) 598-602

Bokoch GM and Knaus UG. 2003. NADPH oxidases: not just for leukocytes anymore!. Trends Biochem Sci 28, 502-508.

Boonstra J and Post JA. 2004. Molecular events associted with reactive oxygen species and cell cycle progression in mammalian cells. Gene 337, 1-13.

Chamners AF and Matrisian LM. 1997. Changing views of the role of matrix metalloproteinases in metastasis. J Natl Cancer Inst 89, 1260-1270.

Chen XJ, Duan FD, Zhang HH, Xiong Y and Wang J. 2012. Sodium selenite-induced apoptosis mediated by ROS attack in human osteosarcoma U2OS cells. Biol Trace Elem Res 145, 1-9.

Chiarugi P. 2003. Reactive oxygen species as mediators of cell adhesion. Ital J Biochem 52, 28-32.

Coussens LM and Werb Z. 2002. Inflammation and cancer. Nature 420, 860-867.

Datta R, Yoshinaga K, Kaneki M, Pandey P and Kufe D. 2000. Phorbol ester-induced generation of reactive oxygene species is protein kinase cβ-dependent and required for SAPK activation. J Biol Chem 275, 41000-41003.

Debidda M, Sanna B, Cossu A, Posadino AM, Tadolini B, Ventura C and Pintus G. 2003. NAMI-A inhibits the PMA-induced ODC gene expression in ECV304 cells: involvement of PKC/Raf/Mek/ERK signaling pathway. Int J Oncol 23, 477-482.

Gourlay CW and Ayscough KR. 2005. The actin cytoskeleton: a key regulator of apoptosis and ageing?. Nat Rev Mol Cell Biol 6, 583-589.

Hauben EI, Arends J, Vandenbroucke JP, van Asperen CJ, Van Marck E and Hogendoorn PC. 2003. Multiple primary malignancies in osteosarcoma patients: incidence and predictive value of osteosarcoma subtype for cancer syndromes related with osteosarcoma. Eur J Hum Genet 11, 611-618.

Kähäri VM and Saarialho-kere U. 1999. Matrix metalloproteinases and their inhibitors in tumour growth and invasion. Ann Med 31, 34-45.

Kaste SC, Pratt CB, Cain AM, Jones-Wallace DJ and Rao BN. 1999. Metastases detected at the time of diagnosis of primary pediatric extremity

osteosarcoma at diagnosis: imaging features. Cancer 86, 1602-1608.

Kiguchi T, Niiya K, Shibakura M, Miyazono T, Shinagawa K, Ishimaru F, Kiura K, Ikeda K, Nakata Y and Harada M. 2001. Induction of urokinase-type plasminogen activator by the anthracycline antibiotic in human RC-K8 lymphoma and H69 lung-carcinoma cells. Int J Cancer 93, 792-297.

Kim S, Choi MG, Lee HS, Lee Sk, Kim SH, Kim WW, Hur SM, Kim JH, Choe JH, Nam SJ, Yang JH, Kim S, Lee JE and Kim JS. 2009. Silibinin suppresses TNF- α-induced MMP-9 expression in gastric cancer cells through inhibition of the MAPK pathway. Molecules 14, 4300-4311.

Kimura R, Ishikawa C, Rokkaku T, Janknecht R and Mori N. 2011. Phosphorylated c-Jun and Fra-1 induce matrix metalloproteinase-1 and thereby regulate invasion activity of 143B osteosarcoma cells. Biochim Biophys Acta 1813, 1543-1553

Klauing JE, Xu Y, Isenberg JS, Bachowski S, Kolaja KL, Jiang J, Stevenson DE and Walborg EF Jr. 1998. The role of oxidative stress in chemical carcinogenesis. Environ Health Perspect 106 Suppl 1, 289-295.

Klein G, Vellenga E, Fraaije MW, Kamps WA and de Bont ES. 2004. The possible role of matrix metalloproteinase (MMP)-2 and MMP-9 in cancer, e.g. acute leukemia. Crit Rev Oncol Hematol 50, 87-100.

Liotta LA and Kohn EC. 2001. The microenvironment of the tumor-host

interface. Nature 411, 375-379.

Mehta P, Lawson D, Ward MB, Lee-Ambrose L and Kimura A. 1986. Effects of thromboxane A2 inhibition on osteogenic sarcoma cell-induced platelet aggregation. Cancer Res 46, 5061-5063.

Miki H, Yamaguchi H, Suetsugu S and Takenawa T. 2000. IRSp53 is an essential intermediate between Rac and WAVE in the regulation of membrane ruffing. Nature 408, 732-735.

Mori K, Shibanuma M and Nose K. 2004. Invasive potential induced under long-term oxidative stress in mammary epithelial cells. Cancer Res 64,7464-7472.

Nelson AR, Fingleton B, Rothenberg ML and Matrisian LM. 2000. Matrix metalloproteinases: biologic activity and clinical implications. J Clin Oncol 18, 1135-1149.

Nelson KK and Melendez JA. 2004. Mitochondrial redox control of matrix metalloproteinases. Free Radic Biol Med 37, 768-784.

Nimnual AS, Taylor LJ and Bar-Sagi D. 2003. Redox-dependent down-regulation of Rho by Rac. Nat Cell Biol 5, 236-241.

Otani H. 2004. Reactive oxygen species as mediators of signal transduction in ischemic preconditioning. Antioxid Redox Signal 6, 449-469.

Pancholi V and Fichetti VA. 1998. α-Enolase, a novel strong plasmin(ogen) binding protein on the surface of pathogenic Streptococci. J Biol Chem 273,

14503-14515.

Poli G, Leonarduzzi G, Biasi F and Chiarpotto E. 2004. Oxidative stress and cell signaling. Curr Med Chem 11, 1163-1182.

Radisky DC, Levy KK, Littlepage LE, Liu H, Nelson CM, Fata JE, Leake D, Godden EL, Alvertson DG, Nieto MA, Werb Z and Bissell MJ. 2005. Rac1b and reactive oxygen species mediate MMP-3-induced EMT and genomic instability. Nature 436, 123-127.

Ryu B, Qian ZJ and Kim SK. 2010. SHP-1, a novel peptide isolated from seahorse inhibits collagen release through the suppression of collagenases 1 and 3, nitric oxide products regulated by NF-kB/p53 kinase. Peptides 31, 79-87.

Thomas NV and Kim SK. 2010. Metalloproteinase inhibitors: status and scope from marine organisms. Biochem Res Int 2010, 845975.

Thompson RC Jr, Cheng EY, Clohisy DR, Perentesis J, Manivel C and Le CT. 2002. Results of treatment for metastatic osteosarcoma with neoadjuvant chemotherapy and surgery. Clin Orthop Relat Res (397), 240-247.

Tian L, Yin D, Ren Y, Gong C, Chen A and Guo FJ. 2012. Plumbagin induces apoptosis via the p53 pathway and generation of reactive oxygen species in human osteosarcoma cells. Mol Med Report 5, 126-132.

Vanegas G, Quiñones W, Carrasco-López C, Concepción JL, Albericio F and

Avilán L. 2007. Enolase as a plasminogen binding protein in *Leishmania mexicana*. Parasitol Res 101, 1511-1516.

Werner E and Werb Z. 2002. Integrins engage mitochondrial function for signal transduction by a mechanism dependent on Rho GTPases. J Cell Biol 158, 357-368.

Woo JH, Lim JH, Kim YH, Suh SI, Min DS, Chang JS, Lee YH, Park JW and Kwon TK. 2004. Resveratrol inhibits phorbol myristate acetate-induced matrix metalloproteinase-9 expression by inhibiting JNK and PKC δ signal transduction. Oncogene 23, 1845-1853.

Zhang P, Yang Y, Zwidler-McKay PA and Hughes DP. 2008. Critical role of notch signaling in osteosarcoma invasion and metastasis. Clin Cancer Res 14, 2962-2969.

Acknowledgement

지난 석사과정은 저에게 있어서 뜻깊은 시간이자 경험이었습니다. 제일먼저 부족한 저에게 끊임없는 지원과 격려를 해주시며, 지속적인 관심을 가져주시고, 세심한 배려로 돌보아주신 박선주 지도교수님께 감사드리며, 모자란 제 논문을 넓은 안목으로 심사해주시고 많은 충고와 따뜻한 격려를 해주신 김세권 교수님과 심현관 교수님께 진심으로 감사를 드립니다.

처음으로 석사과정생을 교육하고 인도하시느라 힘든 역경도 많으셨지만, 끝까지 포기하지 않으시고, 손을 놓지 않고 이끌어 주신 박선주 지도교수님께 진심으로 감사드립니다. 또, 이번에 박사학위를 받으시는 안별님선배님, Zafer 선배님, 석사학위를 받는 김민정님에게도 감사와 함께 축하의 말씀을 전하며, 끝으로 지금까지 곁에서 믿음과 변함없는 사랑을 준 가족들에게 진심으로 감사의 말씀을 전하고 부족한 저를 믿고 따라와주는 대학원의 후배님들과 많은 조언을 해주시는 선배님들에게도 감사드립니다. 이외에 언급하지 못한, 많은 격려와 도움을 주신 분들께 감사드리며, 더욱더 노력하겠습니다.