A Thesis for the Degree of Master of Science

PKCι is a molecular target suppressing UVB-induced MMP-1 expression by 7,8,4'-trihydroxyisoflavone in human dermal fibroblasts

7,8,4'-Trihydroxyisoflavone의 인간 진피섬유아세포에서 자외선에 의한 MMP-1 발현 저해효과 및 표적발굴

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Abstract

Abnormal matrix metalloproteinase-1 (MMP-1) is a representative mediator in photoaging which degrades collagen in human skin tissue. Solar UV induces the activation of various inflammatory signaling pathways such as mitogen-activated protein kinases (MAPKs) and consequently MMP-1 expression. In this paper, 7,8,4'-trihydroxyisoflavone (7,8,4'-THIF), a metabolite of daidzin in soy is suggested to be a possible anti-photoaging agent. 7,8,4'-THIF reduces UVB-induced MMP-1 expression in transcriptional level in human dermal fibroblasts. The effect of 7,8,4'-THIF on UVB-induced MAPKs signaling pathway is evaluated. 7,8,4'-THIF decreases UVB-induced phosphorylation of MEK, MKK4, and MKK3/6 as well as ERK, JNK, and p38. Based on Kinase profiling analysis, protein kinase C iota (PKCι) is suggested as a direct target of 7,8,4'-THIF. In addition, pull-down assay shows that 7,8,4'-THIF directly binds PKCι competitively with ATP. In results,

7,8,4’-THIF produces the anti-photoaging effect by directly targeting PKCι in human dermal fibroblasts and suppressing UVB-induced MMP-1 expression. Thus, 7,8,4’-THIF could be beneficial agent for reducing UV-induced wrinkle formation.

Keywords: 7,8,4’-THIF; Kinase profiling; MMP-1; PKCι; UVB

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I. Introduction

Skin aging can be divided into extrinsic (chronologic) and intrinsic aging. Skin aging is a process of senescence that is commonly related to wrinkling, sagging and laxity [1, 2]. Extrinsic aging is generally considered as photoaging since it is caused by intense and chronic ultraviolet (UV) light exposure. Intrinsic aging is characterized by smooth, dry, pale and finely wrinkled skin. On the other hand, photoaging is characterized by severe wrinkling and pigmentary changes [3].

Solar UV is divided into three wavelength spectra: UVA (320-400nm), UVB (280-320nm), and UVC (200-280nm) [4]. While UVC is blocked by an ozone layer, UVA and UVB penetrate the ozone layer and cause many pathological changes on the human skin [5]. Especially, UVB is responsible for numerous biological effects on human skin such as sunburn, immune-suppression [6], skin cancer as a long term consequence [7], and premature skin aging or photoaging [8]. The matrix metalloproteinases (MMPs) are

a large family of zinc-dependent endoprotease with having a broad range of substrate specificities, and are responsible for the degradation of collagenous extracellular matrix (ECM) in connective tissues such as dermis of the skin [9, 10]. Various MMPs including MMP-1 (collagenase), MMP-2 (gelatinase), and MMP-9 (gelatinase) are expressed in the human skin [11, 12]. Among those MMPs, MMP-1 is the most important MMP in the degradation of the ECM [13]. Repeated exposure to UVB increases MMP-1 expression in dermis tissue and consequently forms skin wrinkle [14].

Many studies have suggested that UV activates growth factor receptor and induces the activation of protein kinase cascades such as the MAPKs signal transduction pathways in the cells [15, 16]. And the MAPKs signaling pathway rapidly induces activator protein-1 (AP-1) activity, which regulates transcription of MMP-1. Increased MMP-1 level interrupts the metabolism ECM and causes the characteristic changes of photoaging in histopathology and

clinical manifestation [17, 18].

Protein kinase C (PKC) structurally relates to serine/threonine protein kinases and plays key roles in fundamental cellular processes such as cell survival and apoptosis [19], proliferation and migration [20], as well as malignant transformation [21]. The PKC family is divided into three groups according to their domain structure: Conventional PKC (cPKC), novel PKC (nPKC), and atypical PKC (aPKC). The different domain structure determines their requirement for allosteric activator. cPKC isoforms (α, β_I, β_{II} and γ) requires Ca^{2+} for activation, as well as diacylglycerol (DAG) and the lipid phosphatidyl serine. nPKC isoforms (δ, ε, η and θ) require DAG and phosphatidyl serine but not Ca^{2+}. aPKC isoforms (ξ and ι) only require a lipid cofactor [22].

PKC is considered as a therapeutic target for various human diseases because several PKC isozymes activate multiple survival pathway [23]. Aberrant PKC activation and/or expression

have been represented in various human diseases. And several studies have shown that PKC is related to MMP-1 [24, 25]. In particular, cPKC has been suggested as a potential regulator of MMP-1.

Many studies have reported that UV irradiation induces various cellular changes by regulating PKC. *Zigang Dong et al.* represented that translocation of PKCξ in membrane regulates UV-mediated signaling pathway in mouse epidermal cell line [26]. Also, recent previous study has reported that heat-induced MMP-1 expression is regulated by PKCα in human skin tissue [27]. Overall, PKC-mediated multiple inflammatory signaling pathways are associated with MMP expression. However, it is still unclear for the role of aPKC isoforms in MMP-1 expression.

Daidzin, 7-O-glucosude of daidzein, is a natural organic compound in the soybean and soy food with high concentration [28, 29]. *Iovine B et al.* reported that daidzin exerts anti-inflammatory and photo-protective effect in human skin cell [30]. Although

daidzin has been regarded as a potential anti-skin aging compound, bio-converted product of daidzein (aglycone of daidzin) has not been studied enough. 7,8,4'-THIF is a bio-converted isoflavone, which is synthesized from daidzein using *N. farcinica*mutant [31]. In this paper, anti-skin aging effects of 7,8,4'-THIF is compared to daidzin on UVB-induced MMP-1 expression, and firstly report that 7,8,4'-THIF decreases UVB-induced MMP-1 expression in human dermal fibroblast by directly suppressing PKCι kinase activity.

Ⅱ. Materials and Methods

2.1. Chemicals and reagents

7,8,4'-THIF was obtained from Indofine chemical company, Inc. (United Kingdom). Dulbecco's modified eagle medium (DMEM) was purchased from Hycolne (Long, UT). Fetal bovine serum (FBS), β-actin antibody were obtained from Sigma-Aldrich (St.Louis, MO). The MMP-1 antibody was obtained from

EPITOMICS, Inc. (Burlingame, CA). Antibodies against phosphorylated extracellular-signal regulated kinase 1/2 (ERK1/2) (Thr^{202}/Ty^{r204}), total ERK1/2, total mitogen-activated protein kinase kinase 4 (MKK4), total c-Jun N-terminal kinase 1/2 (JNK1/2), phosphorylated-p38 (Thr^{180}/Tyr^{182}), and total p38 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against phosphorylated JNK1/2 (Thr^{183}/Tyr^{185}), phosphorylated MKK4 (Ser^{257}/Th^{r261}), phosphorylated mitogen-activated protein kinase/extracellular signal-regulated kinase 1/2 (MEK1/2) ($Ser^{217/221}$), phosphoylated mitogen-activated protein kinase kinase 3/6 (MKK3/6) ($Ser^{189/207}$), total MEK, and total MKK3 were obtained from Cell Signaling Biotechnology (Beverly, MA). 3-[4,5-dimethylatiazol-2-yl]-2,5 diphenyltetrazolium bromide (MTT) powder was purchased from USB co. (Cleveland, OH). Penicillin/streptomycin was purchased from Invitrogen (Grand Island, NY). Protein assay kits were obtained from Bio-Rad Laboratories (Hercules, CA).

2.2. UVB irradiation

UVB irradiation was performed in serum-free media. The spectral peak from the UVB source (Bio-Link crosslinker, VilberLourmat, Cedex 1, France) was set at 312 nm. Primary human dermal fibroblasts were exposed to UVB at a dose of 0.02 J/cm^2.

2.3. Cell culture

Primary human dermal fibroblasts were generously provided from Dr. Chung JH laboratory (Seoul National University Hospital, Korea). Cells were isolated from the outgrowth of foreskin obtained from 7 to 30 year old healthy volunteers. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% (v/v) FBS and 2 penicillin/streptomycin at 37℃ and 5% CO_2.

2.4. Cell viability

The cell cytotoxicity was measured using the MTT assay. Primary human dermal fibroblasts were cultured in the 96well plates at a density of 2×10^3 cells/well and incubated in DMEM-10% FBS containing penicillin/streptomycin at 3℃ in a 5% CO_2 atmosphere and then starved in serum-free DMEM for 24 h. The cells and each sample were incubated for 22 h at 37℃, followed by treatment with MTT solution for 4 h. The medium was removed and formazan crystals were dissolved by the addition of dimethyl sulfoxide (DMSO). The absorbance at 570 nm was then measure using a microplate reader (Molecular Devices, CA) and 7,8,4'-THIF treated and non-treated cells were compared.

2.5. Reverse transcription-polymerase chain reaction (RT-PCR)

Human dermal fibroblasts (2×10^3 cells in a 6-cm dish) were treated with 7,8,4'-THIF for 24 h and harvested in RNAiso Plus (Takara Bio, Inc., Shiga, Japan). After RT with oligo-

dTprimers using a PrimeScript^{TM} 1st strand cDNA synthesis Kit (Takara Bio, Inc.), the cDNA was probed using the following primer (Bioner, Daejeon, Korea): MMP-1 forward 5'-ATT CTA CTG ATA TCG GGG CTT TGA-3', MMP-1 reverse 5'-ATG TCC TTG GGG TAT CCC TGT AG-3' (409bp); Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) forward 5'-GAG TCA ACG GAT TTG GTC GT-3', GAPDH reverse 5'-TTG ATT TTG GAG GGA TCT CG-3'(517bp). Before PCR amplification, primers were denatured at 94℃ for 5 min. Amplification consisted of 22 cycles: denaturation at 94℃ for 30 s, annealing at 56℃ for 1 min, and extension at 72℃ for 1 min, followed by a final 5 min extension at 72℃. PCR was performed in a Gene Amp PCR System 2700 (Applied Biosystems, Foster City, CA, USA). The reaction products were separated by 1.5% continuous agarose gel. GAPDH was used as an internal control.

2.6. Western blot analysis

Primary human dermal fibroblasts were cultured for 48 h and then the cells were incubated in serum free-DMEM for 24 h to exclude any potential FBS activation of cell signaling. After starvation, the cells were treated with or without various concentrations of 7,8,4'-THIF (2.5, 5 and 10 μM) or Daidzin (10 μM) for 1 h, followed by UVB (0.02 J/cm^2) irradiation. The cells were lysed with lysis buffer [10 mM Tris (pH 7.5), 150 mM NaCl, 5 mM ethylene diamine tetra acetic acid (EDTA), 1% Triton X-100, 1 mM dithiothreitol (DTT), 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 10% glycerol and protease inhibitor cocktail tablet] on ice for 30 min, scraped, and then centrifuged at 18,620×g for 10 min. The protein concentration was measured using a dye-binding protein assay kit (Bio-Rad Laboratories) as described by the manufacturer. Using a 10% SDS-polyacrylamide gel, the proteins were separated electrophoritically, and transferred to an Immobilon P membrane (Millipore corporation, USA). The membrane was

blocked in 5% fat-free milk for 1 h and then incubated with the specific primary antibody at 4℃ overnight. Protein bands were visualized using a chemiluminescence detection kit (Amersham Biosciences) after hybridization with the HRP-conjugated secondary antibody (Invitrogen, USA).

2.7. Zymography

Zymography was used to determine the activity of secreted MMP-2. Zymography was performed in 10% polyacrylamide gels in the presence of gelatin (0.5 mg/ml) as a substrate for MMP-2. The samples were suspended in loading buffer [10% SDS, 25% glycerol, 0.25 M Tris (pH 6.8) and 0.1% bromophenol blue], and the run on 10% SDS-PAGE gels without denaturation. After electrophoresis, the gels were washed with renaturating buffer (Invitrogen, USA) at room temperature for 30 min, and then incubated for 24 h at 37℃ in developing buffer (Invitrogen, USA). The gels were then stained with 0.5%

Coomassie brilliant blue.

2.8. Kinase profiling analysis

Kinase profiling analysis was conducted by KinaseProfiler™ service (MERCK Millipore). The analysis was followed manufactur's protocol. Briefly, PKCι (h) is incubated with 20 mM HEPES pH 7.4, 0.03% Triton X-100, 50 μM ERMRPRKRQG-SVRRRV, 10 mM MgAcetate and [γ-33P-ATP] (specific activity approx. 500 cpm/pmol, concentration as required). The reaction is initiated by the addition of the MgATP mix. After incubation for 40 min at room temperature, the reaction is stopped by the addition of 3% phosphoric acid solution. 10 μL of the reaction is then spotted onto a P30 filtermat and washed three times for 5 min in 75 mM phosphoric acid and once in methanol prior to drying and scintillation counting.

2.9. Preparation of 7,8,4’-trihydoxyisoflavone-Sepharose 4B

7,8,4’-THIF-sepharose 4B freeze powder (0.3 g) was suspended in 1 mM HCl to activate the compound. The coupled solution [0.1 M $NaHCO_3$ (pH 8.3) and 0.5 NaCl] was then mixed and rotated at 4℃ overnight. The mixture was washed with the coupling buffer and transferred to 0.1 M Tris-HCl buffer (pH 8.0). Finally, to remove excess uncoupled 7,8,4’-THIF, the mixture was washed with 0.1 M acetate buffer (pH 4.0) and 0.1 M Tris-HCl buffer (pH 8.0) containing 0.5 M NaCl.

2.10. *In vitro* and *ex vivo* Pull-down assays

For the *in vitro* and *ex vivo* pull-down assay, active PKCι or human dermal fibroblasts cell lysate was incubated with 7,8,4’-THIF-Sepharose 4B beads (100 μl) or only Sepharse 4B beads (as a negative control) in the reaction buffer [50 mM Tris (pH 7.5), 5 mM EDTA, 150 mM NaCl, 1 mM DTT, 0.01% Nonidet P-40, 2 μg/ml

bovine serum albumin, 0.02 mM PMSF, and 1x protease inhibitor mixture]. After incubation, the mixture was washed with wash buffer [50 mM Tris (pH 7.5), 5 mM EDTA, 150 mM NaCl, 1 mM DTT, 0.01% Nonidet P-40, and 0.02 mM PMSF]. Finally, the proteins were analyzed by immunobloting.

2.11. Molecular modeling

Computer modeling of 7,8,4'-THIF formed by using the Schrödinger Suite 2011 program [32]. First an X-RAY diffraction structure of the catalytic domain of atypical Protein Kinase C-iota with a resolution of 3.0 Å (PDB ID 1ZIZ) [33], was obtained from the RCSB Protein Data Bank [34]. This structure was prepared under the standard procedure of Protein Preparation Wizard in Schrödinger suite 2012, hydrogen atoms were added consistent with a pH 7 and all water molecules were removed. Finally ATP binding site based receptor grid was generated for docking study. 7,8,4'-THIF was prepared under the program of LigPrep of Schrödinger

for docking by default parameters. Then 7,8,4'-THIF-protein docking was accomplished using the program Glide by default parameters under the extra precision (XP). Herein best-docked representative structure was analysis.

2.12. Statistical analysis

When necessary, the data are expressed as means ± standard deviation (S.D.). The Student's *t*-test was used for statistical comparisons and $p < 0.05$, $p < 0.01$ and $p < 0.001$ were used as the criterion for statistical significance.

Ⅲ. Results

3.1. Effect of 7,8,4’-trihydoxyisoflavone and daidzin on viability of human dermal fibroblast

The effect of 7,8,4’-THIF (Fig. 1A, right panel) and daidzin (Fig. 1A, left panel) on human dermal fibroblasts viability was evaluated using MTT assay. The cells were treated with different concentrations of the 7,8,4’-THIF and daidzin. Only 7,8,4’-THIF represented reduction of viability at 40 μM of 7,8,4’-THIF. However, daidzin did not show cytotoxicity on human dermal fibroblasts at concentrations up to 100 μM (Fig. 1B).

3.2. 7,8,4’-Trihydoxyisoflavone inhibits UVB-induced MMP-1 expression in human dermal fibroblast.

Previous study has demonstrated that MMP-1 expression is closely involved in wrinkle formation [35]. Thus to examine the effect of 7,8,4’-THIF on MMP-1 expression levels, RT-PCR and

western blot analysis were performed. 7,8,4'-THIF dramatically decreased MMP-1 mRNA expression compared with the UVB-irradiated control (Fig. 2A). And, I also confirmed the effect of 7,8,4'-THIF on UVB-induced MMP-1 protein expression in human dermal fibroblasts. I could find that 7,8,4'-THIF markedly inhibited UVB-induced MMP-1 expression in a dose-dependent manner (Fig. 2C). Furthermore, 7,8,4'-THIF exhibited better inhibitory effect on MMP-1 expression compared with daidzin known as isoflavone glycoside at the same dose (10 μM). Taken together, these results represented that daidzin metabolite, 7,8,4'-THIF suppresses transcriptional expression level of UVB-induced MMP-1.

3.3. 7,8,4'-Trihydoxyisoflavone suppresses UVB-induced MAPKs phosphorylation in human dermal fibroblasts.

In Fig. 2A and 2C, 7,8,4'-THIF showed inhibitory effect on UVB-induced MMP-1 expression in human dermal fibroblasts. I thus next examined molecular mechanisms of 7,8,4'-THIF in

human dermal fibroblasts. Based on the previous papers, UVB-induced MAPKs signaling pathway plays an important role in regulating MMPs expression [17]. To investigate the molecular mechanisms by which 7,8,4'-THIF reduces MMP-1 levels, the effect of 7,8,4'-THIF on UVB-induced MAPKs signaling pathway was evaluated. In Fig. 3, I could find that 7,8,4'-THIF decreased UVB-induced MEK-ERK (Fig. 3A), MKK4-JNK (Fig. 3B) and MKK3/6-p38 (Fig. 3C) signaling pathways. Overall, I found that 7,8,4'-THIF suppresses UVB-induced MMP-1 expression by inhibiting the MAPKs signaling pathway in human dermal fibroblasts.

3.4. 7,8,4'-Trihydoxyisoflavone suppresses the PKCι kinase activity

To elucidate the direct target of 7,8,4'-THIF, Kinase profiling analysis was conducted by using KinaseProfiler^{TM} service (MERCK Millipore). The kinase profiling procedure was described

at Materials and Methods part. Prior to KinaseProfiler^{TM} service, I selected 20 kinases for profiling purpose based on three criteria, which are UV-activated kinases [36, 37], upstream regulator of MAPKs [38-41] and MMP-1 regulating proteins [27, 42, 43]. The candidates which belong to the first criteria are c-RAF, cSRC and EGFR whereas candidates that belong to the second criteria are Fyn, PKA, PKB, PKCβ, PKCδ, PKCι, PKCθ, SGK, SIK and Syk. Lastly, PKCα, CaMKI, CaMKIIβ, GSK3β and TGFBR1 were selected based on the third criteria. Interestingly, among these kinases, only PKCι activity was markedly decreased to 62% (Fig. 4) by 7,8,4'-THIF. Taken together, based on Kinase profiling analysis, PKCι is suggested as a direct target of 7,8,4'-THIF (Fig. 4).

3.5. 7,8,4'-Trihydoxyisoflavone directly binds to ATP binding site of PKCι

To determine whether the inhibition of PKCι activity by 7,8,4'-THIF was caused by direct interaction, I performed *in vitro*

pull-down assay using active PKCι. From this analysis, I confirmed that there was a direct binding interaction between PKCι and 7,8,4'-THIF (Fig. 5A). Additionally, I also observed that 7,8,4'-THIF could directly bind with PKCι in cell lysate (Fig. 5B). Therefore the results above indicated that 7,8,4'-THIF inhibited PKCι kinase activity by direct interaction. Next, we evaluated whether the binding between PKCι and 7,8,4'-THIF is ATP competitive or not by using ATP competition binding assay. In Fig. 5C, 7,8,4'-THIF attenuated PKCι activity in an ATP competitive dose-dependent manner. In summary, 7,8,4'-THIF directly binds to ATP pocket of PKCι, to promote the inhibition of PKCι kinase activity.

Ⅳ. Discussion

Chronic sun exposure is a major cause of premature skin aging process, and this phenomenon is called photoaging [44]. Among the solar UV rays, UVB (280-320 nm) accelerates various physiological changes in the cells. By this, photoaging is occurred in result of wrinkle formation, pigmentary changes, and increases of laxity [1, 2]. In wrinkle formation, MMP-1 is a key enzyme because it breakdowns collagen structure in the skin tissue [45]. Thus inhibition of MMP-1 expression is a promising strategy for the photoaging prevention.

Accumulative evidence has reported that soy isoflavones are helpful ingredients for suppressing wrinkle formation [46-48]. Especially, *Toru Izumi et al.* reported that oral intakes of soy isoflavone improves the skin condition in aged skin. In this literature, oral intake group of soy isoflavone represented a statistically significant improvement of wrinkle and skin elasticity compared to the untreated group [49]. Among the soy isoflavones,

daidzin (daidzein 7-glucoside) is one of the representative compounds for nutritional food or cosmetics. Previous papers have reported the anti-photoaging effect and underlying mechanisms of daidzin [30]. However, there is no paper which represents the anti-skin aging effects of metabolites of daidzin.

7,8,4'-THIF is a major bio-converted product from daidzein (aglycone of daidzin), made by a whole cell *Nocardiafarcinica* IFM10152 [31, 50]. This chemical is converted through the hydroxylation of the A-ring of daidzein by enzymatic reaction using the mutants of *N. farcinica* IFM10152 [51]. In this paper, the suppression effect of MMP-1 between daidzein and 7,8,4'-THIF was compared. 7,8,4'-THIF revealed better inhibitory effect on UVB-induced MMP-1 expression than daidzein in human dermal fibroblasts.

Upon UV irradiation exposure, MAP kinase signal transduction pathway is activated, which mediates downstream cellular response [52]. Since previous studies have demonstrated

that MMP-1 expression is regulated by MAPKs activation, the effect of 7,8,4'-THIF on UVB-induced MAPKs activations was investigated. The results showed that UVB-induced phosphorylation of MEK-ERK, MKK4-JNK1/2 and MKK3/6-p38 were decreased in 7,8,4'-THIF treated cells. These observations suggest that 7,8,4'-THIF attenuates UVB-induced MMP-1 expression by inhibiting MAPK signaling pathway (Fig. 3). To elucidate the direct target, the effect of 7,8,4'-THIF on selected 20 kinases activity were investigated by KinaseProfiler^{TM} service (MERCK Millipore). Selected 20 kinases were chosen based on three criteria; which are UV-activated kinases [36, 37], upstream regulator of MAPKs [38-41] and MMP-1 regulating proteins [27, 42, 43]. This result demonstrated that PKCι kinase activity is inhibited by 7,8,4'-THIF (Fig. 4). Furthermore, pull-down assay showed that 7,8,4'-THIF directly binds to ATP binding site of PKCι.

To understand better on how 7,8,4'-THIF interacts with PKCι, the computational docking model was performed by using

Glide docking program of Schrödinger suite 2012. According to the docking models, 7,8,4'-THIF binds well at the ATP binding pocket of PKCι (Fig. 6). Some important hydrogen bonds were formed between 7,8,4'-THIF and (Val^{326} and Asn^{327} at backbone; Lys^{274} and Glu^{293} at side chain). This suggests that 7,8,4'-THIF is considered as a potential inhibitor of PKCι (Some images were generated with UCSF Chimera program [53]).

Recently, several papers have demonstrated the multiple inhibition of cellular signaling pathway by natural chemicals [54, 55]. Therefore, another target proteins of 7,8,4'-THIF cannot be excluded, because only 20 kinases were screened in the current paper. Thus, in order to get more molecular targets of 7,8,4'-THIF, a larger scale screening system is required.

In summary, 7,8,4'-THIF is shown to inhibit UVB-induced MMP-1 expression in human dermal fibroblasts, and PKCι is suggested as a direct target of PKCι. To the best of our knowledge, this is the first indication of an anti-photoaging effect

and the target validation of 7,8,4'-THIF. To expand this finding, *in vivo* or clinical studies will be necessarily conducted. According to these findings, 7,8,4'-THIF exerts a significant photo-protective effect on UVB-induced wrinkle formation. Therefore, 7,8,4'-THIF could be beneficial as a preventing agent for UVB-induced skin aging.

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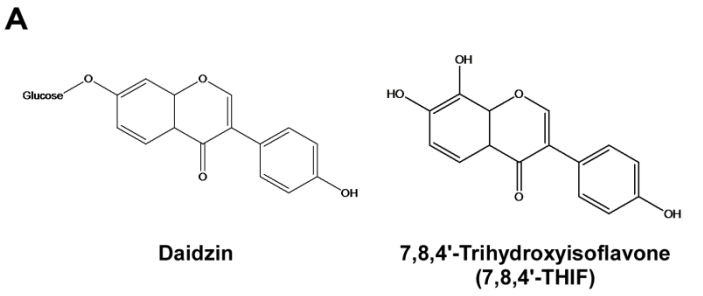
Figure 1


A
Glucose
O
O
O
OH
Daidzin
OH
HO
O
O
OH
7,8,4'-Trihydroxyisoflavone
(7,8,4'-THIF)

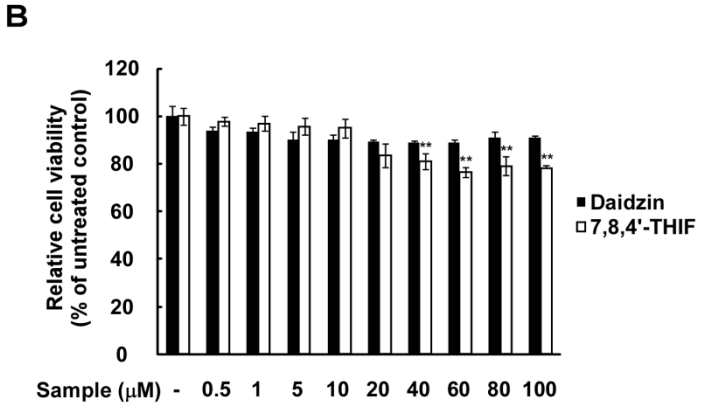
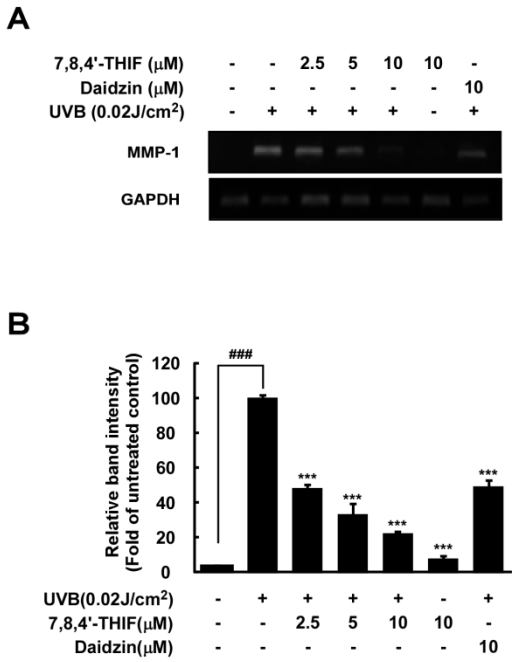

B
Relative cell viability
(% of untreated control)
120
100
80
60
40
20
0
Sample (μM) - 0.5 1 5 10 20 40 60 80 100
Daidzin
7,8,4'-THIF
**

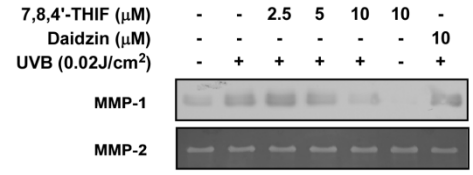
Figure 1. Effect of 7,8,4'-trihydoxyisoflavone and daidzin on viability of human dermal fibroblast.

A, Chemical structure of daidzin and 7,8,4'-Trihydoxyisoflavone (7,8,4'-THIF). **B**, Cell viability in human dermal fibroblasts. The cell cytotoxicity of daidzin and 7,8,4'-THIF was measured using the MTT assay as described in the Materials and Methods. ** $p < 0.01$ versus the non-treated control. Data are shown as the means ± S.D. of three independent experiments.

Figure 2


A
7,8,4'-THIF (μM) - - 2.5 5 10 10 -
Daidzin (μM) - - - - - - 10
UVB (0.02J/cm2) - + + + + - +
MMP-1
GAPDH
B
Relative band intensity (Fold of untreated control)
120
100
80
60
40
20
0
###
***
UVB(0.02J/cm2) - + + + + - +
7,8,4'-THIF(μM) - - 2.5 5 10 10 -
Daidzin(μM) - - - - - - 10

C


7,8,4'-THIF (μM) - - 2.5 5 10 10 -
Daidzin (μM) - - - - - - 10
UVB (0.02J/cm2) - + + + + - +
MMP-1
MMP-2

D

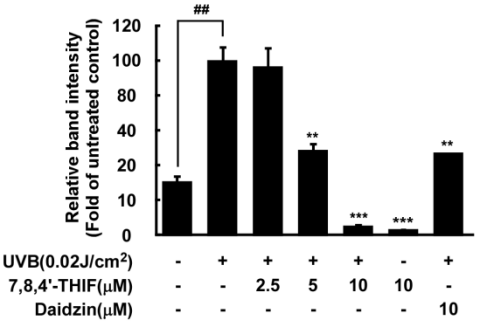

Relative band intensity
(Fold of untreated control)
120
100
80
40
20
10
0
##
**
***
***
**
UVB(0.02J/cm2) - + + + + - +
7,8,4'-THIF(μM) - - 2.5 5 10 10 -
Daidzin(μM) - - - - - - 10

Figure 2. Effects of 7,8,4'-trihydoxyisoflavone on UVB-induced MMP-1 expression in human dermal fibroblast.

A, 7,8,4'-THIF inhibits UVB-induced MMP-1 mRNA levels in human dermal fibroblasts. HDF were pretreated with 7,8,4'-THIF and daidzin at the indicated concentration for 1 h and then exposed to UVB irradiation (0.02 J/cm^2). After 12 h of UVB exposure, MMP-1 mRNA levels were determined by RT-PCR. GAPDH was used as loading control. **B**, The relative band intensity of MMP-1/GAPDH was determined by densitometry. **C**, After 48 h of UVB irradiation, MMP-1 protein secretion into the culture media was determined by western blot analysis. MMP-2 was used as loading control. **D**, The relative band intensity of MMP-1/MMP-2 was determined by densitometry. ## $p < 0.01$, ### $p < 0.001$ significant differences between non-treated control and group treated with UVB alone respectively. ** $p < 0.01$ and *** $p < 0.001$ significant differences between group treated with both 7,8,4'-THIF and UVB and the group treated with UVB alone respectively. Data are shown

as the means ± S.D. of three independent experiments.

Figure 3

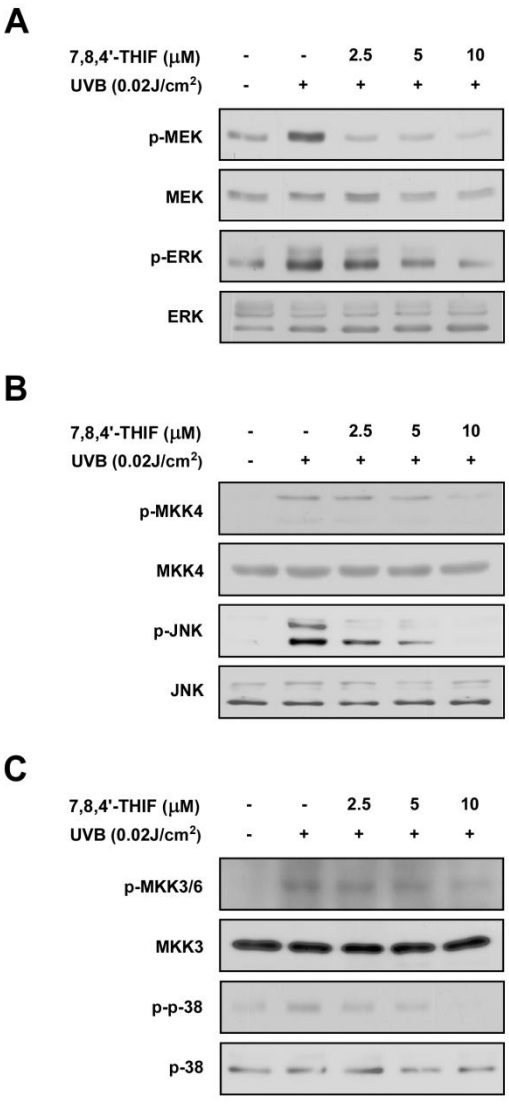
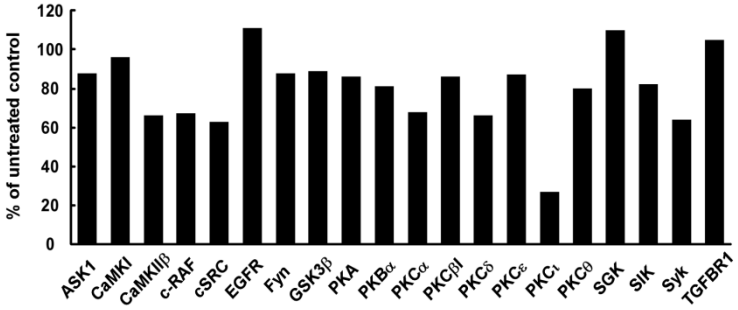

A
7,8,4'-THIF (μM) - - 2.5 5 10
UVB (0.02J/cm²) - + + + +
p-MEK
MEK
p-ERK
ERK
B
7,8,4'-THIF (μM) - - 2.5 5 10
UVB (0.02J/cm²) - + + + +
p-MKK4
MKK4
p-JNK
JNK
C
7,8,4'-THIF (μM) - - 2.5 5 10
UVB (0.02J/cm²) - + + + +
p-MKK3/6
MKK3
p-p-38
p-38

Figure 3. Effect of 7,8,4'-trihydoxyisoflavoneon UVB-induced signaling pathways in human dermal fibroblasts.

A, 7,8,4'-THIF inhibits UVB-induced phosphorylation of MEK-ERK. **B**, 7,8,4'-THIF decreases UVB-induced phosphorylation of MKK4-JNK signaling pathway. **C**, 7,8,4'-THIF reduces UVB-induced phosphorylation of MKK3/6-p38. The phosphorylated- and total protein levels were determined by Western blot analysis using specific antibodies described in the Materials and Methods. Data are representative of three independent experiments that gave similar result.

Figure 4



kinase	% of untreated control
ASK1(h)	88
CaMKI(h)	96
CaMKIIβ(h)	66
c-RAF(h)	67
cSRC(h)	63
EGFR(h)	111
Fyn(h)	88
GSK3β(h)	89
PKA(h)	86
PKBα(h)	81

kinase	% of untreated control
PKCα(h)	68
PKCβI(h)	86
PKCδ(h)	66
PKCε(h)	87
PKCι(h)	27
PKCθ(h)	80
SGK(h)	110
SIK(h)	82
Syk(h)	64
TGFBR1(h)	105

Figure 4. Effect of 7,8,4’-trihydoxyisoflavoneon PKCι kinase activity.

PKCι is suggested the direct target of 7,8,4’-THIF. Kinase profiling analysis was carried out to elucidate the target of 7,8,4’-THIF by KinaseProfiler^{TM} service (MERCK Millipore). The effect of 7,8,4’-THIF (20 μM) on 20 kinases activity was estimated. Data are representative of two independent experiments that gave similar result.

Figure 5

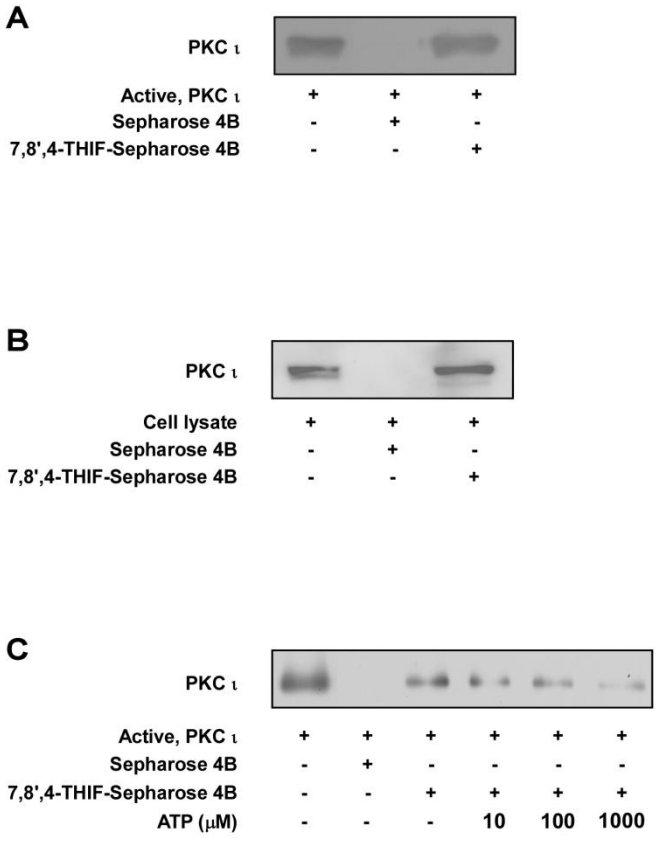

A
PKC ι
Active, PKC ι + + +
Sepharose 4B - + -
7,8',4-THIF-Sepharose 4B - - +
B
PKC ι
Cell lysate + + +
Sepharose 4B - + -
7,8',4-THIF-Sepharose 4B - - +
C
PKC ι
Active, PKC ι + + + + + +
Sepharose 4B - + - - - -
7,8',4-THIF-Sepharose 4B - - + + + +
ATP (μM) - - - 10 100 1000

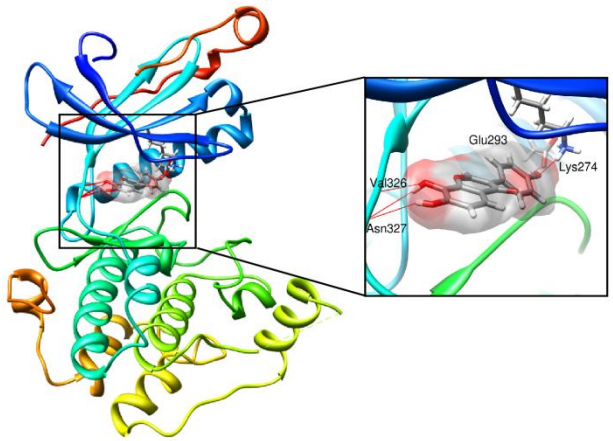
Figure 5. Direct binding between 7,8,4'-trihydoxyisoflavone and PKCι.

A, 7,8,4'-THIF directly binds with PKCι. 7,8,4'-THIF binding was evaluated by immunoblotting using an antibody against PKCι. Lane 1 (input control), active PKCι; lane 2, active PKCι precipitated with Sepharose 4B, and lane 3, active PKCι precipitated with 7,8,4'-THIF-Sepharose 4B. **B**, 7,8,4'-THIF directly interacts with PKCι in cell lysate. The 7,8,4'-THIF-PKCι binding in human dermal fibroblasts was evaluated by immunoblotting; Lane 1, whole cell lysate from human dermal fibroblasts; lane 2, lysate precipitated with Sepharose 4B; lane 3, whole cell lysates from human dermal fibroblasts precipitated by 7,8,4'-THIF-Sepharose 4B beads. **C**, 7,8,4'-THIF binds to ATP pocket of PKCι. Active PKCι was incubated with ATP at different concentrations (10, 100 and 1000 μM) for 1 h, and 100 μl of 7,8,4'-THIF-Sepharose 4B or Sepharose 4B (as negative control) were added. The 7,8,4'-THIF-PKCι binding in human dermal fibroblasts was confirmed by

immunoblotting; Lane 1, (input control), active PKCι; lane 2, active PKCι precipitated with Sepharose 4B; lane 3, active PKCι precipitated with 7,8,4'-THIF-Sepharose 4B; lanes 4, 5 and 6, active PKCι precipitated with 7,8,4'-THIF-Sepharose 4B with different concentrations of ATP. Each experiment were performed three times.

Figure 6

A


Glu293
Lys274
Val326
Asn327

B

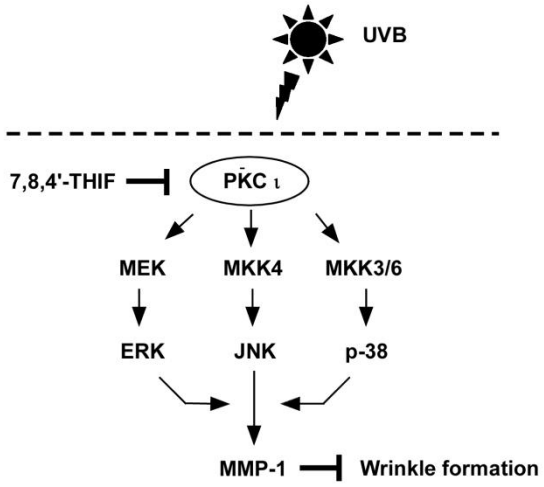

UVB
7,8,4'-THIF
PKC ι
MEK
MKK4
MKK3/6
ERK
JNK
p-38
MMP-1
Wrinkle formation

Figure 6. Hypothetical model of PKCι in complex with 7,8,4'-trihydoxyisoflavone and proposed mechanism of 7,8,4'-trihydoxyisoflavone.

A, The binding pose of 7,8,4'-THIF (Val^{326} and Asn^{327} at backbone; Lys^{274} and Glu^{293} at side chain) the ATP-binding site of PKCι. Here, only the kinase domain of PKCι was shown for clarity. The interaction between 7,8,4'-THIF and several residues in the ATP-binding pocket. Box indicates an enlarged view. 7,8,4'-THIF binds with P38 at the ATP binding pocket and forms some hydrogen bonds (shown as red line). **B**, 7,8,4'-THIF inhibits on UVB-induced MMP-1 expression by directly suppressing PKCι kinase activity in human dermal fibroblasts.

VI. 국문초록

자외선 노출에 의해 유도된 피부노화는 MMP-1의 비정상적인 발현으로 인해 피부 내 콜라겐이 과다하게 분해되는 과정이다. 자외선에 의해 활성화된 MAP kinase 효소는 MMP-1 발현을 증가시켜 노화를 유발시킨다. 본 연구를 통해 다이드제인의 대사체인 7,8,4'-THIF이 콜라겐 분해효소의 발현을 직접적으로 저해함으로써 피부노화를 예방할 수 있음을 제시하였다.

7,8,4'-THIF은 인간 섬유아세포에서 전사인자의 활성을 억제시킴으로써 UVB에 의해 유도된 MMP-1의 발현을 농도 의존적으로 억제하였다. 또한 Western blot을 통해 전사인자를 조절한다고 알려진 MAPKs 신호전달 체계를 확인하였다. 그 결과, 7,8,4'-THIF이 UVB에 의해 유도된 ERK, JNK, p38 뿐만

아니라 MEK, MKK4, MKK3/6의 인산화를 억제시키는 것을 확인하였다. 이후 Kinase profiling analysis를 통해 PKCι가 7,8,4'-THIF의 직접적인 표적 단백질로 제시되었고, 7,8,4'-THIF 농도 의존적으로 PKCι의 활성이 억제되는 것을 확인하였다. PKCι의 활성 억제는 7,8,4'-THIF의 직접적인 결합에 의해서 이루어지고, ATP 경쟁적으로 결합함을 밝혔다.

본 연구 결과를 통해 7,8,4'-THIF은 UVB에 의해 유도된 피부노화를 억제하는 강력한 화학적 효능을 가지고 있음을 밝혔고, 이는 7,8,4'-THIF이 PKCι의 활성을 직접적으로 억제하여 이루어지는 것을 규명하였다. 따라서 7,8,4'-THIF은 자외선 노출에 의해 발생되는 피부 노화를 개선할 수 있는 천연물로 이용될 수 있을 것이다.

주요어: 7,8,4'-THIF; MMP-1; PKCι; Kinase profiling; UVB

감사의 말씀

풋풋한 20 살, 어슴푸레 시작된 저의 꿈은 자신감이 되었고 저를 앞으로 나아가게 만드는 이상이 되었습니다. 그리고 이 결과물을 만들기 위해 쏟았던 시간과 노력 속에서 저는 회의할 줄 알게 되었고, 저만의 꿈을 이루기 위해 늘 깨어있어야 한다는 것을 알게 되었습니다. 이러한 경험 덕분에 저는 진정으로 사는 법을 조금이나마 맛보게 되었을 것입니다. 꿈은 꾸되 늘 깨어있으라는 말의 진정한 뜻을 깨닫기까지 도와주신 수많은 분들께 논문 앞부분을 빌어 감사의 말씀드립니다,

먼저, 저의 지도교수님인 이기원 교수님께 마음 깊이 감사의 말씀을 드립니다. 대학원 첫 입학을 앞두고 무엇부터 시작해야 할지 몰라 전전긍긍하던 저를 부르셔서 격려의 말씀을 해주셨던 날, 저는 24 살 이후 제 인생계획을 세울 수 있는 기회를 얻었습니다. 항상 말보다는 행동으로 몸소 보여주시며 저희가 우러러 볼 수 있게 해주셔서 감사합니다. 이러한 교수님의 가르침을 통해 기회는 찾아오는 것이 아니라 찾아가야 함을 깨닫게 되었습니다. 때로는 범처럼 엄하게, 때로는 어버이처럼 다정하게 저를 이끌어 주셨던 교수님이 계셨기에 2 년이라는 시간 동안 발전하는 학생이 될 수 있었습니다. 2012 년 1 월 1 일 "너희들이 빛날 수 있도록 노력하마"라고 말해주셨던 교수님의 말씀이 기억에 오래도록 남아있습니다. 작은 별이 어둠속에서

빛날 수 있도록 안내자의 역할을 해주신 교수님의 노고를 잊지 않고 더욱 더 빛나는 제자가 되도록 노력하겠습니다.

다음으로 제 대학원 생활에 도움을 주신 분들께 감사의 인사를 드리고자 합니다. 항상 상냥한 웃음으로 저를 대해주셨던 종란언니, 이제는 김종란박사님^^ 축하드립니다. 또한 제가 힘들 때 묵묵히 어깨를 두드려 주시던 수진언니, 언니의 격려 덕분에 힘이 났어요. 실험실 꼬꼬마 시절부터 저를 봐왔던 보경언니, 모르는 것 투성이인 제게 언제나 안내자 역할을 해주셔서 감사해요. 실험실에서 엄마역할을 하시며 항상 저희를 다독여 주시던 희언니, 배울 점이 많은 선배님이자 언니였어요. 언제나 힘차게 파이팅! 하며 하루를 시작하는 언니가 많이 그리울거에요. 실험, 노래, 춤, 외모 어느 것 하나 빠짐없이 완벽한 시영오빠, 투덜대는 날이 많았던 저에 비해 항상 변함없는 자세로 열심히 공부하는 오빠의 모습에서 많은 것을 배웠어요. 항상 제게 좋은 말씀 많이 해주시고, 본보기가 되어주셔서 감사해요. 제게 큰 산처럼 보였던 진환오빠, 낯설고 복잡했던 실험실 생활에 적응할 수 있게 도와주셔서 항상 고마웠어요. 선배가 되어 후배를 바라볼 때 항상 진환오빠 같은 선배가 되어야겠다는 생각을 많이 했었어요. 그만큼 오빠는 제게 좋은 선배님이었어요^^ 나의 하나 뿐인 동기 lai yee, "윤아 힘내요"라는 말을 가장 많이 해준 언니. 언니와 함께한 2 년이라 웃음이 끊이지 않았던 것 같아요.

서로 국적은 다르지만 마음만은 누구보다 잘 통했던 언니를 잊지 못할거에요. 언니 결혼할 때 청첩장 보내는 거 잊지 말아요~ 한 학기 후배인 희정이, 남은 한 학기 힘내서 꼭 좋은 논문 쓰길 바래. 밥한끼 제대로 먹어보지 못한 현경언니, 언니의 다정한 웃음이 많이 기억날 것 같아요. 편하게 대하래도 항상 존댓말을 쓰던 승용오빠, 다음에 만날 땐 편하게 대해주세요! 그리고 내가 가장 힘들 때 짠! 하고 나타난 가은이 원범이. 너희들은 어떠한 것을 줘도 바꿀 수 없는 소중한 후배였어. 고된 몸을 이끌고 집으로 걸어가는 길에 많은 이야기를 나누기도 하고, 한 달이라는 시간동안 동고동락 하며 친 자매처럼 지냈던 가은아. 항상 내편이었던 네가 있어서 큰 힘이 됐어. 언니~언니~하던 네 모습을 잊지 못할 것 같아. 정말 고마워. 피부팀의 든든한 버팀목이 돼주었던 원범아, 항상 선배와 후배를 위하는 너의 따뜻한 마음 잊지 않을게. 표현은 서툴러도 항상 내게 힘을 주었던 네가 있어 큰 힘이 됐어. 이렇게 끝이나는 것 같았던 인연이 너희 형을 통해 다시 이어져서 정말 기뻐. 앞으로 자주보자~ 피부팀 막내로 들어온 은희언니, 다솜아. 힘들어도 항상 웃으며 할 수 있다고 말해주는 후배들이 있어 든든했었어. 앞으로 힘든 일도 많겠지만 꼼꼼하고 책임감 있는 너희이기에 꼭 좋은 결과 있을 거라고 생각해. 피부팀 파이팅! 생글생글 웃는 모습이 예쁜 희림이, 뚝부러진 헤인이. 같은 공간에서 지내지

못해 아쉬운 점이 많지만 후배로써 많은 일들을 묵묵히 해줘서 정말 고마워. 제게는 한분도 빠짐없이 고마운 분들이기에 글이 많이 길어졌습니다. 2년 동안 제게 힘을 주셔서 감사 드립니다.
나의 모든 것을 함께하고 나의 모든 것을 사랑해주는 친구 소정이, 혜원이, 은혜, 혜진이, 혜민이. 그녀들의 격려로 제가 많은 난관을 이겨낼 수 있었습니다. 그녀들과 함께한 시간 속에는 우리의 열정이 고스란히 남아 숨 쉬고 있고 앞으로도 그녀들과 함께 살아나갈 이세상에서 우리의 열정은 식지 않을 것이라는 것을 믿습니다.
석사논문을 쓰는 시간동안, 나에게 가장 가까운 사람으로써 그리고 가장 이성적인 눈으로 조언을 아끼지 않았던, 사랑하는 사람이자 같이 학문하는 사람으로써 훌륭한 본보기가 되어준 승재오빠에게 감사드립니다.
마지막으로 항상 응원해주시는 저의 가족에게 감사드립니다. 언제나 나의 자랑이라 말씀해 주시던 어머니, 그리고 언제나 저의 선택이 옳다고 말해주시던 아버지 감사합니다. 늦은 밤 야식 심부름까지 불평 없이 해준 동생 정규야. 누나를 굳게 믿는 동생이 있어 항상 힘이 났었어. 지금까지도 누구보다 편한 대화의 상대이며 나의 영원한 role model 인 어머니. '도전하는 것에는 젊고 늙고는 없다. 다만 열정이 크고 작음만이 있을 뿐이다'를

모소 실천하고 계시는 아버지. 아버지 딸임이 부끄럽지 않도록 저는 부단히 노력하고 또 노력할 것입니다.

하나의 매듭은 또다시 그 다음 매듭으로 이어질 것입니다. 석사논문은 이제 하나의 시작에 불과할 것입니다. 다음 번에는 좀 더 완성된 모습으로 여러분들께 감사의 말씀을 드릴 수 있게 되길 기원해 봅니다.

2013년 여름, 연구실에서 김윤아올림