p53 이 결손 된 유선상피세포에서의 aromatase 발현 증폭 및 작용기전 연구

Studies on the Aromatase Induction in p53-Inactivated Mammary Epithelial Cells and its Mechanism

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이 논문을 약학 석사학위신청 논문으로 제출함.

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(국문 초록)

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노 상 희

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폐경기 여성에 있어서 p53 결손과 aromatase 과다발현은 유방암 발병에 중요한 인자로 작용한다. 이 논문에서 본인은 p53 유전자의 exon 5,6 번이 결손 된 생쥐의 유선상피세포로부터 1 차 배양되어 얻어진 세포(p53$^{\triangle 5,6}$MEC)에서 aromatase 발현이 정상세포에 비하여 상향 조절되어 있음과 그 신호전달경로를 규명하였다. 정상 유선상피세포와 비교했을 때, p53$^{\triangle 5,6}$MEC 의 aromatase 기저활성과 발현이 확연히 증가되어 있었다. 또한 aromatase promoter 또는 CRE minimal

promoter 가 삽입된 reporter gene 활성 역시 $p53^{\triangle 5,6}$MEC 가 정상세포보다 유의성 있게 높았다. $p53^{\triangle 5,6}$MEC 또는, in vitro 상에서 p53 유전자를 결손 시 CREB 이 인산화되어 핵 내로 축적되었다. $p53^{\triangle 5,6}$MEC 에서 ERK 또는 Src 신호를 선택적 차단제로 억제하면 aromatase 유전자의 과다발현과 CREB 의 핵 내 이동이 차단되었다. 이러한 결과들은 유선상피세포에서 p53 결손이 ERK 또는 Src 신호의 활성을 통해 aromatase 유전자의 발현을 증폭시킨다는 사실을 시사하며, 이는 aromatase 유전자의 발현이 유선상피세포의 p53 활성과 밀접한 관련이 있음을 보여준다.

핵심어 : aromatase; CREB; ERK; Src; p53; 유선상피세포; 에스트로겐 수용체

Studies on the Aromatase Induction in p53-Inactivated Mammary Epithelial Cells and its Mechanism

Abstract

Both the functional loss of p53 and the overexpression of aromatase are important for the progression of breast cancer in postmenopausal women. Here, we found that aromatase expression was up-regulated in primary cultures of mammary epithelial cells ($p53^{\Delta 5,6}$ MEC) isolated from mice with a defect in exons 5 and 6 of the p53 gene. Aromatase basal activity and expression levels were significantly increased in $p53^{\Delta 5,6}$ MEC compared with wild-type MEC. Reporter gene activity in $p53^{\Delta 5,6}$ MEC transfected with the aromatase promoter or the CRE minimal promoter was higher than wild-type MEC. p53 inactivation increased both Ser133-phosphorylated CREB and the nuclear accumulation of CREB. Inhibition of extracellular signal-regulated kinase (ERK) or Src tyrosine kinase blocked *aromatase* gene transactivation and CREB activation in the $p53^{\Delta 5,6}$ MEC. These results support the hypothesis that a genetic defect in the function of p53 enhances the expression of aromatase via ERK or Src activation in MEC, which suggests that aromatase expression is closely related to the p53 status in MEC.

Key words: aromatase; CREB; ERK; mammary epithelial cell; estrogen receptor; Src; p53

1. Introduction

Breast cancer is one of the most common malignancies in Western women. Human breast tumors arise from normal cells after the accumulation of multiple mutations of tumor suppressor genes, such breast cancer susceptibility gene 1/2 (BRCA1/2) and p53 (Mann and Borgen, 1998). Approximately 30-50% of breast cancers carry a mutation in the *p53* gene (Sjogren et al., 1996). Germ-line mutations in the DNA binding domain of *p53* gene present in 70% of patients with Li-Fraumeni syndrome, which shows development of early-onset breast cancer (Malkin et al., 1990; Srivastava et al., 1990).

Breast cancer is also characterized by hormone-dependent proliferation. Ovarian steroid hormones, including estrogen, are critical in both mammary gland development and breast carcinogenesis (Petrangeli et al., 1994; Muller et al., 2002). Clinical application of aromatase (CYP19) inhibitors in adjuvant therapy for hormone-sensitive breast cancer has grown rapidly over the past few years. Unlike tamoxifen, which antagonizes estrogen receptors (ER) and subsequently inhibits transcription of estrogen-responsive genes, aromatase inhibitors block synthesis of estrogens by blocking the

catalysis of C_{19} androgens to estrogens. Although tamoxifen has been approved for the chemoprevention of breast cancer and is the most widely used anti-estrogen in ER-positive breast cancer patients, serious side effects can occur, including endometrial cancer, thrombosis and embolism (Mourits et al., 2001; Cuzick et al., 2003). Additionally, tamoxifen resistance is a serious problem for long-term tamoxifen treatment (Clemons et al., 2002). Hence, there is a shift towards treatment with aromatase inhibitors, especially after third-generation inhibitors (anastrozole, letrozole and exemestane) have shown superiority to tamoxifen (Smith and Dowsett, 2003; Koberle and Thurlimann, 2005).

Two mouse lines have been established with conditional knockouts of the *p53* gene in mammary glands using the Cre–loxP recombination system (Lin et al., 2003). The system requires two mouse lines: one carrying the floxed p53 alleles ($p53^{fp/fp}$) and the other expressing Cre recombinase under the regulation of the whey acidic protein (WAP) promoter (WAP-Cre). Cre expression leads to recombination within the lox P sequences in introns 4 and 6 of the *p53* gene, which deletes part of the DNA binding domain (exon 5 and 6) of p53 and inactivates the gene ($p53^{\Delta 5,6}$). Using the $p53^{fp/fp}$ and $p53^{\Delta 5,6}$

mammary epithelial cells (MEC), we recently showed that ErbB2 expression level is higher in the p53-inactivated MEC through AP-2α activation (Yang et al., 2006).

The expression of aromatase is stimulated by diverse pathophysiological factors such as prostaglandin E2 and leptin (Richard et al., 2003; Catalano et al., 2003). Recent studies also revealed that the transcription of *aromatase* gene is affected by a tumor suppressor, BRCA1 (Hu et al., 2005). Although both p53 mutation and aromatase expression are important for the progression of breast cancer in postmenopausal women, the interaction of aromatase expression with p53 inactivation is unknown. Here, we show for the first time that p53 inactivation enhances aromatase expression and the enzyme activity in primary cultured MEC. We also show that Src- or extracellular signal-regulated kinase (ERK)-dependent cAMP-responsive element binding protein (CREB) activation is required for the aromatase induction in p53-inactivated MEC.

2. Materials and methods

2-1. Materials

The anti-CREB and aromatase antibodies were purchased from Cell Signaling Technology (Beverly, MA) and Abcam (Cambridge, UK), respectively. The alkaline phosphatase and horseradish peroxidase-conjugated donkey anti-mouse, anti-rabbit and anti-goat IgGs were acquired from Jackson ImmunoResearch (West Grove, PA). The 5-bromo-4-chloro-3-indoylphosphate/nitroblue tetrazolium and phRL-SV40 plasmid were purchased from Promega (Madison, WI). PP2 was supplied from Calbiochem (La Jolla, CA). The anti-actin antibody and the other reagents in the molecular studies were obtained from Sigma Chemical (St. Louis, MO). pGRE-Luc plasmid was a gift from Dr. Lee KY (Chonnam National University, Gwangju, South Korea). pCRE-Luc was purchased from Stratagene (La Jolla, CA). The aromatase-Luc was kindly provided by Dr. Jameson (Northwestern University Medical School, Chicago, IL) (Ito et al., 2000).

2-2. Generation of WAP-Cre p53$^{fp/fp}$ mice and mammary epithelial cell (MEC) culture

WAP-Cre mice were mated with p53$^{fp/fp}$ mice, and Cre-

mediated recombination and p53 deletion were confirmed by PCR analysis and x-gal staining, as published previously (Lin et al., 2003). MEC were isolated from the number 4 mammary gland from 6-month-old $p53^{fp/fp}$ or WAP-Cre $p53^{fp/fp}$ mice using a slight modification of a previously published method (Deome et al., 1959). After washing with sterile phosphate-buffered saline (PBS), the mammary gland was minced gently with two knives and dissociated in the presence of 0.15% collagenase for 12 h at 37 °C. The cells were collected and embedded in a fetuin-coated plastic dish and maintained in F-12/DMEM containing 10 ng/ml epidermal growth factor (EGF), 1 μg/ml insulin, 15% fetal calf serum, 100 units/ml penicillin and 100 μg/ml streptomycin at 37 °C in a humidified atmosphere containing 5% CO_2. The cells were used for experiments up to 10 passages.

2-3. Preparation of the nuclear fraction

Cells in dishes were washed with ice-cold PBS, scraped, transferred to microtubes, and allowed to swell after adding 100μl of a lysis buffer containing 10mM HEPES (pH 7.9), 0.5% Nonidet P-40, 10mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol, and 0.5 mM phenylmethylsulfonylfluoride. Cell membranes were disrupted by

vortexing, and the lysates were incubated for 10 min on ice and centrifuged at 7,200 *g* for 5 min. Pellets containing crude nuclei were resuspended in 100 μl of an extraction buffer containing 20 mM HEPES (pH 7.9), 400 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol and 1 mM phenylmethylsulfonylfluoride, and then incubated for 30 min on ice. The samples were centrifuged at 15,800 *g* for 10 min to obtain the supernatants containing the nuclear extracts. The nuclear extracts were stored at -80 °C until needed.

2-4. Immunoblot analysis

Sodium dodecylsulfate (SDS)-polyacrylamide gel electrophoresis and immunoblot analysis were performed according to the procedures reported in the literature (Kang et al., 2003). Cells were lysed in a buffer containing 20 mM Tris·Cl (pH 7.5), 1% Triton X-100, 137 mM sodium chloride, 10% glycerol, 2 mM EDTA, 1 mM sodium orthovanadate, 25 mM β-glycerophosphate, 2 mM sodium pyrophosphate, 1 mM phenylmethylsulfonylfluoride and 1 μg/ml leupeptin. The cell lysates were centrifuged at 10,000 *g* for 10 min to remove debris. The proteins were fractionated using a 10% separating gel and then transferred electrophoretically to nitrocellulose paper for

immunoblotting. The secondary antibodies used were horseradish peroxidase- or alkaline phosphatase-conjugated anti-IgG antibody. The nitrocellulose paper was developed using 5-bromo-4-chloro-3-indolylphosphate/4-nitroblue tetrazolium chloride or developed using an ECL chemiluminescence system.

2-5. Determination of aromatase activity

Aromatase activity was measured in $p53^{fp/fp}$ MEC and $p53^{\Delta 5,6}$ MEC with a tritiated water release assay, described previously (Kinoshita and Chen, 2003). Experiments were conducted when cells reached 80% confluence. $p53^{fp/fp}$ MEC and $p53^{\Delta 5,6}$ MEC were incubated in serum-free medium for 36 h and the cells were treated with [1β-^{3}H] androst-4-ene-3,17-dione (100 nM) for an additional 1 h. The medium was then mixed thoroughly with 5% charcoal/0.5% dextran for 12 h and centrifuged at 10,000 g for 30 min at 4 °C to remove any residual androst-4-ene-3,17-dione. A 1 ml volume of the supernatant was added in a scintillation vial containing 3 ml of scintillation cocktail, and ^{3}H was measured as disintegrations per minute using a liquid scintillation counter (LS 6500, Beckman Coulter, Inc., Fullerton, CA).

2-6. Reporter gene assay

A dual-luciferase reporter assay system (Promega, Madison, WI) was used to determine promoter activity. Briefly, cells were transiently transfected with 0.5 μg of aromatase-Luc, pGRE-Luc, pCRE-Luc or pERE-Tk-Luc plasmid and 0.2 μg of pCMV-β-gal plasmid (β-galactosidase expression for normalization) using the LipofectAMINE Plus Reagent. The luciferase and β-galactosidase activities were determined as described previously (Choi et al., 2001). The reporter activity was calculated by normalizing the reporter-driven firefly luciferase activity to that of β-galactosidase activity.

2-7. cAMP determination

A commercial chemiluminescent enzyme-linked immunosorbent assay (ELISA) kit (Promega, Madison, WI) was used to determine 3',5'-cyclic AMP levels in cell lysates, according to the manufacturer's protocol.

2-8. Gel shift assay

A double-stranded DNA probe (1.75 *p*mole/μl) for the consensus sequences of cAMP response element (CRE) (5'-CAGTCATTTCGTCACATGGG-3') was obtained from Promega (Madison,

WI) and used for gel shift analyses after end-labeling the probe with [γ-^{32}P]ATP and T_4 polynucleotide kinase. The reaction mixture contained 2 μl of 5× binding buffer with 20% glycerol, 5 mM $MgCl_2$, 250 mM NaCl, 2.5 mM EDTA, 2.5 mM dithiothreitol, 0.25 mg/ml poly dI-dC, 50 mM Tris-Cl (pH 7.5), 10 μg of nuclear extracts, and sterile water to a total volume of 10 μl. Incubations were performed at room temperature for 20 min by adding 1 μl probe (10^6 cpm) after a 10 min pre-incubation. The specificity of DNA/protein binding was determined through competition reactions using a 20-fold molar excess of unlabeled CRE oligonucleotide. For the immunodepletion assay, the antibody against CREB(1 μg) was added to the reaction mixture. Samples were loaded onto 5% polyacrylamide gels at 100 V. After electrophoresis, the gels were removed, dried, and imaged using autoradiography.

2-9. Data analysis

One way analysis of variance (ANOVA) was used to assess significant differences between the treatment groups. The Newman-Keuls test was used to compare multiple group means for each significant effect of treatment. Statistical significance was set at either $p<0.05$ or $p<0.01$.

3. Results

3-1. Enhanced activity and expression of aromatase in p53-inactivated MEC

MEC were isolated from 6-month-old $p53^{fp/fp}$ or WAP-Cre $p53^{fp/fp}$ mice. We previously showed that the presence of loxP sites in introns 4 and 6 did not interfere with the transcription of p53, and full-length p53 protein was detected in the doxorubicin-treated $p53^{fp/fp}$ MECs (Yang et al., 2006). In contrast, a smaller protein product with the expected mass of 39 kDa, which was designated $p53^{\Delta5,6}$ by Cre recombination, was found in the WAP-Cre $p53^{fp/fp}$ MECs, and p53-dependent transcription was inhibited (Lin et al., 2003; Yang et al., 2006).

We first tested whether aromatase levels were changed in $p53^{fp/fp}$ MEC and $p53^{\Delta5,6}$ MEC were compared. The basal expression of aromatase in the $p53^{\Delta5,6}$ MEC was significantly higher than in $p53^{fp/fp}$ MEC (Fig. 1A). Consistent with differences in expression, the conversion of [^{3}H] androstenedione to [^{3}H] estrogen in the $p53^{\Delta5,6}$ MEC was 3.2 fold higher than wild-type MEC (Fig. 1B). These results demonstrate that aromatase activity is enhanced through the induction

of aromatase protein in p53-inactivated MEC.

This enhanced aromatase expression in $p53^{\Delta 5,6}$ MEC could result from additional genetic mutations in the mice. An adenovirus over-expressing Cre recombinase (Ad-Cre) was introduced to the $p53^{fp/fp}$ MECs to directly remove exons 5 and 6 out of the *p53* gene. The truncated 39 kDa p53 band was found in the Ad-Cre-treated $p53^{fp/fp}$ MEC, but not in β-galactosidase expressing adenovirus (Ad-gal)-treated $p53^{fp/fp}$ MECs (Fig. 2), which shows that Ad-Cre infection to $p53^{fp/fp}$ MECs efficiently removes the transactivation domains of p53 (Fig. 2). We then determined the expression levels of aromatase in Ad-Cre treated MECs. Exposing the $p53^{fp/fp}$ MECs to Ad-Cre for 24 h resulted in a concentration-dependent increase in aromatase protein levels (Fig. 2), suggesting that aromatase expression is directly coupled with p53 activity in MECs.

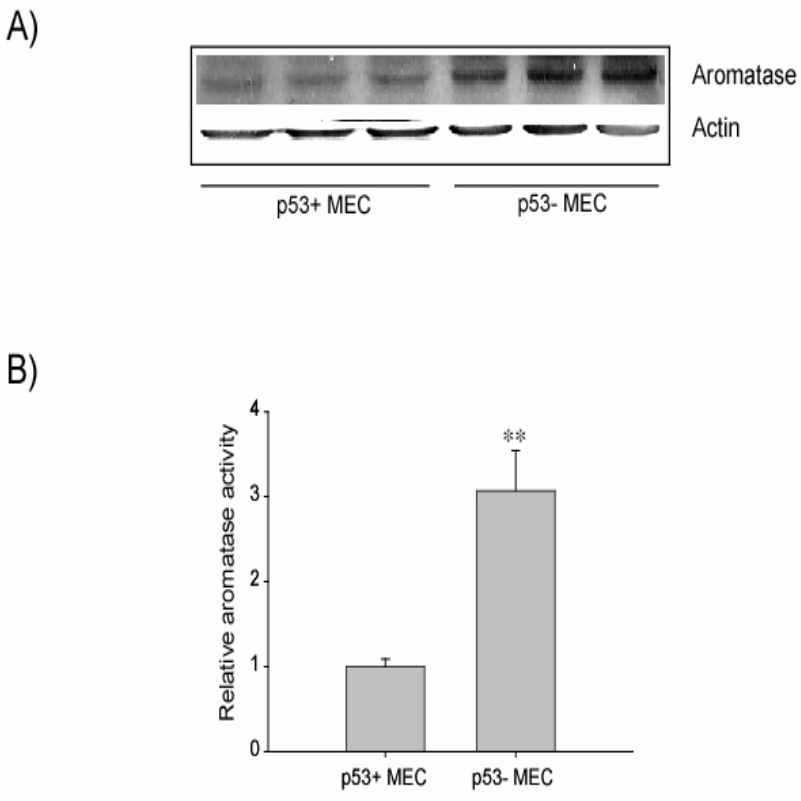

Figure 1. Effect of p53 inactivation on the expression of aromatase in mammary epithelial cells (MEC). (A) Immunoblot analysis of aromatase. Each lane was loaded with 15 μg of the cell lysates. Equal loading of proteins was verified by actin immunoblot. (B) Aromatase activities in $p53^{fp/fp}$ MEC (p53+ MEC) and $p53^{\Delta 5,6}$ MEC (p53- MEC). Aromatase activity was expressed as a relative change to that of p53+ MEC. The data represents the mean ± SD of 6 separate samples (significant as compared to the p53+ MEC, **$p<0.01$).

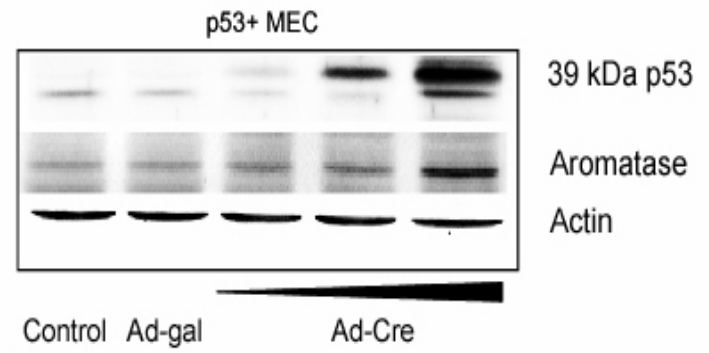

Figure 2. Immunoblot analysis of p53 (upper band) and aromatase (middle band) after Cre expressing adenovirus (Ad-Cre) exposure to $p53^{fp/fp}$ MEC (p53+ MEC) (24 h). β-galactisidase expressing adenovirus (Ad-gal) was used for a mock infection purpose.

3-2. CRE activation is required for aromatase overexpression in p53-inactivated MECs

Aromatase gene expression is regulated by multiple tissue-specific promoters (e.g., promoters I.1 and I.2 in the placenta; promoter I.4 in skin fibroblasts and adipocytes; promoters I.3 and PII in the ovary) (Means et al., 1991). The promoter I.4 and PII are predominantly used in normal mammary epithelial tissues and breast cancer tissues, respectively (Utsumi et al., 1996). Both CREB binding to cAMP-responsive element (CRE) or glucocorticoids binding to glucocorticoid response element (GRE) can regulate the transcription of the *aromatase* gene (Young et al., 1998; Simpson and Zhao, 1996), so we examined whether the activation of GR and CREB mediated p53 activation.

We first compared reporter activity in both the $p53^{fp/fp}$ MEC and $p53^{\Delta 5,6}$ MEC transfected with an aromatase-Luc plasmid containing a –294/+20 bp promoter region of rat *aromatase* gene (Ito et al., 2000). The aromatase-Luc reporter activity in $p53^{\Delta 5,6}$ MEC was higher than in $p53^{fp/fp}$ MEC (Fig. 3), which suggests that the enhanced aromatase levels are mainly due to the transcriptional activation of the *aromatase* gene. In addition, the basal CRE reporter activity in $p53^{\Delta 5,6}$ MEC was

5.6 fold higher than in $p53^{fp/fp}$ MEC (Fig. 4A). Moreover, cAMP levels in $p53^{\Delta 5,6}$ MEC were also 2.6 fold higher than in $p53^{fp/fp}$ MEC (Fig. 4B). These results suggest that the transactivation of *aromatase* gene is associated with aromatase overexpression in p53-inactivated MEC.

Glucocorticoid receptor binding to the putative GRE binding site in the I.4 promoter region is involved in the expression of human *aromatase* gene (Shozu et al., 2000). However, GRE-luciferase reporter activity was not significantly altered in $p53^{\Delta 5,6}$ MEC (Fig. 5). The data suggest that GR activation in not involved in transactivation of *aromatase* gene in p53-inactivated MEC.

Increases in CRE reporter activity might result from changes in the nuclear migration of CREB. Nuclear levels of CREB were significantly higher in $p53^{\Delta 5,6}$ MEC compared with $p53^{fp/fp}$ MEC (Fig. 6A). Moreover, levels of Ser 133-phosphorylated CREB in total cell lysates from $p53^{\Delta 5,6}$ MEC were higher than that from the $p53^{fp/fp}$ MEC (Fig. 6A). To determine whether the nuclear CREB binding to CRE was increased in the p53-inactivated MEC, we isolated nuclear extracts from $p53^{\Delta 5,6}$ MEC or $p53^{fp/fp}$ MEC and probed them with a radiolabeled CRE binding sequence. The band of the slow migrating complex was higher in samples from the $p53^{\Delta 5,6}$ MEC than in the wild-type MEC

(Fig. 6B). Competition experiments using an excess (20X) of the unlabeled CRE oligonucleotides or immunodepletion experiment using 1 μg anti-CREB antibody confirmed that the increased band resulted from enhanced binding of the nuclear CREB protein (Fig. 6B). Thus, CREB phosphorylation and nuclear translocation in p53-inactivated MEC are essential for CRE binding in the promoter region of the *aromatase* gene.

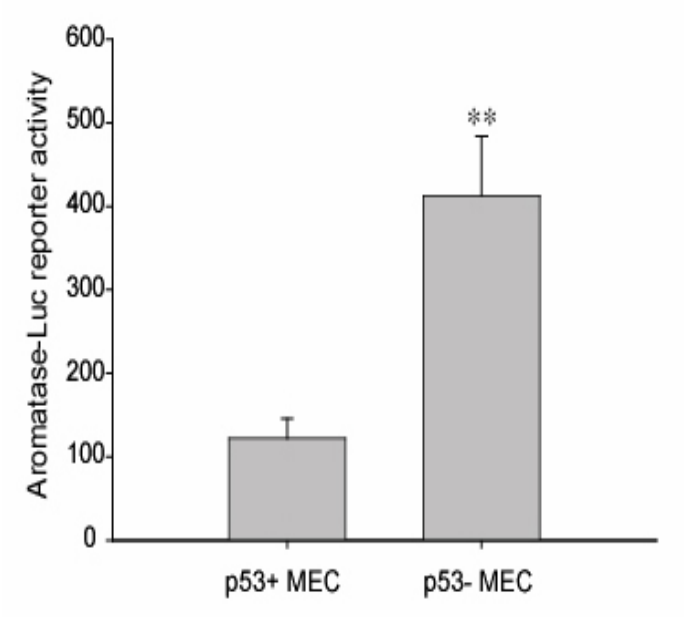

Figure 3. Aromatase reporter activity in both p53+ MEC and p53- MEC. Induction of luciferase activity in $p53^{\Delta 5,6}$ MEC (p53- MEC) transiently transfected with aromatase-Luc, which contained the 294 bp promoter region of the rat *aromatase* gene and luciferase cDNA. Activation of the reporter gene was calculated as a relative change to β-galactosidase activity. Data represented the mean ± SD with 3 different samples (significant as compared to the $p53^{fp/fp}$ MEC (p53+ MEC), **$p<0.01$).

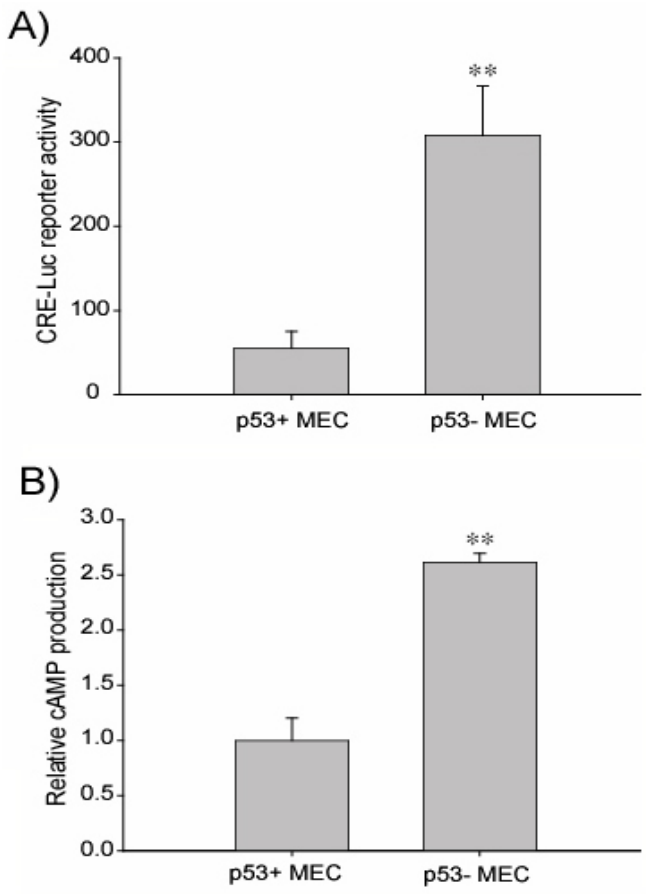

Figure 4. CRE reporter activity in both p53+ MEC and p53- MEC. (A) Increase of CRE reporter activity in p53- MEC. Data represent the mean ± SD with 3 different samples (significant as compared to the p53+ MEC, **$p<0.01$). (B) Increase of cAMP in p53- MEC. The cell lysates were obtained from both the p53+ and p53- MEC incubated in serum-free medium for 18 h. cAMP levels were determined using ELISA kit. Data represent the mean ± SD with 3 different samples (significant as compared to the p53+ MEC, **$p<0.01$).

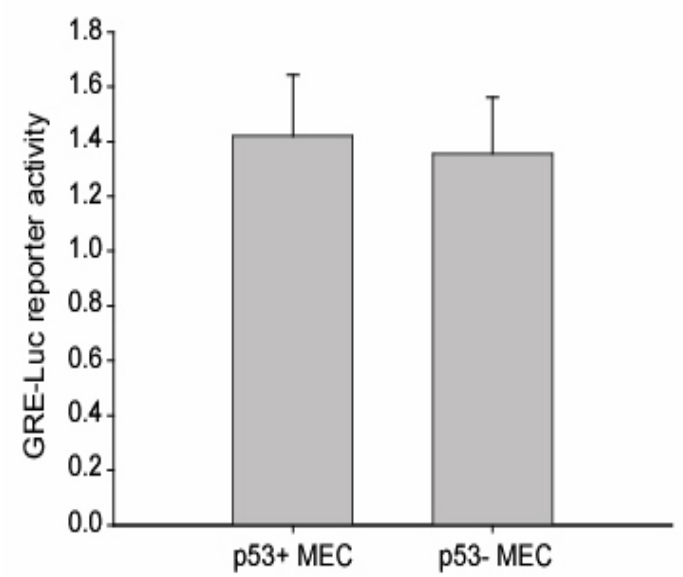

Figure 5. GRE reporter activity in both p53+ MEC and p53- MEC. No increase of GRE reporter activity in p53- MEC. Data represent the mean ± SD with 3 different samples.

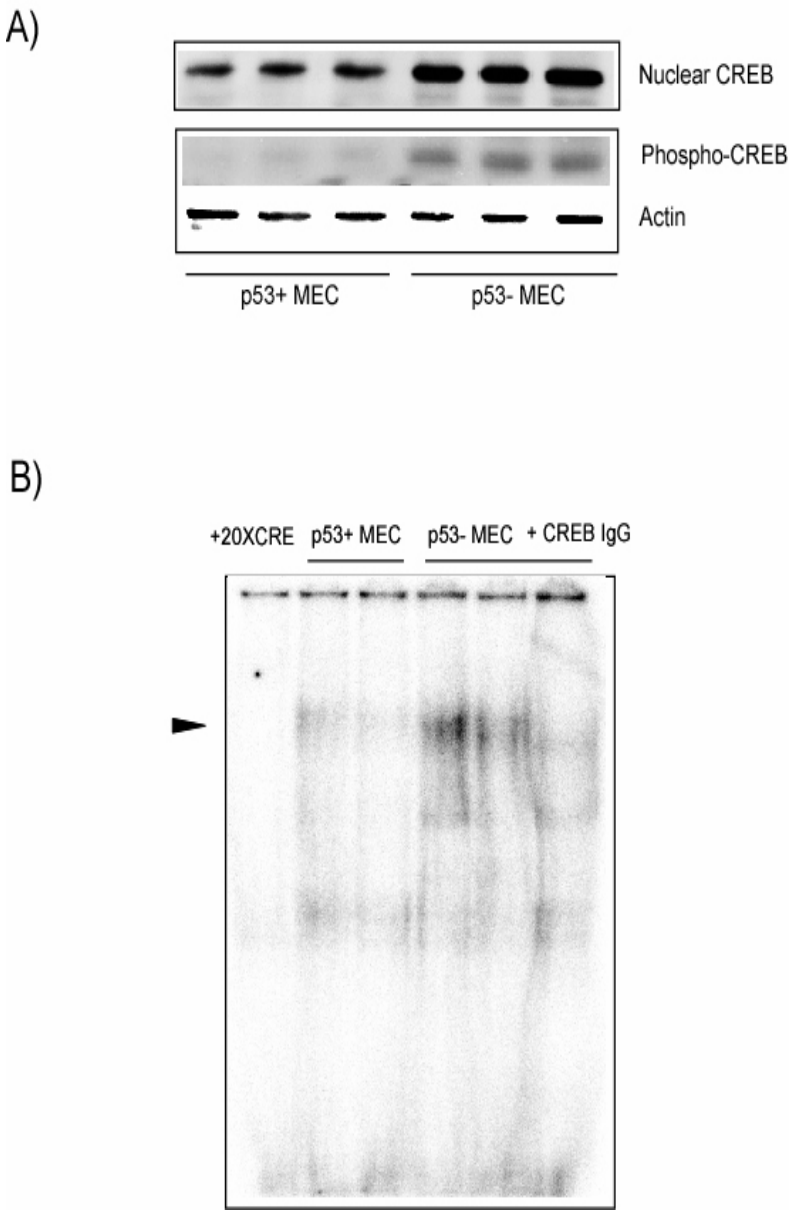

Figure 6. CREB activation in p53-inactive MEC. (A) CREB activities in both $p53^{\Delta 5,6}$ MEC (p53- MEC) and $p53^{fp/fp}$ MEC (p53+ MEC). Upper band; Immunoblot analysis of nuclear CREB in both p53+

MEC and p53- MEC. Middle band; Immunoblot analysis of phosphorylated CREB in the total cell lysates of both p53+ MEC and p53- MEC. (B) Gel shift analysis of the CRE binding. Nuclear extracts were prepared from both p53+ MEC and p53- MEC. All lanes contained 10 μg of nuclear extracts and the labeled CRE consensus sequence. Competition studies were carried out by adding a 20-fold excess of unlabeled CRE consensus sequence (First lane). Immunodepletion studies were carried out by adding a CREB antibody (1 μg) to the nuclear extracts of p53- MEC (Last lane).

3-3. Role of Src and ERK in CREB-mediated aromatase expression in p53-inactivated MEC

The activities or expression levels of most transcription factors are regulated by members of the kinase family, which are triggered in response to a variety of stimuli (Treisman, 1996). Two mitogen-activated protein (MAP) kinases, extracellular signal-regulated kinase (ERK) and p38 kinase, are involved in Ser-133 phosphorylation (and activation) of CREB (Cammarota et al., 2001; Hokari et al., 2005; Gelain et al., 2006). The incubation of cells with PD98059 (30 μM), a specific inhibitor of MAPK/ERK kinase 1/2 (MEK1/2, upstream kinase of ERK), for 24 h significantly suppressed both aromatase expression and the nuclear CREB levels in the $p53^{\Delta 5,6}$ MEC (Fig. 7A). In contrast, p38 kinase inhibition by SB203580 (10 μM, a p38 kinase inhibitor) did not affect the levels of aromatase and nuclear CREB (Fig. 7A).

Src tyrosine kinase is coupled with p53 status (Pal et al., 2001) and Src can phosphorylate CREB (Kawasaki et al., 2004). Pretreatment of cells with PP2 (10 μM), a Src inhibitor, completely suppressed aromatase expression by p53 inactivation (Fig. 7B) and blocked nuclear CREB localization (Fig. 7B). These results indicate that Src tyrosine kinase is also involved in CREB-dependent aromatase expression in

p53$^{\Delta 5,6}$ MEC. In addition, the enhanced reporter activity in p53$^{\Delta 5,6}$ MEC was 76% and 85% inhibited by PD98059 and PP2, respectively (Fig. 8). These results suggest that Src and ERK regulate CREB activation and subsequent *aromatase* gene expression in p53-inactivated MEC.

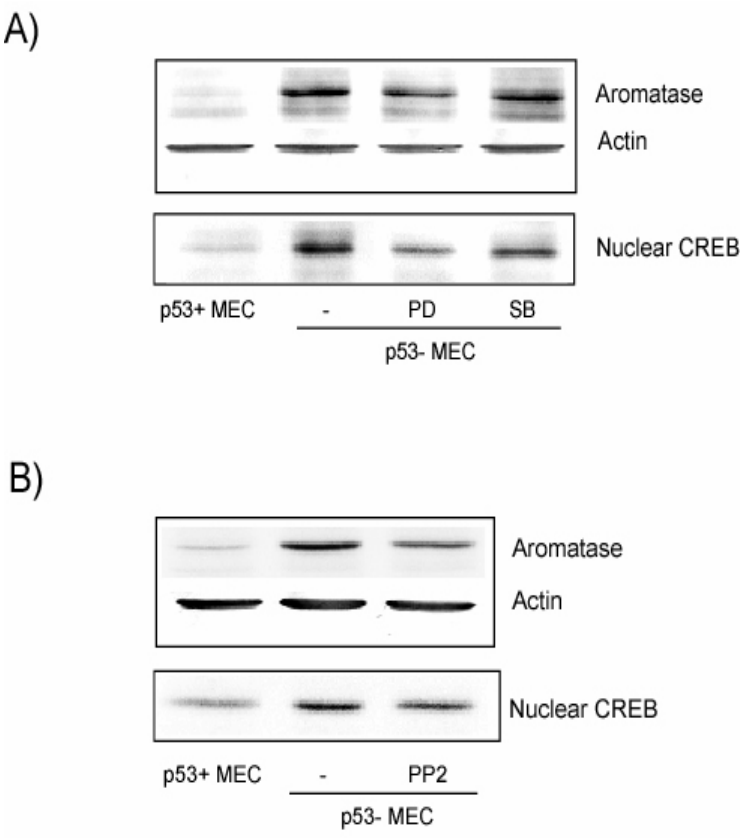

Figure 7. Effects of the chemical inhibitors of ERK, p38 kinase and Src tyrosine kinase on the CREB-dependent aromatase expression in p53-inactivated MEC. (A) Effects of ERK and p38 kinase inhibitors on the increase in the aromatase level. $p53^{\Delta 5,6}$ MEC (p53- MEC) were incubated with PD98059 (PD, 30 μM), SB203580 (SB, 10 μM) for 24 h and the levels of aromatase and nuclear CREB were immunochemically assessed. (B) Effect of Src tyrosine kinase inhibitor on the increase in the aromatase level. $p53^{\Delta 5,6}$ MEC were incubated with PP2 (10 μM) for 24 h and the levels of aromatase and nuclear CREB were immunochemically assessed.

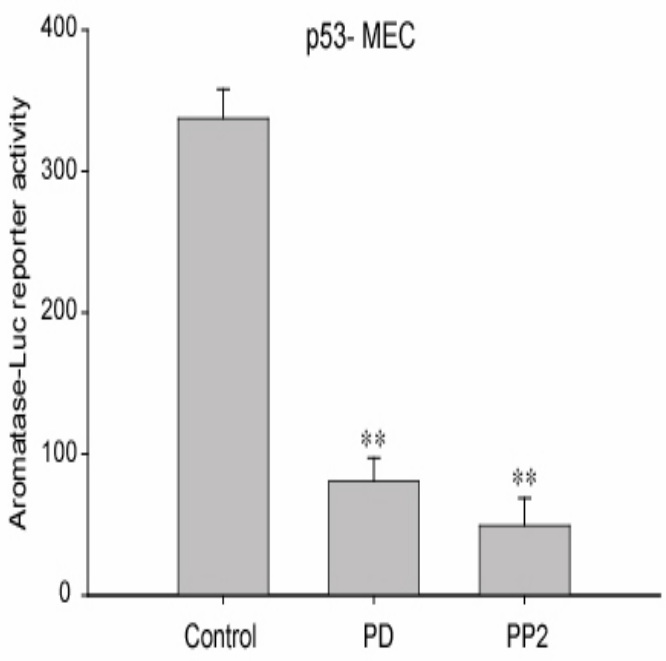

Figure 8. Effects of the chemical inhibitors of ERK and Src tyrosine kinase on the aromatase-Luc reporter activity in p53-inactivated MEC. Activation of the reporter gene was calculated as a relative change to β-galactosidase activity. Data represented the mean ± SD with 3 different samples (significant as compared to the vehicle-treated control (p53- MEC), **$p<0.01$).

4. Discussion

p53 is a key tumor suppressor gene that is mutated or lost in approximately 50% of all human cancer cases (Levine et al., 1991). Because p53 target genes are involved in mediating cell-cycle arrest and apoptosis, p53 dysfunction results in uncontrolled cell proliferation and ultimately carcinogenesis (Vousden and Lu, 2002). In postmenopausal women, locally-produced estrogen through aromatase promotes proliferation of estrogen receptor-positive breast cancer (Simpson, 2003). Estrogens produced by aromatase in breast carcinomas or mammary epithelial cells may be sufficient to stimulate cell proliferation (Brodie et al., 1997). Although the expression of the tumor suppressor genes, p53 or retinoblastoma, was decreased in aromatase transgenic mammary glands (Kirma et al., 2001), the interaction of aromatase expression and p53 status is still unknown. We therefore focused on testing whether aromatase expression was up-regulated in p53-inactivated MEC and exploring the controlling signal transduction pathways. Aromatase expression was enhanced in p53 inactivated MEC, demonstrating p53 dysfunction could cause overproduction of estrogens in mammary glands. A representative tumor suppressor gene, BRCA1, inhibits transcription of the *aromatase*

gene (Hu et al., 2005; Lu et al., 2006), and aromatase expression is inversely correlated with BRCA1 abundance in human adipose stromal cells (Ghosh et al., 2007). Since p53 and BRCA1 both inhibit *aromatase* gene expression, their inactivation in MEC could lead to a local increase in estrogen production, which subsequently may cause estrogen-dependent hyper-proliferation of mammary glands or breast carcinomas.

Because there are no putative binding site(s) for p53 in the *aromatase* gene promoter, the stimulatory effect of *aromatase* gene transcription by p53 inactivation may result from the activation of other transcription factor(s). In particular, CRE and GRE binding sites can regulate aromatase expression in MEC and breast cancer cells (Kijima et al., 2006; Young et al., 1998; Simpson and Zhao, 1996). We found that CRE, but not GRE, activity was enhanced in p53-inactivated MEC. CREB phosphorylation and nuclear translocation was also increased in $p53^{\Delta 5,6}$ MEC, which strongly supports the essential role of CREB activation in aromatase overexpression in p53-inactivated MEC.

Ser-133 phosphorylation of CREB, which can be mediated by p38 kinase or ERK is important for binding to CRE in the promoter region of the *GSTP1* gene (Lo and Ali-Osman, 2002). Although a p38 kinase inhibitor did not suppress aromatase expression, ERK inhibition

by PD98059 significantly reduced the enzyme expression and nuclear levels of CREB in p53-inactivated MEC. Src-mediated tyrosine phosphorylation also causes CREB activation (Wu et al., 2005). Here, Src inhibition by PP2 significantly suppressed CREB activation and blocked aromatase expression. How is CREB-dependent aromatase expression simultaneously regulated by ERK and the Src pathway? Since ERK activity depends on upstream Src tyrosine kinase (Barthet et al., 2007), p53 inactivation may persistently activate Src tyrosine signaling to stimulate ERK-dependent CREB phosphorylation and subsequent aromatase induction.

We previously showed that Her-2/ErbB2 was over-expressed in p53-inactivated MEC and suggested that exaggerated EGF receptor signaling through ErbB2 induction is responsible for the uncontrolled proliferation of MEC in response to EGF (Yang et al., 2006). Our current data indicate that p53 inactivation in MEC enhances aromatase activity through the induction of the *aromatase* gene through Src/ERK-dependent CREB activation. Thus, the increased de novo synthesis of local estrogen via aromatase overexpression may be associated with the uncontrolled estrogen-dependent growth of p53-inactivated mammary glands. (Fig. 9)

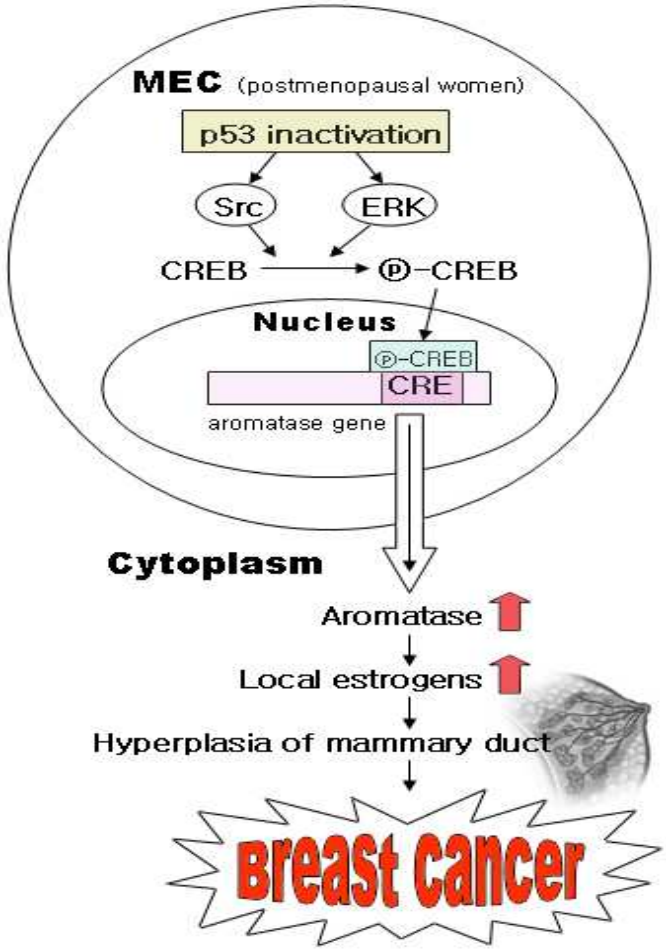

Figure 9. p53 inactivation in MEC enhances aromatase activity through the induction of the *aromatase* gene, which is mediated via Src/ERK-dependent CREB activation. The increased de novo synthesis of local estrogen via aromatase overexpression may be associated with the uncontrolled estrogen-dependent growth of p53-inactivated mammary ducts.

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저작물 이용 허락서

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논문제목	한글 : p53 이 결손 된 유선상피세포에서의 aromatase 발현 증폭 및 작용기전 규명 영어 : Studies on the aromatase induction in p53-inactivated mammary epithelial cells and its mechanism				

본인이 저작한 위의 저작물에 대하여 다음과 같은 조건아래 -조선대학교가 저작물을 이용할 수 있도록 허락하고 동의합니다.

- 다 음 -

1. 저작물의 DB 구축 및 인터넷을 포함한 정보통신망에의 공개를 위한 저작물의 복제, 기억장치에의 저장, 전송 등을 허락함
2. 위의 목적을 위하여 필요한 범위 내에서의 편집·형식상의 변경을 허락함. 다만, 저작물의 내용변경은 금지함.
3. 배포·전송된 저작물의 영리적 목적을 위한 복제, 저장, 전송 등은 금지함.
4. 저작물에 대한 이용기간은 5 년으로 하고, 기간종료 3 개월 이내에 별도의 의사표시가 없을 경우에는 저작물의 이용기간을 계속 연장함.
5. 해당 저작물의 저작권을 타인에게 양도하거나 또는 출판을 허락을 하였을 경우에는 1 개월 이내에 대학에 이를 통보함.
6. 조선대학교는 저작물의 이용허락 이후 해당 저작물로 인하여 발생하는 타인에 의한 권리 침해에 대하여 일체의 법적 책임을 지지 않음
7. 소속대학의 협정기관에 저작물의 제공 및 인터넷 등 정보통신망을 이용한 저작물의 전송·출력을 허락함.

동의여부 : 동의(○) 반대( )

2008 년 2 월 25 일

저작자: 노 상 희 (서명 또는 인)

조선대학교 총장 귀하

< 감사의 글 >

지난 2 년동안 저는 제 인생에 있어서 네 가지의 새로운 역할을 시작했습니다. 약학석사를 준비하는 학생으로서, 한 남자의 아내로서, 한 집안의 며느리로서 그리고 한 아이의 엄마로서... 하지만 제 욕심처럼 모든 역할을 잘해내기엔 제가 너무나도 부족했고, 특히 학업과 육아를 함께 하기가 힘들었습니다. 많은 분들의 도움이 있었기에 해낼 수 있었고, 그 분들께 진심으로 감사의 인사를 전하고자 이렇게 글을 남깁니다. 감사인사를 남길 수 있는 공간이 있어서 참으로 다행입니다.

어느 덧 시간이 흘러 졸업을 앞두고 있는 시점에서, 그동안 열심히 준비해 온 연구를 졸업논문으로 완성시키지 못하고, 다른 분들의 도움을 받아서 진행해 온 연구로 졸업논문을 완성하게 되어 아쉬움이 큽니다. 하지만 이 아쉬움은 다음 발전을 위한 밑거름이 될 테고, 아쉬움보다는 도와주신 분들께 죄송하고 감사한 마음이 더욱 큰 게 사실입니다.

제일 먼저, 오늘에 이르도록 학문적 바탕을 마련해 주시고 학문과 인생의 선배로서 이끌어 주신 약학과의 모든 교수님들께 감사드립니다. 늘 건강하시길 바랍니다.

특히 강건욱 교수님. 교수님의 가르침을 받아 졸업하는 첫 여제자인데, 입학하자마자 결혼하고, 아기까지 낳느라 교수님과 함께 연구도 많이 못 해보고 졸업하게 되어 정말 아쉽습니다. 실수했을 때는 격려해 주시고, 작은 성과에도 큰 칭찬으로 자신감을 불러 일으켜 주셨던 교수님의 멋진 배려는 저에게 언제나 큰 힘이 되었습니다. 교수님 아니었으면 전 제 자신을 이겨내지 못했을 것 같아요. 정말 감사드립니다. 교수님의 연구생활에 언제나 훌륭한 성과가 있기를 바라며, 그 과정에 제가 꼭 다시 함께 할 수 있는 날이 오길 바랍니다.

그리고 이 논문을 낼 수 있게 부족한 부분을 채워주신 독성학 실험실의 정혜광 교수님께도 진심으로 감사드립니다. 함께 실험도 많이 하고, 이래 저래 신경 많이 써주신 독성학 실험실 선생님들 모두 기억에 남네요. 특히, 이번 논문의 실험을 도와주신 김형균 선생님과 한은희 선생님께도 머리 숙여 감사드립니다. 이 논문은 선생님들 덕분에 완성 될 수 있었습니다. 정말 감사합니다.

항상 관심 가져주시며 격려해주신 고옥현 교수님과 따끔하지만 따뜻한 말씀 많이 해주신 최후균 교수님, 학부생 시절 때부터 많은 가르침 주신 한효경 교수님께도 감사드립니다. 앞으로도 많은 충고와 관심 부탁드립니다.

혼자서 어린 후배 둘을 이끌어 주시느라 고생하신 진원오빠도 정말 고맙고, 바쁘신 와중에도 찾아오셔서 격려해주신 정용오빠도 고맙습니다. 언제나 진심으로 축하해주고 격려해 준 Yuba 와 Sindu, 그들의 예쁜 딸 Shreeya 도 잊지 못할 겁니다. 제 빈자리까지 채워가며 열심히 후배들 이끌어주고 저에게도 많은 도움 준 너무나도 착한 동기 창엽이(네겐 어떠한 말로도 표현 못 할 고마움이 한가득이야!), 부족한 선배지만 함께 해준 고마운 후배 상은오빠와 경빈, 미라. 그리고 행복한 옥이언니까지! 모두들 정말 고맙고, 언젠가 꼭 다시 함께 연구할 수 있는 시간이 오길 바랍니다. 꼭! 꼭!

든든하고 성실한 동기인 기수와 기특한 후배 영빈이도 고맙고, 가까이에서, 멀리서 저를 응원해주고 한없는 사랑 보내준 선배, 후배들. 그리고 친구들도 정말 고맙습니다. 모두들 각자의 자리에서 꼭 성공하길 바랍니다.

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언제나 제 편이 되어주시고, 끝없는 사랑주시는 부모님, 철없는 막내며느리를 딸처럼 아껴주시고 격려해주시는 시부모님. 부모님들의 보살핌이 없었다면 전 아무것도 해내지 못했을꺼에요. 부모님들께서 믿어주시는 만큼 더욱 장한 딸, 기특한 며느리가 되도록 늘 노력할께요. 정말 감사드리고 사랑합니다. 오래오래 건강하세요.

지금쯤 열심히 후반기 훈련 받고 있을 하나뿐인 남동생 명훈이(앞으로의 군생활도 무사히 잘 끝내길!), 막내 동생처럼 잘 챙겨주시고 힘들 때마다 도와주시는 시숙님들과 형님들. 눈에 넣어도 아프지 않을 사랑스러운 조카 민서와 도엽이. 모두들 제게 큰 힘이 됩니다. 더 큰 사랑으로 조금씩 보답해 갈께요. 정말 사랑합니다.

마지막으로, 비록 몸은 떨어져있지만 마음만은 늘 함께하며 사랑으로 격려해 주고, 함께 웃고 울어 준 사랑하는 남편 재환씨(남은 군생활도 열심히 잘 해내길!)와 제게 '엄마'라는 소중한 이름표를 달아 준 예쁜 딸 예서(예서야, 엄마는 너의 사랑스러운 미소와 귀여운 옹알이 덕분에 자신감을 얻었고, 엄마의 선택을 후회하지 않겠다고 결심했단다. 넌 그 무엇과도 바꿀 수 없는, 아빠와 엄마의 소중한 보물이야.)에게 고맙다고, 사랑한다고 전하며 이 글을 맺을까합니다.

감사의 글만으로도 논문 한 권 더 쓸 수 있을 것 같네요.

전 참 행복한 사람입니다.