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Studies on the molecular mechanism of epithelial mesenchymal transition in tamoxifen-resistant breast cancer cells

조선대학교 대학원

약학과

김 미 라

타목시펜저항성유방암세포주에서의 EMT 현상 유도 및 분자조절 기작 연구

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타목시펜 저항성 유방암 세포주에서의 EMT 현상 유도 및 분자조절 기작 규명

김 미 라

지도 교수 : 강 건 욱

조선대학교 대학원

약학과

유방암 환자에게 있어서 종양의 타목시펜에 대한 저항성 획득은 치료학적으로 중요한 문제중의 하나이다. 상피간질 형질전환 (EMT)는 상피 세포의 표현형을 보이던 세포들이 높은 이동성을 지닌 간질 세포의 표현형으로 전환되는 현상으로, 암세포의 침윤 및 전이와 관련되어 있다.

본 연구에서는 타목시펜저항성유방암세포주 (TAMR-MCF-7)에서 이러한 상피간질 형질전환 (EMT) 현상이 일어남을 밝혔다. 대조군유방암세포주 (MCF-7)와 비교 시, 타목시펜저항성유방암세포주 (TAMR-MCF-7)는 간질세포 같은 표현형을 지니고 있었고, E-cadherin 의 발현량이 감소된 반면 N-cadherin 및 vimentin 은 과다 발현되어 있었다. 또한 이 세포주에서 Snail 의 전사 활성 및 단백 발현량이 증가되어 있었음을 확인하였다. 상피간질형질전환 (EMT) 시 E-cadherin 의 감소에 대한 glycogen synthase kinase-3β (GSK-3β) 및 NF-κB/snail 의 역할을 알아보기 위하여 먼저 이들 신호의 활성을 정량하였다. 그 결과, TAMR-MCF-7 세포에서는 PTEN/PI3K/Akt 경로가 활성화되어 있음을 밝혔다. Pin1 (Peptidyl-prolyl isomerase)은 타목시펜저항성유방암세포주 (TAMR-MCF-7)에서 과다 발현된 단백질로, SiRNA 를 처치 시 snail 유전자의 전사 및 EMT 지표 발현량 감소를 부분적으로 역전시켰다. 이러한 결과로 미루어 보아, 타목시펜저항성유방암 세포주 (TAMR-MCF-7)에서의 Pin1 이라는 인자가 상피간질전환현상에 관여하는 인자 중 하나이며, 이는 PTEN/PI3K/Akt/GSK-3β 그리고/또는 GSK-3β-NF-κB 경로를 경유하여 Snail 을 활성화시킴을 알 수 있었다. 따라서, 본 연구 결과는 유방암 발달과정 중에 일어나는 상피간질세포전환 (EMT) 현상에 Pin1 이 치료 타켓이 될 수 있음을 시사하며, 이는 유방암 치료에 일조할 것으로 사료된다.

ABSTRACT

Studies on the mechanism of epitheial mesenchymal transition of tamoxifen-resistant breast cancer cells

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Acquisition of resistance to tamoxifen (TAM) is a critical therapeutic problem in breast cancer patients. Epithelial mesenchymal transition (EMT), where cells undergo a developmental switch from a polarized epithelial phenotype to a highly motile mesenchymal phenotype, is associated with invasion and motility of cancer cells. Here, we found that TAMR-MCF-7 cells had undergone EMT, evidenced by mesenchymal-like cell shape, down-regulation of the basal E-cadherin expression and overexpression of N-cadherin and vimentin, as well as increased Snail transcriptional activity and protein expression. Given the roles of glycogen synthase kinase-3β (GSK-3β)

and NF-κB in the snail-mediated E-cadherin deregulation during EMT, we examined the role of these signaling pathways in the EMT of TAMR-MCF-7 cells. Both Ser9-phosphorylated GSK-3β (inactive form) and NF-κB reporter activity were increased in TAMR-MCF-7 cells, as was activation of PTEN/PI3K/Akt pathway in TAMR-MCF-7 cells. Pin1, a peptidyl-prolyl isomerase, was overexpressed in TAMR-MCF-7 cells, and Snail transcription and the expression of EMT markers could be decreased by Pin1 SiRNA treatment. These results imply that Pin1 overexpression in TAMR-MCF-7 cells is involved in the EMT process via PTEN/PI3K/Akt/GSK-3β and/or GSK-3β-NF-κB-dependent Snail activation, and suggest the potential involvement of Pin1 in EMT during breast cancer development.

Key Words*:* Tamoxifen resistant breast cancer; EMT; Snail; Pin1; Signaling pathway

1. Introduction

Breast cancer is the most common malignancy in Western women. Hormonal-dependent control of growth is a characteristic of breast cancer. Ovarian steroid hormones, including estrogen, are essential for both mammary gland development and breast carcinoma formation (1, 2). Hence, the administration of anti-estrogens to reduce breast tumor growth has played a key role in the endocrine therapy of breast cancer. Tamoxifen (TAM), a non-steroidal antiestrogen, is the most widely used antiestrogen in estrogen receptor-positive breast cancer patients (3). Despite an initial response to tamoxifen, the majority of patients will ultimately relapse and present with disease progression. Therefore, resistance to TAM is a major challenge in the management of breast cancer patients (4, 5). To mimic this condition, we and others have established an MCF-7 derived TAM-resistant cell line (TAMR-MCF-7 cells) by long-term (> 9 months) culture of MCF-7 cells with 4-hydroxytamoxifen (6, 7).

Epithelial tumors metastasize by initially invading the adjacent tissues, a process involving the loss of their cell-cell adhesions and the acquisition of migratory capabilities. These

processes coincide with phenotype changes associated with epithelial mesenchymal transitions (EMT), similar to those taken place during certain steps of embryonic development (8). EMT is involved in the efficient invasion and motility of cancer cells, because migration of single cancer cells is elicited by using either mesenchymal or amoemoid phenotypes (9, 10). The invasive and metastatic phenotype is associated with downregulation of E-cadherin expression (11). Several mechanisms have been implicated in the regulation of E-cadherin expression during tumor progression, including genetic, epigenetic, and transcriptional changes (12, 13). Snail transcription factor is a direct repressor of E-cadherin expression in epithelial cells; the expression of Snail induces a full EMT and increases migration/invasion in different physiological and pathological situations (14, 15, 16). Moreover, Snail expression occurs in different invasive carcinoma and melanoma cell lines and, importantly, in invasive regions of squamous cell carcinomas and de-differentiated ductal breast cancer carcinomas and hepatocarcinomas (16, 17). Upon acquisition of tamoxifen resistance, breast cancer cells impart greater *in vitro* metastatic ability to these cells, as demonstrated by an increase in their motile and invasive behavior (18). Therefore, in this

report, we examined the molecular mechanism of EMT in TAMR-MCF-7 cells.

Overexpresion of peptidyl-prolyl isomerase Pin1 is a prognostic marker in several cancer types, including breast and prostate cancers (19, 20). Activation of Pin1 affects oncogenic pathways and/or contributes to the transformation of breast epithelial cells. In this study, we found that Pin1 is overexpressed in TAMR-MCF-7 cells and we used Pin1 SiRNA to clarify its contribution to EMT. Given the important roles of the glycogen synthase kinase-3β (GSK-3β)/Snail and NF-κB/Snail axis in E-cadherin de-regulation during EMT, we also investigated the role of GSK-3β and NF-κB/Snail signaling in EMT induction in TAMR-MCF-7 cells. Herein, we report for the first time Pin1 involvement in EMT induction of TAMR-MCF-7 cells through changes in the activities of PTEN/PI3K/Akt and/or NF-κB pathways.

2. Materials and Methods

2-1. Materials

Anti-E-cadherin, anti-N-cadherin, and anti-snail antibodies were supplied by BD transduction (San Jose, CA) and Abcam (Cambridge Science Park, UK), respectively. Anti-vimentin, anti-Pin1, and anti-GSK-3β antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies for phosphorylated Akt, Akt, and serine-9 phosphorylated GSK-3β were purchased from Cell Signaling Technology (Beverly, MA). Horseradish peroxidase-conjugated donkey anti-rabbit IgG, anti-goat IgG, and alkaline phosphatase-conjugated donkey anti-mouse IgG were purchased from Jackson Immunoresearch Laboratories (West Grove, PA). Anti-actin antibody and most of the reagents used for molecular studies were obtained from Sigma (St. Louis, MO). SiRNA targeting human Pin1 and control SiRNA were purchased from Ambion (Austin, TX). Snail-Luc plasmids and GSK-3β overexpression vector were kindly donated from Dr. Lee KY (Chonnam National University, Gwangju, Korea)

2-2. Cell culture

MCF-7 cells were cultured at 37 °C in 5% CO_2/95% air in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 mg/ml streptomycin. TAMR-MCF-7 cells were established using the methodology reported elsewhere (7). Briefly, MCF-7 cells were washed with PBS, and the culture medium was changed to phenol-red-free DMEM containing 10% charcoal-stripped, steroid-depleted FBS (Hyclone, Logan, UT) and 4-hydroxytamoxifen (0.1 μM). The cells were continuously exposed to this treatment regimen for 2 weeks and the concentration of 4-hydroxytamoxifen was gradually increased to 3 μM over a 9-month period. Initially, the cell growth rates were reduced. However, after exposure to the medium for 9 months, the rate of cell growth gradually increased, showing the establishment of a tamoxifen-resistant cell line (6).

2-3. Immunoblot analysis

After washing with sterile PBS, the MCF-7 or TAMR-MCF-7 cells were lysed in EBC lysis 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 the debris, and the proteins were fractionated using a 10% separating gel. The fractionated proteins were then transferred electrophoretically to nitrocellulose paper, and the proteins were immunoblotted. Horseradish peroxidase- or alkaline phosphatase-conjugated anti-IgG antibodies were used as the secondary antibodies. The nitrocellulose papers were developed using 5-bromo-4-chloro-3-indolylphosphate (BCIP)/4-nitroblue tetrazolium (NBT) or an ECL chemiluminescence system. For ECL chemiluminescence detection, the LAS3000-mini system (Fujifilm, Tokyo, Japan) was used.

2-4. Reporter gene assay

Promoter activity was determined using a dual-luciferase reporter assay system (Promega, Madison, WI). Briefly, the cells (3×10^5 cells/well) were re-plated in 12-well plates overnight and transiently transfected with the Snail-Luc plasmids containing -1.5 kb or –2.5 kb human snail promoter/phRL-SV plasmid (*hRenilla* luciferase expression for normalization) (Promega, Madison, WI) or the NF-κB minimal reporter plasmid/phRL-SV plasmid using

Hilymax® reagent (Dojindo Molecular Technologies, Gaithersburg, MD). The cells were then incubated in the culture medium without serum for 18 h, and the firefly and *Renilla* luciferase activities in the cell lysates were measured using a luminometer (LB941, Berthold Technologies, Germany). The relative luciferase activities were calculated by normalizing the promoter-driven firefly luciferase activity versus *Renilla* luciferase.

2-5. Establishment of stably Pin1-overexpressing MCF-7 cells (Pin1-MCF-7 cells)

MCF-7 cells stably over-expressing Pin1 were established using an MSCV-GFP retrovirus system. Briefly, Pin1 cDNA was subcloned into the MSCV-GFP retroviral vector, and phoenix cells (a packaging cell line) were transfected with either the MSCV-GFP (Control) or the MSCV-Pin1-GFP (Pin1 over-expression) plasmid. Supernatants containing amphotrophic replication-incompetent retroviruses were collected and stored at -80 °C until required. Thirty percent-confluent MCF-7 cells were multiply infected (12 times) with retrovirus particles. Intensities of infection were monitored by GFP-fluorescence and Western blot analysis using a Pin1 antibody.

2-6. Data analysis

Paired Student's *t*-test was used to examine the significant inter-group differences. Statistical significance was accepted at either $p<0.05$ or $p<0.01$.

3. Results

3-1. EMT induction in TAMR-MCF-7 cell lines

TAMR-MCF-7 cells, which has acquired resistance to tamoxifen, displays enhanced invasive capacity *in vitro* (18) and a morphology distinct from the parent MCF-7 cells. While MCF-7 cells show highly organized cell-cell adhesion and cell contact, TAMR-MCF-7 cells had a refractive and elongated appearance, with cell scattering and loss of cell-cell contacts (Fig. 1A). The cobblestone-like morphology of MCF-7 cells at confluence was changed in TAMR-MCF-7 cells to a spindle-like fibroblastic morphology (Fig. 1A). Loss of the epithelial adherence junction protein, E-cadherin, and up-regulation of mesenchymal proteins such as N-cadherin and vimentin are biochemical markers of EMT. In fact, the loss of E-cadherin is a common marker of EMT in mammary epithelial cells and is also associated with an aggressive phenotype with poor prognosis during mammary carcinogenesis (21). The levels of total cellular E-cadherin were significantly reduced in TAMR-MCF-7 compared to MCF-7 cells (Fig. 1B). Conversely, the expression N-cadherin and vimentin were elevated in TAMR-MCF-7 cells (Fig. 1B). Therefore, both the morphological changes and

molecular changes in the TAMR-MCF-7 cells demonstrated that these cells had undergone EMT.

3-2. Up-regulation of Snail in TAMR-MCF-7 cells

Snail, a zinc finger transcriptional repressor, inhibits E-cadherin transcription and its increase is closely related with EMT during cancer cell growth and invasiveness (22, 23). Snail is absent in epithelial cells but expressed in tumors, and its expression correlates with tumor grade (24). Snail levels were increased in TAMR-MCF-7 cells (Fig. 2A), as was the transcriptional activity of the *snail* gene (Fig. 2B), suggesting that Snail up-regulation in TAMR-MCF-7 cells is associated with the transcriptional activation of *snail* gene.

3-3. *Role of GSK-3β inactivation in snail up-regulation*

GSK-3 is a ubiquitously expressed serine/threonine kinase initially identified as a kinase phosphorylating glycogen synthase. Snail protein levels and its repressor functions are regulated by GSK-

3β during EMT (25, 26). In resting cells, GSK-3β is constitutively active as a mitogenic signaling pathway; Akt inactivates the enzyme through Ser-9 phosphorylation (27, 28). The levels of phosphorylated (inactive) GSK-3β in TAMR-MCF-7 cells were significantly higher than in MCF-7 cells (Fig. 2C). To test whether GSK-3β inactivation regulates snail in TAMR cells, we determined the snail reporter activity in TAMR-MCF-7 cells co-transfected with a wild-type GSK-3β expression plasmid. GSK-3β overexpression significantly decreased Snail reporter activity/expression (Fig. 2D), which supports the notion that GSK-3β inactivation causes snail up-regulation in TAMR-MCF-7 cells.

3-4. GSK-3β-dependent snail increase is associated with deregulated PTEN/PI3K/Akt in TAMR-MCF7 cells

The PTEN/PI3-kinase/Akt pathway involved in carcinogenesis (29, 30). Oncogenic transformation can be associated with the abnormal activation of various signaling pathways that induce EMT; abnormal PI3-kinase and Akt activation is frequent in various

epithelial cell tumors (31, 32) and their clinical features are consistent with Akt playing roles in modification of cell morphology, tumorigenicity, cell motility, and invasiveness (33). Akt is also an upstream kinase for controlling GSK-3β activity, so we compared Akt activity in both MCF-7 and TAMR-MCF-7 cells. Akt phosphorylation was increased in TAMR-MCF-7 cells (Fig. 3A), but total Akt levels were lower, consistent with our previous report (6). Increase in the basal Akt phosphorylation could indicate deregulation of an Akt-targeting phosphatase. Phosphatase and tensin homolog depleted on chromosome ten (PTEN) is an upstream negative regulator of Akt. PTEN levels were lower in TAMR-MCF-7 cells (Fig. 3B). To confirm whether defects in PTEN-mediated Akt inactivation up-regulates Snail, we measured snail promoter activity after transfection of TAMR-MCF-7 cells with PTEN or myc-p85 (a dominant negative mutant form of PI3-kinase) overexpression vectors. Inhibition of PI3-kinase by PTEN or myc-p85 decreased Snail promoter activity (Fig. 3C and 3D). These results suggest that PTEN down regulation and subsequent activation of PI3-kinase/Akt regulate EMT in TAMR-MCF-7 cells.

3-5. Role of Pin1overexpression in EMT of TAMR-MCF7 cells

Pin1, a peptidyl-prolyl isomerase, isomerizes the peptide bond pSer/Thr-Pro, to regulate cell cycle progression and other functions (34, 35). Overexpresion of Pin1 also contributes to cell transformation (36, 37) and β-catenin upregulation, which is frequently observed in EMT (38). Therefore, we hypothesized that sustained Pin1 activation was associated with EMT of TAM-resistant breast cancer cells. Interestingly, we could observe a constitutive increase in Pin1 levels in TAMR-MCF-7 cells compared to MCF-7 cells (Fig. 4A).

To ensure Pin1 overexpression in TAMR-MCF-7 cells is involved in EMT, we then evaluated Snail promoter activity and E-cadherin expression after exposure of TAMR-MCF-7 cells to control or Pin1 SiRNA. Pin1 SiRNA decreased Pin1 protein levels (Fig. 4B, upper panel). The increased Snail promoter activity was partially, but significantly, inhibited and E-cadherin expression was also restored by Pin1 SiRNA treatment (Fig. 4B, lower panel). In addition, Pin1 SiRNA reduced the phosphorylation of Akt, but increased PTEN expression in TAMR-MCF-7 cells (Fig. 4C). Therefore, Pin1 affects

PTEN expression and the subsequent PI3-kinase/Akt-dependent GSK3β inactivation is involved in EMT of TAMR-MCF-7 cells.

3-6. *NF-κB involvement in Pin1-mediated EMT in TAMR-MCF-7 cells*

NF-κB is an essential regulator of EMT in breast cancer cells (39, 40). NF-κB can bind to the promoter region of *snail* gene and increase its transcriptional activity (39, 41). Because GSK-3β activity and NF-κB activity are related (42), we measured NF-κB reporter activities in both cell types. The basal NF-κB activity was 6.5 fold enhanced in TAMR-MCF-7 compared to MCF-7 cells (Fig. 5A). Treatment of TAMR-MCF-7 cells for 24 h with an NF-κB inhibitor, N-a-tosyl-L-phenylalanine chloromethyl ketone (TPCK), partially reversed the induction of Snail expression and its promoter activity (Fig. 5B). TPCK treatment also slightly increased E-cadherin expression (Fig. 5C).

Pin1 directly binds to the Thr254-Pro motif of p65 (43) and increases nuclear levels of p65. Basal NF-κB promoter activity was

significantly suppressed by Pin1 knock-down (Fig. 5D), implying that Pin1 overexpression in TAMR-MCF-7 cells is related to NF-κB activation. To sum up, the induction of EMT in TAMR-MCF-7 is also partly dependent on NF-κB activation.

3-7. No induction of EMT in Pin1-overexpressed MCF-7 cells

In order to test whether the sustained Pin1 overexpression alone results in EMT in breast cancer cells, we determined several EMT markers in stably Pin1 overexpressed MCF-7 (Pin1-MCF-7) cells. After multiple infections of MCF-7 cells with Pin1 retrovirus, Pin1 expression levels in Pin-MCF-7 were higher than those in GFP-MCF-7 cells (Fig. 6A). However, the basal expression levels of E-cadherin, N-cadherin and vimentin were not affected by Pin1 overexpression. Moreover, both the snail reporter activity and cell morphology of Pin1-MCF-7 cells were not distinct from GFP-MCF-7 cells (Fig. 6B and 6C). These results suggest that Pin1 overexpression alone is not enough to cause EMT in breast cancer cells.

4. Discussion

Phosphorylation of proteins on serine/threonine residues preceding proline (Ser/Thr-Pro) is a key signaling mechanism in breast cancer cell lines (44, 45, 46). Pin1 is essential for diverse cellular processes, such as proliferation, differentiation, and apoptosis (36, 19, 20). Moreover, Pin1 overexpression influences oncogenic pathways and/or contributes to the transformation of breast epithelial cells. Our findings suggest a new pathological role of Pin1 overexpression in EMT induction in TAM-resistant breast cancer. Pin1 overexpression induced EMT phenomena, as evidenced by drastic changes in cell morphology and expression of vimentin, E-cadherin, and N-cadherin. Although increased migration occurs in TAMR-MCF-7 cells (18), with the role of Pin1 in EMT in these cells is unclear, but Pin1 can partially transform mammary epithelial cells (47).

The Pin1-mediated EMT is associated with increases in the transcription and protein stability of Snail, since Snail blocks E-cadherin expression at multiple levels. GSK-3β constitutively

suppresses transactivation of *snail* gene as well as Snail protein stability by phosphorylating Snail to promote its degradation and cytoplasmic localization (25, 48). Here, we found that Ser9-phosphorylated GSK-3β, an inactive form of the kinase, was enhanced in TAMR-MCF-7 cells. An important implication of our findings is that endogenous suppressors of GSK-3β, such as Akt, which are frequently activated in carcinoma cells (28), may also inhibit E-cadherin transcription and promote an EMT. Also, Akt activation in epithelial cells leads to substantial morphological modification, induction of cell migration and invasiveness (50). Akt can phosphorylate and inactive GSK-3β (51), and we found that the basal Akt phosphorylation was increased in TAMR-MCF-7 cells, suggesting that its regulation is disrupted. PTEN is an upstream antagonistic phosphatase of PI3-Kinase. It removes the 3′ phosphate of PIP3 and inhibits downstream signaling of activated PI3-kinase (52-57). We confirmed that PTEN expression in TAMR-MCFR-7 cells is down-regulated. Through these data, we may infer that PTEN loss induced the consistent activation of Akt. Furthermore, PTEN loss and subsequent Akt activation may significantly reduce GSK-3β activity and transcriptional activation of *snail* gene. Activation of

oncogenes, including Pin1, controls PI3-kinase activity during EMT (58). Indeed, blocking Pin with SiRNA treatment disrupted PI3-kinase activity and Pin1-mediated EMT. Moreover, Pin1-mediated EMT seems to be derived from the consistent PTEN down-regulation, as both Akt expression and its upstream negative regulator PTEN was reversed by Pin1 RNA (Fig. 5C). Pin1 may change PTEN expression, which subsequently promotes the activation of PI3-Kinase/Akt/GSK-3β-dependent EMT in TAMR-MCF-7 cells.

NF-κB is essential for EMT and metastasis in a breast cancer cell model (39). NF-κB affects cell proliferation and various cancers, including breast cancer cells (59, 60). High levels of NF-κB are found in the majority of primary human and rodent breast tumor tissue samples, breast cancer cell lines, and carcinogen-transformed mammary epithelial cells (59, 61). There are reports that GSK-3β regulates Snail via NF-κB (42), and NF-κB directly binds to the *snail* promoter to increase its activity (41). NF-κB is a central mediator of EMT (39), and NF-κB function is regulated by Pin1-mediated prolyl isomerization and ubiquitin-mediated proteolysis of the p65/RelA subunit (43). Upon cytokine treatment, Pin1 binds to the pThr254-Pro

motif in p65 and inhibits p65 binding to IκBα, resulting in increased nuclear accumulation and protein stability of p65 and enhanced NF-κB activity (43). Our study also showed that NF-κB is partly associated with Pin1-mediated EMT.

Although Pin1 can trigger EMT in TAMR-MCF-7 cells, Pin1 overexpression alone is not sufficient to induce EMT or Snail activity in MCF-7 cells. In fact, Pin1 overexpression alone does not seem to affect cell growth or cell morphology under normal growth conditions (48). Pin1 may become oncogenic only after activation of other oncogenic pathways leading to phosphorylation of its substrates. Indeed, Pin1 greatly enhances and facilitates transformation induced by oncogenic Her-2/Neu and Ras in mammary epithelial cells (48). Thus, Pin1 can cooperate with oncogenic pathways in a 'post-phosphorylation' regulation where the prompt *cis-trans* isomerization of prolyl residues adjacent to phosphorylated serine or threonine residues promotes cell proliferation and transformation. EMT is therefore under the control of several signaling pathways.

In this study, we have revealed that Pin1 overexpression partly contributes to the EMT process via PTEN/PI3-kinase/Akt/GSK-3β-

dependent and/or NF-κB-dependent Snail activation and suggests potential Pin1 involvement in EMT during acquisition of resistance to anti-hormones in breast cancers.

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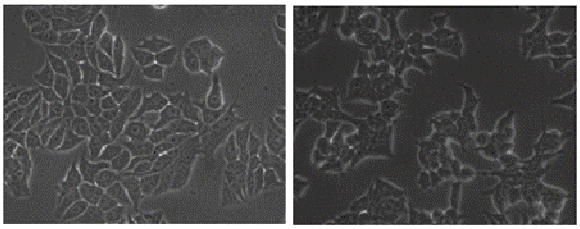
6. Figure Legends

A)



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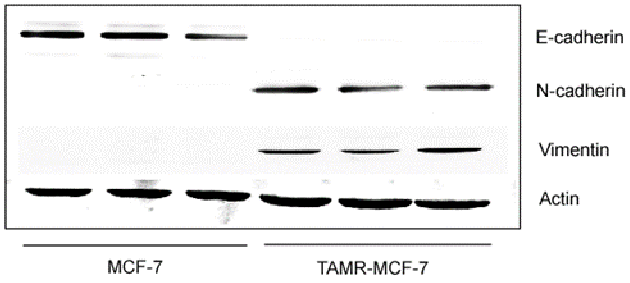


Figure 1. EMT induction in TAMR-MCF-7 cell lines. (A) Cell morphology of MCF-7 and TAMR-MCF-7 cells. (B) Immunoblot analysis of E-cadherin, N-cadherin, and vimentin. Equal loading of proteins was verified by actin immunoblot.

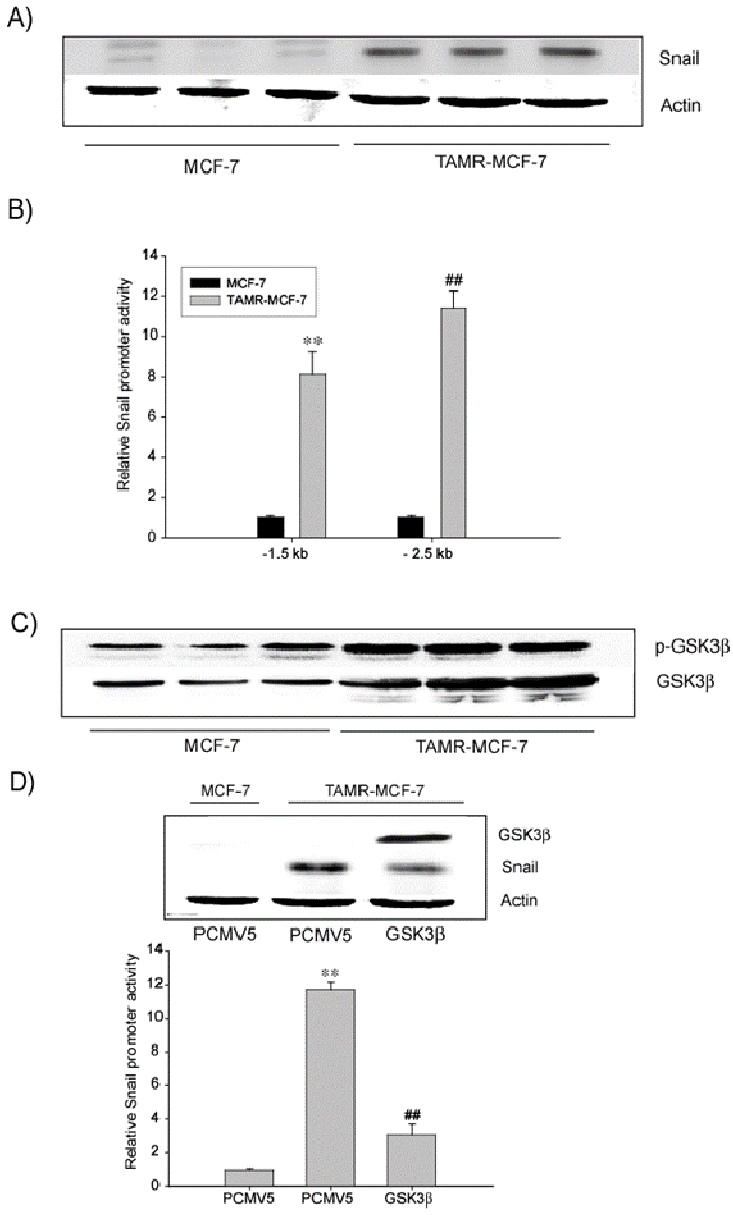

A)
Snail
Actin
MCF-7
TAMR-MCF-7
B)
Relative Snail promoter activity
MCF-7
TAMR-MCF-7
**
##
-1.5 kb
- 2.5 kb
C)
p-GSK3β
GSK3β
MCF-7
TAMR-MCF-7
D)
MCF-7
TAMR-MCF-7
GSK3β
Snail
Actin
PCMV5
PCMV5
GSK3β
Relative Snail promoter activity
**
##
PCMV5
PCMV5
GSK3β

Figure 2. Up-regulation of Snail in TAMR-MCF-7 cells. (A) Immunoblot analysis of Snail. A representative immunoblot shows Snail protein in both MCF-7 and TAMR-MCF-7 cells serum-deprived for 18 h. (B) Basal reporter activity of Snail promoters (Snail-Luc) in MCF-7 and TAMR-MCF-7 cells. Each cell type was transiently transfected with -1.5kb or -2.5kb Snail-Luc plasmid. Dual luciferase reporter assays were performed on the lysed cells co-transfected with Snail-Luc plasmids (firefly luciferase) and phRL-SV (*hRenilla* luciferase) (a ratio of 100:1) 18 h after transfection. Reporter gene activation was calculated as a relative ratio of firefly luciferase to h*Renilla* luciferase activity. Data represent means ± SD with 4 different samples (significant versus MCF-7 cells, **p<0.01, $^{\#\#}$p<0.01). Role of GSK-3β inactivation in snail up-regulation. (C) Immunoblot analysis of p-GSK-3β and GSK-3β. Representative immunoblots show each protein in both MCF-7 and TAMR-MCF-7 cells serum-deprived for 18 h. (D) Inhibition of Snail transactivation by GSK3β overexpression vector. Upper panel: Levels of GSK3β and Snail were determined by immunoblotting in TAMR-MCF-7 cells transfected with GSK3β overexpression vector (1 μg/ well) or mock vector PCMV5 (1 μg/well). Lower panel: MCF-7 and TAMR-MCF-7 cells were co-

transfected with Snail -2.5kb reporter plasmid in combination with GSK3β overexpression vector or control mock vector PCMV5 (1 μg/well). Data represents means ± SD with 4 different samples (significant versus the control, **p<0.01; significant versus PCMV5-transfected group, ##p<0.01; control level = 1).

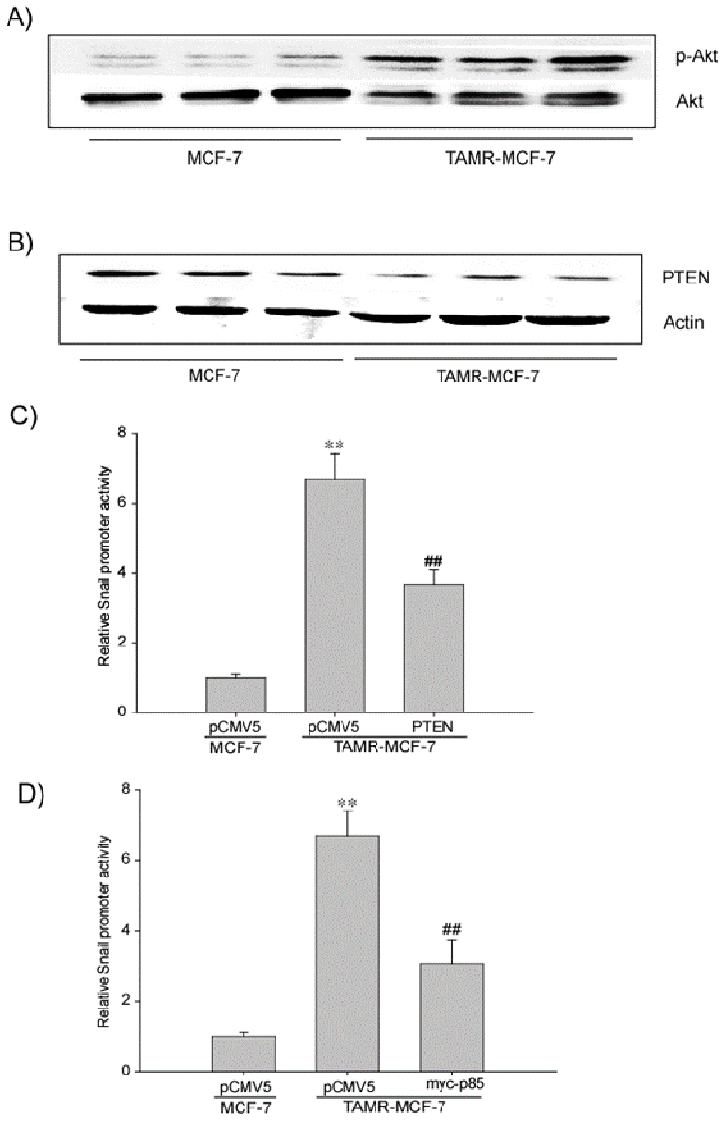
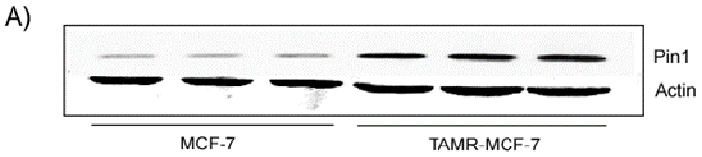
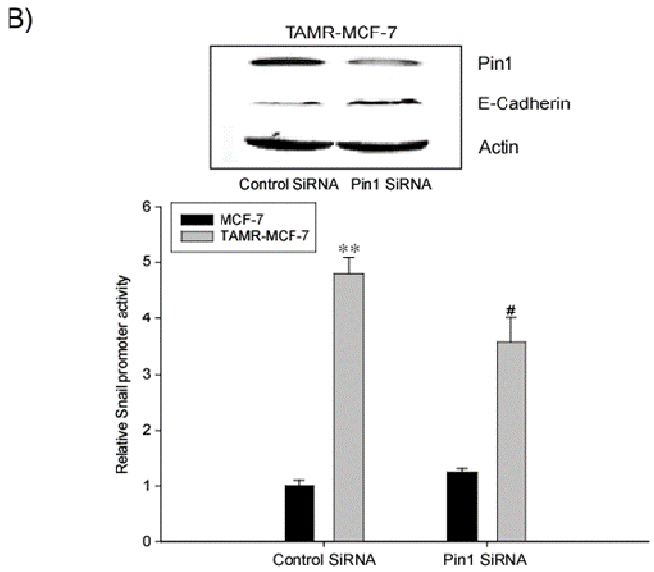

A)
p-Akt
Akt
MCF-7
TAMR-MCF-7
B)
PTEN
Actin
MCF-7
TAMR-MCF-7
C)
Relative Snail promoter activity
0
2
4
6
8
**
##
pCMV5
pCMV5
PTEN
MCF-7
TAMR-MCF-7
D)
Relative Snail promoter activity
0
2
4
6
8
**
##
pCMV5
pCMV5
myc-p85
MCF-7
TAMR-MCF-7

Figure 3. Role of PTEN/PI3-kinase/Akt in Snail *u*pregulation of TAMR-MCF7 cells. (A) Immunoblot analyses of p-Akt (A) and PTEN (B). Equal loading of proteins was verified by actin immunoblot. (C) Inhibition of Snail transactivation by PTEN overexpression vector. MCF-7 and TAMR-MCF-7 cells were co-transfected with -2.5 kb Snail-Luc reporter plasmid in combination with PTEN overexpression vector (0.1 μg/well) or mock vector PCMV5 (0.1 μg/well). Data represents means ± SD with 4 different samples (significant versus the MCF-7 cells, **p<0.01; significant versus PCMV5-transfected TAMR-MCF-7 cells, $^{\#\#}$p<0.01). (D) Inhibition of Snail transactivation by myc-p85 overexpression vector. MCF-7 and TAMR-MCF-7 cells were co-transfected with -2.5 kb Snail-Luc reporter plasmid in combination with myc-p85 overexpression vector (0.1 μg/well) or control mock vector PCMV5 (0.1 μg/well). Data represents means ± SD with 4 different samples (significant versus the MCF-7 cells, **p<0.01; significant versus PCMV5-transfected TAMR-MCF-7 cells, $^{\#\#}$p<0.01).


A)
Pin1
Actin
MCF-7
TAMR-MCF-7


B)
TAMR-MCF-7
Pin1
E-Cadherin
Actin
Control SiRNA
Pin1 SiRNA
MCF-7
TAMR-MCF-7
Relative Snail promoter activity
0
1
2
3
4
5
6
**
#
Control SiRNA
Pin1 SiRNA

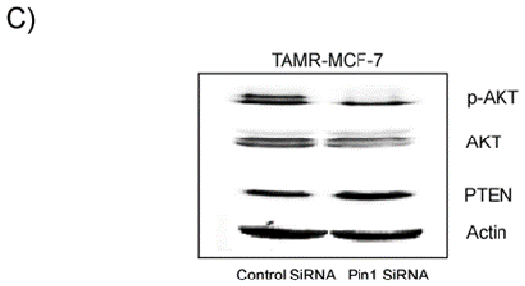
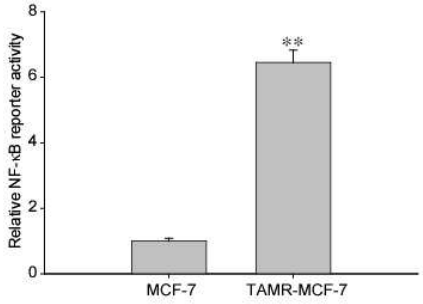
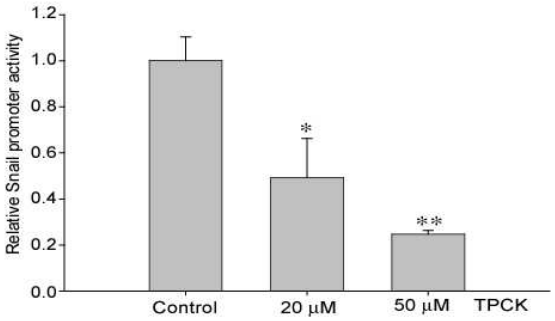

C)
TAMR-MCF-7
p-AKT
AKT
PTEN
Actin
Control SiRNA
Pin1 SiRNA

Figure 4. Role of Pin1 overexpression in EMT of TAMR-MCF7 cells. (A) Immunoblot analysis of Pin1. A representative immunoblot shows Pin1 protein in both MCF-7 and TAMR-MCF-7 cells serum-deprived for 18 h. Equal loading of proteins was verified by actin immunoblot. (B) Effects of Pin1 SiRNA on the E-cadherin expression and Snail transcription activity in TAMR-MCF-7 cells. Upper panel: Levels of Pin1 and E-cadherin were determined by immunoblottings in TAMR-MCF-7 cells transfected with Pin1 siRNA (60 *p*mole) or control siRNA. Lower panel: TAMR-MCF-7 cells were co-transfected with -2.5 kb Snail-Luc reporter plasmid in combination with Pin1 siRNA (20 *p*mole) or control siRNA. Data represents means ± SD with 4 different samples (significant versus the control, **$p<0.01$; significant versus the control SiRNA-transfected group, $^{\#}p<0.05$). (C) Decrease in p-Akt expression and increase in PTEN level by Pin1 SiRNA introduction. Protein levels of p-Akt and PTEN were determined by immunoblottings in TAMR-MCF-7 cells transfected with Pin1 siRNA (60 *p*mole) or control siRNA.

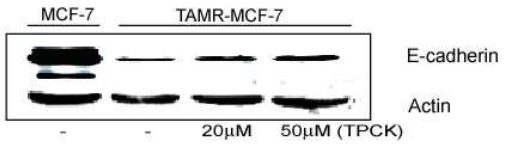
A)


Relative NF-κB reporter activity
8
6
4
2
0
**
MCF-7
TAMR-MCF-7

B)


Relative Snail promoter activity
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
**
Control
20 μM
50 μM
TPCK

C)


MCF-7
TAMR-MCF-7
E-cadherin
Actin
-
-
20μM
50μM (TPCK)

D)

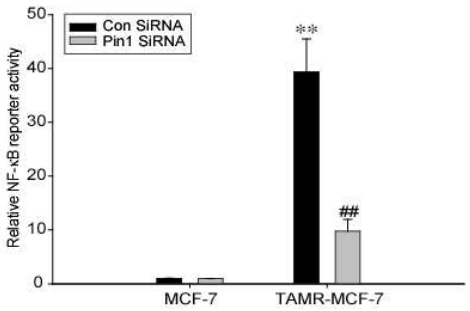
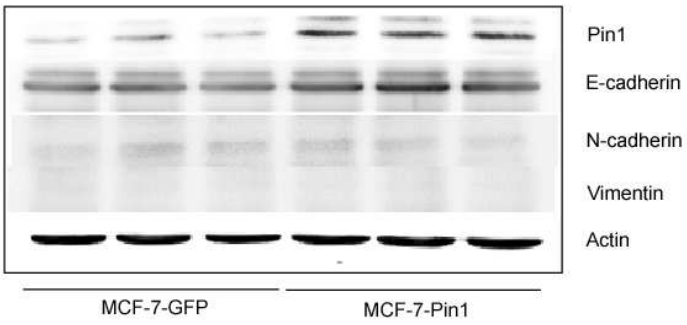

Relative NF-κB reporter activity
50
40
30
20
10
0
Con SiRNA
Pin1 SiRNA
**
##
MCF-7
TAMR-MCF-7

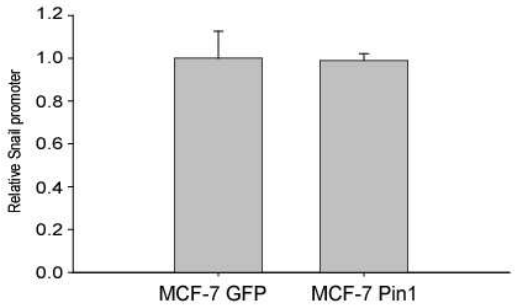
Figure 5. Involvement of NF-κB in Pin1-mediated EMT of TAMR-MCF-7 cells. (A) NF-κB reporter activity in TAMR-MCF-7 cells. Dual luciferase reporter assays were performed on the lysed cells co-transfected with NF-κB plasmid (firefly luciferase) and phRL-SV (*hRenilla* luciferase) (a ratio of 100:1) 18 h after transfection. Reporter gene activation was calculated as a relative ratio of firefly luciferase to h*Renilla* luciferase activity. Data represent means ± SD with 4 different samples (significant versus the MCF-7 cells, **p<0.01). (B) Inhibition of NF-κB transactivation by TPCK, a NF-κB inhibitor. MCF-7 and TAMR-MCF-7 cells were transfected with -2.5kb Snail-Luc reporter plasmid and treated TPCK for 24hr. Data represents means ± SD with 4 different samples (significant versus the control, *p<0.05, **p<0.01; control level = 1). (C) Decrease in E-cadherin level by TPCK treatment. Level of E-cadherin was determined by immunoblotting in TAMR-MCF-7 cells treated TPCK (0, 20, 50 μM). (D) Inhibition of NF-κB transactivation by Pin1 siRNA. TAMR-MCF-7 cells were co-transfected with NF-κB reporter plasmid in combination with Pin1 SiRNA (20 *p*mole) or control SiRNA. Data represents means ± SD with 4 different samples (significant versus the control SiRNA-transfected MCF-7 cells, **p<0.01; significant versus the

control SiRNA-transfected TAMR-MCF-7 cells, $^{\#\#}p<0.01$).

A)


Pin1
E-cadherin
N-cadherin
Vimentin
Actin
MCF-7-GFP
MCF-7-Pin1

B)


Relative Snail promoter
1.2
1.0
0.8
0.6
0.4
0.2
0.0
MCF-7 GFP
MCF-7 Pin1

C)

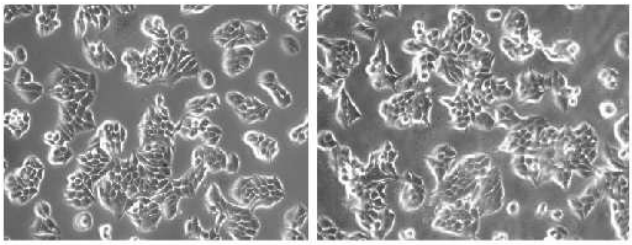

MCF-7-GFP
MCF-7-Pin1

Figure 6. No induction of EMT in Pin1-overexpressed MCF-7 cells. (A) Effect of Pin1 overexpression on the EMT phenotypes in MCF-7 cells. Representative immunoblots show E-cadherin, N-cadherin and vimentin protein levels in both GFP-MCF-7 (Control) and Pin1-MCF-7 cells serum-deprived for 18 h. (B) Basal reporter activity of Snail promoters (Snail-Luc) in GFP-MCF-7 and Pin1-MCF-7 cells. Each cell type was transiently transfected with -2.5 kb Snail-Luc plasmid. Dual luciferase reporter assays were performed on the lysed cells co-transfected with Snail-Luc plasmids (firefly luciferase) and phRL-SV (*hRenilla* luciferase) (a ratio of 100:1) 18 h after transfection. Reporter gene activation was calculated as a relative ratio of firefly luciferase to h*Renilla* luciferase activity. Data represent means ± SD with 6 different samples. (C) Cell morphology of GFP-MCF-7 and Pin1-MCF-7 cells.

저작물 이용 허락서

학 과	약학과	학 번	20077050	과 정	**석사**, 박사
성 명	한글: 김 미 라 한문 : 金 美 羅 영문 : Kim, Mi Ra				
주 소	광주광역시 서구 치평동 금호쌍용아파트 209 동 201 호				
연락처	E-MAIL : goldlig@hanmail.net				
논문제목	한글 : 타목시펜저항성유방암세포주에서의 EMT 현상 유도 및 분자 조절 기작 연구 영어 : Studies on the molecular mechanism of epithelial mesenchymal transition in tamoxifen-resistant breast cancer cells				

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( )

2009 년 2 월 25 일

저작자: 김 미 라 (서명 또는 인)

조선대학교 총장 귀하

감사의 글

이제 제법 차가운 바람이 불어오고 겨울이 왔습니다. 매년 맞는 겨울이지만 대학원에서 맞는 마지막 겨울이라 생각하니 아쉬운 생각이 듭니다. 길다면 길고 짧다면 짧은 2년이라는 시간이 지나고 어느덧 졸업의 문턱에 섰습니다. 졸업을 앞두고 감사의 글을 쓰려고 하니 감사할 분들을 찾기도 전에 떠오르는 얼굴들이 많습니다. 그 분들께서 항상 곁에 있어주셨기에, 항상 지켜봐 주시며 응원해 주셨기에 지금 이 자리까지 올 수 있었습니다. 하지만 저는 부끄럽게도 지금까지 그런 것들을 그저 당연한 거라고 생각해 왔던 것 같습니다. 이번 기회를 빌어 앞으로는 더 잘 해드리리라 다짐하며 감사의 마음을 몇 자 적어볼까 합니다.

먼저, 가장 가까운 자리에서 말 없이 항상 절 믿고 아껴 주시며 응원해 주신 우리 가족들께 감사 드리고 싶습니다. 지금까지 제가 공부에 매진할 수 있었던 것은 그 분들께서 계셨기에 가능할 수 있었습니다. 공부가 잘 될 때에는 칭찬으로, 공부가 힘들었을 때에는 격려로써 저에게 큰 힘이 되어 주셨습니다. 가장 사랑하는 분들임에도 불구하고 공부한다는 핑계로 그 동안 더 잘 해 드리지 못해서 송구스럽습니다. 연로하심에도 불구하고 항상 저를 위해 애써주시는 할아버지, 할머니, 공부하는데 지장이 없도록 항상 돌봐주시고 애써 주신 아빠, 엄마 모두 건강하시고 행복하세요. 하나뿐인 내 동생 영환이, 누나로서 더 잘 해 주지 못해서 미안

해. 앞으로 의지를 가지고 전진하길 바래. 그 외에도 항상 저를 예뻐해 주시고, 좋은 말씀을 해 주시며 용기를 북돋아 주신 고모, 고숙, 이모, 그리고 작은 아버지와 작은 어머니들께도 감사의 말씀을 드리고 싶습니다.

다음으로 멋진 지도 교수님이신 강건욱 교수님, 정말 감사합니다. 학생들 입장에서 최대한 이해해 주시고 작은 일에도 칭찬을 아끼지 않아주셨던 교수님 덕에 가끔은 힘들고 지쳐도 대학원 생활을 잘 해 올 수 있었습니다. 교수님의 연구에 대한 열정과 연구에 매진하시는 모습은 저에게 귀감이 되었습니다. 그리고 '항상 꿈을 가지고 전진하라', '자신감을 가지고 자신이 최고라 생각하라'는 그 말씀들을 자신감이 부족하고 미래에 대한 두려움이 많은 저에게 큰 힘이 되었습니다. 교수님의 열정과 그 말씀들을 가슴 깊이 새겨 더욱 전진하여 자랑스러운 제자가 되겠습니다. 또한, 밝게 웃어주시며 저를 격려해 주셨던 최후균 교수님, 정혜광 교수님, 최준식 교수님, 문영희 교수님, 고옥현 교수님, 오원근 교수님, 최홍석 교수님을 비롯한 여러 교수님께 감사의 말씀을 드리고 싶습니다.

2년 동안 하루의 절반 이상을 함께 지내왔던 실험실 사람들에게도 고맙습니다. 항상 열심히 하는 모습이 보기 좋았던 경빈이, 연구에 몰두하기 좋아하는 상은 오빠, 배울 점이 많았던 창엽 오빠, 환하게 웃는 모습이 너무 예쁜 옥이씨(신랑분과 현민이와 행복하셔요^^), 그리고 우리 실험실에 웃음을 되찾게 해 준 정우 오빠 (정우 오빠와 더불어서 나를 챙겨 준 지연 언니~ 고마워)… 함께 했던 시간들이 소중할 수 있도록 해

주어서 모두모두 고맙습니다. 저에게 외국 생활에 대한 조언도 해 주고 용기를 북돋아 주었던 멋진 외국인 친구 유바씨, 신두씨, 그리고 귀여운 쓰리아와 유신이, 새로운 친구가 된 푸헝… 다가올 미래가 멋지고 행복하길 기원합니다. 또, 가끔씩 실험실을 찾아오셔서 격려해 주신 상희 언니, 정용이 오빠, 진원 오빠에게도 감사하다는 말씀을 전하고 싶습니다.

실험실 생활을 같이 하지 않음에도 불구하고 저에게 큰 힘이 되어준 운정이, 친언니같이 저를 잘 챙겨주고 다독여준 은진 언니, 늘 티격태격하지만 소중한 친구 승재, 대학원동기인 나연이, 영빈이, 영진이, 명학 오빠 등에게도 고맙습니다. 진로 문제로 방황하던 시기에 많은 도움을 주신 승식 오빠, 열심히 공부에 정진하라며 충고를 아끼지 않던 현택 오빠에게도 감사 드립니다. 그리고 물리약학실 선자씨, 선임씨, 로바쓰씨, 위생약학 실험실 선생님들께도 감사 드리고 싶습니다. 이외에도 여러 물품을 납품해 주신 회사측 분들, 기계를 수리해 주시던 분들, 학교를 지켜주시는 수위 아저씨, 주변을 깨끗하게 해 주시던 청소부 아주머니들께도 감사 드립니다.

그리고 힘든 시간 함께 보내주며 웃음으로 이겨낼 수 있도록 해 준 우리 이쁜이들 영선이, 지영이, 나를 항상 최고라 해 주며 용기를 북돋아 준 최고로 멋진 친구 여진이, 오랜 친구지만 특히 시험 준비 기간 동안 정신적으로 많은 힘이 되어준 현경이, 자주 보지는 못하지만 의지가 되어준 부전이를 비롯한 보물 같은 내 친구들에게도 고맙습니다. 마지막으로

항상 내 걱정해 주며 멀리서 응원해 주고 있는 창현 오빠에게도 너무너무 고맙습니다. 함께 하지 못해 조금은 힘에 부쳤지만 마음만은 함께 했기에 든든했습니다. 이 외에도 항상 저를 기억해주시고 사랑해 주시는 모든 분들께 감사 드리고 싶습니다.

저를 아껴주시는 분들이 제 주변에 이렇게 많았다는 사실을 이 기회를 빌어 다시 한 번 깨닫게 되었습니다. 이런 분들께서 계시지 않았다면 오늘의 저도 존재할 수 없었을 것입니다. 다시 한 번 고개 숙여 감사 드립니다. 앞으로 더욱 분발하여 훌륭한 사람이 되어 그 분들께 보답하겠습니다.

'모든 일의 끝은 새로운 시작이다' 라는 말이 있습니다. 이제 하나의 과정을 마치고 새로운 발걸음을 할 때가 온 것 같습니다. 그 발걸음도 그 분들의 수고가 헛되지 않도록 열심히 노력하겠습니다. 그럼 모두 행복하시고 건강하십시오.

-김 미라 올림