Master of Science

The role of the corepressor N-CoR1 related to TNF-alpha-mediated apoptosis in Breast cancer cells

The Graduate School
Of the University of Ulsan

Department of Medicine
Seung-Ho Park

The role of the corepressor N-CoR1 related to TNF-alpha-mediated apoptosis in Breast cancer cells

Supervisor: Kyung-Chul Choi

A Dissertation

Submitted to
The Graduate School of the University of Ulsan
In partial Fulfillment of the Requirements
For the Degree of

Master of Science

by

Seung-Ho Park

Department of Medicine
Ulsan, Korea
February 2018

The role of the corepressor N-CoR1 related to TNF-alpha-mediated apoptosis in Breast cancer cells

This certifies that the dissertation of Seung-Ho Park is approved.

Committee Chair Dr. Yong Shin

Committee Member Dr. Chang Hoon Ha

Committee Member Dr. Kyung-Chul Choi

Department of Medicine

Ulsan, Korea

February 2018

Abstract

We have investigated the role of Nuclear Receptor Corepressor1 (NCoR1) in Estrogen Receptor alpha (ERα) (+) breast cancer cells. The NCoR1 plays an important role in the Tumor Necrosis Factor-alpha (TNF-α)-mediated apoptosis. But, mechanism of ERα-NCoR1 complex is little known in breast cancer. Thus, we studied that TNF-α significantly down-regulates NCoR1 protein levels in ERα (+) MCF7 breast cancer cells. In addition, the reduced NCoR1 expression by TNF-α was induced the down-regulation of ERα protein levels in the MCF7 cells. This result suggests that NCoR1 expression may be involved in the TNF-α-mediated apoptosis of ERα (+) breast cancer cells. Interestingly, the phosphorylation of NCoR1 by Casein Kinase2 (CK2) inhibited the ubiquitin-dependent proteasomal degradation of NCoR1 in the TNF-α-treated MCF7 breast cancer cells. In conclusion, the down-regulation of NCoR1 and ERα by TNF-α induced the activation of Tumor suppressor p53 (p53). Thus, our results show that TNF-α treatment induces dissociation of ERα-NCoR1-p53 complex and activates p53-dependent pro-apoptosis target genes through the acetylation of p53.

These findings indicate that ERα-NCoR1-p53 complex represses the transcription activity of cell death genes, such as cyclin-dependent kinase inhibitor1 (p21), Bcl2-associated-X protein (Bax) and p53 upregulated modulator of apoptosis (Puma).

Keyword : Tumor Necrosis Factor-alpha (TNF-α), Estrogen Receptor alpha (ERα), Nuclear Receptor Corepressor1 (NCoR1), Tumor suppressor p53 (p53), Apoptosis, Breast cancer

CONTENTS

LIST OF FIGURES

Introduction

Breast cancer is the most frequently diagnosed cancer worldwide. It is a major cause of female cancer-related deaths. Advances in the treatment and early diagnosis of breast cancer have helped survival. But cancer recurrence and drug resistance are the reasons for the high mortality rate.

Breast cancer has pathological markers. It has three types of estrogen receptor (ER), progesterone receptor (PR) and human epithelial cell growth factor receptor2 (HER-2). Understanding the pathological features of breast cancer can provide a basis for choosing better treatment. We compared ERα (+) breast cancer with ERα (-) breast cancer to study differences in pathologic features according to the presence of ER. Several reports have s that estrogen suppresses the apoptosis induced by TNF-α, and chemotherapeutic drugs in MCF7 cells. But, we have not been shown that estrogen suppresses the apoptosis induced by TNF-α. The Ligand ERα initiates transcription either directly by binding to the estrogen response elements (EREs) of genes, or indirectly though the phosphorylation of the signaling proteins involved in cell cycle progression.

The nuclear co-repressor contains two major subunits. One is histone deacetylase (HDAC) and the other is HDAC activator. HDAC catalyzes the removal of acetyl group from substrate protein, and HDAC activators are silencing mediator for retinoic acid receptor and thyroid hormone receptor (SMRT or NCoR2) and nuclear receptor co-repressor1 (NCoR1). Its presence is necessary for catalytic activation of HDAC. In particular, NCoR1 and NCoR2 were served as a cofactor scaffolding platform. Transcription factors and co-repressors are important mediators in biological homeostasis, because they are involved in transcriptional regulation (Desvergne et al., 2006, Chen et al., 1995).

Although HDAC3 has been reported to contribute many biological processes, the relationship of its function to NCoR1 and SMRT has been unclear. Recently published reports show that ERα-NCoR1-p53 clearly acts as regulator of p53-mediated apoptosis. However, how ERα and NCoR1 are involved in apoptosis in largely unknown. We have

found that ERα-NCoR1-p53 regulatory complex plays an important role in cancer cell apoptosis in breast cancer.

Tumor necrosis factor-alpha (TNF-α), a potent inflammation cytokine, is involved in a series of cellular functions such as cell proliferation, differentiation, and apoptosis. TNF-α has been shown to play a paradoxical role in the evolution and treatment of breast cancer. Some studies have shown that TNF-α binds to specific receptors on tumor cell membrane, inhibiting cell survival and inducing apoptosis. We have continuously shown that TNF-α-induced apoptosis has different results depending on the presence of ERα. When cells are exposed to TNF-α treatment, pathological features of breast cancer are down-regulated. Regulation of pathological features by TNF-α is helpful to understand the mechanisms of apoptosis pathway in ERα positive breast cancer. The stimulation of TNF-α in osteoclast differentiation is known to be mediated by the induction of the cyclin-dependent kinase inhibitor1 (p21). In breast cancer, we hypothesized that an increase in p21 by TNF-α was associated with the presence of ER.

Each phase of the cell cycle is regulated by regulatory factor such as CDKs and its regulatory subunits, cyclins and CDK inhibitors. Tumor cell proliferation was inhibited by increased expression of p21, and apoptosis was induced by activated expression of Bcl-2-assaociated-X protein (Bax). The p21 and Bax genes are direct downstream target genes for p53, the tumor suppressor. In addition, up-regulation of p21 expression is involved in the process of cell senescence as well as cell cycle arrest induced by DNA damage in various tumor cell culture studies.

In this study, we demonstrate that TNF-α induces apoptosis in breast cancer cell, such as ERα positive MCF7, by inducing dissociation of ERα-NCoR1-p53 complex. And down-regulation of NCoR1 and up-regulation of p53 leads to the activation of p21 in MCF7.

.

Materials and Methods

1. Cell culture

The breast cancer MCF7, MDA-MB-231 cells maintained in Dulbecco's modified Eagle's medium (DMEM, Corning, USA) containing 10% fetal bovine serum (FBS, Corning, USA) and 1% antibiotics (100units/ml Penicillin and 100ug/ml Streptomycin, Hyclone). Cells were maintained at 37℃ in a humidified atmosphere with 5℃ CO_2.

2.Antibodies

ERα, p21, Bcl-2, Bax, p53, pro-Caspase3, pro-Caspase7, Caspase8, Caspase9 antibodies were purchased from Santa Cruz Biotechnology. CK2 antibody was purchased from upstate. NCoR1 antibody was purchased from abcam. β-actin, p-NCoR1 antibodies were purchased from Thermo Fisher Scientific. PARP-1 antibody was purchased from Cell Signaling.

3. Western blotting

Cells were harvested with cold PBS, scrapper. Cell extracts were prepared with lysis buffer[50mM Tris-HCl (pH7.5), 150mM NaCl, 1% NP-40, 10mM NaF, 10mM sodium pyrophosphate, and protease inhibitors], incubated for 30min on ice. The lysates were centrifuged at 13,000rpm (or 20,000xg) for 20min at 4℃. The supernatant was collected and protein concentration was determined by using protein assay buffer (Pierce™ 660nm Protein Assay Reagent, Thermo Fisher Scientific). Equal protein amount of cell lysates were loaded into 8% and 12% SDS-PAGE gel and separated by electrophoresis and transferred to nitrocellulose membranes. The membranes were blocked in 5% skim milk (BD Biosciences, Franklin Lake, NJ, USA) with 1X PBST by incubating for 1h. The blocked membranes were incubated overnight at 4℃ with the primary antibody. After washing three times with 1X PBST, the membranes were incubated with secondary HRP-conjugated antibody for 1h 30min. The membranes were then subjected to western blot analysis and were visualized using an ECL solution.

4. RT-PCR analysis

Total RNA were extracted with RNA extraction kit (RNA EasySpin kit, Intron Biotechnology, Korea). Total RNA was then reversely transcribed with Poly(rA)–oligo$(dT)_{18}$ using Reverse Transcriptase (PrimeScript^{TM} Reverse Transcriptase, Takara) according to the protocols of the manufacturer. All samples were normalized to human GAPDH and expressed as fold changes. All reactions were done in triplicate.

5. Report assay

In order to determine the transcriptional activity of the promoters of ERE-luc, MCF7 and MDA-MB-231 cells were transiently transfected with reporter constructs (ERE-Luc). The Renilla luciferase reporter plasmid was included as an internal control. Cells were harvested, total cell extracts were prepared, and dual luciferase activity was measured according to the manufacturer's instructions (Promega). All reporter activities were normalized to *Renilla* luciferase activity.

6. Cell viability assay

Cell viability was analysis by MTT assay (EZ-cytox cell viability assay). The cells (5000 per well) were grown in completely medium in 96-well flat-bottomed plates. After seeding for 24h, cells were exposed to various concentrations of drugs and incubated at 37℃ in a CO_2 incubator. After treatment, medium was replaced by 110μl of MTT solutions, and incubated for 2h at 37℃ in a CO_2 incubator. The absorption was measured at 450nm with a micro plate reader.

7. Caspase-3/7 activity assay

Caspase-3/7 activity assay was determined by colorimetric assay using a caspase-3/7 activation kit according to the manufacturer's protocol. The cells were prepared with lysis buffer. The supernatants were collected and incubated with the supplied reaction buffer containing dithiothreitol and substrates at 37℃. Caspase activity was determined by measuring changes in absorbance at 405nm using a micro-plate reader.

8. Flow cytometric analysis

After treatment, Cells were collected and treated with 1U/ml of RNaseA (DNase free) and resuspended in 10ug/ml PI solution at room temperature for 30min before analysis in the dark. A FACS flow cytometer was used to analyze the population of sub-G1 phase.

Results

Expression of ERα decreases by TNF-α in MCF7

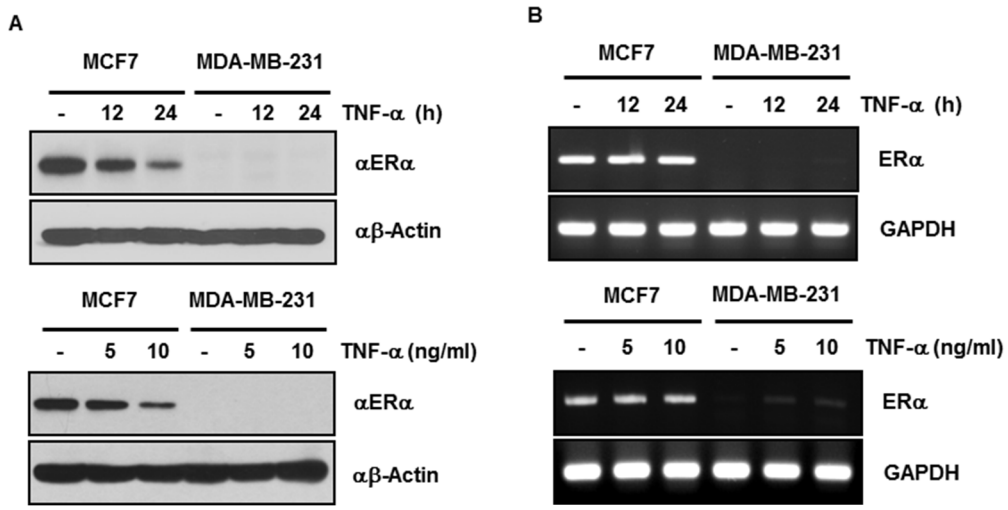

Fig. 1. TNF-α regulates ERα expression in breast cancer cells

(A) TNF-α decreased ERα expression in ERα (+) MCF7 breast cancer cell at time-dependent and dose-dependent manner, but did not detect ERα expression in ERα (-) MDA-MB-231.

(B) TNF-α did not decrease ERα mRNA level in ERα (+) MCF7 breast cancer cell, also did not detect ERα mRNA level in ERα (-) MDA-MB-231.

Breast cancer cells were treated with recombinant human TNF-α. The response experiment showed that treatment with doses of 0, 5, 10ng/ml of TNF-α. TNF-α was treated in MCF7 and MDA-MB-231. TNF-α strongly decreased ERα expression at a dose-and time-dependent manner in ERα (+) MCF7 breast cancer cell. In addition, treatment of MCF7 cells showed decreased expression of ERα with 5ng/ml of TNF-α for a period (0, 12, 24h). But ERα expression in ERα (-) MDA-MB-231 did not show a significant impact. ERα in MCF7 was decresed in Figure 1A. MDA-MB-231 is negative ERα breast cancer cell, so it doesn't express ERα.

We showed the alteration of ERα protein expression with TNF-α in MCF7 and MDA-MB-231 breast cancer cells. Additionally, we wanted to confirm what the results would be in mRNA level. Breast cancer cells were treated with TNF- α at a dose- and time-dependent manner. When ERα (+) MCF7 was treated with TNF-α, there was no decrease in mRNA of ERα. And mRNA of ERα was not detected in ERα (-) MDA-MB-231 breast cancer cell. As a result, this experiment showed that TNF-α affects ERα expression but not mRNA in ERα (+) MCF7 breast cancer cell. ERα mRNA expression were assessed by semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR) using specific primers for amplification. In this RT-PCR, by using easy-blue (Intron), total RNA was extracted from MCF7 and MDA-MB-231. Amplification of ERα, which was established by electrophoresis is presented in Figure 1B.

ERE reporter assay shows down-regulation of ERα signaling activity in MCF7

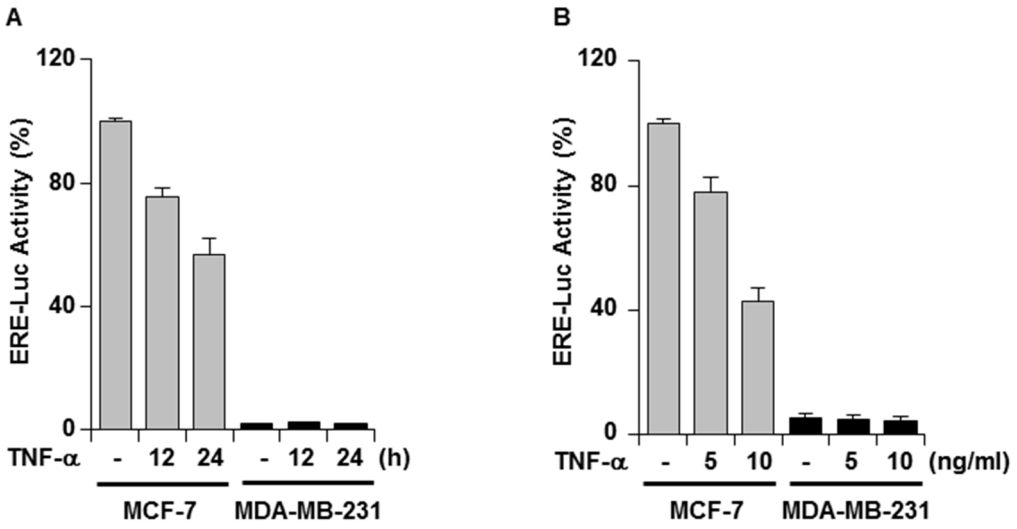

Fig. 2. TNF-α time- and dose-response analysis of ERE reporter transcriptional activity. TNF-α down-regulate ERE-luc activity

(A,B) TNF-α decreased the activity of ERE (estrogen response element) in ERα (+) MCF7 breast cancer at time dependent and dose dependent, but had a remarkably low the activity of ERE in ERα (-) MDA-MB-231.

We have shown in previous results that TNF-α down-regulates ERα expression. ERα directly binds to the estrogen response element (EREs) of genes, and ERα initiates transcription factor. Additionally, ERα indirectly induces phosphorylation of the signaling proteins involved in cell cycle progression. To determine whether ERα reduction affects the activity of EREs, we performed a reporter assay.

TNF-α decreased the activity of EREs (Estrogen response element) in ERα (+) MCF7 breast cancer at a time-dependent or dose-dependent manner. In a time-dependent experiments, TNF-α reduced the activity of EREs by 50% at 24h in ERα (+) MCF7 breast cancer cell. Furthermore, in dose-dependent experiments, TNF-α was shown to reduce the activity of ERE by 60% at 10ng/ml in ERα (+) MCF7 breast cancer cell. This result shows that reduction of ERα by TNF-α in MCF7 affects the decrease of the ERE activity. MDA-MB-231 lacking expression ERα, showed no the significant activity of ERE reporter assay compared with the control in dose dependent manner. Thus, in this experiment MDA-MB-231 did not show any significant activity ERE-luc reporter assay.

Cytotoxicity determination by MTT assay

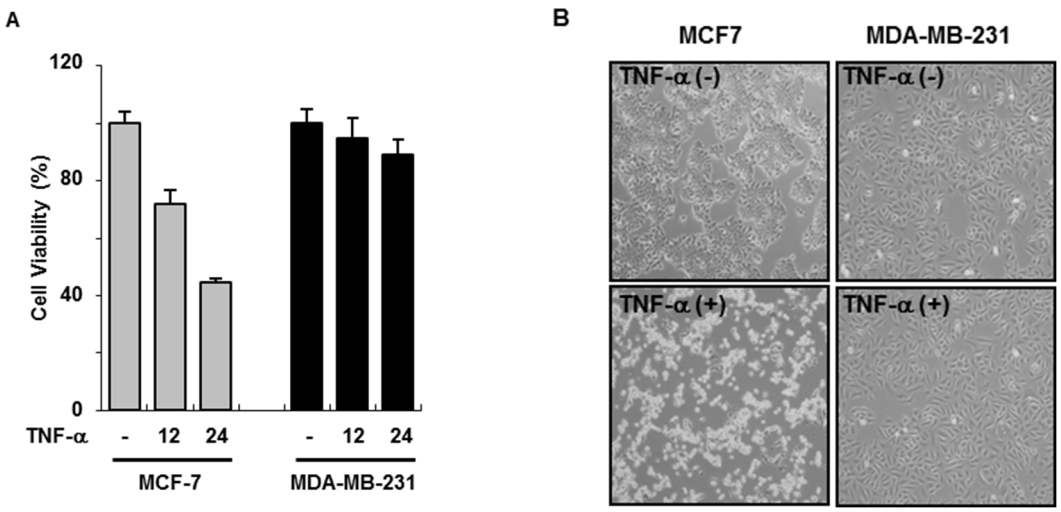

Fig. 3. Cell viability with TNF-α in breast cancer cells

(A) TNF-α decreased cell viability in ERα (+) MCF7 at time-course dependent, but did not little decrease cell viability in ERα (-) MDA-MB-231 breast cancer cell.

(B) TNF-α changed the morphology for bad conditions in ERα (+) MCF7 breast cancer cell, but did not change the morphology in ERα (-) MDA-MB-231 breast cancer cell.

In the previous results for TNF-α, TNF-α only affected expression of ERα and the activity of EREs in MCF7, which is positive ERα, but not in MDA-MB-231, which is negative ERα. TNF-α is cytokine that causes inflammation and apoptosis. Cell viability was measured to determine the difference in TNF-α-induced apoptosis between MCF7 and MDA-MB-231. TNF-α decreased cell survival to 40% for 24h in ERα (+) MCF7 breast cancer cell. So, TNF-α decreased cell viability in ERα (+) MCF7 in a time-dependent manner. However, When MDA-MB-231 was treated by TNF-α, it was no significant decrease in the cell survival.

In order to confirm morphological changes that cell viability decreased, we observed with a microscope. Treatment with TNF-α significantly induced morphological shrinkage in ERα (+) MCF7 breast cancer cell. However, TNF-α-induced morphological change was not observed in ERα (-) MDA-MB-231 breast cancer cell. As a result, MTT assay and microscopy demonstrated that TNF-α-induced apoptosis is dependent on ERα expression.

TNF-α induces cell cycle arrest and apoptosis in MCF7 cells

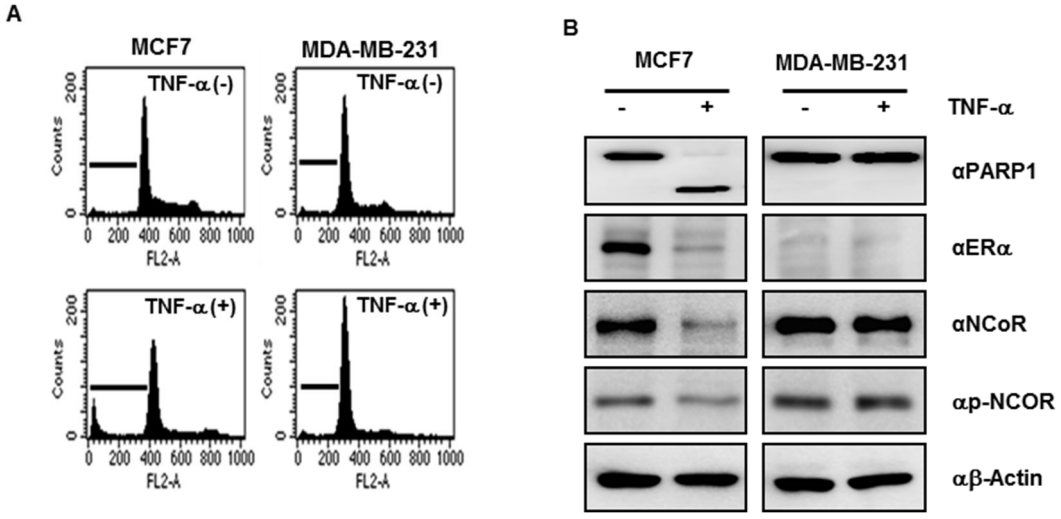

Fig. 4. Flow cytometry in analysis cell cycle and apoptosis with TNF-α treatment in breast cancer.

(A) TNF-α decreased the G1 phase of ERα (+) MCF7 breast cancer cell, but did not decrease the G1 phase of ERα (-) MDA-MB-231 breast cancer cell.

(B) TNF-α led to cleave PARP1, and decreased expression of ERα, NCoR1 and p-NCoR1 in ERα (+) MCF7 breast cancer cell, but did not affect expression in ERα (-) MDA-MB-231 breast cancer cell.

Furthermore, we used flow cytometry to investigate the amount of cells with sub-G1 DNA cells. Cell cycle analysis was performed to confirm cell cycle arrest by TNF-α in breast cancer cells. TNF-α decreased the G1 phase of ERα (+) MCF7 breast cancer cell, and increased the sub-G1 phase. TNF-α has been shown to induced cell cycle arrest by CDK inhibitor. We also found that PARP1 was cleaved by TNF-α, and expression of ERα was also significantly reduced in ERα (+) MCF7 breast cancer cell. However, the cleavage of PARP1 by TNF-α did and ERα did not detect in MDA-MB-231.

We have proposed a model to suggest that NCoR1 act as co-repressors that form an inhibitory complex with ERα. Therefore, it was expected that TNF-α-induced apoptosis would affect the expression of NCoR1. As expected, TNF-α decreased expression of ERα and decreased expression of NCoR1 and p-NCoR1, but did not affect expression in ERα (-) MDA-MB-231 breast cancer cell.

TNF-α regulates cell apoptosis proteins in MCF7

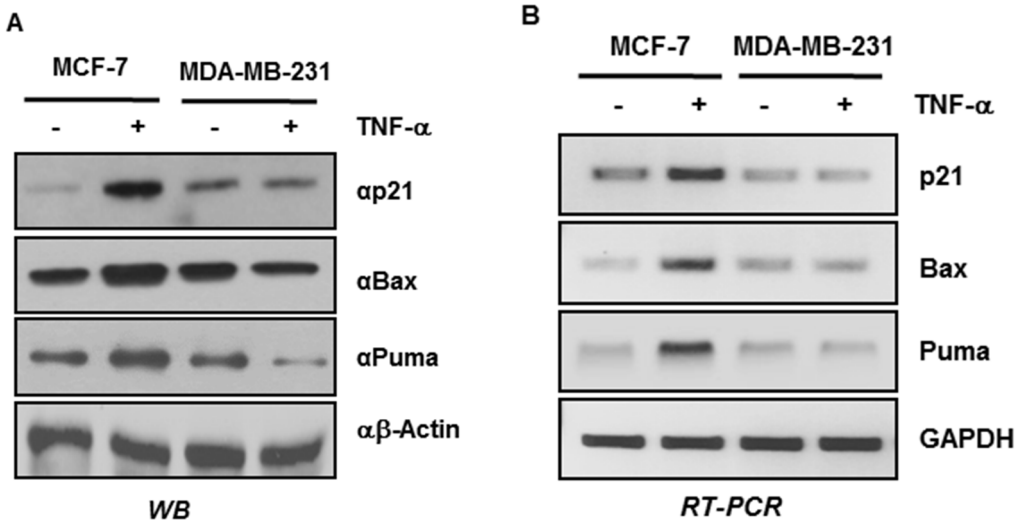

Fig. 5. Expression of p21, Bax and Puma with TNF-α in breast cancer cells.

(A) TNF-α induced up-regulation of p21, Bax and Puma expression in ERα (+) MCF7 breast cancer cell, but did not affect expression in ERα(-) MDA-MB-231 breast cancer cell.

(B) TNF-α induced up-regulation of p21, Bax and Puma mRNA level in ERα (+) MCF7 breast cancer cell, but did not affect mRNA level in ERα (-) MDA-MB-231 breast cancer cell.

We next studied the expression and function of apoptosis marker with TNF-α in the breast cancer. Previously, we showed a cell cycle arrest caused by TNF-α in MCF7, and an increase in p21 expression support and complement previous results. TNF-α could regulate the expression of p21 in ERα (+) MCF7 breast cancer cell. p21 is transcriptional target of the tumor suppressor gene p53, and also known as cyclin-dependent kinase inhibitor 1(CKI). Thus p21 was shown that associated with linked DNA damage to cell cycle arrest.

In addition, TNF-α up-regulated that the expression of pro-apoptosis proteins, such as Bax and Puma. Puma is pro-apoptotic protein, also known as p53 up-regulated modulator of apoptosis. The expression of Puma is regulated by p53. ERα (-) MDA-MB-231 did not increase expression of p21, Bax and Puma by TNF-α. Even at MDA-MB-231, Bax and Puma slightly decreased by TNF-α. In previous experiments, TNF-α did not reduce mRNA level of ERα, but increased mRNA level of Bax and Puma in MCF7 breast cancer cell. However, there was no change mRNA level of pro-apoptosis genes in MDA-MB-231.

TNF-α induces caspases-dependent apoptosis in MCF7

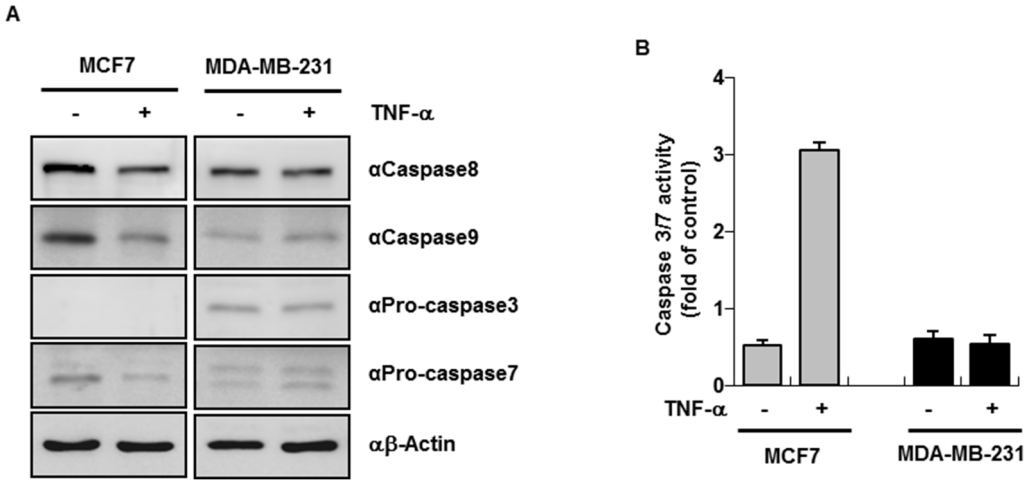

Fig. 6. Caspase signaling were checked for apoptotic pathway with TNF-α in breast cancers.

(A) TNF-α decreased Caspase8, caspase9 and pro-caspase7 expression and did not detect pro-caspase3 in ERα (+) MCF7 breast cancer cell, but did not effect caspase8, caspase9, pro-caspase3 and pro-caspase7 expression in ERα (-) MDA-MB-231 breast cancer cell.

(B) TNF-α up-regulated the activity of caspase3/7 in ERα (+) MCF7 breast cancer cell, but did not affect the activity of caspase3/7 in ERα (-) MDA-MB-231 breast cancer cell.

We thought that this is necessary to further study the cell apoptosis by TNF-α. So we checked the apoptotic pathway with TNF-α. In TNF- α-induced apoptosis, Caspase-8 is recruited and has activation. TNF-α-induced apoptosis is known to cleave caspase-8. The active Caspase-8 then initiates a caspase cascade, which results in apoptosis (Faleiro et al. 1997; Cryns and Yuan 1998). In ERα (+) MCF7 with TNF-α, caspase-8, caspase-9 and pro-caspase-7 were cleaved by TNF-α treatment. This result indicates that apoptosis by TNF-α is caused by a cascade for cleavage of other caspases. However, the cleavage of caspases by TNF-α was not observed in ERα (-) MDA-MB-231 breast cancer cell. To determine whether the degradation of caspase increased cell apoptosis, we measured activation of caspase-3/7 by caspase-3/7 activity assay. In ERα (+) MCF7 cell, we found that the activity of caspase-3/7 by TNF-α was increased. The activity of caspase-3/7 was not increased in MDA-MB-231 without caspases degradation. This result indicate that TNF-α increases the activity of caspase-3/7 in depending on whether ERα is expressing breast cancer cell.

TNF-α causes cell apoptosis through knockdown of NCoR1

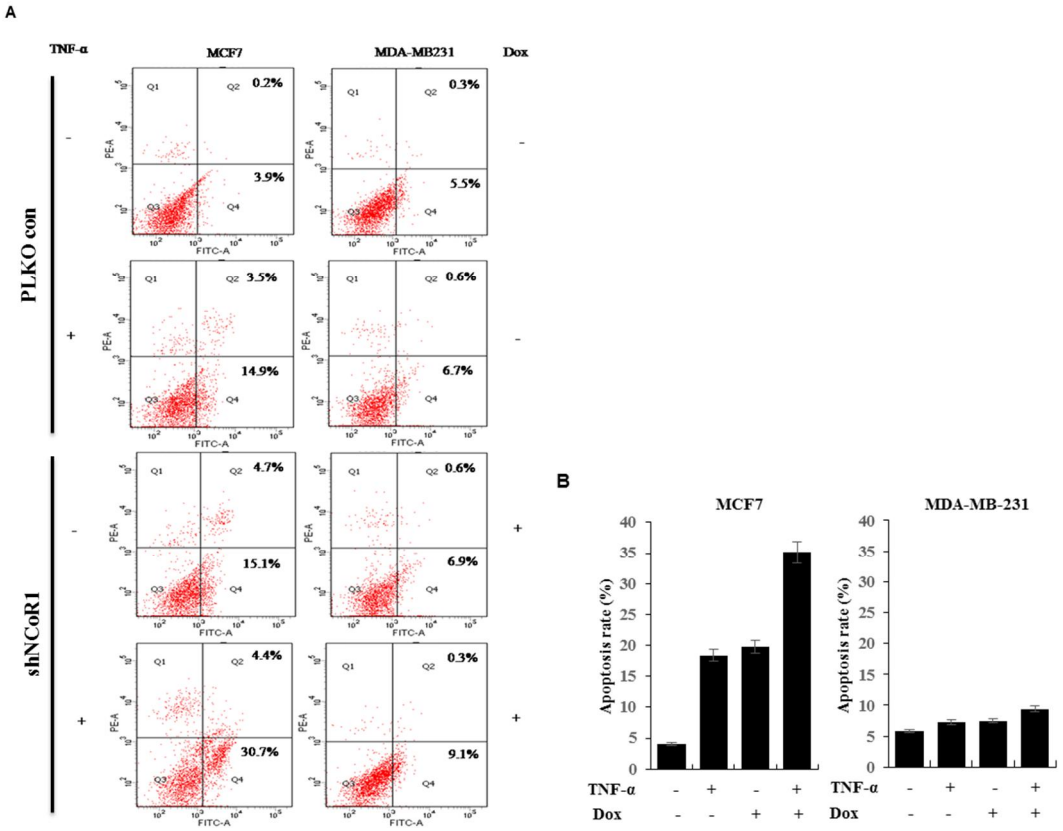

Fig. 7. Knockdown of NCoR1 affects the apoptosis with TNF-α in breast cancer cells.

(A) TNF-α caused cell death in PLKO control ERα (+) MCF7 breast cancer cell and shNCoR1 ERα (+) MCF7 breast cancer cell. Also, TNF-α induced more apoptosis in NCoR1 knock-down stable cell than PLKO control.

(B) TNF-α increased apoptosis rate, in addition co-treatment was more apoptosis rate in ERα (+) MCF7 breast cancer cell. However, TNF-α little effected cell death in ERα (-) MDA-MB-231 breast cancer cell.

Previous studies have shown that ERα interacts with NCoR1. When ERα-dependent breast cancer cells were knockdown of NCoR1, they would be more sensitive to TNF-α-induced apoptosis. In MCF7 breast cancer cell, apoptosis rate increased to 19% by TNF-α, but MDA-MB-231 did not increase. Knockdown of NCoR1 MCF7 without TNF-α showed the basic 20% apoptosis rate and knockdown of NCoR1 with TNF-α showed significantly high cell apoptosis rate. However, knockdown of NCoR1 MDA-MB-231 without TNF-α and with TNF-α did not affect to cell apoptosis. As a result, we found that TNF-α-induced apoptosis in dependent on ERα expression, and it has greater effect on knockdown NCoR1 in breast cancer cells.

TNF-α-induced ERα-NCoR1 complex down-regulation leads to the activation of p53 target genes

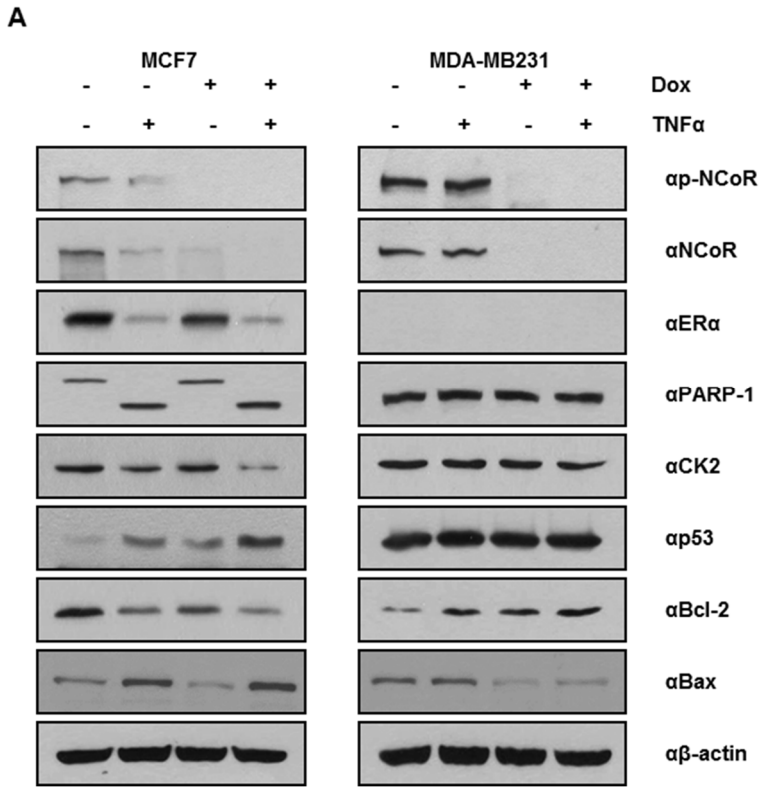

Fig. 8. Knockdown of NCoR1 effects the expression of apoptosis markers with TNF-α in breast cancer cells

(A) MCF7-PLKO con with TNF-α was induced with reduction of expression NCoR1, p-NCoR1, ERα, CK2, Bcl-2, and up-regulation of expression p53, Bax, and cleavage PARP1. MCF7-shNCoR1 did not detect expression of NCoR1, p-NCoR1. Also, it induced reduction of expression ERα, CK2, Bcl-2, and up-regulation of p53, Bax, and cleavage PARP1.

As in the previous experiments, we showed that the expression of NCoR1 and ERα affects TNF-α-induced apoptosis. Furthermore, we examined to confirm if apoptosis markers, p53 and CK2 were affected by TNF-α. p21 is a transcriptional target of the tumor suppressor gene p53. In previous experiments, we observed increased expression of p21 by TNF-α-induced apoptosis, and in this experiment, we observed an increase of p53 expression by increased p21. MCF7 depending on ERα and NCoR1 with TNF-α induced that cleaved PARP1 and degraded NCoR1, ERα and Bcl-2, and increased p53, and Bax.

In specific studies, they have described the interaction of CK2 and NCoR1. CK2 can exert anti-apoptosis effects through various mechanisms. CK2-mediated NCoR1 phosphorylation promotes cell survival in tumor cells. So degradation of CK2 and NCoR1 in MCF7 influenced apoptosis pathway.

However, we showed that only a slight increase in Bcl-2 by TNF-α and knockdown NCoR1, and no effect on the apoptosis pathway and CK2.

Discussion

Breast cancer is the most common type of cancer in women worldwide, but its incidence in continuously decreasing in the year 2000. Breast cancer is the only cancer that is considered universal among women worldwide. About one-eighth U.S women (about 12%) will develop invasive breast cancer over the course of her lifetime. In 2017, 252,710 new cases of invasive breast cancer are expected in U.S women and 63,410 new cases of non-invasive breast cancer occur. Progressive cancer spreads beyond the breast to nearby tissues, such as skin or bone or liver [7].

The estrogen receptor α (ERα) belongs to the superfamily of nuclear receptors. And it regulates the transcription of specific target genes. The ERα plays a crucial role in tumor development and progression, which is about two-thirds of breast cancer [4, 5, 29]. TNF-α is a potent pro-apoptosis cytokine, and anti-cancer properties. It may aid in the development of mechanism-based cancer-prevention approaches.

In this study, we demonstrated the mechanisms of TNF-α-induced apoptosis by studying its pro-apoptotic effect on depending ERα expression breast cancer cell. Expression of ERα was decreased in MCF7 with TNF-α at time- and dose-dependent manner. However, MDA-MB-231 which is negative ERα breast cancer didn't detect expression (Figure 1). Therefore, our results demonstrate that TNF-α response is dependent on ERα expression and only affects protein expression. In addition, we discovered that TNF-α exposure decreased the activity of ERE-Luc in MCF7 breast cancer cell (Figure 2). In a time- and dose- dependent experiment, TNF-α reduced the activity of EREs in ERα positive MCF7 breast cancer cell. Since MDA-MB-231 is negative for ERα, the activity of EREs was not observed. Breast cancer cells lacking ERα did not affect the activity and expression in the presence of TNF-α.

TNF-α is pro-apoptosis cytokine in breast cancer cell. We predict that TNF-α induces apoptosis in ERα positive MCF7, which has reduced ERα expression by TNF-α. We examined the results of cell viability on the apoptosis in TNF-α-treated breast cancer cells. We conclude that TNF-α enhances the apoptotic effect in MCF7 breast cancer cell, and not effect in MDA-MB-231. Degraded ERα by TNF-α was induced apoptosis in MCF7, but MDA-MB-231 became resistant to TNF-α-induced cytotoxicity (Figure 3). ERα pathway

induces the progression in ERα positive breast cancer cells [9, 16]. TNF-α inhibits the ERα-dependent pathway by decreasing ERα expression. ERα negative breast cancer cell is independent of the ERα pathway leading to cancer progression.

A further finding of this study was that breast cancer cells with TNF-α induced cell arrest depending on expression ERα. TNF-α decreased the G1 phase of MCF7, but it didn't not decrease the G1 phase of MDA-MB-231. The proposed signaling pathway could be viewed as apoptosis to degradation ER that is initiated by TNF-α in MCF7. This result showed that cell apoptosis was cleaved PARP1 by TNF-α-induced apoptosis, and was degraded ERα, NCoR1 in MCF7. Other results which didn't change also show that MDA-MB-231 has the resistant by TNF-α (Figure 4). NCoR1 complex are transcriptional co-repressors that regulate cell cycle arrest, cell differentiation, and apoptosis in cancer cell lines. Degradation of NCoR1 has been shown to enhance pro-apoptosis in MCF7, such as positive ERα. TNF-α can regulate the expression of p21, Bax and Puma in MCF7 breast cancer cell. TNF-α treatment led to the significant increase in the expression of the pro-apoptotic proteins p21, Puma and p21 (Figure 5). In addition TNF-α treatment up-regulates mRNA levels of pro-apoptotic genes, such as p21, Bax and Puma in MCF7. The stability of p53 determines the fate of cancer cells in MCF7 treated TNF-α.

We also examined the activity of caspases on breast cancer cells by TNF-α. TNF-α treatment stimulate caspase3/7 signal in MCF7 breast cancer. MCF7 with TNF-α induced the activation of initiator caspases and effector caspases. In MCF7 breast cancer cell, TNF-α leads to decrease in the expression of caspase-8, caspase-9 and pro-caspase-7. However, No changes were observed in MDA-MB-231 breast cancer cell. This results show that TNF-α-induced apoptosis was initiated by caspase-8 activation. To confirm, we examined caspase-3/7 activity assay whether TNF-α-induced apoptosis increased the activity of caspase cascade. Caspase-3/7 activation was 3-fold increased by TNF-α in MCF7 (Figure 6). This analysis shows that TNF-α-induced apoptosis was initiated by caspase cascade.

Previous work showed that degradation of ERα and NCoR1 by TNF-α induced cell apoptosis, suggesting that the NCoR1 complex regulates cell apoptosis. The results showed that TNF-α-induced-apoptosis increased the annexin-V cell population in PLKO con MCF7 cell. In addition, knockdown NCoR1 of MCF7 with TNF-α led to increase the annexin-V

cell population by 35%. In this study, we have demonstrated that knockdown NCoR1 increases sensitive of TNF-α-induced apoptosis in MCF7 (Figure 7). However, Knockdown NCoR1 of MDA-MB-231 did not affect to cell apoptosis by TNF-α. Moreover, The CK2 and p53 is a key regulatory factor for caspase cascade during cancer cell apoptosis. The CK2-NCoR signaling promotes invasive growth of PC-3 cells. We demonstrated that TNF-α-induced apoptosis induced pro-apoptosis signaling, such as cleaved PARP1, increased Bax. Knockdown control of MCF7 with TNF-α was induced the activation of p53, and knockdown NCoR1 of MCF7 with TNF-α was more induced the activation of p53 (Figure 8).

These results, TNF-α-mediated-CK2 is dependent the presence of NCoR1 expression. And, p21-mediated-p53 led to the cleavage of PARP1 and degradation of Bcl-2 in ERα positive MCF7 breast cancer cell. Thus, it is possible to understand that ERα-NCoR1-p53 complex is very important for the regulation of viability of breast cancer cells. Furthermore, the based on these studies, we will study to determine the effect of tumorigenesis, whether ERα-NCoR1-p53 is important for the mechanism of TNF-α-induced signaling

.

Conclusion

We have studied the role of corepressor N-CoR1 in ERα (+) and ERα (-) breast cancer cells. The nuclear receptor corepressor NCoR1 involved in the TNF-alpha-mediated apoptosis. TNF-α mediated apoptosis increased in ERα (+) breast cancer cells. We have found that TNF-α markedly down-regulates NCoR1 protein levels in ERα (+) MCF7 breast cancer cells. NCoR1 expression by TNF-α was induced the down-regulation of ERα protein levels in the MCF7 cells. This result suggests that NCoR1 expression may be involved in the TNF-α-mediated apoptosis of ERα (+) breast cancer cells. Interestingly, the phosphorylation of NCoR1 by CK2 inhibited the ubiquitin-dependent proteasomal degradation of NCoR1 in the TNF-a-treated MCF7 breast cancer cells. Eventually, the down-regulation of NCoR1 and ERα by TNF-α was increased the activation of p53. Thus, our results show a mechanism by which the TNF-α treatment induces the dissociation of ERα-NCoR-p53 complexes and activate the p53-dependent pro-apoptosis target genes through the activation of caspases. In conclusion, we demonstrated the function of ERα-p53-NCoR1 complexes in TNF-α induced apoptosis. The TNF-α treatment destabilizes ERα-p53-NCoR1 complexes via ubiquitination in apoptosis signal. Importantly, we found that knockdown of NCoR1 promotes TNF-α-induced apoptosis. In the absence of NCoR1 and ERα, the disrupt of complex enhance to sensitize TNF-α induced apoptosis signal in ERα (+) breast cancer.

Reference

1. Albuquerque, R.C., Baltar, V.T., and Marchioni, D.M. (2014). Breast cancer and dietary patterns: a systematic review. Nutr Rev 72, 1-17.
2. Cesi, V., Casciati, A., Sesti, F., Tanno, B., Calabretta, B., and Raschella, G. (2011). TGFbeta-induced c-Myb affects the expression of EMT-associated genes and promotes invasion of ER+ breast cancer cells. Cell Cycle 10, 4149-4161.
3. Chen, J.D., and Evans, R.M. (1995). A transcriptional co-repressor that interacts with nuclear hormone receptors. Nature 377, 454-457.
4. Chien, P.Y., Ito, M., Park, Y., Tagami, T., Gehm, B.D., and Jameson, J.L. (1999). A fusion protein of the estrogen receptor (ER) and nuclear receptor corepressor (NCoR) strongly inhibits estrogen-dependent responses in breast cancer cells. Mol Endocrinol 13, 2122-2136.
5. Cooper, C., Liu, G.Y., Niu, Y.L., Santos, S., Murphy, L.C., and Watson, P.H. (2004). Intermittent hypoxia induces proteasome-dependent down-regulation of estrogen receptor alpha in human breast carcinoma. Clin Cancer Res 10, 8720-8727.
6. Desvergne, B., Michalik, L., and Wahli, W. (2006). Transcriptional regulation of metabolism. Physiol Rev 86, 465-514.
7. Dickler, M.N., Tolaney, S.M., Rugo, H.S., Cortes, J., Dieras, V., Patt, D., Wildiers, H., Hudis, C.A., O'Shaughnessy, J., Zamora, E., et al. (2017). MONARCH 1, A Phase II Study of Abemaciclib, a CDK4 and CDK6 Inhibitor, as a Single Agent, in Patients with Refractory HR+/HER2- Metastatic Breast Cancer. Clin Cancer Res 23, 5218-5224.
8. Dreschers, S., Gille, C., Haas, M., Grosse-Ophoff, J., Schneider, M., Leiber, A., Buhring, H.J., and Orlikowsky, T.W. (2013). Infection-induced bystander-apoptosis of monocytes is TNF-alpha-mediated. PLoS One 8, e53589.
9. Ford, C.H., Al-Bader, M., Al-Ayadhi, B., and Francis, I. (2011). Reassessment of estrogen receptor expression in human breast cancer cell lines. Anticancer Res 31, 521-527.

10. Fozzatti, L., Park, J.W., Zhao, L., Willingham, M.C., and Cheng, S.Y. (2013). Oncogenic Actions of the Nuclear Receptor Corepressor (NCOR1) in a Mouse Model of Thyroid Cancer. PLoS One 8, e67954.
11. Gupta, N., Jung, K., Wu, C., Alshareef, A., Alqahtani, H., Damaraju, S., Mackey, J.R., Ghosh, S., Sabri, S., Abdulkarim, B.S., et al. (2017). High Myc expression and transcription activity underlies intra-tumoral heterogeneity in triple-negative breast cancer. Oncotarget 8, 28101-28115.
12. Huang, X., Wang, F., Chen, W., Wang, N., Chen, Y., and Sun, L. (2014). DaoTan decoction (DTD) inhibits tumor necrosis factor-alpha (TNF-alpha)-induced expression of intercellular adhesion molecule-1 (ICAM-1), p53 and p21, in human umbilical vein endothelia cells (HUVECs). Pharm Biol 52, 1320-1326.
13. Kao, J., Salari, K., Bocanegra, M., Choi, Y.L., Girard, L., Gandhi, J., Kwei, K.A., Hernandez-Boussard, T., Wang, P., Gazdar, A.F., et al. (2009). Molecular profiling of breast cancer cell lines defines relevant tumor models and provides a resource for cancer gene discovery. PLoS One 4, e6146.
14. Kim, M.S., Lee, W.S., Jeong, J., Kim, S.J., and Jin, W. (2015). Induction of metastatic potential by TrkB via activation of IL6/JAK2/STAT3 and PI3K/AKT signaling in breast cancer. Oncotarget 6, 40158-40171.
15. Kwak, H.B., Jin, H.M., Ha, H., Kang, M.J., Lee, S.B., Kim, H.H., and Lee, Z.H. (2005). Tumor necrosis factor-alpha induces differentiation of human peripheral blood mononuclear cells into osteoclasts through the induction of p21(WAF1/Cip1). Biochem Biophys Res Commun 330, 1080-1086.
16. Lee, S.H., and Nam, H.S. (2008). TNF alpha-induced down-regulation of estrogen receptor alpha in MCF-7 breast cancer cells. Mol Cells 26, 285-290.
17. Malhotra, G.K., Zhao, X., Band, H., and Band, V. (2010). Histological, molecular and functional subtypes of breast cancers. Cancer Biol Ther 10, 955-960.
18. Meiyanto, E., Hoshijima, M., Ogawa, T., Ishida, N., and Takeya, T. (2001). Osteoclast differentiation factor modulates cell cycle machinery and causes a delay in s phase progression in RAW264 cells. Biochem Biophys Res Commun 282, 278-283.

19. Moon, D.O., Kim, M.O., Kang, S.H., Choi, Y.H., Park, S.Y., and Kim, G.Y. (2010). HA14-1 sensitizes TNF-alpha-induced apoptosis via inhibition of the NF-kappaB signaling pathway: involvement of reactive oxygen species and JNK. Cancer Lett 292, 111-118.
20. Mottis, A., Mouchiroud, L., and Auwerx, J. (2013). Emerging roles of the corepressors NCoR1 and SMRT in homeostasis. Genes Dev 27, 819-835.
21. Park, S.Y., Kim, M.J., Park, S.A., Kim, J.S., Min, K.N., Kim, D.K., Lim, W., Nam, J.S., and Sheen, Y.Y. (2015). Combinatorial TGF-beta attenuation with paclitaxel inhibits the epithelial-to-mesenchymal transition and breast cancer stem-like cells. Oncotarget 6, 37526-37543.
22. Perissi, V., Jepsen, K., Glass, C.K., and Rosenfeld, M.G. (2010). Deconstructing repression: evolving models of co-repressor action. Nat Rev Genet 11, 109-123.
23. Prat, A., and Perou, C.M. (2011). Deconstructing the molecular portraits of breast cancer. Mol Oncol 5, 5-23.
24. Rao, R., Balusu, R., Fiskus, W., Mudunuru, U., Venkannagari, S., Chauhan, L., Smith, J.E., Hembruff, S.L., Ha, K., Atadja, P., et al. (2012). Combination of pan-histone deacetylase inhibitor and autophagy inhibitor exerts superior efficacy against triple-negative human breast cancer cells. Mol Cancer Ther 11, 973-983.
25. Sankar, U., Patel, K., Rosol, T.J., and Ostrowski, M.C. (2004). RANKL coordinates cell cycle withdrawal and differentiation in osteoclasts through the cyclin-dependent kinase inhibitors p27KIP1 and p21CIP1. J Bone Miner Res 19, 1339-1348.
26. Shimizu, H., Astapova, I., Ye, F., Bilban, M., Cohen, R.N., and Hollenberg, A.N. (2015). NCoR1 and SMRT play unique roles in thyroid hormone action in vivo. Mol Cell Biol 35, 555-565.
27. Sundararajan, R., Cuconati, A., Nelson, D., and White, E. (2001). Tumor necrosis factor-alpha induces Bax-Bak interaction and apoptosis, which is inhibited by adenovirus E1B 19K. J Biol Chem 276, 45120-45127.
28. Thakur, R., Trivedi, R., Rastogi, N., Singh, M., and Mishra, D.P. (2015). Inhibition of STAT3, FAK and Src mediated signaling reduces cancer stem cell load, tumorigenic potential and metastasis in breast cancer. Sci Rep 5, 10194.

29. Vanneste, M., Hanoux, V., Bouakka, M., and Bonnamy, P.J. (2017). Hyaluronate synthase-2 overexpression alters estrogen dependence and induces histone deacetylase inhibitor-like effects on ER-driven genes in MCF7 breast tumor cells. Mol Cell Endocrinol 444, 48-58.
30. Wang, R., Lv, Q., Meng, W., Tan, Q., Zhang, S., Mo, X., and Yang, X. (2014). Comparison of mammosphere formation from breast cancer cell lines and primary breast tumors. J Thorac Dis 6, 829-837.
31. Wolf, J., Dewi, D.L., Fredebohm, J., Muller-Decker, K., Flechtenmacher, C., Hoheisel, J.D., and Boettcher, M. (2013). A mammosphere formation RNAi screen reveals that ATG4A promotes a breast cancer stem-like phenotype. Breast Cancer Res 15, R109.
32. Yan, J., Gao, Z., Yu, G., He, Q., Weng, J., and Ye, J. (2007). Nuclear corepressor is required for inhibition of phosphoenolpyruvate carboxykinase expression by tumor necrosis factor-alpha. Mol Endocrinol 21, 1630-1641.
33. Ye, Y., Xiao, Y., Wang, W., Yearsley, K., Gao, J.X., and Barsky, S.H. (2008). ERalpha suppresses slug expression directly by transcriptional repression. Biochem J 416, 179-187.
34. You, S.H., Lim, H.W., Sun, Z., Broache, M., Won, K.J., and Lazar, M.A. (2013). Nuclear receptor co-repressors are required for the histone-deacetylase activity of HDAC3 in vivo. Nat Struct Mol Biol 20, 182-187.
35. Zhang, B., Wu, T., Wang, Z., Zhang, Y., Wang, J., Yang, B., Zhao, Y., Rao, Z., and Gao, J. (2015). p38MAPK activation mediates tumor necrosis factor-alpha-induced apoptosis in glioma cells. Mol Med Rep 11, 3101-3107.
36. Zhao, X.P., Huang, Y.Y., Huang, Y., Lei, P., Peng, J.L., Wu, S., Wang, M., Li, W.H., Zhu, H.F., and Shen, G.X. (2010). Transforming growth factor-beta1 upregulates the expression of CXC chemokine receptor 4 (CXCR4) in human breast cancer MCF-7 cells. Acta Pharmacol Sin 31, 347-354.

Abstract (In Korean)

유방암 세포에서 종양괴사인자알파(TNF-α)에 의한 세포사멸에서의 N-CoR1 역할

유방암은 여성의 암 중 매우 흔한 악성 종양이며, 전 세계적으로 발생률이 지속적으로 증가하고 있다. 수십 년 간에 걸친 기초연구 및 임상 시험 연구에도 불구하고 유방암의 사망률은 아직도 높다. 최근 연구에서는, 전이성 유방암을 극복하기 위해 복합적인 치료법과 종합적인 치료법이 제시되고 있다. 에스트로겐 수용체 (ERα) 양성 유방암은 유방 종양의 70% 이상을 차지하며 선택적인 에스트로겐 수용체 조절치료법 및 내분비 치료제는 여전이 종양의 표준 보조 치료법이다. 그러나 대다수의 환자는 호르몬 요법에 대한 저항성을 보이며 대체 요법이 필요하다. Nuclear receptor co-repressor1 (NCoR1) 과 같은 전사 보조억제자는 에스트로겐 수용체가 상호작용하는 유전자에 적대자(antagonist)로써 경쟁하여 전사 침묵을 유지한다. 그래서 우리는 에스트로겐 수용체 양성 유방암 세포에서 NCoR1 의 역할을 연구하였다.

NCoR1 은 종양괴사인자알파 (TNF-α) 에 의해 유도되는 세포사멸에 중요한 역할을 한다. 그러나 유방암에서 어떻게 ERα-NCoR1 복합체를 조절하는지는 거의 알려져 있지 않다. 우리는 에스트로겐 수용체 양성 MCF7 유방암 세포에서 종양괴사인자알파가 NCoR1 의 발현조절에 관여한다는 것을 발견했다. 하지만 에스트로겐 수용체 음성 MDA-MB-231 유방암 세포에서는 종양괴사인자알파에 의한 세포사멸이 나타나지 않았는데, 이는 종양괴사인자알파에 의한 저항성이 에스트로겐 수용체 단백질의 결핍 때문이라고 생각했다. 결과적으로, 종양괴사인자알파에 의한 NCoR1 및 에스트로겐 수용체의 하향조절은 p53 의 발현과 활성을 증가시켰고, 증가된 p53 이 p21 의 활성과 발현을 증가시켰다. 그로 인해, p21 유전자는 세포주기를 정지시키고 세포자살을 일으켰다.

최종적으로, 우리는 p21, Bax 및 Puma 와 같은 세포사멸 유전자의 활성화는 종양괴사인자알파에 의해 해리된 ERα-NCoR1-p53 복합체가 핵심 역할을 한다는 것을 보여주었다.

중심단어 : 종양괴사인자알파 (TNF-α), 에스트로겐 수용체 알파 (ERα), Nuclear Receptor Corepressor1 (NCoR1), p53, 세포사멸, 유방암