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석사학위논문

The Prolyl-isomerase Pin1 induces LC-3 expression and mediates tamoxiffen resistance in breast cancer

조선대학교 대학원

약 학 과

남궁 광 모

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이 논문을 약학 석사학위신청 논문으로 제출함.

2010 년 10 월

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List of Abbreviations

AP-1	Activator protein-1
BME	Basal Medium Eagle
ER	Estrogen receptor
DMSO	Dimethylsulfoxide
DMEM	Dulbecco's Modified Eagle Medium
ERK1/2	Extracellular signal-related kinase1/2
FBS	Fetal bovine serum
JNK1/2	c-Jun N-terminal kinase1/2
MAPK	Mitogen-activated protein kinase
MEF	Mouse embryonic fibroblasts
MEM	Eagle's minimal essential medium
MSCV-Pin1	Pin1-overexpressing MCF7 cells
PARP	Poly(ADP-ribose) polymerase)
RT-PCR	Reverse transcriptase polymerase chain reaction
SDS-PAGE	Sodium dodecyl sulfate-polyacryamide gel electrophoresis
TAMR-MCF7	Tamoxifen-resistant MCF7 breast cancer cells
TPA	S12-O-Tetradecanoylphorbol-13-acetate
TUNEL	Terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick-end

국문 초록

Prolyl-이성질화효소 Pin1 의 LC-3 발현 유도 및 타목시펜 저항성에 관한 연구

남궁 광모

지도교수: 최홍석

조선대학교 대학원 약학과

에스트로겐 수용체 신호전달과정을 억제하는 내분비 치료요법은 ER 알파 양성 유방암에 대한 가장 일반적이고 효율적인 치료법이다. 그러나 이러한 약물들의 사용은 빈번한 저항성의 형성으로 제한되고 내분비치료 저항성에 대한 정확한 기전 역시 완벽히 규명되지 않았다. 이 논문에서 우리는 peptidyl-prolyl isomerase Pin1 이 타목시펜 저항성에 대한 중요한 결정요인이라는 것을 입증하였고 Pin1 이 MEK1/2 의 인산화와 상호작용을 통해 E2F-4 와 Egr-1 에 의한 LC-3 의 발현을 증가시킨다는것을 밝혀내었다. 사람 타목시펜-저항성 유방암에서,

우리는 Pin1 의 과다발현과 LC-3 의 양적 증가가 강한 상관관계를 보임을 관찰할 수 있었다. control MCF7 세포보다 타목시펜-저항성 MCF7 세포에서 Pin1 의 발현수준 뿐만 아니라 promoter 활성까지 뛰어나게 증가하였고, autophagy marker 인 LC-3 mRNA 와 단백질 역시 같은 패턴을 보였다. Pin1-/- mouse embryonic fibroblasts (MEF)는 Pin1+/+ MEF 보다 TPA 에 의한 MEK1/2 인산화 수준이 낮았고, MCF7 세포에서 Pin1 발현의 silencing 은 TPA 에 의한 MEK1/2 인산화를 억제시켰다. 더군다나, MEK1/2 의 특이적 억제제인 PD98059 와 Pin1 의 억제제인 Juglone 은 TPA 에 의해 유도되는 LC-3 유전자 발현을 조절할 수 있는 Egr-1 뿐만 아닌 E2F4 전사조절인자를 억제시킨다. 중요한것은, Pin1 이나 LC-3 의 silencing 시킨 후 병용한 4-OH 타목시펜은 MCF7 세포의 콜로니 성장을 억제시키기 위해 cleaved PARP 와 DNA fragmentation 을 증가시킨다. 따라서 우리는 Pin1/MEK 경로와 LC-3 를 매개로한 타목시펜-저항성을 연관짓고 타목시펜-저항성 유방암의 치료에서 Pin1 의 가능성을 보여주었다.

ABSTRACT

The Prolyl-isomerase Pin1 induces LC-3 expression and mediates tamoxifen resistance in breast cancer

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Endocrine therapies, which inhibit estrogen receptor (ER) signaling, are the most common and effective treatments for ERα positive breast cancer. However, the utility of these agents is limited by the frequent development of resistance and the precise mechanisms underlying endocrine therapy resistance remain incompletely understood. Here, we demonstrate that peptidyl-prolyl isomerase Pin1 is an important determinant of resistance to tamoxifen and show that Pin1 increases E2F-4- and Egr-1-driven expression of LC-3 as a result of an increased interaction with and phosphorylation of MEK1/2. In human tamoxifen-resistant breast cancer, our results show a significant correlation between Pin1 overexpression and high levels of LC-3. Promoter activity as well as expression levels of Pin1 were drastically higher in tamoxifen resistant MCF7 cells than control MCF7 cells, as were levels of LC-3 mRNA and protein, an autophagy marker. *Pin1*-/- mouse embryonic fibroblasts (MEF) showed lower TPA-

induced MEK1/2 phosphorylation than *Pin1*+/+ MEF. Silencing of Pin1 expression inhibited TPAinduced MEK1/2 phosphorylation in MCF7 cells. Moreover, PD98059, a specific inhibitor of MEK1/2, and Juglone, a potent Pin1 inhibitor, significantly suppressed the TPA-induced expression of E2F4 as well as Egr-1 transcription factors, which control LC-3 gene expression. Importantly, 4-OH tamoxifen, when used in combination with silencing of Pin1 or LC-3, increased cleaved PARP and DNA fragmentation to inhibit cologenic growth of MCF7 cells. We therefore link the Pin1/MEK pathway and LC-3-mediated tamoxifen resistance, and show the therapeutic potential of Pin1 in the treatment of tamoxifenresistance breast cancer.

I. Introduction

Breast cancer is one of the most common malignancies in women and the second most common cause of female cancer-related deaths (1). However, deaths due to breast cancer have decreased because of the development of targeted therapies, including hormone therapy, in addition to conventional chemotherapy and surgical interventions (1). The majority of breast cancers in post-menopausal women express the estrogen receptors (ER), and after surgery they can be treated with hormonal therapy alone, in the absence of more toxic chemotherapy, resulting in a relatively favorable prognosis (2). However, a significant fraction of these hormone-sensitive breast cancer patients will experience disease progression because of resistance to endocrine agents, such as tamoxifen, resulting in mortality (3). Tamoxifen is currently the most widely prescribed, orally active, selective ER modulator for the treatment of breast cancer (4). Although tamoxifen is an ER antagonist in breast tissue, it can also be a partial agonist. Antagonist activity enables the drug to block ER-mediated transcription and cancer cell growth in ERpositive breast cancer cells (5). However, tamoxifen resistance might occur when its agonistic activity overcomes its ntagonistic effect (4). This variability could be related, in part, to the cellular milieu of ER co-activators and co-repressors (6). For example, increased levels of co-activators, such as SRC-3, enhance the estrogen agonist properties of

tamoxifen, whereas decreased levels of co-repressors, such as SMRT and N-CoR, correlate with acquired tamoxifen resistance (6).

Pin1 has two domains: a peptidyl-prolyl cis/trans isomerase (PPIase) domain at its COOH terminus responsible for isomerization, and a WW domain at the NH2 terminus, which functions as a binding element specific for pSer/Thr-Pro motifs (7). Through these two domains, Pin1 binds to and isomerizes specific pSer/Thr-Pro motifs and catalytically induces conformational changes after phosphorylation (7).

Recently, Stanya and colleagues showed that CDK2 (cyclin dependent kinase 2)-mediated phosphorylation of SMRT (silencing mediator for retinoid and thyroid receptors), an ER corepressor, creates a binding site for Pin1 PPIase, which in turn induces conformational changes to promote SMRT degradation (8). Moreover, this event mediates human epidermal growth factor receptor-2 (HER-2)-dependent SMRT protein degradation and resultant endocrine resistance (8). These findings help elucidate the molecular mechanism of ER regulation and indicate that further investigation is needed of the role and therapeutic potential of Pin1 in the treatment of endocrine-resistant breast cancers.

Here, we have determined for the first time a significant correlation between Pin1 overexpression and high levels of autophagy-related protein LC-3 in human tamoxifen-resistant breast cancer. We found that Pin1 binds directly to MEK1/2 and regulates its activity, resulting in inducing the expression of E2F-4, Egr-1, and

ultimately LC-3 to affect tamoxifen resistance in breast cancer cells. Moreover, we show that 4-OH tamoxifen, when used in combination with silencing of Pin1 or LC-3, induces robust, caspase-dependent apoptosis of breast cancer cells. These results may have relevance to the development of tamoxifen resistance, as shown by the strong correlation, observed in tamoxifen-resistance breast cancer, between Pin1 overexpression and the presence of high levels of LC-3.

II. Materials & Methods

1. Reagents and Antibodies

Dulbecco's Modified Eagle Medium (DMEM), Eagle's minimal essential medium (MEM), L-glutamine, gentamicin, and FBS were purchased from Invitrogen (Carlsbad, CA). Charcoal/dextran treated FBS was obtained from Hyclone (Logan, UT). 12-*O*-Tetradecanoylphorbol-13-acetate (TPA) and PD98059 were obtained from Calbiochem–Novabiochem (San Diego, CA). The dual-luciferase reporter assay kit was obtained from Promega (Madison, WI). Basal Medium Eagle (BME), Juglone, 4-hydroxytamoxifen, 3-[4,5-Dimethylthiazol-2-thiazoyl]-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO). The antibodies against phospho-MEK1/2, phospho-ERK1/2 and total antibodies against MEK1/2, ERK1/2, Beclin-1, Atg-5, Atg-12, and LC-3 were purchased from Cell Signaling Technology Inc. (Beverly, MA). The antibodies against Pin1, E2F4, and Egr-1 were acquired from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-X-press antibody was obtained from Invitrogen. The anti-ErbB2 antibody was purchased from Dako Cytomation (Glostrup, Denmark)

2.Tumor samples -

Breast cancer patients selected for immunohistochemical staining consisted of two groups: tamoxifen resistant group (4 patients, age range: 42-72) and non tamoxifen

resistant group (8 patients, age range: 47-58). The eligibility for tamoxifen resistant group was mammary infiltrating duct carcinoma patient who had undergone mastectomy with adjuvant hormone therapy and had subsequently bone metastasis, and that for non tamoxifen resistant group was mammary infiltrating duct carcinoma patient who had undergone mastectomy with adjuvant hormone therapy and had no subsequently local recurrence or metastasis within 5 years.

3.Immunohistochemical staining-

All tumors investigated in the study were tested for, LC-3 and Pin1. Immunolocalization for LC-3 and Pin1 was performed using a Polink-2 HRP plus anti-rabbit DAB detection kit (Golden Bridge International, Inc. Mukilteo, WA) according to the supplier's protocol. Slides were incubated for 1h with anti-Pin1 antibody and for overnight with anti-LC-3 antibody, respectively. An isotype matched control antibody was also used. Positive control for LC-3 was capsaicin treated WI38 cell, those for Pin-1 were mammary infiltrating duct carcinoma with strong nuclear staining in another study. Instead of the primary antibody, normal goat serum was used in negative control. Distinct nuclear staining was considered as positive immunoreactivity.

4.Cell culture

MCF7 human breast cancer cells and *Pin1*+/+ *and Pin1*-/- mouse embryonic fibroblast (MEF) cells, which were kindly provided by Dr. Kun Ping Lu (Beth Israel Deaconess Medical Center, Harvard Medical School), were grown in DMEM

supplemented with 10% fetal bovine serum. DMEM containing 10% charcoal/dextran treated FBS and 3μM 4-OH tamoxifen were used to culture the Tamoxifen-resistant MCF7 breast cancer cells (TAMR-MCF7), which were provided by Dr. Kwon-Wook, Kang (College of Pharmacy, Chosun University).

5. Construction of mammalian expression and small interfering RNA

The cDNA of full sequence of Pin1, which was a gift from Dr. Kun Ping Lu were subcloned to the pcDNA4/Xpress vector (Invitrogen). The mutant of Pin1 S16A was given by Dr. Jeong-Hyun Shim (Chonbuk National University). A segment encoding the full sequence of MEK1 was amplified by PCR and cloned in-frame into the BamHI/XbaI sites of the pCMV-HA (Mountain View, Clontech, CA) or pGEX-5X-1 (GE Healthcare Biosciences, Piscataway, NJ) vector to produce the plasmid pCMV-HA-MEK1 or pGEX-5X-1-MEK1, respectively. All MEK1 deletion mutants (pGEX-5X-1-D1, -D2, or D3) were generated from pGEX-5X-1-MEK1 by PCR and subcloned inframe into the BamHI/XbaI sites of pGEX-5X-1 vector, respectively. The silencing of human Pin1 (accession number: NM_006221), human LC-3 (accession number: NM_022818), human E2F-4 (NM_001950), and human Egr-1 (NM_001964) were performed by transfecting the ONTARGET plus siRNA SMART pool-specific or nonspecific control pool double-stranded RNA oligonucleotides (Dharmacon, Chicago, IL) using LipofectamineTM 2000 (Invitrogen).

6. Infection of GFP and Pin1 into JB6 Cl41 cells

Pin1 was overexpressed stably in MCF7 cells using the MSCV-GFP retrovirus system. Human Pin1 was subcloned into the MSCV-GFP retroviral vector (Clontech Inc., Mountain View, CA), and phoenix cells (a packaging cell line) were transfected with the MSCV-GFP or MSCVPin1-GFP plasmids. The supernatants containing amphotropic, replication-incompetent retroviruses were collected and stored at -80°C until needed. MCF7 cells (20% confluent) were multiply infected (8 times) with retrovirus particles.

7. Cell proliferation by MTT assay

Cells seeded on 96-well microplates at 10,000 cells per well were incubated with 4-OH tamoxifen for indicated times. Respective medium was removed and then incubated with MTT solution (5 μg/ml) for 3 h. Absorbance was determined using microplate reader.

8. Protein extract and immunoblotting

Cells were disrupted in immunoprecipitation assay buffer [50 mM Tris (pH 7.5), 5 mM EDTA, 150 mM NaCl, 1 mM DTT, 0.01% Nonidet P-40, 0.2 mM phenylmethylsulfonyl fluoride, and 1×protease inhibitors cocktail] or RIPA lysis buffer [50 mM Tris (pH 7.5), 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% Nonidet P-40, 0.1% SDS, 0.2 mM phenylmethylsulfonyl fluoride, and 1× protease inhibitors cocktail] and subjected to the immunoblotting.

9. Reporter gene assays

Pin1 promoter luciferase activity was measured with the Dual-Luciferase® Reporter Assay System (Promega, Madison, WI) according to manufacture's instructions. Briefly, cells were cotransfected with a plasmid mixture containing the *Pin1* promoter luciferase reporter gene (a gift from Dr. Kun Ping Lu) with the phRL-SV40 gene (Promega). At 48 h after transfection, firefly luciferase activity was measured using GloMax®-Multi Detection System (Promega). Subsequently, renilla luciferase activity was measured in order to normalize the firefly luciferase data.

10. RNA isolation and semi-quantitative RT-PCR

Total RNA was isolated from cells using the RNeasy Micro Kit (Qiagen, Valencia, CA). Reverse transcription-PCR (RT-PCR) was performed using the AcessQuick RT-PCR system (Promega, WI) on a Gradient Thermal Cycler (Bio-Rad Lab., Hercules, CA) using the following PCR primers: *human LC-3* (sense, 5'-AGCAGCATCCAA CCAAAATC-3'; antisense, 5'-CTGTGTCCGTTCACCAACAG-3'), *human Pin1* (sense, 5'-AGCAGCAGTGGTGGCAAAAA-3'; antisense, 5'-GGCCAGAGACTC AAAGTCCT-3), and β-*actin* (Promega), respectively. All data were normalized to β-*actin* as an internal control according to the manufacturer's instructions.

11. In vitro binding assay and GST protein expression

For expression of the Xpress-epitope tagged Pin1, the appropriate plasmids (pcDNA4/Xpress-Pin1) were translated *in vitro* with L-methionine using the TNT Quick coupled transcription/translation system (Promega). For the GST pull-down

assay, 5μg GST fusion protein of MEK1 and deletion mutants were collected on glutathione-Sepharose beads (GE Healthcare Biosciences), incubated for 4 h at 4°C with Xpress-Pin1. The bound proteins were denatured in sample buffer and separated by 10-20% SDS-PAGE and expression was detected by immunoblotting.

12. Detection of apoptosis

Apoptosis assay was performed by terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick-end labeling (TUNEL) and was detected with an in situ Cell Death detection Kit (Roche Applied Science, Indianapolis, IN) according to manufacturer's instructions. Briefly, cells were transfected with siRNA-sc, siRNA-Pin1, or siRNA-LC-3 and incubated for 48 h. The cells were then starved with serum-free DMEM for 24h, treated with tamoxifen for an additional 24 h. Then cells were stained with TUNEL solution and incubated at 37 ºC for 2 h, washed twice with PBS, and mounted with crystal mount reagent for 4 h in the dark. The amount of DNA fragmentation was detected by using an Axiovert 200 M fluorescence microscope and Axio Vision software (Carl Zeiss Inc., Thornwood, NY).

13. Apoptosis assessed by flow cytometry

The induction of early and late apoptosis was analyzed by flow cytometry using the Becton Dickinson FACS Calibur Flow Cytometer (BD Biosciences). TAMR-MCF7 cells (5 □105 per dish), were grown in 6-cm dishes for 12 h in 10% FBS/DMEM.

Then, cells were transfected with siRNA-sc or -Pin1, incubated for 48 h, and then treated with 4-OH tamoxifen. After additional incubation for 24 h, the medium was collected and attached cells were harvested with 0.025% trypsin in 5 mM EDTA in PBS. Cells were washed by centrifugation at 1,000 rpm for 5 min and processed for detection of early and late apoptosis using Annexin V-FITC and propidium iodide staining according to the manufacturer's protocol.

14. Anchorage-independent cell transformation assay (soft agar assay)

Briefly, the cells (8×103) were exposed to PD98058 or Juglone with or without TPA in 1 ml of 0.3% basal Eagle's agar containing 10% FBS. The cultures were maintained at 37 °C for 10-15 days, and the cell colonies were scored using an Axiovert 200M florescence microscope and Axio Vision software (Carl Zeiss Inc., Thornwood, NY).

15. Statistical Analysis

The Fisher's Exact test, two sided values of probability (*P*) are given, was used to analyze the correlation between Pin1 expression and level of LC-3 in tamoxifenresistant breast cancer patients. Data from cell viability, promoter activity, RT-PCR, or soft agar assay were statistically analyzed using unpaired *t*test and *P* values < 0.05 were considered to be significant. Statistical calculations were carried out with Prism 4 for Macintosh software (GraphPad Software. Inc., La Jolla, CA).

III. Results

1. Pin1 and LC-3 levels correlate in tamoxifen-resistant breast cancer

To investigate the pathological relevance of the relationship between Pin1 and LC-3 expression in tamoxifen-resistant breast cancers, we analyzed these 2 proteins in 8 human non-tamoxifen-resistant and 4 human tamoxifen-resistant breast cancer tissue samples. Immunohistochemical staining showed that there was a positive correlation between the levels of LC-3 and Pin1 in human tamoxifen-resistant breast cancer samples as tumors with high levels of LC-3 (in 3 of 4 samples) also had high levels of Pin1 protein levels (in all 4 samples) in tamoxifen-resistant cancers, whereas low levels of LC-3 (in 7 of the 8 samples) were paralleled by low levels of Pin1 (in 7 of the 8 samples) in nontamoxifen-resistant cancers ($P < 0.010$, Fisher's Exact test; Fig. 1*A*).

To further examine whether Pin1 or LC-3 facilitates the development of antiestrogen resistance, we did a drug selection in which MCF7 cells were exposed to small, incremental increases of 4-OH tamoxifen. The stepwise drug selection was continued until the MCF7 cell population could sustain viability and proliferate when challenged with 3.0 μM 4-OH tamoxifen. The acquisition of 4-OH tamoxifen resistance in TAMR-MCF7 cells was verified using a MTT assay. 4-OH tamoxifen caused a concentration-dependent decrease in the cell viability of MCF7 cells but not TAMR-MCF7 up to 5 μM (Fig. 1*B*).

To examine the effects on Pin1 promoter activity, MCF7 or TAMR-MCF7 cells were transfected with mixtures of the Pin1 promoter luciferase construct and phRL-

SV-40 gene. The promoter activity of Pin1 was significantly increased in TAMR-MCF7 cells compared with MCF7 cells (Fig. 1*C*, *upper panels*). Pin1 expression was higher in TAMR-MCF7 cells than MCF7 cells in immunoblots (Fig. 1*C*, *lower panels)*. These results suggested that Pin1 is likely to be involved in MCF7 resistance to tamoxifen. To determine whether autophagy proteins affect tamoxifen resistance, we next monitored the expression levels of autophagy proteins in TAMR-MCF7 cells. TAMR-MCF7 cells showed higher levels of LC-3 and, a lesser extent, Beclin-1, ATG5, and ATG12 than control MCF7 cells (Fig. 1*D, third lane*). To further confirm whether overexpression of LC-3 in TAMR-MCF7 cells are affected by Pin1, pcDNA4/Xpress-Pin1 were transfected into MCF7 cells and the cells were incubated for 48 h. Pin1-overexpressing MCF7 cells showed increased levels of LC-3 (Fig. 1*D, second lane*).

To examine the effect of Pin1 on the expression level of LC-3, which is an autophagy marker, we then analyzed mRNA levels of *LC-3* followed by overexpression of Pin1 in MCF7 cells or knockdown of Pin1 in TAMR-MCF7 cells, respectively. Semi-quantitative RT-PCR results showed that *LC-3* mRNA level was increased by up to 5 folds in Pin1-overexpressing MCF7 cells compared to MCF-7 cells, whereas the increased LC-3 mRNA level in TAM-MCF7 cells significantly decreased with knockdown of Pin1 (Fig. 1*E*). Collectively, these data suggest that Pin1-mediated overexpression of LC-3 may be responsible for the 4-OH tamoxifen resistance in breast cancer.

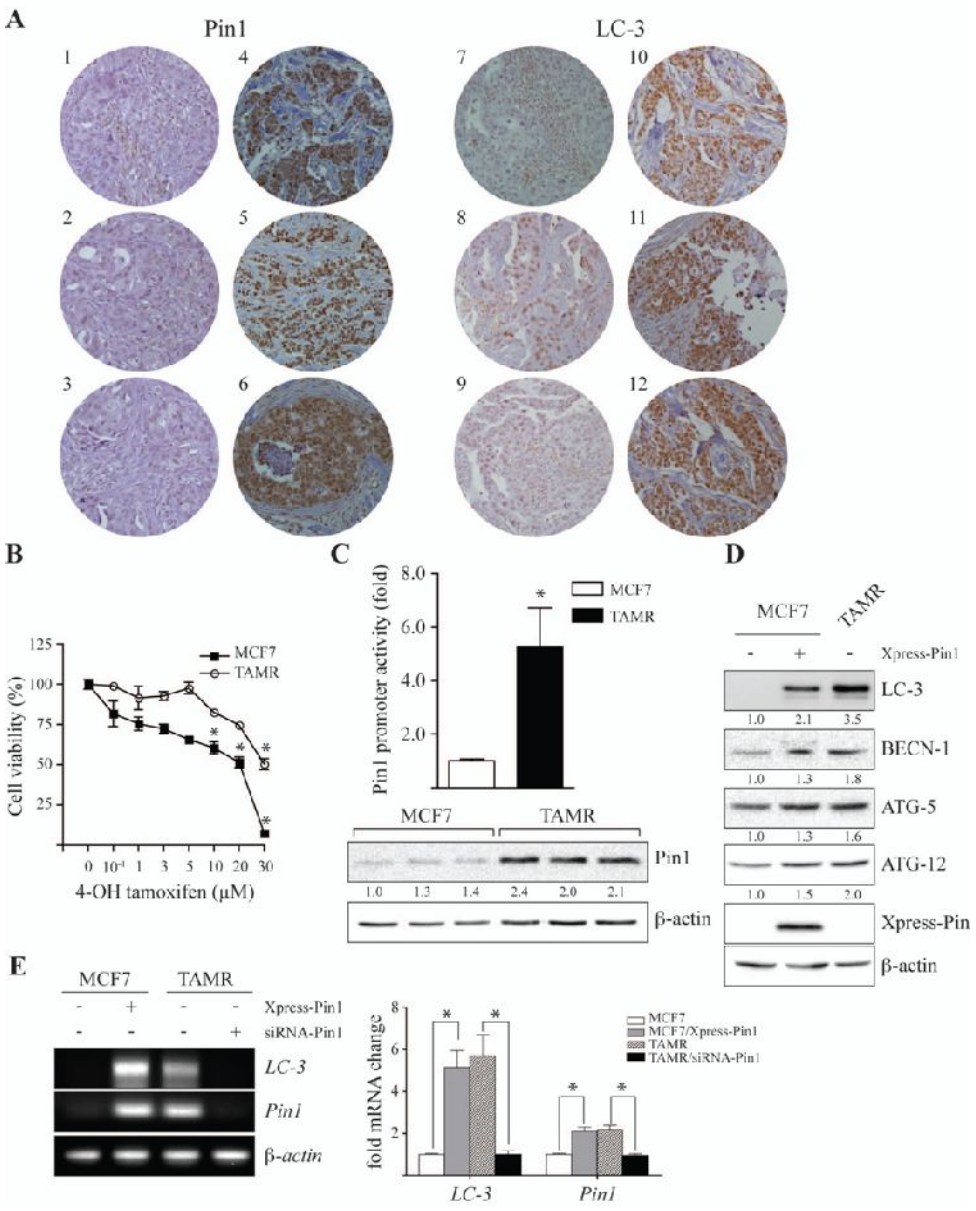

A
Pin1
LC-3
1
2
3
4
5
6
7
8
9
10
11
12
B
Cell viability (%)
125
100
75
50
25
MCF7
TAMR
0 10⁻³ 1 3 5 10 20 30
4-OH tamoxifen (μM)
C
Pin1 promoter activity (fold)
8.0
6.0
4.0
2.0
MCF7
TAMR
MCF7
TAMR
Pin1
1.0 1.3 1.4 2.4 2.0 2.1
β-actin
D
MCF7
TAMR
- + -
Xpress-Pin1
LC-3
1.0 2.1 3.5
BECN-1
1.0 1.3 1.8
ATG-5
1.0 1.3 1.6
ATG-12
1.0 1.5 2.0
Xpress-Pin
β-actin
E
MCF7
TAMR
- + - -
Xpress-Pin1
- - - +
siRNA-Pin1
LC-3
Pin1
β-actin
fold mRNA change
8
6
4
2
MCF7
MCF7/Xpress-Pin1
TAMR
TAMR/siRNA-Pin1
LC-3
Pin1

Fig. 1. Expression of LC-3 correlates with Pin1, which is associated with tamoxifen resistance of human breast cancer

A, representative samples showing results of immunohistochemical analysis of breast infiltrating duct carcinoma performed with the indicated antibodies on adjacent sections of the sample. Shown are examples of non-TAMR breast carcinoma for Pin1 (1-3) and LC-3 (7-9) and examples of moderate to strong expression for Pin1 (4-6) and LC-3 (10-12). *B,* cells were treated with 4-OH tamoxifen for 72 h and then cell viability were measured by MTT assay. *, $P < 0.05$, compared with control cells. *C, (upper panel)* human Pin1 promoter luciferase reporter was used for luciferase assays in MCF7 or TAMR-MCF-7 cells. *, $P < 0.05$, compared with MCF7 cells. *(lower panel)* whole cell lysates from MCF7 and TAMR-MCF7 cells were separated by SDS–PAGE and immunoblotted with antibodies against Pin1 and β-actin. *D,* MCF7 cells were transfected with Xpress-Pin1 plasmid to overexpress Pin1. After 48 h of transfection, whole cell lysates from MCF7 or TARMR-MCF7 cells were separated by SDS–PAGE and immunoblotted with specific antibodies, respectively. Corresponding signal intensities of each protein were densitometrically determined and normalized to β-actin in each lane and given below in each data. *E, the levels of LC-3, Pin1, and* β-*actin* mRNA in MCF7 or TAMR cells cultured after transfection of Xpress-Pin1 or siRNA-Pin1,

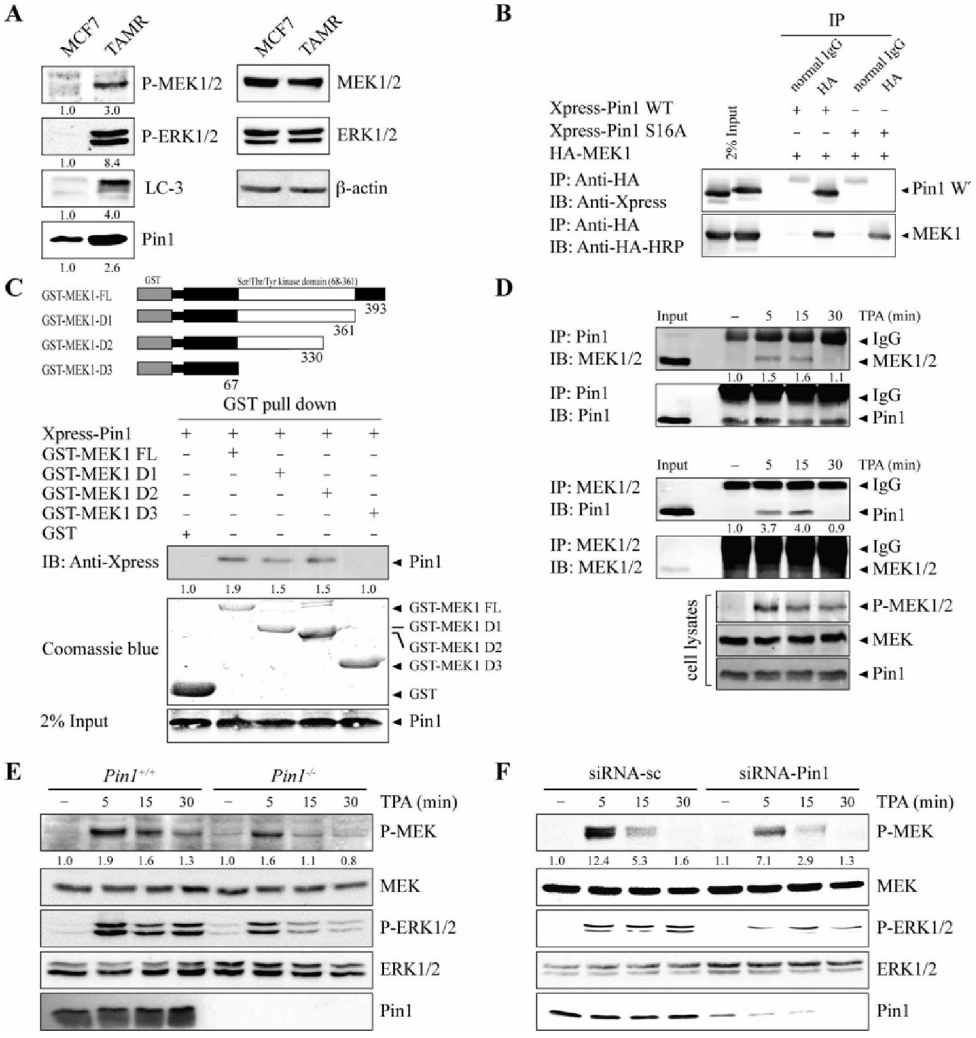
respectively, were assessed by RT-PCR. *Columns*, mean of triplicate samples; *bars*, SE. *, $P < 0.05$, compared with control cells

2. Pin1 enhances MEK1/2 phosphorylation induced by TPA

Activation of Ras/mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) signaling induced by TPA plays an important role in autophagy (9). Additionally, the inhibition of autophagy accelerates imatinib-induced cell death of imatinib-resistant cells (9). To determine whether MAPK signaling affects tamoxifen resistance, we monitored the activity of MAPK proteins in MCF7 and TARMR-MCF7 cells. TAMR-MCF7 cells showed a marked increase in MEK1/2 and ERK1/2 phosphorylation as well as LC-3 and Pin1 compared to control MCF7 cells (Fig. 2*A*).

In order to confirm the interaction between Pin1 and MEK1, we next co-transfected HAtagged MEK1 with Xpress-tagged Pin1 or Pin1 mutant affecting the WW domain (S16A) in HEK 293 cells. The cells lysates were immunoprecipitated (IP) using the normal IgG or anti-HA antibody, and blotted with the anti-Xpress antibody, respectively. The results showed that the exogenously expressed MEK1 only bound to the wild-type Pin1, but not to the Pin1 S16A mutant affecting the WW domain (Figure 2*B*). To determine the region of MEK1 that was responsible for its interaction with Pin1, GST fusion protein of full length MEK1 (MEK1-WT) or each of respective MEK1 deletion fragments (MEK1-D1, D2, D3) was incubated with *in vitro* translated Pin1, respectively, and the interaction was examined by GST-pull down assay. The results suggested that residues 68-330 of MEK1, which is included in Ser/Thr kinase domain, were required for its interaction with Pin1 (Fig. 2*C*).

Next, we examined the time course for the TPA-induced interaction of Pin1 and MEK1/2 in MCF7 cells. Reciprocal immunoprecipitation /immunoblotting showed that Pin1 was detectable in MEK1/2 immunoprecipitates from 5 min to 15 min after TPA treatment (Fig. 2*D*). To assess whether MAPK signaling was regulated by Pin1, we exposed *Pin1+/+ and Pin1-/-* MEF cells to TPA and immunoblotted with antibodies against phospho-MEK1/2 and phospho-ERK1/2. We found significantly decreased phosphorylation of MEK1/2 and ERK1/2 in *Pin1-/-* MEF cells compared to in *Pin1+/+* MEF cells (Fig. 2*E*). Next, to determine whether knock down of Pin1 suppresses the phosphorylation of MEK1/2 and ERK1/2 induced by TPA, we transfected small interfering RNA (siRNA) for Pin1 or control siRNA in MCF7 cells. TPA induced less phosphorylation of MEK1/2 and ERK1/2 induced by TPA in Pin1-knockdown cells (Fig. 2*F*). Taken together, these data indicate that Pin1 enhanced the phosphorylation of MEK1/2 as well as ERK1/2 induced by TPA, resulting from its interaction with MEK1/2, and suggest that Pin1-enhanced phosphorylation of MEK1/2 may regulate a high level of LC-3.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MCF7
TAMR
MCF7
TAMR
P-MEK1/2
MEK1/2
1.0 3.0
P-ERK1/2
ERK1/2
1.0 8.4
LC-3
β-actin
1.0 4.0
Pin1
1.0 2.6
B
IP
normal IgG
HA
normal IgG
HA
Xpress-Pin1 WT
Xpress-Pin1 S16A
HA-MEK1
2% Input
IP: Anti-HA
IB: Anti-Xpress
Pin1 W
IP: Anti-HA
IB: Anti-HA-HRP
MEK1
C
GST
Ser/Thr/Tyr kinase domain (68-361)
GST-MEK1-FL
393
GST-MEK1-D1
361
GST-MEK1-D2
330
GST-MEK1-D3
67
GST pull down
Xpress-Pin1
GST-MEK1 FL
GST-MEK1 D1
GST-MEK1 D2
GST-MEK1 D3
GST
IB: Anti-Xpress
Pin1
1.0 1.9 1.5 1.5 1.0
Coomassie blue
GST-MEK1 FL
GST-MEK1 D1
GST-MEK1 D2
GST-MEK1 D3
GST
2% Input
Pin1
D
Input
TPA (min)
IP: Pin1
IB: MEK1/2
IgG
MEK1/2
1.0 1.5 1.6 1.1
IP: Pin1
IB: Pin1
IgG
Pin1
Input
TPA (min)
IP: MEK1/2
IB: Pin1
IgG
Pin1
1.0 3.7 4.0 0.9
IP: MEK1/2
IB: MEK1/2
IgG
MEK1/2
cell lysates
P-MEK1/2
MEK
Pin1
E
Pin1+/+
Pin1-/-
TPA (min)
P-MEK
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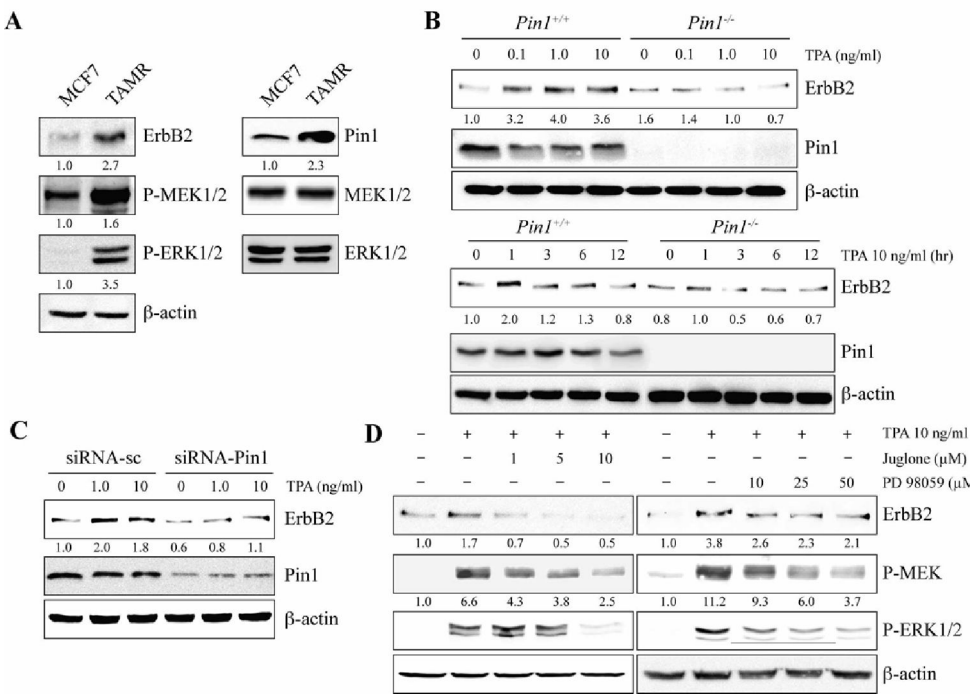
MEK
P-ERK1/2
ERK1/2
Pin1
F
siRNA-sc
siRNA-Pin1
TPA (min)
P-MEK
1.0 12.4 5.3 1.6 1.1 7.1 2.9 1.3
MEK
P-ERK1/2
ERK1/2
Pin1

Fig. 2. Pin1 associates with MEK1, which depends on MEK1 phosphorylation by TPA

A, enhanced phosphorylation of MEK1/2 in TAMR-MCF7 cells. *B,* MEK1 (HA-tag) was transfected into HEK 293 cells with Pin1 WT or WW domain mutant, Pin1 S16A (Xpress-tag), respectively, and then MEK1 were IP from transfeted HEK 293 cells and subjected to immunoblotting. *C,* the cDNA of Xpress-Pin1 was translated *in vitro*, then the Xpress-Pin1 was mixed with GST-MEK1-FL or each respective deletion mutants (GST-MEK1-D1, -D2, or -D3) and a pulldown assay was performed Proteins were visualized by immunoblotting or Coomassie blue staining. *D,* immunoprecipitation (IP) was performed to precipitate endogenous MEK1/2 and Pin1 after treatment of 10 ng/ml TPA, and immunoblotting (IB) analysis was performed using antibodies against Pin1 and MEK1/2, respectively. *E, Pin1*+/+ and *Pin1*-/- MEF cells were treated with or without 10 ng/ml TPA, harvested, lysed, and immunoblotted. *D,* MCF7 cells were cultured for 24 h and then transfected with siRNA-Pin1 or siRNA-sc. At 48 h after transfection, the cells were starved with serum-free DMEM for 24 h then treated with TPA, harvested, lysed, and immunoblotted. Corresponding signal intensities of each protein were densitometrically determined and normalized in each lane and given below in each data.

3. Pin1 regulates TPA-induced ErbB2 expression

Resistance associated with the activated ErbB system in breast cancer cells is reversed by inhibiting MAPK or PI3K/Akt signaling (10). In fact, targeting the ErbB2 pathway through blocking antibodies (Herceptin) has been suggested in the context of tamoxifen resistance based on the link between activation of growth factor signaling pathways and estrogen-independent tumor growth (11). As our expectation, TAMR-MCF7 cells showed high level of ErbB2 compared with control MCF7 cells, similarly to Pin1 (Fig. 3*A*). Given the role of Pin1 in activating MEK1/2 (Fig. 2), we further determined whether ablation or knockdown of Pin1 suppresses the ErbB2 expression induced by tumor promoters, such as TPA. Therefore, we exposed *Pin1*+/+ *and Pin1*-/- MEF cells to TPA and performed immunoblotting with an antibody against ErbB2. *Pin1*-/- MEF showed significantly lower ErbB2 levels than *Pin1*+/+ MEF cells treated with TPA in a dose-dependent analysis (Fig. 3*B, upper panels*) and time-dependent analysis (Fig. 3*B, lower panels*). To confirm the effect of Pin1 knockdown on the ErbB2 expression induced by TPA, we transfected siRNA-Pin1 or siRNA-sc (control) in MCF7 cells and then performed immunoblotting with antibodies against ErbB2 and Pin1. TPA-induced ErbB2 expression was significantly suppressed in siRNA-Pin1-transfected cells compared to siRNA-sc-transfected cells (Fig. 3*C*). Similarly, pretreatment with PD98059, a MEK1/2 inhibitor, or Juglone, a Pin1 specific inhibitor (12), dramatically decreased TPA-induced ErbB2 expression (Fig. 3*D*). Taken together, these results indicate that Pin1 regulates TPA-induced ErbB2 expression in MCF7 cells through enhancing MEK1/2 and ERK1/2 phosphorylation.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MCF7
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1.0 1.6
P-ERK1/2
1.0 3.5
β-actin
MCF7
TAMR
Pin1
1.0 2.3
MEK1/2
ERK1/2
B
Pin1+/+
Pin1-/-
0 0.1 1.0 10 0 0.1 1.0 10 TPA (ng/ml)
ErbB2
1.0 3.2 4.0 3.6 1.6 1.4 1.0 0.7
Pin1
β-actin
Pin1+/+
Pin1-/-
0 1 3 6 12 0 1 3 6 12 TPA 10 ng/ml (hr)
ErbB2
1.0 2.0 1.2 1.3 0.8 0.8 1.0 0.5 0.6 0.7
Pin1
β-actin
C
siRNA-sc
siRNA-Pin1
0 1.0 10 0 1.0 10 TPA (ng/ml)
ErbB2
1.0 2.0 1.8 0.6 0.8 1.1
Pin1
β-actin
D
− + + + + − + + + + TPA 10 ng/ml
− − 1 5 10 − − − − − Juglone (μM)
− − − − − − − 10 25 50 PD 98059 (μM
ErbB2
1.0 1.7 0.7 0.5 0.5 1.0 3.8 2.6 2.3 2.1
P-MEK
1.0 6.6 4.3 3.8 2.5 1.0 11.2 9.3 6.0 3.7
P-ERK1/2
β-actin

Fig. 3. Pin1 enhances ErbB2 expression induced by TPA.

A, increased expression of ErbB2 in TAMR-MCF7 cells. *B, Pin1*+/+ and *Pin1*-/- MEF cells were cultured for 24 h, starved in serum-free DMEM for 24 h, and then treated with different doses of TPA for 12 h (*upper panels*) or 10 ng/ml TPA for the indicated times (*lower panels*), harvested, lysed, and imunoblotted. *C,* MCF7 cells were transfected with either siRNA-Pin1 or the siRNA-sc. At 48 h after transfection, the cells were starved with serum-free DMEM for 24 h, treated with 10 ng/ml TPA for 12 h, harvested, lysed, and immunoblotted. *D,* MCF7 cells were serum-starved for 24 h, then treated with either Juglone (*left panels*) or PD98059 (*right panels*), and incubated for 2 h. Cells were then exposed to TPA and incubated for 12 h. The lysates were resolved by SDS-PAGE and immunoblotted. Corresponding signal intensities of each protein were densitometrically determined and normalized in each lane and given below in each data.

4. Pin1 is essential for TPA-induced LC-3 expression via E2F-4 and Egr-1

The E2F-4 and Egr-1 regulate LC-3 transcription and may be novel targets for regulating autophagy in mammalian systems (13). We therefore examined whether Pin1 is necessary for E2F-4 and Egr-1 to induce LC-3. As expected, E2F-4 and Egr-1 levels were higher in TAMR-MCF7 cells than MCF7 cells, consistent with high LC-3 and Pin1 levels (Fig. 4*A*). Similarly, TPA could induce E2F-4 and Egr-1 expression in *Pin1*+/+ MEF cells, but not in *Pin1*-/- MEF cells (Fig 4*B*). Finally, the siRNAPin1, but not the siRNA-sc, blocked TPA-induced E2F-4 and Egr-1 expression (Fig. 4*C*). Juglone (Fig 4*D*, *left panel*) and PD98059 (Fig 4*D*, *right panel*) dose-dependently decreased TPA-induced expression of E2F-4 and Egr-1.

Next, we determined the effect of knockdown of E2F-4 or Egr-1 on the TPA-induced LC-3, respectively. Interestingly, these results showed that both E2F-4 and Egr-1 are essential for the TPA-induced expression of LC-3 in MCF7 cells (Fig. 4*E*). Therefore, we further examined whether ablation of the E2F-4 or Egr-1 gene in Pin1-overexpressing MCF7 cells (MSCV-Pin1) affects the LC-3 levels induced by TPA. MSCVPin1 cells were transfected with siRNA-sc, -E2F4, or -Egr-1, respectively, and then either treated or not treated with TPA. The results showed that TPA markedly induced the LC-3 expression in MSCV-Pin1 cells compared to MSCV-GFP cells, whereas knockdown of E2F-4 or Egr-1 in MSCV-Pin1 cells suppressed TPA-induced LC-3 expression compared to siRNA-sc-transfected MSCV-Pin1 cells (Fig. 4*F*). Taken together, these results strongly support our notion that the regulation of

MEK1/2 by Pin1 is critical for regulating LC-3 expression via increases in transcriptional factors E2F-4 and Egr-1.

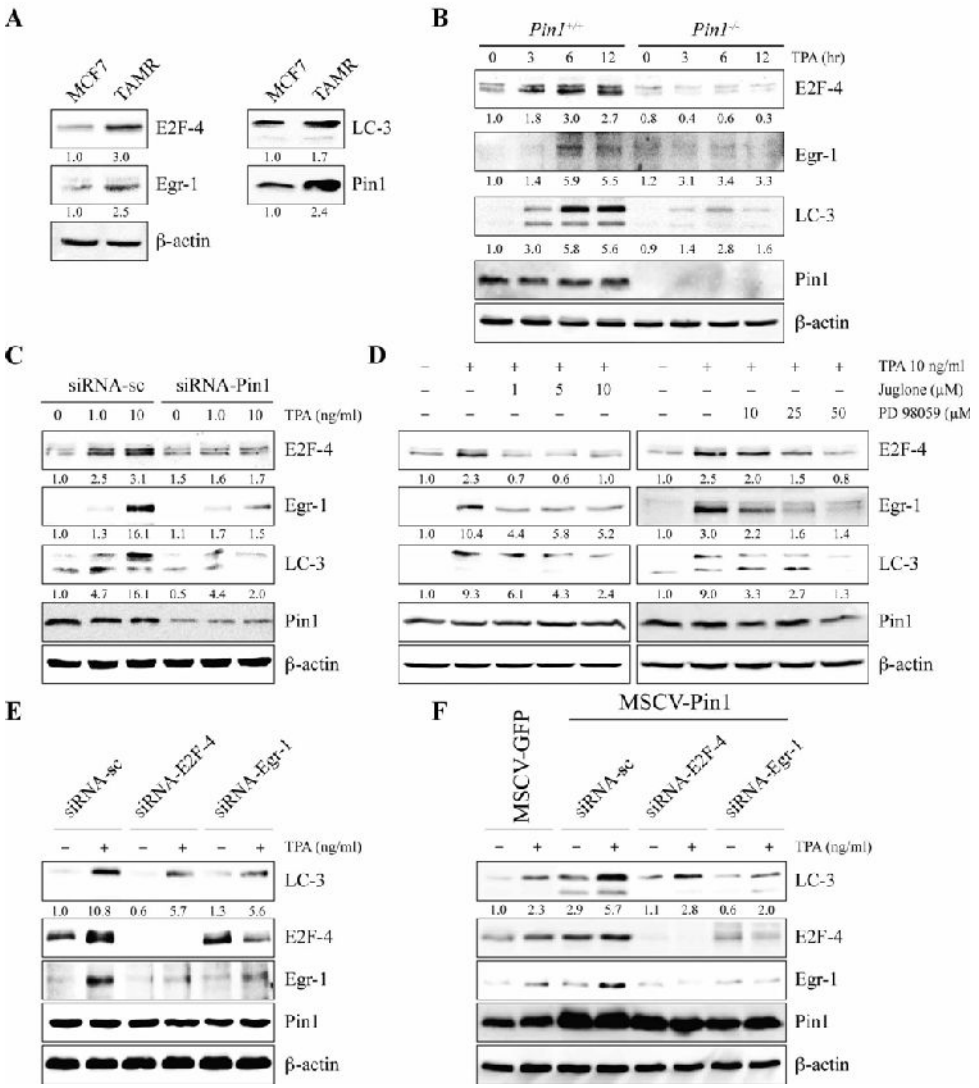

A
MCF7
TAMR
E2F-4
1.0 3.0
Egr-1
1.0 2.5
β-actin
MCF7
TAMR
LC-3
1.0 1.7
Pin1
1.0 2.4
B
Pin1+/+
Pin1-/-
0 3 6 12 0 3 6 12 TPA (hr)
E2F-4
1.0 1.8 3.0 2.7 0.8 0.4 0.6 0.3
Egr-1
1.0 1.4 5.9 5.5 1.2 3.1 3.4 3.3
LC-3
1.0 3.0 5.8 5.6 0.9 1.4 2.8 1.6
Pin1
β-actin
C
siRNA-sc
siRNA-Pin1
0 1.0 10 0 1.0 10 TPA (ng/ml)
E2F-4
1.0 2.5 3.1 1.5 1.6 1.7
Egr-1
1.0 1.3 16.1 1.1 1.7 1.5
LC-3
1.0 4.7 16.1 0.5 4.4 2.0
Pin1
β-actin
D
TPA 10 ng/ml
Juglone (μM)
PD 98059 (μM)
E2F-4
1.0 2.3 0.7 0.6 1.0 1.0 2.5 2.0 1.5 0.8
Egr-1
1.0 10.4 4.4 5.8 5.2 1.0 3.0 2.2 1.6 1.4
LC-3
1.0 9.3 6.1 4.3 2.4 1.0 9.0 3.3 2.7 1.3
Pin1
β-actin
E
siRNA-sc
siRNA-E2F-4
siRNA-Egr-1
− + − + − + TPA (ng/ml)
LC-3
1.0 10.8 0.6 5.7 1.3 5.6
E2F-4
Egr-1
Pin1
β-actin
F
MSCV-GFP
MSCV-Pin1
siRNA-sc
siRNA-E2F-4
siRNA-Egr-1
− + − + − + − + TPA (ng/ml)
LC-3
1.0 2.3 2.9 5.7 1.1 2.8 0.6 2.0
E2F-4
Egr-1
Pin1
β-actin

Fig. 4. Pin1-enhanced expression of E2F-4 and Egr-1 induced by TPA.

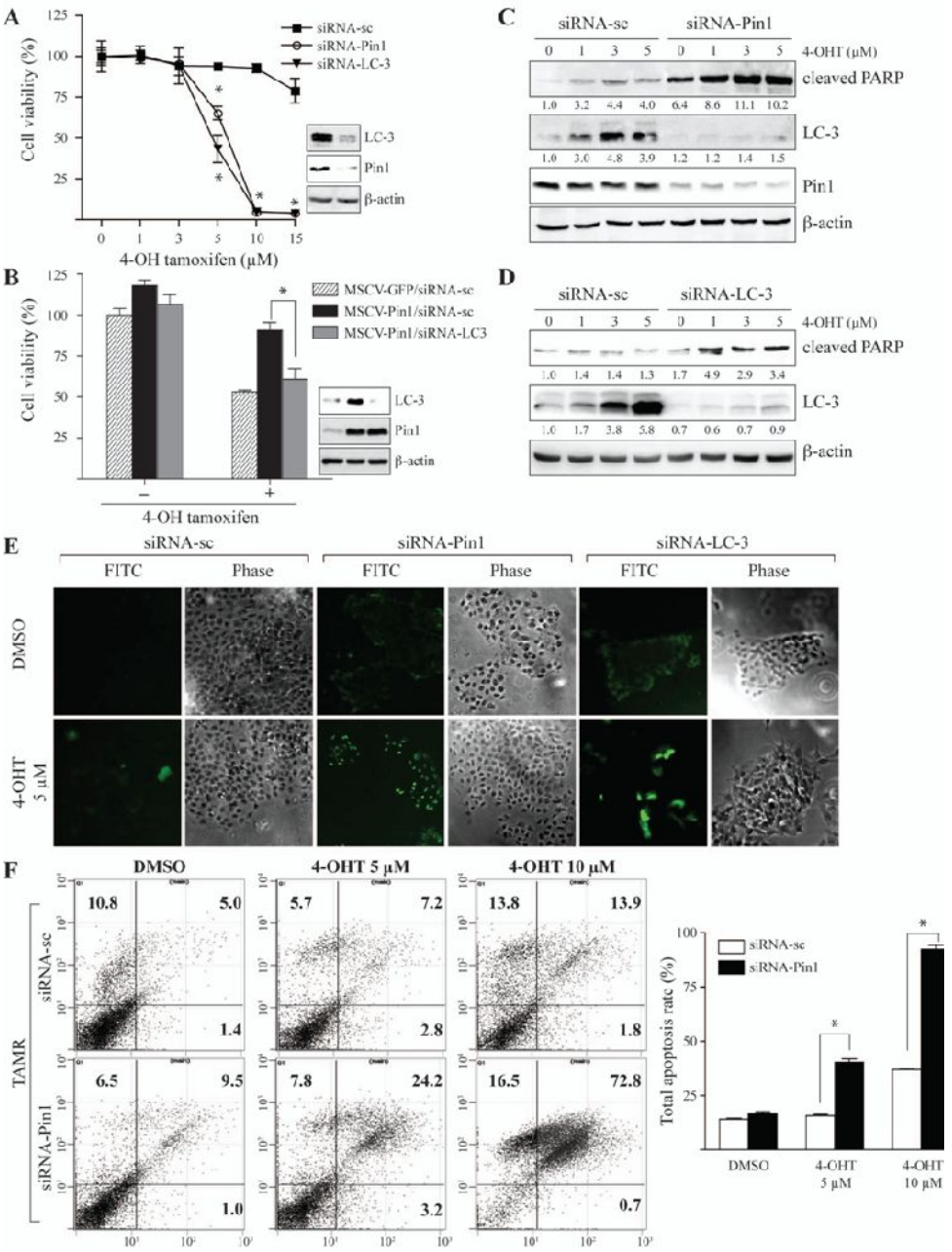
A, increased expression of E2F-4 and Egr-1 in TAMR-MCF7 cells. *B, Pin1*+/+ and P*in1*-/- MEF cells were serum-starved for 24 h, treated with 10 ng/ml TPA, harvested, lysed, and immunoblotted. *C,* MCF7 cells were transfected with either siRNA-sc or -Pin1. After 48 h, cells were serum-starved, treated with TPA for 12 h, and then harvested and lysed. The lysates were resolved by SDS-PAGE and immunoblotted. *D,* MCF7 cells were serum-starved for 24 h, treated with either Juglone (*left panels*) or PD98059 (*right panels*), and incubated for 2 h. Following TPA treatment for 12 h, cells were harvested in ice cold PBS and lysed. The lysates were resolved by SDS-PAGE and immunoblotted. *E and F,* MCF7 cells (*E*) or MSCV-Pin1 MCF7 cells (*F*) were transfected with siRNA-sc, -E2F-4 or -Egr-1, respectively. After 48 h, cells were serum-starved, treated or not treated with 10 ng/ml TPA for 12 h, harvested, lysed, and immunoblotted. Corresponding signal intensities of each protein were densitometrically determined and normalized in each lane and given below in each data.

5. Pin1 silencing enhances tamoxifen-induced apoptotic signaling in MCF7 and TAMR-MCF7 cells

We next tested whether Pin1 and LC-3 knockdown would potentiate tamoxifen-induced cell death. 4-OH tamoxifen treatment reduced MCF7 cell viability by 20%, whereas Pin1-knockdown with siRNA produced almost complete sensitivity (Fig. 5*A*, *open circles*), as did LC-3-knockdown (Fig. 5*A*, *black triangle*) in MTT assays. To further examine whether ablation of the LC-3 gene in Pin1-overexpressing MCF7 cells affect tamoxifen sensitivity, we next transfected siRNA-sc or -LC-3 in MSCV-Pin1 MCF7 cells, respectively, and then either treated or not treated with 4-OH tamoxifen. Pin1 overexpression caused 4-OH tamoxifen resistance in cell viability, whereas LC-3-silenced MSCVPin1 MCF7 cells resulted in significant decrease in cell viability (Fig. 5*B*).

Because 4-OH tamoxifen induces cell death by poly(ADP-ribose) polymerase (PARP) cleavage (14), we next tested the effects of knockdown of Pin1 or LC-3 on PARP cleavage. 4-OH tamoxifen induced dramatically higher cleavage of PARP after Pin1-knockdown (Fig. 5*C*) or LC-3-knockdown (Fig. 5*D*), providing a mechanism for the increased cell death. 4-OH tamoxifen-induced DNA fragmentation was also higher in Pin1 or LC-3 knockdowns, as measured in the TUNEL assay (Fig. 5*E*). These data indicate that 4-OH tamoxifen induces more cell death through PARP cleavage and DNA fragmentation if Pin1 or LC-3 expression is downregulated. Furthermore, 4-OH tamoxifen induced apoptosis of control siRNA-transfected TAMR-MCF7 cells, which were characterized by a marked rate of total apoptosis that

reached 15.7% and 36.9% at 5μM and 10μM 4-OH tamoxifen, respectively (Fig. 5*F*). Importantly, tamoxifen sensitivity in TAMR-MCF7 cells was highly increased by Pin1 silencing, which the rate of total apoptosis reached 40.3% and 92.4% at 5μM and 10μM 4-OH tamoxifen, respectively (Fig. 5*F*).


A
Cell viability (%)
siRNA-sc
siRNA-Pin1
siRNA-LC-3
LC-3
Pin1
β-actin
4-OH tamoxifen (μM)
B
Cell viability (%)
MSCV-GFP/siRNA-sc
MSCV-Pin1/siRNA-sc
MSCV-Pin1/siRNA-LC3
LC-3
Pin1
β-actin
4-OH tamoxifen
C
siRNA-sc
siRNA-Pin1
4-OHT (μM)
cleaved PARP
LC-3
Pin1
β-actin
D
siRNA-sc
siRNA-LC-3
4-OHT (μM)
cleaved PARP
LC-3
β-actin
E
siRNA-sc
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siRNA-LC-3
FITC
Phase
DMSO
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F
DMSO
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1.4
6.5
9.5
1.0
5.7
7.2
2.8
7.8
24.2
3.2
13.8
13.9
1.8
16.5
72.8
0.7
Total apoptosis rate (%)
siRNA-sc
siRNA-Pin1
DMSO
4-OHT 5 μM
4-OHT 10 μM

Fig. 5. Silencing of Pin1 and LC-3 increases tamoxifen-induced apoptosis.

A, MCF7 cells were transfected with siRNA-sc, Pin1, or -LC-3, respectively. At 48 h after incubation, cells were serumstarved for 24 h, and treated with 4-OH tamoxifen for 24 h. Cell viability was measured by MTT assay and was plotted. The inserted figure in the right side indicates expression levels of Pin1 and LC-3 following transfection of siRNA-Pin1 or -LC-3. *, $P < 0.05$, compared with control cells. *B,*MSCV-Pin1 MCF7 cells were transfected with either siRNA-sc or -LC-3. After 48 h, cells were treated or not treated with 15μM 4-OH tamoxifen for 24 h. Cell viability was measured by MTT assay. The inserted figure in the right side indicates expression levels of Pin1 and LC-3 following transfection of siRNA-LC-3. *Columns*, mean of triplicate samples; *bars*, SE. *, $P < 0.05$, compared with control cells. *C and D,* MCF7 cells were transfected with either siRNA-Pin1 (*C*) or siRNA-LC-3 (*D*), and then incubated with 48 h. Following 24 h serum starvation, cells were treated with 4-OH tamoxifen for 24h, lysed, and immunoblotted. Corresponding signal intensities of each protein were densitometrically determined and normalized to β-actin in each lane and given below in each data. *E,*MCF7 cells were transfected with siRNA-sc, siRNA-Pin1, or siRNA-LC-3, and then incubated for 48 h. Following 24 h serum starvation, cells were treated with 4-OH tamoxifen for 24 h. DNA fragmentation induced by 4-OH tamoxifen was detected. *F,* TAMR-MCF7 cells were transfected with siRNA-sc or -Pin1, and then incubated for 48 h. Cells

were treated with 4-OH tamoxifen for 24 h. The induction of early and late apoptosis rate induced by 4-OH tamoxifen was analyzed by flow cytometry. *Columns*, mean of triplicate samples; *bars*, SE. *, $P < 0.05$, compared with control cells.

6. The inhibition of Pin1 or MEK1 suppresses TPA-induced neoplastic cell transformation

To assess whether inhibition of Pin1 or MEK1/2 suppresses Pin1-enhanced cell transformation induced by TPA, GFP-JB6 and Pin1-JB6 cells were treated with TPA with Juglone or PD98059 in soft agar. TPA treatment increased colony number and size more in Pin1-JB6 cells than GFP-JB6 cells (Fig 6*A* and *B*). Juglone (Fig. 6*A*) and PD98059 (Fig. 6*B*) dose-dependently blocked this increase in Pin1-JB6 cells.

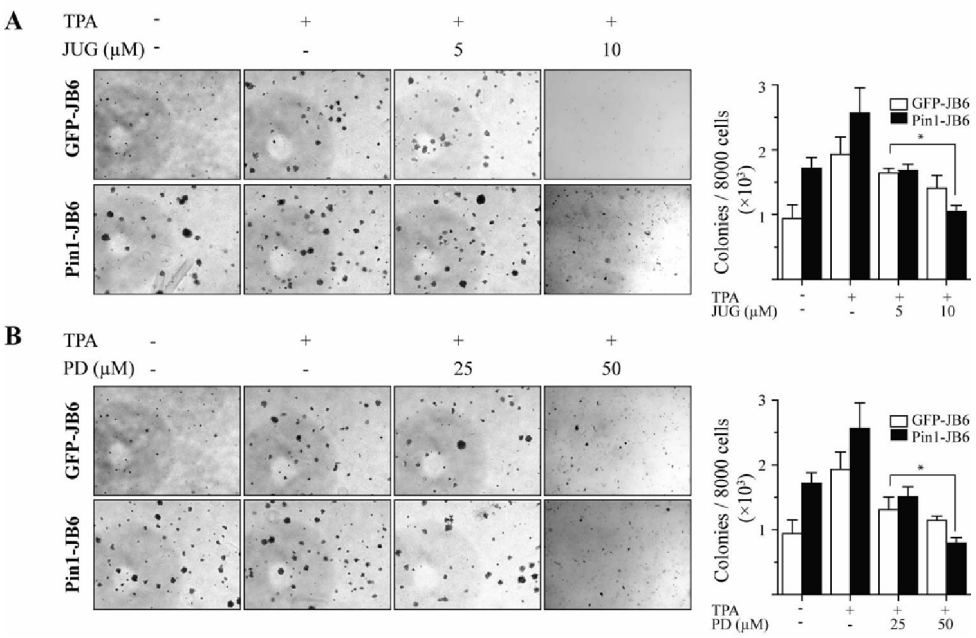


Fig. 6. Juglone and PD98059 inhibit Pin1-enhanced neoplastic cell transformation promoted by TPA.

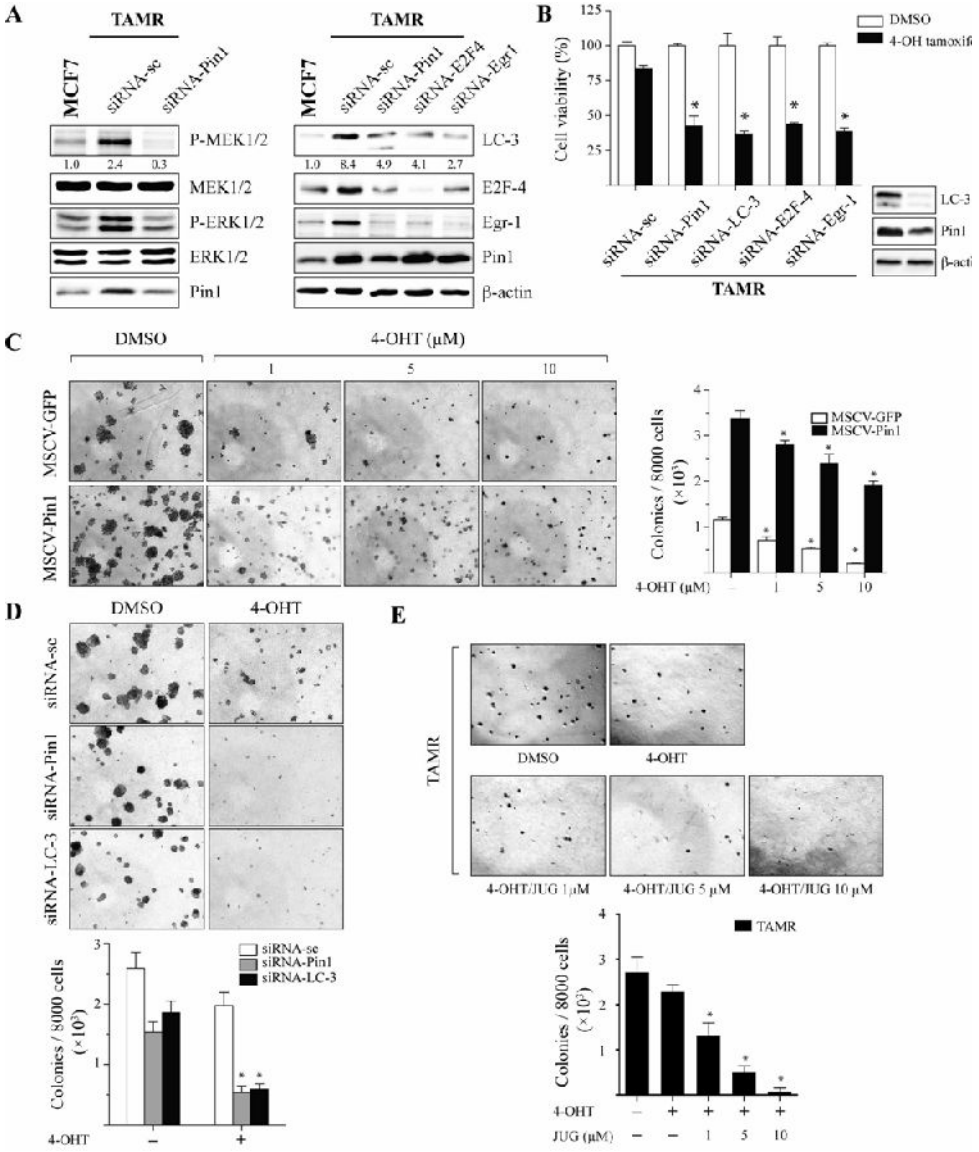
A and B, GFP-JB6 and Pin1-JB6 cells were exposed to TPA with/without treatment with Juglone (*A*) or PD98059 (*B*) in soft agar. The average colony number was calculated and the colonies from three separate experiments were photographed. *Columns*, mean of triplicate samples; *bars*, SD. *, $P < 0.05$, compared with control cells.

7. Pin1 silencing increases tamoxifen-induced inhibition of cologenic growth

To determine the mechanism by which Pin1, E2F-4, or Egr-1 silencing regulates the expression of LC-3, resulted in increased tamoxifen sensitivity in TAMR-MCF7 cells, we next transfected siRNA-Pin1, -E2F4, or -Egr-1 in TAMR cells, respectively. As expected, LC-3 expression levels were decreased by knockdown of Pin1, E2F4, Egr-1 in TAMR-MCF7 cells, respectively, compared to control TAMR-MCF7 cells (Fig. 7*A*, *right 1st panel*). In addition, Pin1 silencing decreased the activity of MEK1/2 and ERK1/2, as well as the expression levels of E2F-4, Egr-1, and LC-3 in TARMR-MCF7 cells, consistent with total MEK1/2, ERK1/2, and β-actin levels (Fig. 7*A*).

We next tested whether knockdown of Pin1 LC-3, E2F-4, and Egr-1 would increase tamoxifen sensitivity in TAMR-MCF7 cells. 4-OH tamoxifen treatment reduced the viability of TAMR-MCF7 cells by up to 17%, whereas Pin1, LC-3, E2F-4, and Egr-1 silencing with siRNA produced more significant sensitivity by up to 58%, 64%, 57%, and 62%, respectively in MTTPin1 overexpression reduced 4-OH tamoxifen sensitivity in MCF7 cells, GFP-MCF7 and Pin1-MCF7 cells were treated with 4-OH tamoxifen in a dose-dependent manner in soft agar. 4-OH tamoxifen treatment decreased colony number and size in both cell lines, but Pin1-MCF7 cells showed greater resistance (Fig. 7*C*). In addition, silencing of Pin1 and LC-3 with siRNA in MCF7 cells significantly increased the sensitivity to 4-OH tamoxifen (Fig. 7*D*). We then combined 4-OH tamoxifen and Pin1 inhibitor, Juglone, to treat TAMR-MCF7 cells and found that it significantly sensitized the tamoxifen-resistant cell to the 4-OH tamoxifen in a dose-dependent manner (Fig. 7*E*).

Overall, these results illustrate that Pin1 increases LC-3 expression through MAPK signaling, promoting resistance to tamoxifen, which suggests that the combination chemotherapy of tamoxifen and Pin1 inhibitor may be a promising treatment for overcoming breast cancer chemoresistance in clinic.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MEK1/2
P-ERK1/2
ERK1/2
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TAMR
MCF7
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siRNA-E2F4
siRNA-Egr1
LC-3
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E2F-4
Egr-1
Pin1
β-actin
B
DMSO
4-OH tamoxif
Cell viability (%)
125
100
75
50
25
siRNA-sc
siRNA-Pin1
siRNA-LC-3
siRNA-E2F-4
siRNA-Egr-1
TAMR
LC-3
Pin1
β-acti
C
DMSO
4-OHT (μM)
1
5
10
MSCV-GFP
MSCV-Pin1
Colonies / 8000 cells (×10³)
MSCV-GFP
MSCV-Pin1
4-OHT (μM)
D
DMSO
4-OHT
siRNA-sc
siRNA-Pin1
siRNA-LC-3
Colonies / 8000 cells (×10³)
siRNA-sc
siRNA-Pin1
siRNA-LC-3
4-OHT
E
TAMR
DMSO
4-OHT
4-OHT/JUG 1 μM
4-OHT/JUG 5 μM
4-OHT/JUG 10 μM
Colonies / 8000 cells (×10³)
TAMR
4-OHT
JUG (μM)

Fig. 7. Silencing of Pin1 and LC-3 increases tamoxifen sensitivity to inhibit the tumorigenicity of MCF7 cells.

A and B, TAMR-MCF7 cells was transfected with siRNA-sc, -Pin1, -E2F-4, or -Egr-1, respectively. *A,* after 48 h, cells were harvested and lysed, and immunoblotted. Corresponding signal intensities of each protein were densitometrically determined and normalized to β-actin in each lane and is given below in each data. *B,* cells were treated or not treated with 15μM 4-OH tamoxifen for 72 h. Cell viability was measured by MTT assay. The inserted figure in the bottom indicates expression levels of LC-3 following transfection of siRNA-LC-3. *Columns*, mean of triplicate samples; *bars*, SE. *, $P < 0.05$, compared with control cells. *C,* MSCV-GFP or MSCV-Pin1 cells were treated with 4-OH tamoxifen in soft agar, and average colony numbers were measured. *Columns*, mean of triplicate samples; *bars*, SD. *, $P < 0.05$, compared with control cells. *D,* MCF7 cells were transfected with siRNA-sc, -Pin1, or -LC-3, respectively, and subjected to soft agar assays as described above in the absence or presence of 5-μM 4-OH tamoxifen. *Columns*, mean of triplicate samples; *bars*, SD. *, $P < 0.05$, compared with control cells. *E,* TAMR-MCF7 cells were treated with 5μM 4-OH tamoxifen with/without Juglone in soft agar, and average colony numbers were measured. *Columns*, mean of triplicate samples; *bars*, SD. *, $P < 0.05$, compared with control cells.

Ⅴ. Discussion

The phosphorylation of proteins on serine or threonine residues that immediately precede proline resides (Ser/Thr-Pro) is an important signaling mechanism for cell cycle regulation, transcription, cell differentiation, and proliferation (7,15). The prolyl isomerase Pin1 binds to and isomerizes the peptidyl-prolyl bond in specific phosphorylated Ser/Thr-Pro motifs to induce conformational changes in its target proteins (7). These conformational changes can have profound effects on the function of Pin1 substrates, modulating their activity, phosphorylation status, protein-protein interaction, subcellular localization, and stability (16). Interestingly, it was reported that Pin1 regulates SMRT and SRC-3, as downstream effectors of HER2 signaling (8,17), which is often increased in endocrine-resistant breast tumors and contributes to activate proliferation and/or survival and hormone resistance (6,18). Here, we show that there was a positive correlation between the levels of LC-3 and Pin1 in human tamoxifen-resistant breast cancer samples (Fig. 1*A*). Although the small numbers, unmatched sample groups are the inevitable of our study, Fisher's Exact tests revealed that there was a significant association between expression of LC-3 and Pin1 ($P < 0.010$). Recently, quantitative immunohistochemical analysis of human melanomas showed a strong correlation between the levels of B-Raf protein and LC-3, suggested that high oncogenic B-Raf levels trigger autophagy, which may have a role in tumor progression (19). In the context of B-Raf signaling, the oncogenic activity of B-Raf was increased in cells

overexpressing WT Pin1, whereas their transforming activity was reduced in cells that overexpress a dominant negative Pin1 (20). These reports supported our hypothesis that high levels of Pin1 may regulate LC-3 expression, resulted in tumor progression as well as tamoxifen resistance in breast cancer. In this study, we further found that the expression level and promoter activity of Pin1 were significantly increased in tamoxifen resistance MCF7 breast cancer cells established by long-term exposure to tamoxifen (Fig. 1*C*). Autophagy is a key mechanism of cell survival in ER positive breast cancer cells, resulting in the development of tamoxifen resistance (21). Also, antiestrogen resistance could be reduced by targeting autophagosome function, which is regulated by LC-3, beclin-1, Atg-5, and Atg-12 (21-23), suggested that high Pin1 expression in tamoxifen-resistant MCF7 cells may enhance autophagy through increased expression of autophagy related proteins, such as LC-3, to produce tamoxifen resistance. As expected, LC-3 levels were higher in tamoxifen resistant MCF7 cells, and Pin1 overexpression produces the same expression patterns as tamoxifen-resistant cells, suggesting that Pin1 regulates tamoxifen resistance via enhanced LC-3 expression (Fig. 3*D*).

Overexpression of many growth factor receptors, as well as growth factors, confers varying degrees of estrogen-independent growth on ER positive breast cancer cells (24). Transfection of constitutively active MEK1 or c-RAF into MCF7 cells, which results in hyperactivation of ERK1/2, causes loss of ERα-mediated gene expression, characterized by acquisition of antiestrogen resistance (24,25). In addition, activation of MAPK signaling, circumventing the reliance upon ER-α signaling, causes tamoxifen resistance by reducing CDK10 expression (26). Interestingly, the

induction of an autophagy marker, LC-3, a mammalian homolog of yeast Atg8 (Aut7/Apg8), by TPA is mediated by the Ras/MAPK pathway (9,27). Recent study shows that depletion of ERK partially inhibited autophagy, whereas specific inhibition on MEK completely inhibited autophagy (28). As shown in this study, TPA induced the interaction of Pin1 with MEK and resulted in expression levels of LC-3 mRNA and protein (Fig. 2D). Thus, our findings propose that the Pin1-MEK/ERK pathway regulation of LC-3 may induce autophagy and tamoxifen resistance in MCF-7 cells.

LC-3 expression is directly regulated by the E2F-4 and Egr-1 transcription factors, which have putative binding sites at the LC-3 promoter, and Egr-1 also indirectly regulates LC-3 conversion through regulation of Atg4B (13). We have shown here that phosphorylation of MEK1/2 and ERK1/2 is markedly increased in tamoxifen-resistant MCF7 cells compared with MCF7 cells (Fig. 2A). Furthermore, TPA dramatically induces the interaction of Pin1 with MEK1/2 in MCF7 cells (Fig. 2D), and enhances the phosphorylation of MEK1/2 and ERK1/2 in *Pin1*+/+ MEF or siRNA-control-transfected MCF7 cells, but the TPA-induced phosphorylation of MEK1/2 and ERK1/2 is suppressed in *Pin1*-/- MEF or siRNAPin1-transfected cells (Fig. 2E and 2F). Interestingly, E2F-4 and Egr-1 are also highly expressed in tamoxifen-resistant MCF7 cells and in response to TPA in MCF7 cells (Fig. 4A and 4C). TPA-induced E2F-4 and Egr-1 expression are suppressed by Pin1 knockdown, Juglone, or PD98059 in MCF7 cells, resulting in downregulation of LC-3 expression (Fig. 4C and 4D). Theses results indicated that Pin1 interacts with

MEK1/2 to play a pivotal role in LC-3 expression through regulating E2F4 and Egr-1 transcriptional factors.

Overexpression of ErbB2 is a predictor for tamoxifen resistance in ER+ disease, and ErbB2+ and ER+ breast cancer is a subgroup with poor prognosis in premenopausal breast cancer (29). The induction of autophagy is closely related to the cell survival triggered by ErbB2-gene amplified human breast cancer cells in response to the anti-ErbB2 monoclonal antibody, trastuzumab (Tzb) (30). Knockdown of autophagy, in combination with tamoxifen in tamoxifen-resistant ErbB2-overexpressing MCF7 cells, reduced cell viability with increased mitochondrial-mediated apoptosis (29). Interestingly, a majority of breast cancers overexpressed Pin1 (54%), and Pin1 overexpression was more prevalent in the HER-2-overexpressing tumors (62.5%) than in HER-2-negative breast cancers (31). Therefore, we hypothesized that Pin1-enhanced phosphorylation of MEK1/2, which increases LC-3 expression, may also regulate ErbB2 expression, resulting in tamoxifen resistance in MCF7 cells. ErbB2 levels are higher in tamoxifen-resistant MCF7 cells and also in response to TPA in MCF7 cells (Fig. 3A and 3C). TPA dramatically induces ErbB2 expression in *Pin1*+/+ MEF or siRNA-control-transfected MCF-7 cells, whereas the TPA-induced phosphorylation of MEK1/2 and ERK1/2 was suppressed in *Pin1*-/- MEF or siRNA-Pin1-transfected cells (Fig. 3*B* and 3*C*). Furthermore, the TPA-induced expression of ErbB2 is almost totally inhibited by PD98059 or Juglone (Fig. 3*D*), suggesting that the TPA-induced expression of ErbB2 may be mediated by an interaction between Pin1 and MEK1/2 in breast cancer.

We show here that knockdown of Pin1 and LC-3 expression with siRNA enhances apoptotic pathways after tamoxifen treatment, as measured by the MTT assay (Fig. 5*A*), PARP cleavage, and DNA fragmentation (Fig. 5*C* and 5*D*). Tamoxifen markedly increased LC-3 expression, but not PARP cleavage, in siRNA-sc-transfected MCF7 cells, indicating that LC-3 may play an important role in terms of cancer cell survival. Our findings are consistent with earlier reports showing that autophagy inhibition activates the mitochondrial apoptotic pathway and increases apoptosis, at least in part through caspase-9 (32,33). In addition, overexpression of Pin1 in JB6 Cl41 cells enhanced TPA-induced cell transformation (Fig. 6*A* and *6B*). We also confirmed that the Pin1 inhibitor, Juglone, as well as the MEK1/2 inhibitor, PD98059, dramatically suppressed TPA-induced cell transformation in Pin1-overexpressing cells, indicating a role for MEK1/2 (Fig. 6*A* and *6B*). Overexpression of Pin1 attenuated tamoxifen-suppressed tumorigenicity in MCF7 cells, whereas loss of Pin1 and LC-3 increased tamoxifen activity, indicating that Pin1 and LC-3 have an important role in tamoxifen resistance in breast cancer cells (Fig. 7*D* and 7*E*).

In summary, our data indicate that Pin1 induces the expression of LC-3 via regulation of E2F-4 and Egr-1 and facilitates the progression of breast cancer to tamoxifen resistance. Our findings also indicate that autophagy inhibition targeting Pin1 and LC-3 might be advantageous in a combination therapy setting to sensitize breast cancer to tamoxifen, which is in complete agreement with two recent studies (29,34) in which autophagy was shown to reduce the efficacy of chemotherapy and tamoxifen therapy, respectively, in ER+ breast cancer cells. Further, to our

knowledge, our work is the first to show that Pin1 inhibition can sensitize tamoxifen-resistant cells and indicates that this approach may be a viable strategy to sensitize therapyresistance cancers.

V. References

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논문제목	한글 : Prolyl-이성질화효소 Pin1 의 LC-3 발현 유도 및 타목시펜 저항성에 관한 연구				
	영문 : The Prolyl-Isomerase Pin1 induces LC-3 expression and mediates tamoxifen resistance in breast cancer				

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

- 다 음 -

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

동의 여부 : 동의 (○) 반대 ( )

2010년 8월 25일

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