Research Article

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# The Prolyl Isomerase Pin1 Enhances HER-2 Expression and Cellular Transformation via Its Interaction with Mitogen-Activated Protein Kinase/Extracellular Signal-Regulated Kinase Kinase 1

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## Abstract

The HER-2 oncogene, a member of the erythroblastosis oncogene B (ERBB)-like oncogene family, has been shown to be amplified in many types of cancer, including breast cancer. However, the molecular mechanism of HER-2 overexpression is not completely understood. The phosphorylation of proteins on the serine or threonine residues that immediately precede proline (pSer/Thr-Pro) is specifically catalyzed by the prolyl isomerase Pin1 and is a key signaling mechanism in cell proliferation and transformation. Here, we found that Pin1 interacts with mitogen-activated protein kinase/extracellular signal-regulated kinase kinase (MEK) protein kinase 1, resulting in the induction of HER-2 expression. *Pin1*$^{-/-}$ mouse embryonic fibroblasts exhibited a decrease in epidermal growth factor (EGF)–induced MEK1/2 phosphorylation compared with *Pin1*$^{+/+}$ mouse embryonic fibroblast. In addition, a knockdown of Pin1 resulted in the inhibition of MEK1/2 phosphorylation induced by EGF in MCF-7 cells. Furthermore, PD98059, a specific inhibitor of MEK1/2, and Juglone, a potent Pin1 inhibitor, markedly suppressed the expression of activator protein-2α and the HER-2 promoter activity induced by EGF or 12-*O*-tetradecanoylphorbol-13-acetate in MCF-7 cells. Importantly, these inhibitors inhibited the neoplastic cell transformation induced by EGF in Pin1-overexpressing JB6 Cl41 cells, which showed enhanced cellular formation compared with the control cells. Therefore, Juglone and PD98059 inhibited the colony formation of MCF-7 breast cancer cells in soft agar. These results indicate that Pin1 amplifies EGF signaling in breast cancer cells through its interaction with MEK1 and then enhances HER-2 expression, suggesting that Pin1 plays an important role in the overexpression of HER-2 through Pin1-MEK1-activator protein-2α signaling in breast cancer. *Mol Cancer Ther; 9(3); 606–16. ©2010 AACR.*

## Introduction

Subclass I of the receptor tyrosine kinase superfamily contains the erythroblastosis oncogene B (ERBB) or epidermal growth factor (EGF) receptors (EGFR) and consists of four members: EGFR/HER-1, HER-2, HER-3, and HER-4 (1). All members have an extracellular ligand-binding region, a single membrane-spanning region, and a cytoplasmic tyrosine kinase–containing domain (1). HER-2, which is also known as ERBB2, has been implicated in the development of many human cancers, including breast cancer (2–4). There is considerable evidence suggesting that *HER-2* overexpression is associated with elevated tumorigenicity (5), enhanced metastatic potential (6), increased resistance to tumor necrosis factor-α (7), and, in certain circumstances, resistance to chemotherapy (8, 9). Overexpression of the *HER-2* gene occurs in ~25% to 30% of breast cancer patients and is indicative of a poor prognosis (10). However, the underlying molecular mechanism of its overexpression is still unknown.

Activator protein-2 (AP-2) transcription factors are involved in the regulation of cell proliferation, differentiation, apoptosis, and carcinogenesis (11, 12). AP-2α and AP-2γ, as well as AP-2β to some extent, are expressed in HER2/neu–overexpressing breast cancer cell lines alone or in combination and activate the *HER-2* promoter *in vitro* (13, 14). Cooperation between the two functional AP-2 binding sites of the *HER-2* promoter is needed for the maximal *HER2/neu* transcriptional activity in breast cancer cells (12). In addition to HER-2, AP-2 is involved in the transcriptional control of other EGFR family members, including *EGFR*, and *HER-3* (15, 16). On the other hand, the Ets protein PEA3 binds directly to the consensus motif and prevents *HER-2* overexpression by suppressing the promoter activity (17). The downregulation of *HER-2* expression inhibits

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cell growth *in vitro* and, in a preclinical gene therapy setting, blocks tumor formation among *HER-2*–overexpressing cancer cells and prolongs the survival of the treated animals (17). This suggests that transcriptional activation or repression on the promoter of *HER-2* might be a target in breast cancer caused by the overexpression of pathogenic genes, such as *HER-2*.

Pin1, a recently identified peptidyl-prolyl cis/trans isomerase (PPIase), has two domains: a PPIase domain at its COOH terminus responsible for isomerization and a WW domain at the $NH_2$ terminus, which functions as a binding element specific to pSer/Thre-Pro motifs (18–20). Through these two domains, Pin1 binds to and isomerizes specific pSer/Thr-Pro motifs and catalytically induces conformational changes after phosphorylation. Such conformational changes can have profound effects on the function of many Pin1 substrates, such as p53 (21), cyclin D1 (22), c-Jun (23), NF-κB (24), C-myc (25), β-catenin (26), and Cdc25C (27), thereby playing important roles in many cellular events, such as cell cycle progression and differentiation. Also, Pin1 is overexpressed in breast cancer and cooperates with Ras signaling in increasing catalysis for oncogenesis (23). Upon growth factor stimulation, the pathway from Ras through Raf and mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) kinase (MEK) to ERK/MAPK regulates many fundamental cellular processes that govern cell transformation, differentiation, proliferation, and survival (28, 29). Moreover, the active Ras/Raf/MEK/ERK pathway inevitably accounts for a hallmark of malignant phenotypes: abnormal cell growth, invasion, and angiogenesis (30). Interestingly, the hyperphosphorylated/desensitized Raf-1 interacts with the protein phosphatase PP2A and the Pin1, both of which are required for the recycling of Raf-1 to an activation-competent state (31). Although many details of the Ras signaling process have been elucidated, little is known regarding how the phosphorylation events are regulated. Uncovering the mechanisms responsible for the prolonged phosphorylation of MAPK signaling–related proteins is an important goal, given that constitutive signaling from Pin1 results in tumorigenesis.

This study examined the molecular events that are mediated by Pin1 after mitogen activation by EGF in MCF-7 cells. The data provide the first evidence that Pin1 regulates the EGF signaling pathway in such a way as to increase the phosphorylation of MEK1/2, resulting in the overexpression of HER-2 and a cellular transformation. In this signaling pathway, Pin1 binds to MEK1, which is downstream of Ras signaling in MCF-7 cells. Moreover, Pin1 affects the AP-2α levels induced by EGF, resulting in increased *HER-2* promoter activity and neoplastic cellular transformation. Overall, these findings suggest that MEK1 is positively regulated by a direct interaction with Pin1. In addition, the prolonged phosphorylation of MEK1/2 results in *HER-2* overexpression, and Pin1 plays an important role in promoting EGF-induced carcinogenesis in breast cancer.

## Materials and Methods

### Reagents and Antibodies

DMEM, Eagle's MEM, L-glutamine, gentamicin, and fetal bovine serum were purchased from Invitrogen. EGF, 12-*O*-tetradecanoyl phorbol-13-acetate (TPA), and PD98059 were obtained from Calbiochem-Novabiochem. Polyvinylidene difluoride membrane was supplied by Millipore. The jetPEI cationic polymer transfection reagent and the jetSI-ENDO were purchased from Polyplus Transfection, Inc. The dual-luciferase reporter assay kit was obtained from Promega. 5-Hydroxy-1,4-naphthoquinone (Juglone) was purchased from Sigma-Aldrich Co. The antibodies against phospho-MEK1/2, phospho-ERK1/2, and total antibodies for each respective protein were purchased from Cell Signaling Technology, Inc. The antibodies against Pin1, AP-2β, AP-2γ, mouse IgG, and rabbit IgG were acquired from Santa Cruz Biotechnology. Anti-Xpress antibody was obtained from Invitrogen. The anti-ErbB2 and anti-AP-2α antibodies were purchased from Dako Cytomation and Upstate, respectively.

### Cell Culture and Transfection

MCF-7 human breast cancer cells, human embryonic kidney cells (HEK293), and $Pin1^{+/+}$ and $Pin1^{-/-}$ mouse embryonic fibroblast (MEF) cells were grown in DMEM supplemented with 10% fetal bovine serum, glutamine, and antibiotics (100 units/mL penicillin, 100 μg/mL streptomycin). MEM (5%) was used to maintain the JB6-Pin1 cells. The $Pin1^{+/+}$ and $Pin1^{-/-}$ MEF cells were kindly provided by Dr. Kun Ping Lu (Beth Israel Deaconess Medical Center). The p756 (HER-2 promoter)–luciferase construct was kindly provided by Dr. Keon-Wook Kang (Chosun University). The DNA transfection of the cells was done using jetPEI cationic polymer transfection reagent.

### Construction of Mammalian Expression and Small Interfering RNA Vectors

For the mammalian two-hybrid system, the cDNAs of human protein kinases were amplified by a PCR, and each was introduced into the pACT or pBIND two-hybrid system vector. In this system, pBIND and pACT (Promega) are fusion vectors used to link the proteins to the GAL4-DNA binding domain and to the VP16 transactivation domain. pBIND-Pin1 was generated by PCR and subcloned into the pcDNA/*Xpress* vector (Invtrogen). A segment encoding the full encoding sequence of the selected human kinase candidate, MEK1, was amplified by PCR and cloned in-frame into the *Bam*HI/*Xba*I sites of the pACT fusion to produce the plasmid pACT-MEK1. The cDNA of Pin1–wild type (WT), -WW domain (WW), and -PPIase domain (PPIase), which was a gift from Dr. Kun Ping Lu (Beth Israel Deaconess Medical Center, Harvard Medical School), were subcloned to either the pBIND vector or pcDNA4/*Xpress* vector (Invitrogen). pcDNA-Myc-cRaf was a gift from Dr. Kwang-Youl Lee

(College of Pharmacy, Chonnam National University). The small interfering RNA (siRNA)–mediated downregulation of human Pin1 (accession number NM_006221) was done by transfecting the SMART pool-specific or nonspecific control pool double-stranded RNA oligonucleotides (Dharmacon, Inc.) using Lipofectamine 2000 (Invitrogen).

### Mammalian Two-Hybrid Assay

pACT-MEK1, pBind-Pin1-WT, pBind-Pin1-WW, or pBind-Pin1-PPIase was combined with pG5-luciferase in an equal molar ratio, and the total amount of DNA was at most 100 ng/mL. The transfections were carried out using the jetPEI reagent, as described by the manufacturer. The cells were disrupted by the direct addition of 100 μL of a passive lysis buffer (Promega) into each well of the 48-well plates and then incubated at 4°C for 1 h. The cells were lysed completely by freezing and thawing twice. After centrifugation, 50 μL of the cell lysates were added to the respective wells of a 96-well luminescence plate. The luminescence activity was measured using a luminometer (Berthold Technologies LB941). The relative luciferase activity was calculated and normalized to the pG5-luciferase basal control. The transfection efficiency and protein concentration were assessed using the *Renilla* luciferase assay and Lowry protein assay, respectively.

### Infection of Green Fluorescent Protein and Pin1 into JB6 Cl41 Cells

Pin1 was expressed stably in JB6 Cl41 cells using the Murine Stem Cell Virus (MSCV)–green fluorescent protein (GFP) retrovirus system. Human Pin1 was subcloned into the MSCV-GFP retroviral vector (Clontech, Inc.), and phoenix cells (a packaging cell line) were transfected with the MSCV-GFP (control) or MSCV-Pin1-GFP (Pin1-overexpressing) plasmid. The supernatants containing amphotropic replication-incompetent retroviruses were collected and stored at −80°C until needed. The JB6 Cl41 cells (20% confluent) were multiply infected (eight times) with retrovirus particles. The intensities of infection were monitored by GFP fluorescence and Western blotting analysis using the Pin1-specific antibody.

### Immunoblotting

The cells were disrupted in a lysis buffer [50 mmol/L Tris (pH 7.5), 5 mmol/L EDTA, 150 mmol/L NaCl, 1 mmol/L DTT, 0.01% Nonidet P-40, 0.2 mmol/L phenylmehylsulfonyl fluoride, and 1 × protease inhibitors cocktail]. The proteins were resolved by SDS-PAGE and transferred to polyvinylidene difluoride membranes. The membranes were blocked and hybridized with the appropriate primary antibody overnight at 4°C. The protein bands were visualized using Amersham ECL Advance Western blotting detection kit (GE Healthcare) after hybridization with the horseradish peroxidase–conjugated secondary antibody from rabbits or mice. The LAS3000-mini (Fujifilm) was used for chemiluminescence detection.

### *In vitro* Binding Assay

DNA transfection and immunoprecipitation were done to evaluate the biochemical interaction between Pin1 and MEK1 *in vitro*. Human embryonic kidney cells (HEK293) were cotransfected with the Xpress epitope–tagged Pin1-WT (pcDNA4/Xpress-Pin1-WT) and VP16 epitope–tagged MEK1 (pACT/VP16-MEK1) using the jetPEI reagent as described by the manufacturer. Xpress-Pin1-WT or VP16-MEK1 was immunoprecipitated with the antibody against anti-Xpress or the anti-VP16 antibody, respectively, and immunoblotting was then done. The bound proteins were denatured in a sample buffer and separated by 10% to 20% SDS-PAGE, and expression was detected using a LAS-3000mini.

### Reporter Gene Assays

A reporter gene assay for HER-2 promoter activity was done using the lysates from the cells transfected with p756-luciferase vector. The reporter gene vector phRL-SV40 was also transfected into each cell line. The *Renilla* luciferase activity generated by this vector was used to normalize the transfection efficiency. The cell lysates were prepared by washing the transfected cells once with 1 × PBS. Two hundred microliters of a 1 × passive lysis buffer (Promega Dual-Luciferase Reporter Assay system) were then added after removing the PBS completely. The cells were then incubated for 1 h with gentle shaking at room temperature. After the cell lysates (50 μL from each) were mixed with the 50 μL Luciferase Assay II reagent and firefly luciferase, the luminescence emission was measured using a luminometer. Subsequently, 50 μL of the *Renilla* luciferase substrate were added to normalize the firefly luciferase data.

### Anchorage-Independent Cell Transformation Assay (Soft Agar Assay)

An EGF-induced cell transformation was investigated in GFP-, Pin1-infected JB6 Cl41 cells or MCF-7 cells in the presence or absence of Juglone, Pin1 inhibitor, and PD98059, MEK1/2 inhibitor. Briefly, the cells ($8 \times 10^3$) were exposed to either Juglone (0, 1, 5, and 10 μmol/L) or PD98059 (0, 10, 25, and 50 μmol/L) with or without EGF in 1 mL of 0.3% basal medium Eagle's agar containing 10% fetal bovine serum. The cultures were maintained at 37°C in a 5% $CO_2$ incubator for 10 to 15 d, and the cell colonies were scored using an Axiovert 200 M florescence microscope and Axio Vision software (Carl Zeiss, Inc.).

## Results

### EGF Enhances the Interaction of Pin1 with MEK1

To understand the contribution of Pin1 to the processes that lead to HER-2 overexpression in breast cancer, the potential protein-binding partners of Pin1 were first investigated by screening with the M2H system (Promega). Among the protein kinases screened, MEK1 was found to

interact with Pin1 *in vitro* (data not shown). To further confirm the interaction between Pin1 and MEK1, the HEK293 cells were next cotransfected with VP16-tagged MEK1 and Xpress-tagged Pin1. The cell lysates were immunoprecipitated using the normal IgG or anti-VP16 antibody and blotted with the anti-Xpress antibody. Reciprocal immunoprecipitation/immunoblotting using anti-Xpress and anti-VP16 antibodies showed that the exogenously expressed MEK1 interacted with the Xpress-tagged full-length Pin1 (Fig. 1A). Among the components of the MAPK pathways, the Raf/MEK/ERK signaling cascade has been identified as a key downstream effector of Ras, which is a frequently mutated oncogene in 30% or more of human cancers (32). To examine whether Pin1 can bind with other components, including MEK1, among the Ras/Raf/MEK/ERK cascade, Myc-Raf-1, Myc-ERK, HA-MEK1, or Flag-MKK7, which is an upstream of c-Jun $NH_2$-terminal kinase 1/2, was cotransfected with Xpress-Pin1, and the interaction was examined using the immunoprecipitation/immunoblotting analysis. Pin1 interacted with MEK1 and Raf-1, but not ERK or MKK7 (Fig. 1B). To determine the region of Pin1 that is responsible for its interaction with MEK1, the full-length Pin1 (Pin1-WT), the WW domain of Pin1 (Pin1-WW), or the PPIase domain of Pin1 (Pin1-PPIase; Fig. 1C, top) was cotransfected with pACT-MEK1, and the interaction was examined using a mammalian two-hybrid assay (Promega). The Pin1 binding site on MEK1 encompasses amino acids 1 to 44, which are located on the WW domain (Fig. 1C, bottom). Next, the time course analysis for the EGF-induced interaction of Pin1 and MEK1/2 was examined in human breast cancer MCF-7 cells to determine if endogenous Pin1 and MEK1/2 also interact *ex vivo*. An antibody against MEK1/2 was used to immunoprecipitate MEK1/2, and Pin1 was then detected by immunoblotting. The results showed that Pin1 could be detected in the MEK1/2 immunoprecipitates from 5 to 15 minutes after the EGF treatment, concomitant with increased phosphorylation of MEK1/2 (Fig. 1D). To further examine whether Pin1 may interact with Raf-1 as well as MEK1 induced by EGF and/or TPA, MCF-7 cells were treated with EGF or TPA and immunoprecipitated with antibody against Raf-1 or MEK1/2, respectively, and Pin1 was then detected by immunoblotting. The results showed that EGF as well as TPA induced its interaction with MEK1/2, but EGF could not induce its interaction with Raf-1 (Fig. 1E). These results suggest that the WW domain of Pin1 binds to the protein MEK1, which was further enhanced in the presence of EGF as well as TPA.

### Pin1 Enhances the EGF-Induced Phosphorylation of MEK1/2 and ERK1/2

To determine if EGF signaling is regulated by Pin1, the $Pin1^{+/+}$ and $Pin1^{-/-}$ MEF cells were exposed to EGF (1 ng/mL) and immunoblotted with the antibodies against phospho-MEK1/2 and phospho-ERK1/2. The level of MEK1/2 and ERK1/2 phosphorylation was considerably lower in the $Pin1^{-/-}$ MEF cells than in the $Pin1^{+/+}$ MEF cells (Fig. 2A). To further confirm the regulatory role of Pin1 on the phosphorylation of MEK1/2 and ERK1/2, GFP or Pin1 stably overexpressing C2C12 cells were exposed to EGF (1 ng/mL) and immunoblotting was done with the respective antibodies. There was an increase in the level of MEK1/2 and ERK1/2 phosphorylation in Pin1 stably overexpressing C2C12 cells compared with the control GFP-C2C12 cells (Fig. 2B). To determine if knockdown of Pin1 suppresses the EGF-induced phosphorylation of MEK1/2 and ERK1/2, MCF-7 cells were transfected with siRNA Pin1 or control siRNA and treated or not treated with EGF at 10 ng/mL in a time-dependent manner. The decrease in phosphorylation of MEK1/2 observed coincided with the phosphorylation of ERK1/2 induced by EGF in Pin1 knockdown cells (Fig. 2C). Overall, these data indicate that Pin1 enhances the EGF-induced phosphorylation of MEK1/2 and ERK1/2 through its interaction with MEK1.

### Pin1 Regulates the EGF-Induced Expression of HER-2

Pin1 is strongly expressed in 62% of HER-2–positive breast cancers and regulates its protein stability (33). To determine if the ablation or knockdown of Pin1 suppresses the HER-2 expression induced by tumor promoters, such as EGF, the $Pin1^{+/+}$ and $Pin1^{-/-}$ MEF cells were first exposed to EGF in a dose-dependent manner and the immunoblotting was done with the antibody against HER-2. The decreased HER-2 expression was observed in the $Pin1^{-/-}$ MEF cells compared with $Pin1^{+/+}$ MEF cells (Fig. 3A). A similar pattern was observed in the time course analysis with EGF in $Pin1^{+/+}$ and $Pin1^{-/-}$ MEF cells (Fig. 3B). Next, to confirm the effect of Pin1 knockdown on the expression of HER-2 induced by EGF, MCF-7 cells transfected with siRNA-Pin1 or control siRNA were exposed to EGF in dose-dependent manner, and then immunoblotting was done with the antibody against HER-2 and Pin1, respectively. This result showed that EGF-induced HER-2 expression was suppressed in the siRNA-Pin1–transfected cells compared with control-siRNA–transfected cells (Fig. 3C). To further determine if the inhibition of MEK1/2 or Pin1 downregulates HER-2 expression induced by EGF, the MCF-7 cells were pretreated with PD98059, a MEK1/2 inhibitor, or Juglone, an irreversible inhibitor of *cis/trans* isomerization activity of Pin1 (34), for 2 hours, and either exposed or not exposed to EGF at 10 ng/mL for 12 hours, and immunoblotting was then done with antibodies against phospho-MEK1/2, phospho-ERK1/2, and HER-2, respectively. PD98059 and Juglone markedly decreased the EGF-induced HER-2 expression (Fig. 3D). Overall, these results suggest that Pin1 plays a key role in regulating the EGF-induced HER-2 expression in MCF-7 cells by enhancing the phosphorylation of MEK1/2 and ERK1/2 due to its interaction with MEK1.

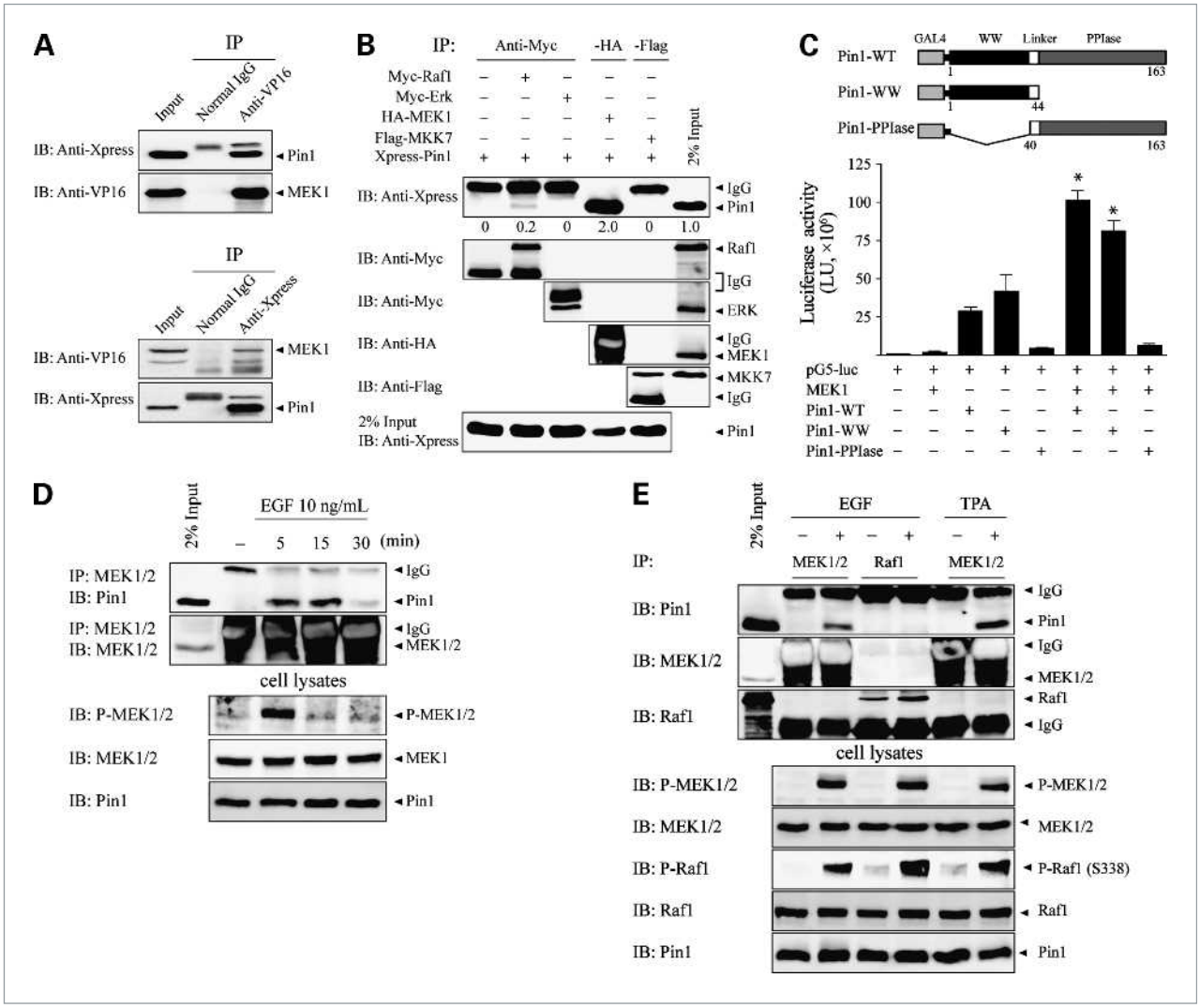


Figure 1. The interaction of Pin1 with MEK1. A, HEK293 cells were cotransfected with the pcDNA4-Xpress-Pin1 plasmid and pACT-VP16-MEK1. After 48 h of transfection, proteins were extracted and used for immunoprecipitation (IP) with anti-VP16 against MEK1 (top) or anti-Xpress against Pin1 (bottom). Normal IgG antibody from a mouse was used as a negative control for immunoprecipitation. Pin1 or MEK1 was visualized by immunoblotting (IB) with the anti-Xpress or anti-VP16 antibody, respectively. B, pcDNA4-Xpress-Pin1 plasmid were cotransfected with pcDNA-Myc-Raf-1, -Myc-ERK, -HA-MEK1, or -Flag-MKK7, respectively. After 48 h of transfection, proteins were extracted and used for immunoprecipitation with anti-Myc against Raf-1 or ERK, anti-HA against MEK1, or anti-Flag antibody against MKK7. Each protein was visualized by immunoblotting with the anti-Xpress, -Myc, -HA, -Flag antibody. Corresponding signal intensities were densitometrically determined and normalized to 2% input control and were given below in each data. C, top, schematic diagram of full-length (1–163) pBIND-pin1 WT (Pin1-WT), the WW domain (1–44) of pBIND-pin1 (Pin1-WW), or the PPIase domain (40–163) of pBIND-Pin1 (Pin1-PPIase). As a negative control, the HEK293 cells were transfected with pACT-MEK1 along with the pG5-luc reporter plasmid. The pACT-MEK1 and pBIND-Pin1 (WT, WW, or PPIase) were cotransfected with the pG5-luc plasmid to confirm the binding site for MEK1 in Pin1. All experiments were carried out at least twice with triplicate samples and are reported as the mean ± SEM. The asterisk (*) indicates a significant increase in activity compared with the negative control, pACT-MEK1 only (*, $P < 0.05$). Data are shown in relative luciferase activity units. D, the MCF-7 cells were starved in serum-free DMEM for 24 h, either exposed or not exposed to EGF (10 ng/mL), and harvested after incubation for the indicated time. Immunoprecipitation was done to precipitate the endogenous MEK1/2. The proteins in the whole-cell lysates were separated by SDS-PAGE, and immunoblotting analysis was done. E, the MCF-7 cells were starved in serum-free DMEM for 24 h and either exposed or not exposed to EGF (10 ng/mL) or TPA (10 ng/mL) for 5 min. Immunoprecipitation was done to precipitate the endogenous MEK1/2 or Raf-1, respectively, and immunoblotting analysis was done using antibodies against Pin1, MEK1/2, and Raf-1. Immunoblotting analysis was also done for phospho-MEK1/2, phospho-Raf-1, Pin1, MEK1/2, and Raf-1 by taking the protein from the total cell lysate.

## Pin1 Is Essential for the HER2/Neu Promoter Activity Induced by EGF and TPA

Considering the role of Pin1 in HER-2 expression, experiments were carried out to determine if Pin1 is essential for the HER-2 promoter induced by TPA or EGF. AP-2α and AP-2α/γ coexpression is positively associated with HER-2 expression, whereas the expression of AP-2γ alone is not related to HER-2 expression (35). In addition,

AP-2α is expressed at elevated levels in a most HER-2–overexpressing mammary tumor cells (13). The level of AP-2α induced by EGF or TPA in the presence or absence of PD98059 was first examined to determine if HER-2 expression regulated by Pin1 is associated with AP-2α. The levels of AP-2α induced by EGF or TPA were decreased markedly by the PD98059 treatment (Fig. 4A). Next, we determined the effect of knockdown of Pin1 on the EGF- or TPA-induced AP-2α. Interestingly, these results suggest that Pin1 is essential for the EGF- and TPA-induced expression of AP-2α (Fig. 4B). Therefore, we further examined whether ablation of the *Pin1* gene, inhibition of Pin1, or inhibition of MEK1/2 affects the *HER-2* promoter activity (p756-luciferase promoter) induced by EGF and TPA. The $Pin1^{+/+}$ and $Pin1^{-/-}$ MEF cells were transfected with mixtures of the p756-luciferase promoter plasmid and *phRL*-SV40 gene in each of the 24-well plates and then either treated or not treated with EGF and TPA (Fig. 4C). The results showed that either EGF (Fig. 4C, left) or TPA (Fig. 4C, right) significantly induced the p756-luciferase activity in $Pin1^{+/+}$ MEF cells, whereas there was no any significant change in $Pin1^{-/-}$ MEF cells compared with the control (Fig. 4C). The p756-luciferase promoter activity induced by EGF or TPA in the presence or absence of Juglone and PD98059 in MCF-7 cells was next determined. The MCF-7 cells were transfected with mixtures of the p756-luciferase promoter plasmid and *phRL*-SV40 gene in each of a 24-well plate. At 24 hours after transfection, the cells were starved for an additional 24 hours in serum-free DMEM at 37°C in a 5% $CO_2$ incubator. Cells were then treated with or without EGF or TPA in the presence or absence of Juglone and PD98059. PD98059 and Juglone decreased significantly the EGF- (Fig. 4D, left) and TPA-induced p756-luciferase promoter activity (Fig. 4D, right) in a dose-dependent manner. Overall, these results strongly support the hypothesis that the regulation of MEK1 by Pin1 plays a key role in the induction of HER-2 expression via an increase in the level of AP-2α transcriptional factor.

### Pin1 Enhances EGF-Induced Neoplastic Cell Transformation through MAPK Signaling

The mouse skin epidermal JB6 Cl41 cell system is a well-developed model for examining tumor promotion under anchorage-independent growth conditions (36). To determine if the inhibition of Pin1 or MEK1/2 suppresses Pin1-enhanced cell transformation induced by EGF, the GFP-overexpressing JB6 Cl41 control cells (MSCV-GFP) and Pin1-overexpressing JB6 Cl41 cells (MSCV-Pin1) were treated with EGF (1 ng/mL) in the presence or absence of Juglone (Fig. 5A) or PD98059 (Fig. 5B) in soft agar and incubated at 37°C in a 5% $CO_2$ incubator for 2 weeks. The colony numbers and colony sizes were significantly higher in the MSCV-Pin1 cells than in the MSCV-GFP cells (Fig. 5A and B). Importantly, Juglone and PD98059 significantly decreased the colony numbers in the cells treated with either Juglone (Fig. 5A) or PD98059 (Fig. 5B) in a dose-dependent manner compared with the only EGF-treated MSCV-Pin1 cells, respectively. To further assess whether the Juglone or PD98059 inhibited the tumorigenicity of MCF-7 breast cancer cells, MCF-7 cells were treated with Juglone or PD98059 in soft agar and incubated under the same

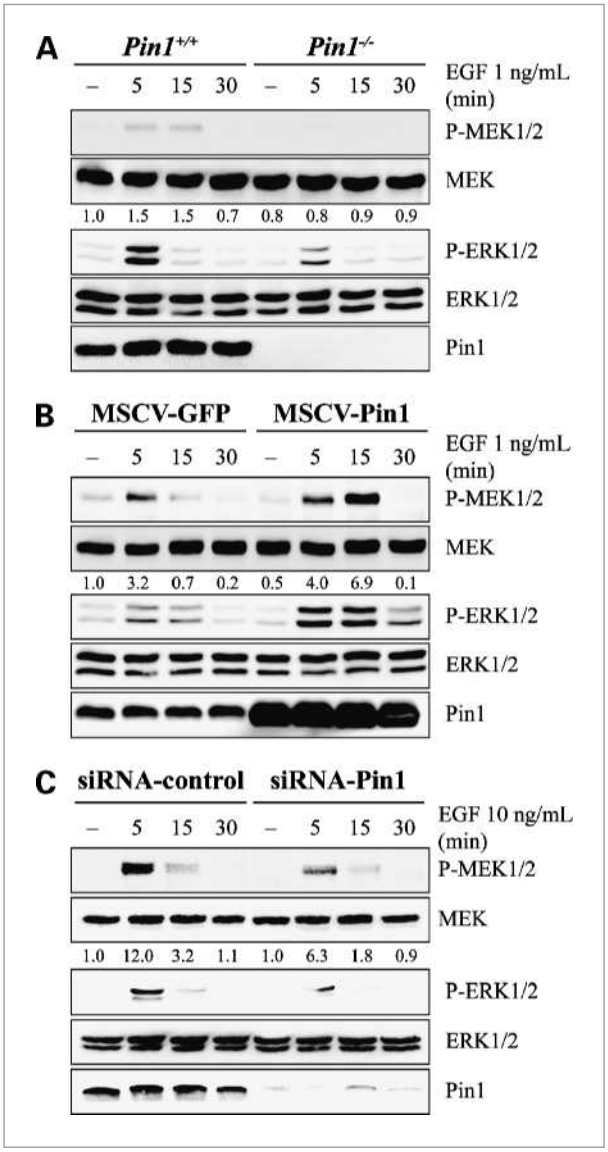


Figure 2. Pin1 regulates the EGF-induced phosphorylation of MEK1/2 and ERK1/2. A, the $Pin1^{+/+}$ and $Pin1^{-/-}$ MEF cells were starved in serum-free DMEM for 24 h. The cells were then treated with or without 1 ng/mL EGF for the indicated times, harvested, lysed, and immunoblotted. B, MSCV-GFP– or MSCV-Pin1–overexpressing C2C12 cells were starved in serum-free DMEM and treated or not treated with EGF (1 ng/mL) for the indicated times, harvested, lysed, and immunoblotted. C, MCF-7 cells were transfected with siRNA-Pin1 (50 nmol) or the siRNA-control (50 nmol). At 48 h after transfection, the cells were starved with serum-free DMEM for an additional 24 h. The cells were then treated with or without 10 ng/mL EGF for the indicated times, harvested, lysed, and immunoblotted. Corresponding signal intensities of phospho-MEK1/2 were densitometrically determined and normalized to total MEK1/2 in each lane and is given below in each data.

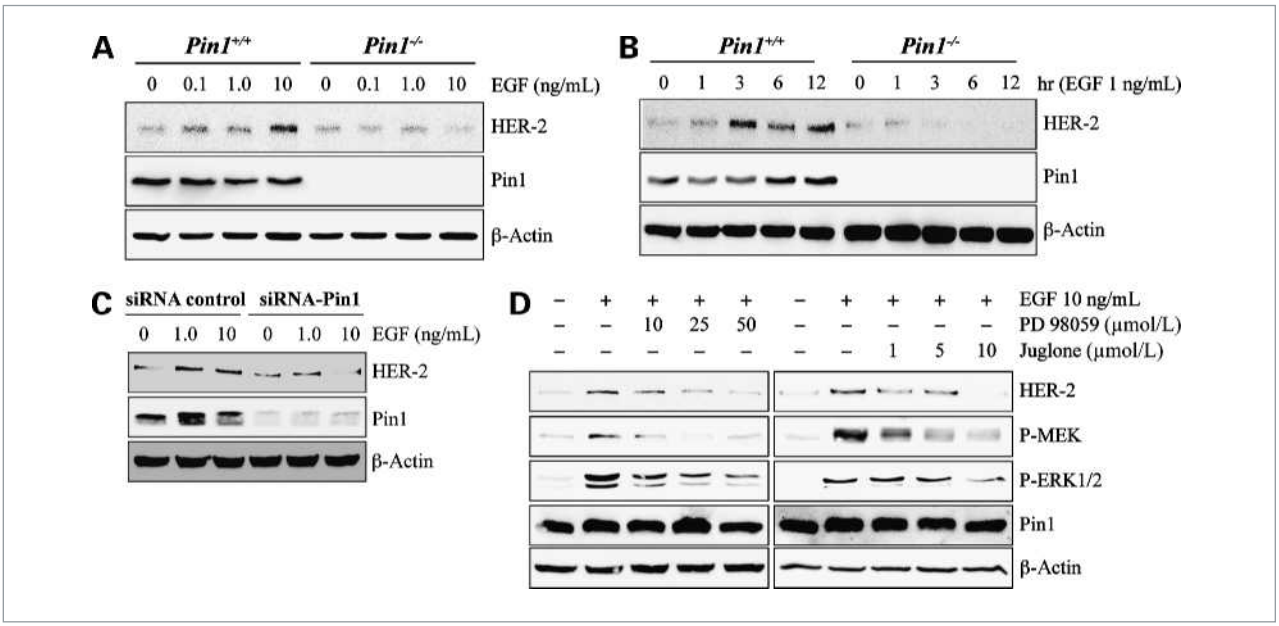


Figure 3. Pin1 regulates the EGF-induced HER-2 expression. A and B, the *Pin1*$^{+/+}$ and *Pin1*$^{-/-}$ MEF cells were starved in serum-free DMEM for an additional 24 h. The cells were treated with or without EGF at the indicated dose for 12 h (A) or EGF 1 ng/mL for the indicated times (B), harvested, and lysed. Immunoblotting analyses were done using the antibodies against the respective proteins. C, MCF-7 cells were transfected with either siRNA-Pin1 (50 nmol) or the siRNA-control (50 nmol). At 48 h after transfection, the cells were starved with serum-free DMEM for 24 h. The cells were then treated with or without the indicated amounts of EGF for 12 h, harvested, lysed, and immunoblotted. D, MCF-7 cells were starved with serum-free DMEM for an additional 24 h. The cells were then treated with either PD98059 (left; 10, 25, and 50 μmol/L) or Juglone (right; 1, 5, and 10 μmol/L) and incubated for a further 2 h. Subsequently, the cells were exposed to 10 ng/mL EGF and incubated for 12 h, harvested, lysed, and immunoblotted.

conditions as shown in Fig. 5A. Both inhibitors decreased the colony numbers and colony sizes of MCF-7 cells compared with control cells (Fig. 5C and D), respectively, indicating that the Pin1-MEK1/2 interaction might be a potential treatment target in HER-2–positive breast cells.

## Discussion

A growth factor receptor gene, human EGFR-2 (HER-2), is amplified in 25% to 30% of breast cancers. Moreover, in these cases, the encoded protein is present in abnormally high levels in the malignant cells, suggesting that it is a clear predictor of the response to HER-2 therapy (37). Although the humanized monoclonal antibody trastuzumab (Herceptin) is initially as effective as a mono and combination treatment, almost all patients develop resistance (37). To overcome trastuzumab resistance, the underlying molecular mechanism and development of strategies are important. However, the factors that regulate HER-2 protein expression are unclear. The prolyl isomerase Pin1 catalyzes the isomerization of the specific pSer/Thr-Pro motifs that have been phosphorylated as a response to mitogenic signaling (20). Recently, it was reported that 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 (37.5%; refs. 10, 33). This suggests that Pin1 might be a potential treatment target for HER-2 breast cancer. The interaction between Pin1 and several protein kinases was screened using an *in vitro* mammalian two-hybrid assay to identify new specific substrates that might be associated with the distinct role of Pin1 in HER-2–positive breast cancers. The results showed that MEK1 strongly interacts with Pin1 *in vitro* (Fig. 1A). In addition, the WW domain of Pin1 was found to be essential for its interaction with MEK1 *in vitro* (Fig. 1C). In addition, our results showed that the EGF treatment for the activation of MEK1/2 enhanced an interaction between Pin1 and MEK1 (Fig. 1D). The Raf-1 kinase is an important signaling molecule, functioning in the Ras pathway to transmit mitogenic, differentiative, and oncogenic signals to the downstream kinases MEK and ERK (38). Therefore, we examined whether Pin1 may interact with human Raf-1, also known as c-Raf, ERK, or MKK7, an upstream of c-Jun $NH_2$-terminal kinase 1/2, directly. Our results showed that Pin1 could bind with MEK1 as well as Raf-1, but not ERK or MKK7. In previous report, Raf-1 was maximally phosphorylated on $S^{338}$ after 5 minutes of platelet-derived growth factor treatment; however, Raf-1 could be phosphorylated on $S^{642}$, which is its inhibitory site, and shifted to a lower migrating form after 30 minutes of platelet-derived growth factor treatment. Interestingly, Pin1 interacts with Raf-1 phosphorylated at $S^{642}$ and cooperates with the PP2A protein phosphatase to return the desensitized Raf-1 to activation-competent state (31). We did the *ex vivo* experiment whether the Pin1 binds with MEK1/2 or Raf-1 after EGF treatment in MCF7 cells. The results

showed that both MEK1/2 and Raf1 ($S^{338}$) were phosphorylated after 5 minutes of EGF treatment and Pin1 interacted with MEK1/2, but not Raf-1 (Fig. 1E). These results suggest that Pin1 enhances MEK1/2 phosphorylation via its direct interaction with MEK1 in the early stage of growth factor signaling and also contributes to the sensitivity of Raf-1 through dephosphorylation of Raf-1 at $S^{642}$ in the late stage of growth factor signaling, resulting in the human malignancies of the Ras/RAF/MEK/ERK signaling cascade.

The ERK signaling pathway has been studied extensively and is a key MAPK pathway that plays a role in cell proliferation, differentiation, and transformation (39). The ERK cascade is activated by a large number of extracellular stimuli including EGF, which stimulates Ras/Raf/MEK/ERK signaling cascade in an EGF-receptor–dependent pathway. The first model proposed to explain that the signaling specificity by the ERK cascade involved changes in the duration and strength of the signals transmitted by the cascade (40). In addition,

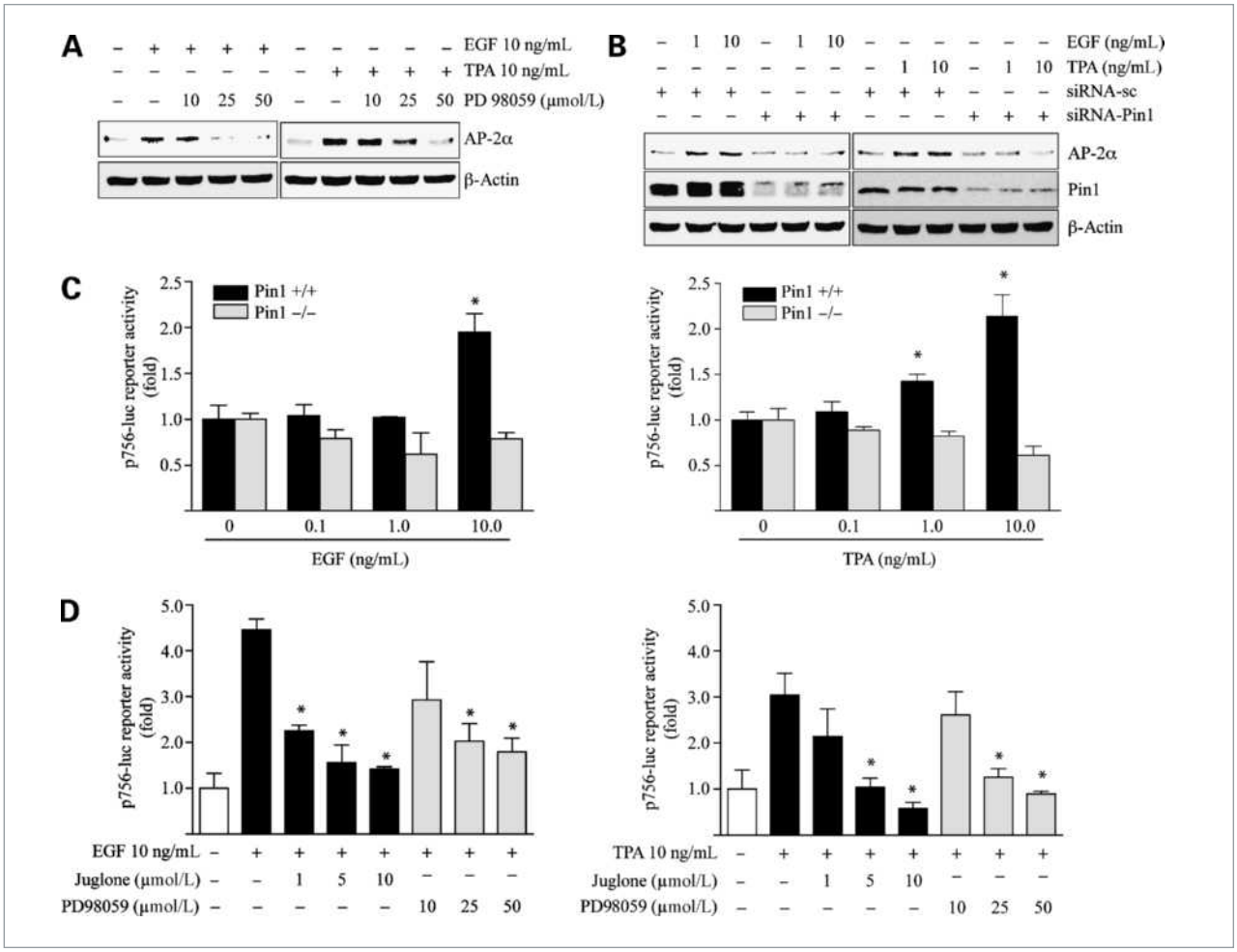


Figure 4. Juglone and PD98059 inhibit EGF- or TPA-induced AP-2α expression as well as HER-2 promoter activity. A, the MCF-7 cells were starved with serum-free DMEM for an additional 24 h. The cells were then treated with PD98059 (10, 25, and 50 μmol/L) and incubated for a further 2 h. Subsequently, the cells were exposed to either 10 ng/mL EGF (left) or 10 ng/mL TPA (right) and incubated for 12 h. Immunoblotting analyses were carried out using the antibodies against the respective proteins. B, MCF-7 cells were transfected with either siRNA-Pin1 (50 nmol) or siRNA-control (50 nmol). At 48 h after transfection, the cells were starved with serum-free DMEM for 24 h. The cells were then treated with or without the indicated amount of either EGF (left) or TPA (right) for 12 h, harvested, lysed, and immunoblotted. C, the $Pin1^{+/+}$ and $Pin1^{-/-}$ MEF cells were transfected with a mixture containing the p756-luciferase plasmid (0.5 μg) and phRL-SV40 gene (0.5 ng). After 24 h incubation, the cells were then starved with serum-free DMEM for 12 h and exposed to EGF (left; 0.1, 1.0, and 10 ng/mL) or TPA (right; 0.1, 1.0, and 10 ng/mL) for additional 12 h. Subsequently, the firefly luciferase activity was measured in the cell lysates and normalized against the *Renilla* luciferase activity. *, $P < 0.05$, significant increases in p756-luciferase activity compared with control cells. D, Juglone and PD98059 decreases EGF- or TPA-induced p756-luciferase activity. MCF-7 cells were transfected with a mixture containing the p756-luciferase plasmid (0.5 μg) and phRL-SV40 gene (0.5 ng). After 24 h incubation, the cells were then starved with serum-free DMEM for 12 h and pretreated with Juglone (1, 5, and 10 μmol/L) or PD98059 (10, 25, and 50 μmol/L) for 2 h. Subsequently, the cells were exposed to either EGF (left; 10 ng/mL) or TPA (right; 10 ng/mL) and incubated at 37°C in a 5% $CO_2$ incubator. The firefly luciferase activity was measured in the cell lysates and normalized against the *Renilla* luciferase activity. *, $P < 0.05$, significant decreases in p756-luciferase activity compared with control cells.

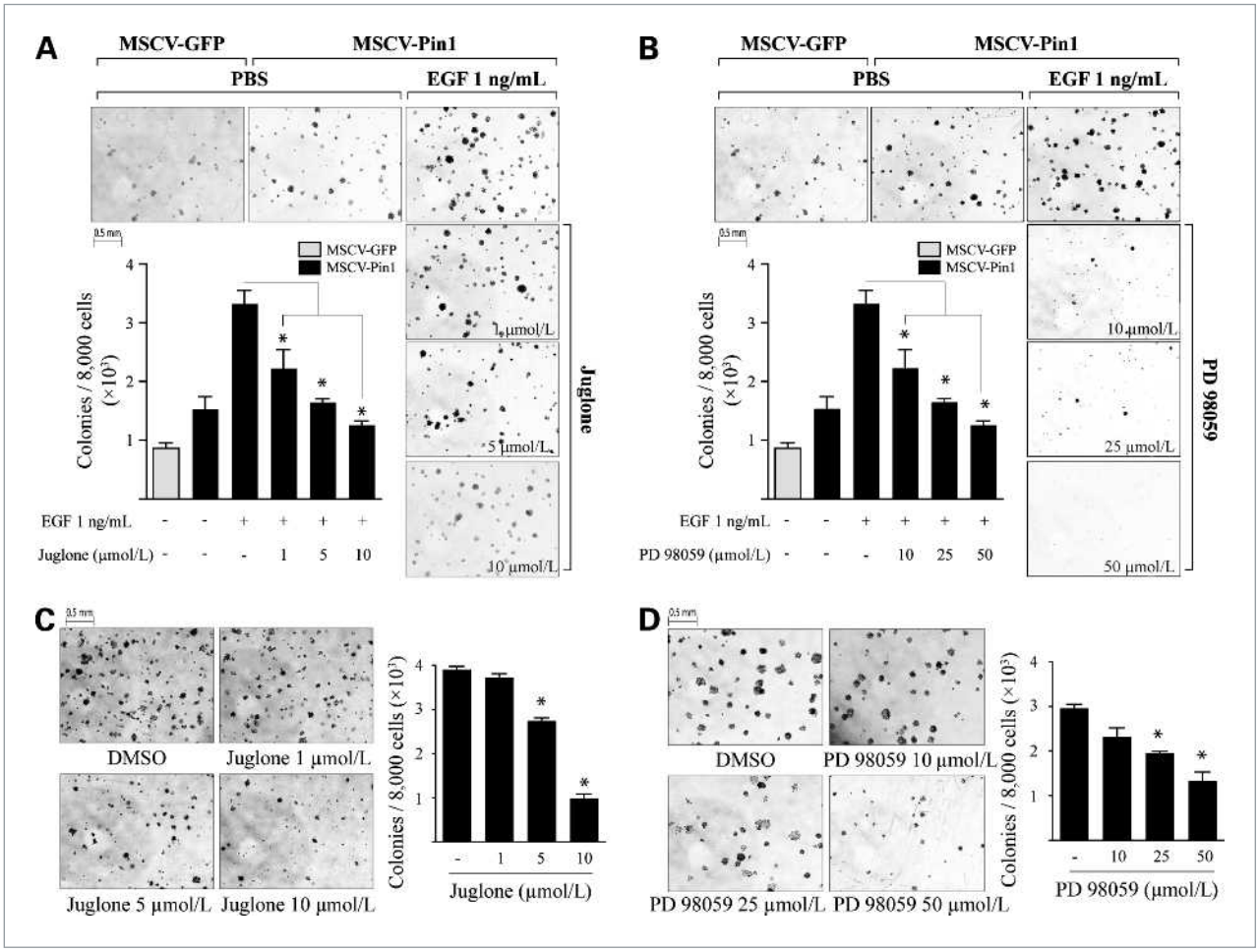


Figure 5. Juglone and PD98059 suppress the Pin1-enhanced neoplastic cell transformation. A and B, GFP- (MSCV-GFP) or Pin1-infected (MSCV-Pin1) JB6 Cl41 cells were exposed to 1 ng/mL EGF with/without a treatment with Juglone (A; 1, 5, and 10 μmol/L) or PD98059 (B; 10, 25, and 50 μmol/L) in soft agar. After 2 wk incubation, the average colony number (diameter > 80 μmol/L) was calculated, and the colonies from three separate experiments were photographed. Columns, mean of triplicate samples; bars, SEM. *, $P < 0.05$, significant inhibition of the EGF-induced cell transformation compared with control cells. C and D, The MCF-7 cells were exposed to Juglone (C; 1, 5, and 10 μmol/L) or PD98059 (D; 5, 10, and 25 μmol/L) in soft agar. After 2 wk incubation, the average colony number (diameter > 80 μmol/L) was calculated, and the colonies were photographed in three separate experiments. Columns, mean of triplicate samples; bars, SEM. *, $P < 0.05$, significant inhibition of tumorigenicity of MCF-7 cells compared with control cells.

the MAPK pathway can increase the level of HER-2 expression, indicating that these pathways are important for modulating cell proliferation, cellular transformation, and breast carcinoma (41, 42). However, the role of Pin1 on HER-2 overexpression via enhancement of the MAPK pathway is not completely understood. Our analysis showed that EGF markedly induces the phosphorylation of MEK1/2 and ERK1/2 in *Pin1*$^{+/+}$ MEF, Pin1-overexpressing C2C12, or siRNA-control–transfected MCF-7 cells, whereas the EGF-induced phosphorylation of MEK1/2 and ERK1/2 was suppressed in *Pin1*$^{-/-}$ MEF, GFP-overexpressing C2C12, or siRNA-Pin1–transfected cells (Fig. 2). These results indicate that Pin1 may regulate the phosphorylation of MEK1/2 and ERK1/2 induced by EGF. Furthermore, the EGF-induced expression of HER-2 is almost totally inhibited by ablation or knockdown of Pin1 and treatment of PD98059 or Juglone, suggesting that the EGF-induced phosphorylation of MEK1/2 is mediated by an interaction between Pin1 and MEK1 (Fig. 3). Hence, Pin1 regulates HER-2 expression by controlling MEK1 in MCF-7 cells.

The transcription factor AP-2 plays a key role in regulating *HER-2* gene transcription in breast cancer (11, 12). It was reported that AP-2α and AP-2γ are more active than AP-2β in the transcriptional activation of the *HER-2* gene induced by multiple growth factor signaling pathways (35). In the human *HER-2* gene, two putative AP-2α binding sites are involved in the expression of the *HER-2* gene at the potent enhancer elements. Therefore, the role of AP-2α in EGF-inducible HER-2 expression was examined in MCF-7 cells. Our results show that the nuclear level of AP-2α is enhanced by an EGF and TPA treatment (Fig. 4A). In addition, EGF- or TPA-induced expression of AP-2α in MCF-7 cells was suppressed by the MEK1/2

inhibitor PD98059 (Fig. 4A). Moreover, the knockdown of Pin1 suppressed the AP-2α induced by EGF or TPA (Fig. 4B), suggesting that Pin1 could regulate HER-2 overexpression via EGF- or TPA-induced expression of AP-2α in breast cancer. Reporter gene assays using the human *HER-2* promoter strongly suggest an important role for AP-2α expression in HER-2 overexpression in EGF- or TPA-stimulated MCF-7 cells (Fig. 4C and D). Overall, these results indicate that the AP-2α in the nucleus is associated with the induction of HER-2 in EGF- or TPA-stimulated MCF-7 cells.

There is considerable evidence suggesting that HER-2 overexpression induces neoplastic cell transformation and oncogenic tumorigenesis (43–45). In addition, TPA and EGF are associated with a neoplastic cell transformation in JB6 Cl41 cells (46–48). Our results show that the overexpression of Pin1 induced a neoplastic cell transformation in JB6 Cl41 cells, and EGF enhanced the Pin1-induced cell transformation (Fig. 5A). Interestingly, the EGF-induced neoplastic cell transformation in MSCV-Pin1–overexpressing JB6 Cl41 was inhibited by Juglone and PD98059, respectively (Fig. 5A and B). These results support our hypothesis that Pin1 enhances the EGF-induced cell transformation through its interaction and prolonged activity toward MEK1. Pin1 overexpression is found in HER-2–positive breast cancer, and Pin1 inhibition suppresses the growth of HER-2–positive breast cancer cells, indicating that Pin1 regulates HER-2 stability by interfering with its proteasomal degradation (33). Whereas Pin1 inhibition greatly increased the sensitivity of HER-2–positive breast cancer cell to the mTOR inhibitor rapamycin, it did not increase their sensitivity to trastuzumab (33). Our results showed that Juglone or PD98059 suppressed the tumorigenicity of MCF-7 cells in soft agar, indicating that a blockade of Pin1 or MEK1/2 might inhibit carcinogenesis in HER-2–positive breast cancer through the mechanism of downregulation of HER-2 expression (Fig. 5C and D).

Our group previously reported that Pin1 amplifies insulin signaling in hepatocarcinoma cells via its interaction with p70S6K. This suggests that Pin1 plays an important role in insulin-induced tumorigenesis and is a potential therapeutic target in hepatocarcinoma (49). However, the development of prolyl-isomerase inhibitors as a cancer treatment is still in the early stages. Pin1 itself has been suggested to be a therapeutic target. However, aside from Juglone (34), no specific inhibitors have been identified. For therapeutic purposes, the diversity of the signaling cascades involving Pin1 may be an advantage and provide broad inhibitory coverage of targets that may be needed to treat cancer efficiently (50). In summary, Pin1 plays a pivotal role in EGF-induced HER-2 expression through its interaction with MEK1 and prolongs the activity of MEK1/2 in MCF-7 cells. In addition, the interaction of the two proteins promotes a cellular transformation in JB6 Cl41 cells. Moreover, Juglone and PD98059 suppresses the tumorigenicity of MCF-7 cells, suggesting that the specific inhibition of phospho-specific prolyl isomerization by Pin1 may provide a means of simultaneously blocking the multiple signal transduction pathways and enhancing the efficacy of specific target-directed medications.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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