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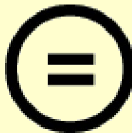

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석사학위논문

Effects of interleukin-22 on epithelial cell transformation and breast tumorigenesis

조선대학교 대학원

약 학 과

김 가 람

Interleukin-22가 상피세포의 형질전환과 유방암 형성에 미치는 영향

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2014년 2월 25일

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List of Abbreviations

AP-1	Activator protein-1
BME	Basal medium Eagle
CAM	Chorioallantoic membrane
JNK	c-Jun N-terminal kinase
ERK	Extracellular signal-regulated kinase
IL-1	Interleukin-1
IL-22	Interleukin-22
IL-22R1	Interleukin-22 receptor 1
LPS	Lipopolysaccharide
MAP	Mitogen-activated protein
MKK	Mitogen-activated protein kinase kinase
MAP3K8	Mitogen-activated protein kinase kinase kinase 8
MEF	Mouse embryonic fibroblasts
PPIase	Peptidyl-prolyl cis/trans isomerase
Pin1	Peptidyl-prolyl cis/trans isomerase NIMA interacting 1
PBS	Phosphate-buffered saline
PVDF	Polyvinylidene difluoride
Ser/Thr-Pro	Serine or threonine residues that immediately precede proline residues

siRNA	Small interfering RNA
Th 17	T helper 17
TNFα	Tumor necrosis factor α

국문 초록

Interleukin-22가 상피세포의 형질전환과 유방암 형성에 미치는 영향

김가람

지도교수: 최홍석

조선대학교 대학원 약학과

인터루킨 22 (IL-22)는 T helper 17 (Th 17) 세포에서 분비되는 cytokine의 일종으로서, IL-22 수용체 1 (IL-22R1)과 IL-10R2를 포함하는 class II cytokine 수용체에 결합을 하며 다양한 면역 반응에 영향을 끼친다. 또한, IL-22가 extracellular signal-regulated kinase (ERK)나 c-Jun N-terminal kinase (JNK)와 같은 세포주기 및 증식 매개자들을 조절하는 것으로 보여지지만, 종양 형성에서의 IL-22의 근본적인 분자적 메커니즘에 관해서는 알려진 것이 거의 없다. 이 논문에서 우리는 유방에서의 암 형성과 내피세포의 증식을 조절하는 데 있어 IL-22가 중요한 역할을 수행하고 있다고 제안하려 한다. IL-22는 IL-22R1을 경유하여 MAP3K8의 인산화를 증가시켰으며 이는 MEK-ERK, JNK-c-JUN, STAT3 신호 경로의 유도를 일으켰다. 더욱이, IL-22-IL-

22R1 신호 경로는 activator protein-1 (AP-1)과 Her2의 프로모터 활성을 활성화시켰다. 더불어 Pin1이 IL-22에 의해 유도되는 MEK, c-Jun, STAT3의 인산화 의존적 활성에 관해 중요한 긍정적 조절자란 것을 확인할 수 있었다. Pin1-/-mouse embryonic fibroblasts (MEF) 세포는 $Pin1^{+/+}$ MEF 세포에 비해 IL-22에 의해 유도되는 MEK1/2, c-Jun, STAT3의 인산화의 감소를 상당히 나타내었다. 더불어, Pin1의 knockdown은 IL-22에 의해 유도되는 MEK1/2, c-Jun, STAT3의 인산화를 막았다. *In vivo* chorioallantoic membrane (CAM) assay 또한 IL-22가 JB6Cl41 세포의 종양 형성을 증가시키는 것을 보여주었다. 더욱이, MAP3K8과 Pin1의 knockdown은 MCF7 세포에서의 종양 형성을 경감시켰다. 이러한 관찰들과 일관성을 보이며, IL-22의 수준은 인간의 유방암에서 MAP3K8과 Pin1의 발현과 긍정적으로 연관성을 보이고 있다. 전반적으로, 우리의 발견은 IL-22에 의해 유도되는 MAP3K8 신호 경로가 종양 미세환경에서의 암과 연관된 면역반응을 촉진시키는 데 있어 중요한 역할을 한다는 것을 가리키고 있다.

I. Introduction

Interleukin-22 (IL-22) is produced by innate lymphoid cells, Th17 cells, and Th22 cells, particularly at mucosal surfaces (1-3). IL-22 signaling takes place through a distinct class 2 receptor (IL-22R) that is composed of the subunits IL-22R1 (IL-22RA1) and IL-10R2 (IL-10RB2), which are independently shared with IL-20 and IL-24 and with IL-10 and IL-26, respectively (4,5). First, IL-22 binds to IL-22R1 and then the IL-22–IL-22R1 complex binds to IL-10R2 to propagate downstream signals (6). IL-22R1 is absent in immune cells but is expressed within tissues, such as epithelial cells of pancreas, intestine, liver, lung, and skin (1). The important function of IL-22 in the promotion of antimicrobial immunity is induction of antimicrobial peptides and tissue repair by induction of epithelial cell proliferation and survival. However, IL-22 can also promote pathological inflammatory responses in the skin or intestine in mouse models, and its concentration is found to be increased in a variety of human diseases including psoriasis, rheumatoid arthritis, inflammatory bowel disease, and cancer (1,7). In line with the pleiotropic roles of IL-22, it is known that this cytokine uses the Jak-STAT signal transduction pathway, inducing phosphorylation of the kinases Jak1 and Tyk2 and the STAT1, STAT3, and STAT5 transcription factors (4,8), which is important for tumor development (9). However, the pathway regulated by IL-22 during tumor development and its functions needs to

be understood, because both tumor-inhibiting and tumor-promoting effects have been reported.

A serine/threonine protein kinase, mitogen-activated protein kinase kinase kinase 8 (MAP3K8), also known as COT, was originally identified as a target for proviral integration in MoMuLV-induced rat T cell lymphomas and MMTV-induced mammary carcinomas (10,11). MAP3K8 belongs to the MAP3K family of proteins (12). When overexpressed in cell lines, MAP3K8 activates the extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 MAPK pathways, all due to the ability of MAP3K8 to induce phosphorylation and activation of the respective MAP2K (12-14). The overexpression of MAP3K8 also induces transcription of the along with an ectopic expression of MAP3K8 significantly enhances IL-2 production (16). In addition, MAP3K8 plays an important role in IL-12 production and Th cell differentiation (17), giving rise to a possibility that MAP3K8 may be an interesting target for the development of drugs to treat chronic inflammatory diseases. However, it is largely unknown whether MAP3K8 is also involved in the signaling pathway of IL-22 during tumor development.

Pin1, peptidyl–prolyl *cis*/*trans* isomerase (PPIase), consists of two domains: a PPIase domain at its C-terminus responsible for isomerization and a WW domain at its N–terminus that acts 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, cyclin D1, c-Jun, MEK1, nuclear factor-κB, and STAT3, which results in its important roles in many cellular events, such as cell cycle progression and differentiation (21-26). Furthermore, Pin1 is overexpressed in breast cancer and together with Ras signaling results in increased tumorigenesis (23). Although many details of the Pin1 function have been elucidated, little is known regarding whether Pin1 influences IL-22 signaling pathway during tumorigenesis. Therefore, uncovering the mechanisms responsible for the prolonged phosphorylation of IL-22-signaling-related proteins becomes an important goal, given that constitutive signaling from Pin1 results in tumorigenesis.

In this paper, we have determined for the first time that MAP3K8 regulates the IL-22 signaling pathway. In this signaling pathway, IL-22 induced MAP3K8 phosphorylation, which increased the activity of ERK1/2, JNK1/2, and STAT3, resulting in the transcriptional activation of *c-fos* and *c-jun*. Furthermore, these IL-22-induced signaling pathways were enhanced by prolyl isomerase Pin1, whereas Pin1 knockdown attenuated IL-22-induced AP-1 activity and neoplastic cellular transformation. These results suggest that both MAP3K8 and Pin1 play an important role in promoting IL-22-induced tumorigenesis.

II. Materials & Methods

1. Reagents and antibodies

Recombinant human interleukin-22 (IL-22) was obtained from R&D Systems (Minneapolis, MN). MAP3K8 inhibitor [TKI (Tpl2 kinase inhibitor); 4-(3-chlor-4-fluorophenylamino)-6-(pyridin-3-yl-methylamino)-3-cyano-1, 7-naphthylridine], PD9 8059, and SP600125 were purchased from Calbiochem-Novabiochem (San Diego, CA). Juglone (4-hydroxytamoxifen) and Basal medium Eagle were procured from Sigma-Aldrich (St. Louis, MO). Cell Proliferation ELISA and BrdU (colorimetric) were from Roche Applied Science (Indianapolis, IN). The Dual-Luciferase Reporter Assay Kit was purchased from Promega (Madison, WI). The phospho-specific and total antibodies against -MEK1/2, -ERK1/2, -JNK1/2, and -STAT3 (Tyr 705) and phospho-specific antibodies against MAP3K8 and -c-Jun (Ser 63) were acquired from Cell Signaling Technology, Inc. (Beverly, MA); antibody against IL-22R1 was from Millipore (Bedford, MA); and antibodies against Pin1, MAP3K8, and c-Jun was from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-Xpress antibody was obtained from Invitrogen. Anti-IL22 antibody was from Abcam (Cambridge, MA).

2. Cell culture and small interference RNA (siRNA)

JB6 Cl41 mouse epidermal cells were maintained in Eagle's minimal essential medium (MEM) supplemented with 5% fetal bovine serum (FBS). MCF7 human breast cancer cells 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 Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS. All these cell lines were cultured and maintained at 37ºC in humidified air containing 5% CO_2. Mouse IL-22RA1 (accession number: NM_178257), Pin1 (accession number: NM_006221), and MAP3K8 (accession number: NM_005204) were silenced by transfecting the ON-TARGET plus small interfering RNA (siRNA) SMART pool-specific or nonspecific control pool double-stranded RNA oligonucleotides (Dharmacon, Chicago, IL) using LipofectamineTM 2000 (Invitrogen).

3. Immunoblot analysis

The cells were disrupted in RIPA lysis buffer. The proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto PVDF. The membranes were blocked and hybridized with the appropriate primary antibody overnight at 4°C. After hybridization with HRP-conjugated secondary antibody from rabbits or mice, the protein bands were visualized using a chemiluminescence detection kit (Amersham HRP Chemiluminescent Substrates, Amersham Biosciences, Piscataway, NJ). For detecting chemiluminescence, LAS4000 (GE Healthcare Biosciences, Pittsburgh, PA) was used.

4. Cell proliferation assay (BrdU incorporation)

Cells were seeded (5×10^4 cells/well) in 96-well plates in 100 µl of 5% FBS-MEM.

After 24 h, they were treated with or without IL-22 for 48 h, labeled with 10 μl/well BrdU labeling solution, and then incubated for 4 h at 37 °C in a 5% CO_2 atmosphere. After the media was removed, FixDenat solution was added to each well and the contents were incubated at room temperature (RT) for 30 min. FixDenat solution was removed after 30 min, anti-BrdU-POD working solution was added to each well, and the contents were incubated for 90 min at RT. The cells were then washed thrice with a washing solution and 100 μl of substrate solution was added to each well and incubated for 30 min. Cell proliferation was estimated by measuring the absorbance at 370 nm.

5. Anchorage-independent cellular transformation assay (soft agar assay)

The effect of IL-22 on cell transformation was investigated in JB6 Cl41 cells and MCF7 cells. Briefly, 8×10^3 cells were exposed to different doses of IL-22 in 1 ml of 0.3% basal medium Eagle (BME) agar containing 10% FBS, 2 mM L-glutamine, and 25 μg/ml gentamicin. The cultures were maintained at 37°C in a 5% CO_2 incubator for 14-18 days, and cell colonies were scored using an Axiovert 200 M fluorescence microscope and Axio Vision software (Carl Zeiss, Inc., Thornwood, NY).

6. Reporter gene assays

For detecting firefly luciferase activity, the reporter gene assay was performed using lysates from AP-1-, c-jun-, c-fos-, or Her2-luc-transfected JB6 Cl41 cells. The reporter gene vector pRL-TK-luciferase plasmid (Promega) was co-transfected into each cell line and the renilla luciferase activity generated by this vector was used to

normalize the results for transfection efficiency. Cell lysates were prepared by washing the transfected JB6 Cl41 cells once with phosphate-buffered saline (PBS) at RT. Then PBS was removed from the lysate and passive lysis buffer (Promega) was added and the cells were incubated at RT for 1 h with gentle shaking. The supernatant fraction was used for the measurement of firefly and renilla luciferase activities. Cell lysates were mixed with luciferase assay II reagent, and firefly luciferase light emission was measured by GloMax®-Multi Detection System (Promega). Subsequently, renilla luciferase substrate was added to normalize the firefly luciferase data. The c-fos-luc promoter (pFos-WT GL3) and c-jun-luc promoter (JC6GL3) constructs were kindly provided by Dr. Ron Prywes (Columbia University, New York, NY). The AP-1 luciferase reporter plasmid (-73/+63 collagenase-luciferase) was kindly provided by Dr. Dong Zigang (Hormel Institute, University of Minnesota, Austin, MN).

7. Chorioallantoic membrane (CAM) assay

Briefly, fertilized chicken eggs were transferred to an egg incubator and allowed to grow for 10 days. After this, The IL-22-treated JB6 Cl41 cells (1×10^7) were placed on the exposed CAM, and the eggs were incubated in a humidified incubator at 37°C for 5–6 days. After 5–6 days, images were digitally recorded at 15× magnification with an SZ-61 zoom stereomicroscope (Olympus, Center Valley, PA). Tumor areas were analyzed with ImageJ digital imaging software (download from the NIH website).

8. Tumor samples

The breast tissues that were selected for immunohistochemical staining were collected from a breast cancer group of 20 patients (age range: 42–72). The normal breast group included mammary infiltrating duct carcinoma patients who had undergone mastectomy with adjuvant hormone therapy and had no subsequent local recurrence or metastasis within 5 years, and the breast cancer group included mammary infiltrating duct carcinoma patients who had undergone mastectomy with adjuvant hormone therapy and lapsed into a subsequent bone metastasis. Informed consent was obtained from all the patients, and research protocols were approved by the ethics committee of Chosun University Hospital (South Korea).

9. Immunohistochemical staining

All tumors investigated in the study were tested for IL-22, Pin1, and MAP3K8. Immunolocalization for each protein was performed using a Polink-2 HRP plus anti-rabbit DAB detection kit (Golden Bridge International, Inc., Mukilteo, WA) according to the manufacturer's protocol. Slides were incubated for 1 h with anti-Pin1 and -MAP3K8 antibodies (Santa Cruz Biotechnology) and overnight with anti-IL-22 antibody (Santa Cruz Biotechnology). Instead of the primary antibody, normal goat serum was used in the negative control. Distinct nuclear staining was considered as positive immune reactivity.

10. Statistical analysis

Fisher's exact test with two-sided probability values ($P < 0.001$) was used to analyze the correlation between IL-22, MAP3K8, and Pin1 in breast cancer patients. Data from reporter gene assay and soft agar assay were analyzed statistically using unpaired *t*-tests, and P values < 0.05 were considered significant. Statistical calculations were carried out with Prism Macintosh 4.0 (GraphPad Software, Inc., La Jolla, CA). Results are expressed as the mean ± standard error of triplicate measurements of three independent experiments.

III. Results

1. IL-22 promotes anchorage-independent transformation and tumorigenesis of JB6 Cl41 cells.

To examine the effects of IL-22 on cell proliferation and anchorage-independent cell transformation, BrdU incorporation assay and soft agar assay were performed. We found that IL-22 treatment significantly and dose-dependently induced the cell proliferation of JB6 Cl41 cells (Figure 1A). Furthermore, IL-22 treatment dose-dependently increased not only colony numbers but also colony sizes in JB6 Cl41 cells (Figure 1B-D). To determine whether IL-22 affects tumorigenesis in vivo, CAM assay with JB6 Cl41 cells was performed. Representative images of CAM are shown (Figure 1E). For statistical evaluation, tumor section images were digitally recorded and tumor areas were analyzed (Figure 1F). The results showed that IL-22 significantly induced an increase in the tumor area compared to untreated control groups. Collectively, these results suggest that IL-22 induces the neoplastic cell transformation and tumorigenesis of JB6 Cl41 cells *in vitro* and *in vivo*.

Figure 1

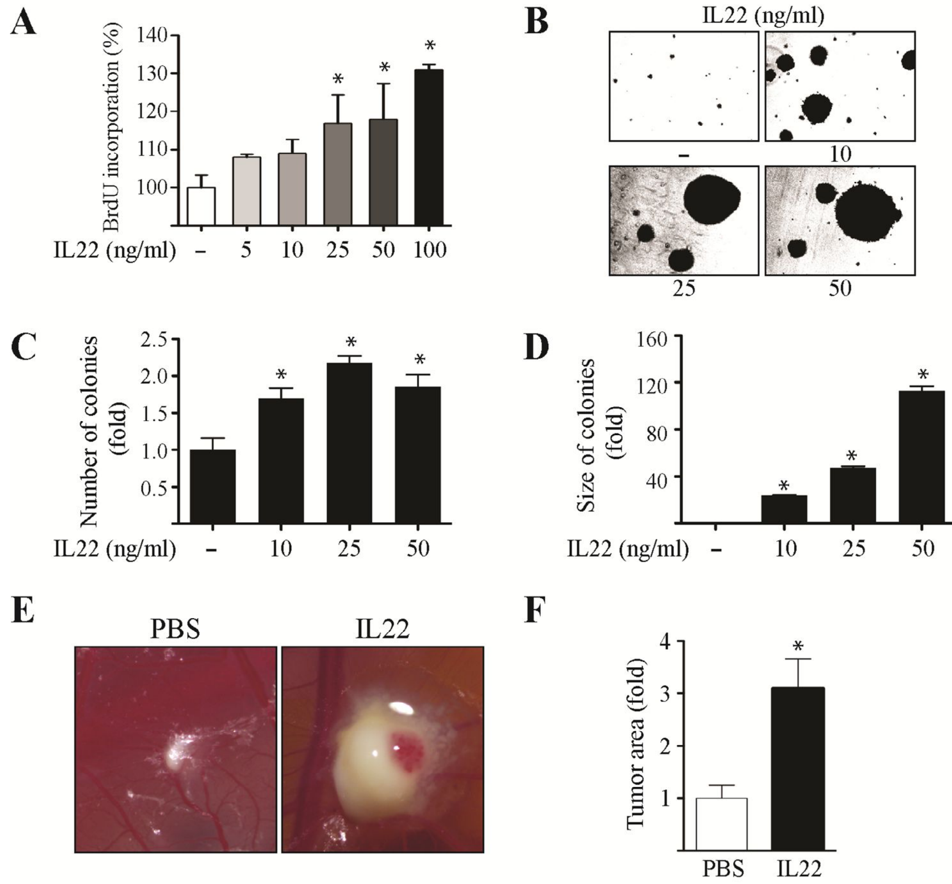

Figure 1. IL-22 promotes neoplastic cellular transformation of JB6 Cl41 cells *in vitro* and *in vivo.*

(**A**) Cell proliferation was estimated by BrdU incorporation assay. Cells were seeded and treated with different concentrations of IL-22 for 48 h. *Columns*, mean of triplicate measurement of two experiment; *bars*, standard deviation. $*P<0.05$, compared with control cells.

(**B-D**) Cells were exposed to various concentrations of IL-22 in soft agar matrix, and incubated at 37°C in a 5% CO_2 atmosphere for 17 days. The colonies from three separate experiments are photographed (B). Then, the average colony number was calculated (C), and colony size was measured (D) under a microscope. Column, mean of triplicate samples; bars, standard deviation. $P<0.05$, compared with control cells.

(**E and F**) Fertilized chicken eggs were grown for 10 days, then cells were placed on the CAM. The implanted eggs were incubated with/without treatment of IL-22 (100 ng/ml) for 6 days. The representative pictures of the CAM (E) and measured tumor area (F) are shown. Error bars indicate the means ± standard deviation of six samples per group from two independent experiments. $*P<0.05$, compared with control groups.

2. IL-22 activates MEKs-ERKs and JNKs-cJun signaling via MAP3K8

The MAPK signaling pathway not only promotes cell proliferation but also mediates cell survival and is upregulated in various cancer cells (27). To determine whether IL-22 regulates MAPK signaling pathways through MAP3K8 activation, JB6 Cl41 cells were treated with IL-22 and immunoblotting was conducted with specific antibody for phospho-MAP3K8 (Ser400). The results showed that IL-22 markedly induced the phosphorylation of MAP3K8 in a dose- and time-dependent manner (Figure 2A and B). To further examine whether MAP3K8 activation is mediated by IL-22R1, JB6 Cl41 cells were transfected with siRNA-IL-22R1, and then treated with IL-22. The results showed that the knockdown of IL-22R1 suppressed the phosphorylation of MAP3K8 induced by IL-22 compared with control cells (Figure 2C). Since MAP3K8 is an upstream kinase of MEK1/2 and JNK1/2 (12,28), we examined the effects of IL-22 on the MEKs-ERKs and JNKs-c-Jun signaling pathway. The results showed that IL-22 induced phosphorylation of MEK1/2 and ERK1/2 both in terms of dose and time dependence (Figure 2D and E) as well as that of JNK1/2 and c-Jun (Figure 2F and G). Furthermore, treatment with TKI, a MAP3K8 kinase inhibitor, inhibited the phosphorylation of MEK1/2, ERK1/2, JNK1/2, and c-Jun induced by IL-22 (Figure 2H). Similarly, PD98059 (a specific MEK1/2 inhibitor) and SP600125 (a specific JNK inhibitor) also inhibited the phosphorylation of ERK1/2 and c-Jun induced by IL-22 (Figure 2I), thus suggesting that MAP3K8 regulates the signaling pathways of

MEKs-ERKs and JNKs-c-Jun induced by IL-22 through the IL-22 receptor in JB6 Cl41 cells.

Figure 2

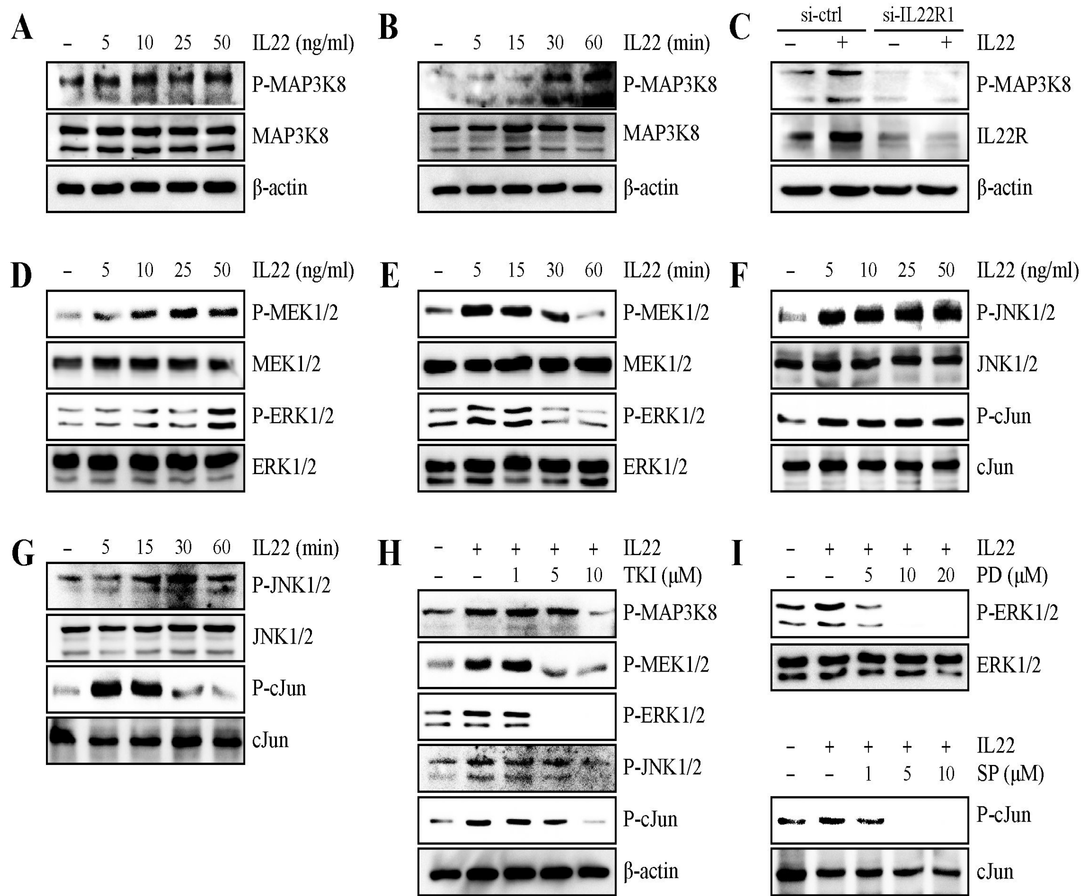

Figure 2. MAP3K8 mediates MEKs-ERKs and JNKs-cJun signaling induced by IL-22 in JB6 Cl41 cells

(**A and B**) Cells were serum-starved for 24 h, treated with the indicated doses of IL-22 for 15 min (A) or 25 ng/ml IL-22 for the indicated times (B), harvested and lysed. Proteins in whole cell lysates were separated by SDS-PAGE and immunoblotted.

(**C**) Cells were transfected with siRNA-IL-22R1. After 48 h, cells were serum-starved for 24 h, treated with 25 ng/ml IL-22 for 15 min, harvested, and lysed. Proteins in whole cell lysates were separated by SDS-PAGE and immunoblotted.

(**D-G**) Cells were serum-starved for 24 h, treated with the indicated doses of IL-22 for 15 min (D and F) or 25 ng/ml IL-22 for the indicated times (E and G), harvested and lysed. The lysates were resolved by SDS-PAGE and immunoblotting analysis was performed using specific antibodies against the corresponding proteins.

(**H**) Cells were serum-starved for 24 h, pretreated with the indicated concentrations of TKI for 2 h, exposed to 25 ng/ml IL-22 for 15 min, harvested and lysed. The lysates were resolved by SDS-PAGE and immunoblotting analysis was performed using specific antibodies against the corresponding proteins. (**I**) Cells were serum-starved for 24 h, treated with either PD98059 (*upper panel*) or SP600125 (*lower panel*). At 1 h after pretreatment, cells were exposed to 25 ng/ml IL-22 for 15 min, harvested and lysed. The lysates were resolved by SDS-PAGE and immunoblotting analysis was performed using specific antibodies against the corresponding proteins.

3. IL-22 activates STAT3 through activation of MAP3K8

Accumulated evidences suggest that IL-22 promotes tumor growth and metastasis by STAT3 activation (7,29). To determine whether STAT3 activation induced by IL-22 is mediated by MAP3K8, we first examined the IL-22-induced phosphorylation of STAT3 in JB6 Cl41 cells. The results showed that IL-22 dose- and time-dependently increased the phosphorylation of STAT3 in JB6 Cl41 cells (Figure 3A and B). In addition, the IL-22-induced phosphorylation of STAT3 was suppressed in siRNA-IL-22R1-transfected cells compared with siRNA-control-transfected cells (Figure 3C). To examine the effects of knockdown of MAP3K8 on the STAT3 activation, JB6 Cl41 cells were transfected with either siRNA-control or siRNA-MAP3K8, and then exposed to IL-22. The results showed that the knockdown of MAP3K8 inhibited the IL-22-induced phosphorylation of STAT3 (Figure 3D). Furthermore, TKI inhibited the phosphorylation of STAT3 induced by IL-22 in a dose-dependent manner (Figure 3E), indicating that MAP3K8 positively regulates STAT3 signaling pathway induced by IL-22.

Figure 3

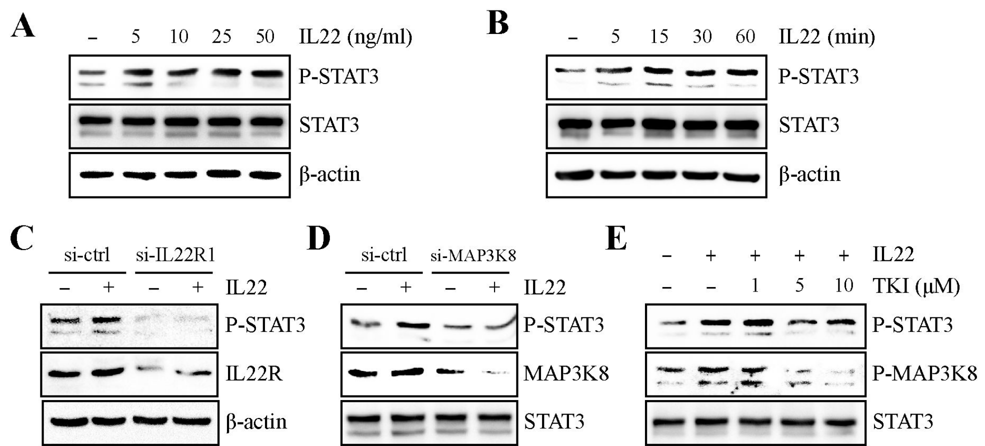

Figure 3. MAP3K8 mediates STAT3 signaling induced by IL-22 in JB6 Cl41 cells

(**A and B**) Cells were serum-starved for 24 h, treated with the indicated doses of IL-22 for 15 min (A) or 25 ng/ml IL-22 for the indicated times (B), harvested and lysed. The lysates were resolved by SDS-PAGE, and phosphorylation of STAT3 was evaluated by immunoblotting analysis. After stripping, this same blot was stained using an antibody for total STAT3.

(**C and D**) Cells were cultured for 24 h, then transfected with either siRNA-IL-22R1 (C) or siRNA-MAP3K8 (D). After 48 h, cells were serum-starved for 24 h, treated or not treated 25 ng/ml IL-22 for 15 min, harvested, and lysed. The lysates were resolved by SDS-PAGE and immunoblotted using specific antibodies against the corresponding proteins.

(**E**) Cells were serum-starved for 24 h, pretreated with the indicated concentrations of TKI for 2 h and then exposed to 25 ng/ml IL-22 for 15 min, harvested and lysed. The lysates were resolved by SDS-PAGE and immunoblotted using specific antibodies against the corresponding proteins.

4. Pin1 required for the phosphorylation-dependent MEKs, c-Jun, and STAT3 activity induced by IL-22

Given that peptidyl–prolyl *cis*/*trans* isomerase 1 (Pin1), which specifically recognizes the pSer/Thr-Pro motifs on its target proteins, interacts with MEK1 (30), c-Jun (23) and STAT3 (26), we next examined whether IL-22 signaling is regulated by Pin1. The $Pin1^{-/-}$ MEF cells were transfected with Xpress-Pin1 and exposed to IL-22. The results showed that the levels of MEK1/2, cJun, and STAT3 phosphorylation were considerably increased by Pin1 overexpression in the $Pin1^{-/-}$ MEF cells (Figure 4A). In addition, Pin1 overexpression was found to markedly enhance IL-22-induced phosphorylation of MEK1/2, cJun, and STAT3 (Figure 4A). To further confirm the regulatory role of Pin1 on the phosphorylation of MEK1/2, cJun, and STAT3, mock- or Pin1-overexpressing JB6 Cl41 cells were exposed to IL-22 and immunoblotting was performed with the respective antibodies. There was an increase in the level of MEK1/2, cJun, and STAT3 phosphorylation in Pin1-overexpressing cells compared to control cells (Figure 4B). To determine whether knockdown of Pin1 suppresses the IL22-induced phosphorylation of MEK1/2, cJun, and STAT3, JB6 Cl41 cells were transfected with siRNA-Pin1 and treated or not treated with IL-22. The IL-22-induced phosphorylation of MEK1/2, cJun, and STAT3 was attenuated in Pin1-knockdown cells (Figure 4C). Also, on treatment with juglone, a Pin1 inhibitor, the IL22-induced phosphorylation of MEK1/2, cJun, and STAT3 in JB6 Cl41 cells was found to be

inhibited (Figure 4D). Overall, these results indicate that Pin1 enhances the IL22-induced phosphorylation of MEK1/2, cJun, and STAT3.

Figure 4

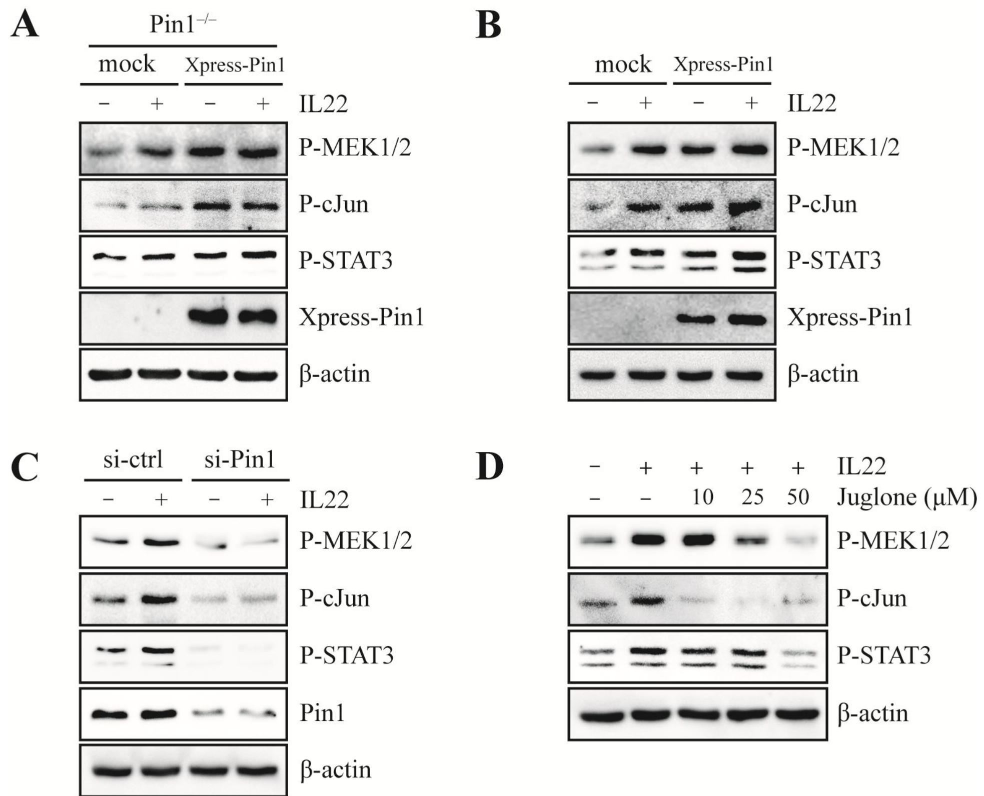

Figure 4. Pin1 enhances the IL-22-induced phosphorylation of MEKs, cJun, and STAT3

(**A**) Pin1$^{-/-}$ MEF cells were transfected with Xpress-Pin1. At 24 h after transfection, cells were serum-starved for 24 h, treated with 25 ng/ml IL-22 for 15 min, harvested, and lysed. The lysates were resolved by SDS-PAGE and immunoblotting analysis was performed using specific antibodies against the corresponding proteins.

(**B and C**) JB6 Cl41 cells were transfected with either Xpress-Pin1 (B) or siRNA-Pin1 (C), respectively. At 48 h after transfection, cells were serum-starved for 24 h, treated or not treated 25 ng/ml IL-22 for 15 min, harvested, and lysed. The lysates were resolved by SDS-PAGE and immunoblotting analysis was performed using specific antibodies against the corresponding proteins.

(**D**) JB6 Cl41 cells were serum-starved for 24 h, pretreated with the indicated concentrations of juglone for 2 h and then exposed to 25 ng/ml IL-22 for 15 min, harvested and lysed. The lysates were resolved by SDS-PAGE and immunoblotting analysis was performed using specific antibodies against the corresponding proteins.

5. MAP3K8 and Pin1 are required for IL-22-induced AP-1 and cell transformation in JB6 Cl41 cells

The AP-1 transcription factor is a dimeric complex of homo- or heterodimers of Jun, Fos, activating transcription factor, and musculoaponeurotic fibrosarcoma protein family members that are activated by MAPK signaling pathway (31,32). To determine the effects of IL-22 on the AP-1 activity, we examined the effects of IL-22 on the *c-fos* and *c-jun* promoter. The transcriptional activity of *c-fos* and *c-jun* was significantly increased by treatment with IL-22 in JB6 Cl41 in a dose-dependent manner (Figure 5A and B). In addition, treatment with IL-22 was found to significantly induce AP-1 transactivation in JB6 Cl41 (Figure 5C). Since a *cis*-acting element of HER2 promoter is regulated by STAT3 signaling (33), we next examined the effect of IL-22 on HER2 promoter activity in JB6 Cl41 cells. The results showed that IL-22 treatment significantly enhanced HER2 promoter activity (Figure 5D). To further confirm that MAP3K8 and Pin1 cooperatively regulated AP-1 and HER2 promoter activated by IL-22, we assessed IL-22-induced AP-1 and HER2 activity in the presence or absence of TKI and/or juglone in JB6 Cl41 cells. As expected, the TKI and juglone treatment inhibited the IL-22-induced AP-1 (Figure 5E) and HER2 (Figure 5F) activity in JB6 Cl41 cells. Taken together, these results strongly support the idea that IL-22-induced AP-1 and HER2 promoter activity is mediated by MAP3K8 signaling and enhanced by Pin1.

To investigate whether MAP3K8 and Pin1 are essential for IL-22-induced epithelial cell transformation, we first examined the effect of TKI and juglone on IL-22-induced transformation of JB6 Cl41 cells. The results showed that the TKI and juglone treatment significantly inhibited the IL-22-induced cell transformation (Figure 5G). Furthermore, TKI and juglone decreased not only colony numbers (Figure 5H) but also colony sizes (Figure 5I). To further determine the effects of TKI and juglone on the IL-22-induced tumorigenesis *in vivo*, a CAM assay was performed with JB6 Cl41 cells. Representative images of CAM (Figure 5J) are shown. For statistical evaluation, tumor section images were digitally recorded and tumor areas were analyzed (Figure 5K). The results indicated that both TKI and juglone significantly suppressed IL-22-induced tumorigenesis compared to untreated control groups in CAM (Figure 5J and K). Overall, these results suggest that MAP3K8 and Pin1 are essential for IL-22-induced epithelial transformation and tumorigenesis of JB6 Cl41 cells *in vitro* and *in vivo*.

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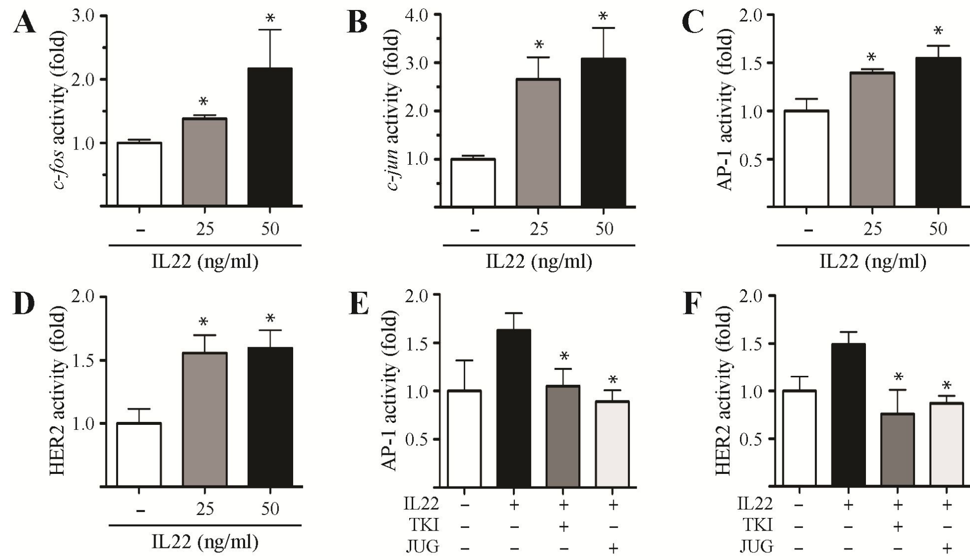
Figure 5



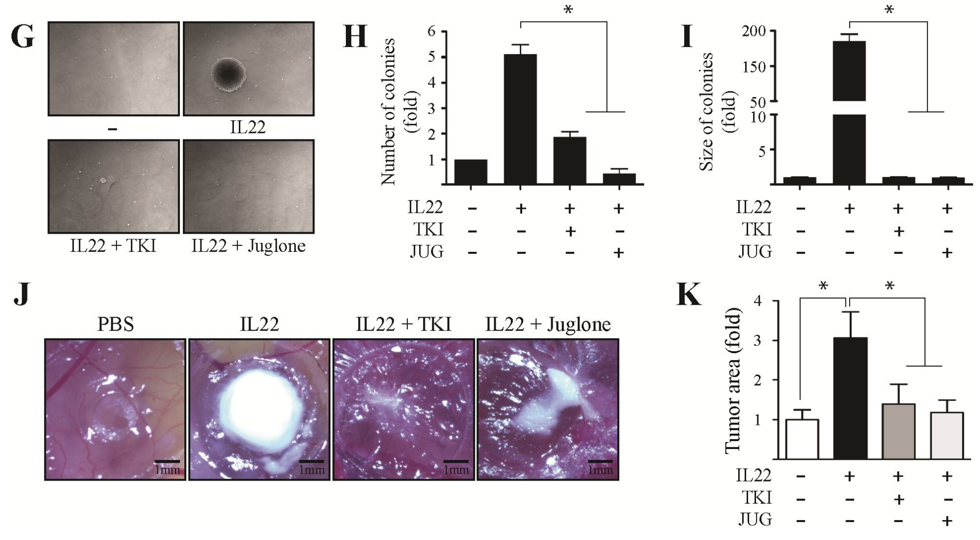

G
–
IL22
IL22 + TKI
IL22 + Juglone
H
Number of colonies (fold)
IL22
TKI
JUG
I
Size of colonies (fold)
IL22
TKI
JUG
J
PBS
IL22
IL22 + TKI
IL22 + Juglone
1mm
K
Tumor area (fold)
IL22
TKI
JUG

Figure 5. Effects of TKI and juglone in IL-22-induced AP-1 and cell transformation in JB6 Cl41 cells

(**A-D**) Cells were seeded and co-transfected with the luciferase reporters, *c-fos*-luc (A), *c-jun*-luc (B), AP-1-luc (D), or HER2-luc (D), and the pRL-TK vector, respectively. At 30 h after transfection, cells were serum-starved for 24 h, and then treated with the indicated doses of IL-22 for 30 h before a luciferase assay was performed.

(**E and F**) Cells were seeded and co-transfected with the luciferase reporter, AP-1-luc (E) or HER2-luc (F), and the pRL-TK vector, respectively. After 30 h, cells were serum-starved for 24 h and then treated with the indicated doses of IL-22 in the presence or absence of 10 μM TKI or 25 μM juglone as indicated for 30 h before a luciferase assay was performed. In all of the promoter assays, the firefly luciferase activity was determined in cell lysates and normalized against renilla luciferase activity, and these luciferase activities are expressed relative to control cells. Columns, mean of triplicate measurements of two experiments; bars, standard deviation. *, $P < 0.05$, compared with control cells.

(**G-I**) Cells were treated with 50 ng/ml IL-22 with/without treatment of 10 μM TKI or 25 μM juglone as indicated in soft agar matrix, and incubated at 37°C in a 5% CO_2 atmosphere for 17 days. The colonies from three separate experiments are photographed (G). Then the average colony number was calculated (H), and colony

size was measured (I) under a microscope. Column, mean of triplicate samples; bars, standard deviation. *, *P*<0.05, compared with control cells.

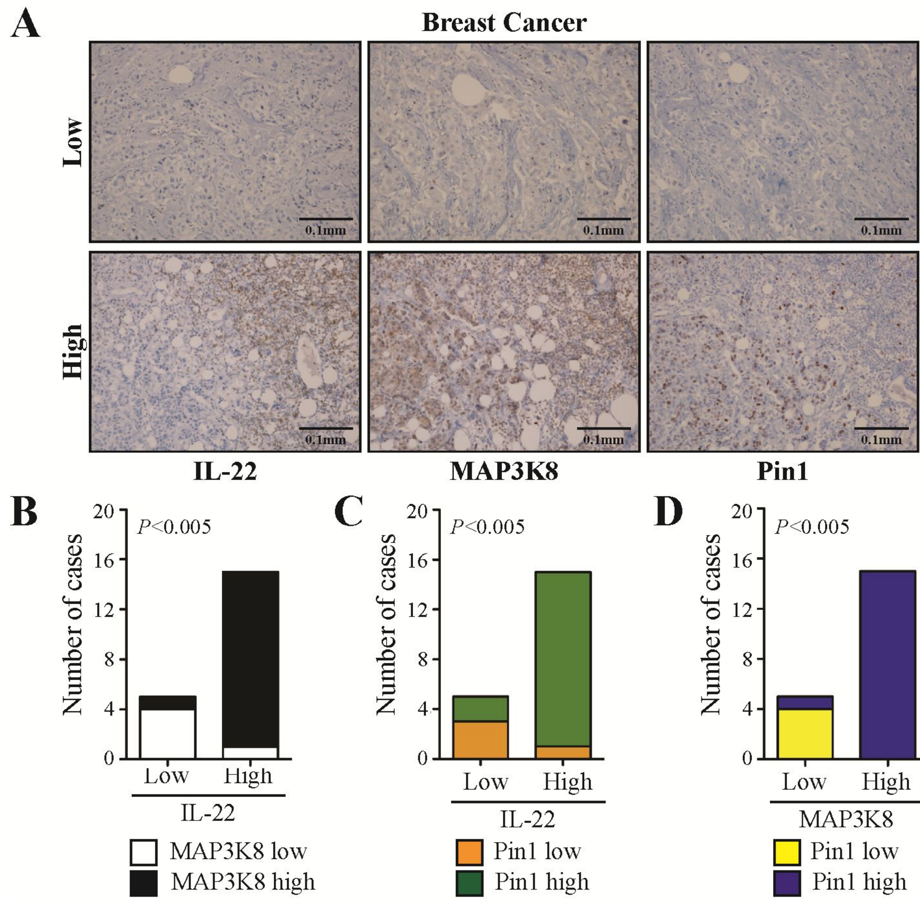
(**J and K**) Fertilized chicken eggs were grown for 10 days and then cells were placed on the CAM. The implanted eggs were incubated with/without treatment of 100 ng/ml IL-22 in the presence or absence of 10 μM TKI or 25 μM juglone for 4 days. The representative pictures of the CAM (J) and measured tumor area (K) are shown. Error bars indicate the means ± standard deviation of six samples per group from two independent experiments. *, *P*<0.05, compared with control groups.

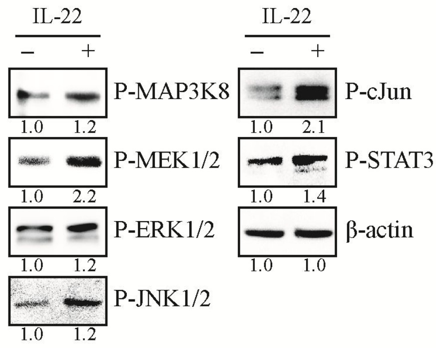
6. IL-22 levels positively correlate with MAP3K8 and Pin1 expression in human breast cancer

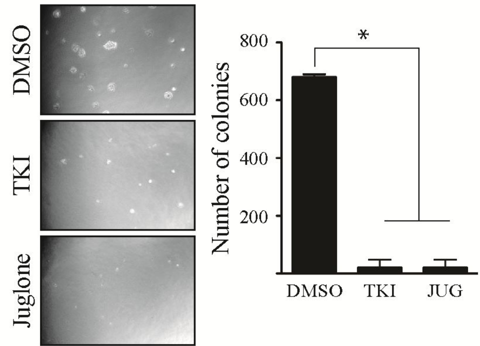
To further understand the pathological relevance of IL-22 in breast tumorigenesis, we next examined the expression of IL-22 in human breast tumors. Knowing that MAP3K8 mediated the IL-22-induced MAPK signaling and Pin1 enhanced such signaling, immunohistochemistry was performed on human breast cancer tissues using IL-22, MAP3K8, and Pin1 antibodies. Four of the 5 breast cancer samples that had a low amount of IL-22 presented with a lower expression MAP3K8 and Pin1, whereas 14 of the 15 breast cancer samples that contained a high amount of IL-22 correspondingly had a higher expression of both MAP3K8 and Pin1 (Figure 6A-C). Four of the 5 breast cancer samples that contained a low amount of MAP3K8 also had a lower expression of Pin1, whereas all 15 breast cancer samples that contained a higher amount of MAP3K8 had a correspondingly high expression of Pin1, indicating that most tumors containing high levels of MAP3K8 also had markedly increased levels of Pin1 (Figure 6A and D). Therefore, we examined the effect of IL-22 on the signaling pathway of MAP3K8, MEKs, JNKs, and STAT3 in MCF7 cells. The results showed that IL-22 markedly induced the phosphorylation of MAP3K8, resulting in activation of MEKs-ERK, JNKs-cJun, and STAT3 signaling pathways (Figure 6E). Thereafter, we studied the correlation between MAP3K8 as well as Pin1 activity and IL-22 on tumorigenicity of MCF7 cells using soft agar assay. The results showed that

treatment of TKI and juglone significantly inhibited the colony formation of MCF7 cells (Figure 6F). Consistent with these results, depletion of MAP3K8 and Pin1 in MCF7 cells profoundly reduced the tumor growth induced by IL-22 compared with untreated control group (Figure 6G).

Figure 6




E
IL-22
–
+
P-MAP3K8
1.0
1.2
P-MEK1/2
1.0
2.2
P-ERK1/2
1.0
1.2
P-JNK1/2
1.0
1.2
IL-22
–
+
P-cJun
1.0
2.1
P-STAT3
1.0
1.4
β-actin
1.0
1.0


F
DMSO
TKI
Juglone
Number of colonies
800
600
400
200
*
DMSO
TKI
JUG

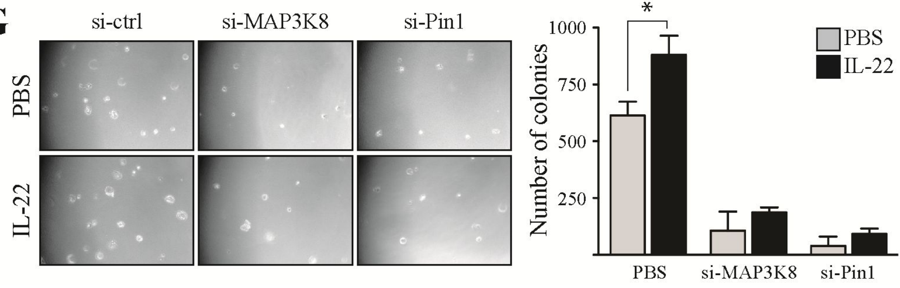

G
si-ctrl
si-MAP3K8
si-Pin1
PBS
IL-22
Number of colonies
1000
750
500
250
*
PBS
IL-22
PBS
si-MAP3K8
si-Pin1

Figure 6. Involvement of MAP3K8 or Pin1 on breast tumorigenesis induced by IL-22

(**A-D**) Representative samples showing results of immunohistochemical analysis of breast infiltrating duct carcinoma were performed with the indicated antibodies on adjacent sections of the samples. In each sample, IL-22, MAP3K8, and Pin1 levels were semiquantified in a double-blind manner as high or low according to the standards presented and statistically analyzed in A. Their correlation was analyzed by Fisher's exact test ($P < 0.05$).

(**E**) MCF7 cells were serum-starved for 24 h, treated with or without 25 ng/ml IL-22 for 15 min, harvested, and lysed. The lysates were resolved by SDS-PAGE and immunoblotting analysis was performed using specific antibodies against the corresponding proteins.

(**F**) MCF7 cells were treated with either 10 μM TKI or 25 μM juglone as indicated in soft agar matrix, and incubated at 37°C in a 5% CO_2 atmosphere for 14 days. The colonies from three separate experiments are photographed (left) and the average colony number was calculated (right). Column, mean of triplicate samples; bars, standard deviation. *, $P<0.05$, compared with control cells.

(**G**) MCF7 cells were transfected with either siRNA-MAP3K8 or siRNA-Pin1. At 48 h after transfection, cells were treated with/without 50 ng/ml IL-22 in soft agar matrix and incubated at 37°C in a 5% CO_2 atmosphere for 17 days. The colonies from three

separate experiments are photographed (left) and the average colony number was calculated (right). Column, mean of triplicate samples; bars, standard deviation. *, $P<0.05$, compared with control cells.

IV. Discussion

Tumor microenvironments that lead to a growth stimulation effect in malignancy include a large population of tumor cells, malignant transforming cells, immune cells, and macrophages (34). Given that there is an increasing amount of evidence for the tumor microenvironment, it has recently been proposed as a target for therapy (35,36). But the application of chemoprevention to control the tumor microenvironment during the early stages of carcinogenesis has not received concerted attention so far. In our study, we provide the first direct evidence that IL-22 leads to the activation of MAP3K8, which results in the induction of epithelial transformation through its downstream AP-1 signaling pathways. We also demonstrate that Pin1 enhances the phosphorylation of MEK1/2, cJun, and STAT3 induced by IL-22. Taken together with our increasing understanding of IL-22–IL-22R1 signaling pathway, this work highlights how MAP3K8 as well as Pin1 regulate IL-22-induced tumorigenicity.

Upon growth factor stimulation, the pathway from Ras through Raf and mitogen-activated protein (MAP) kinase kinase (MKK, also called MEK) to ERK/MAPK regulates many fundamental cellular processes that govern the cell transformation, differentiation, proliferation, and survival (37,38). Moreover, the active Ras/Raf/MEK/ERK pathway invariably presents the hallmark of malignant phenotypes: abnormal cell growth, invasion, and angiogenesis (39). Interestingly,

lipopolysaccharide (LPS), a component of Gram-negative bacteria, and the proinflammatory cytokines tumor necrosis factor α (TNFα) and interleukin-1 (IL-1) do not require Raf but distinct MAP3K8 to promote tumor development and growth (40-43). Moreover, the activation of MAP3K8 is required for the LPS-induced production of TNFα or IL-1β in T cell lines, which is achieved by activating the transcription factors NFAT and NF-κB (43,44), suggesting that MAP3K8 may be an interesting target for the development of drugs to treat chronic inflammatory diseases and cancer. It was reported that MAP3K8 is a potent serine threonine kinase, which, when overexpressed, activates the MAPK and SAPK pathway (12) and is overexpressed in human breast cancer (45). MAP3K8 also induces cell transformation through upregulation of *c-fos* transcriptional activity (46). Despite the vital role of MAP3K8 in tumor development, the precise mechanisms that regulate IL-22 signaling pathway in carcinogenesis have not been well explored yet. In our study, we found that IL-22 strongly induced phosphorylation of MAP3K8 at Ser400, followed by the activation of MEKs-ERKs and JNKs-c-Jun signaling pathways. In addition, the knockdown of IL-22R1 lowered the MAP3K8 phosphorylation induced by IL-22, indicating that MAP3K8 activation depends on the IL-22 signaling pathway through the IL-22 receptor. Moreover, TKI, PD98059, and SP600125, which are MAP3K8, MEK and JNK inhibitors, respectively, strongly suppressed the signaling pathways of MEK-ERK and JNK-c-Jun induced by IL-22. Overall, these data indicated that

MAP3K8 functions as an important mediator of IL-22–IL-22R1 signaling pathway. A previous study had demonstrated that IL-22 increases the expression of many anti-apoptotic and mitogenic proteins following the activation of STAT3 (29). This observation is in agreement with a recent report showing that IL-22 can be found in the microenvironment of hepatocellular carcinoma, and it results in tumor growth, inhibition of apoptosis, and promotion of metastasis due to STAT3 activation (7). In the current study, we found that IL-22-induced STAT3 signaling pathway through the phosphorylation of MAP3K8 resulted in the induction of HER2 promoter activity. These results suggested that MAP3K8 might play a pivotal role in IL-22-induced STAT3 activation for tumor growth.

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 (47). By binding to and isomerizing peptidyl–prolyl bond in specific phosphorylated Ser/Thr-Pro motifs, prolyl isomerase Pin1 induces conformational changes in its target proteins (20). These conformational changes can have profound effects on the Pin1 substrate functioning, resulting in modulation of their activity, change in phosphorylation status, modification of protein–protein interaction, altered subcellular localization, and decreased stability (48). It was reported that Pin1 regulates MAPK and STAT3 signaling pathway in breast tumor development through its interaction with MEK1

(24), c-Jun (23), and STAT3 (26). These reports supported our hypothesis that high levels of Pin1 may regulate IL-22-induced signaling pathway, leading to tumor progression in breast cancer. Therefore, we examined the molecular events that are mediated by Pin1 after mitogen activation by IL-22 in MCF-7 cells. The data provide the first evidence that Pin1 regulates the IL-22-induced signaling pathway in such a way as to increase the phosphorylation of MEK1/2, c-Jun, and STAT3, thus resulting in an increased AP-1 and HER2 promoter activity and neoplastic cellular transformation. Subsequently, the *in vivo* CAM assay with JB6 cells showed that IL-22-induced tumorigenesis and the inhibition of Pin1 activity significantly reduced the tumor growth induced by IL-22.

Although the role of IL-22 in tumorigenesis has been demonstrated, the physiological significance of IL-22 in breast cancer is not yet elucidated. To further understand the role of IL-22 in breast tumorigenesis, we performed immunohistochemical analysis on human breast cancer tissues using IL-22, MAP3K8, and Pin1 antibodies. Our results show that IL-22 levels correlate positively with MAP3K8 as well as Pin1 expression in breast cancer tissues. These results define an IL-22 signaling that conveys an oncogenic signal to promote aggressiveness in human breast cancer through the induction of MAP3K8 and Pin1 activity. Consistent with these observations, our results also showed that knockdown of MAP3K8 and Pin1 decreased tumorigenicity of MCF7 cells, suggesting that MAP3K8 and Pin1 might be

important molecular targets for the development of breast cancer by IL-22. This study documents a previously unknown role of IL-22 in human breast cancer by showing its promotional role in proliferation, cell survival, transformation, and tumor development. Moreover, these results make MAP3K8 and Pin1 attractive therapeutic targets in tumor microenvironment, inhibition of which could potentially attenuate the aggressiveness of breast cancer. Hence, our study not only reveals a novel mechanism underlying the IL-22 activity but also provides a novel treatment strategy against metastatic breast cancer.

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ABSTRACT

Effects of interleukin-22 on epithelial cell transformation and breast tumorigenesis

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Interleukin-22 (IL-22), one of the cytokines secreted by T helper 17 (Th17) cells, binds to a class II cytokine receptor containing an IL-22 receptor 1 (IL-22R1) and IL-10R2 and influences a variety of immune reactions. IL-22 has also been shown to modulate cell cycle and proliferation mediators such as extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK), but little is known about the underlying molecular mechanisms of IL-22 in tumorigenesis. In this paper, we propose that IL-22 has a crucial role to play in controlling tumorigenesis and epithelial cell proliferation in the breast. IL-22 increased MAP3K8 phosphorylation through IL-22R1, followed by the induction of MEK-ERK, JNK-c-Jun, and STAT3 signaling pathways. Furthermore, IL-22-IL-22R1 signaling pathway activated

activator protein-1 (AP-1) and HER2 promoter activity. In addition, Pin1 was identified as a key positive regulator for the phosphorylation-dependent MEK, c-Jun, and STAT3 activity induced by IL-22. $Pin1^{-/-}$ mouse embryonic fibroblasts (MEF) exhibited significantly a decrease in IL-22-induced MEK1/2, c-Jun, and STAT3 phosphorylation compared to $Pin1^{+/+}$ MEF. In addition, a knockdown of Pin1 prevented phosphorylation induced by IL-22. The *in vivo* chorioallantoic membrane (CAM) assay also showed that IL-22 increased tumor formation of JB6 Cl41 cells. Moreover, the knockdown of MAP3K8 and Pin1 attenuated tumorigenicity of MCF7 cells. Consistent with these observations, IL-22 levels positively correlate with MAP3K8 and Pin1 expression in human breast cancer. Overall, our findings point to a critical role for the IL-22-induced MAP3K8 signaling pathway in promoting cancer-associated inflammation in the tumor microenvironment.

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