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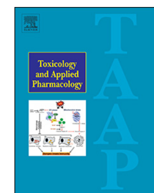
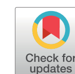


H-*Ras* induces Nrf2-Pin1 interaction: Implications for breast cancer progression



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A B S T R A C T

Aberrant activation of H-*Ras* is often associated with tumor aggressiveness in breast cancer. Peptidyl-prolyl *cis-trans* isomerase NIMA-interacting 1 (Pin1) is a unique enzyme that interacts with phosphorylated serine or threonine of a target protein and isomerizes the adjacent proline residue. Pin1 is prevalently overexpressed in human cancers, and its overexpression correlates with poor prognosis. Nuclear factor E2-related factor 2 (Nrf2) is a master regulator of cellular redox homeostasis. The sustained activation/accumulation of Nrf2 has been observed in many different types of human malignancies, conferring an advantage for growth and survival of cancer cells. The activated form of H-Ras (GTP-H-Ras) is highly overexpressed in human breast cancer tissues. In our present study, silencing of H-*Ras* decreased the invasiveness of MDA-MB-231 human breast cancer cells and abrogated the interaction between Pin1 and Nrf2 in these cells. *Pin1* knockdown blocked the accumulation of Nrf2, thereby suppressing proliferation and clonogenicity of MCF10A-*Ras* human mammary epithelial cells. We found that Pin1 binds to Nrf2 which stabilizes this transcription factor by hampering proteasomal degradation. In conclusion, H-Ras activation in cooperation with the Pin1-Nrf2 complex represents a novel mechanism underlying breast cancer progression and constitutive activation of Nrf2 and can be exploited as a therapeutic target.

1. Introduction

Activating mutations of *Ras* oncogene have been implicated in the development and progression of many different forms of human malignancies (Forbes et al., 2011; Prior et al., 2012). So far three *Ras* isoforms were identified. These include K-*Ras*, N-*Ras* and H-*Ras*. Ras belongs to small G protein family with intrinsic GTPase activity and is a major regulator of a plethora of pathophysiological events including growth, proliferation, cytoskeleton integrity, adhesion, migration, differentiation, and survival of cells (Khan et al., 2019).

A point mutation in codon 12 which substitutes Asp for Gly (G12D) has been found in H-*Ras* and N-*Ras* (Franks and Teich, 1997). Although both H-*Ras* and N-*Ras* can transform human breast epithelial MCF10A cells, only H-*Ras* induces invasive and migratory phenotypes in these cells (Moon et al., 2000). Thus, aberrant activation of H-*Ras* signaling has been suggested as a prognostic marker of breast cancer (Clark and Der, 1995; Geyer et al., 2018; Moon et al., 2008; Yong et al., 2011). Moreover, H-*Ras* and K-*Ras* oncogenes regulate different biological processes, which may differentially impact the overall process of carcinogenesis. While H-*Ras* is mostly involved in regulation of genes controlling cell morphology related to the epithelial-mesenchymal transition, K-*Ras* preferentially modulates gene expression responsible for cytokine signaling, cell adhesion, and colonic development. (Roberts et al., 2006).

Peptidyl-prolyl *cis-trans* isomerase NIMA-interacting 1 (Pin1), consisting of an N-terminal WW domain and a C-terminal peptidylprolyl isomerase (PPIase) domain, interacts with a protein harbouring phosphorylated serine/threonine (pSer/Thr) residues that precede proline (Pro). As a consequence, the conformation of the bound proteins is altered, which influences their subcellular localization, stability,

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interaction with other proteins, and biological activities (Lu, 2004; Lu et al., 2002a, 2002b; Lu and Zhou, 2007; Ryo et al., 2001; Ryo et al., 2002).

Pin1 has been shown to be upregulated in several different types of cancer tissues (Bao et al., 2004; Lu, 2004; Wulf et al., 2001). Pin1 overexpression is associated with neoplastic transformation and uncontrolled growth of tumors (Han et al., 2017; Xu et al., 2016). The oncogenic activity of Pin1 is largely attributed to its ability to stabilize/activate oncoproteins and/or to destabilize/inactivate tumor suppressors (Girardini et al., 2011; Han et al., 2016a; Takahashi et al., 2007). Ablation of Pin1 in H-*Ras* transgenic mice or *p53*-knockout mice suppressed tumorigenesis (Hu et al., 2017; Nicole Tsang et al., 2013; Yeh et al., 2004). It has been reported that H-Ras signaling cooperates with Pin1, which leads to enhanced transcriptional activity of c-Jun towards Cyclin D1 (Wulf et al., 2001).

Nuclear factor E2-related factor 2 (Nrf2) is a leucine zipper transcription factor that plays an essential role in maintaining the cellular redox balance against oxidative stress. In basal conditions, Nrf2 forms an inactive complex with the inhibitory protein Keap1. Keap1 facilitates degradation of Nrf2 through the ubiquitin-proteasome system. Some electrophilic molecules and reactive oxygen species (ROS) modify the critical sensor cysteine residues of Keap1, which can disrupt its sequestration of Nrf2 in the cytoplasm. As a result, Nrf2 is released from Keap1 and translocates to the nucleus where it binds to the antioxidant response element (ARE) or electrophile response elements (EpRE) present in the promoter regions of target genes (Itoh et al., 1999; Motohashi and Yamamoto, 2004; Taguchi and Yamamoto, 2017; Yu et al., 2000).

In recent years, Nrf2 and some of its target proteins have been shown to play differential roles in multi-stage carcinogenesis, acting either as tumor suppressors or tumor promotors (Taguchi and Yamamoto, 2017). While transient induction of Nrf2 in normal cells activates a broad spectrum of cellular defense signaling pathways against various carcinogenic insults, constitutively elevated accumulation of Nrf2 in transformed or malignant cells can create a redox environment that favours tumor growth and promotes resistance to anticancer therapy (Cheng et al., 2019; Ge et al., 2017; Lu et al., 2017; Rojo de la Vega et al., 2018; Wang et al., 2008). As such, persistent activation of Nrf2 in tumors is generally correlated with poor prognosis (Ge et al., 2017).

Nrf2 harbours multiple pSer/Thr-Pro motifs (Sun et al., 2009), and can hence be a putative substrate of Pin1. The expression of Nrf2 and Pin1 has been reported to be induced by H-Ras activation (Funes et al., 2014; Han et al., 2016b; Kitamura et al., 2017; Lim and Leprivier, 2019; Liang et al., 2019; Ryo et al., 2002; Ryo et al., 2009). This prompted us to explore the possibility that Pin1 binds and structurally modify Nrf2 in H-*Ras* transformed mammary epithelial cells, thereby influencing the proliferation and survival of these cells.

2. Materials and methods

2.1. Reagents and antibodies

Dulbecco's Modified Eagle Medium (DMEM) Nutrient mixture F-12 (Ham), DMEM, penicillin/streptomycin mixtures and fetal bovine serum (FBS) were obtained from Gibco BRL (Grand Island, NY, USA). TRIzol® reagent and Stealth™ RNAi negative control duplexes were purchased from Invitrogen Life Technologies, Inc. (Carlsbad, CA, USA). Primary antibodies for Pin1 and vascular endothelial growth factor (VEGF) were supplied by Santa Cruz Biotechnology (Santa Cruz, CA, USA). Primary antibodies for Nrf2 and GTP-H-Ras were supplied by Abcam (Abcam; Cambridge, UK). Secondary antibodies were purchased from Zymed Laboratories Inc. (San Francisco, CA, USA). Dithiothreitol (DTT) and cycloheximide (CHX) were purchased from Sigma-Aldrich (St. Louis, MO, USA). A Western blot detection kit (Absignal) was obtained from Abclon (Seoul, South Korea). Control siRNA and *Pin1* targeting siRNA were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All other chemicals used were in the purest form available commercially. Human breast cancer tissue slides (including both adjacent and malignant tissues), obtained from the biorepository of Lab of Breast Cancer Biology at the Cancer Research Institute, Seoul National University (IRB No., 1405-088-580), were used to detect GTP-H-Ras, Nrf2 and Pin1 proteins.

2.2. Cell culture

An immortalized human breast epithelial MCF10A and H-*Ras* transformed MCF10A (MCF10A-*Ras*) cells were cultured in DMEM/F12 supplemented with 5% horse serum, 0.5 μg/ml hydrocortisone, 10 μg/ml insulin, 20 ng/ml epidermal growth factor, 0.1 μg/ml cholera enterotoxin, 100 units/ml penicillin-streptomycin, 2 mM L-glutamine, and 0.5 μg/ml amphotericin. Cells were maintained in a humidified atmosphere with 95% air and 5% CO_2 at 37 °C. The human breast cancer (MDA-MB-231) cell line obtained from American Type Culture Collection was maintained in DMEM containing 5% FBS at 37 °C in a 5% CO_2/95% air incubator.

2.3. Lentiviral production and infection

Lentiviruses were produced by transfecting HEK293T cells using lentiviral vectors. In brief, HEK293T cells transfected with Pin1 shRNA lentiviral vector were re-transfected with VSV-G-, pLP1- and pLP2-expressing plasmids, and lentiviral supernatants were collected at 48 h and 72 h post-transfection. MCF10A-*Ras* cells were infected with Pin1 shRNA or control virus with 5 μg/ml polybrene, and stable clones were selected using 1 μg/ml puromycin.

2.4. Anchorage-independent growth assay

To prepare the hard agar layer, 2.5 ml of the boiled agarose solution (3.3%) dissolved in phosphate-buffered saline (PBS) was added immediately to 60-mm dishes using a pre-warmed pipette and then kept in the 37 °C incubator to solidify. To prepare the soft agar layer containing the cells, MCF10A-*Ras* (1×10^5) or the same number of MCF10A cells were suspended in the 0.33% agarose solution with gentle mixing, and 2.5 ml of this solution was inoculated on top of the hard agar layer. After allowing the solution to harden as a soft agar for 4 h, 2.5 ml of the fresh medium was added to the top of the hardened soft agar layer. After 3 to 4 weeks of incubation, anchorage-independent growth (spherical formation containing > 10 cells) was scored using a light microscope. The total number of foci per 1×10^5 cells in a well was counted. For experiments with shPin1 stable MCF10A-*Ras* cells or siH-Ras MDA-MB-231 cells, cells were plated in 6-well plates at a density of 150 cells per well. The DMEM/F-12 medium was changed every other day. After one week of incubation, the colonies were fixed in cold methanol and stained by 0.5% crystal violet for 4 h. The stained colonies were washed with PBS to remove the excess dye. Quantitative changes in clonogenicity were determined by extracting stained dye with 10% acetic acid, and the absorbance at 570 nm was measured.

2.5. Wound healing assay

Pre-treated MCF10A-*Ras* transfected with control or Pin1 shRNA Lentiviral vector were plated into the ibidi culture insert on 6 well dishes. After 5 h of incubation for appropriate cell attachment, the culture-insert was gently removed by using sterile tweezers. Cell migration was observed under the microscope.

2.6. Preparation of cytosolic and nuclear extracts

Cells were pelleted by centrifugation at 1700 xg for 5 min after washing with cold PBS and suspended in ice-cold hypotonic buffer A

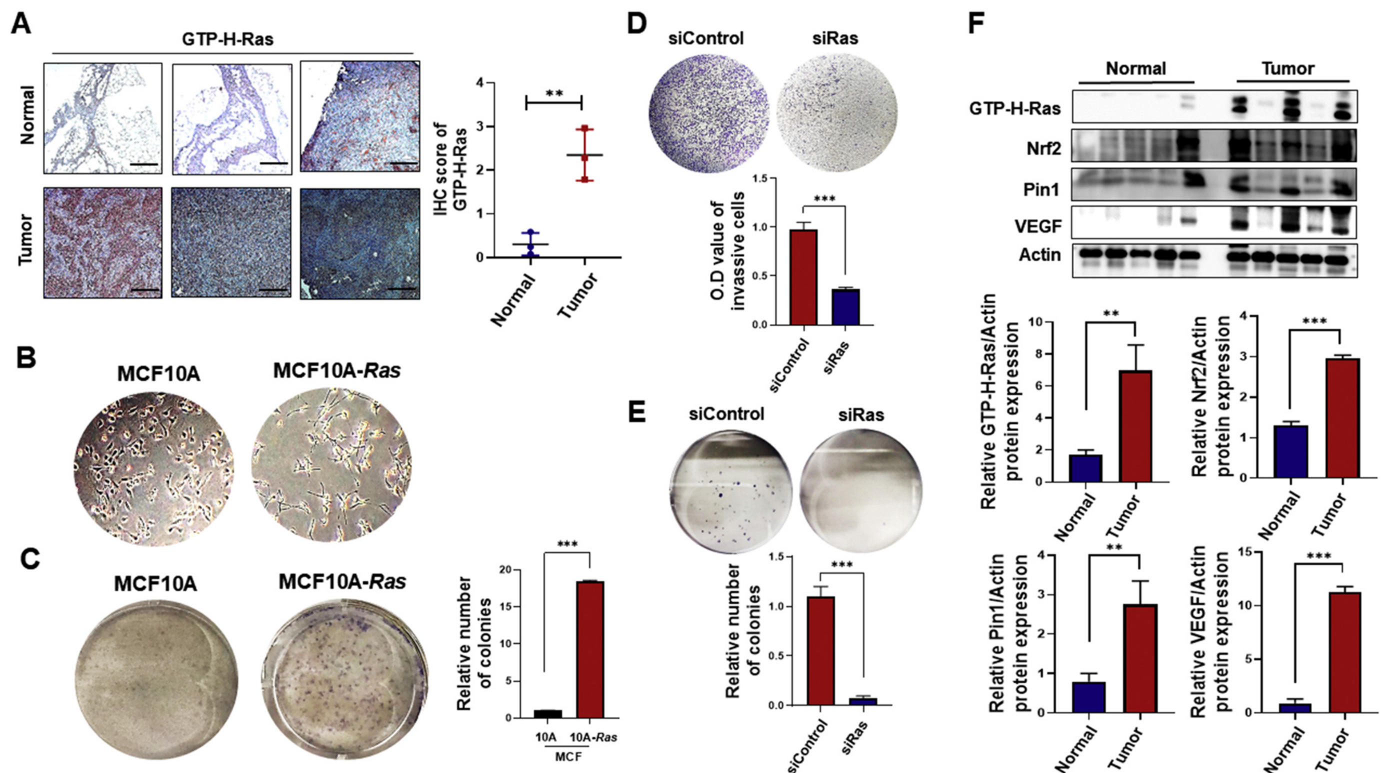

Fig. 1. Overexpression and functional role of H-Ras in breast cancer.
(A) Immunohistochemical analysis of GTP bound H-Ras in breast cancer specimens and adjacent normal tissues. **Significantly different from the control ($p < .01$). Scale bar: 200 μm. (B) The morphology of H-*Ras* transformed human mammary epithelial cells compared with the non-transformed MCF10A cells. (C) Comparison of anchorage-independent growth of MCF10A-*Ras* cells and MCF10A cells. Cells were treated as described in Materials and Methods. Attached cells were photographed, and the proportion of attached cells was quantified by counting the number of colonies. A representative set of images from three independent experiments is shown. Data are shown as the mean ± SD, and the statistical significance was determined by Student's *t*-test. ***$p < .001$. (D) Invasiveness of MDA-MB-231 cells was measured using 24-well microchemotaxis chambers. MDA-MB-231 cells were treated with control or H-Ras siRNA as described in Materials and Methods. The randomly chosen fields were photographed, and the number of cells migrated to the lower surface was counted. Data are shown as the mean ± SD of three independent experiments, and the statistical significance was determined by Student's *t*-test. ***$p < .001$. (E) MDA-MB-231 cells seeded in 6-well plates were treated with control or H-Ras siRNA as described in Materials and Methods. Attached cells were photographed after crystal violet staining, and the proportion of attached cells was quantified by counting the number of colonies. A representative set of images from three independent experiments is shown. Data are shown as the mean ± SD, and the statistical significance was determined by Student's *t*-test. ***$p < .001$. (F) Comparative expression of GTP-H-Ras, Pin1, Nrf2 and VEGF in breast cancer tissues and adjacent normal tissues measured by Western blot analysis. **$p < .01$, and ***$p < .001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

[10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT and 0.2 mM phenylmethylsulfonyl fluoride (PMSF)]. Following incubation in an ice bath for 15 min, cells were centrifuged again at 6000 x*g* for 5 min and the supernatant was collected as a cytosolic fraction. The remaining cell pellets were resuspended in ice-cold buffer C containing 20 mM HEPES (pH 7.9), 20% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, and 0.2 mM PMSF and were incubated at 0 °C for 1 h. After vortex mixing, the resulting suspension was centrifuged at 18,000 x*g* for 15 min, and the supernatant was collected as a nuclear extract and stored at −70 °C.

2.7. In situ proximity ligation assay (PLA)

PLA was carried out using the DUOLinkTM kit (OLINK; Uppsala, Sweden) according to the manufacturer's instructions. In brief, MCF10A-*Ras* cells on glass coverslips were fixed, permeabilized, and blocked with blocking solution [0.1% Triton in PBS containing 5% bovine serum albumin (BSA)] and incubated with the antibodies against Pin1 (1:100) and Nrf2 (1:100) overnight at 37 °C. PLA plus and minus affinity probes were then added and incubated for an additional 1 h at 37 °C. The probes were hybridized using a ligase to be a closed circle. The DNA was then amplified (a rolling-circle amplification) and detected by fluorescence microscopy.

2.8. Western blot analysis

MCF10A, MCF10A-*Ras* and MDA-MB-231 cells were lysed in lysis buffer [250 mM sucrose, 50 mM Tris-HCl (pH 8.0), 25 mM KCl, 5 mM $MgCl_2$, mM EDTA, 2 mM NaF, 2 mM sodium orthovanadate, and 1 mM PMSF for 1 h on ice followed by centrifugation at 18,000 x*g* for 20 min. The protein concentration of the supernatant was measured by using the BCA reagents (Pierce; Rockford, IL, USA). Protein (30 μg) was separated by running through 8% or 12% SDS-PAGE gel and transferred to the PVDF membrane (Gelman Laboratory; Ann Arbor, MI, USA). The blots were blocked with 5% non-fat dry milk PBST buffer for 1 h at room temperature. The membranes were incubated overnight at 4 °C with 1:1000 dilution of Pin1 antibody, 1:2000 dilution of Nrf2 antibody, 1:1000 dilution of VEGF antibody, or 1:5000 dilution of GTP-H-Ras. Equal lane loading was assured using β-actin (Sigma-Aldrich Co.; St. Louis, MO, USA). The blots were rinsed three times with PBST buffer for 10 min each. Washed blots were treated with 1:5000 dilution of the horseradish peroxidase (HRP)-conjugated secondary antibody (Pierce Biotechnology; Rockford, IL, USA) for 1 h and washed again three times with PBST buffer. The transferred proteins were visualized with an enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech; Buckinghamshire, UK).

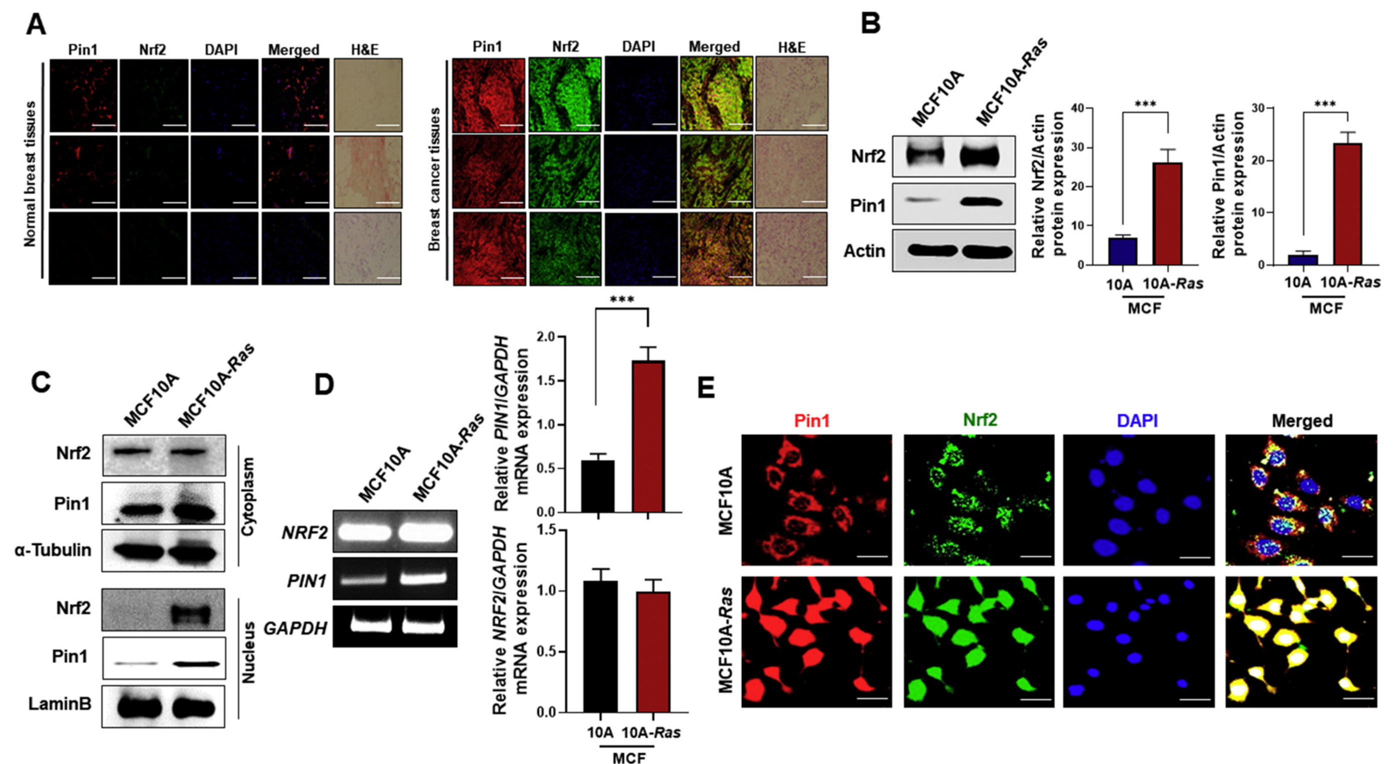

Fig. 2. Co-localization of Pin1 and Nrf2 in human breast tumor tissues and H-Ras transformed human breast epithelial cells in culture.
(A) Co-localization of Pin1 and Nrf2 in human breast cancer tissues determined by immunofluorescence analysis. Breast cancer specimens were exposed to anti-Nrf2 and anti-Pin1 antibodies. Scale bar: 200 μm. (B and C) Comparative expression of Pin1 and Nrf2 proteins in the whole lysate, cytoplasmic and nuclear fractions of MCF10A and MCF10A-*Ras* cells. ****p* < .001. (D) Comparison of mRNA expression of Pin1 and Nrf2 in non-oncogenic MCF10A and MCF10A-*Ras* cells. ****p* < .001. (E) Immunofluorescence staining of Pin1 and Nrf2 in MCF10A and MCF10A-*Ras* cells. Scale bar: 200 μm.

2.9. Immunoprecipitation

Cells were lysed in 250 mM sucrose, 50 mM Tris-HCl (pH 8.0), 25 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 2 mM NaF, 2 mM sodium orthovanadate, and 1 mM PMSF. Total protein (100 μg) was subjected to immunoprecipitation by shaking with Nrf2 primary antibody at 4 °C overnight followed by the addition of protein A/G-agarose bead suspension (40 μl). After centrifugation at 1000 *xg* for 1 min, immunoprecipitated beads were collected by discarding the supernatant and washed with cell lysis buffer. The immunoprecipitate was then resuspended in 24 μl of sample buffer plus 4 μl of 5 x dye and boiled for 5 min. The supernatant from each sample was collected by centrifugation and loaded on SDS-polyacrylamide gel.

2.10. Immunohistochemistry and immunofluorescence analysis

For immunohistochemical analysis of the expression of GTP-H-Ras, 4 mm sections of 10% formalin-fixed, paraffin embedded tissues from breast cancer patients were placed on glass slides and deparaffinized 3 times with xylene and rehydrated through graded alcohol bath. The deparaffinized sections were heated by using microwave and boiled twice for 6 min in 10 mM citrate buffer (pH 6.0) for antigen retrieval. To diminish non-specific staining, each section was treated with 3% hydrogen peroxide and 4% peptone casein blocking solution for 15 min. For the detection of respective protein expression, slides were incubated with GTP-H-Ras antibody (1:500) at room temperature for 40 min in TBST followed by treatment with respective HRP-conjugated secondary antibody (rabbit). The peroxidase binding sites were detected by staining with 3,3'-diaminobenzidine tetrahydrochloride. Finally, counterstaining was performed using Mayer's hematoxylin. For immunofluorescence staining of human paraffin-embedded breast cancer tissues and matched adjacent normal breast tissues, a standard protocol for deparaffinization, antigen retrieval, and permeabilization was followed. After overnight incubation at 4 °C with anti-Nrf2 (1:200) and anti-Pin1 (1:100) antibodies, the tissue sections were washed with PBS and then labeled with TRITC or FITC-conjugated secondary antibody for 1 h at room temperature. The slides were then visualized under a fluorescent microscope. For immunofluorescence analysis of Nrf2 and Pin1 in shControl or shPin1 MCF10A-Ras stable cells, cells were plated on the 8-well chamber slide (10^5 cells/well). Cells were fixed in 95% methanol for 10 min at −20 °C. After rinse with 1× diluted PBS, cells were incubated in 0.2% Triton X-100 in PBS for 5 min. After three washing steps with 1× PBS, cells were blocked for 2 h in fresh blocking buffer [(1× PBS, pH 7.4, containing 5% bovine serum albumin (BSA)] and incubated overnight at 4 °C with anti-Nrf2 or anti-Pin1 antibody. After three washing steps with 1× PBS, the cells were incubated with a diluted (1:1000) TRITC-conjugated anti-mouse or FITC-conjugated anti-rabbit IgG secondary antibody in 1× PBS with 1% BSA at room temperature for 1 h. Cells were also stained with 4′,6-diamidino-2-phenylindole (DAPI) and rinsed with 1× PBS. Stained cells were visualized under a microscope and photographed.

2.11. Small interfering RNA (siRNA) and plasmid transient transfection

siRNA specifically targeting *Pin1* and non-specific si-control RNA were purchased from Santa Cruz Biotechnology (sc-36,230). siRNA specifically targeting H-*Ras* was purchased from Thermo Fisher Scientific. Transient transfections with Pin1 siRNA or H-Ras siRNA were performed using the Lipofectamine RNAi-MAX transfection reagents according to the instructions supplied by the manufacturer (Invitrogen; Carlsbad, CA, USA). After 48- to 72-h transfection, cells were lysed for Western blot analysis.

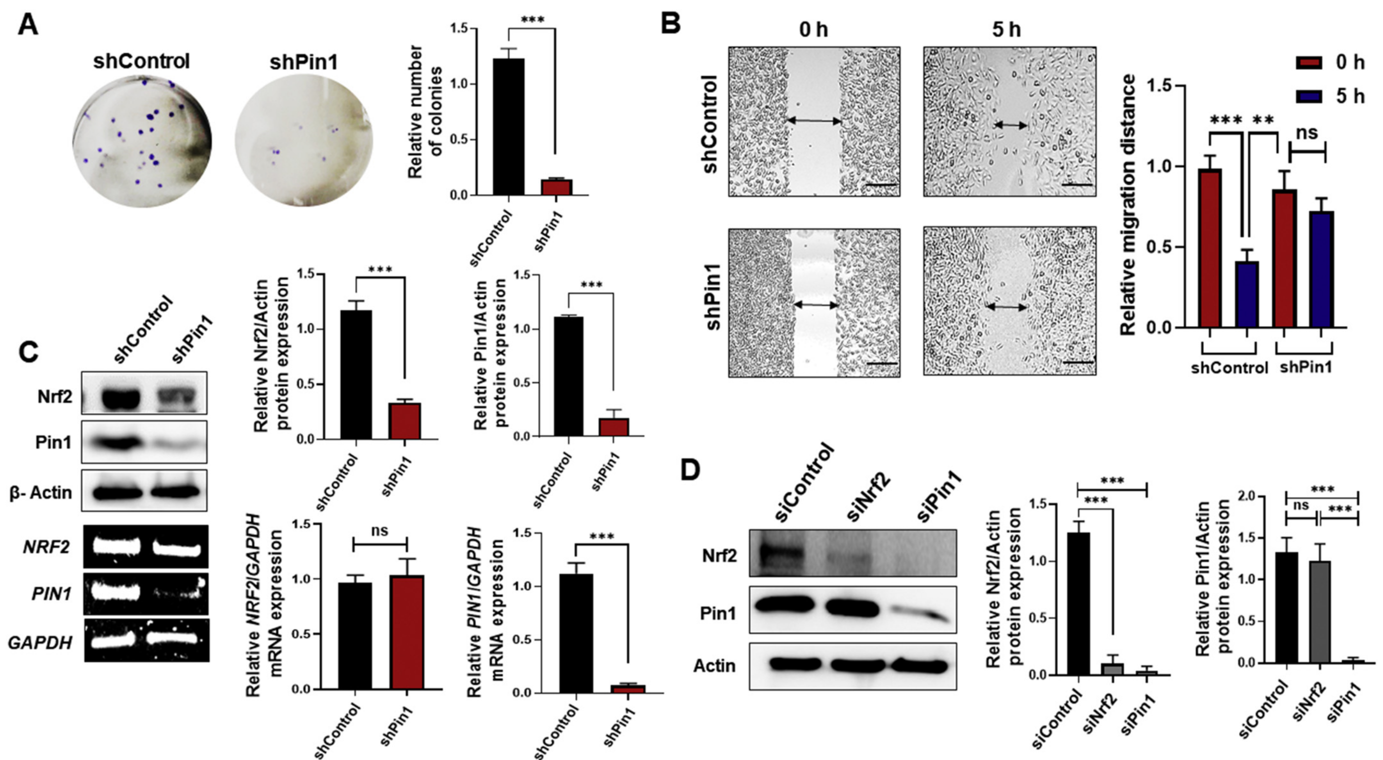

Fig. 3. Effects of Pin1 on the clonogenicity and migration of MCF10A-*Ras* cells.
(A) Control or shPin1 MCF10A-*Ras* stable cells were seeded in 6-well plates and treated as described in Materials and Methods. Attached cells were photographed after crystal violet staining, and the proportion of attached cells was quantified by counting the number of colonies. A representative set of images from three independent experiments is shown. Data are shown as the mean ± SD, and the statistical significance was determined by Student's *t*-test. $^{***}p < .001$. (B) Stable MCF10A-*Ras* cells were generated with control or Pin1 Lentivirus shRNA. Then, cell migration was visualized under a microscope. $^{**}p < .01$ and $^{***}p < .001$. ns, not significant. Scale bar: 200 μm. (C) Effects of Pin1 knockdown on Nrf2 protein and mRNA expression in MCF10A-*Ras* cells. $^{***}p < .001$. ns, not significant. (D) Differential effects of silencing *Pin1* and *Nrf2* on expression of Nrf2 and Pin1, respectively. $^{***}p < .001$. ns, not significant. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.12. Protein stability assay

The MCF10A-*Ras* cells transfected with control or Pin1 Lentiviral shRNA were treated with 10 μM CHX to block protein synthesis. The cells were collected at different time intervals for Western blot analysis.

2.13. Statistical analysis

All data are presented as the mean ± SD. Experiments were repeated at least three times. Two-tailed unpaired Student's *t*-tests or one-way ANOVA were used to evaluate the data. Statistical differences were considered significant at $^{*}p < .05$; $^{**}p < .01$, and $^{***} p < .001$.

3. Results

3.1. H-Ras is overexpressed and implicated in pathogenesis of human breast cancer

To investigate the correlation between H-Ras and breast cancer progression, we compared the expression of H-Ras in tumor and adjacent normal tissues from breast cancer patients. As illustrated in Fig. 1A, expression of the active form of H-Ras (GTP-bound) was upregulated in the tumor, compared with that in the normal tissues. We have demonstrated that H-*Ras* oncogene transforms non-oncogenic MCF10A human mammary epithelial cells and consequently changes their phenotypic characteristics. Thus, MCF10A-*Ras* cells are considerably elongated, whereas MCF10A cells have a round shape (Fig. 1B). The number of colonies formed by anchorage-independent cell growth was dramatically increased in MCF10A-*Ras* cells compared to parental MCF10A cells (Fig. 1C). Silencing of H-*Ras* resulted in marked reduction in migration (Fig. 1D) and clonogenicity (Fig. 1E) of MDA-MB-231 cells. Next, we investigated the expression of Nrf2 and Pin1 in human breast cancer tissues. As shown in Fig. 1F, the levels of both proteins were significantly higher in the tumor tissues than in the adjacent normal tissues, which correlated with activated H-Ras expression. Tumor tissues also exhibited significant upregulation of VEGF, a representative pro-angiogenic marker (Fig. 1F).

3.2. Pin1 and Nrf2 co-localize in nucleus of breast cancer cells

In order to explore the cooperative role of Nrf2 and Pin1 in breast cancer development and progression, we examined their co-localization in human breast cancer specimens. While normal tissues exhibited relatively low protein expression and immunofluorescence signals from antibodies recognizing Nrf2 and Pin1, the tumor tissues showed highly enhanced intensities and co-localization of both proteins (Fig. 2A). We also found that MCF10A-*Ras* cells express Nrf2 and Pin1 to a greater extent than the MCF10A parental cells (Fig. 2B). The protein level of Pin1 was higher in both cytoplasmic and nuclear fractions of MCF10A-*Ras* cells than that in MCF-10A cells (Fig. 2C). Nrf2 protein was found to be expressed at a similar level in the cytoplasmic fraction of both cell lines, but there was marked elevation in its nuclear accumulation in MCF10A-*Ras* cells (Fig. 2C). We found that both MCF10A-*Ras* and MCF-10A cells express equivalent levels of *Nrf2* mRNA, but a higher level of *Pin1* mRNA in the former cells (Fig. 2D).

In another experiment, the subcellular distribution of Nrf2 and Pin1 in both MCF10A and MCF10A-*Ras* cells was examined by immunofluorescence analysis. Consistent with the immunoblot data (Fig. 2C),

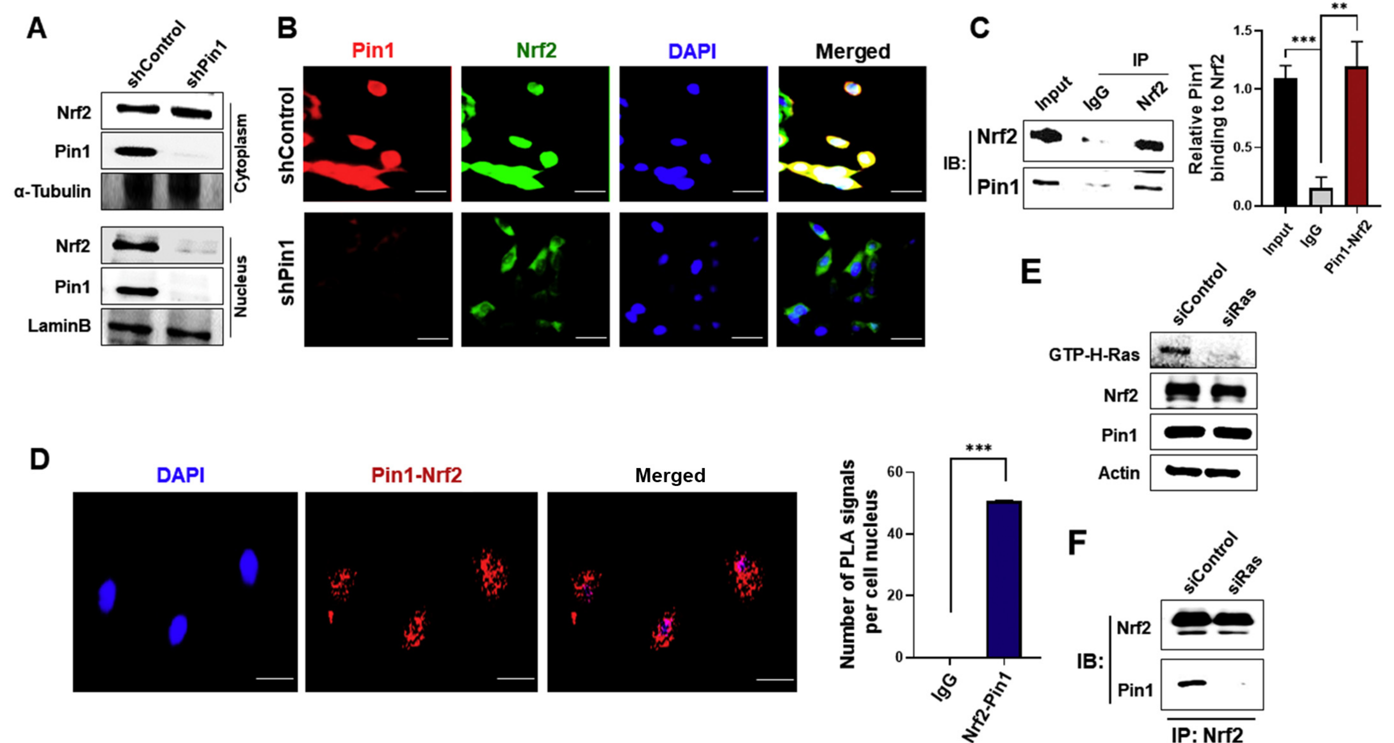

Fig. 4. Physical interaction between Pin1 and Nrf2.
(A) Effects of stable knockdown of *Pin1* on Nrf2 protein expression in the cytoplasmic and nuclear fractions of MCF10A-*Ras* cells. (B) Immunofluorescence staining of Nrf2 in control and *Pin1* silenced MCF10A-*Ras* cells. Scale bar: 200 μm. (C) Interaction between Pin1 and Nrf2 in MCF10A-*Ras* cells. The Pin1-Nrf2 complex was detected by immunoprecipitation with anti-Nrf2 antibody followed by immunoblot analysis with an antibody against Pin1. $^{**}p < .01$ and $^{***}p < .001$. (D) Detection of Pin1-Nrf2 interaction *in situ*. The interaction of Pin1 with Nrf2 was visualized by Duolink analysis (PLA) that allows *in situ* detection protein interactions with high specificity and sensitivity. Pin1 and Nrf2 were co-labeled with corresponding antibodies. Each red spot represents a single interaction, and nucleus was stained with DAPI. Scale bar: 200 μm. $^{***}p < .001$. (E and F) Effects of H-*Ras* silencing on the protein expression of Nrf2 and Pin1 as well as H-Ras (E) and interaction between Pin1 and Nrf2 (F) in the MDA-MB-231 cells. Scale bar: 200 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Nrf2 and Pin1 were predominantly co-localized in the nucleus in MCF10A-*Ras* cells while they exist mainly in the cytoplasm in MCF10A cells (Fig. 2E).

3.3. Silencing of Pin1 attenuates the clonogenic and migratory capability of MCF10A-Ras cells

The regulation of MCF10A-*Ras* cell growth and proliferation by Pin1 was evidenced by marked reduction in colony formation (Fig. 3A) and migration (Fig. 3B) upon stable shRNA knockdown of its expression. Further, silencing of *Pin1* decreased the expression of Nrf2 protein, but not its mRNA transcript (Fig. 3C). In contrast, knockdown of *Nrf2* failed to suppress Pin1 expression (Fig. 3D).

3.4. Pin1 depletion inhibits the nuclear accumulation of Nrf2

At the subcellular levels, *Pin1* knockdown did not affect the expression of cytoplasmic Nrf2 protein (Fig. 4A upper). However, the nuclear accumulation of Nrf2 was abolished in the *Pin1* silenced cells (Fig. 4A lower). An immunofluorescence assay also verifies that Pin1 depletion inhibits the nuclear translocation of Nrf2 (Fig. 4B).

3.5. Pin1 directly interacts with Nrf2

The co-localization of Pin1 and Nrf2 prompted us to examine whether both proteins could physically interact each other. An immunoprecipitation assay revealed that there was a pronounced interaction between Pin1 and Nrf2 in MCF10A-*Ras* cells (Fig. 4C). The direct interaction was verified by the PLA, a powerful tool which detects an enhanced fluorescent signal as a discrete spot when two proteins are localized in proximity (Fig. 4D). Next, we explored the role of H-*Ras* oncogene in the expression/accumulation of Nrf2 and Pin1. Silencing of H-*Ras* had no effect on the expression of both proteins in MDA-MB-231 breast cancer cells (Fig. 4E). However, H-*Ras*-silenced cells exhibited no interaction between Nrf2 and Pin1 in these cells (Fig. 4F).

3.6. Pin1 stabilizes Nrf2 in human breast cancer cells

After confirmation of the direct binding of Pin1 to Nrf2, we investigated whether this could affect the stability of Nrf2 in MCF10A-*Ras* cells. MCF10A-*Ras* cells express a higher level of Nrf2 than the parental MCF10A cells (Fig. 2B and Fig. 2C), but equivalent levels of its mRNA transcript (Fig. 2D). Moreover, silencing of *Pin1* had no effect on the expression of *Nrf2* mRNA, but markedly inhibited its protein expression (Fig. 3C). These findings suggest that Pin1-induced accumulation of Nrf2 is mediated through stabilization of the Nrf2 protein rather than stimulation of gene transcription. In order to test this possibility, shControl and shPin1 stable cells were treated with CHX to block *de novo* synthesis of proteins. As shown in Fig. 5A, *Pin1*-silenced MCF10A-*Ras* cells exhibited a significantly elevated degradation rate of pre-existing Nrf2 compared with the shControl group. Treatment with a proteasome inhibitor, MG-132 abolished the degradation of Nrf2 induced by all-*trans* retinoic acid (ATRA), a pharmacologic inhibitor of Pin1 (Fig. 5B). The knockdown of *Pin1* substantially increased the level of ubiquitinated Nrf2 (Fig. 5C), corroborating the stabilization of Nrf2 by Pin1.

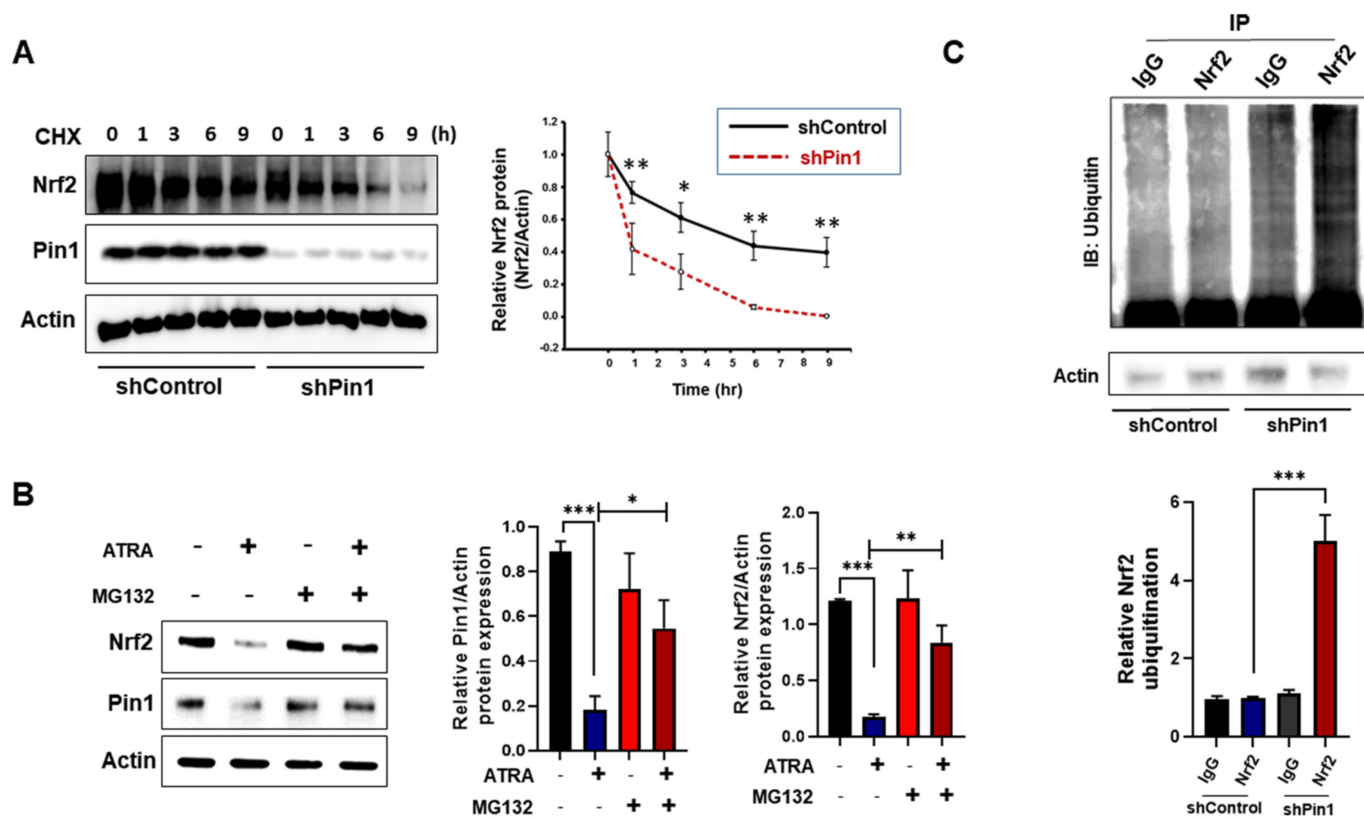

Fig. 5. Stabilization of Nrf2 by Pin1.
(A) The reduced stability of Nrf2 by *Pin1* silencing. shControl and shPin1 MCF10A-*Ras* stable cells were exposed to CHX (10 μM) for the indicated time periods. Cell lysates were subjected to Western blot analysis with anti-Pin1 and anti-Nrf2 antibodies. $^{*}p < .05$ and $^{**}p < .01$. (B) Effects of pharmacologic inhibition of Pin1 on Nrf2 protein accumulation in MCF10A-*Ras* cells with or without exposure to the proteasome inhibitor MG-132. After treatment with ATRA (50 μM) for 48 h, cells were exposed to MG-132 (20 μM) for additional 4 h, and subjected to Western blot analysis. $^{*}p < .05$, $^{**}p < .01$ and $^{***}p < .001$. (C) Effects of *Pin1* silencing on ubiquitination of Nrf2 in control and *Pin1* knockdown MCF10A-*Ras* cells. Nrf2 ubiquitination was determined by immunoprecipitation of Nrf2 with anti-ubiquitin antibody. $^{***}p < .001$.

4. Discussion

Breast cancer is a heterogeneous malignancy consisting of different subtypes that are characterized by distinct histopathological features, specific genetic and epigenetic alterations, and diverse aggressive behaviors (Vargo-Gogola and Rosen, 2007). Mutations of the *Ras* oncogene are among the most frequent genetic alterations in human tumors. The single point mutation at amino acid residue 12 (Gly to Asp) of H-*Ras* is frequently found in mammary carcinoma (Franks and Teich, 1997). In contrast, K-*Ras* and N-*Ras* mutations are more predominant in other types of cancers, such as bladder, ovarian, thyroid, lung, colon and rectum, and pancreatic carcinoma; neuroblastoma (Bentires-Alj et al., 2006; Cichowski and Jacks, 2001; Rochlitz et al., 1989; Tartaglia and Gelb, 2005; Watzinger and Lion, 1999).

It has been reported that non-cancerous human mammary epithelial MCF10A cells transfected with H-*Ras* acquires invasive and migratory phenotypes (Moon et al., 2000). Manifestation of an invasive phenotype by H-*Ras* was also observed in the MDA-MB-453 human breast cancer cell line (Yong et al., 2011). The divergence among the Ras isoforms is attributable to the 23 to 24C-terminal amino acids, so called ‘hypervariable region (HVR)’ that retains the signals responsible for correct plasma membrane localization of Ras (Jaumot et al., 2002). The C-terminal HVR of H-Ras, especially the flexible linker domain with two consecutive proline residues (Pro173 and Pro174), has been shown to play a critical role in the activation of H-Ras and its invasive potential in human breast epithelial cells (Yong et al., 2011).

Pin1 is involved in the majority of main cellular processes of breast cancer development and progression. Pin1 is often overexpressed in breast cancers and associated with worse clinical outcome (Reineke et al., 2008). The WW domain of Pin1 binds to a pSer-Pro or pThr-Pro motif, in a sequence-specific manner (Lu et al., 1999; Lu et al., 2002a, 2002b). It is noticeable that the C-terminal HVR of H-Ras harbours Pro173 preceded by Ser, which may comprise a WW binding motif, providing a potential binding site for Pin1. Overexpression of Pin1 correlates with upregulation/activation of distinct oncoproteins, such as Cyclin D1, β-catenin, AKT, NF-κB/p65 and PKM2. Pin1 not only binds to phosphorylated c-Jun, but also dramatically increases its ability to transactivate the Cyclin D1 promoter in cooperation with either activated JNK or oncogenic H-Ras (Gianni et al., 2009; Liao et al., 2009; Lu and Hunter, 2014; Moretto-Zita et al., 2010; Wei et al., 2015).

H-Ras and some other oncogenic signaling molecules, such as HER2, PI3K and p38, have been shown to induce *Pin1* mRNA expression (Kitamura et al., 2017; Ryo et al., 2002; Ryo et a.l, 2009). Notably, Pin1 overexpression in non-transformed human breast epithelial cells led to neoplastic transformation and also greatly enhanced the acquisition of the transformed phenotype induced by oncogenic H-*Ras* (Ryo et al., 2002). Moreover, Pin1 overexpression disrupts cell cycle coordination leading to centrosome amplification, chromosome instability and breast cancer development (Wei et al., 2015). In contrast, *Pin1* knockout mice were resistant to mammary tumorigenesis, even that induced by activated oncogenes including *Ras* (Wulf et al., 2004). In breast cancer, overexpression of both Pin1 and Cyclin E contributes to centrosome amplification and oncogenic H-Ras activity (Rustighi et al., 2017). Our current study also reveals that Pin1 plays a role in clonogenicity and migration of MCF10A-*Ras* cells as its knowckdown attenuated both events.

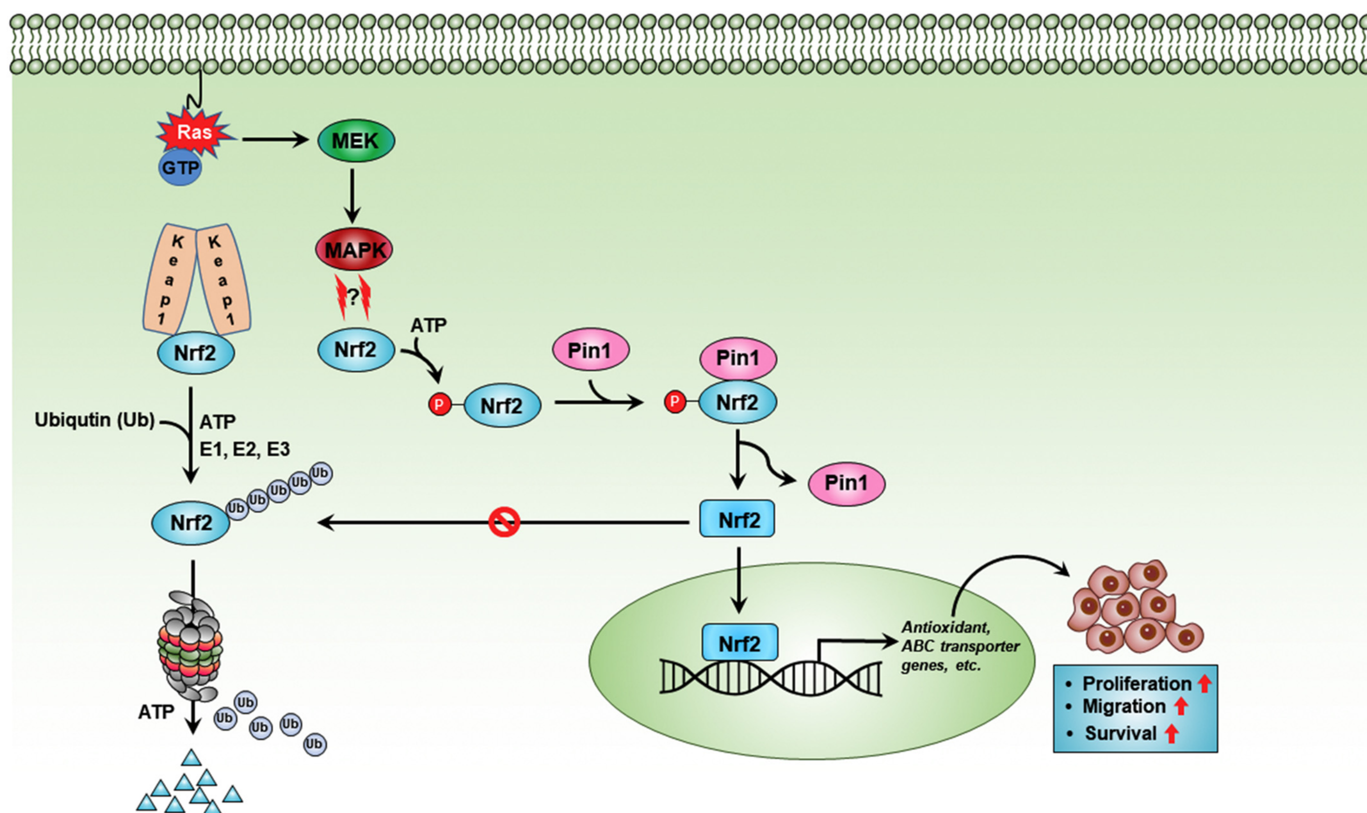

Fig. 6. Proposed scheme for Nrf2 stabilization by complex formation with Pin1 in breast cancer cells with activated H-Ras.
In a resting state, Nrf2 is sequestered in the cytoplasm as an inactive complex with Keap1 (Kelch-like-ECH-associated-protein 1), an adaptor for a cullin-3 (Cul3)-based ubiquitin ligase, responsible for proteasomal degradation of Nrf2. H-Ras in a GTP bound form activates mitogen-activated protein kinases (MAPKs) via the MEK pathway. Some MAPKs (e.g., ERK) have been known to phosphorylate Nrf2 at specific serine/threonine residues, some of which are present in the WW domain binding motif. This facilitates Pin1 binding to Nrf2, which may alter conformation of this transcription factor. As a result, Nrf2 escapes from sequestration by Keap1 and hence Cul3-mediated ubiquitination and degradation. The stabilized Nrf2 translocates to nucleus where it regulates the transcription of antioxidant and other stress responsive genes encoding proteins (e.g., glutamate cysteine ligase, heme oxygenase, ABC transporters, etc.) essential for cancer cell proliferation, migration, and survival.

Nrf2 plays a central role in cellular stress response. In unstressed conditions, Nrf2 is ubiquitinated by the Keap1-Cul3 complex and subsequently degraded by the proteasomes. Once the cells are exposed to electrophiles or ROS, some sensor cysteine residues of Keap1 is modified, which instigates its inactivation and consequently renders Nrf2 stabilized. Nrf2 then translocates to the nucleus and activates the transcription of cytoprotective genes by binding to ARE/EpRE. However, aberrant overactivation of Nrf2 in tumor tissues is significantly associated with a poor clinical outcome in various cancers (Itoh et al., 1999; Lu et al., 2017; Motohashi and Yamamoto, 2004; Taguchi and Yamamoto, 2017; Wang et al., 2008; Yu et al., 2000).

Because Nrf2 potentiates the cellular antioxidant capacities, constitutive activation of Nrf2 in cancer cells promotes their survival against oxidative stress and confers resistance to chemo- and radiotherapy which are mainly dependent on the ROS generation (Itoh et al., 1999; Lu et al., 2017; Taguchi and Yamamoto, 2017). It is speculated that oncogenic Ras may regulate both pro-oxidant and antioxidant programs depending on the redox status of the tumor cells in order to promote their growth and progression (Lim and Leprivier, 2019). In maintaining the redox balance through the complementary role of both pro- and antioxidant pathways, Ras may cooperate with Nrf2. Further studies will be necessary to explore the coordinated function of H-Ras and the Nrf2-Pin1 complex in the breast cancer progression.

In this study, we have shown that the expression of Pin1 is positively correlated with the accumulation of Nrf2 in human breast tumor tissues as well as in H-*Ras* transformed human breast epithelial cells. It has recently been reported that Pin1 interacts with Nrf2, and the resulting complex co-localizes in the nucleus in pancreatic cancer cells (Liang et al., 2019). Consistent with this observation, we have also found that Pin1-Nrf2 complex predominantly accumulates the nucleus in MCF10A-*Ras* cells. The genetic ablation of *Pin1* markedly reduced the levels of Nrf2 protein. However, silencing of *Pin1* had no significant effect on the mRNA expression of Nrf2, indicating that Pin1 may regulate Nrf2 in a post-translational way. Our data indicate that Pin1 stabilizes Nrf2 by inhibiting its ubiquitination and degradation. Although silencing of *Pin1* expression by siRNA decreased the Nrf2 accumulation, knockdown of *Nrf2* had no significant effect on the expression of Pin1, indicating that the Pin1-Nrf2 interaction is unidirectional. Further, H-*Ras* deficiency abolished the interaction between Pin1 and Nrf2 in breast cancer cells. It is likely that H-Ras activation facilitates the association between Pin1 and Nrf2, and thereby stimulates growth and progression of human mammary epithelial cells. We speculate that H-Ras activates some mitogen-activated protein kinases (e.g., ERK), which in turn phosphorylates Nrf2 at specific serine/threonine residues, especially those present in the WW domain binding motif. This promotes Pin1 binding to Nrf2, which may cause a conformational change and stabilization of Nrf2 (Fig. 6).

In conclusion, Nrf2 can be a novel partner protein of Pin1 and the Pin1-Nrf2 interaction facilitated in the presence of H-*Ras* may contribute to human breast cancer development and progression (Fig. 6). Therefore, the Pin1-Nrf2 axis can be considered a novel therapeutic target, especially for H-*Ras* mutated breast cancer.

Declaration of Competing Interest

The authors declare no potential conflicts of interest.

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