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Modulation of ERK5 Is a Novel Mechanism by Which Cdc42 Regulates Migration of Breast Cancer Cells

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ABSTRACT

Members of Rho family GTPases including Cdc42 are known to play pivotal roles in cell migration. Cell migration is also known to be regulated by many protein kinases. Kinetworks KPSS 11.0 phospho-site screening of Cdc42-silenced Hs578T breast cancer cells revealed most dramatic change in ERK5 MAP kinase. In the present study, we set out to determine the relationship between Cdc42 and ERK5 and its significance in breast cancer cell migration and invasion. Specific siRNAs were used for knocking down Cdc42 or ERK5 in breast cancer cells. Increased ERK5 phosphorylation in breast cancer cells was achieved by infection of constitutively active MEK5 adenovirus. The cells were then subjected to cell migration or invasion assay without the presence of serum or any growth factor. We found that Cdc42 negatively regulated phosphorylation of ERK5, which in turn exhibited an inverse relationship with migration and invasiveness of breast cancer cells. To find out some in vivo relevance of the results of our in vitro experiments we also examined the expression of ERK5 in the breast cancer tissues and their adjacent normal control tissues by real-time RT-PCR and immunocytochemistry. ERK5 expression was found to be reduced in breast cancer tissues as compared with their adjacent uninvolved mammary tissues. Therefore, Cdc42 may promote breast cancer cell migration and invasion by inhibiting ERK5 phosphorylation and ERK5 expression may be inversely correlated with the progression of some breast tumors. J. Cell. Biochem. 116: 124–132, 2015.

KEY WORDS: BREAST CANCER; Cdc42; CELL MIGRATION; ERK5; Rho GTPase

Members of Rho GTPase family play key roles in many cellular functions essential for tumor progression, which include cell migration [Merajver and Usmani, 2005; Bustelo et al., 2007]. RhoA, Rac1, and Cdc42 are the most extensively studied members of this family, though the family consists of more than 20 members [Ridley et al., 2003]. A Rho GTPase is at on-state when bound with GTP and at off-state when bound with GDP, thus making it function as molecular switch to modulate downstream signaling pathways (Ridley, 2004). The activities of Rho GTPases can be governed by three families of proteins: guanine nucleotide exchange factors (GEFs), GTPases activating proteins (GAP) and guanine-nucleotide dissociation inhibitors (GDIs) [Ridley et al., 2003]. GEFs promote the exchange of GDP for GTP to activate Rho GTPases. GAPs facilitate the hydrolysis of the bound GTP to inactivate Rho GTPases. GDIs negatively regulate the activity of Rho proteins through binding to Rho GTPases for preventing them from interacting with GEFs and GAPs [Walker and Olson, 2005; Villalonga and Ridley, 2006]. GTP-bound Rho GTPases can activate the downstream effector molecules subsequently leading to a cellular response [Madaule and Axel, 1985; Ridley and Hall, 1992]. To date, more than 80 GEFs, 70 GAPs, and 60 effectors have been isolated for RhoA, Rac1, and Cdc42. Because any one of these three members is able to participate in multiple cellular functions by interacting with so many proteins, dissecting signaling pathways of individual Rho GTPases in one particular cellular function such as 'migration' is a challenging task. Cell type-dependent regulation of a cellular function by Rho GTPase further adds to the complexity of the task.

RhoA, Rac1, and Cdc42 play pivotal roles in cell migration by coordinately regulating rearrangements of cytoskeletal actin and microtubule. RhoA is known to contribute to cell migration

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through stress fiber formation and cell contraction, whereas Rac1 and Cdc42 contribute through lamellipodial and filopodial protrusions, respectively. In order to find out the involvement of protein kinase(s) in the inhibition of the cell migration resulting from down-regulation of Cdc42, we utilized the Kinetworks™(KPSS 11.0) phosphoprotein analysis of Cdc42-silenced breast cancer cells and compared the results with those of control siRNA-treated cells (Zuo et al., 2012). Phosphoprotein analysis of Cdc42-silenced Hs578T cells showed significantly enhanced phosphorylation of big MAP kinase1(BMK1/ERK5) without exhibiting any other change on KPSS 11.0 screen (Zuo et al., 2012). In the present report, we confirm the finding of enhanced ERK5 phosphorylation resulting from Cdc42-silencing and explore its possible consequence on cell migration/invasion. We demonstrate that in breast cancer cells, Cdc42 negatively regulates phosphorylation of ERK5, which in turn exerts a negative regulatory influence on their intrinsic migratory and invasive abilities. In keeping with these findings, real-time RT-PCR and immunohistochemistry performed in clinical samples of primary breast tumors and adjacent non-tumor tissues show that ERK5 expression is significantly decreased in tumors. These data, therefore, suggest ERK5 modulation as a novel mechanism of Cdc42 regulation of breast cancer cell migration.

MATERIALS AND METHODS

REAGENTS AND ANTIBODIES

RPMI 1640 and FBS were obtained from Invitrogen Canada Inc. (Burlington, Ontario, Canada). Multiporous transwells were purchased from Costar (Corning, New York). Phospho-ERK5 antibody (#3371) and HA-Tag (6E2) Mouse mAb (#2367) were purchased from Cell Signaling (Cell Signaling Technology, Inc. Danvers, MA). Anti-ERK5 antibody (Rabbit monoclonal ab40809) was purchased from Abcam Inc. (Cambridge, MA), anti-ERK5 goat polyclonal antibody (sc-1284) was purchased from Santa Cruz Biotech. Anti-Cdc42 primary antibody (mouse monoclonal MAB3707) was purchased from Chemicon (Chemicon, Temecula, CA). Anti-Rac1 primary antibody (clone 23A8) was purchased from Upstate (Upstate Biotechnology, New York). Anti-RhoA antibody (sc-418) was purchased from Santa Cruz (Santa Cruz biotechnology, Inc. Santa Cruz, CA). Anti-GAPDH antibody (mouse monoclonal clone Mab 6C5) was purchased from HyTest (HyTest, Findland). Alexa Fluo 488 donkey anti-goat IgG was obtained from Molecular Probes (Invitrogen, Eugene, OR).

BREAST TUMOR SPECIMENS

Frozen tumor tissues and adjacent uninvolved mammary tissues and formalin-fixed paraffin-embedded blocks of the same tissues from nine breast cancer patients were obtained from the tumor bank of London Health Sciences Centre (London, Ontario, Canada). All the patients were women. The average age of the patients was 69 years. Each tissue sample was examined by a Canadian Board Certfied Pathologist. Four samples were of SBR (Scarff-Bloom Richardson) grade III, three were of grade II and one was of grade I. Lymph node, ER (estrogen receptor) and PR (progesterone receptor) statuses of five samples were positive. The study was approved by the University of Western Ontario Ethics Committee.

CELL CULTURE

The human breast cancer cell lines MDA-MB-231 and Hs578T were kindly provided by Dr. Dale Laird (University of Western Ontario, Canada) who originally obtained them from the American Type Culture Collection (Rockville, MD). Each of the breast cancer cell lines was maintained in RPMI 1640 containing penicillin (100 U/ml), streptomycin (100 U/ml), and 10% fetal bovine serum (FBS) at 37°C under 5% CO_2 in air, and given passage at 80–90% confluence.

INFECTION OF CONSTITUTIVELY ACTIVE MEK5 ADENOVIRUS

Constitutively active human recombinant MEK5 (CAMEK5) adenovirus was purchased from Cell Biolabs (Cell Biolabs, San Diego, CA) and amplified according to manufacture's protocol. Briefly, Cells were seeded in 6-well plate, cultured overnight and infected with adenovirus for 48 h. A non-specific GFP adenovirus with the same multiplicity of infection (MOI) was used as a negative control.

TRANSFECTIONS OF siRNA

Two sets of siRNA oligonucleotides duplex specific for human Cdc42 were used to knock down Cdc42 in cancer cells. The first set of siRNA was made according to Wilkinson et al. [2005] by Dharmacon Inc. The second set was Dharmacon ON-TARGETplus Duplex J-005057–05-0050, human Cdc42 siRNA (Catalog # J-043087–09, NM_002046). siRNA oligonucleotides duplexes specific for Rac1 were designed according to Noritake et al. [2004] and Deroanne et al. [2003] and for RhoA was designed according to Pille et al. [2005] To knock down ERK5 in human cancer cells, siRNA specific for ERK5 (Catalog # J-003513–08-0050, NM_139032) was purchased from Dharmacon Inc. (Dharmacon Lafayette, Colorado). For control, siCONTROL non-targeting siRNA Pool reagent (Catalog # D-001206–13-05) was also purchased from Dharmacon Inc. Transfection of siRNA was carried out using DharmaFECTTM (Dharmacon Lafayette, Colorado) as described before [Zuo et al., 2006].

RNA ISOLATION AND REAL-TIME RT-PCR

Total RNA from breast cancer cell lines or clinical breast cancer tissue samples was isolated using Trizol reagent following the manufacturer's protocol. Real-time RT-PCR was performed using the LightCycler™ (Roche Diagnistic, Laval, Canada) following the procedure used before [Zuo et al., 2006]. Primer sequences were (Cdc42) forward: GTG CCT GAG ATA ACT CAC CA, reverse: GTA GGT GCA GGG CAT TTG TC; (GAPDH) forward: ACC ACA GTC CAT GCC ATC AC, reverse: TCC ACC ACC CTG TTG CTG TA. Sequence of ERK5 primer forward: ACG AGT ACG AGA TCA TCG AGA CC, reverse: GGT CAC CAC ATC GAA AGC ATT AGG. Sequence of MEK5 primer forward: CAG ATA TTT CCA AGA GCC TGC AAG CC. reverse: GCT TTG TAG ACT GTG CCT CCG TTG CC. Cycling parameters were optimized as follows: denaturation 95°C (0 s), annealing 55°C (5 s), extension 72°C (24 s), and detection 80°C (1 s). Quantification of Cdc42 and ERK5 mRNA levels relative to GAPDH mRNA levels were performed using the cycler software.

WESTERN BLOTTING

For Western blot analysis, cell extracts (50 μg/lane) were denatured and resolved in 10% SDS-PAGE, and then electrotransferred onto Immuno-Blot™ PVDF membrane (Bio-Rad). After blocking the non-specific proteins with 5% BSA, the membranes were incubated with anti-phospho ERK5 (1:1000), anti-ERK5 (1:1000), anti-Cdc42 (1:250), anti-RhoA (1:1000), anti-HA (1:1000) or anti-GAPDH (1:10,000). After three washes, the blots were incubated with 1:20,000 anti-mouse horseradish peroxidase-conjugated secondary antibodies or 1:40,000 anti-rabbit horseradish peroxidase-conjugated secondary antibodies and detection was performed using enhanced chemiluminescence (ECL).

INTRINSIC CELL MIGRATION AND INVASION ASSAYS

Following transfection of adenovirus or siRNA, migration of MDA-MB-231 and Hs578T cells was assessed using 24-well transwell permeable supports with multiporous (8 μm pore size/6.6mm diameter) polycarbonate membranes (Corning). Cells (1×10^5cells/ml) were suspended in RPMI 1640 media supplemented with 0.1% BSA. One hundred micro liter of cell suspension from treatment group or control group was plated on the upper chambers of the transwell system already containing 100 μl of serum free media (SFM) and the lower chamber containing 800 μL of SFM. The cells were then allowed to migrate for 24 h at 37°C, 5% CO_2. Non-migrant cells were gently swabbed off the insert membranes, and then the remaining migratory cells were fixed and stained using Harleco Hematocolor staining kit (EM Science, Gibbstown, New Jersey) and counted using light microscopy (100× magnification). Cells throughout the membrane were counted. For invasion assay, cells were allowed to migrate through the filter coated with 50 μl of 18 mg/ml Cultrex® basement membrane extract (BME) (Trevigen, Gaithersburg, Maryland) of a transwell system. Experiments were performed in triplicate. For plotting the data, migration and invasion scores were converted to migration/invasion indices.

IMMUNOFLUORESCENCE STAINING FOR ERK5

Formalin-fixed, paraffin-embedded tumor samples and its adjacent normal tissue samples of breast cancer patients were obtained from London Health Sciences Centre, London, Ontario. Representative 4 μm sections from formalin-fixed, paraffin-embedded tumor samples and its adjacent normal tissue were warmed at 60°C for 20 min. Then the slides were deparaffinized by xylene (10 min × 3) and rehydrated. The slides were then blocked with 10% horse serum in PBS for 30 min and incubated with first antibody goat anti-ERK5 (sc-1284) from Santa Cruz (Santa Cruz biotechnology, Inc. Santa Cruz, California) at 4°C for over night. After rinsing with PBS, the slides were incubated with anti-goat IgG. Nucleic acid was stained with Hoechst for 3 min. The slides were mounted in Vectashield Mounting Medium (Vector Laboratories). Images were captured using fluorescence microscopy at 40× magnification.

CELL SURVIVAL/PROLIFERATION ASSAY

The effects of 48 h of siRNA transfections into breast cancer cells on cell survival/proliferation were assessed using cell proliferation kit WST-1 (Roche Diagnostics; Laval, Quebec, Canada).

STATISTICAL ANALYSIS

Migration and invasion assay results were converted to indices by normalizing each value as a percent of the control. The data were expressed as the mean ± SD and analyzed by analysis of variance (ANOVA), followed by Newman–Keuls test whenever appropriate. Student's *t* tests were used to compare the mRNA levels after gene-specific siRNA transfections with those of their respective controls, as obtained by real-time RT-PCR. Differences at P values <0.05 were considered significant. Gene expression changes between cancer and benign samples were assessed using a paired two-sample *t*-test. All statistical analyses were performed using GraphPad Prism for Windows version 4.0 (GraphPad).

RESULTS

Cdc42 REGULATES ERK5 PHOSPHORYLATION LEVEL IN BREAST CANCER CELLS

The Kinetworks™(KPSS 11.0) signal transduction protein profiling of breast cancer cell line Hs578T following Cdc42 siRNA treatment indicated that downregulation of Cdc42 in these cells increased ERK5 phosphorylation by fivefold without exhibiting any significant change in the phosphorylation of other MAP kinases such as p38, JNK and ERK1/2 MAP kinases (Zuo et al., 2012). We validated these results by using two sets of Cdc42 siRNA to knockdown Cdc42 in Hs578T cells. Western blot analysis showed that Cdc42 siRNAs markedly reduced Cdc42 protein expression in Hs578T cells (Fig. 1A). Down-regulation of Cdc42 by both sets of siRNA also inhibited GTP-Cdc42 levels without exhibiting any change in the levels of GTP-Rac1 or GTP-RhoA (data not shown). Down-regulation of Cdc42 by both sets of siRNA significantly increased the phosphorylation of ERK5 (Fig. 1A). We sought to investigate whether other members of Rho GTPase family, that is, RhoA and Rac1, could also regulate ERK5 phosphorylation in breast cancer

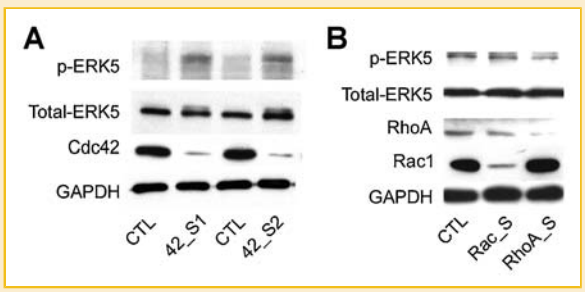

Fig. 1. Negative regulation of ERK5 phosphorylation by Cdc42. Western blot showing that down-regulation of Cdc42 increases the phosphorylation of ERK5 in breast cancer cell line Hs578T (A). Cdc42 protein was significantly knocked down by two sets of siRNA (42_S1, 42_S2). Knocking down Cdc42 resulted in significantly increased phosphorylaiton of ERK5 (p-ERK5) without exhibiting any significant change in the level of total ERK5 (A). Down-regulation of Rho GTPases Rac1 or RhoA by siRNA (Rac_S, RhoA_S) did not increase the levels of phosphorylated or total ERK5 in these cells (B). The experiments were reproduced in three separate occasions.

cells. RhoA and Rac1 siRNAs were used to specifically knockdown the expressions of RhoA and Rac1, respectively (Fig. 1B). Western blots showed that RhoA siRNA significantly decreased RhoA protein level, but had no effect on Rac1 or Cdc42 protein level. Likewise Rac1 siRNA decreased Rac1 protein level, but not RhoA or Cdc42 protein level. Downregulation of neither Rac1 nor RhoA increased p-ERK5 and total ERK5 protein levels (Fig. 1B). RhoA downregulation rather showed modest downregulation of p-ERK5 level (Fig. 1B). Altogether, the results suggest inhibitory influence of Cdc42 on ERK5 phosphorylation.

UPREGULATION OF ERK5 PHOSPHORYLATION DECREASES AND SILENCING ERK5 INCREASES MIGRATION AND INVASION OF BREAST CANCER CELLS MDA-MB-231 AND Hs578T.

ERK5 is known to be involved in migration of endothelial cells, keratinocyte, aortic smooth muscle cells and hepatic stellate cells [Izawa et al., 2007; Arnoux et al., 2008; Rovida et al., 2008; Spiering et al., 2009]. To investigate the role of MEK5-ERK5 signaling pathway in the migration of breast cancer cells, ERK5 activity was upregulated in Hs578T and MDA-MB-231 breast cancer cells by constitutively active MEK5 adenovirus. Infection of adenovirus was confirmed by measuring the HA-tag expression, which was fused to the mutant MEK5 protein (Fig. 2A). Infection of constitutively active MEK5 adenovirus resulted in significant decrease of migration/invasion (Figs. 2B and C) of both Hs578T and MDA-MB-231 cells.

To complement the gain of function study and to confirm the negative regulation of ERK5 in migration and invasion of breast cancer cells, we used siRNA to specifically knockdown ERK5 protein in breast cancer cells. Real time RT-PCR results showed that siRNA significantly decreased ERK5 mRNA level more than 80% in the two cells lines (Fig. 3A shows results of Hs578T cell line). ERK5 protein levels were also markedly decreased by ERK5 siRNA in both the cell lines (Fig. 3B). Following ERK5 siRNA transfection, migration and invasion assays were performed for the two breast cancer cell lines. The results showed that knocking down ERK5 by siRNA caused significant increase in the migratory and invasive capacities of Hs578T and MDA-MB-231 cells (Figs. 3C and D). Taken together, our results confirm ERK5 as a negative regulator of intrinsic migration of breast cancer cells.

SILENCING OF ERK5 INCREASES VIMENTIN EXPRESSION IN BREAST CANCER CELLS

The acquisition of a migratory phenotype of an epithelial cell is associated with the expression of mesenchymal cell markers. One of the most important epithelial–mesenchymal transition (EMT) markers is vimentin [Franke et al., 1982]. To find out whether increased migration of ERK5-silenced cells is associated with increased expression of vimentin, we measured its mRNA levels by real time RT-PCR and protein levels by immunocytochemical localization in control siRNA-transfected and ERK5 siRNA-transfected Hs578T cells. Indeed, ERK5-down-regulation in Hs578T cells resulted in enhancement of both mRNA (Fig. 4A) and protein levels of vimentin (Fig. 4B). While these results do not prove vimentin as a mediator of the effect of ERK5 knockdown in cell migration, they certainly provide a supporting evidence for the regulatory role of ERK5 on EMT of breast cancer cells.

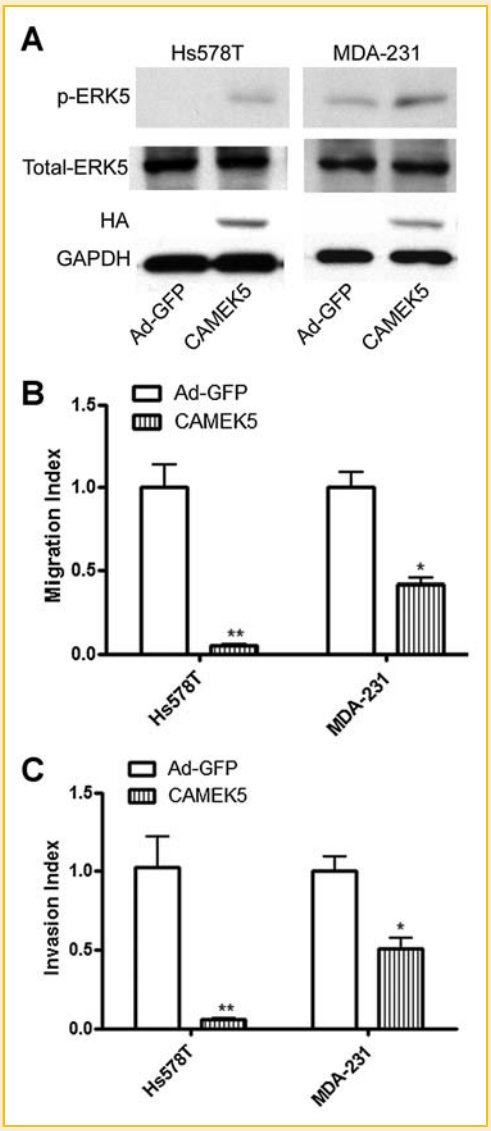

Fig. 2. Enhanced ERK5 phosphorylation decreases migration and invasion of breast cancer cells MDA-MB-231 and Hs578T. Overexpression of constitutively activated MEK5 (CAMEK5) by infection of CAMEK5 adenovirus was confirmed by expression of CAMEK5 fusion protein HA using Western blot (A). Overexpression of CAMEK5 significantly increased ERK5 phosphorylation in breast cancer cell lines Hs578T and MDA-MB-231 without exhibiting any significant change of their total ERK5 levels (A). CAMEK5 overexpression was associated with decreased migration (B) and invasiveness (C) of Hs578T and MDA-MB-231 cells. $^{*}P < 0.05$; $^{**}P < 0.01$ compared with Ad-GFP (control) and CAMEK5-transfected groups. The experiments were reproduced in three separate occasions.

ERK5 EXPRESSION WAS REDUCED IN BREAST CANCER TISSUES COMPARED WITH THEIR ADJACENT NORMAL BREAST TISSUES

On the basis of our in vitro results, we hypothesized that breast cancer cells in the tumor tissues show reduced ERK5 expression acquiring more migratory ability. To test this, we compared the expressions of MEK5 and ERK5 in tumor tissues with its adjacent uninvolved mammary tissues from nine breast cancer patients (A-I)

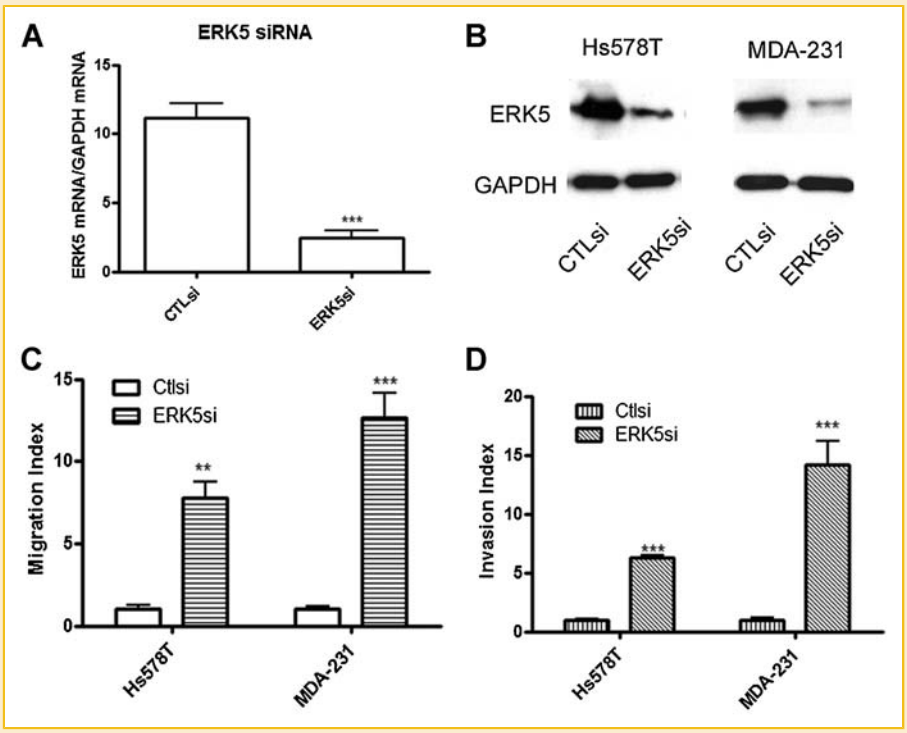

Fig. 3. ERK5 silencing significantly increases migration and invasiveness of breast cancer cells. Transfection of ERK5 siRNA significantly reduced the level of ERK5 mRNA in Hs578T cells (A). ***$P < 0.001$ compared with control siRNA (CTLsi) and ERK5 siRNA (ERK5si). ERK5 siRNA transfection in MDA-MB-231 cells also resulted in similar reduction of its mRNA level (data not shown). Western blot results showed that ERK5 siRNA significantly reduced ERK5 protein expression in Hs578T and MDA-MB-231 cells (B). Knocking down ERK5 by siRNA significantly increased cell migration (C) and invasiveness (D) of Hs578T and MDA-MB-231 cells. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ compared with control siRNA (Ctlsi) and ERK5 siRNA (ERK5si); n = 3 per group. The experiments were reproduced on two separate occasions.

whose profiles are shown in Table I. Real-time RT-PCR results showed that MEK5 mRNA levels in tumor tissues were not significantly different from the controls (adjacent uninvolved mammary tissues) (Fig. 5A). However, ERK5 mRNA levels were shown to be downregulated in eight out of nine tumor tissues in comparison with their adjacent normal controls (Fig. 5B). Due to the lack of a specific antibody that would only detect p-ERK5 in immunohistochemical analyses, we assessed total ERK5 protein levels. We stained ERK5 in breast cancer tissue sections and its adjacent uninvolved tissue sections by immuofluorescence staining. In the adjacent normal control tissue sections, ERK5 was diffusely expressed in the epithelial duct cells of breast tissues (Fig. 6). However, in most of the cancer tissues, ERK5 staining was negative or much less than the adjacent normal tissues (Fig. 6). This result was consistent with the mRNA level of ERK5 in breast cancer tissue and adjacent normal tissue, suggesting that ERK5 negatively regulates breast tumorigenesis.

DISCUSSION

In this study, we have demonstrated for the first time that the modulation of ERK5, a member of the MAPK family, is an important mechanism responsible for Cdc42-induced cell migration. Silencing of Cdc42, but not that of RhoA or Rac1 induced phosphorylation of ERK5. ERK5, also known as big MAP kinase 1 (BMK1), is a member of MAPK family, which also includes extracellular-regulated protein kinases 1 and 2 (ERK1/2), c-Jun NH_2-terminal protein kinases (JNKs), and p38 MAPKs [Pearson et al., 2001]. Like other members of MAPK, the N-terminus of ERK5 contains MAPK catalytic TEY sequence domain. Upon activation, ERK5 can be phosphorylated at Thr (219) and Tyr (221) by a variety of stimuli such as mitogens and stress stimuli [Abe et al., 1996; Kato et al., 1997, 1998; Yan et al., 1999; Watson et al., 2001; Suzaki et al., 2002; Hayashi et al., 2004; Kesavan et al., 2004]. ERK5 can be activated by a wide variety of molecules including Ras [Hayashi and Lee, 2004]. We, however, have not explored the potential involvement of Ras signaling pathway in the Cdc42 regulation of ERK5. ERK5 has been implicated in many physiological functions such as cell survival, proliferation, differentiation and migration. The role of ERK5 in cell migration remains elusive. While keratinocyte and vascular smooth muscle cell migrations were shown to be positively regulated [Izawa et al., 2007; Arnoux et al., 2008], hepatic and endothelial cell migrations [Pi et al., 2005; Rovida et al., 2008; Spiering et al., 2009] were negatively regulated by ERK5. Thus, cell migration regulation by ERK5 may be cell-type specific and/or environmentally sensitive. In the present study, we have investigated the role of ERK5 in migration and invasion of breast cancer cells. Upon stimulation, MEK5 specifically activates ERK5 via dual phosphorylation of its TEY motif [Mody et al., 2003]. We found that

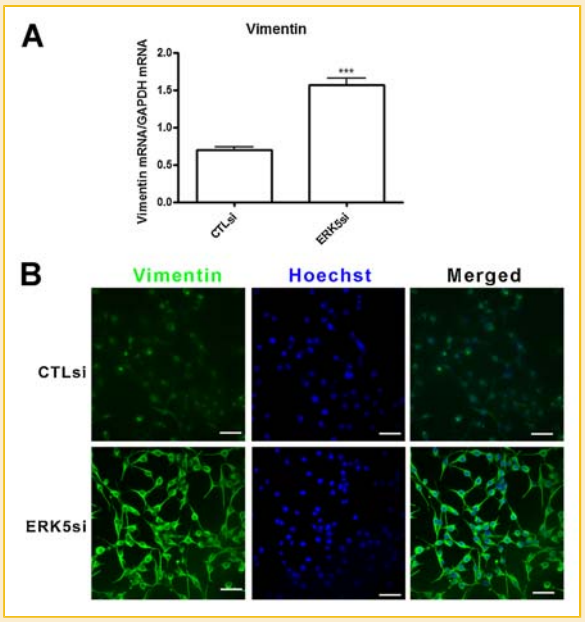

Fig. 4. ERK5 siRNA increases Vimentin expression in breast cancer cells. Vimentin expression was examined in Hs578T cells by Real-time PCR and immunoflurescence staining. Knocking down ERK5 by siRNA significantly increased expression of Vimentin at mRNA level (A). ***$P < 0.001$ compared with control siRNA (CTLsi) and ERK5 siRNA (ERK5si). n = 3 per group. By immunoflurescence staining, weakly positive staining (green) was observed in the cytoplasm of control siRNA (CTLsi) treated cells. In contrast, a much stronger vimentin staining (green) was observed in the cytoplasm of ERK5 siRNA treated cells in comparison with control cells. Nuclear was stained using Hoechst dye (blue) (B). The experiments were reproduced on two separate occasions. The scale bar represents 20 μm.

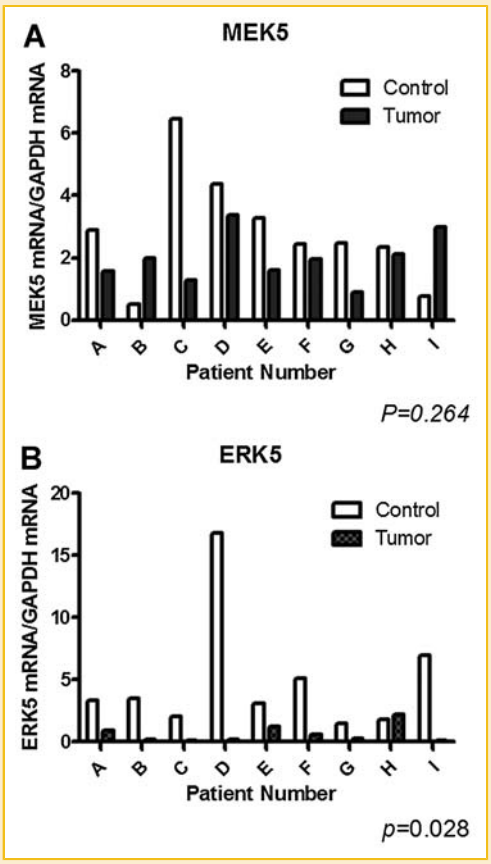

Fig. 5. Expression of ERK5 in tumor samples and their adjacent normal tissues from the breast cancer patients. ERK5 and MEK5 expressions in breast cancer tumors and its adjacent controls from nine patients were examined by Real-time RT-PCR. ERK5 expression was significantly decreased in tumor tissues than their adjacent control tissues ($P = 0.028$, paired t test) (A). ERK5 upstream activator MEK5 had no significant change in tumor tissue in comparison with its normal control ($P = 0.264$, paired t test) (B).

activation of ERK5 by a constitutively active MEK5 adenovirus significantly decreased migration and invasion of both MDA-MB-231 and Hs578T cells. Conversely, knocking down ERK5 by siRNA significantly increased migration and invasion of breast cancer cells. In one study, ERK5 phosphorylation was shown to positively regulate micromotion of MDA-MB-231 cells, as measured by the electric cell-substrate impedance sensing (ECIS) technique [Sawhney et al., 2009]. This technique however, is more sensitive for measuring cell adhesion and spreading [Keese et al., 2004] than cell migration [Noiri et al., 1998; Lee et al., 2004]. In some studies, increased micromotion of cells as measured by ECIS technique were found to be associated with decreased migration or invasion in Transwell assays [Noiri et al., 1998; Lee et al., 2004]. Like the previous studies in other cell lines, we also found ERK5 to positively

TABLE I. Clinicopathological Parameters of Nine Breast Cancer Cases

Case no.	Age (year)	Tumor diameter	Tumor type	SBR grade (score)	Positive axillary lymph node (%)	Nuclear pleomorphism score	Tubular score	Adjacent non-tumor ratio for Erk5 mRNA
A	97	5 cm	IMC	III (9)	1 of 9	ND	ND	3.7
B	60	4 cm	IMC	II (7)	15 of 15	3	3	18.31
C	67	0.5 cm	IMC	III (8)	2 of 9	2	3	12.62
D	56	4.2 cm	IMC	II (6)	4 of 14	ND	ND	80.0
E	79	4.5 cm	IMC	III (9)		3	3	2.4
F	59	4.2 cm	IMC	II (?)	2 of 11	2	3	8.42
G	48	1.5 cm	IMC	I (5)	0 of 15	ND	ND	4.77
H	64	1.4 cm	IMC	III (9)	0 of 21	3	3	0.82
I	78	1.6 cm	IMC	II (6)	0 of 13	2	2	46.46

IMC, invasive mammary carcinoma; SBR, Scarff-Bloom-Richardson; ND, not determined: Gr. III is the highest grade ('9' is the highest score).

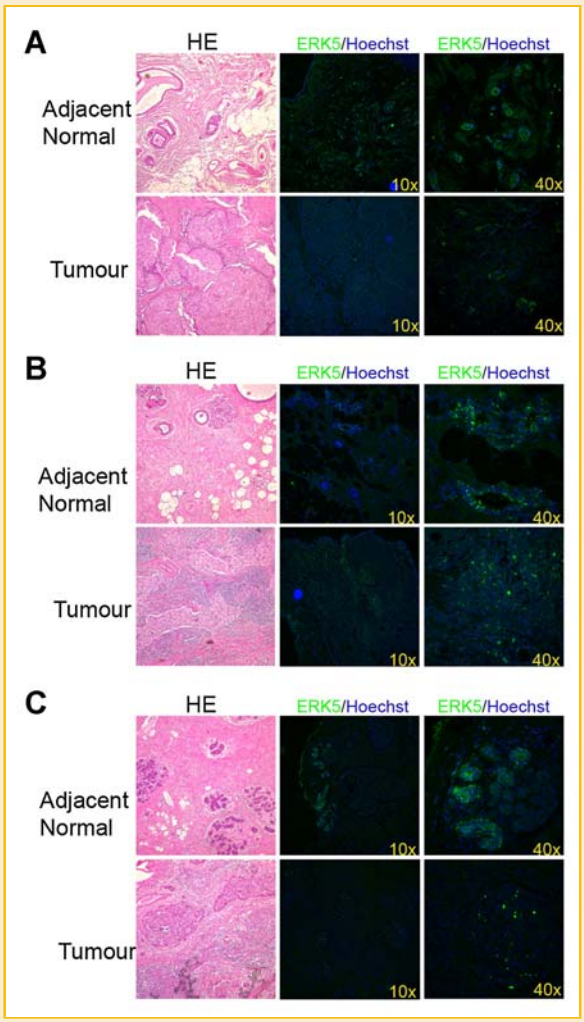

Fig. 6. Immunohistochemical staining analyzes expression of ERK5 in breast cancer tumor samples and the adjacent control samples. ERK5 protein expression in breast cancer tumor tissues were examined by immunofluorescence using specific antibody against ERK5 (green). Nuclei were stained by DAPI (blue). HE staining showed the structure of tumor cells in tumor samples and the normal cells in the adjacent control tissues. Three sections from tumor and three sections from control tissues of each patient were stained but the results of three representative patients (A–C) are shown here. In all nine patients, ERK5 expression was much higher in adjacent control than the tumor tissue. ERK5 was diffusely expressed in the cytoplasm of epithelial cells of breast ducts of all unaffected control mammary tissues.

regulate proliferation of breast cancer cells (data not shown). Thus, down-regulation of ERK5, while decreasing the proliferative capacity of breast cancer cells, may enhance their migratory and invasive abilities. Activated ERK5 is known to exert its physiological and pathological effects through its substrates such as MEF2, Sap1a, SGK, Cx43, and Bad [Hayashi and Lee, 2004]. We have not explored whether any of these downstream substrates has been used by ERK5 to modulate migration/invasion of breast cancer cells.

Vimentin is an intermediate filament protein in mesenchymal cells and a marker for EMT, which plays a pivotal role in cancer progression [Franke et al., 1982; Guarino et al., 2007]. In human breast cancer cell lines the expression of vimentin was found to be induced in invasive cell lines [Sommers et al., 1989, 1992]. Expression of vimentin was closely associated with poor prognosis in breast cancer [Thomas et al., 1999]. Furthermore, in vitro studies directly showed that downregulation of vimentin impaired breast cancer cell migration and adhesion [McInroy and Maatta, 2007]. Therefore, an increased expression of vimentin in breast cancer cell line Hs578T following downregulation of ERK5 in the present study is suggestive of ERK5 as negative modulator of EMT.

We found that the expression of ERK5 in human breast tumor tissue was decreased as compared to their adjacent uninvolved breast tissue of most cancer patients. These results along with the previous reports [Fritz et al., 1999, 2002] showing over-expression of Cdc42 protein by the breast tumor tissue as compared with the adjacent control tissue further supports the findings of our in vitro experiments of the inverse correlations of ERK5 and Cdc42 expression in breast cancer cells. Therefore, breast cancer cells may acquire more migratory ability by decreasing expression of ERK5. We were not able to find any correlation between ERK5 expression and any other parameters that were studied in these patients, which included tumor grade, estrogen and progesterone receptor (ER and PR) status, presence of lymph node metastasis etc. However, a case control study by Montero et al. [2009] showed overexpression of ERK5 in 20% breast cancer patients. According to these authors, the patients with ERK5 overexpressions had poor prognosis [Montero et al., 2009]. Surprisingly, they did not find correlation of any other well established parameters with the prognosis of breast cancer patients. Whereas Montero et al. [Montero et al., 2009] found no correlation of lymph node status with the prognosis of the patients, de Boer et al. [2009] in a large cohort study found strong correlation of the presence of regional lymph nodes with a reduced 5-year rate of disease-free survival of breast cancer patients. While we cannot predict the prognosis of our nine patients with low ERK5 expressions in cancer tissues, the findings by Montero et al. [2009] are likely to be confounded by factors such as adjuvant therapy.

In summary, we have shown that Cdc42 but not RhoA or Rac1 mediates ERK5 signaling in the regulation of breast cancer cell migration and invasion. Though many effector pathways are shared by Rac1 and Cdc42, the fact that Rac1 does not modulate ERK5 may suggest involvement of effector pathways that are unique to Cdc42 in this process. Ack1 & 2 [Yang and Cerione, 1997; Modzelewska et al., 2006], Borg proteins [Joberty et al., 2001] and MRCK [Wilkinson et al., 2005] are examples of such pathways. Further studies are required to find out whether any one of these effector pathways is used by Cdc42 to regulate ERK5 expression in breast cancer cells.

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