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Activation of H-Ras and Rac1 correlates with epidermal growth factor-induced invasion in Hs578T and MDA-MB-231 breast carcinoma cells

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ABSTRACT

There is considerable experimental evidence that hyperactive Ras proteins promote breast cancer growth and development including invasiveness, despite the low frequency of mutated forms of Ras in breast cancer. We have previously shown that H-Ras, but not N-Ras, induces an invasive phenotype mediated by small GTPase Rac1 in MCF10A human breast epithelial cells. Epidermal growth factor (EGF) plays an important role in aberrant growth and metastasis formation of many tumor types including breast cancer. The present study aims to investigate the correlation between EGF-induced invasiveness and Ras activation in four widely used breast cancer cell lines. Upon EGF stimulation, invasive abilities and H-Ras activation were significantly increased in Hs578T and MDA-MB-231 cell lines, but not in MDA-MB-453 and T47D cell lines. Using small interfering RNA (siRNA) to target H-Ras, we showed a crucial role of H-Ras in the invasive phenotype induced by EGF in Hs578T and MDA-MB-231 cells. Moreover, siRNA-knockdown of Rac1 significantly inhibited the EGF-induced invasiveness in these cells. Taken together, this study characterized human breast cancer cell lines with regard to the relationship between H-Ras activation and the invasive phenotype induced by EGF. Our data demonstrate that the activation of H-Ras and the downstream molecule Rac1 correlates with EGF-induced breast cancer cell invasion, providing important information on the regulation of malignant progression in mammary carcinoma cells.

1. Introduction

Ras proteins relay extracellular signals to cytoplasmic signaling networks, causing diverse cellular functions [1]. The activation of Ras contributes to malignant phenotypic conversion of cells including invasiveness and angiogenesis [2,3]. In spite of low frequency (<5%) of mutated forms of *ras* in breast cancer, there is growing evidence that suggests hyperactive Ras proteins promote breast cancer growth and development [1,4].

The Ras family consists of three isoforms, H-Ras, K-Ras (A and B), and N-Ras, which share almost complete sequence homology between amino acid 1–165 but the carboxy-terminal hypervariable region, which consists of residues 166–189, is highly divergent [2,5,6]. We have previously shown that H-Ras, but not N-Ras, induces an invasive phenotype in MCF10A human breast epithelial cells, whereas both induce a transformed phenotype [7]. In addition, H-Ras activation mediated by the Rac1-MKK3/6-p38 MAPK pathway is essential for cell invasion and motility, while the Raf-MEK-ERK and PI3K-Akt pathways are common signaling pathways activated by H-Ras and N-Ras in MCF10A human breast epithelial cells [8,9]. Among widely used human breast cancer cell lines, Hs578T and MDA-MB-231 cell lines are known to express an active mutant of H-Ras (G12D) and K-Ras (G13D), respectively [10].

EGF and its cognate receptor EGFR play important roles in the aberrant growth of many tumor cell types, including breast, lung, and colorectal cancers [11]. EGF signaling has been implicated in tumor invasion and metastasis through the activation of Ras downstream pathways. Ras-MEK1/2-ERK and Ras-Cdc42-Rac signaling pathways are major downstream pathways for EGF-induced invasion and motility [12–15]. Although the crucial role of EGF in Ras activation and malignant cancer progression has been demonstrated, differential activation of Ras isoforms in response to EGF stimulation in breast cancer cell lines has not been reported yet.

The present study aims to investigate the involvement of H-Ras in the invasive phenotype induced by EGF using four human breast cancer cell lines, Hs578T, MDA-MB-231, MDA-MB-453, and T47D. Hs578T and MDA-MB-231 cells are invasive cell lines whereas MDA-MB-453 and T47D cells are originally not invasive [1,16]. Here, we show that EGF enhanced H-Ras activation and invasiveness in Hs578T and MDA-MB-231 cells, but not in MDA-MB-453 and T47D cells. Using siRNA-induced knock-down of H-Ras and Rac1, we also show a crucial role of H-Ras and Rac1 in the invasive phenotype induced by EGF in Hs578T and MDA-MB-231 cells.

Abbreviations: EGF, epidermal growth factor; siRNA, small interfering RNA.

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2. Materials and methods

2.1. Cell lines and culture conditions

Hs578T and MDA-MB-453 breast cancer lines were purchased from the Korean Cell Line Bank (KCLB). MDA-MB-231, T47D cells were kindly provided by Dr. Dong Young Noh (Seoul National University, Seoul, Korea). MDA-MB-231, Hs578T, MDA-MB-453 cells were cultured in DMEM supplemented with 10% FBS and 100 μg/ml penicillin–streptomycin, whereas T47D cells were cultured in RPMI1640 supplemented with 10% FBS and 100 μg/ml penicillin–streptomycin. Cells were maintained in humidified atmosphere with 95% air and 5% CO_2 at 37 °C.

2.2. In vitro invasion assay

The *in vitro* invasion assay was modified from a previously described method [17]. The assay was performed using a 24-well transwell unit with polycarbonate filters (Corning Costar, Cambridge, MA, USA). The lower side of the filter was coated with type I collagen, and the upper side was coated with 100 μg Matrigel (Collaborative Research, Lexington, KY, USA). The lower compartment was filled with serum-free media containing 0.1% BSA and 10 ng/ml EGF. Cells were placed in the upper part of the transwell plate containing 0.1% BSA, incubated for 17 h for invasive Hs578T and MDA-MB-231 cells or 24 h for non-invasive MDA-MB-453 and T47D cells, fixed with methanol and stained with hematoxylin for 10 min followed briefly by eosin. The cells on the upper surface of the filter were removed using a cotton swab. The invasive phenotypes were determined by counting the cells that had invaded the lower side of the filter using light microscopy (Olympus CKX31, Tokyo, Japan) at 400× optical resolution. Thirteen fields were counted for each filter and each sample was assayed in triplicate.

2.3. Immunoblot analysis

Immunoblot analysis was performed as previously described [8]. Protein extracts in MLB buffer were subjected to 12% SDS–PAGE analysis and transferred to nitrocellulose membrane. Polyclonal anti-H-Ras, and monoclonal anti-N-Ras, monoclonal anti-K-Ras antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Anti-Rac1 antibodies were from Upstate Biotechnology, Inc. (Lake Placid, NY, USA). The enhanced chemiluminescence system (ECL, Amersham-Pharmacia, UK) was used for detection. Relative band intensities were determined by quantitation of each band with a Gel Logic 200 Imaging System (Kodak, Rochester, USA).

2.4. Ras activity assay

The levels of Ras-GTP were measured by affinity precipitation using a GST fusion protein containing the Ras-binding domain (GST-Raf-RBD) (Upstate Biotechnology, Inc., Lake Placid, NY, USA) following the manufacturer's instructions. The cells were exposed to 10 ng/ml EGF (Sigma–Aldrich, St. Louis, MI, USA) for 30 min and then immediately lysed in MLB buffer. Raf-1 RBD-agarose was added to 300–400 μg of proteins present in lysates and incubated for 45 min at 4 °C. The bead pellet was washed three times with MLB buffer and proteins were immunoblotted with specific Ras isoform antibodies.

2.5. Rac1 activity assay

The levels of Rac1-GTP were measured by affinity precipitation using the PAK-1 p21-binding domain Rac Assay Reagent (GST-PAK-RBD) (Upstate Biotechnology, Inc., Lake Placid, NY, USA). After exposure of EGF (10 ng/ml, 30 min), the cell lysates (100–150 μg proteins) were subjected to a pull-down analysis with agarose beads. The subsequent experimental procedures were the same as the Ras activity assay described above except that the incubated time was 1 h and proteins were immunoblotted with anti-Rac1 antibody.

2.6. siRNA preparation and transfection

The siRNA molecules targeting H-Ras (VHS40291), Rac1 (VHS40448) and Stealth™ RNAi-negative control duplex (12935-300) were purchased from Invitrogen (Validated Stealth RNAi, Carlsbad, CA, USA). K-Ras siRNA (sc-35731), N-Ras siRNA (sc-36004), control siRNA (sc-37007) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Hs578T and MDA-MB-231 cells were plated in six-well plates at 1.5×10^5 cells/well, grown for 24 h, then transfected with 100 pmol of siRNA for 6 h using Lipofectamine 2000 reagent and OPTIMEM reduced serum medium (Invitrogen, Carlsbad, CA, USA).

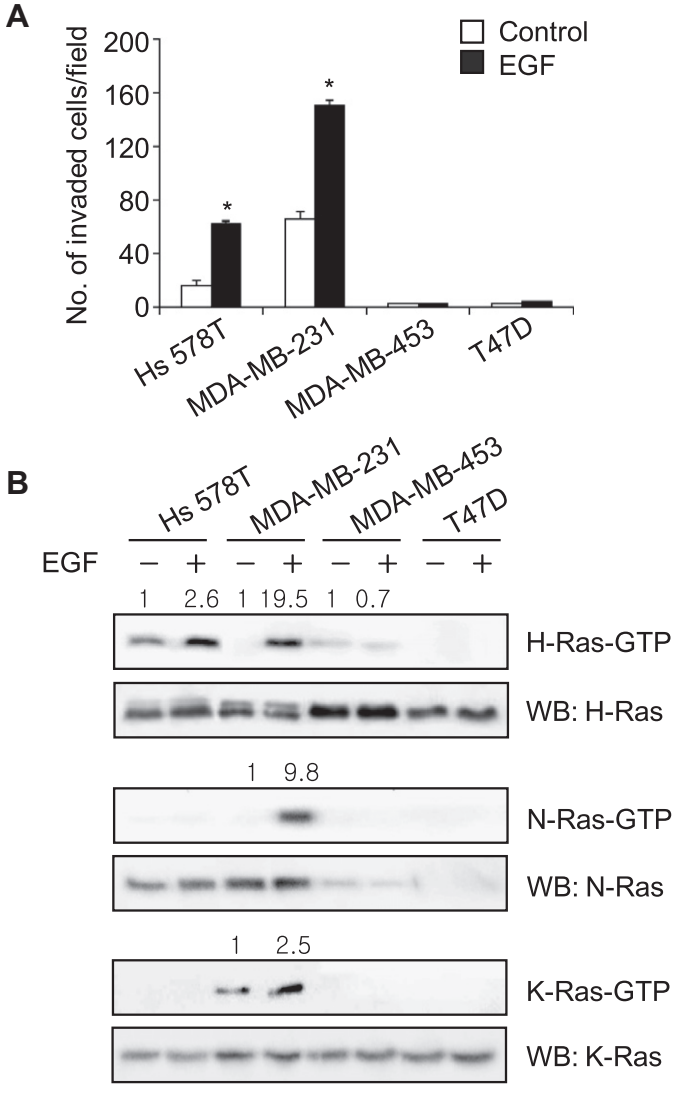

Fig. 1. EGF enhances H-Ras activation and invasion in Hs578T and MDA-MB-231 cells. (A) Breast cancer cells (1×10^5/well) were subjected to *in vitro* invasion assay. The number of invaded cells per field was counted (400× optical resolution) in thirteen arbitrary visual fields. ∗, Statistically different from control at $p < 0.01$. (B) Serum-starved breast cancer cells were exposed to 10 ng/ml EGF for 30 min and then subjected to Ras activity assay and immunoblot analysis.

3. Results

3.1. EGF enhances invasion and H-Ras activation in Hs578T and MDA-MB-231 cells

We investigated the effect of EGF on the invasive phenotype of four commonly used breast cancer cell lines. As shown in Fig. 1A, treatment of EGF (10 ng/ml) significantly enhanced the invasive abilities of Hs578T cells and MDA-MB-231 cells. In contrast, invasive phenotypes of MDA-MB-453 and T47D cells, which are non-invasive cell lines, were not significantly affected by EGF. This demonstrates that EGF enhances invasion in Hs578T and MDA-MB-231 cells, but not in MDA-MB-453 and T47D cells.

In order to examine the differential activation of Ras isoforms in breast cancer cell lines by EGF stimulation, we conducted a pull-down assay using a GST fusion protein containing the Ras-binding domain (GST-Raf-RBD). In Hs578T cells where H-Ras is originally activated, treatment with EGF significantly increased the active form of H-Ras (Fig. 1B). Neither K-Ras nor N-Ras was activated by EGF in Hs578T cells. In MDA-MB-231 cells, which originally contain the active mutant of K-Ras, EGF treatment markedly increased activation of H-Ras as well as that of K-Ras and N-Ras. No Ras isoform activation was detected in MDA-MB-453 and T47D cells upon EGF treatment. This demonstrates that EGF activates H-Ras in Hs578T and MDA-MB-231 cells, in which invasive abilities were enhanced by EGF, implying that H-Ras activation correlates with EGF-induced invasive properties of breast cancer cells.

3.2. H-Ras is critical for the invasive phenotype of breast cancer cells by EGF

To investigate if H-Ras activation is responsible for the EGF-induced invasive capacities of Hs578T and MDA-MB-231 cells, we knocked-down H-Ras by siRNA targeting H-Ras. Transfection with siRNA effectively inhibited both the active form of H-Ras and total H-Ras (Fig. 2A). EGF-induced invasive abilities of Hs578T and MDA-MB-231 cells were significantly inhibited by the knock-down of H-Ras by 70% and 38%, respectively (Fig. 2B). These results demonstrate that H-Ras is crucial for the EGF-induced invasive phenotype in Hs578T and MDA-MB-231 cells.

Since EGF activated not only H-Ras, but also N-Ras and K-Ras in MDA-MB-231 cells, we examined the involvement of N-Ras and/or K-Ras in the EGF-induced invasive phenotype. N-Ras and K-Ras of MDA-MB-231 cells were effectively knocked-down by siRNA molecules as confirmed by immunoblot analysis (Fig. 2C). As shown in Fig. 2D, the knock-down of K-Ras, but not that of N-Ras, significantly inhibited the EGF-induced invasive phenotype in MDA-MB-231 cells, implying that K-Ras is additionally required for the invasive abilities of MDA-MB-231 cells upon EGF stimulation.

3.3. Rac1 is required for the EGF-induced invasive phenotype of breast cancer cells

Rac1 is responsible for Ras-induced phenotype changes by regulating motility and invasion in mammary epithelial cells [18]. We have previously reported that enhanced invasion induced by H-Ras is mediated by Rac1 in MCF10A cells [8,9]. To assess the involvement of Rac1 in the EGF-induced invasive phenotype of Hs578T and MDA-MB-231 cells, we first examined if EGF activated Rac1 in these cells. As shown in Fig. 3A, Rac1 activity increased in response to EGF stimulation. The level of the active form of Rac1(Rac1-GTP) decreased by the knock-down of H-Ras using siRNA targeting H-Ras, demonstrating that activation of Rac1 by EGF was dependent on H-Ras in Hs578T and MDA-MB-231 cells (Fig. 3B). Since both N-Ras and K-Ras were activated by EGF treatment in MDA-MB-231 cells, we next examined if N-Ras and K-Ras

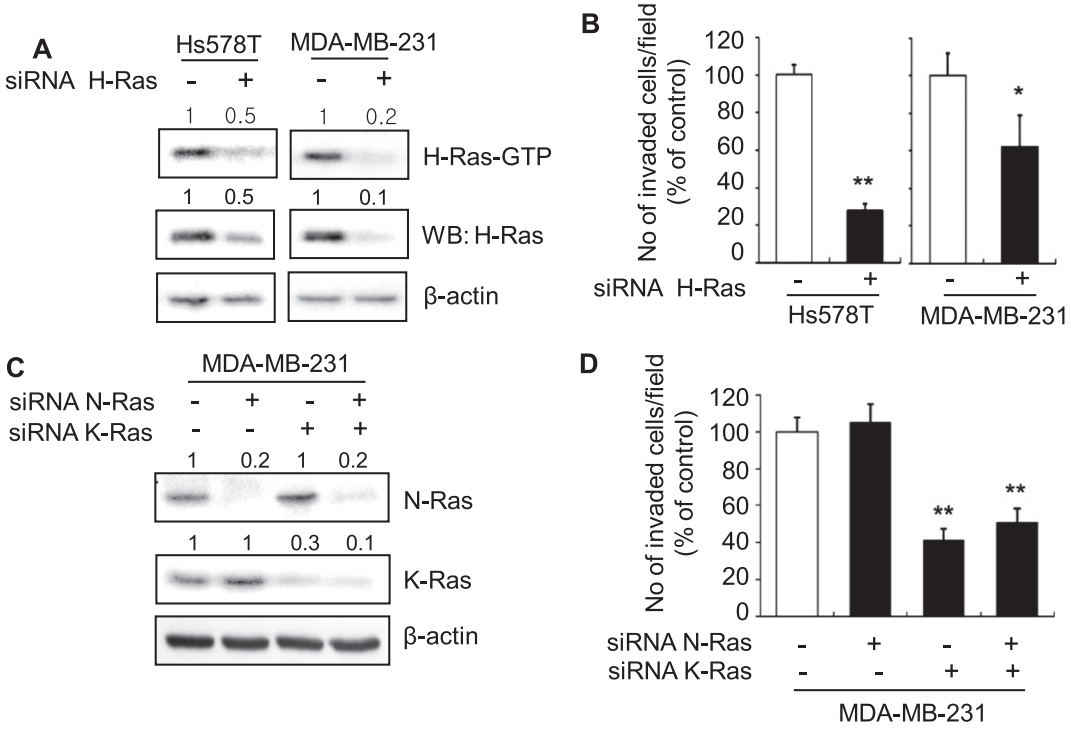

Fig. 2. H-Ras is critical for the invasive phenotype of breast cancer cells induced by EGF. (A) Hs578T and MDA-MB-231 cells were transfected with control siRNA or siRNA H-Ras. Serum-starved cells were exposed to 10 ng/ml EGF for 30 min and then subjected to Ras activity assay and immunoblot analysis. (C) MDA-MB-231 cells were transfected with siRNA molecules targeting N-Ras and K-Ras. Then serum-starved cells were subjected to immunoblot analysis. (B and D) Hs578T (1×10^5 cells/well) and MDA-MB-231 (5×10^4 cells/well) cells were subjected to *in vitro* invasion assay. The number of invaded cells per field was counted (400× optical resolution) in thirteen arbitrary visual fields. *,** Statistically different from control at $p < 0.05$ and $p < 0.01$, respectively.

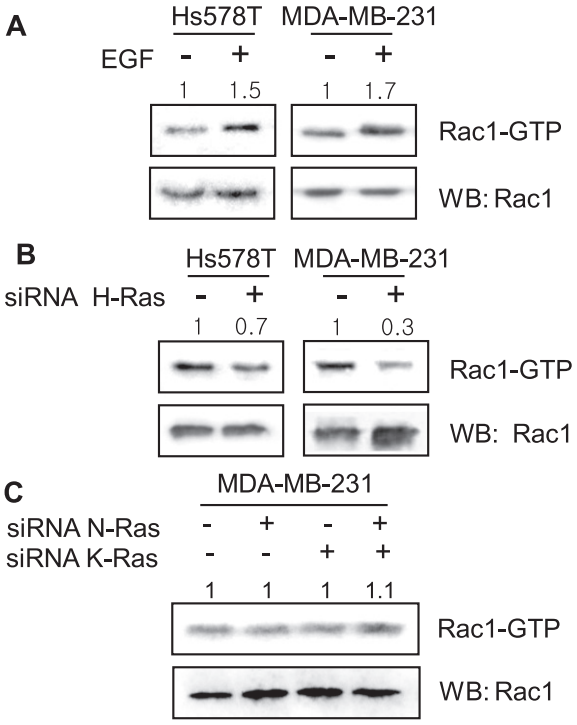

Fig. 3. Activation of Rac1 by EGF is dependent on H-Ras. (A) Serum-starved Hs578T and MDA-MB-231 cells were exposed to 10 ng/ml EGF for 30 min and then subjected to Rac1 activity assay. (B) Hs578T and MDA-MB-231 cells were transfected with control siRNA or siRNA H-Ras. (C) MDA-MB-231 cells were transfected with siRNA molecules targeting N-Ras and K-Ras. Serum-starved cells were exposed to 10 ng/ml EGF for 30 min and subjected to Rac1 activity assay and immunoblot analysis.

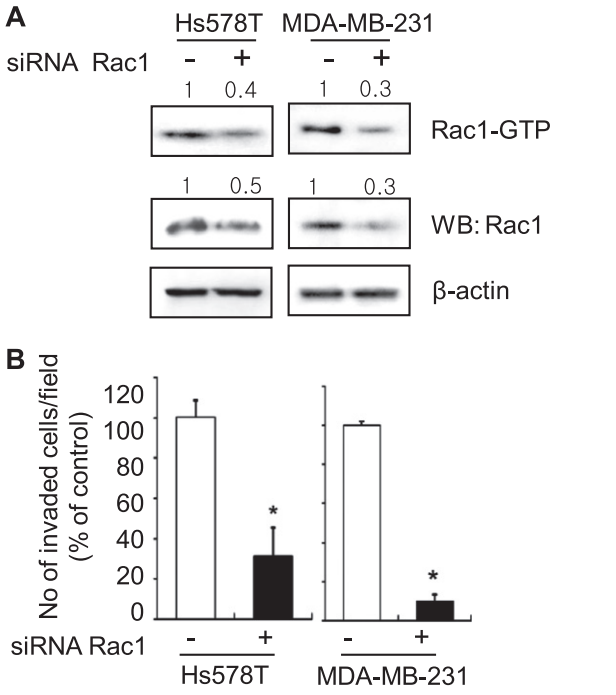

Fig. 4. Rac1 is required for the EGF-induced invasive phenotype of breast cancer cells. (A) Hs578T and MDA-MB-231 cells were transfected with control siRNA or siRNA Rac1. Serum-starved these cells were exposed to 10 ng/ml EGF for 30 min and then subjected to Rac1 activity assay and immunoblot analysis. (B) These Hs578T (1×10^5 cells/well) and MDA-MB-231 (5×10^4 cells/well) were subjected to *in vitro* invasion assay. After incubation, the number of invaded cells per field was counted (400× optical resolution) in thirteen arbitrary visual fields. ∗, Statistically different from control at $p < 0.01$.

also contributed to the activation of Rac1. The knock-down of neither N-Ras nor K-Ras affected the level of the active form of Rac1, demonstrating that the activation of Rac1 was not dependent on N-Ras or K-Ras in MDA-MB-231 cells (Fig. 3C).

To examine the role of Rac1 in EGF-enhanced invasive abilities of breast cancer cells, we knocked-down Rac1 by siRNA. Both active Rac1 and total Rac1 levels were effectively inhibited by siRNA targeting Rac1 as confirmed by immunoblot analysis (Fig. 4A). Data from the *in vitro* invasion assay clearly demonstrated that knock-down of Rac1 significantly inhibited the invasive abilities of Hs578T and MDA-MB-231 cells (Fig. 4B), suggesting that Rac1, a downstream molecule of H-Ras, may play a crucial role in the invasive process during EGF stimulation in Hs578T and MDA-MB-231 human breast cancer cells.

4. Discussion

Breast cancer has been estimated as one of the most common causes of cancer-related death among women and metastasis has been one of the major causes of mortality in breast cancer patients [19]. In response to EGF, the invasive process can be enhanced by chemotaxis to blood vessels, which facilitates the systemic spread of cancer cells [15]. In this study, we show that EGF increased the invasive abilities of MDA-MB-231 and Hs578T cell lines which are originally invasive. Interestingly, EGF did not affect the invasiveness of originally non-invasive MDA-MB-453 and T47D cell lines. These data suggest that EGF alone was not sufficient to induce the invasive phenotype and other stimulators may be required for the induction of invasion in MDA-MB-453 and T47D cells [20,21].

Although mutated *ras* genes are not associated with the majority of breast cancers, it has been reported that aberrant Ras activation may be a common feature of breast cancers [1]. Ras isoforms exhibit differential and cell type-specific biological responses including transforming potential [(reviewed in 22),23]. It would be important to define the role of Ras isoforms that are required for EGF-mediated invasion in breast cancer cells. The present study used four breast cancer cell lines and showed that among Ras isoforms, activation of H-Ras correlates with invasive abilities of breast cancer cells during EGF stimulation. Consistent with these results, a significant role of H-Ras in breast cell invasion has been reported from several laboratories including ours [7–9,24–26].

Inhibition of H-Ras activity by siRNA significantly reduced the EGF-mediated invasion of Hs578T and MDA-MB-231 cells (Fig. 2B). A greater inhibition was observed in Hs578T cells (70%) compared to MDA-MB-231 cells (38%). Since H-Ras was the only Ras activated by EGF in Hs578T cells, the contribution of H-Ras to the EGF-induced invasive phenotype would be more significant in Hs578T cells than in MDA-MB-231 cells where EGF activated not only H-Ras but K-Ras and N-Ras. To address this issue, we examined the involvement of N-Ras and/or K-Ras in the EGF-induced invasiveness of MDA-MB-231 cells and showed that K-Ras, but not N-Ras, played an essential role in the EGF-induced invasive abilities of MDA-MB-231 cells (Fig. 2D), suggesting the contribution of both H-Ras and K-Ras in EGF-mediated invasion of MDA-MB-231 cells (Fig. 1A). Consistent with these results, we have previously reported that N-Ras did not play a role in invasive phenotype of MCF10A human breast epithelial cells [7,9].

Activation of the EGF receptor leads to the activation of Rho family proteins (Rho, Cdc42, Rac), which enable cells to migrate via chemotaxis towards EGF [15]. We have previously revealed a

crucial role of Rac1, which is activated in an H-Ras-specific manner, in breast cell invasion and migration [8]. The present study clearly demonstrates that the activation of Rac1 by EGF plays an important role in the EGF-induced invasive phenotype in Hs578T and MDA-MB-231 cells. Consistent with our findings, the activation of Rac1 is required for the EGF-induced morphological changes by regulating the organization of the actin cytoskeleton [27].

Taken together, this study demonstrates that in response to EGF, H-Ras and the downstream component Rac1 were activated and cell invasion was significantly enhanced in Hs578T and MDA-MB-231 cells, while originally non-invasive MDA-MB-453 and T47D cells did not respond to EGF. These results elucidate a correlation between H-Ras activation and breast cell invasion, providing useful information on the role of EGF and Ras in the regulation of the invasive process of breast cancer cells.

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