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# Activation of the MEK pathway is required for complete scattering of MCF7 cells stimulated with heregulin-β1

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ABSTRACT

Rac1 is important for dissociation of cells during scattering, but whether its activation alone is sufficient to induce complete scattering is not known. To test this, we created an inducible MCF7 cell line that expresses dominant active Rac1. Although induction of dominant active Rac1 resulted in dissociation of cells, their scattering was incomplete. We co-expressed dominant active MKK1a, an activator of ERK, and dominant active Rac1. In this case, cells completely scattered. These results suggest that not only Rac1 but also the MEK1 pathway is required for dissociation and complete scattering of MCF7 cells treated with HRG-β1.



## 1. Introduction

Neighboring epithelial cells form adherens and tight junctions, and coordinately participate in various processes [1,2]. Both types of junctions interact with F-actin [2]. The adherens junction complex consists of E-cadherin, α-catenin, β-catenin, and p120 catenin. Among these proteins, α-catenin directly binds to F-actin and E-cadherins from neighboring cells dimerize ensuring cohesion of epithelial cells and maintenance of tissue architecture [1]. Alternatively, tight junctions are selective barriers that prevent leakage of materials between cells [2,3]. Tight junctions also separate plasma membranes and proteins into apical or basolateral regions [4].

MCF7 cells were derived from breast cancer epithelial cells [4]. Previous studies have shown that heregulin (HRG)-β1 treatment stimulates cell scattering. HRG-β1 also promotes membrane ruffling for several hours in MCF7 cells, unlike most other cells, which terminate ruffling after about ten minutes. The p38 MAP kinase signaling pathway is essential in the scattering process [5]. About 15–18 h after HRG-β1 stimulation, adherens and tight junction proteins translocate from plasma membrane to the cytoplasm enabling the cells to dissociate and scatter. The function of scattering is not well understood in physiological terms. It probably reflects an *in vivo* response to cell division signaling, which triggers breakdown of cell–cell contacts. During the initial phase of scattering, the MEK1 pathway promotes F-actin detachment from adherens and tight junctions [6]. A few hours after F-actin detaches from junctions, cells begin to dissociate [6]. It is widely accepted that Rac1 is a major factor in the dissociation of cells [7,8].

The receptors for HRG-β1 are ErbB3/Her3 and ErbB4/Her4 [9,10]. Because ErbB4 is not expressed in MCF7 cells, it is likely that ErbB3 is the receptor for HRG-β1 in these cells. ErbB3 is a member of the EGF tyrosine kinase receptor family but its tyrosine kinase activity has yet to be detected certainly. In most cases it heterodimerizes with another member of the EGF receptor family with tyrosine kinase activity. The phosphatidylinositol (PI) 3-kinase and MEK pathways are the major signaling pathways activated by ErbB3 [11,12].

In this paper, we sought to determine whether Rac1 activity alone is sufficient to induce cell scattering induced by HRG-β1. The results indicated that Rac1 signaling controls cell–cell dissociation but fails to induce complete MCF7 cell scattering. To achieve complete scattering, the combined activities of MEK1 signaling and Rac1 are necessary.

## 2. Materials and methods

### 2.1. Reagents used in this study

Anti-E-cadherin, anti-β-catenin, and anti-α-catenin antibodies were purchased from BD Biosciences (Franklin Lakes, NJ, USA) and Millipore Co. Ltd. (Billerica, MA, USA). Anti-phospho-p38 MAP kinase, anti-ERK, and anti-phospho-ERK antibodies were from Cell Signaling Technology Inc. (Danvers, MA, USA). Anti-p38 MAP kinase antibody was from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Anti-Rac1 antibody was from Millipore Co. Ltd. TRITC-conjugated rhodamine–phalloidin was from Sigma–Aldrich

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Corp. (St. Louis, MO, USA). EGF domain of HRG-β1 was from R&D Systems (Minneapolis, MI, USA). The secondary antibody conjugated to Alexa 488 was from GE Healthcare (London, UK).

### 2.2. Cell culture

MCF7 cells, kindly provided by Dr. Takao Yamori, were cultured in Dulbecco's modified minimal essential medium supplemented with 5% fetal calf serum. The cells carrying the inducible dominant, active MKK1a gene, a kind gift from Dr. Yukiko Gotoh, and/or HA-tagged, dominant, and constitutively active Rac1 gene, a kind gift from Dr. Kozo. Kaibuchi, were produced using the Cre-*LoxP* system established by Kanegae et al. [13]. In this system, infection of adenoviruses encoding Cre recombinase allow for the expression of the indicated genes.

### 2.3. Immunofluorescence

Cells were fixed with 10% formaldehyde and permeabilized by 0.2% Triton X100 in PBS. The samples were treated with the primary antibody for 1 h and then with secondary antibody for 30 min. The samples were observed using a confocal laser microscope equipped with a 40X UPlanFL N objective. *Z*-plane sectioning (0.25 μm slices) was applied to the samples, and the images with the clearest planes were presented (Olympus FV300, Olympus, Tokyo).

### 2.4. Western blotting and immunoprecipitation

Western blotting was performed using 1% skim milk in TBS containing 0.1% Tween 20 as a blocking reagent and detected by an ECL system as previously described [14]. For immunoprecipitation, cells were lysed in a buffer containing 10 mM Tris–HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, and 1 mM PMSF. Protein A Sepharose bound to appropriate antibodies was added to the lysate. After washing the beads with the lysis buffer three times, the proteins bound to the beads were detected by Western blotting with appropriate antibodies.

### 2.5. Detection of active Rac1

GST-PAK-CRIB, a probe that selectively binds to active Rac1, was expressed in E. coli strain BL21 and suspended in a buffer containing 50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 5 mM $MgCl_2$, 1 mM

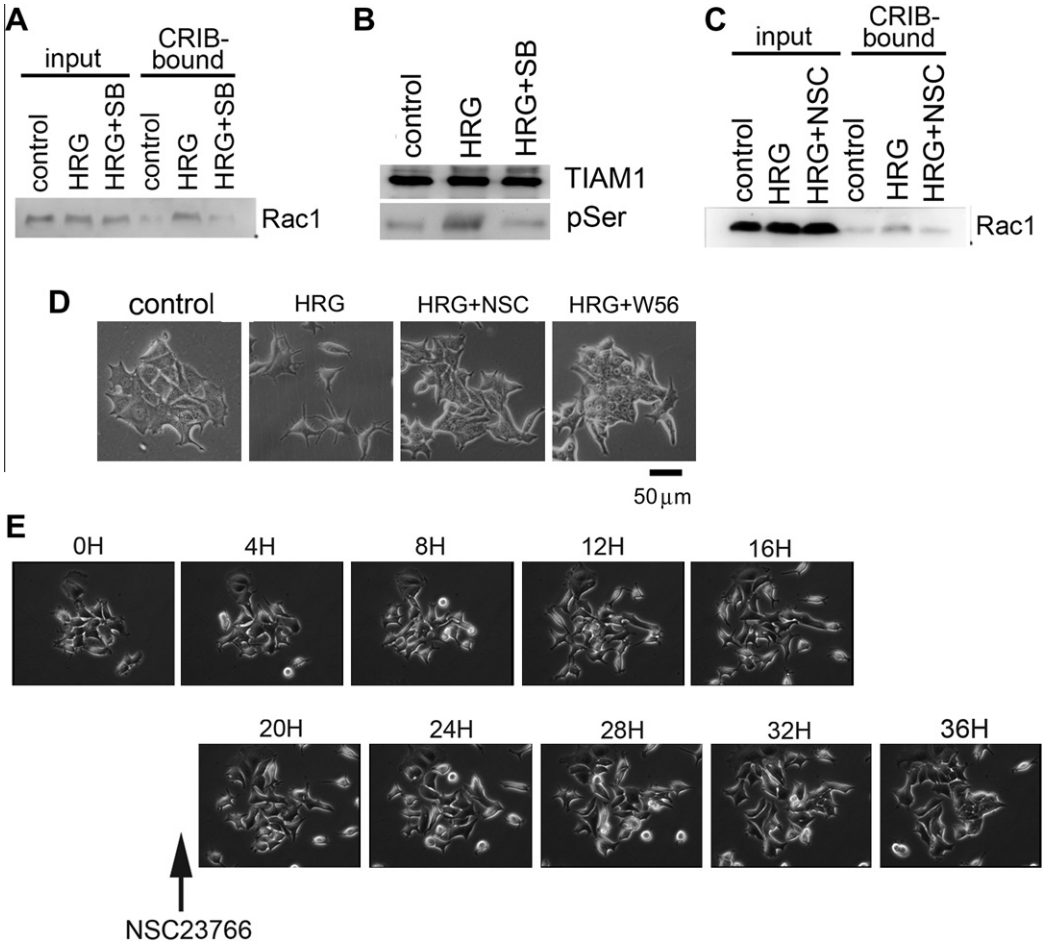


**Fig. 1.** The p38 MAP kinase pathway is connected to Rac1. (A) MCF7 cells were stimulated with HRG-β1 for 10 h and activated Rac1 levels (CRIB-bound) were examined. Input shows the levels of Rac1 used in the assay. HRG + SB, SB202190 was added 15 min before harvesting the cells. (B) MCF7 cells were stimulated with HRG-β1 for 10 h in the presence or absence of NSC23766. Cells were lysed, TIAM1 was immunoprecipitated and the phosphorylation of TIAM1 was analyzed by Western blotting with an anti-phophoserine antibody. The amounts of TIAM1 in the immunoprecipitates are shown in the upper panel. (C) MCF7 cells were stimulated with HRG-β1 in the presence or absence of TIAM1 inhibitors, NSC23766 (10 μM) or W56 (500 μM). After 20 h, cells were observed under the microscope. (D) MCF7 cells were stimulated with HRG-β1 for 10 h in the presence or absence of NSC23766 and activated Rac1 levels were analyzed. (E) MCF7 cells were treated with HRG-β1 for 24 h to allow dissociation. Then NSC23766 and another dose of HRG-β1 were added. Cells were monitored by time-lapse imaging. Cells were dissociated; but after addition of NSC23766, they began to re-aggregate within 10 min.

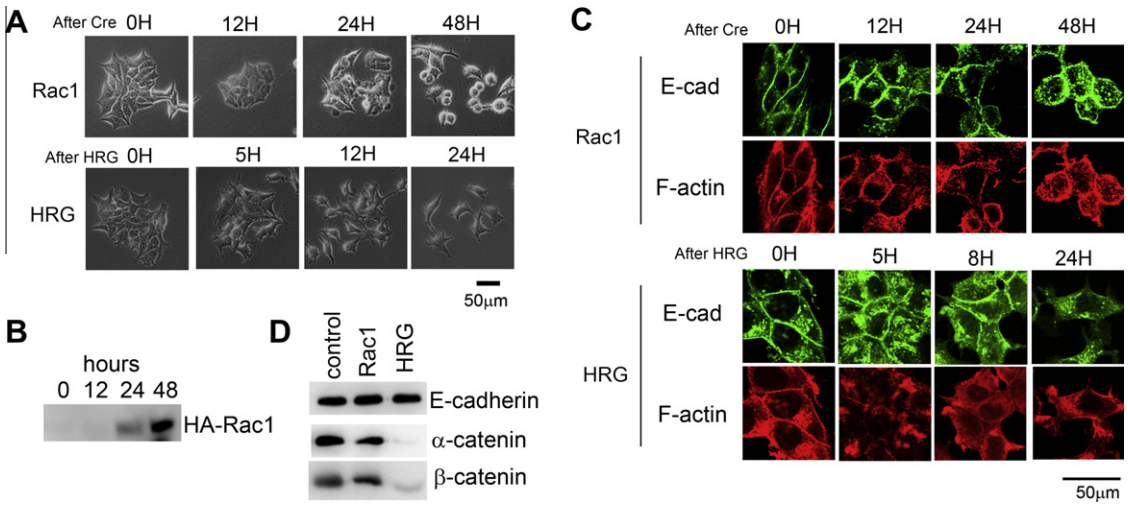


**Fig. 2.** Activation of Rac1 alone induces incomplete dissociation of cells. (A) Upper panels: MCF7-R5-7 cells were infected with adenovirus encoding Cre-recombinase (m.o.i. = 10) and incubated for the indicated times. Bottom panels: wild type MCF7 cells were stimulated with HRG-β1 and were incubated for the indicated times. Cells were observed under the microscope. (B) MCF7-R5-7 cells were infected with adenovirus encoding Cre recombinase and incubated for the indicated times. Expression of dominant active Rac1 was detected by Western blotting using anti-Rac1 antibody. (C) The cells shown in (A) were stained for E-cadherin and F-actin. (D) MCF7-R5-7 cells were infected with adenovirus encoding Cre recombinase and incubated for 48 h or stimulated with HRG-β1 for 24 h. E-cadherin was immunoprecipitated and α-catenin and β-catenin in the immunoprecipitates were detected by Western blotting with the relevant antibodies.

EDTA, 1 mM PMSF, and 1 mg/ml lysozyme. After incubation on ice for 10 min, the bacteria were sonicated and cellular debris was clarified by centrifugation. GST-PAK-CRIB was absorbed on glutathione Sepharose 4B beads and washed with the buffer mentioned above. MCF7 cells were lysed in a buffer containing 10 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1% Triton X100, and 1 mM PMSF. After removal of cellular debris, the lysate was mixed with the GST-PAK-CRIB-loaded beads and incubated for 1 h. After washing with the lysis buffer three times, Rac1 bound to the beads was analyzed by Western blotting with anti-Rac1 antibody.

### 2.6. Time-lapse imaging

Time-lapse imagin was performed using a Leica A7 6000 LX time-lapse imager. Cells were cultured in 3.5 cm dishes at appropriate density and applied to the imager. If addition of HRG-βl was required, it was added just prior to loading the dishes into the imager. The p38 MAP kinase inhibitor SB202190 was added while the imager was under operation. Pictures were captured every 10 min. Representative photos are shown in the figures.

## 3. Results

### 3.1. Rac1 plays an important role in the dissociation of MCF7 cells prior to scattering

Activation of p38 MAP kinase causes HRG-β1-induced scattering of MCF7 cells [5]. Within this pathway, Rac1 is required to induce dissociation of cells. As shown in Fig. 1A, activation of Rac1 was detected when p38 MAP kinase was activated by HRG-β1 stimulation. Because maximal activation of p38 MAP kinase occurs 10 h after HRG-β1 stimulation, we examined Rac1 at this time. Rac1 activity was inhibited by the p38 MAP kinase inhibitor SB202190, confirming that the p38 MAP kinase pathway is connected to Rac1. In line with these results, serine phosphorylation of the Rac1-GEF, TIAM1, was observed and was sensitive to SB202190 (Fig. 1B). A selective inhibitor of TIAM1, NSC23766, also inhibited Rac1 activation in HRG-β1 treated cells (Fig. 1C). Therefore, it is likely that there is a protein kinase cascade downstream of p38 MAP kinase that phosphorylates (and activates) TIAM1, which leads to Rac1 activation. Indeed, the TIAM1 inhibitors NSC23766 and W56 blocked scattering of MCF7 cells (Fig. 1D).

### 3.2. Continuous activation of Rac1 is required for scattering of MCF7 cells

As mentioned above, MCF7 cells begin to dissociate at about 15 h and completely scatter by 18 h after HRG-β1 stimulation (Fig. 1E). To examine the effect of TIAM1 inhibition at this late stage of dissociation, NSC23766 was added. As shown in the Fig. 1E, the cells immediately started to re-aggregate in the presence of NSC23766, indicating that continuous activation of Rac1 is required for the scattering phenotype. These results are in line with previous work showing that continuous activation of p38 MAP kinase is required for the maintenance of scattering [6]. The present data reinforce this pathway and further confirm that the p38 MAP kinase pathway is connected to Rac1.

### 3.3. Rac1 activation alone confers incomplete scattering of cells

Rac1 has been suggested to be a major contributor to the scattering process. Therefore, we generated a cell line, MCF7-R5-7, that expressed HA-tagged, dominant active Rac1 that can be induced by infection with an adenovirus harboring the Cre recombinase gene. In this system, there is a stuffer sequence (the neomycin resistant gene) flanked by *loxP* sites and positioned between the CMV promotor and the coding sequence of active Rac1. Expression of the Cre recombinase extrudes the stuffer sequence and unites the CMV promotor with the dominant active Rac1 coding sequence. This system allows almost all of the cells to express dominant active Rac1, which facilitates the analysis of scattering on a population basis. When dominant active Rac1 was expressed, cells dispersed, however they were notable differences between cells treated with HRG-β1 (Fig. 2A and B). Specifically, some cells remained associated and scattering appeared to be incomplete.

Staining of the cells for E-cadherin and F-actin revealed that that these proteins were bound to the plasma membrane during dominant active Rac1-induced scattering. In contrast, E-cadherin and F-actin in HRG-β1-stimulated cells localized diffusely to the

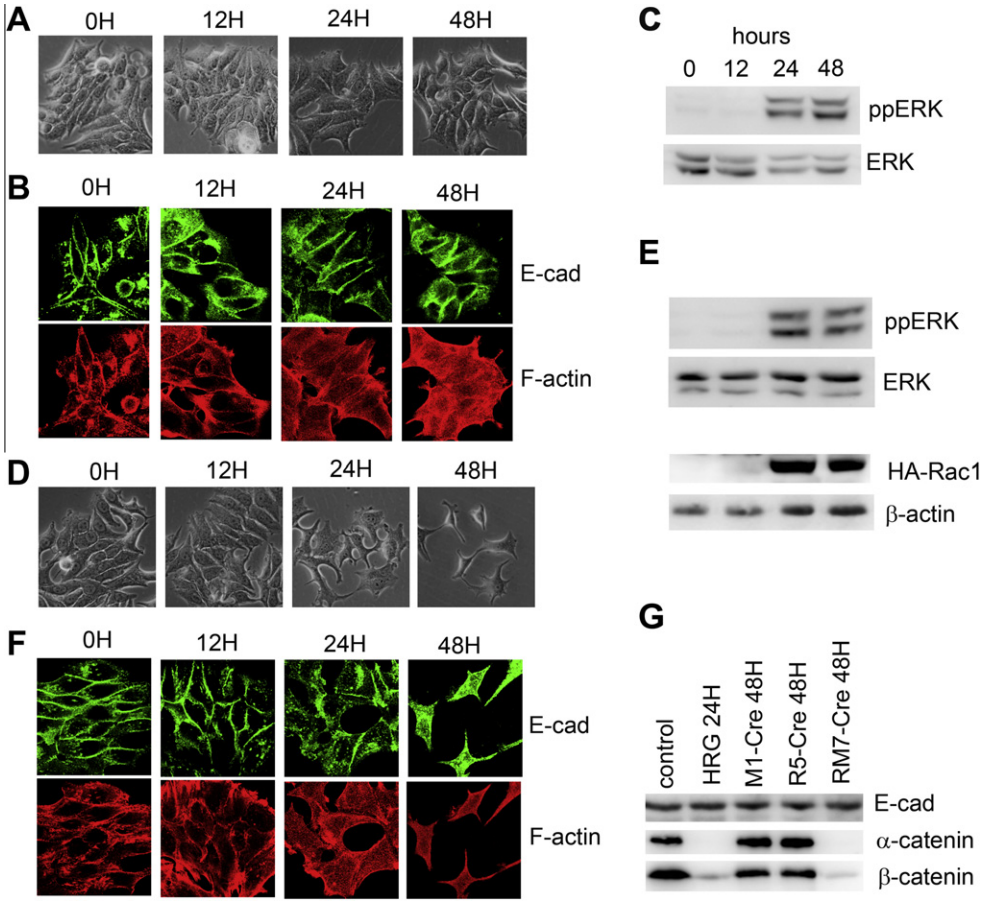


**Fig. 3.** Cooperation of the MEK1 pathway and Rac1 results in complete dissociation of MCF7 cells. (A) MCF7-M1 cells were infected with adenovirus encoding Cre-recombinase, incubated for the indicated times, and observed under the microscope. (B) The cells shown in (A) were stained for E-cadherin and F-actin. (C) MCF7-M1 cells were infected with adenovirus encoding Cre recombinase and incubated for the indicated times. Expression of dominant, active MKK1a was monitored by ERK activation. ERK and phospho-ERK were detected by Western blotting using anti-ERK and anti-phospho-ERK antibodies. (D) MCF7-RM7 cells were infected with adenovirus encoding Cre-recombinase, incubated for the indicated times, and observed under the microscope. (E) MCF7-RM7 cells were infected with adenovirus encoding Cre recombinase and incubated for the indicated times. Expression of dominant active MKK1a and dominant active Rac1 were monitored by ERK activation and the presence of HA-Rac1. ERK and phospho-ERK were detected by Western blotting using anti-ERK and anti-phospho-ERK antibodies. Expression of dominant active Rac1 was detected by Western blotting using an anti-Rac1 antibody. (F) The cells shown in (D) were stained for E-cadherin and F-actin. (G) Various MCF7 cells were infected with adenovirus encoding Cre recombinase and incubated for 48 h or stimulated with HRG-β1 for 24 h. E-cadherin was immunoprecipitated, and the levels of α-catenin and β-catenin in the immunoprecipitates were detected by Western blotting with relevant antibodies.

cytoplasm (Fig. 2C). Therefore, sole expression of dominant active Rac1 may not be sufficient to induce complete scattering.

E-cadherin was immunoprecipitated and the associated proteins were examined. As shown in Fig. 2D, α-catenin and β-catenin separated from E-cadherin in HRG-β1 stimulated cells, but remained associated in cells expression dominant active Rac1. These results suggested that the adherens junction complex did not break down with expression of dominant active Rac1.

### *3.4. Activation of MEK1 induces dissociation of F-actin from cell–cell contacts*

The inability of cells to completely scatter after dominant active Rac1 expression was likely a consequence of F-actin remaining at the plasma membrane. Previously, we have shown that the detachment of F-actin from the plasma membrane invovles activation of MEK1 [6]. Therefore, we produced a Cre recombinase-inducible cell line, MCF7-M1, that expresses dominant active MKK1a, which an upstream activator of ERK. Dissociation of cells was not detected after expression of MKK1a, which was monitored by phosphorylation of ERK (Fig. 3A). However, staining of the cells with TRITC-phalloidin revealed that F-actin disappeared from the plasma membrane (Fig. 3B). Activation of the MEK1 pathway was detected 24 h after infection of the virus harboring the Cre recombinase gene, which coincides with the timing of F-actin detachment from cell–cell contacts (Fig. 3C). E-cadherin remained at the cell–cell contacts, suggesting that adherens junctions lacking an F-actin backbone can still maintain cell–cell adhesion.

The results above suggested that both Rac1 and MEK1 activity are required to induce complete cell scattering. To test this, we established an inducible cell line, MCF7-RM7, that expresses dominant active MKK1a and dominant active Rac1. As shown in Fig. 3D, activation of both the MEK1 pathway and Rac1 resulted in complete dissociation and scattering of cells. Scattering coincided with the expression of MKK1a and Rac1 (Fig. 3E). As expected, F-actin and E-cadherin were diffusely distributed in the cytoplasm of these dissociated cells, as seen in HRG-β1 treated cells (Fig. 3F). Additionally, α-catenin and β-catenin were no longer associated with E-cadherin, suggesting that the adherens junction complex broke down (Fig. 3G). HRG-β1 mainly activates the MEK1pathway and the PI 3-kinase pathway, which is upstream of p38 MAP kinase and Rac1 signaling. Therefore, it is likely that the complete scattering of MCF7 cells requires the participation of these two signaling pathways.

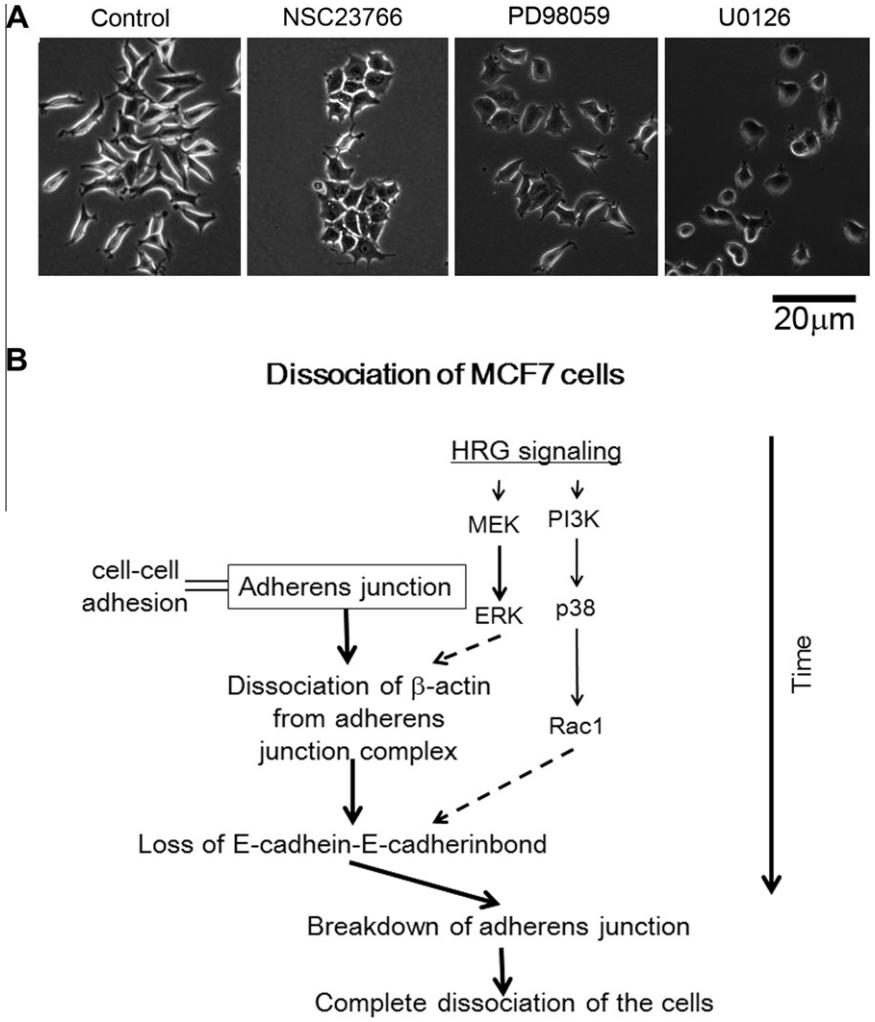


**Fig. 4.** (A) MCF7 cells were stimulated with HRG-β1 for 18 h. Then, the indicated drugs were added to the medium and further incubated for 6 h. Cells were observed under the microscope. (B) A model for signaling associated with MCF7 cell scattering.

As mentioned above, dissociated cells re-aggregated when Rac1 was inhibited (Fig. 4A). However, inhibition of the MEK1 pathway did not yield a similar outcome, suggesting that MEK1-mediated F-actin rearrangement was not sufficient to promote cell–cell association. The rounded morphology of HRG-β1 and MEK inhibitor treated cells, which was distinct from the control HRG- β1 treated cells, suggested that F-actin arrangement had occurred.

## 4. Conclusion

The results shown in this paper suggest that Rac1 regulates E-cadherin-mediated cell–cell association and the MEK pathway regulates actin rearrangement. The mechanism of adherens junction complex break down is not known, however, we propose that F-actin binding to α-catenin at the plasma membrane may function to stabilize the adherens junction complex.

We have shown that activation of Rac1 alone is not sufficient to induce complete scattering of MCF7 cells. Instead, complete scattering is achieved when MEK1 and Rac1 are activated. Previously we showed that actin detaches from tight and adherens junction prior to the dissociation of cells [6]. MEK inhibition blocked this process, suggesting that the MEK pathway was required for cell scattering. Previous studies have also implicated the MEK pathway in the regulation of F-actin dynamics [15–17]. In line with these results, we showed that direct activation of MEK1 resulted in the detachment of F-actin from tight and adherens junctions (Fig. 4B).

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