EGFR phosphorylation-dependent formation of cell-cell contacts by Ras/Erks cascade inhibition

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Summary

Cell-cell contacts play important roles in the homeostasis of normal epithelium and in the steps of metastasis of tumor cells, although signaling mechanisms to regulate cell-cell contacts are unclear. In this study, we observed that phenotype of no cell-cell contacts in rat intestinal epithelial cell subline (RIE1-Sca) correlated with increased Erk1/2 signaling activity, compared to that of parental RIE1 cells growing in colonies. Furthermore, cell-cell contacts between RIE1-Sca cells were reformed by treatment with a specific MEK inhibitor (U0126), with translocation of ZO1 and β-catenin to cell-cell contacts, without changes of their expression levels. U0126 treatment also increased EGFR phosphorylation in a ligand-independent manner. Pretreatment with EGFR kinase inhibitor abolished U0126 treatment-mediated EGFR phosphorylation, and expression of dominant negative H-Ras N17 allowed EGFR phosphorylation and cell-cell contacts even without U0126 treatment. Furthermore, the expression of a nonphosphorylatable EGFR Y5F mutant abolished U0126-mediated cell-cell contacts. U0126 treatment also caused less efficient wound healing by keeping monolayer integrity intact, compared to control untreated cells. This U0126-mediated reduction in wound healing was further altered either by pretreatment of EGFR kinase inhibitor or expression of H-Ras N17 or EGFR Y5F. Taken together, this study supports a unique mechanism of cell-cell contact formation through MEK/Erks inhibition-mediated EGFR phosphorylation.

Introduction

The architectural integrity of the adhesive epithelial monolayer is maintained by integrin engagement to extracellular matrix (ECM) proteins at basal membranes and cell-cell contacts between a cell and adjacent cells [1]. Disruption of this monolayer integrity not only impairs functions of normal epithelium but also allow the dissemination of cancerous cells from the primary tumor bodies during the early steps of cancer metastasis [2, 3]. Moreover, the dissemination of individual cells from epithelial monolayer results in a transition of epithelial cell types with well-established cell contacts to mesenchymal-like cells (i.e., elongated spindle-type cells) with few or no cell contacts [4]. This loss of cell-cell contacts is important clinically, since disseminated tumor cells might be facilitated in cell migration and invasion leading to tumor metastasis [3, 5]. Therefore, it is interesting to reveal the mechanisms of how cell-cell contact loss can involve the regulation of intracellular signal transduction and the suppression of E-cadherin [6].

Cell-cell contacts at tight and adherence junctions are linked to intracellular actin filaments through diverse protein-protein interactions [1]. Therefore, epithelial monolayer integrity and intracellular actin organization influence each other bi-directionally. That is, the disruption of cell-cell contacts can cause alterations in actin organization, and conversely abnormal actin organization can cause changes in cell contact status [7]. In addition, several signaling molecules stimulated by integrin receptor, growth factor receptor, or cross-talks between these receptors are known to be involved in the regulation of cell-cell contact formation [6]. These molecules include focal adhesion kinase (FAK), c-Src family kinase [6], Rho GTPases [8], Akt/PKB [9], Ras/Raf1/MEK/Erks cascade [10, 11], c-Met [12], and others. That is, the activation of these molecules disrupts cell-cell contacts, by releasing E-cadherin, β-catenin, and

zonula occludens-1 (ZO1) from cell-cell contact sites [13].

Meanwhile, the opposite phenomenon, namely, cell-cell contact formation may also be clinically important, as normal epithelial cells maintain homeostasis at a monolayer integrity and tumor cells that have moved from primary tumor bodies to other distal sites through lymph nodes or blood vessels would undergo cell-cell contact formation and acquire growth for metastatic tumors [3]. However, the manner in which cells form cell-cell contacts via the regulation of intracellular signaling pathways beyond the regulation of E-cadherin expression is largely unknown. It has been shown that the disruption or inhibition of the signaling activities of the molecules responsible for cell-cell contact loss leads to the abolishment of the loss. However, it is not known whether such disruptions and inhibitions always cause formation of cell-cell contacts. Moreover, an understanding of the mechanisms of cell-cell contact formation could lead to the development of reagents or strategies not to allow dissemination of tumor cells from primary tumors and to deal with settle-down for metastatic tumors.

In this study, we used a normal rat intestinal epithelial sub-cell line growing in a scattered pattern (RIE1-Sca) to examine how scattered-growth patterns can be converted to colony-forming patterns. We observed that the inhibition of the Ras/Raf1/MEK/Erks cascade reverted the scattered to colony-forming patterns and this revert required phosphorylation of EGFR. Therefore, the Ras/Erks cascade inhibition-mediated phosphorylation of EGFR appears to be important for cell-cell contact formation in the normal rat epithelial cell system.

Materials and Methods

Cells: RIE1-Sca cells growing in a scattered pattern were prepared by selection and enrichment from normal rat intestinal epithelial (RIE1) cells growing in a colony-forming pattern. The multiple clones with scattered-growing patterns were selected and mixed prior to enrichment. Subculture of the RIE1-Sca cells showed consistently scattered-growing patterns, while maintaining cells for 2 months by subculture every 2-3 day. Cells were maintained in DMEM-H (Gibco-BRL) culture media containing 10% (v/v) fetal bovine serum (FBS) at 37^{o}C and 5% CO_2.

Cell lysates preparation and Western blots: Whole cell lysates from cells treated with DMSO or 20 μM U0126 (LC Laboratories®, Woburn, MA) for the indicated periods were prepared as described in previous studies [14, 15]. In cases of EGFR kinase inhibition, AG1478 (LC Laboratories®) at 1 μM was treated for the indicated period or pretreated 30 min or 2 h before U0126 treatment for 2 or 14 h. In cases, cells were infected with retrovirus expressing human H-Ras V12 (active form, a gift from Dr. In-San Kim, Kyungpook National University, Daegu, Korea), H-Ras N17 (dominant negative form), or EGFR Y5F for 12 h. After additional incubation for 22 hr, cells were treated with DMSO or 20 μM U0126 for 14 h. In cases, cells were preincubated with neutralizing anti-EGF (10 μg/ml, Upstate Biotechnology) antibody or serum-starved overnight prior to U0126 treatment for additional 14 h. After incubations, cell lysates were prepared and their protein amounts were normalized using Bicinchoninic acid (BCA) Protein Assay Reagent Kits (Pierce, Rockford, IL). Standard Western blots were performed by using antibodies against phospho-Y^{397}FAK, phospho-Y^{577}FAK, phospho-Y^{861}FAK, phospho-Y^{925}FAK (BioSource International Inc., Camarillo, CA), phospho-Y^{416}Src, c-Src, pY^{1173}EGFR, (Santa Cruz Biotech., Santa Cruz, CA), Erk1/2, phospho-Erk1/2, phospho-

S^{473}Akt/PKB, phospho-Y^{992}EGFR, phospho-Y^{1045}EGFR, phospho-Y^{1068}EGFR (Cell Signaling Tech., Beverly, MA), E-cadherin, phospho-Tyrosine, FAK, α-tubulin (BD Transduction Lab., San Jose, CA), vimentin (Sigma, Saint Louis, MI), Desmoplakin (Serotec Ltd, Oxford, UK), ZO1 (Zymed Lab., South San Francisco, CA), or EGFR (Upstate Biotech., Lake Placid, NY).

Preparation of retroviruses: Human H-Ras V12 in pLNCX vector was used to make dominant negative H-Ras N17 (DN H-Ras N17) via a PCR approach. pRC/CMV-EGFR-Y5F construct in which the sites of tyrosine autophosphorylation (tyrosines 992, 1068, 1086, 1148, and 1173) have been mutated to phenylalanines [16] was also subcloned into a modified pLNCX retroviral vector as a *HindIII* and *XbaI* insert. Sequences of mutants were confirmed by direct sequence analyses. Retroviral vectors were separately transfected into PT67 packaging cells by using Lipofectamine 2000® (Life Technologies, Gaithersburg, MD) following the manufacture's protocol. Two days after separate transfections, cells were selected with G418 (250 μg/ml). The culture soup of the stable PT67 cells was used for retrovirus infections, and reproducible infection rates for similar expressions of target proteins were confirmed by Western blots.

Immunofluorescence microscopy: Cells were replated on normal culture media-precoated glass coverslips and incubated overnight at 37°C to achieve typical cell adhesion and spreading, as described previously [17]. In certain cases, cells were pretreated with AG1478 (1 μM) 30 min or 2 h before U0126 (20 μM) treatment for 14 hr. Pretreatment of AG1478 either 30 min or 2 h prior to U0126 treatment resulted in similar results. In cases, cells were infected with pLNCX-H-Ras V12 or N17 or pLNCX-EGFR Y5F retroviruses for 12 hr. The cells were then normally incubated for an additional 22 hr. Alternatively, cells were preincubated with normal rabbit IgG or neutralizing anti-EGF (10 μg/ml) 60 min before DMSO or U0126 treatment. U0126 at 20 μM was then treated for an additional 14 h to cells infected with H-Ras N17 or EGFR Y5F.

Wounds through cell monolayer on cover glasses were made as below. Cell images were then taken using a phase contrast microscope or cells were fixed with 3.7% formaldehyde in PBS for 10 min, permeabilized with 0.5% Triton X-100 in PBS for 10 min at room temperature, and washed three times with PBS (3 times x 10 min). Cells were then incubated with primary antibody or TRITC-conjugated phalloidin (Molecular Probes, Eugene, OR) for 1 h and washed with PBS as above. The primary antibody used included anti-β-catenin (Santa Cruz Biotech.) and ZO1 (Zymed Lab. Inc.). Cells were then incubated with anti-rabbit or mouse IgG-conjugated TRITC or FITC (Chemicon International, Inc., Temecula, CA) in a dark and humidified chamber for 1 hr. Alternatively, cells were stained for actin by using phalloidin-conjugated with rhodamine, as described earlier [17]. After washings three times with PBS as described above, cells on glass coverslips were mounted with mounting solution (DakoCytomation, Germany) and visualized by fluorescent microscopy (BX51TR, Olympus, Japan).

Wound healing assay: Cells were seeded and cultured to reach a confluent monolayer with or without pLNCX-DN H-Ras N17 or pLNCX-EGFR Y5F retrovirus infection. Wounds were then made by scraping through the cell monolayer with a pipette tip, before washing twice with DMEM-H containing 10% FBS. In certain cases, cells were treated with AG1478 EGFR kinase inhibitor at the indicated concentrations, 20 min before U0126 treatment. After incubation at 37^{o}C for the indicated periods, several images around wounds in each condition were taken and representative wound distance was measured for graphic presentations.

Statistical analysis: Student's *t*-tests were performed for comparisons of mean values to see if the difference is significant. p values ≤ 0.05 were considered significant.

Results

U0126 treatment–mediated formation of cell-cell contacts

To understand the intracellular signaling mechanisms responsible for the regulation of cell-cell contacts, we established a variant cell line (RIE1-Sca) that normally grows in a scattered pattern, from normal rat intestinal epithelial cells (RIE1) that grow in a colony-forming manner, with normal actin organization (Figure 1A). This was done by repeated selection and enrichment processes during traditional cultures. RIE1-Sca cells were more spindle-shaped than the parental RIE1 cells (Figure 1A). Next, we immunoblotted whole cell extracts prepared from RIE1-WT or -Sca cells. Among intracellular signaling molecules, we found that phosphorylations of Erk1/2 and Focal Adhesion Kinase (FAK) Tyr925 (i.e., pY^{925}FAK) were much enhanced in RIE1-Sca cells, as compared with those of RIE1-WT cells (Figure 1B, lines 1 and 3).

We investigated if any pharmacological reagent could convert the scattered RIE1-Sca cells to colony-forming patterns. We examined the effects of diverse commercial inhibitors and activators. U0126, a specific MEK inhibitor, was found to cause a colony-forming pattern in RIE1-Sca cells through cell-cell contact formation (Figure 1C), which is consistent with the observation of an enhanced Erk1/2 activity in RIE1-Sca cells, as compared with that in RIE1-WT cells. When treated with U0126, RIE1-Sca cells showed decreased pY^{397}FAK but no significant changes in other tyrosine phosphorylations, such as of pY^{577}FAK, pY^{861}FAK, and pY^{925}FAK (Figure 1B, lines 3 and 4). In addition, U0126-treatment decreased c-Src phosphorylation at Tyr416 in RIE1-Sca cells (i.e., pY^{416}c-Src, Figure 1B, lines 3 and 4). Moreover, these changes appear to be consistent with previous reports that FAK and c-Src family kinase are involved in loss of cell-cell contact [6]. Therefore, we next examined these U0126-mediated effects by immunostaining for ZO1 protein (a marker of tight junctions) [1]. RIE1-Sca cells did not show

cell-cell contacts with ZO1, but ZO1 localization at cell-cell contacts became obvious after U0126 treatment (Figure 1D, upper). In addition, β-catenin (a marker of adherence junctions) also localized to the cell-cell contacts of RIE1-Sca cells after U0126 treatment, indicating that cell-cell contact formation was induced by U0126 treatment in RIE1-Sca cells (Figure 1D, lower). Moreover, an enhanced vimentin (a mesenchymal maker) level in RIE1-Sca, as compared to that in RIE1-WT cells, was significantly reduced by U0126 treatment (Figure 1E, lanes 1 to 3). However, the immunostaining of E-cadherin (another cell-cell contact marker) in rat cells (i.e., RIE1-Sca) were failed even when we tried diverse anti-E-cadherin or -pan-cadherin antibodies and protocols to fix and permeabilize cells, although the E-cadherin expression was detected unchanged before and after U0126 treatment (Figure 1F). Therefore, U0126 treatment of RIE1-Sca cells caused cell-cell contact formation without altering the expression levels of the cell-cell contact markers tested.

Retarded wound healing by U0126 treatment-mediated cell-cell contact formation

By virtue of a scattered growing pattern and protrusive morphology of RIE1-Sca cells, their wound healing and cell migration can consequently be facilitated, as compared with those of parental RIE1-WT cells. When the wound healing characteristics of the RIE1 and RIE1-Sca cell lines were compared, RIE1-Sca cells were found to move into the middle of wounds faster than the parental RIE1 cells (Figure 2A), although cell numbers after the wound healing assay showed no significant difference (data not shown). Since RIE1-Sca cells healed wounds faster than parental RIE1 cells, U0126 treatment-mediated cell-cell contact formation might retard cell movement. Therefore, we examined the wound healing properties of RIE1-Sca cells before and after U0126 treatment. To minimize the effects of differences in proliferation rates in the absence versus presence of U0126 treatment on the degree of wound healing, wound healings

were analyzed for a short period of 6 h. RIE1-Sca cells healed wounds efficiently even after 6 h, but U0126-treated RIE1-Sca cells were less efficient (Figure 2B). These differences in wound healing rates appeared to be correlated with U0126-mediated cell-cell contact formation (Figure 2C). When cortical actin organization and ZO1-containing cell-cell contacts were visualized, RIE1-Sca cells with well-organized cortical actin filaments were found to be separately oriented toward wounds and cell-cell contacts with ZO1 were not formed, indicating that scattered cells orientated and crawled efficiently toward wounds (Figure 2C, upper). However, U0126-treated RIE1-Sca cells formed cell-cell contacts with ZO1 and cortical actin fibers (Figure 2C, lower), which presumably reduced wound healing efficiency.

U0126 treatment-mediated EGFR phosphorylation

When we analyzed for signaling molecules involved in the U0126 treatment-mediated formation of cell-cell contacts and retardation of wound healing by using whole cell extracts prepared from RIE1 cells with or without U0126 treatment, EGFR was found to be influenced by U0126 treatment; when cells were treated with U0126, RIE1-Sca cells increased phosphorylations of EGFR Tyr992, Tyr1068, and Tyr1173 (i.e., pY^{992}EGFR, pY^{1068}EGFR, and pY^{1173}EGFR, respectively), whereas RIE1 WT cells showed a slightly reduced pY^{992}EGFR but no significant changes in pY^{1068}EGFR or pY^{1173}EGFR (Figure 3A). Moreover, pY^{1045}EGFR required for Cbl binding [18] was decreased in RIE1-Sca, but not in RIE1-WT cells after U0126 treatment (Figure 3A). These U0126-mediated effects were also observed when RIE1-Sca cells were treated with another MEK inhibitor, PD98059 (data not shown). These increases in EGFR phosphorylation in U0126-treated RIE1-Sca cells appeared to be dependent on EGFR kinase activity, since pretreatment with AG1478 (a specific EGFR kinase inhibitor) prior to U0126 treatment abolished U0126-enhanced EGFR phosphorylations including pY^{1173}EGFR and pY^{1068}EGFR (Figure 3B and data not shown). In addition, AG1478-pretreatment also blocked

the U0126-mediated formation of cell-cell contacts, since β-catenin located more distally from the plasma membrane after AG1478 pretreatment (Figure 3C). In addition, AG1478 pretreatment blocked the U0126 treatment-mediated decrease in vimentin level in RIE1-Sca cells (Figure 1E, lanes 2 to 4). Interestingly, U0126 treatment for a short period (i.e., 2 h) caused EGFR phosphorylation (Figure 3B, lanes 1, 3), and this was abolished by pretreatment of EGFR kinase inhibitor (Figure 3B, lanes 3 and 5). However, this short U0126 treatment did not cause cell-cell contact formation (data not shown). This indicates that Erk1/2 inhibition by U0126 treatment requires a longer time (i.e., 14 h) for contact formation between RIE1-Sca cells, and that EGFR phosphorylation kinetically preceded cell-cell contact formation, when RIE1-Sca cells were treated with U0126.

Down-regulation of the Ras/Erks cascade was required for EGFR phosphorylation and cell-cell contact formation

To determine if the effects of U0126 treatment are caused by inhibition of specifically Ras/Raf1/Mek/Erks cascade, we altered activity of this cascade in RIE1-Sca cells prior to analyzing EGFR phosphorylation and cell-cell contact formation, by using retroviruses coding for active or dominant negative H-Ras mutant (H-Ras V12 or N17, respectively, Figure 4A). RIE1-Sca cells were first infected with retroviruses expressing active H-Ras V12, which caused the activation of Erk1/2 but not of Akt/PKB (Figure 4A, left). Cells were then treated with DMSO or U0126 for 14 h, and then immunostained for cell-cell contact markers. RIE1-Sca cells did not form cell-cell contacts by H-Ras V12 expression alone (Figure 4B, left). However, U0126 treatment formed cell-cell contacts in both control and H-Ras V12-expressing RIE1-Sca cells (Figure 4B, right). We then knocked-down Ras/Erks cascade activity using retrovirus expressing dominant negative H-Ras N17, in stead of U0126 treatment. RIE1-Sca cells infected

with H-Ras N17 retrovirus, but not with control virus, formed cell-cell contacts with ZO1 and β-catenin (Figure 4C). Furthermore, the expression of H-Ras N17 reduced Erk1/2 phosphorylation but increased EGFR Tyr1173 phosphorylation (Figure 4D). These observations indicate that indeed the Ras/MEK/Erks cascade inhibited by U0126 treatment appeared to cause cell-cell contact formation involving EGFR phosphorylation.

EGFR Y5F expression abolished cell-cell contact formation mediated by U0126 treatment

Next we examined the significance of EGFR phosphorylation in U0126 treatment-mediated cell-cell contact formation. RIE1-Sca cells were infected with retrovirus coding for a nonphosphorylatable mutant of EGFR (EGFR Y5F; Tyr992/1068/1086/1148/1173Phe, [16], Figure 5B). Twelve hours after infection, media were changed with normal culture media. After an additional 22 h of incubation, cells were treated with DMSO or U0126 for 14 h prior to immunostaining for cell-cell contact markers. Interestingly, the expression of EGFR Y5F alone in RIE1-Sca cells did not form cell contacts (Figure 5A, upper panels). Moreover, U0126 treatment-dependent formation of contacts between RIE1-Sca cells was abolished by EGFR Y5F expression (Figure 5A, lower panels), indicating that U0126 treatment-mediated phosphorylations of EGFR are required for U0126-mediated contact formation.

U0126-mediated EGFR phosphorylation is independent of ligand EGF binding

We then wondered whether the U0126-mediated formation of cell-cell contacts via EGFR phosphorylation depends on ligand (i.e., EGF) binding. To answer this, we first performed experiments in the absence of serum. Even in the absence of serum, treatment with U0126 for 14 h, but not with DMSO, resulted in the formation of cell-cell contacts with ZO1 (Figure 6A, left and middle). On the other hand, U0126 cotreatment with EGF (50 ng/ml) did not cause any significant difference in cell-cell contact formation. As was expected, U0126-treated RIE1-Sca

cells in the absence of serum showed increased EGFR phosphorylation (Figure 6C, middle panels). These findings indicate that the U0126-mediated formation of cell-cell contacts occurs via ligand-independent EGFR phosphorylation. To confirm this alternatively and to rule out the autocrine possibility, we preincubated cells with neutralizing anti-EGF antibody prior to U0126 treatment. In the presence of normal serum-containing culture media, cells were preincubated with control normal rabbit IgG or neutralizing anti-EGF 60 min prior to DMSO or U0126 treatment for 14 h. In the presence of control normal IgG, U0126 treatment caused cell-cell contact formation, as was shown earlier (Figures 1D, 3C, 4B, 5A, and lefts of 6B). Interestingly, U0126-mediated cell-cell contact formation and EGFR phosphorylation were obvious even in the presence of neutralizing anti-EGF (Figures 6B and 6C, rights). These observations also support that U0126-mediated formation of cell-cell contacts requires ligand-independent EGFR phosphorylation, suggesting that regulation of intracellular signaling caused EGFR phosphorylation upon U0126 treatment.

Inhibition of EGFR phosphorylation affects wound healing mediated by U0126 treatment

We observed above that U0126 treatment caused cell-cell contact formation and retarded wound healing (Figure 2). In addition, treatment with AG1478, which inhibited EGFR phosphorylation, abolished U0126-mediated cell-cell contact formation (Figure 3C). It is thus likely that the inhibition of U0126-mediated EGFR phosphorylation facilitates wound healing. To test this, we examined the effects of AG1478 pretreatment on U0126 treatment-mediated wound healing of RIE1-Sca cells. In a dose- and time-dependent manner, AG1478 pretreatment prevented the U0126 treatment-mediated retardation of the wound healing abilities of RIE1-Sca cells (Figure 7A). Furthermore, the expression of H-Ras N17 retarded the wound healing of RIE1-Sca cells even without U0126 treatment (Figure 7B), in the same manner as U0126 treatment-retarded wound healing (Figure 2A). On the other hand, the expression of

nonphosphorylatable EGFR (EGFR Y5F) did not retard the wound healing mediated by U0126 treatment (Figure 7C), presumably because the nonphosphorylation of EGFR prevented cell-cell contact formation even after U0126 treatment.

Discussion

In this study, inhibition of the Ras/Erks cascade increased phosphorylation levels of EGFR, subsequently caused cell-cell contact formation, and inhibited the migratory properties of RIE1-Sca cells. It has previously been reported that Erks activity can negatively regulate EGFR signaling activity, including its tyrosine phosphorylation [19-22], but the biological significance of such a negative feed-back loop between Erks and EGFR was limited to cell proliferation. Thus, the present study provides evidence for the first time that Ras/Erks cascade inhibition-enhanced EGFR phosphorylation levels are involved in cell-cell contact formation and wound healing.

A negative feed-back linkage between Erks and EGFR has been reported to occur through FRS2 (fibroblast growth factor receptor substrate 2) [20-22]. FRS2α/SNT-1 is heavily phosphorylated in Ser/Thr and Tyr residues by various stimuli, and binds to both EGFR via its phosphotyrosine binding (PTB) domain and Erks via its central portion [20]. Thus, FRS2α/SNT-1 transduces signals mediated by liganding of various growth factor receptors including EGFR. Upon EGF stimulation of the human epidermoid carcinoma A431 cells as well as in stable NIH3T3/EGFR transfectants, Erk1/2 are activated and phosphorylate FRS2α/SNT-1 on Ser/Thr residues, with leading to a decrease in its tyrosine phosphorylation. Consequently tyrosine-phosphorylated FRS2α/SNT-1-mediated growth factor signaling is curtailed [20]. However, FRS2α/SNT-1 appears not to be involved in cell-cell contact formation via Ras/Erks inhibition-induced EGFR phosphorylation of RIE1-Sca cells, since the tyrosine-phosphorylation of FRS2α/SNT-1 was unchanged by U0126 treatment (data not shown) and since U0126 treatment-mediated EGFR phosphorylation and cell-cell contact formation were ligand-independent. Meanwhile, FRS2β/SNT-2 is not tyrosine-phosphorylated and binds to

Erk2 and constitutively to EGFR [21]. Activated Erk causes down-regulation of EGFR/Ras/Erk pathway via formation of the FRS2β/SNT-2-Erk2 complex and down-regulation of EGFR activation, presumably either by physical perturbation of autophosphorylation or by mediation of tyrosine phosphatase [22]. Although it is unclear at this time how Ras/Erk cascade inhibition causes EGFR phosphorylation in this study, it is being investigated for another upcoming study.

This study showed that U0126 treatment of RIE1-Sca cells decreased pY^{397}FAK and pY^{416}c-Src, which is consistent with previous reports that FAK and c-Src family kinase are involved in loss of cell-cell contacts [6]. However, FAK phosphorylations at other tyrosine residues of pY^{577}FAK, pY^{861}FAK, and pY^{925}FAK in RIE1-Sca cells were not significantly changed, unlike those observed in RIE1-WT cells after U0126 treatment. Previous studies have indicated that pY^{925}FAK is correlated with the disassembly of cell-cell contacts in certain systems. Whereas pY^{397}FAK and pY^{861}FAK, but not pY^{925}FAK, in HeLa cells correlated with cell-cell contact formation [23], active c-Src-mediated pY^{925}FAK in KM12C colon cancer cells caused the disassembly of cell-cell contacts [23, 24]. However, we observed in this study that pY^{925}FAK appeared not to be involved in U016 treatment-mediated cell-cell contact formation, since pY^{925}FAK was not significantly changed by U0126 treatment. Instead, pY^{397}FAK and pY^{416}Src were concomitantly decreased and this was correlated with cell-cell contact formation after U0126 treatment. Therefore, observations from these previous studies and the present study indicate different significance of FAK phosphorylation at distinct tyrosine residues during regulation of cell-cell contacts.

On the other hand, RIE1-Sca cells significantly increased pY^{992}EGFR, pY^{1068}EGFR, and pY^{1173}EGFR, and decreased pY^{1045}EGFR, whereas RIE1-WT cells non-significantly altered pY^{1045}EGFR and pY^{1068}EGFR and slightly reduced pY^{992}EGFR and pY^{1173}EGFR, after U0126

treatment. pY^{992}EGFR is known to recruit protein tyrosine phosphatase (PTP 1B, PTP 2C, or SHP-2) or PLCγ; pY^{1068}EGFR recruits Grb2 and/or Shc; and pY^{1173}EGFR recruits Grb2, Shc, SHP-1, or PLCγ [25]. pY^{1045}EGFR is known to recruit Cbl for EGFR degradation [18], and thus could be oppositely regulated as compared with other EGFR autophosphorylation sites for stimulatory intracellular signaling pathways. Phosphorylated EGFR has previously been shown to localize at cell-cell contact sites, with forming a complex with integrin α2β1, although the biological significance of this localization and complex formation have not been clarified [26]. In addition, both EGFR and E-cadherin were shown to locate at the cell-cell contacts in A431 cells [27] and HaCat keratinocyte cells [28]. In A431 cells, EGF induced tyrosine phosphorylation and relocalization of ZO1 from cytosol to tight junction-like areas in an actin cytoskeleton-dependent manner [29]. EGF treatment of TMK-1 gastric cancer cells also showed the localizations of ZO1 and occludin to cell-cell contact sites of tight junctions in a PKC-dependent manner [30]. It appears that Ras/Erks cascade inhibition-mediated EGFR phosphorylation is required for cell-cell contact formation in the RIE1 system, but that the phosphorylation of EGFR alone is insufficient, since we observed that U0126 treatment for 2 h caused EGFR tyrosine phosphorylation but not cell-cell contact formation. In addition, Ras/Erks inhibition-mediated EGFR phosphorylation and cell-cell contact formation occurred even without ligand binding. Thus, it is likely that U0126 treatment-mediated EGFR phosphorylation without ligand binding may cause biochemical processes to form cell-cell contacts, although it remains to be determined how U0126 treatment-mediated (individual) EGFR tyrosine-phosphorylation is important for cell-cell contact formation. It cannot be ruled out that Ras/Erks cascade inhibition-mediated EGFR phospho-tyrosines may recruit SH2 domain-containing molecules during formation of cell-cell contacts.

Previous studies have shown a correlation between EGFR activation and E-cadherin-mediated cell-cell contacts [31-34]. EGFR was found to interact and phosphorylate β-catenin of the cadherin-catenin complex [34] and to directly down-regulate cell-cell contacts by modulating the linkage of E-cadherin/catenin complex to the actin cytoskeleton [35]. It was also previously reported that cadherin-dependent cell contact formation resulted in Rac1 activation through EGFR signaling [36] and Erk1/2 activation via the recruitment and activation of EGFR [28]. However, other controversial studies previously evidenced that E-cadherin-mediated cell contacts also inhibited receptor tyrosine kinases including EGFR [37] or did not involve EGFR activation [38]. These contradictory studies presumably indicate that the co-relationship between EGFR and cadherin-dependent cell contacts may be dependent on their cell surface expression levels and on differential downstream signaling contexts due to different cell types. Meanwhile, in the present study, we observed that Ras/Erks cascade inhibition-mediated formation of cell-cell contacts required an increase in the phosphorylation of EGFR, based on experiments with either EGFR kinase inhibitor pretreatment or expression of nonphosphorylatable EGFR Y5F. We also observed that the expression of EGFR Y5F alone in RIE1-Sca cells did not form cell contacts, unlike certain previous studies [34, 35]. Therefore, these previous and current observations indicate that EGFR phosphorylation can have different effects on cell-cell contacts depending on cell systems.

Cell-cell contacts together with integrin-extracellular matrix (ECM) interaction and growth factor receptor signaling pathways have been suggested to be an integral part of the morphogenetic programs of cells that control the maintenance of the structural and functional integrity of epithelia [28, 39]. Integrin-mediated cell adhesion signaling appears to be involved in the regulation of cell-cell contact status, indicating the presence of cross-talk between cell

adhesion receptors; integrin $\alpha 6\beta 4$ or $\alpha 2\beta 1$ was shown to be involved in EMT of colon and rat bladder carcinoma cells, respectively [40, 41]. Therefore, information on cell-cell contacts may be linked to integrin-ECM engaging sites (i.e., focal adhesions and contacts), which are dynamically modulated during cell migration. Abnormal functional integrity of epithelia via the disruption of cell-cell contacts can allow the dissemination of migratory cells from (cancer) cell colonies. During the dissemination, integrin interaction with the ECM at focal adhesions should also be dynamically modulated. Although we did not define the involvement of integrins in the system, we observed that Ras/Erks inhibition caused the maintenance of cell-cell contacts between RIE1-Sca cells crawling towards wound centers and consequently retarded wound healing. This correlation between cell-cell contact formation and retarded wound healing may reflect the functional significance of Ras/Erks cascade inhibition-mediated cell-cell contact formation. Thus, we suggest that cell-cell contact status can be regulated by modulating Ras/Raf1/Mek/Erks cascade activity via diverse mechanisms including the loss of integrin-ECM interactions or changes in the cell surface expressions of growth factor receptors under both physiologic and pathologic conditions, such as, during development or tumor metastasis.

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References

[1] J.P. Thiery, Cell adhesion in cancer, Comptes Rendus Physique 4 (2003) 289-304.

[2] S. Hirohashi and Y. Kanai, Cell adhesion system and human cancer morphogenesis, Cancer Sci. 94 (2003) 575-581.

[3] J.P. Thiery, Epithelial-mesenchymal transitions in tumour progression, Nat. Rev. Cancer 2 (2002) 442-454.

[4] B. Boyer, A.M. Valles and N. Edme, Induction and regulation of epithelial-mesenchymal transitions, Biochem. Pharmacol. 60 (2000) 1091-1099.

[5] P. Savagner, Leaving the neighborhood: molecular mechanisms involved during epithelial-mesenchymal transition, Bioessays 23 (2001) 912-923.

[6] E. Avizienyte and M.C. Frame, Src and FAK signalling controls adhesion fate and the epithelial-to-mesenchymal transition, Curr. Opin. Cell Biol. 17 (2005) 542-547.

[7] C. Jamora and E. Fuchs, Intercellular adhesion, signalling and the cytoskeleton, Nat. Cell Biol. 4 (2002) E101-108.

[8] V.M. Braga, M. Betson, X. Li and N. Lamarche-Vane, Activation of the small GTPase Rac is sufficient to disrupt cadherin- dependent cell-cell adhesion in normal human keratinocytes, Mol. Biol. Cell 11 (2000) 3703-3721.

[9] L. Larue and A. Bellacosa, Epithelial-mesenchymal transition in development and cancer: role of phosphatidylinositol 3' kinase//AKT pathways, Oncogene 24 (2005) 7443-7454.

[10] M. Lan, T. Kojima, M. Osanai, H. Chiba and N. Sawada, Oncogenic Raf-1 regulates epithelial to mesenchymal transition via distinct signal transduction pathways in an immortalized mouse hepatic cell line, Carcinogenesis 25 (2004) 2385-2395.

[11] L. Xie, B.K. Law, A.M. Chytil, K.A. Brown, M.E. Aakre and H.L. Moses, Activation of the Erk pathway is required for TGF-β1-induced EMT in vitro, Neoplasia 6 (2004) 603-610.

[12] C. Birchmeier, W. Birchmeier, E. Gherardi and G.F. Vande Woude, Met, metastasis, motility and more, Nat. Rev. Mol. Cell Biol. 4 (2003) 915-925.

[13] C. Birchmeier and W. Birchmeier, Molecular aspects of mesenchymal-epithelial interactions, Annu. Rev. Cell Biol. 9 (1993) 511-540.

[14] J.W. Lee and R.L. Juliano, The α5β1 integrin selectively enhances epidermal growth factor signaling to the phosphatidylinositol-3-kinase/Akt pathway in intestinal epithelial cells, Biochim. Biophys. Acta 1542 (2002) 23-31.

[15] M.S. Lee, S.G. Ko, H.P. Kim, Y.B. Kim, S.Y. Lee, S.G. Kim, H.S. Jong, T.Y. Kim, J.W. Lee and Y.J. Bang, Smad2 mediates Erk1/2 activation by TGF-β1 in suspended, but not in adherent, gastric carcinoma cells, Int. J. Oncol. 24 (2004) 1229-1234.

[16] S. Decker, Transmembrane signaling by epidermal growth factor receptors lacking autophosphorylation sites, J. Biol. Chem. 268 (1993) 9176-9179.

[17] M.-S. Lee, T.Y. Kim, Y.-B. Kim, S.-Y. Lee, S.-G. Ko, H.-S. Jong, T.-Y. Kim, Y.-J. Bang and J.W. Lee, The Signaling Network of Transforming Growth Factor β1, Protein Kinase Cδ, and Integrin Underlies the Spreading and Invasiveness of Gastric Carcinoma Cells, Mol. Cell. Biol. 25 (2005) 6921-6936.

[18] L.M. Grovdal, E. Stang, A. Sorkin and I.H. Madshus, Direct interaction of Cbl with pTyr 1045 of the EGF receptor (EGFR) is required to sort the EGFR to lysosomes for degradation, Exp. Cell Res. 300 (2004) 388-395.

[19] I. Griswold-Prenner, C.R. Carlin and M.R. Rosner, Mitogen-activated protein kinase

regulates the epidermal growth factor receptor through activation of a tyrosine phosphatase, J. Biol. Chem. 268 (1993) 13050-13054.
[20] Y. Wu, Z. Chen and A. Ullrich, EGFR and FGFR signaling through FRS2 is subject to negative feedback control by ERK1/2, Biol. Chem. 384 (2003) 1215-1226.
[21] L. Huang, N. Gotoh, S. Zhang, M. Shibuya, T. Yamamoto and N. Tsuchida, SNT-2 interacts with ERK2 and negatively regulates ERK2 signaling in response to EGF stimulation, Biochem. Biophys. Res. Commun. 324 (2004) 1011-1017.
[22] L. Huang, M. Watanabe, M. Chikamori, Y. Kido, T. Yamamoto, M. Shibuya, N. Gotoh and N. Tsuchida, Unique role of SNT-2/FRS2β/FRS3 docking//adaptor protein for negative regulation in EGF receptor tyrosine kinase signaling pathways, Oncogene 25 (2006) 6457-6466.
[23] E. Avizienyte, A.W. Wyke, R.J. Jones, G.W. McLean, M.A. Westhoff, V.G. Brunton and M.C. Frame, Src-induced de-regulation of E-cadherin in colon cancer cells requires integrin signalling, Nat. Cell Biol. 4 (2002) 632-638.
[24] V.G. Brunton, E. Avizienyte, V.J. Fincham, B. Serrels, C.A. Metcalf, 3rd, T.K. Sawyer and M.C. Frame, Identification of Src-specific phosphorylation site on focal adhesion kinase: dissection of the role of Src SH2 and catalytic functions and their consequences for tumor cell behavior, Cancer Res. 65 (2005) 1335-1342.
[25] B. Linggi and G. Carpenter, ErbB receptors: new insights on mechanisms and biology, Trends Cell Biol. 16 (2006) 649-656.
[26] X. Yu, S. Miyamoto and E. Mekada, Integrin α2β1-dependent EGF receptor activation at cell-cell contact sites, J. Cell Sci. 113 (Pt 12) (2000) 2139-2147.
[27] R. Fukuyama and N. Shimizu, Detection of epidermal growth factor receptors and E-cadherins in the basolateral membrane of A431 cells by laser scanning fluorescence microscopy, Jpn. J. Cancer Res. 82 (1991) 8-11.
[28] S. Pece and J.S. Gutkind, Signaling from E-cadherins to the MAPK pathway by the recruitment and activation of epidermal growth factor receptors upon cell-cell contact formation, J. Biol. Chem. 275 (2000) 41227-41233.
[29] C.M. Van Itallie, M.S. Balda and J.M. Anderson, Epidermal growth factor induces tyrosine phosphorylation and reorganization of the tight junction protein ZO-1 in A431 cells, J. Cell Sci. 108 (Pt 4) (1995) 1735-1742.
[30] K. Yoshida, S. Kanaoka, T. Takai, T. Uezato, N. Miura, M. Kajimura and A. Hishida, EGF rapidly translocates tight junction proteins from the cytoplasm to the cell-cell contact via protein kinase C activation in TMK-1 gastric cancer cells, Exp. Cell Res. 309 (2005) 397-409.
[31] P. Reddy, L. Liu, C. Ren, P. Lindgren, K. Boman, Y. Shen, E. Lundin, U. Ottander, M. Rytinki and K. Liu, Formation of E-cadherin-mediated cell-cell adhesion activates AKT and mitogen activated protein kinase via phosphatidylinositol 3 kinase and ligand-independent activation of epidermal growth factor receptor in ovarian cancer cells, Mol. Endocrinol. 19 (2005) 2564-2578.
[32] X. Shen and R.H. Kramer, Adhesion-mediated squamous cell carcinoma survival through ligand-independent activation of epidermal growth factor receptor, Am. J. Pathol. 165 (2004) 1315-1329.
[33] M. Fedor-Chaiken, P.W. Hein, J.C. Stewart, R. Brackenbury and M.S. Kinch, E-cadherin binding modulates EGF receptor activation, Cell Commun. Adhes. 10 (2003) 105-118.
[34] H. Hoschuetzky, H. Aberle and R. Kemler, β-catenin mediates the interaction of the

cadherin-catenin complex with epidermal growth factor receptor, J. Cell Biol. 127 (1994) 1375-1380.
[35] R.B. Hazan and L. Norton, The epidermal growth factor receptor modulates the interaction of E-cadherin with the actin cytoskeleton, J. Biol. Chem. 273 (1998) 9078-9084.
[36] M. Betson, E. Lozano, J. Zhang and V.M. Braga, Rac activation upon cell-cell contact formation is dependent on signaling from the epidermal growth factor receptor, J. Biol. Chem. 277 (2002) 36962-36969.
[37] X. Qian, T. Karpova, A.M. Sheppard, J. McNally and D.R. Lowy, E-cadherin-mediated adhesion inhibits ligand-dependent activation of diverse receptor tyrosine kinases, EMBO J. 23 (2004) 1739-1748.
[38] V. Sovova, D. Kucerova, M. Vojtechova, E. Sloncova and Z. Tuhackova, Transactivation of E-cadherin is not involved in the activity of EGF receptor in colorectal carcinoma cells, Int. J. Oncol. 25 (2004) 1459-1464.
[39] B.M. Gumbiner, Cell adhesion: the molecular basis of tissue architecture and morphogenesis, Cell 84 (1996) 345-357.
[40] R.C. Bates, Colorectal cancer progression: integrin αvβ6 and the epithelial-mesenchymal transition (EMT), Cell Cycle 4 (2005) 1350-1352.
[41] A.M. Valles, B. Boyer, G. Tarone and J.P. Thiery, α2β1 integrin is required for the collagen and FGF-1 induced cell dispersion in a rat bladder carcinoma cell line, Cell Adh. Commun. 4 (1996) 187-199.

Figure legends

Figure 1. U0126 treatment-mediated formation of cell-cell contacts in RIE1-Sca cells. (A) Parental RIE1 wildtype cells and its scattered variant RIE1-Sca cells in 60 mm culture dishes or serum-containing culture media-precoated coverglasses at subconflence were imaged or stained for actin by using phalloidin-conjugated rhodamine. (B) RIE1-WT or -Sca cells were treated with 20 μM U0126 for 14 h, prior to harvests of whole cell lysates. Lysates were normalized and used in immunoblots for indicated molecules. (C) Vehicle DMSO or U0126 at 20 μM were treated to sub-confluent RIE1-Sca cells for 14 h, prior to taking images. (D) RIE1-Sca cells were replated onto glass coverslips precoated with serum-containing normal culture media. After cells became confluent, DMSO or U0126 were treated for 14 h. After treatment, cells were processed for immunofluorescent staining for ZO1 (upper) or β-catenin (lower), as explained in the Materials and Methods. (E and F) Whole cell lysates were prepared from cells treated with DMSO or 20 μM U0126, as explained above, and used in standard Western blots by using antibodies against the indicated molecules. AG1478 was pretreated 30 min before 20 μM U0126 treatment for 14 h (E). The data shown are representative of 3 isolated experiments.

Figure 2. U0126 treatment-mediated cell contact formation appeared to retard wound healing. (A and B) Confluent RIE1 WT or RIE1-Sca cells in 60 mm culture dishes were wounded by scraping through the cell monolayer with a yellow pipette tip. Cells were then washed twice with DMEM-H containing 10% FBS. DMSO or U0126 (20 μM) were treated immediately after wounding and washing cells. Twenty (A) or 6 hours (B) later, phase contrast images were taken. Dotted lines indicate the start lines of wound healings. (C) RIE1-Sca cells were seeded onto cover glasses precoated with 10% FBS-containing normal culture media.

When cells were confluent, wounds were made through monolayers using a yellow pipette tip. Cells were then washed twice with the culture media and immediately treated with DMSO or U0126 (20 μM). Fourteen hours later, cells were stained for actin (left) or immunostained for ZO1 (right), as explained in the Materials and Methods. Arrows indicate wounds centers. The data shown represent 3 independent experiments.

Figure 3. Signaling activities involved in U0126 treatment-mediated contact formation of RIE1-Sca cells. (A and B) Whole cell lysates prepared from cells treated with DMSO or U0126 (20 μM) for 14 h in the absence (A) or presence of AG1478 pretreatment (2 h prior to U0126 treatment, B) were immunoblotted for the indicated molecules. (C) RIE1-Sca cells on glass coverslips under normal serum-containing culture conditions were pretreated with 1 μM AG1478, 2 h before 20 μM U0126 treatment for 14 h (AG1478 + U0126). Cells were then immunostained for β-catenin (D). The band intensity ratio between experimental conditions (A) is measured by a densitometer and shown as following; 4.56, 1.23, 1.0, and 5.12 (from lane 1 to 4, respectively) for pY^{992}EGFR, 0.08, 1.1, 1.0, and 0.09 for pY^{1045}EGFR, 2.1, 2.82, 1.0 and 2.04 for pY^{1068}EGFR, 3.02, 1.97, 1.0, and 5.4 for pY^{1173}EGFR. The data shown are representative of 3 different experiments.

Figure 4. Regulation of the Ras/Raf-1/MEK/Erks cascade via Ras mutant expression affected cell-cell contacts. RIE1-Sca cells were infected with retroviruses expressing control pLNCX plasmid (Retro-Cont), pLNCX-human H-Ras V12 (Retro-V12) or N17 (Retro-N17) mutants for 12 h. After an additional normal culture for 22 h, DMSO or U0126 (20 μM) were treated for 14 h before preparation of cell lysates (A) or immunostaining for β-catenin (B and

upper panels of C) or ZO1 (C, lower panels), as explained in the Materials and Methods. (D) Whole cell lysates were prepared from cells infected with control (Retro-Cont) or H-Ras N17 (Retro-N17) retroviruses as detailed above, prior to immunoblottings against the indicated molecules. Data shown are representative of 3 independent experiments. 'Retro-' indicates retrovirus expressing the indicated molecules.

Figure 5. Expression of EGFR Y5F in RIE1-Sca cells abolished U0126-mediated cell contact formation. RIE1-Sca cells on glass coverslips or on 60 mm culture dishes were infected with either control retrovirus (Retro-Cont) or retrovirus expressing EGFR Y5F (Retro-Y5F), in which the 5 autophosphorylation tyrosine residues were mutated to phenylalanines to be nonphosphorylatable. After an infection period of 12 h and an additional normal culture for 22 h, cells were treated with DMSO or U126 (20 μM) for 14 h, prior to processing for immunofluorescent staining for β-catenin (A, lefts) or ZO1 (A, rights) or harvest of cell lysates for immunoblottings against the indicated molecules (B). Data shown are representative of 3 isolated experiments.

Figure 6. U0126-mediated EGFR phosphorylation and cell-cell contact formation occur in a ligand-independent manner. (A and left/middle panels of C) Serum was removed from cell cultures prior to the DMSO or U0126 treatment without (U0126) or with EGF (50 ng/ml, U0126 + EGF). Treatments were continued for 14 h prior to immunofluorescent staining for ZO1 (A) or cell lysates harvests for immunoblottings (C, left and middle panels). (B and right panels of C) Cells on either 60 mm culture dishes or coverglasses were preincubated with normal IgG or anti-EGF (10 μg/ml) 60 min before DMSO or U0126 treatment for 14 h. After incubations, the

cells were processed for ZO1 immunofluorescent staining (B) or harvested for whole cell lysates and then immunoblottings (C, right panels). The data shown represent 3 independent experiments.

Figure 7. Regulation of U0126 treatment-mediated signaling activities affects wound healing. (A) RIE1-Sca cells were seeded and wounded with or without treatments of pharmacological inhibitors, as explained in the Materials and Methods. Fourteen or eighteen hours after wounding and treatment, phase contrast images were taken. Dotted lines indicate the starting lines for wound healings of a representative experiment. * and ** indicate statistic significance, as compared to DMSO treatment for 14 h (* of $p < 0.001$) and with U0126-alone treatment for 14 h (** of $p < 0.05$), respectively. (B & C) Cells in 60 mm culture dishes were infected with retrovirus for empty pLNCX (Retro-Cont), pLNCX-H-Ras N17 (Retro-N17), or pLNCX-EGFR Y5F (Retro-Y5F) plasmids for 12 h. After an additional incubation for 22 h, confluent cells were then wounded and washed, and cell images around wounds were taken at the indicated times (B) or at 14 h post-wounding (C). The dotted lines are start lines. Representative wound distances were measured for graphic presentations, and higher bars represent less wound healing. * and ** in (B) indicate statistic significance ($p < 0.001$), as compared with 10 h wound healing analysis of control-virus infected cells. * or ** in (C) also indicate statistic significance, as compared with control virus-infected cells treated with DMSO (* of $p < 0.001$), or with control virus-infected cells treated with U0126 (** of $p < 0.001$), respectively. The data shown are representative of three independent experiments.

Figure 1

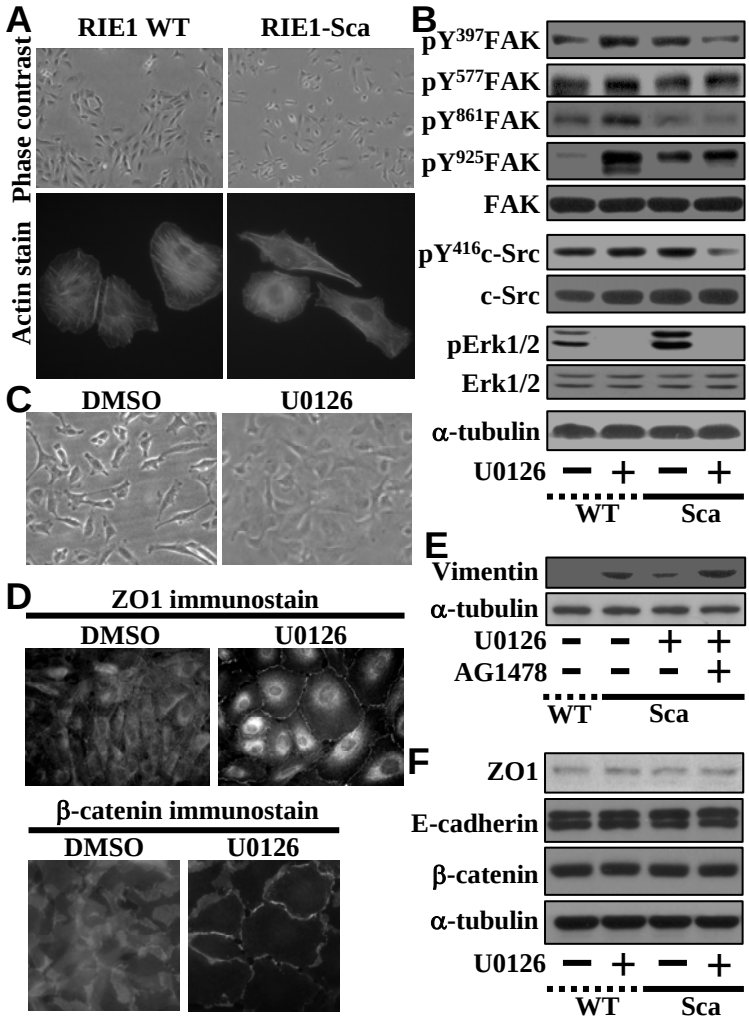
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Figure 2

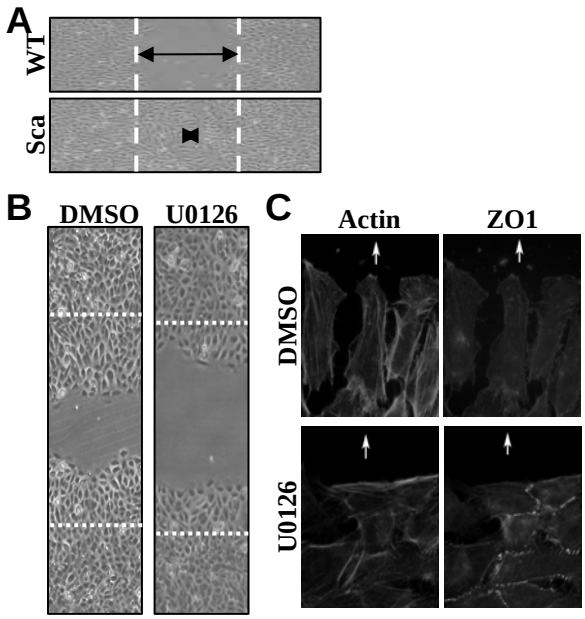

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Figure 3

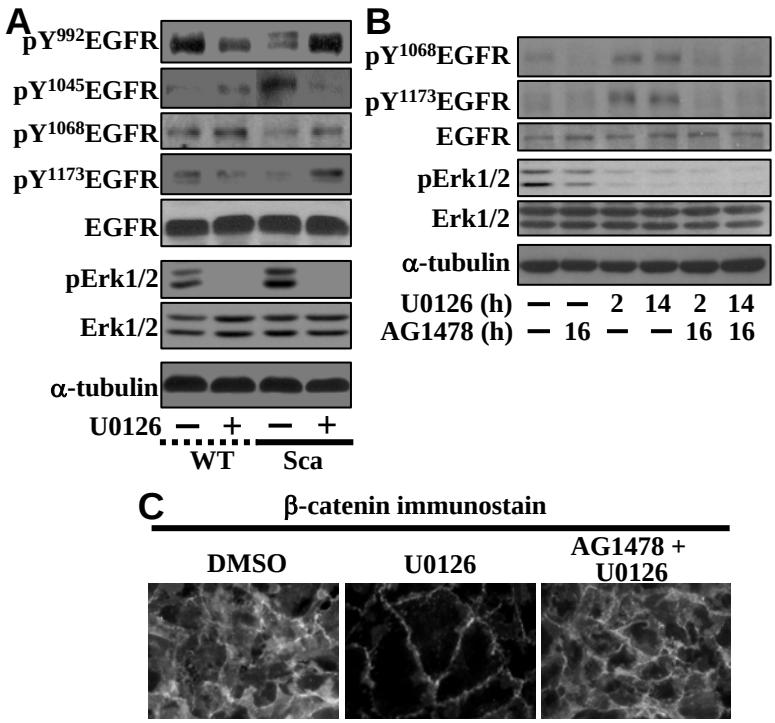

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Figure 4

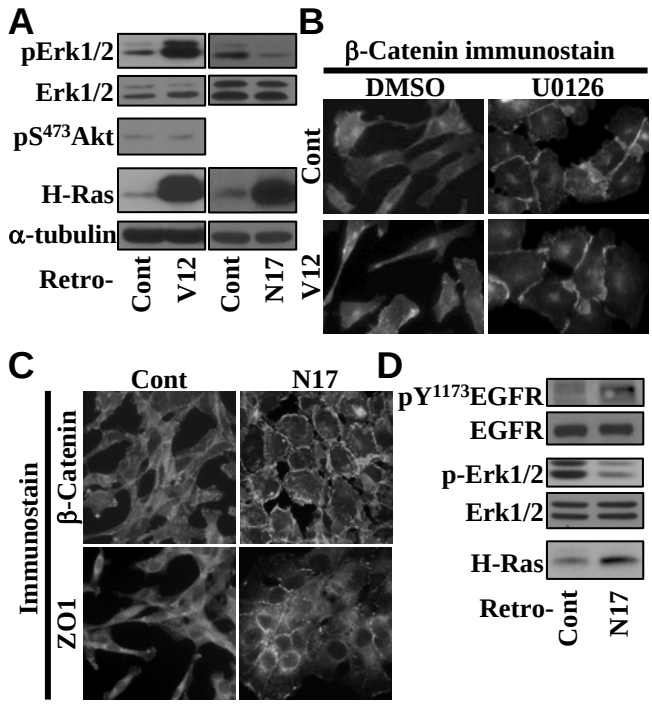

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Figure 5

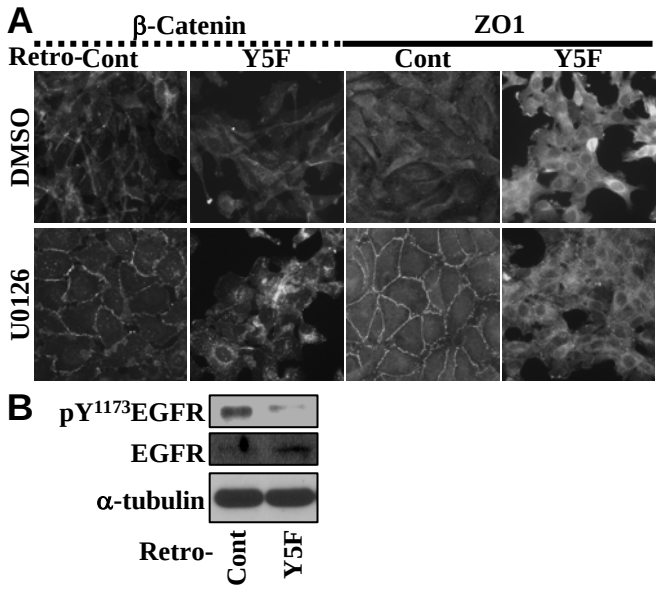

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Figure 6

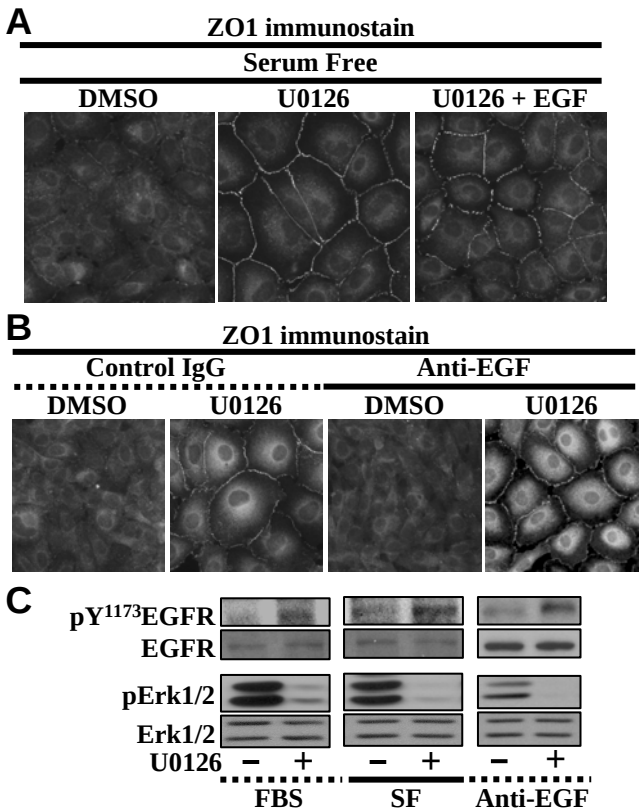

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Figure 7