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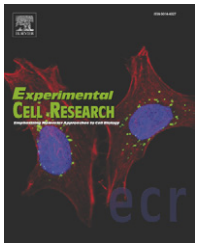

Research Article

PKC-δ binds to E-cadherin and mediates EGF-induced cell scattering

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ABSTRACT

EGF is known to affect adherens junctions and disrupt cell–cell adhesion in a variety of carcinomas but the underlying mechanisms are not completely understood. Using human tumor epithelial cells overexpressing EGFR we demonstrated that EGF-induced cell scattering was mediated by protein kinase C-delta (PKC-δ). PKC-δ knockdown by siRNA significantly inhibited EGF-induced internalization of E-cadherin into the cytoplasm and blocked cell scattering. EGF phosphorylated PKC-δ at Y311 and ectopic expression of the mutant Y311F prevented PKC-δ binding to E-cadherin and EGF-induced cell scattering. Moreover, depletion of Src using siRNA decreased EGF-induced phosphorylation of PKC-δ at Y311 and blocked scattering. Finally, EGF reduced expression of the tight junction protein, occludin, and this effect was also mediated by PKC-δ through Src. In summary, PKC-δ mediated the effects of EGF on adherens and tight junctions thereby playing an important role in cell–cell adhesion with possible wider implications in tumor metastasis or epithelial-to-mesenchymal transition.

Introduction

Members of the EGF receptor family, EGFR and HER2 are major targets of anti-cancer therapy as tumors overexpressing these receptors exhibit poor prognosis [1]. EGFR and HER2 have been shown to be strong prognostic indicators of several tumors including bladder, cervical, oesophageal, head and neck and ovarian cancers [2,3]. EGFR overactivity leads to increased growth [4] as well as spread of tumors [5]. Increased activity of the receptor-mediated signaling may be caused by mutations [6,7], misregulation of EGFR gene [8,9] or the tumor microenvironment e.g. hypoxia [10] or high concentration of EGFR ligands such as EGF and TGF-α [9].

EGF causes cell migration and invasion by breaking cell–cell adhesion and modulating cell–ECM interactions. Expression of ECM proteins and integrins as well as the phosphorylation of kinases that relay integrin signals e.g. integrin-linked-kinase (ILK) and focal adhesion kinase (FAK) is affected by EGF [11,12]. EGF also induces E-cadherin internalization and disruption of cell–cell adhesion through caveolin-dependent endocytosis that

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Abbreviations: PKC, protein kinase C; EGFR, epidermal growth factor receptor; EGF, epidermal growth factor; GFP, green fluorescence protein

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leads to β-catenin nuclear import and transcriptional activation [13]. In addition to adherens junctions, EGF affects epithelial polarization and cell–cell adhesion through tight junction proteins ZO-1 [14] and claudins [15]. Thus, the dynamic changes initiated by binding of EGF to its receptor result in reduced cell–cell interaction and increased cell motility leading to epithelial-to-mesenchymal transformation (EMT) of normal cells or invasion of cancer cells.

Adherens junctions are calcium-dependent cell–cell adhesion junctions mediated by the transmembrane glycoprotein E-cadherin that participates in homophilic interactions with E-cadherin of adjacent cells. The cytoplasmic domain of E-cadherin directly interacts with β-catenin, which in turn binds to α-catenin linking adherens junctions to the actin cytoskeleton. However, this view has been challenged by two recent studies which suggested that α-catenin can either bind the E-cadherin/β-catenin complex or homodimerize and crosslink actin filaments, but it cannot bind both E-cadherin/β-catenin and F-actin at the same time [16,17]. Other proteins e.g. p120-catenin, vinculin, α-actinin, talin, vasp, ZO-1, F-actin and occludin could also be part of the junction [18].

Tight junctions, on the other hand, are composed of claudin, occludin and junctional adhesion molecule (JAM) that are anchored in the membranes of two cells and interact with each other to hold the cells together and regulate passage of molecules through intercellular space [19]. Occludin and claudins interact with ZO-1, which in turn interacts with actin thereby providing a direct link to the cytoskeleton. Interestingly, occludin also binds to α-catenin [20], while E-cadherin is required for the formation of functional tight junctions in vivo [21]. Moreover, dissolution of tight junctions in EMT is followed by down-regulation of E-cadherin and dissolution of adherens junctions [22]. Taken together these observations provide strong evidence that these two families of adhesion molecules are functionally linked under normal or pathological conditions.

The protein kinase C (PKC), a family of serine–threonine kinases, contains at least 10 members that are classified into three groups: classical (α, β1, β2, γ), novel (δ, ε, η and θ) and atypical (ζ and λ/ι). In general, PKCs have been implicated in diverse cellular functions including proliferation, differentiation [23] and apoptosis [24] through multiple signaling pathways. In particular, PKC-δ has been shown to play a role in regulation of cell proliferation and programmed cell death in response to DNA damage and oxidative stress [25–28]. Recent studies identified a distinct proteolytic activation of PKC-δ involving its tyrosine phosphorylation by the Src family kinases and p38 [29–31]. Despite an explosion of information in the field of PKC signaling, there have been no studies implicating PKC-δ in EGF-induced cell scattering and EMT.

In this study we hypothesize that PKC-δ may mediate – at least in part – the effects of EGF on cell scattering. To this end, we provide evidence that EGF phosphorylates PKC-δ at tyrosine 311 within minutes after administration. Phosphorylation at Y311 is necessary for binding of PKC-δ to E-cadherin, internalization of adherens junctions and cell scattering. PKC-δ also mediates the decrease in occludin triggered by EGF treatment, suggesting that in addition to adherens junctions PKC-δ may play a key role in controlling tight junctions. Therefore, our results suggest that PKC-δ may be important in cell–cell adhesion and epithelial-to-mesenchymal transition by modulating adherens and tight junctions.

Materials and methods

Cell culture and chemical reagents

Primary human keratinocytes were isolated from neonatal foreskins following the protocol of Green et al. [32] and expanded on feeder layers of mitomycin-C treated 3T3-J2 mouse fibroblasts (ATCC, Manassas, VA) as described previously [33,34]. After the initial expansion, primary keratinocytes were cultured in keratinocyte serum-free medium (SFM) supplemented with 50 μg/ml bovine pituitary extract (BPE) and 5 ng/ml EGF (Invitrogen, Carlsbad, CA). A431 epidermoid carcinoma cells and human embryonic kidney cell-derived retrovirus-packaging cell line HEK 293T/17 (ATCC) were cultured in DMEM (Invitrogen) containing 10% certified fetal bovine serum (Gibco BRL, Grand Island, NY), 100 U of penicillin and 100 μg/ml streptomycin (Gibco BRL).

Chemical inhibitors for Erk1/2 (PD98059 and U0126), p38 (SB203580), JNK inhibitor (SP600125), Src (PP2), protein kinase C (Gö6976 and Gö6983) and EGFR inhibitor (PD153035) were purchased from EMD Biosciences, San Diego, CA. Cetuximab (clone C225), a monoclonal antibody generated against the extracellular domain of EGFR was a generous gift from ImClone Systems Incorporated, New York, NY.

Cell scattering

Cells were plated in 6-well plates (100,000 cells/well; ~10% confluency) and the next day EGF was added at 100 ng/ml. Cells without EGF treatment served as negative control. After 24 h, the cells were visualized using a Zeiss Axio Observer.Z1 microscopy (Carl Zeiss MicroImaging, Thornwood, NY) and pictures of several fields of view per well (300–700 cells per well) were taken at random using an ORCA-ER CCD camera (Hamamatsu Photonics, Hamamatsu City, Japan). Cells that fell under either of the following two categories were designated as scattered cells: (1) peripheral cells of a colony that appeared to be moving away (appeared elongated radially) from the colony; or (2) isolated cells that appeared to spread less and were highly reflective under the microscope. The total number of scattered cells was divided by total number of cells and the ratio was plotted as % scattered cells under the indicated conditions.

Western blotting

Cells plated in 6-well plates (1.5×10^6 cells/well) were lysed using ice-cold lysis buffer: 62.5 mM Tris–HCl (pH 6.8 at 25 °C), 2% (w/v) SDS, 10% glycerol and 0.01 (w/v) bromophenol blue (Cell Signaling, Danvers, MA) containing 41.67 mM dithiothreitol (DTT) (Cell Signaling) and protease inhibitor cocktail (Complete Mini; Roche Applied Science, Indianapolis, IN). Lysates were frozen at −80 °C until use.

For gel electrophoresis the samples were passed through 26.5-gauge needle five times, then heated at 95 °C for 5 min, placed on ice for 5 min, centrifuged for 5 min at 10,000 ×g, loaded onto a 8–10% denaturing gel (SDS-PAGE) and run for 60–90 min at 150–200 V (Mini-protean 3 system; BioRad Laboratories, Hercules, CA). The proteins were then transferred onto a nitrocellulose membrane (Trans-Blot® Transfer Medium, BioRad Laboratories) for 1 h at 350 mA using an electrophoretic transfer cell (Mini Trans-Blot®; BioRad Laboratories). The membrane was incubated in blocking

buffer (5% (w/v) non-fat milk in wash buffer (TBS/0.1% TWEEN 20)) for 1 h at room temperature (RT). Then, the membrane was incubated with rabbit or mouse anti-human (phospho-PKC-δ-Y311, phospho-PKC-δ-T505, total Erk1/2 or β-catenin: 1/1000 dilution, Cell Signaling; E-cadherin: 1/2500 dilution, PKC-δ: 1/1000 dilution, BD Biosciences, San Jose, CA; β-actin: 1/5000 dilution, Sigma-Aldrich, St. Louis, MO; GFP: 1/5000 dilution, Clontech, Mountain View, CA; Occludin, ZO-1: 1/500 dilution, Invitrogen, Carlsbad, CA). The primary antibody incubation was performed in 2% (w/v) non-fat milk in wash buffer overnight at 4 °C. The membrane was washed three times for 5 min each with wash buffer and incubated with HRP-conjugated goat anti-rabbit or mouse IgG polyclonal secondary antibody (1/2000 dilution in blocking agent for 1 h at RT; Cell Signaling). The membrane was washed three times for 5 min each with wash buffer and the bands were detected using chemiluminescence (LumiGLO; KPL, Gaithersburg, MD) according to the manufacturer's instructions. Luminescence intensity was quantified using Kodak 1D v.3.6.1 software (Kodak Scientific Imaging Systems, New Haven, CT).

Co-immunoprecipitation

Cells plated in 6-well plates (1.5×10^6 cells/well) were lysed using ice-cold non-denaturing cell-lysis buffer (#9803, Cell Signaling) containing protease inhibitor cocktail (Complete Mini) and 1 mM AEBSF (EMD Chemicals). Lysates were passed through 26.5-gauge needle six times and were centrifuged at 10,000 ×*g* for 10 min. The supernatants were incubated overnight with antibodies (E-cadherin: 1/40 dilution, BD Biosciences; PKC-δ: 1/40 dilution, Cell Signaling; GFP: 1/40 dilution, Clontech) at 4 °C with gentle rotation. Following antibody incubation, lysates were further incubated with 40 μl/per sample of Protein A-Sepharose 4B (Invitrogen) for 3 h at 4 °C.

The protein–antibody–protein A complex was precipitated by centrifugation at 10,000 ×*g* for 2 min and the complex was washed three times with 500 μl of lysis buffer. After washing the complex was resuspended in 6% SDS loading buffer (#7722, Cell Signaling), vortexed 3 times, 5 s each, heated at 95 °C for 5 min, cooled on ice, and centrifuged for 1 min at 17,000 ×*g*. The supernatants were loaded on 8–10% SDS-PAGE gels and western blotting was performed as described above.

Immunofluorescence

Cells were plated on tissue culture treated glass slides. After treatment with EGF, they were fixed with ice-cold methanol for 8 min. Following three washes with PBS, the samples were blocked with 1% BSA in PBS for 45 min. Then samples were incubated with primary antibody (phospho-PKC-δ-(Y311): 1/50 dilution in blocking agent, overnight at 4 °C, Santa Cruz Biotechnology, Santa Cruz, CA; E-cadherin: 1/100 dilution in blocking agent, overnight at 4 °C, BD Biosciences). Following three washes with PBS, samples were then incubated with Alexa 488 (green) or 594 (red) conjugated goat anti-rabbit or goat anti-mouse secondary antibodies for 45 min at RT. Samples were washed three times with PBS and incubated with Hoechst 33342 (blue, nuclear stain) for 5 min at RT. After two more washes, slides were mounted with an antifade agent (Gel/Mount; Biomeda, Foster City, CA) and images were acquired using a confocal microscope (LSM 510; Zeiss) equipped with a digital camera.

siRNA inhibition of PKC-δ

siRNA targeting the same nucleotides of *pkc*-δ mRNA as described by Kiyotsugu Yoshida et al. [25] was incorporated using pSIREN-RetroQ vector according to manufacturer's instructions (Clontech)

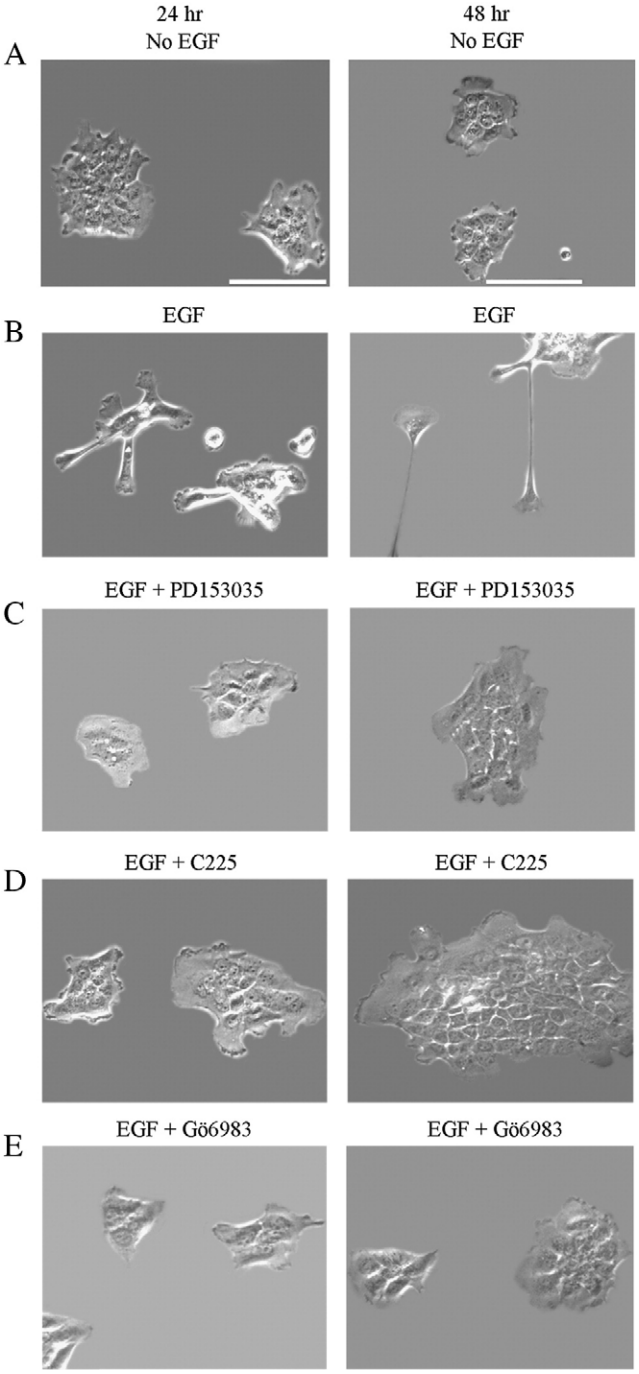


Fig. 1 – EGF-induced cell scattering is inhibited by a PKC-δ inhibitor. (A) A431 cells were seeded in 6-well tissue culture treated plates at 10^5 cells per well. On the next day, cells were treated with (A) an IgG isotype antibody (control); (B) EGF (100 ng/ml) + IgG isotype antibody; (C) EGF + PD153035 (300 nM); (D) EGF + C225 (50 μg/ml) or (E) EGF + Gö6983 (10 μM) for 24 or 48 h. Phase contrast photomicrographs are shown from a representative experiment ($n=3$). Bar = 100 μm.

as described previously [35]. Briefly, to deplete PKC-δ three 21-nucleotide regions of PKC-δ mRNA were targeted using siRNA. The target sequences were: 5′-AAGAAGCCGACCATGTATCCT-3′ (aa 492–512, human PKC-δ, Gen Bank# NM_006254), 5′-AAGATGAAG-GAGGCGCTCAGC-3′ (aa 444–464, human PKC-δ, Gen Bank# NM_006254), and 5′-AAGGCTGAGTTCTGGCTGGAC-3′(aa 654–674, human PKC-δ, Gen Bank# NM_006254). The 65-nt oligonucleotide sequence containing sense-loop-antisense-stop-codon was annealed into double strand and ligated to pSIREN-RetroQ vector between BamH1 and EcoR1 sites. The sequences of the 65-nt sequences are as follows: 5′-GATCCGAAGCCGACCATGTATCCTTT-CAAGAGAAGGATACATGGTCGGCTTCTTTTTTGCTAGCG-3′, 5′-

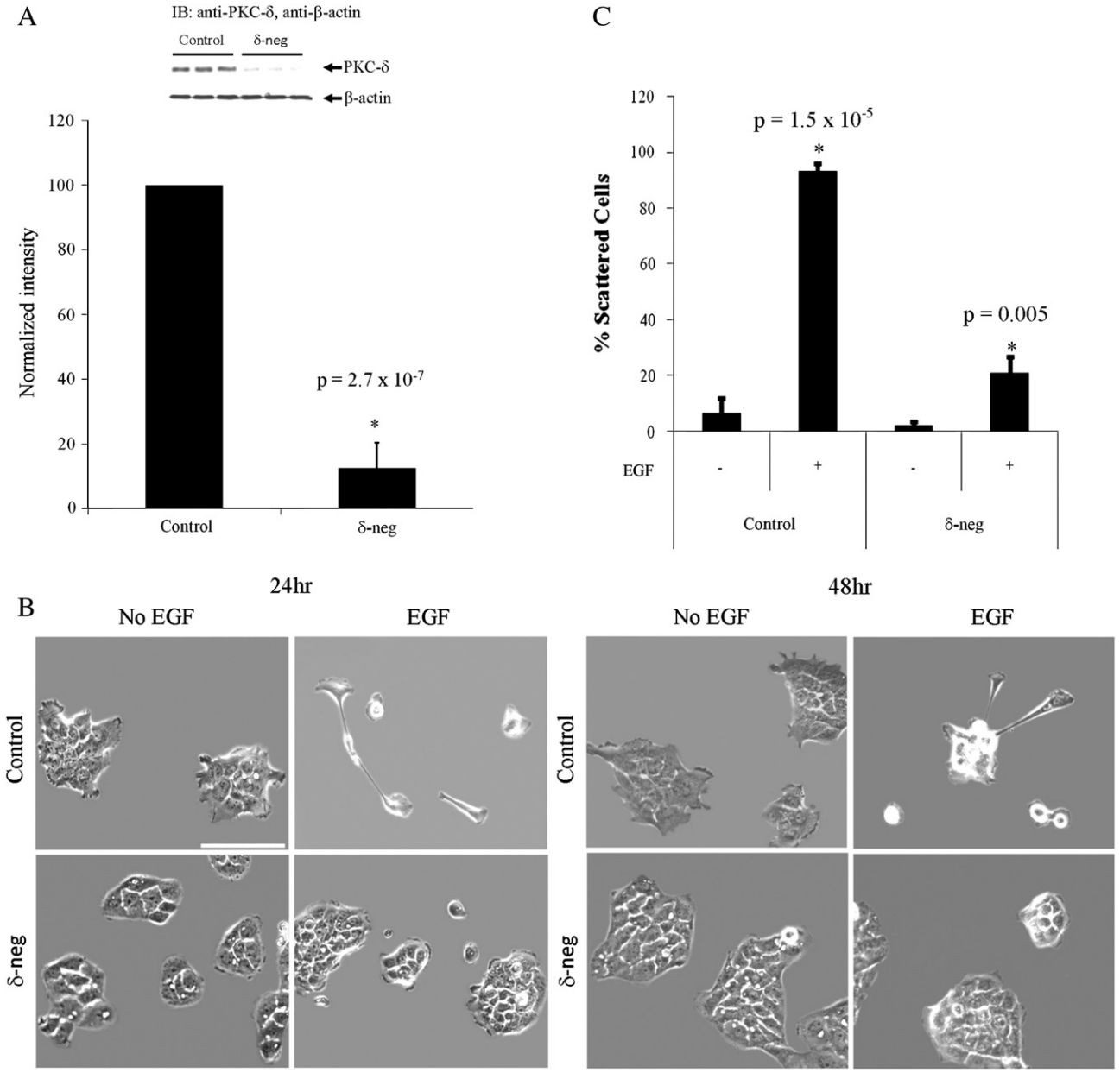


Fig. 2 – EGF-induced cell scattering is mediated through PKC-δ. Knockdown of PKC-δ in A431 cells using siRNA targeting *pkc-δ* mRNA. (A) Western blot showing amount of PKC-δ in cells expressing non-silencing siRNA (Control) or PKC-δ-specific siRNA (δ-neg). The intensity of each PKC-δ band was normalized with that of β-actin (loading control) and plotted as % of normalized PKC-δ in control cells ($n = 4$). (B) Phase contrast images of control or δ-neg cells at 24 or 48 h after treatment with EGF (100 ng/ml). Representative images are shown ($n = 3$). Bar = 100 μm. (C) Cells that appear to break from each colony ("scattered" cells) were counted and normalized to the total number of cells ($n = 300–700$ cells were counted for each condition). The fraction of scattered cells was plotted for the indicated conditions. The *p*-values represent comparison between EGF-treated and untreated cells in each group (control or δ-neg). (D) Confocal immunofluorescence images showing disruption of E-cadherin localization in control but not in δ-neg cells at 24 h after treatment with EGF (100 ng/ml). Images are from a representative experiment ($n = 3$). Bar = 10 μm.

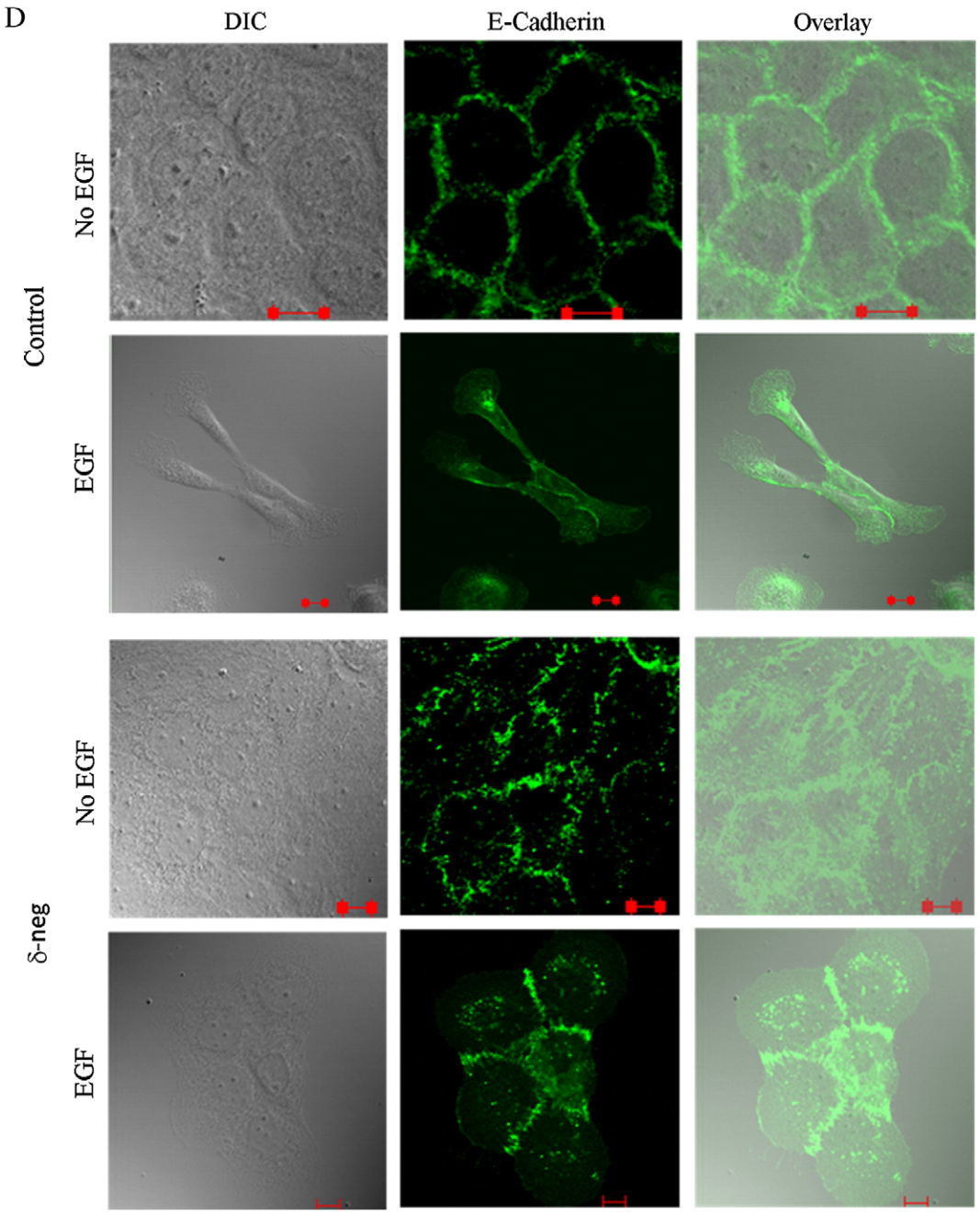


Fig. 2 (*continued*).

GATCCGATGAAGGAGGCGCTCAGCTTCAAGAGAGCTGAGCGCCTCC-TTCATCTTTTTTGCTAGCG-3′, and 5′-GATCCGGCTGAGTTCTGGCTG-GACTTCAAGAGAGTCCAGCCAGAACTCAGCCTTTTTTGCTAGCG-3′, respectively. An oligonucleotide generating a non-silencing control siRNA [36] was also cloned in pSIREN-RetroQ vector and used as negative control. A431 cells were transduced with retrovirus producing PKC-δ or control siRNA and PKC-δ knockdown was confirmed by western blotting.

siRNA inhibition of Src

Lentiviral vector pLKO.1 with Src siRNA was purchased from Sigma (Cat#: TRCN0000038149). The siRNA sequence is: 5′-CCGGGCTCGGCTCATTGAAGACAATCTCGAGATTGTCTTCAATGAGCC-GAGCTTTTTG-3′. Following transduction with lentivirus, A431 cells were selected with puromycin (1 μg/ml) for 2 weeks (Src-neg cells) and knockdown of Src was confirmed by western blot.

Lentiviral vectors and mutagenesis

PKC-δ-FL-GFP (full-length PKC-δ fused with GFP) and PKC-δ-RD-GFP (regulatory domain of PKC-δ fused with GFP) (generously provided by Dr. Christer Larsson, Lund University, Sweden) were cut by Nhe1 and Sal1 sites and ligated to lentiviral vector CSCG between Nhe1 and Xho1 sites. Full-length PKC-δ in vector CSCG-PKC-δ-FL-GFP was mutated at Y311 to phenylalanine (F) using

primer: CAGAGCCTGTTGGGATATTTCAGGGTTTCGAGAAG and site directed mutagenesis kit: QuikChange® II XL (Stratagene, La Jolla, CA). The mutation was confirmed by plasmid sequencing.

Lentivirus production and transduction

Lentivirus was produced by four plasmid co-transfection of 293T cells as follows. HEK 293T/17 cells were plated in T75 flask (5.0×10^6 cells/flask). The next day, cells were co-transfected with three plasmids encoding for lentiviral structural proteins (pMDG (1.5 μg), pMDL (5 μg), and PSRV-Rev (3 μg)) and a lentiviral plasmid encoding the gene of interest (10.5 μg) using the calcium phosphate precipitation method. At 36 h after transfection the medium was replaced with fresh culture medium and the next day, the virus medium was harvested, filtered through 0.45 μm filter and centrifuged (50,000 ×g, 2 h, 4 °C) to pellet the viral particles. The pellet was reconstituted in 1.5 ml of fresh medium and virus was stored at −80 °C in 300 μl aliquots.

For transduction with lentivirus, A431 cells were plated in 24-well plates (5.0×10^5 cells/well). The next day, the culture medium was replaced with 300 μl of lentivirus containing 8 μg/ml of polybrene and transduction was allowed for 12 h, after which the virus containing medium was replaced by fresh culture medium.

Statistical analysis

Statistical analysis of the data was performed using a two-tailed Student's *t*-test using Microsoft Excel (Microsoft, Redwood, CA). The data were considered statistically different when $p<0.05$.

Results

EGF causes cell scattering through PKC-δ

In agreement with previous studies, treatment of A431 epidermoid carcinoma cells with EGF caused rounding of single-cells and scattering of colonies with elongation and detachment of peripheral cells. Both cell-rounding and colony-disorganization were blocked by EGFR tyrosine kinase inhibitor (PD153035) or a monoclonal antibody (Cetuximab, clone C225) that binds to the extracellular domain of EGFR (Figs. 1A–D).

To determine which intracellular pathway(s) may mediate the effect of EGF on cell scattering, we used several pharmacological inhibitors to block known intracellular EGF effectors: Erk1/2 (PD98059, concentration range: 0.25–10 μM), PLCγ (U73122, concentration range: 0.25–10 μM), PI3K (LY294002, concentration range: 1–10 μM) or IP3 activated ER Ca^{2+} channel (2-APB, concentration range: 5–40 μM). To this end, cells were treated with EGF (100 ng/ml) in the absence or presence of these inhibitors and cell scattering was monitored up to 48 h after treatment. Blocking these pathways did not reverse the effect of EGF on cell scattering (data not shown). We also used two different inhibitors of protein kinase C (PKC), namely Gö6976 (concentration range: 1–10 μM) and Gö6983 (concentration range: 1–10 μM). Gö6976 is an inhibitor of classical PKCs i.e. PKC-α and PKC-β, and Gö6983 specifically blocks PKC-δ. While Gö6976 had no effect even at high concentrations (data not shown), Gö6983 reversed the effect of EGF on cell scattering (Fig. 1E).

Since Gö6983 is an effective inhibitor of PKC-δ we examined whether PKC-δ mediated the effect of EGF on cell scattering using siRNA knockdown. In contrast to non-silencing control siRNA, PKC-δ-siRNA reduced the amount of PKC-δ protein by $87.6\pm7.9\%$ ($n=4$) (Fig. 2A) and significantly reduced EGF-induced cell scattering (Figs. 2B, C; also see Fig. S1 for lower magnification images). In addition, confocal immunofluorescence showed that EGF treatment led to translocation of E-cadherin from the cell–cell boundary to the cytoplasm in control cells but not in PKC-δ depleted cells (Fig. 2D). These results suggested that the effect of EGF on adherens junctions may be mediated through PKC-δ.

EGF induces PKC-δ phosphorylation and binding to E-cadherin

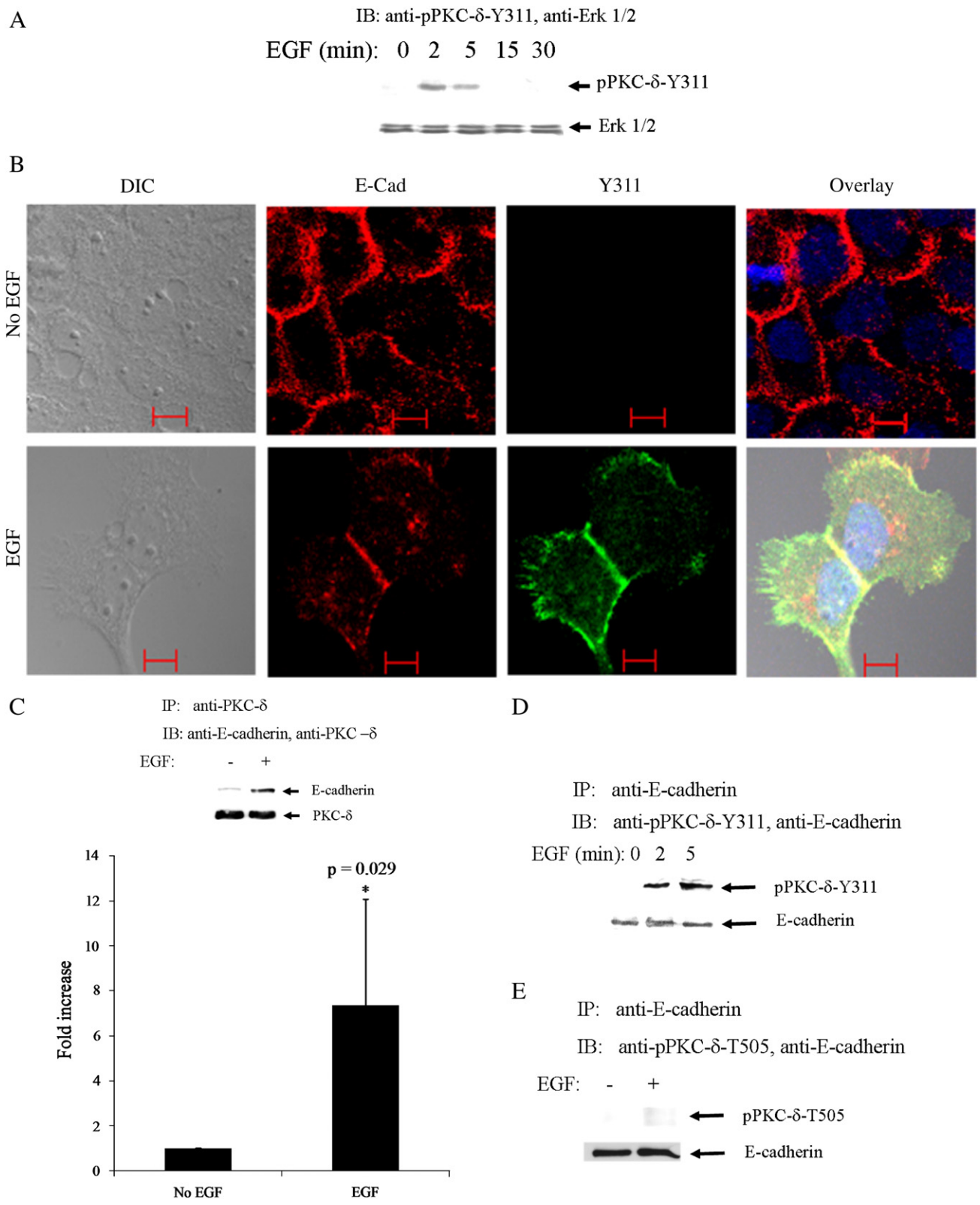
EGF induced transient phosphorylation of PKC-δ at Y311, which peaked at 2 min and disappeared within 15 min of EGF treatment (Fig. 3A). In addition, immunostaining showed that upon treatment with EGF, PKC-δ-Y311 appeared at the cell border, colocalizing with E-cadherin at cell–cell junctions (Fig. 3B).

Since adherens junctions are involved in cell–cell adhesion, which is compromised upon scattering and since PKC-δ-Y311 was localized at the cell border, we hypothesized that PKC-δ may bind to adherens junctions in response to EGF. To test this hypothesis, we immunoprecipitated PKC-δ from lysates of EGF-treated or untreated cells and examined the presence of E-cadherin by immunoblotting. As shown in Fig. 3C a small amount of E-cadherin bound to PKC-δ even in non-stimulated cells. However, treatment with EGF increased binding significantly by 7.3 ± 5.4-fold ($n=3$). Conversely, immunoprecipitation with anti-E-cadherin and western blot for PKC-δ-Y311 also showed that phosphorylated PKC-δ was bound to E-cadherin (Fig. 3D). In contrast, PKC-δ-T505 did not bind to E-cadherin (Fig. 3E), even though it was present in high abundance in cell lysates (data not shown, see also [35]).

Fig. 3 – EGF induces phosphorylation and binding of PKC-δ to E-cadherin. A431 cells were serum starved (0.1% FBS) for 24 h and then treated with EGF (100 ng/ml) for the indicated times. (A) Western blot analysis of cell lysates using anti-pPKC-δ-Y311. The membrane was stripped and re-probed by anti-Erk1/2 antibody that served as a loading control ($n=5$). (B) Confocal immunofluorescence images showing E-cadherin and PKC-δ-Y311 at 2 min after treatment of A431 cells with EGF (100 ng/ml). Fluorescence images from a representative experiment are shown ($n=3$). Bar = 10 μm. (C) A431 cells were treated with EGF (100 ng/ml) for 2 min and cell lysates were immunoprecipitated with anti-PKC-δ and immunoblotted with anti-E-cadherin. The membrane was stripped and re-probed with anti-PKC-δ as immunoprecipitation control ($n=3$). E-cadherin intensity was normalized with that of PKC-δ and plotted as fold increase of EGF-treated compared to untreated cells. The *p*-value represents comparison between EGF-treated and untreated samples. (D) Immunoprecipitation of cell lysates with anti-E-cadherin and immunoblotting with anti-pPKC-δ-Y311 ($n=3$). (E) Immunoprecipitation of cell lysates with anti-E-cadherin and immunoblotting with anti-pPKC-δ-T505. The membrane was stripped and re-probed with anti-E-cadherin as loading control. A representative blot is shown ($n=3$).

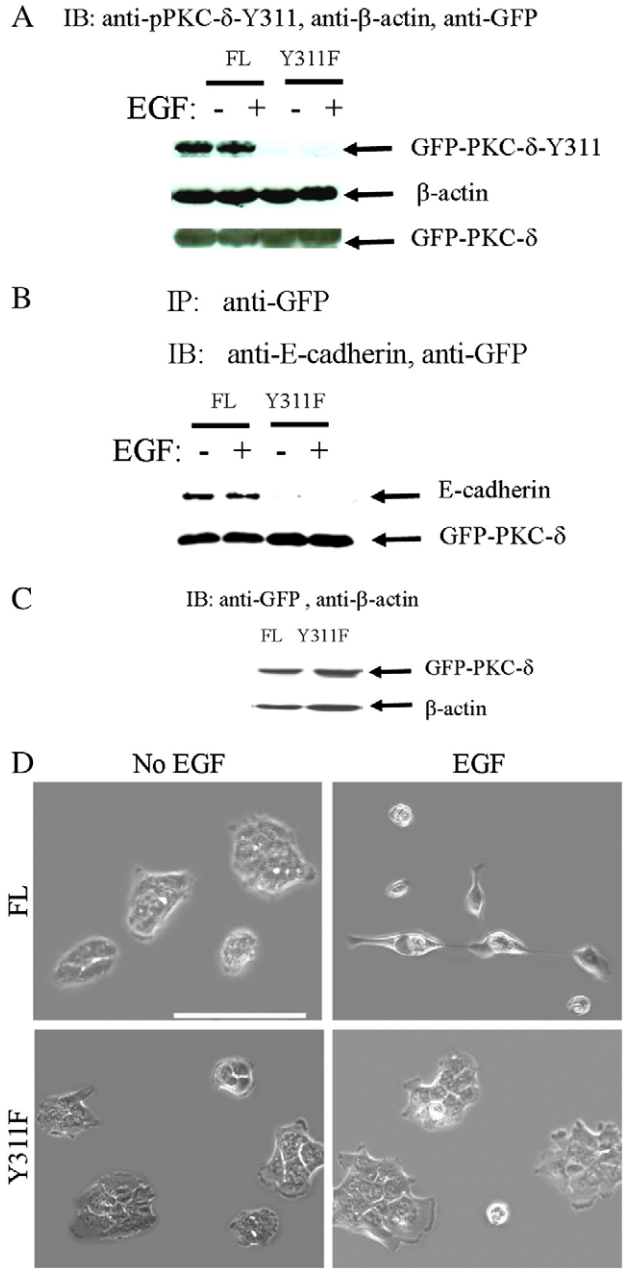
PKC-δ mutant fails to bind E-cadherin and inhibits EGF-induced cell scattering

To examine whether phosphorylation at Y311 was necessary for binding to E-cadherin, we transfected 293-T cells with plasmids encoding for fusions of GFP with full-length, wild-type or mutant (Y311F) PKC-δ, denoted as GFP-PKC-δ-FL or GFP-PKC-δ-Y311F, respectively and used western blot to detect phosphorylated protein. In contrast to A431 cells, GFP-PKC-δ-FL was phosphorylated at Y311 even in the absence of EGF (Fig. 4A), possibly due to



some constitutively active intracellular effector in 293T cells. Consequently, immunoprecipitation of cell lysates with anti-GFP antibody showed that phosphorylated GFP-PKC-δ-FL bound to E-cadherin (Fig. 4B) in the presence or absence of EGF. In contrast, the mutant GFP-PKC-δ-Y311F was not phosphorylated even in EGF-treated cells (Fig. 4A) and failed to bind E-cadherin (Fig. 4B). Taken together, these results suggest that phosphorylation at Y311 may be necessary for binding of PKC-δ to E-cadherin.

To further examine the functional significance of these results in cell scattering, GFP-PKC-δ-FL or GFP-PKC-δ-Y311F was stably expressed in A431 cells using recombinant lentivirus (Fig. 4C). Western blotting showed that the level of GFP-PKC-δ-Y311F expression was 50% higher ($n=3$) than that of endogenous PKC-δ (Fig. S2). Interestingly, expression of GFP-PKC-δ-Y311F decreased EGF-induced cell scattering significantly (Fig. 4D), suggesting that phosphorylation at Y311 may be necessary for disruption of cell–cell contacts.



The regulatory domain of PKC-δ is sufficient for binding to E-cadherin but not for cell scattering

Since Y311 lies in the hinge region between the regulatory and catalytic domains of PKC-δ, we examined whether the regulatory domain containing the hinge region was sufficient for binding of PKC-δ to E-cadherin. To this end, 293T cells were transfected with plasmids encoding fusion proteins of GFP with either the full-length (GFP-PKC-δ-FL) or the regulatory domain (GFP-PKC-δ-RD) of PKC-δ. Immunoprecipitation with anti-E-cadherin followed by western blot using either anti-PKC-δ antibody (Fig. 5A) or by anti-GFP antibody (Fig. 5C) showed that both full-length protein and regulatory domain bound to E-cadherin. Western blots of cell lysates showed that both proteins were expressed at high level in transfected cells (Figs. 5B and D). Interestingly, both anti-PKC-δ and anti-GFP antibodies detected multiple bands of lower molecular weights in the lysates of PKC-δ-RD transfected cells, possibly representing regulatory domain degradation products.

Since the PKC-δ-RD bound to E-cadherin, we examined whether PKC-δ-RD overexpression was sufficient to cause cell scattering even in the absence of EGF. To this end, we transduced A431 cells with recombinant lentiviruses encoding for GFP-PKC-δ-FL or GFP-PKC-δ-RD and protein expression was verified by western blotting (Fig. 5E). Interestingly, ectopic expression of GFP-PKC-δ-RD did not induce cell scattering in the absence of EGF. What is more, PKC-δ-RD reduced EGF-induced cell scattering significantly (Fig. 5F), suggesting that the catalytic domain of PKC-δ may be necessary for dispersion of cell colonies.

Inhibiting Src decreases EGF-induced phosphorylation of PKC-δ at Y311 and cell scattering

Recent studies implicated Src kinases in tyrosine phosphorylation of PKC-δ in cardiomyocytes and epidermal keratinocytes [37,38].

Fig. 4 – PKC-δ mutant fails to bind E-cadherin and inhibits EGF-induced cell scattering. 293T cells were transfected with (1 μg per 10^6 cells) either GFP-PKC-δ-FL (fusion of wild-type full-length PKC-δ with GFP labeled as FL) or GFP-PKC-δ-Y311F (fusion of Y311F mutant with GFP labeled as Y311F) using Lipofectamine™2000. At 36 h after transfection cells were treated with EGF (100 ng/ml) for 2 min. (A) Western blot of cell lysates with anti-pPKC-δ-Y311. The membrane was re-probed with anti-GFP to assess transfection efficiency and anti-β-actin that served as loading control. (B) Cell lysates were immunoprecipitated using anti-GFP and immunoblotted with anti-E-cadherin. The membrane was striped and re-probed with anti-GFP to detect the amount of FL and PKC-δ-Y311F proteins immunoprecipitated and served as loading control ($n=3$). (C) GFP-PKC-δ-FL and GFP-PKC-δ-Y311F were stably expressed in A431 cells using recombinant lentivirus. Western blotting of cell lysates with anti-GFP showed expression of full-length (FL) or dominant negative (Y311F) PKC-δ; β-actin served as loading control. (D) A431 cells expressing GFP-PKC-δ-FL (FL) or GFP-PKC-δ-Y311F (Y311F) were treated with EGF and 24 h later cells were visualized with phase contrast microscopy. Images from a representative experiment are shown ($n=3$). Bar = 100 μm.

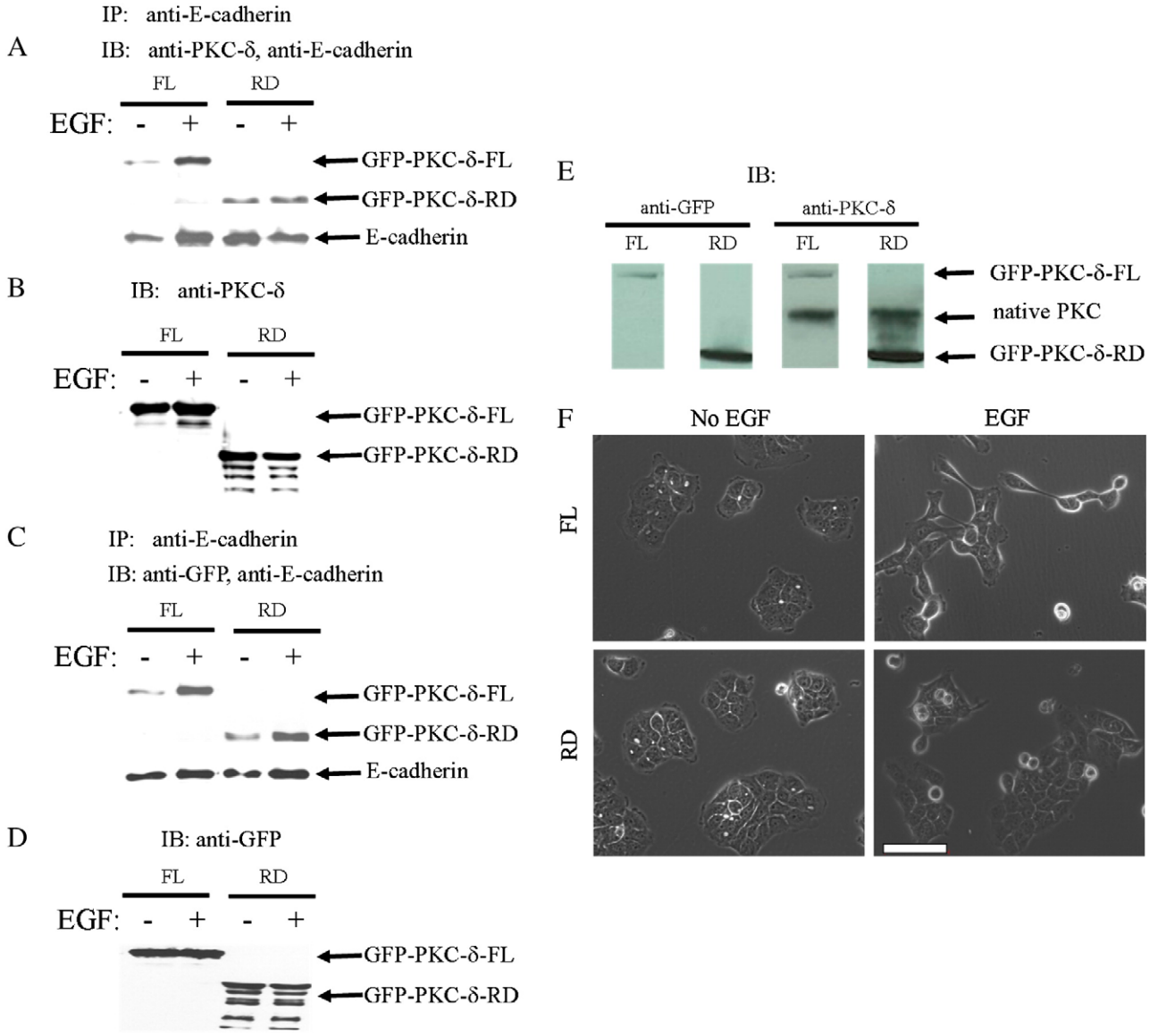


Fig. 5 – The regulatory domain of PKC-δ is sufficient for binding to E-cadherin but not for cell scattering. 293T cells were transfected with (1 μg per 10^6 cells) either GFP-PKC-δ-FL (fusion of wild-type full-length PKC-δ with GFP labeled as FL) or GFP-PKC-δ-RD (fusion of regulatory domain of PKC-δ with GFP labeled as RD) using Lipofectamine™2000. At 36 h after transfection cells were treated with EGF (100 ng/ml) for 2 min. (A) Cell lysates were immunoprecipitated using anti-E-cadherin and immunoblotted with anti-PKC-δ antibody. (B) Western blot analysis of cell lysates using anti-PKC-δ antibody showing that GFP-PKC-δ-FL (107 kDa) or GFP-PKC-δ-RD (66 kDa) were expressed. (C) Cell lysates were immunoprecipitated using anti-E-cadherin and immunoblotted with anti-GFP antibody. (D) Western blot analysis of the lysates using anti-GFP antibody showing that the GFP-PKC-δ-FL or GFP-PKC-δ-RD were expressed. Representative blots from three independent experiments are shown ($n=3$). (E) Western blot using anti-GFP or anti-PKC-δ antibody showing that GFP-PKC-δ-FL or GFP-PKC-δ-RD were expressed in A431 cells. (F) GFP-PKC-δ-FL or GFP-PKC-δ-RD expressing A431 cells were plated at 10^5 cells/well and treated with 100 ng/ml EGF for 24 h. Phase contrast photomicrographs are shown from one representative experiment ($n=3$). Bar = 100 μm.

These data prompted us to examine whether the protein kinase Src, was involved in EGF-induced PKC-δ phosphorylation and cell scattering. Indeed, blocking Src using PP2, significantly reduced EGF-induced phosphorylation of PKC-δ at Y311 (Fig. 6A), binding to E-cadherin (Fig. 6B) and cell scattering (Fig. 6C). In contrast, blocking Erk1/2 or JNK had no effect on cell scattering. Similar results were also obtained with primary keratinocytes, where EGF-induced scattering was inhibited by Gö6983 or PP2, suggesting that the role of PKC-δ in cell adhesion is not limited to A431 carcinoma cells (Fig. 6D).

To further confirm the role of Src, we used lentivirus encoding shRNA to prepare stable Src-depleted A431 cells (Src-neg) in which the level of Src protein decreased by 96.74.7% ($n=2$) (Fig. 6E). Accordingly, phosphorylation of PKC-δ at Y311 by EGF was reduced by $46.1\pm21.5\%$ ($n=3$) (Fig. 6F) and cell scattering by 65.1 ± 5.6 (Fig. 6G), suggesting

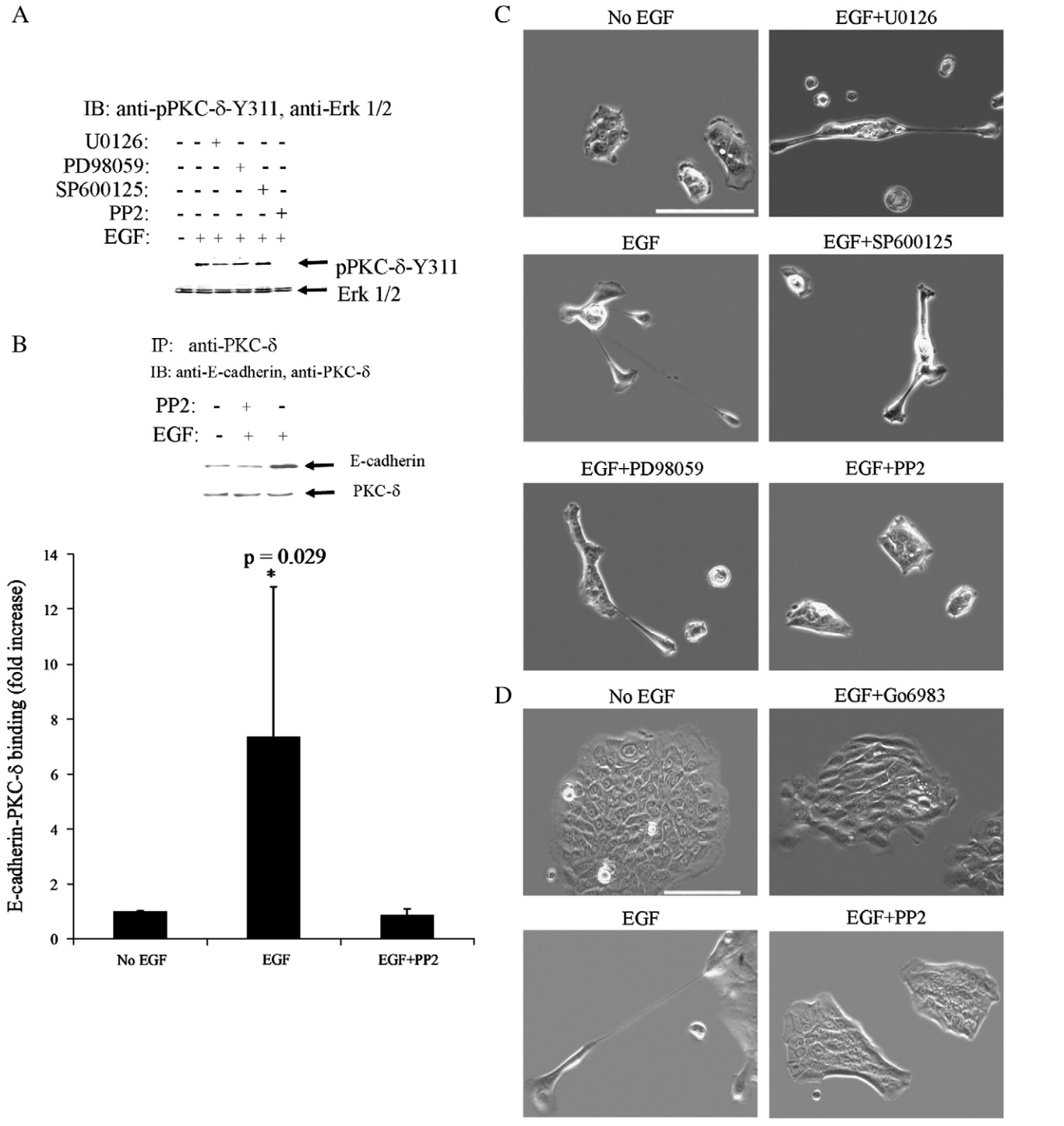
that the Src family of kinases may mediate EGF-induced cell scattering through PKC-δ.

EGF decreases tight junction protein occludin in a PKC-δ dependent manner

In addition to affecting adherens junctions, treatment with EGF decreased the tight junction protein occludin by 60% in cells with control siRNA but this effect was abolished in PKC-δ depleted cells (Fig. 7A). In addition, EGF-induced reduction of occludin was blocked by PP2 (Fig. 7B) and in Src-neg cells (Fig. 7C), suggesting that EGF may also affect tight junctions through a mechanism that involves both PKC-δ and Src.

Discussion

Cell scattering is a complex process that involves integrin and growth factor linked signaling network affecting cell–matrix and cell–cell interactions. Although, these interactions are well studied, their connection with growth factor activated signaling is not completely understood. Many growth factors and extracellular



matrix proteins have been shown to cause cell scattering and migration [39–43]. In particular, EGF was shown to induce scattering in normal and cancer cells and tumor progression correlated with EGFR and ErbB2 overexpression [44–46]. A number of studies have also provided a link between several isoforms of the protein kinase C family and cancer [47–50]. Despite clinical evidence implicating EGFR and the novel PKC-δ in cancer independently, a possible relationship between the two kinases has not been investigated.

Using siRNA knockdown and mutagenesis, we showed that treatment with EGF phosphorylated PKC-δ at Y311 and this phosphorylation was necessary for binding of PKC-δ to E-cadherin, internalization of adherens junctions, and reduction of occludin expression and cell scattering. We interpret these findings as supporting a sequential linear pathway in which activated EGFR results in activation of PKC-δ, which causes many downstream effects, resulting in dissolution of contacts and cell scattering. Interestingly, a low level of PKC-δ was associated with E-cadherin in the absence of EGF treatment (Fig. 3C) – and, since phosphorylation is required for this association (Fig. 4) – this PKC-δ is likely to be phosphorylated (even though this phosphorylation is below the level of detection of the western blot (Fig. 3D)). This basal level of E-cadherin-associated PKC-δ is important because reducing PKC-δ by siRNA resulted in an obvious reduction of membrane ruffling and cell spreading (Fig. 2B; δ-neg, NO EGF). Therefore, the basal level of E-cadherin associated PKC-δ may be essential for some events of cell scattering and activated EGFR may either augment these effects or activate additional parallel pathways by increasing PKC-δ phosphorylation. Identifying other kinases that may phosphorylate PKC-δ in the absence of EGF may reveal additional pathways affecting cell–cell adhesion and epithelial-to-mesenchymal cell transition.

Interestingly, the regulatory domain of PKC-δ bound to E-cadherin and prevented cell scattering in response to EGF, suggesting that the catalytic domain of PKC-δ may be necessary for cell scattering. Therefore, other functions of the full-length molecule such as caspase activation and release of the catalytic domain [29] may be required for disassembly of adherens or tight junctions. On the other hand, PKC-δ-Y311F failed to bind E-cadherin suggesting that phosphorylation at Y311 is necessary for this interaction. Surprisingly, despite its inability to bind E-cadherin, PKC-δ-Y311F prevented endogenous PKC-δ from binding to E-cadherin and cell scattering. Although the mechanism remains unclear, one possible explanation may be that PKC-δ binds to E-cadherin as a complex with another protein (co-factor). This co-factor can bind to both the wild-type and mutant PKC-δ but only the wild-type/co-factor complex can bind to E-cadherin. Hence PKC-δ-Y311F competes with endogenous PKC-δ for the co-factor, ultimately reducing binding of wild-type PKC-δ to E-cadherin.

E-cadherin internalization was previously shown to occur through a mechanism that involved caveolin-1-dependent endocytosis [13] that was followed by nuclear accumulation of β-catenin, induction of the transcriptional repressor, Snail and enhanced β-catenin-TCF/LEF-1 transcriptional activity. Since Snail activation requires serine/threonine phosphorylation [51], it would be interesting to examine whether PKC-δ is involved in Snail activation.

Recent studies identified a distinct pathway of PKC-δ activation involving tyrosine phosphorylation by the Src family kinases [37,38]. In agreement, PKC-δ was shown to play a role in keratinocyte differentiation through a pathway that involved Src and ras^{Ha} [37,52,53]. Src was also implicated in thrombin-induced phosphorylation of PKC-δ at Y311 through a mechanism that involved protease-activated receptors (PAR1/4) [54]. In agreement with these studies, we showed that EGF-induced phosphorylation of PKC-δ at Y311 and cell scattering was mediated in part by Src. In addition, phosphorylation of EGFR was similar in Src-depleted and control cells (Fig. S3). Taken together, these data suggest that Src likely acts downstream of EGFR and upstream of PKC-δ during EGF-induced PKC-δ phosphorylation at Y311. Finally, since depletion of Src using siRNA decreased but did not eliminate phosphorylation of PKC-δ and cell scattering, other Src kinase family members such as Fyn, Yes or others may also mediate EGF-induced PKC-δ-Y311 phosphorylation and cell scattering.

Epithelial-to-mesenchymal transformation (EMT) is characterized by loss of cell–cell adhesion and gain of fibroblast-like morphology by epithelial cells. Cells undergoing EMT lose tight

Fig. 6 – Inhibition of Src decreases EGF-induced phosphorylation of PKC-δ at Y311 and cell scattering. A431 cells were serum starved (0.1% FBS) for 24 h. The next day the cells were pre-treated with the indicated inhibitors for 15 min and then treated for 2 min with EGF (100 ng/ml) in the absence or presence of the same inhibitor as indicated. (A) Cell lysates were immunoblotted with anti-PKC-δ-Y311 and anti-Erk1/2 was used as a loading control ($n=3$). (B) A431 cells remained untreated or treated with EGF (100 ng/ml) in the presence or absence of PP2 for 2 min. PP2 was added 15 min before addition of EGF. Cell lysates were immunoprecipitated with anti-PKC-δ antibody and immunoblotted with anti-E-cadherin. The membrane was striped and re-probed using anti-PKC-δ antibody as IP control. For each condition the intensity of the E-cadherin band was normalized with that of PKC-δ and plotted as fold increase compared to untreated (no EGF) cells ($n=3$). The *p*-value represents comparison between EGF-treated and untreated cells. (C) Phase contrast images of A431 cells at 24 h after treatment with EGF (100 ng/ml) in the presence or absence of inhibitors as indicated ($n=3$) (bar = 100 μm). (D) Primary keratinocytes were plated in the absence of EGF in SFM supplemented with 1 mM Ca^{2+} for 24 h. The next day the cells were pre-treated with the indicated inhibitors for 15 min followed by treatment with EGF (100 ng/ml) in the absence or presence of the same inhibitors. Phase contrast images were acquired 24 h later ($n=2$) (bar = 100 μm). (E) Control and Src-neg A431 cells were lysed and Src expression was measured using western blot. The band intensity of Src was normalized with the intensity of β-actin and plotted as % of normalized Src in control cells. The *p*-value represents comparison between control and Src-neg cells ($n=2$). (F) Control and Src-neg A431 cells were starved in 0.1% FBS for 12 h and then treated with EGF (100 ng/ml) for 2 min. PKC-δ Y311 phosphorylation was measured in cell lysates using western blotting. The band intensity was normalized with the intensity of β-actin and plotted as % of normalized PKC-δ Y311 intensity in control cells. The *p*-value represents comparison between control and Src-neg cells ($n=3$). (G) The fraction of scattered cells at the indicated conditions. The *p*-values represent comparison between EGF-treated or untreated cells ($n=300$–400 cells were counted per condition).

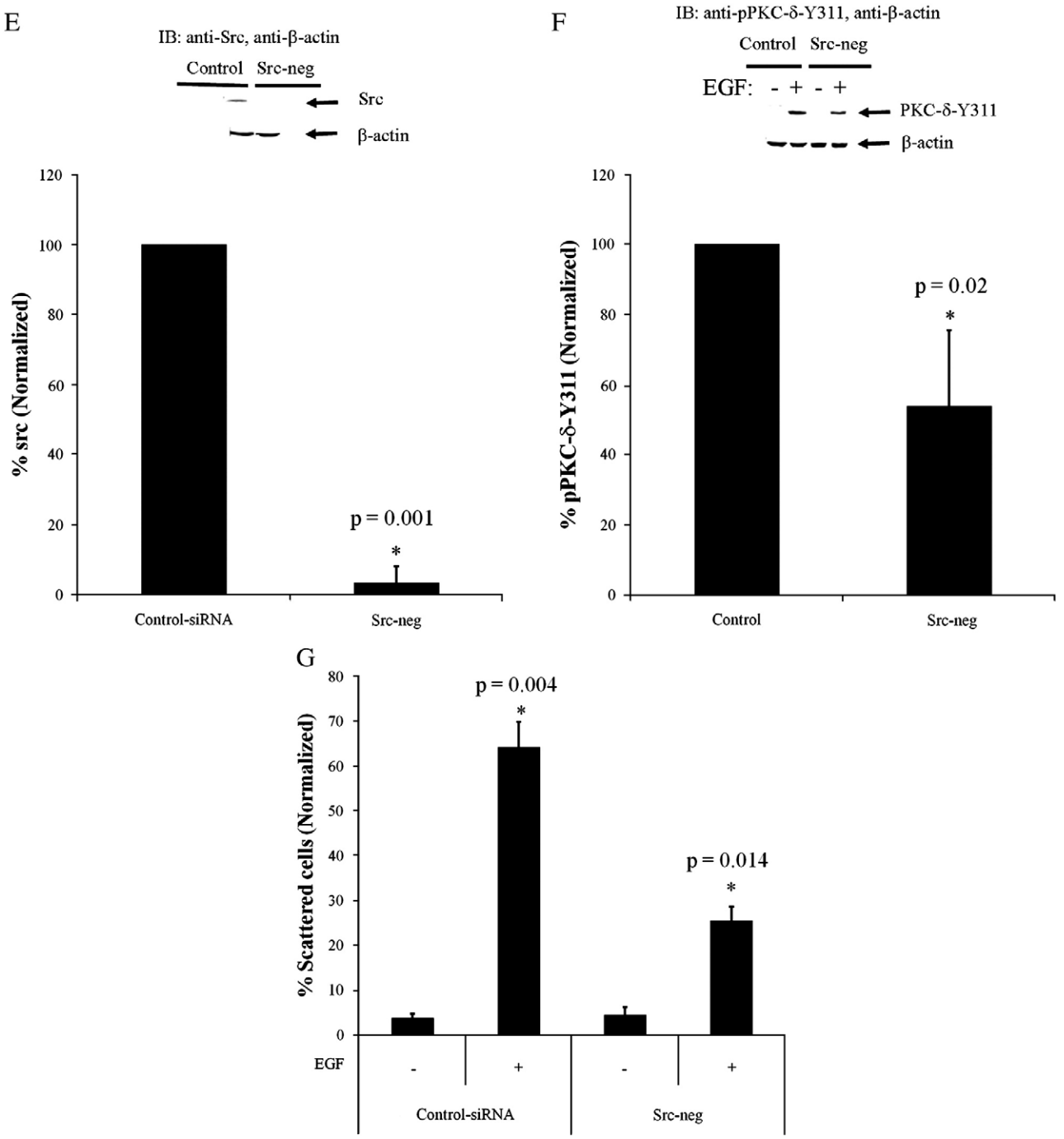


Fig. 6 (*continued*).

junctions [22,55], a phenomenon also observed in metastasis of epithelial tumors [56,57]. In this study, we showed that EGF decreased tight junction protein occludin in a PKC-δ and Src dependent manner. The reduction of occludin could be a direct effect of EGFR signaling but it is also possible that Src and PKC-δ may reduce tight junctions indirectly through inhibition of adherens junctions. Indeed, the latter hypothesis is supported by the kinetics of these processes as phosphorylation of PKC-δ at Y311 and binding to E-cadherin occurred within minutes, while loss of occludin occurred 24 h later and correlated temporally with cell scattering. These results may suggest that phosphorylation and binding of PKC-δ-Y311 to E-cadherin may be the first step in a multi-step process leading to cell scattering. Loss of adherens junctions weakens cell–cell adhesion contacts but subsequent changes such as loss of tight junctions and decreased levels of E-cadherin may involve proteolytic and transcriptional changes including Snail, β-catenin-TCF/LEF-1 [13] and/or c-*jun* activation [58], which may be necessary for breaking cell–cell interactions, re-organizing the cytoskeleton and promoting colony scattering and EMT.

In summary, using biochemical and genetic approaches we present evidence that EGF-induced cell scattering is mediated by

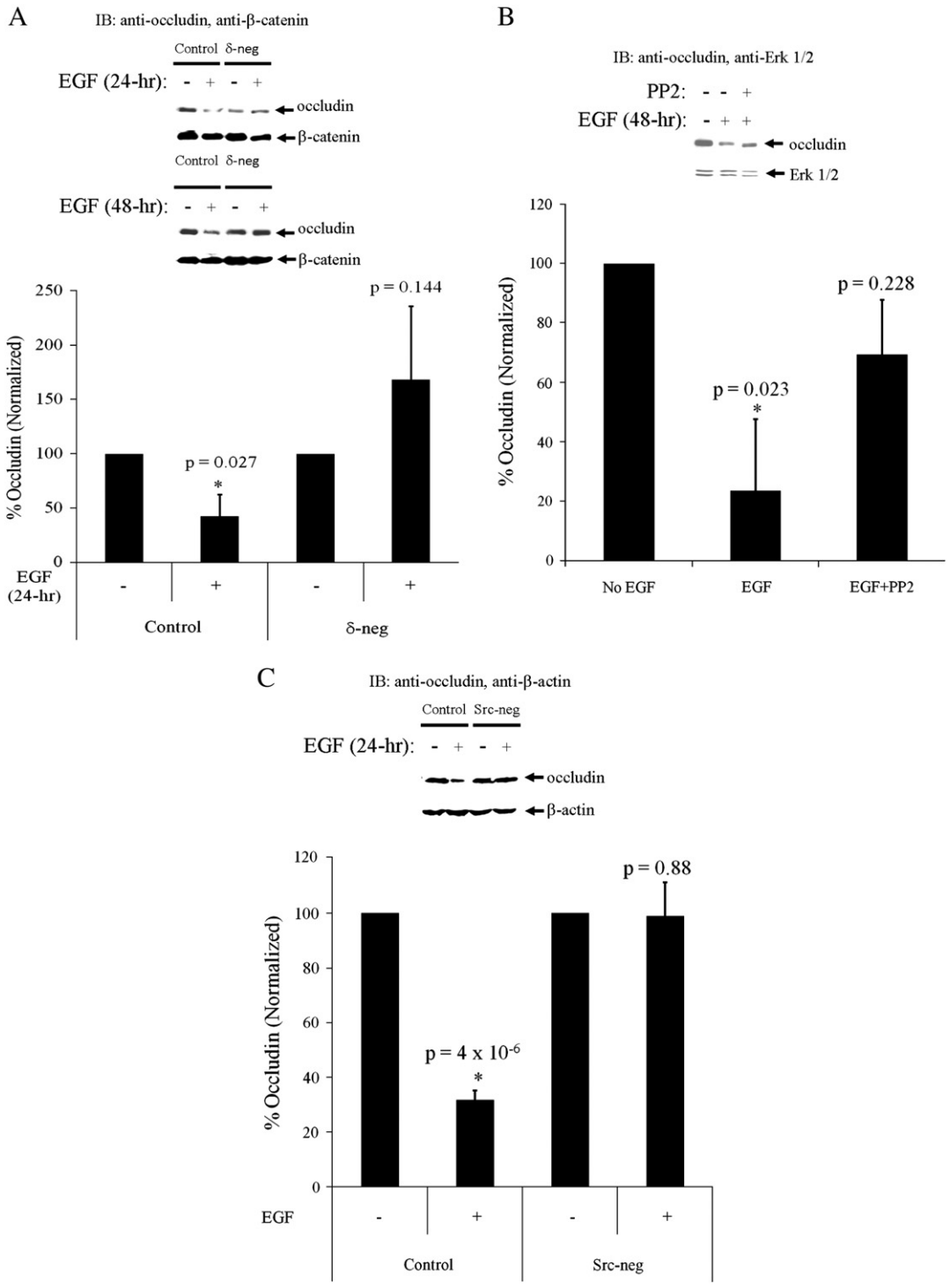


Fig. 7 – EGF decreases tight junction protein occludin in a PKC-δ dependent manner. (A) A431 cells expressing non-silencing siRNA (Control) or PKC-δ siRNA (δ-neg) were treated with EGF (100 ng/ml) for 24 h and cell lysates were subjected to western blotting using anti-occludin antibody. For each condition the intensity of the occludin band was normalized with that of β-catenin and plotted as % of normalized occludin intensity in untreated (no EGF) control or δ-neg cells, respectively ($n = 3$). (B) A431 cells remained untreated or treated with EGF (100 ng/ml) in the presence or absence of PP2 for 48 h. PP2 was added 15 min before addition of EGF. Cell lysates were subjected to western blot analysis using anti-occludin antibody. For each condition the intensity of the occludin band was normalized with that Erk1/2 (loading control) and plotted as % of normalized occludin intensity in untreated (no EGF) cells ($n = 3$). (C) A431 cells expressing non-silencing siRNA (Control) or Src siRNA (Src-neg) were treated with EGF (100 ng/ml) for 24 h and cell lysates were subjected to western blotting using anti-occludin antibody. For each condition the intensity of the occludin band was normalized with that of β-actin and plotted as % of normalized occludin intensity in untreated (no EGF) control or Src-neg cells ($n = 3$). The *p*-value represents comparison between EGF-treated and untreated samples for each cell type (control or Src-neg).

PKC-δ. Specifically, EGF caused PKC-δ phosphorylation at Y311, translocation to the cell border and binding to E-cadherin. Knockdown of PKC-δ using siRNA or blocking of PKC-δ phosphorylation by overexpression of a mutant significantly reduced E-cadherin internalization and cell scattering. These results suggest that PKC-δ may be a possible intracellular target for therapeutic intervention to prevent cancer metastasis, especially in tumors that do not display abnormal levels of EGFR.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.yexcr.2009.07.002.

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