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RhoG/Rac1 signaling pathway involved in migration and invasion of salivary adenoid cystic carcinoma cells

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Abstract

Objectives: This study aimed to explore whether RhoG/Rac1 was involved in migration and invasion of salivary adenoid cystic carcinoma (SACC).

Materials and Methods: RhoG and Rac1 were evaluated in two SACC cell lines, namely SACC-83 and SACC-LM, with low and high rates of lung metastasis, respectively. Functional changes were evaluated using cell proliferation, transwell, and wound-healing assays, and molecular events were investigated using real-time PCR and Western blot assays.

Results: RhoG and Rac1 were highly expressed and more activated in SACC-LM cells than in SACC-83 cells. RhoG overexpression promoted SACC-83 cell migration and invasion through activating Rac1. The knockdown of RhoG or Rac1 partially blocked epiregulin-induced migration and invasion in SACC-83 cells. Epiregulin-induced activation of RhoG/Rac1 in SACC-83 cells was blocked by a Src inhibitor, or an AKT inhibitor or AKT siRNA, or an ERK1/2 inhibitor. Moreover, the epiregulin-induced phosphorylation of AKT and ERK1/2 in SACC-83 cells was blocked by a Src inhibitor, and the epiregulin-induced phosphorylation of ERK1/2 was blocked by an AKT inhibitor or AKT siRNA. Overexpression of activated AKT induced activation of ERK1/2 and RhoG.

Conclusions: RhoG/Rac1 signaling pathway was involved in SACC cell migration and invasion. RhoG/Rac1 at least partially mediated epiregulin/Src/AKT/ERK1/2 signaling to promote SACC cell migration and invasion.

KEYWORDS
AKT, epiregulin, ERK1/2, Rac1, RhoG, SACC

1 | INTRODUCTION

Salivary adenoid cystic carcinoma (SACC) is a clinically common malignant tumor, accounting for ~25% of salivary glands malignancies and ~50% in minor salivary glands (Laurie, Ho, Fury, Sherman, & Pfister, 2011). SACC is characterized by slow local growth, a high

incidence of perineural invasion, and frequent metastasis mostly in the lung (Van Weert et al., 2013). Metastasis is a poor prognostic factor for SACC. Patients without metastasis have overall 5-, 10-, and 20-year survival rates of 85.6%, 67.4%, and 50.4%, respectively, whereas the survival rates for patients with metastasis are 69.1%, 45.7%, and 14.3%, respectively (Gao et al., 2013). To date,

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the mechanism underlying the perineural invasion and metastasis of SACC remains to be fully understood. Therefore, it is still of theoretical and clinical significance to explore the mechanisms of SACC cell migration and invasion.

Potential role of Rho family in cancer metastasis has been excellently reviewed (Jansen, Gosens, Wieland, & Schmidt, 2018). The Rho family of proteins mediates tumor invasion and metastasis by regulating extracellular matrix remodeling, the loss of epithelial polarity, cytoskeleton reorganization, and between-cell junction and adhesion (Jansen et al., 2018). Loss of cell–cell contacts and acquirement of a more motile, migratory phenotype, a process known as epithelial–mesenchymal transition (EMT), are prerequisite for cancer cells to metastasize (Jansen et al., 2018). Cells undergoing EMT show a dissociation of E-cadherin, β-catenin, and p120-catenin in their junctional complexes, known as the adherens junction (Jansen et al., 2018). Rho proteins are a group of small GTPases with homology to Ras-superfamily GTPases, including Rho, Rac, and Cdc42 subfamily (Vega & Ridley, 2008). The activities of Rho proteins are considered to be regulated by the switching between an inactive, GDP-bound form and an active, GTP-bound form. Activation of Rho GTPases requires the GDP-GTP exchange catalyzed by various guanine nucleotide exchange factors (GEFs) and activated GTPases transfer to cell membrane and then bind to their specific effectors that lead to a variety of biological functions (Hanna & El-Sibai, 2013).

RhoG, a member of the Rac subfamily, is most similar to Rac1 in amino acid sequence identity (72%). RhoG induces diverse cellular functions, including neurite outgrowth, macropinocytosis, and cell migration and invasion (Namekata et al., 2012; Valdivia, Goicoechea, Awadia, Zinn, & Garcia-Mata, 2017). Rac1 is activated at the leading edge of motile cells and induces the formation of lamellipodia protrusions, which serve as a major driving force for cell forward movement during wound healing (Kraynov et al., 2000; Nobes & Hall, 1999). RhoG stimulates cell migration by activating Rac1 in a GTP-dependent manner (Katoh, Hiramoto, & Negishi, 2006; Katoh & Negishi, 2003), while the knockdown of RhoG-specific GEFs (Vav3, SGEF, Ephexin4, and Trio) inhibits Rac1 activation and tumor cell migration or invasion (Hiramoto-Yamaki et al., 2010; Hou et al., 2018; Lin et al., 2012; Okuyama, Umeda, Negishi, & Katoh, 2016). However, the upstream factors of RhoG/Rac1 signaling pathway in regulation of cell migration and invasion remain obscure, except that epidermal growth factor (EGF) can rapidly activate RhoG/Rac1 signaling pathway, and the knockdown of RhoG blocks EGF-induced Rac1 activation and cell migration (Ho & Dagnino, 2012; Kwiatkowska et al., 2012; Samson, Welch, Monaghan-Benson, Hahn, & Burridge, 2010).

Epiregulin is a novel EGF receptor ligand encoded by the EREG gene. Epiregulin is a polypeptide composed of 46 amino acid residues, which has only 24%–50% shared identity with other EGF amino acid sequences. The distribution of the mRNA for epiregulin is rarely expressed in normal tissues (except the placenta) but is predominantly expressed in various epithelial-derived tumor cells (Harris, Chung, & Coffey, 2003; Toyoda, Komurasaki, Uchida, &

Morimoto, 1997; Toyoda et al., 1995). Defining genes that enable the metastatic phenotype could yield novel targets for therapy. For example, epiregulin expression in bladder cancer is significantly increased and positively correlates with lung metastasis potential (Nicholson et al., 2005, 2004; Thøgersen et al., 2001). We also previously observed that epiregulin expression is significantly higher in SACC-LM cells, an SACC-derived cell line with a high rate of lung metastasis, than in SACC-83 cells, a SACC-derived cell line with a low rate of lung metastasis (Hu, Li, Gan, Wang, & Yu, 2009). Epiregulin binds to the EGF receptors EGFR/HER1 and HER4 to initiate downstream signal transduction and mediates multiple signaling cascades, including wound healing (Riese & Cullum, 2014). Although both ERK1/2 and AKT are required in the epiregulin-induced migration and invasion of SACC-83 cells, whether AKT is upstream of ERK1/2 or ERK1/2 is upstream of AKT remains to be determined (Hu et al., 2009). Src can promote the activation of Vav proteins and Tiam1, which are responsible for Rac1 activation and, therefore, induce the stimulation of actin-driven protrusive activity (Huveneers & Danen, 2009). In addition, the inhibition of Src family kinases blocks fibronectin-induced ERK1/2 and AKT phosphorylation in lung cancer cell migration, suggesting that Src could be upstream of AKT and ERK1/2 (Meng et al., 2009). Therefore, we first hypothesized that the RhoG/Rac1 signaling pathway might be involved in the epiregulin-promoted migration and invasion of SACC cells, and then further investigated whether epiregulin promoted SACC cell migration and invasion through the Src/AKT/ERK1/2/RhoG/Rac1 signaling pathway.

In this study, we first examined whether the RhoG/Rac1 signaling pathway contributed to the differences in migration and invasion of the two SACC cell lines SACC-83 and SACC-LM, and whether RhoG/Rac1 signaling pathway was involved in the epiregulin-induced migration and invasion of SACC-83 cells. Furthermore, we investigated whether epiregulin activated the RhoG/Rac1 signaling pathway through the Src/AKT/ERK1/2 signaling pathway.

2 | MATERIALS AND METHODS

2.1 | Cell culture

Human SACC cell lines SACC-83 and SACC-LM were obtained as previously reported (Hu et al., 2009). The cells were cultured in RPMI 1640 medium (GIBCO) with 10% fetal bovine serum (FBS, Invitrogen, Carlsbad, CA, USA) and 100 U/ml penicillin/streptomycin (GIBCO) in a humidified incubator (37°C) with 5% CO_2.

2.2 | Reagents and antibodies

Recombinant human epiregulin protein was purchased from Sino Biological. NSC23766, GDC-0994, LY294002, and PP2 were purchased from Selleck Chemicals. The RhoG antibody was purchased

from EMD Millipore (Merck KGaA). The Rac1 antibody was purchased from Proteintech. Antibodies for phospho-AKT (T308), AKT, phospho-ERK1/2, and ERK1/2 were purchased from Cell Signaling Technology. The antibody for β-actin was purchased from Santa Cruz Biotechnology.

2.3 | Transient transfection

Small interfering RNAs (siRNAs) of human RhoG, Rac1, and AKT were purchased from Cell Signaling Technology or commercially synthesized by GenePharma (Chan et al., 2005; Kwiatkowska et al., 2012). siRNAs were transfected using Lipofectamine 3000 (Invitrogen) according to the manufacturer's instructions.

2.4 | Stable transfection with lentivirus

Overexpression of GFP-tagged constitutively active RhoG (RhoG-Q61L) was realized from the recombinant lentiviral plasmids (LV5-GFP-RhoG-Q61L), and LV5-GFP plasmids were used as the control. The plasmids were purchased from GenePharma. Overexpression of GFP-tagged constitutively active AKT (CA-AKT) was realized using the pLVX-AcGFP-N1-CA-AKT plasmids, and pLVX-AcGFP-N1 plasmids were used as the control. The plasmids were each cotransfected into 293T cells using PLP1, PLP2, and VSVG lentiviral packing plasmids (Clontech). Lentiviral supernatants were collected at 48 hr after transfection and then centrifuged (500 g for 10 min at 4°C). The supernatant was added to SACC-83 cells; infected cells were continuously selected by 1 μg/ml puromycin. Overexpression of constitutively active RhoG (RhoG-Q61L) or AKT (CA-AKT) was confirmed by Western blot assay.

2.5 | Real-time quantitative PCR

Total RNA was extracted using TRIzol reagent (Invitrogen). Reverse transcription and real-time PCR were performed as described previously (Gan & Zhang, 2009). The primers for human RhoG are as follows: 5′-CCTGAACCTGTGGGACACTGCGG-3′ (sense) and 5′-CCAGGGTCACAAGAGGATGCAG-3′ (antisense). Real-time PCR was performed using a 7500 real-time PCR system of Applied Biosystems (Invitrogen) with FastStart Universal SYBR Green Master Roche according to the manufacturer's instruction.

2.6 | Protein extraction and Western blot analysis

Whole-cell lysates were extracted using RIPA lysis buffer (Applygen). Membrane proteins were extracted using the Nucl-Cyto-Mem Preparation Kit (Applygen) according to the manufacturer's protocol. Briefly, cells were harvested and resuspended in 500 μl of cytosol extraction reagent and homogenized with 25–30 passes in a 2-ml Dounce homogenizer. The homogenate was centrifuged at 1,000 g for 5 min at 4°C. The supernatant was collected and mixed with 50 μl of membrane extraction reagent; after incubation on ice for 5 min, the mixture was centrifuged at 14,000 g for 30 min at 4°C and the sediment (membrane fraction) was resolved in RIPA lysis buffer. Protein concentrations were determined by BCA protein assay (Thermo Scientific). Forty micrograms of samples were subjected to 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membrane (Millipore). The membrane was blocked with 5% non-fat milk in TBS-T (50 mM Tris, pH 7.5; 150 mM NaCl; 0.05% Tween-20) for 1 hr at room temperature. Following incubation with anti-RhoG or phospho-AKT (T308), or phospho-ERK1/2 antibodies at 1:1,000 in TBS-T for 12 ~ 16 hr at 4°C, the membrane was washed with TBS-T three times and then incubated with secondary horseradish peroxidase-conjugated antibody for 1 hr at room temperature. After extensive washes with TBS-T, the membrane was visualized using FUSION FX imaging system (VILBER, France). The membrane was stripped for the detection of Rac1 or AKT, or ERK1/2 with anti-Rac1 or AKT, or ERK1/2 antibodies, respectively, and finally, the membrane was stripped for the detection of β-actin. The densitometry of the target band was assessed using ImageJ software.

2.7 | Cell proliferation assay

The cell proliferation assay was performed using Cell Counting Kit-8 (CCK-8, Dojindo) following the manufacturer's instructions. Briefly, the cells were seeded into 96-well plates (1.5×10^3 cells/well) and treated with different reagents. Subsequently, 10 μl of CCK-8 reagent was added to each well containing 100 μl of growth medium. After incubation at 37°C for 3 hr, absorbance at 450 nm was determined.

2.8 | Transwell migration and invasion assay

Cell migration and invasion assays were performed in transwell chambers (Corning Costar). For the migration assay, cells were transfected with 100 nM RhoG or Rac1 or scrambled siRNA for 48 hr and then the medium was replaced with serum-free medium for 12 hr. Subsequently, the cells were harvested and seeded at 5×10^4 cells/well in serum-free medium alone or with addition of 2 nM epiregulin in the upper chamber, while the lower chamber was filled with 0.5 ml of growth medium with 10% FBS. After a 16-hr incubation, the cells on the top surface of the membrane were wiped off, whereas those on the bottom surface were fixed with 4% paraformaldehyde, stained with 0.1% crystal violet, and examined under a light microscope (BX60). The cells were counted and averaged by the number of six randomly selected fields. The same procedure was performed for the transwell invasion assay, except that the upper chamber was coated with 20 μg of extracellular matrix gel (BD Biosciences) prior to the seeding of 7.5×10^4 cells/well.

2.9 | Wound-healing assay

SACC-83 cells were seeded at 3×10^5 cells/well in a 12-well culture plate for 24 hr, and then, the medium was replaced with serum-free medium for 12 hr. Subsequently, confluent cells were gently scratched using a 200-µl pipette tip. After washing thrice with phosphate-buffered saline (PBS) to remove the cell debris, the cells were treated with vehicle dimethyl sulfoxide (DMSO) or 3 nM epiregulin for 10 min or 16 hr. The cells treated with 3 nM epiregulin for 10 min were washed thrice with PBS and incubated in serum-free medium. Wounded monolayers were imaged at 0 and 16 hr after scratching. Wound closure was represented by the decreased distance remaining uncovered by cells. An average rate of wound closure was calculated as the equation of decreased distance/incubated time.

2.10 | Immunoprecipitation assay

The cells were washed three times with ice-cold phosphate-buffered saline and placed in non-denaturing lysis buffer (20 mM Tris-HCl, pH 8.0, 137 mM NaCl, 1% non-idet P-40, 2 mM EDTA). Equal amounts (500 µg) of whole-cell lysates were incubated with 5 µg of primary antibodies for 16 hr with gentle rotation at 4°C. After addition of 70 µl of protein A/G agarose beads (Santa Cruz Biotechnology), the mixture was further incubated with gentle rotation for 4 hr at 4°C and then was centrifuged (12,000 g for 1 min). The beads were washed 5 times with wash buffer (10 mM Tris pH 7.4, 1 mM EDTA, 1 mM EGTA pH 8.0, 150 mM NaCl, 1% Triton X-100, 0.2 mM sodium orthovanadate, protease inhibitor cocktail). The protein–bead complex was eluted by boiling in same volume of 2X sodium dodecyl sulfate loading buffer and then analyzed with Western blot.

2.11 | Statistical analysis

Statistical analysis was performed using SPSS 20 for Windows. All experiments were repeated three times, and all data were presented as the mean ± standard deviation (SD). The differences between multiple groups were analyzed by one-way analysis of variance (ANOVA). $p < .05$ was considered to indicate significance.

3 | RESULTS

3.1 | RhoG was involved in SACC cell migration and invasion

We first examined whether RhoG was differentially expressed in the two SACC cell lines: SACC-LM cells with a high rate of lung metastasis and SACC-83 cells with a low rate of lung metastasis. As shown

in Figure 1a, b, RhoG mRNA expression was significantly higher in SACC-LM cells than in SACC-83 cells (Figure 1a), and RhoG protein expression and the membrane-bound RhoG (activated form) were also significantly higher in SACC-LM cells than in SACC-83 cells (Figure 1b).

To evaluate whether RhoG was involved in the migration and invasion of the two SACC cell lines, we knocked down RhoG in SACC-LM cells and overexpressed RhoG in SACC-83 cells. As shown in Figure 1c, d, SACC-LM cell migration and invasion were significantly higher than those of SACC-83 cells, and the knockdown of RhoG in SACC-LM cells significantly inhibited their migration and invasion to levels similar to SACC-83 cells. The knockdown of RhoG was confirmed using Western blot assays (see Figure S1), and the proliferation of SACC-LM cells was not affected by RhoG knockdown (see Figure S2).

Overexpression of RhoG-Q61L (constitutively active RhoG) in SACC-83 cells dramatically promoted their migration and invasion to levels similar to that of SACC-LM cells (Figure 1e, f). RhoG-Q61L overexpression in SACC-83 cells was confirmed using Western blot assays (see Figure S3). Since β-catenin is sequestered with E-cadherin at the adherens junctions, we also examined the interaction of β-catenin and E-cadherin in the SACC-83 and SACC-LM cells, or in SACC83 cells after overexpression of RhoG active mutant (RhoG Q16L) using immunoprecipitation assays. As shown in Figure 1g and H, β-catenin was less interacted with E-cadherin in SACC-LM cells compared to that in SACC83 cells, or in SACC83 cells after overexpression of RhoG active mutant (RhoG Q16L) compared to that in the empty-vector transfected SACC83 cells.

3.2 | Rac1 was involved in SACC cell migration and invasion

RhoG is a key upstream regulator of Rac1 during cell migration. Therefore, we also examined whether Rac1 was differentially expressed in the two SACC cell lines. As shown in Figure 2a, b, RhoG mRNA expression was significantly higher in SACC-LM cells than in SACC-83 cells, and Rac1 protein expression and the membrane-bound Rac1 were significantly higher in SACC-LM cells than in SACC-83 cells.

To detect whether Rac1 was involved in the migration and invasion of the two SACC cell lines, we knocked down Rac1 in SACC-LM cells. As shown in Figure 2b, c, the knockdown of Rac1 in SACC-LM cells significantly inhibited their migration and invasion to levels similar to that of SACC-83 cells. The knockdown of Rac1 was confirmed using Western blot assays (see Figure S4), and the proliferation of SACC-LM cells was not affected by Rac1 knockdown (see Figure S5).

To determine whether Rac1 was downstream of RhoG in the migration and invasion of SACC cells, we knocked down Rac1 in SACC-83 cells stably transfected with RhoG-Q61L. As shown in Figure 2d, e, the knockdown of Rac1 completely blocked RhoG-Q61L
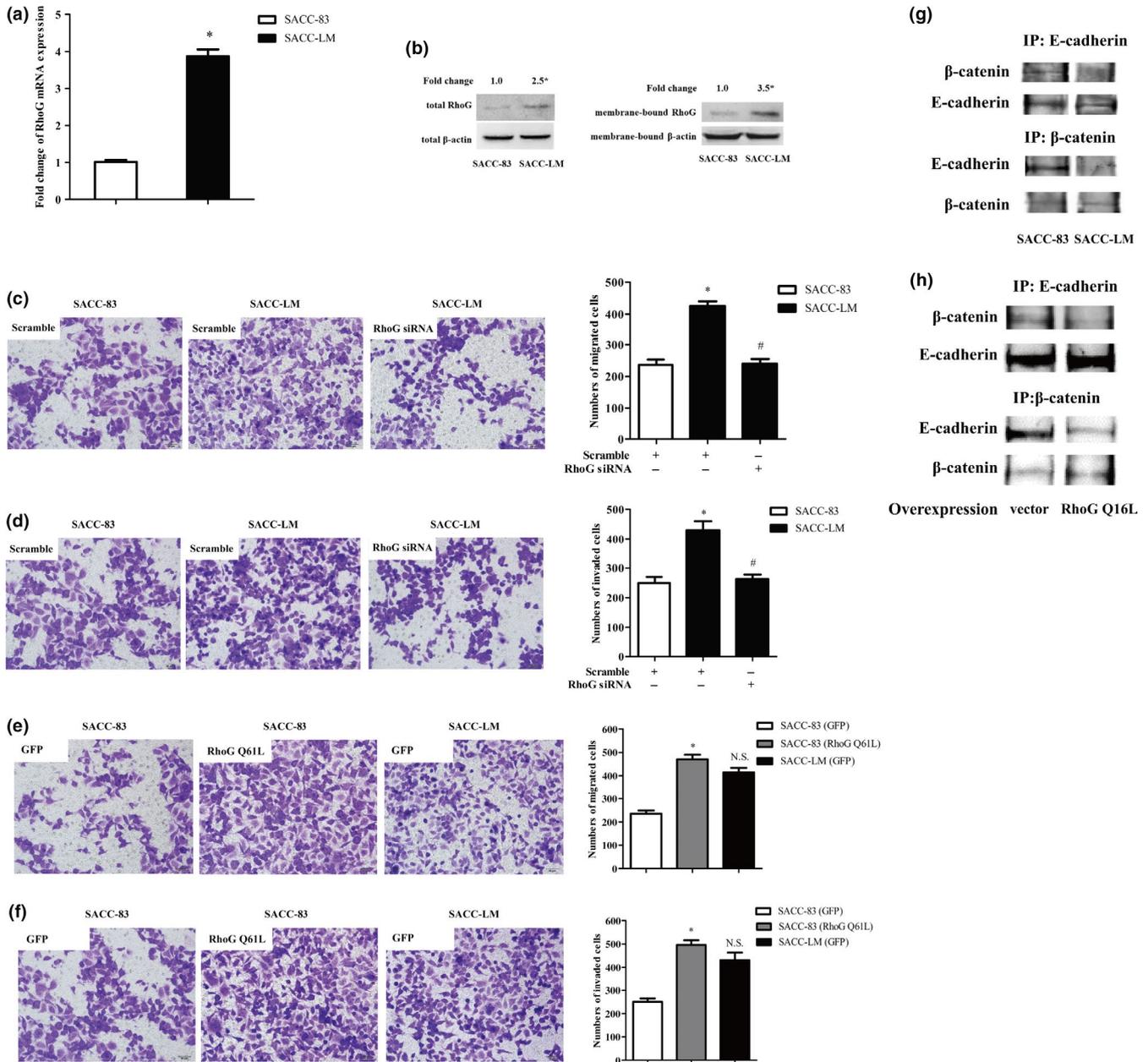
FIGURE 1 RhoG was involved in SACC cell migration and invasion. (a) mRNA expression levels of RhoG in SACC-83 and SACC-LM cells were assessed by real-time PCR. The data were presented as the mean ± SD, *p < .05 versus SACC-83 (n = 3). (b) Protein expression levels of RhoG and membrane-bound RhoG in SACC-83 and SACC-LM cells were assessed by Western blot. *p < .05 versus SACC-83 (n = 3). SACC cells were transfected with 100 nM scrambled or RhoG siRNA for 48 hr and then given serum-free medium for 12 hr. Microphotographs of migration (c) or invasion (d) of cells after different treatment for 16 hr. Bar = 50 μm. The data were presented as the mean ± SD, *p < .05 versus SACC-83; #p < .05 versus the scramble group (n = 3). SACC cells were stably transfected with either GFP-tagged RhoG-Q61L- or GFP-containing plasmids. Microphotographs of migration (e) or invasion (f) of cells after different treatment for 16 hr. Bar = 50 μm. The data were presented as the mean ± SD, *p < .05 versus the GFP group; N.S.: no significant differences versus RhoG-Q61L group (n = 3). Interaction of E-cadherin and β-catenin in SACC-83 and SACC-LM (g), or (h) in SACC-83 transfected with GFP-containing plasmids or GFP-tagged RhoG-Q61L- [Colour figure can be viewed at wileyonlinelibrary.com]

overexpression-induced cell migration and invasion to levels similar to that of the GFP-transfected SACC-83 cells. Overexpression of RhoG-Q61L also induced membrane-bound Rac1 in SACC-83 cells as confirmed using Western blot assays (see Figure S6). Taken together, RhoG was upstream of Rac1 in SACC cells, and the RhoG/Rac1 signaling pathway contributed to the differences in migration and invasion of SACC-83 and SACC-LM cells.

3.3 | Short-term treatment with epiregulin resulted in the activation of RhoG and Rac1 and in the promotion of migration of SACC-83 cells

It was unknown whether epiregulin could activate the RhoG/Rac1 signaling pathway. As shown in Figure 3a, the treatment of SACC-83 cells with epiregulin significantly activated RhoG and Rac1 even

(a)

(b)

(c)

(d)

(e)

(f)
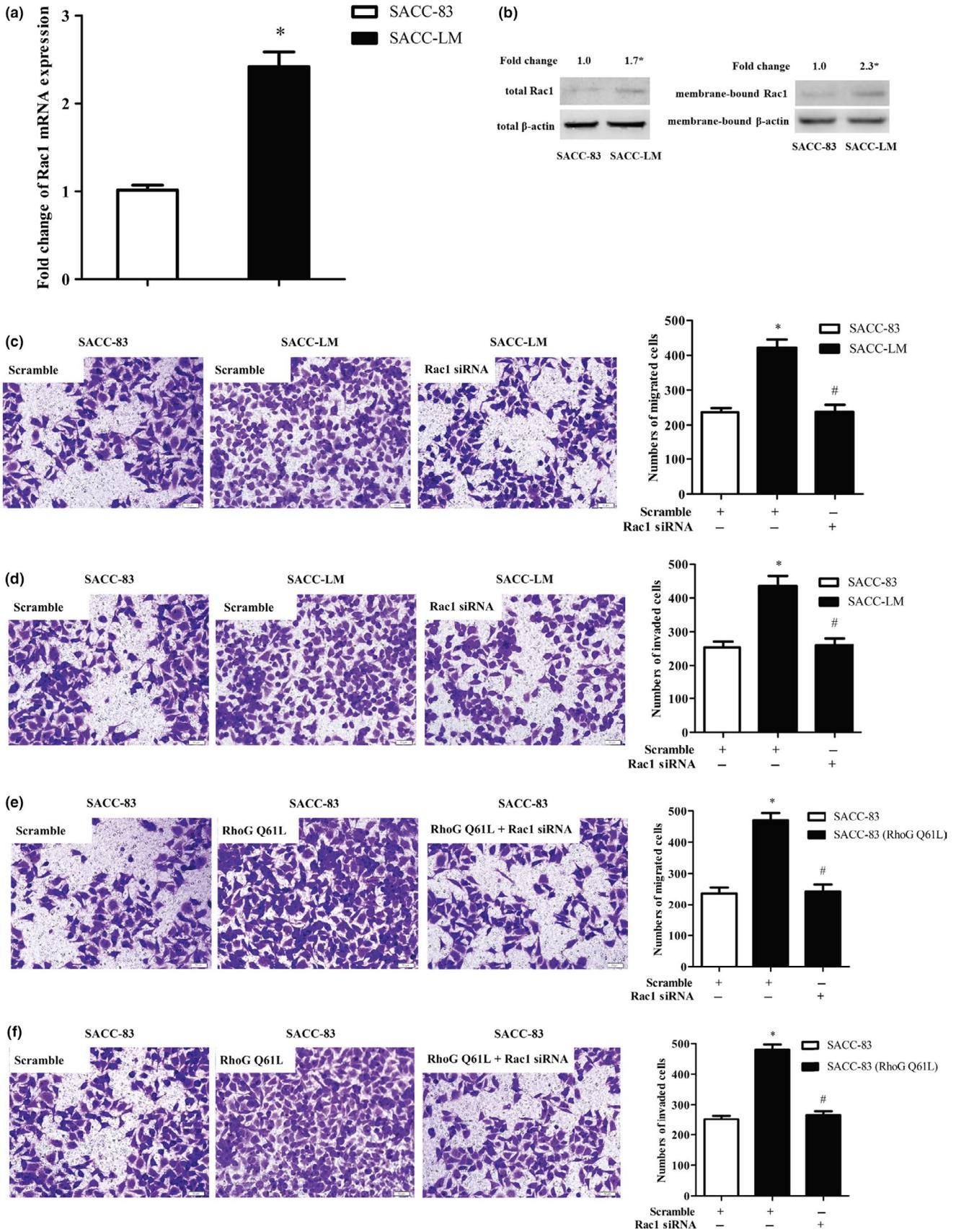
at only 2 min posttreatment, and this activation already showed a decrease at 10 min posttreatment compared to that at 2 min posttreatment and disappeared completely at 30 min posttreatment.

In addition, E-cadherin completely failed to interact with β-catenin in SACC83 cells at 10 min posttreatment with epiregulin compared to that in the solvent-treated SACC83 cells (Figure 3b). We then

FIGURE 2 Rac1 was involved in SACC cell migration and invasion. (a) mRNA expression levels of Rac1 in SACC-83 and SACC-LM cells were assessed by real-time PCR. The data were presented as the mean ± *SD*, *p < .05 versus SACC-83 (*n* = 3). (b) Protein expression levels of Rac1 and membrane-bound Rac1 in SACC-83 and SACC-LM cells were assessed by Western blot. *p < .05 versus SACC-83 (*n* = 3). SACC cells were transfected with 100 nM scrambled or Rac1 siRNA for 48 hr and then given serum-free medium for 12 hr. Microphotographs of migration (c) or invasion (d) of cells after different treatment for 16 hr. Bar = 50 μm. The data were presented as the mean ± *SD*, *p < .05 versus SACC-83; #p < .05 versus the scramble group (*n* = 3). SACC-83 cells stably transfected with RhoG-Q61L- or GFP-containing plasmids were transfected with 100 nM scrambled or Rac1 siRNA for 48 hr and then given serum-free medium for 12 hr. Microphotographs of migration (e) or invasion (f) of cells after different treatment for 16 hr. Bar = 50 μm. The data were presented as the mean ± *SD*, *p < .05 versus SACC-83; #p < .05 versus the scramble group (*n* = 3) [Colour figure can be viewed at wileyonlinelibrary.com]

questioned whether this short-term activation of RhoG and Rac1 was relevant to cell migration.

To address this question, we used a wound-healing assay to assess the migration of SACC-83 cells treated with epiregulin for 10 min or 16 hr. As shown in Figure 3c, the migration of SACC-83 cells treated with epiregulin for 10 min or 16 hr was significantly higher than that of the control group, and there was no difference in migration between the two treatment groups (*p* > .05).

3.4 | Epiregulin promoted SACC-83 cell migration and invasion partially dependent on the RhoG/Rac1 signaling pathway

To further examine whether RhoG was involved in the epiregulin-induced migration and invasion of SACC cells, we knocked down RhoG in epiregulin-treated SACC-83 cells using siRNA. As shown in Figure 4a, b, the knockdown of RhoG partially blocked epiregulin-induced cell migration and invasion.

Similarly, the knockdown of Rac1 partially blocked epiregulin-induced migration and invasion of SACC-83 cells (Figure 4c, d). In addition, specific inhibition of Rac1 activity by NSC23766 also partially blocked epiregulin-induced SACC-83 cell migration and invasion (Figure 4e, f).

3.5 | Epiregulin induced RhoG activation through Src/AKT/ERK1/2 signaling pathway

Epiregulin facilitates SACC-83 cell migration and invasion dependent on both ERK1/2 and AKT (Hu et al., 2009). However, whether AKT is upstream of ERK1/2 or the reverse remains to be determined. As shown in Figure 5a, the inhibition of ERK1/2 by the specific inhibitor GDC-0994 inhibited membrane-bound RhoG and completely blocked epiregulin-induced membrane-bound RhoG, but not AKT phosphorylation, suggesting that ERK1/2 was upstream of RhoG, but not AKT, in the epiregulin signaling pathway. In contrast, the inhibition of AKT by LY-294002 or the knockdown of AKT inhibited
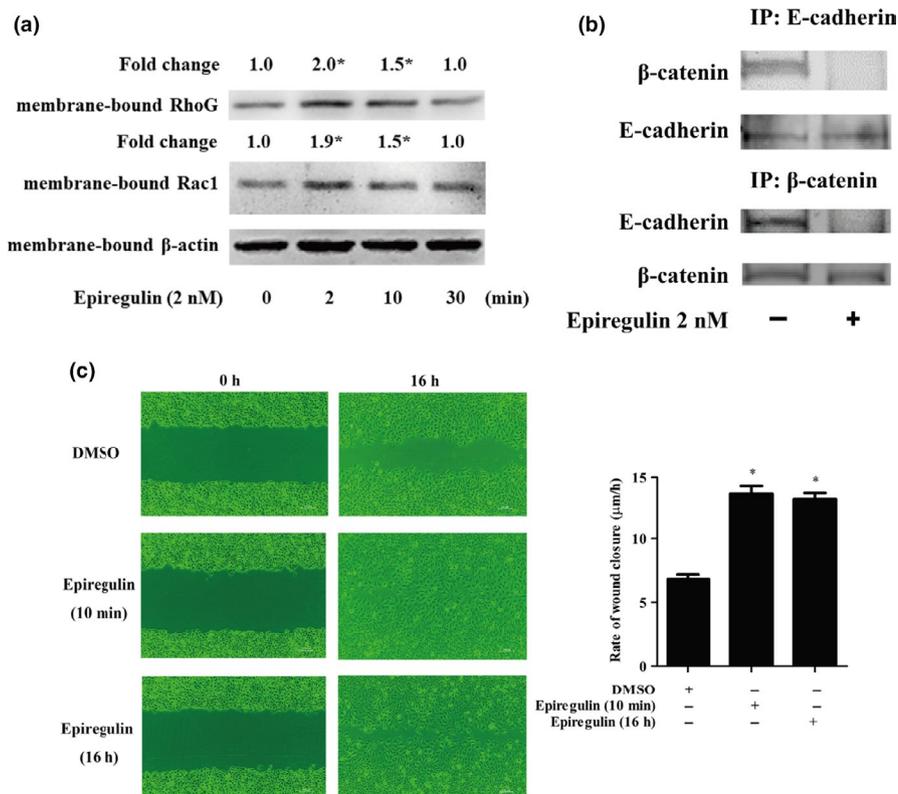
FIGURE 3 Epiregulin promoted SACC-83 cell migration and activated RhoG and Rac1. (a) SACC-83 cells were exposed to epiregulin (2 nM), and membrane proteins were collected at the indicated time points and subjected to Western blot. *p < .05 versus control group (*n* = 3). (b) Interaction of E-cadherin and β-catenin in SACC83 cells at 10 min posttreatment with or without epiregulin. IP: immunoprecipitation; WB: Western blot. (c) Representative images of migrated cells after different treatment for 16 hr. SACC-83 cells starved for 12 hr were exposed to either DMSO or epiregulin (3 nM) for different times. Bar = 100 μm. The data were presented as the mean ± *SD*, *p < .05 versus the DMSO group (*n* = 3). N.S.: no significant differences versus the epiregulin (10 min) group [Colour figure can be viewed at wileyonlinelibrary.com]
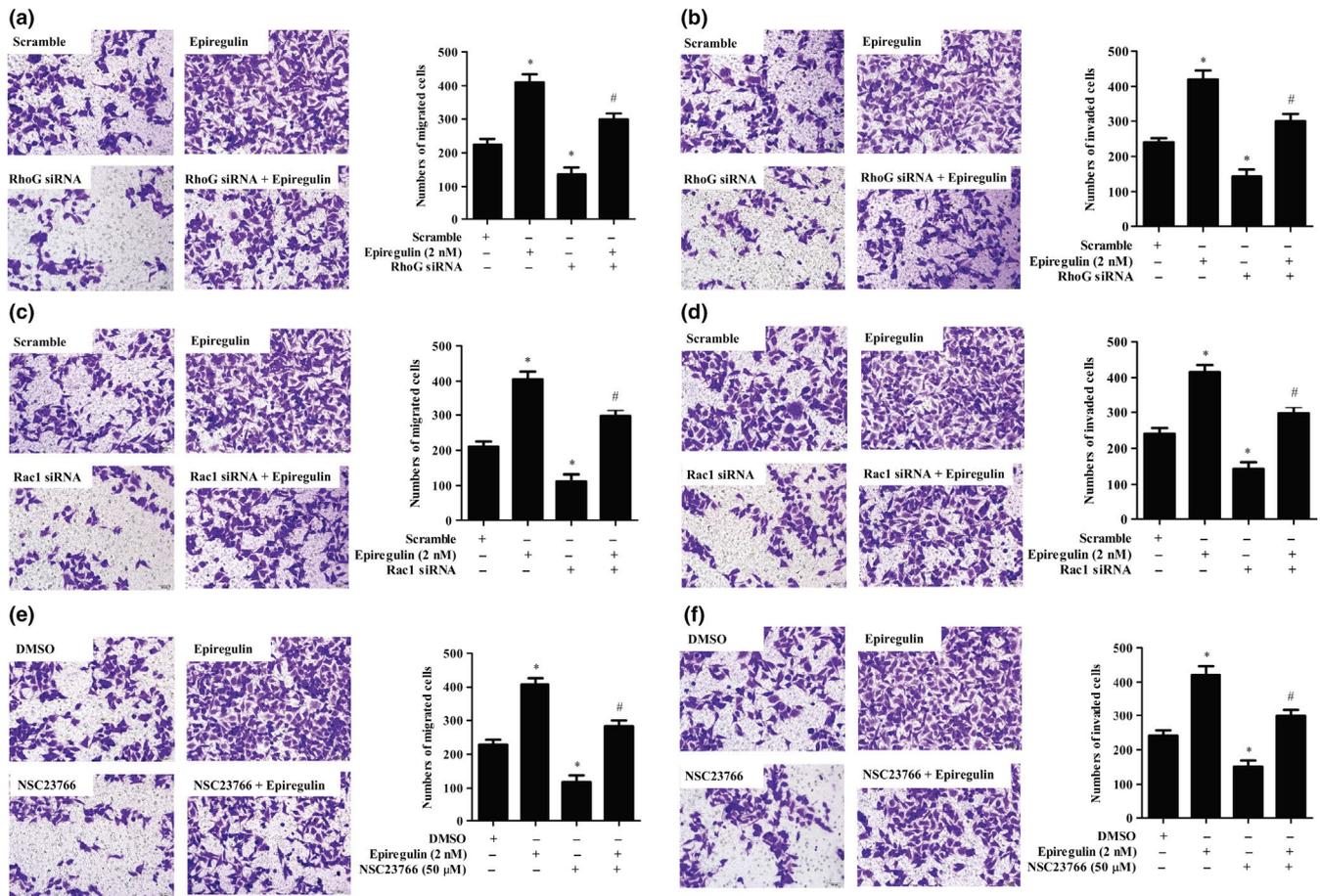
FIGURE 4 Epiregulin promoted SACC-83 cell migration and invasion partially through the RhoG/Rac1 signaling pathway. SACC-83 cells were transfected with 100 nM scrambled or RhoG or Rac1 siRNA for 48 hr and then given serum-free medium for 12 hr. The cells were harvested and seeded in serum-free medium alone or with addition of 2 nM epiregulin in the upper chamber. For pharmacologic inhibitor studies, the cells were supplemented with 50 μM Rac1 inhibitor NSC23766 30 min before seeding. Microphotographs of migration (a, c, e) or invasion (b, d, f) of cells after different treatment for 16 hr. Bar = 50 μm. The data were presented as the mean ± SD, *p < .05 versus scramble or the DMSO group; #p < .05 versus the epiregulin group (n = 3) [Colour figure can be viewed at wileyonlinelibrary.com]

ERK1/2 phosphorylation and membrane-bound RhoG, and completely blocked epiregulin-induced ERK1/2 phosphorylation and membrane-bound RhoG (Figure 5b, c). Moreover, overexpression of AKT active mutant (CA-AKT) upregulated ERK1/2 phosphorylation and membrane-bound RhoG (Figure 5d). Overexpression of RhoG active mutant (RhoG-Q61L) also did not affect AKT phosphorylation (see Figure S7). Taken together, these results suggested that AKT was upstream of ERK1/2, and ERK1/2 was upstream of RhoG, in the epiregulin signaling pathway.

The inhibition of Src blocks fibronectin-induced ERK1/2 and AKT phosphorylation (Meng et al., 2009). These results prompted us to question whether Src was also upstream of AKT/ERK1/2 in the epiregulin-induced RhoG activation. As shown in Figure 5e, the inhibition of Src by specific inhibitor PP2 inhibited phosphorylation of ERK1/2 and AKT and membrane-bound RhoG and completely blocked epiregulin-induced phosphorylation of ERK1/2 and AKT and membrane-bound RhoG, and partially blocked the epiregulin-induced migration and invasion of SACC-83 cells (Figure 5f, g). Therefore, these results suggested that the Src/AKT/ERK1/2

signaling pathway mediated the epiregulin-induced activation of RhoG/Rac1 signaling pathway.

4 | DISCUSSION

In the present study, we demonstrated for the first time that the RhoG/Rac1 signaling pathway was involved in SACC cell migration and invasion and in the epiregulin-induced migration and invasion of SACC cells. We further confirmed that epiregulin promoted SACC cell migration and invasion through the Src AKT/ERK1/2 signaling pathway mediating the RhoG/Rac1 signaling pathway.

The RhoG/Rac1 signaling pathway played an important role in SACC cell migration and invasion. First, the activated RhoG (membrane-bound RhoG) and Rac1 (membrane-bound Rac1) were significantly increased in SACC-LM cells with high rate of lung metastasis compared to that of SACC-83 cells with a low rate of lung metastasis. Second, the knockdown of RhoG in the SACC-LM cells inhibited membrane-bound Rac1 and cell migration and invasion
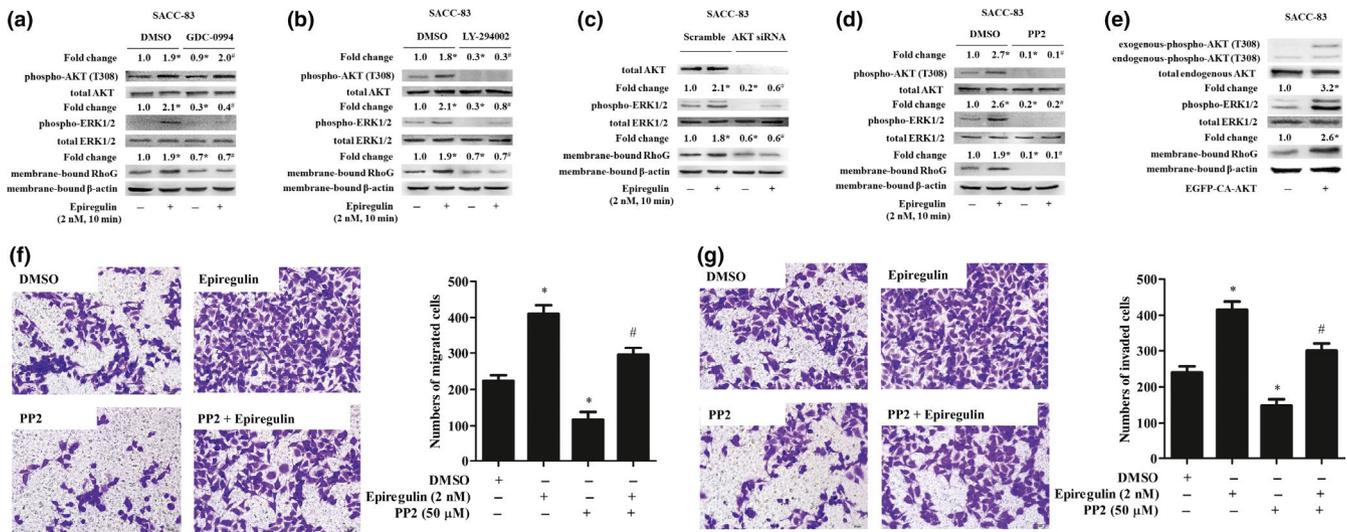
FIGURE 5 Epiregulin induced RhoG activation through the Src/AKT/ERK1/2 signaling pathway. (a) SACC-83 cells were treated with either DMSO or 10 μM ERK1/2 inhibitor GDC-0994 for 2 hr and then exposed to 2 nM epiregulin for 10 min. (b) SACC-83 cells were treated with either DMSO or 50 μM phosphatidylinositol-3-kinase inhibitor LY-294002 for 30 min and then exposed to 2 nM epiregulin for 10 min. (c) SACC-83 cells were transfected with 100 nM scrambled or AKT siRNA for 48 hr and then exposed to 2 nM epiregulin for 10 min. (d) SACC-83 cells were stably transfected with either GFP-tagged CA-AKT- or GFP-containing plasmids. (e) SACC-83 cells were treated with either DMSO or 10 μM Src inhibitor PP2 for 1 hr and then exposed to 2 nM epiregulin for 10 min. Total and membrane proteins were extracted and subjected to Western blot. *$p < .05$ versus DMSO or scramble or GFP group; #$p < .05$ versus epiregulin group ($n = 3$). SACC-83 cells starved for 12 hr were exposed to either DMSO or 10 μM Src inhibitor PP2 for 1 hr. The cells were harvested and seeded in serum-free medium alone or with addition of 2 nM epiregulin in the upper chamber. Microphotographs of migration (f) or invasion (g) of cells after different treatment for 16 hr. Bar = 50 μm. The data were presented as the mean ± SD, *$p < .05$ versus the DMSO group; #$p < .05$ versus the epiregulin group ($n = 3$) [Colour figure can be viewed at wileyonlinelibrary.com]

to levels similar to that of SACC-83 cells. Third, overexpression of RhoG-Q61L in SACC-83 cells enhanced membrane-bound Rac1 and cell migration and invasion to levels similar to that of SACC-LM cells. Fourth, β-catenin was less interacted with E-cadherin in SACC-LM cells compared to that in SACC-83 cells, also implying that β-catenin was less sequestered with E-cadherin at the adherens junctions. These results also suggested that RhoG was upstream of Rac1 and that the RhoG/Rac1 signaling pathway contributed to the higher migration and invasion of SACC-LM cells. Our results that the knockdown of RhoG or overexpression of RhoG-Q61L affected SACC cell migration and invasion were consistent with that in HeLa cells in previous reports (Katoh et al., 2006). Therefore, activation of the RhoG/Rac1 signaling pathway could contribute to the SACC cell migration and invasion.

The RhoG/Rac1 signaling pathway was also involved in the epiregulin-induced migration and invasion of SACC cells. Epiregulin can significantly induce SACC cell migration and invasion and is closely related to lung metastasis (Hu et al., 2009; Yang et al., 2017). However, its downstream effectors remain to be fully understood. In this study, we observed that epiregulin could activate the RhoG/Rac1 signaling pathway during its promotion of SACC cell migration and invasion. The knockdown of RhoG or Rac1 or the inhibition of Rac1 by a specific inhibitor partially blocked the epiregulin-induced SACC cell migration and invasion. These results suggested that the RhoG/Rac1 signaling pathway at least partially mediated epiregulin effects on SACC cell migration and invasion. These results also implied that there could be other signaling pathways that also mediated

the epiregulin effects on SACC cell migration and invasion. Future studies are needed to elucidate those signaling pathways. In addition, epiregulin could activate the RhoG/Rac1 signaling pathway as rapidly as within two min, and this activation lasted for less than 30 min. This finding prompted us to question whether this short-term activation of the RhoG/Rac1 signaling pathway by epiregulin had functional effects. A wound-healing assay demonstrated that the treatment of SACC-83 cells with epiregulin for only 10 min could induce cell migration similar to that of SACC-83 cells treated with epiregulin for 16 hr. An immunoprecipitation assay also showed that E-cadherin completely lost interaction with β-catenin in SACC83 cells at 10 min posttreatment with epiregulin, suggesting that β-catenin was released from the adherens junctions and the cell–cell contacts would be disrupted. These results supported that this short-term activation of the RhoG/Rac1 signaling pathway by epiregulin was functional and their downstream effects could last much longer than RhoG/Rac1 activation themselves. Certainly, it was not ruled out that other signaling pathways might also mediate this effect of the short-term epiregulin treatment. It also appeared that the effect of epiregulin on SACC cell migration could continue for as long as 16 hr once its signaling pathway was initiated.

Epiregulin induced RhoG/Rac1 activation through the Src/AKT/ERK1/2 signaling pathway. We first confirmed that AKT was upstream of ERK1/2 in the epiregulin-induced activation of RhoG by the finding that the inhibition of ERK1/2 completely blocked the epiregulin-induced upregulation of membrane-bound RhoG, but not AKT phosphorylation, whereas the inhibition of AKT or the knockdown

of AKT completely blocked the epiregulin-induced upregulation of ERK1/2 phosphorylation and membrane-bound RhoG. In addition, overexpression of AKT active mutant (CA-AKT) upregulated both ERK1/2 phosphorylation and membrane-bound RhoG, that is, activated both ERK1/2 and RhoG. Therefore, epiregulin-induced RhoG activation was dependent on the AKT/ERK1/2 signaling pathway in SACC cells. Moreover, the inhibition of Src blocked the epiregulin-induced activation of AKT, ERK1/2, and RhoG. These results suggested that Src was upstream of the AKT/ERK1/2/RhoG signaling pathway. Although Src and AKT were previously showed to be downstream of the EGF signaling pathway in the migration of intestinal epithelial cells of mice, AKT was not downstream of Src, and ERK1/2 was not downstream of AKT (Dise, Frey, Whitehead, & Polk, 2007). Those results appear to be different from ours. The reasons could be that the downstream effectors of epiregulin (also a member of EGF family) might be different from those of the other members of EGF family, and the downstream effectors of the EGF signaling pathway may be different in different cells or different species. Taken together, our results suggested that epiregulin induced RhoG/Rac1 activation through the Src/AKT/ERK1/2 signaling pathway in SACC cells.

In conclusion, the RhoG/Rac1 signaling pathway was involved in SACC cell migration and invasion. Epiregulin induced migration and invasion through the Src/AKT/ERK1/2 signaling pathway mediating the RhoG/Rac1 signaling pathway.

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CONFLICT OF INTEREST

None to declare.

AUTHOR CONTRIBUTIONS

YHG designed the study and revised the MS; ZDX and TH performed the expriments and collected the data; ZDH drafted the MS.

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