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ORIGINAL ARTICLE

Rab25 augments cancer cell invasiveness through a β1 integrin/EGFR/VEGF-A/Snail signaling axis and expression of fascin

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The small GTP-binding protein Rab25 is associated with tumor formation and progression. However, recent studies have shown discordant effects of Rab25 on cancer cell progression depending on cell lineage. In the present study, we elucidate the underlying mechanisms by which Rab25 induces cellular invasion. We demonstrate that Rab25 increases β1 integrin levels and subsequent activation of EGFR and upregulation of VEGF-A expression, leading to increased Snail expression, epithelial-to-mesenchymal transition and cancer cell invasiveness. Strikingly, we identify that Snail mediates Rab25-induced cancer cell invasiveness through fascin expression and that ectopic expression of Rab25 aggravates metastasis of ovarian cancer cells to the lung. We thus demonstrate a novel role of a β1 integrin/EGFR/VEGF-A/Snail signaling cascade in Rab25-induced cancer cell aggressiveness through induction of fascin expression, thus providing novel biomarkers and potential therapeutic targets for Rab25-expressing cancer cells.
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INTRODUCTION

Rab25 is a member of the Rab11 subfamily and GTP-binding proteins that is exclusively expressed in epithelial cells.[1] Rab25 mediates recycling of proteins from the endosome to the plasma membrane.[2] The link between Rab25 and cancer progression was identified through high-density array comparative genomic hybridization (CGH), demonstrating amplification with subsequent overexpression in ovarian and breast cancers.[3] However, the role of Rab25 in cancer progression appears to be context dependent. Rab25 suppresses breast cancer initiation and progression in triple negative breast cancer,[4] colorectal adenocarcinoma[5] and esophageal squamous cell carcinoma.[6] Conversely, Rab25 expression is closely associated with invasion and metastasis of gastric,[7] bladder,[8] ovarian[3] and luminal breast[3,9] cancers. Therefore, illumination of the underlying mechanisms by which Rab25 modulates cancer pathophysiology in a context-dependent manner has the potential to reveal novel biomarkers and therapeutic targets for cancer cell progression.

Cancer metastasis is multi-step process that includes epithelial-to-mesenchymal transition (EMT).[10] Tumor cells detach from neighboring epithelial cells through downregulation of factors in adherens junctions including E-cadherin to begin invasion of the surrounding extracellular matrix. The Snail transcription factor contributes to EMT through downregulation of E-cadherin. Recent studies show that Snail expression is an independent prognostic predictor for progression and patient survival of various cancers, including gastric, ovarian and breast cancers.[11–13] Furthermore, overexpression of Snail is associated with lymph node metastasis in patients with breast[14] and gastric cancers.[15]

Fascin is an actin-bundling protein that crosslinks actin filaments into tight, parallel bundles in filopodia and

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invadopodia[16,17] that is closely associated with an increased risk of mortality and progression for various cancers including breast,[18] ovarian[19] and gastric cancer.[19] In addition, fascin expression correlates with repression of E-cadherin.[20] Further, a recent study showed that fascin mediates Slug-induced pancreatic cancer progression,[21] suggesting that fascin might contribute to EMT and thus cancer progression.

Recently, Rab25 was reported to induce Snail expression and bladder cancer metastasis.[22] In addition, Cheng *et al.*[4] claimed that Rab25 enhances apoptosis and suppresses triple-negative breast cancer aggressiveness through modulation of VEGF-A and VEGFR-1 expression. Further, a recent study suggests a context-dependent role of Rab25 in breast cancer.[23] However, the detailed underlying mechanisms by which Rab25 aggravates cancer cell EMT and metastasis have not yet been fully characterized in breast cancer and other cancer cells. In the current study, we demonstrate that Rab25 increases the β1 integrin level and the consequent activation of EGFR and expression of VEGF-A and Snail in cancer cells. Unexpectedly, we demonstrate that fascin is a downstream target of a Rab25-induced β1 integrin/EGFR/VEGF-A/Snail signaling axis that regulates cancer cell aggressiveness. Therefore, our results identify mechanisms by which Rab25 modulates cancer cell invasion through coordinated regulation of β1 integrin to activation of EGFR and expression of VEGF-A to increase the level of Snail protein and subsequent fascin expression.

MATERIALS AND METHODS

Reagents

$VEGF_{165aa}$ human recombinant protein was purchased from Millipore (Temecula, CA, USA). G418 were acquired from Sigma-Aldrich (St Louis, MO, USA). A VEGF-neutralizing antibody was purchased from R&D Systems (Minneapolis, MN, USA). Gefitinib was obtained from Selleckchem (Houston, TX, USA). All other reagents were of the purest grade available.

Cell culture

Breast and ovarian cancer cell lines were purchased from the American Type Culture Collection (Manassas, VA, USA). The gastric cancer cell line MKN-1 was purchased from the Korean Cell Line Bank (Seoul, Korea). MCF-7 cells were cultured in DMEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. SKOV-3 and MKN-1 cells were cultured in RPMI-1640 supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. All cells were incubated at 37 °C under 5% CO_2 in a humidified incubator and routinely tested using a MycoAlert Mycoplasma Detection Kit (LT07-218) from Lonza (Allendale, NJ, USA) for negative mycoplasma contamination.

Plasmid and siRNA transfection

The MCF-7, SKOV-3 and MKN-1 cells were transiently transfected according to the manufacturer's instructions with Lipofectamine 2000 or RNAiMAX (Invitrogen, Carlsbad, CA, USA). Stable overexpression of Rab25 in SKOV-3 cells was established by selecting stable transfectants with G418 (400 μg ml^{-1}). The Snail cDNA in pCR3.1 was kindly provided by Dr. J.I. Yook (Yonsei University college of Dentistry, Korea).[24] The Rab25 cDNA[3] was subcloned into a pcDNA3 vector, and an empty pcDNA3 vector was used as a negative control. β1 integrin constructs were kindly provided by Dr. Y.S Lee (Ewha Woman's University, Seoul). siRNAs of Snail No. 1 (SASI_Hs01_00039785), Snail No. 2 (SASI_Hs01_00039786), VEGF-A No. 1 (SASI_Hs01_00201117), VEGF-A No. 2 (SASI_Hs01_00201118), VEGFR-1 No. 1 (SASI_Hs01_00175950), VEGFR-1 No. 2 (SASI_Hs01_00333027), β1 integrin No. 1 (SASI_Hs01_00333437), β1 integrin No. 2 (SASI_Hs01_00159474) and fascin (SASI_Hs01_00222012) were purchased from Sigma-Aldrich. Control scrambled siRNA was purchased from Invitrogen. A fascin luciferase reporter vector including the fascin gene (+2189 to +2735) subcloned into pGL3 was kindly provided by Dr. Machesky L.M. (CRUK Beatson Institute for Cancer Research, Glasgow, UK).[21]

Quantitative RT-PCR

Briefly, total cellular RNA was isolated using Trizol (Invitrogen), and 1 μg of RNA was reverse transcribed using oligo(dT) and M-MLV reverse transcriptase (Promega, Madison, WI, USA) according to the manufacturer's protocol, as described previously.[25] Complementary DNA was amplified using an iQ5 Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA) with the following primer sets: Snail, 5′-TTT ACC TTC CAG CAG CCC TA-3′ (forward) and 5′-GGA CAG AGT CCC AGA TGA GC-3′ (reverse); fascin, 5′-ACC TGT CTG CCA ATC AGG AC-3′ (forward) and 5′-AGT ACT TGC CCG TGT GGG TA-3′ (reverse); VEGF-A, 5′-CAA GGC CAG CAC ATA GGA GA-3′ (forward), 5′-ACG CGA GTC TGT GTT TTT GC-3′ (reverse); VEGF-B, 5′-AGC ACC AAG TCC GGA TG-3′ (forward) and 5′-GTC TGG CTT CAC AGC ACT G-3′ (reverse); VEGF-C, 5′-TGC CGA TGC ATG TCT AAA CT-3′ (forward) and 5′-TGA CAG GTC TCT TCA TCC AGC-3′ (reverse); VEGF-D, 5′-GTA TGG ACT CTC GCT CAG CAT-3′ (forward) and 5′-AGG CTC TCT TCA TTG CAA CAG-3′ (reverse); VEGFR-1, 5′-CAG GCC CAG TTT CTG CCA TT-3′ (forward) and 5′-TTC CAG CTC AGC GTG GTC GTA-3′ (reverse); VEGFR-2, 5′-TGC CTA CCT CAC CTG TTT C-3′ (forward) and 5′-GGC TCT TTC GCT TAC TGT TC-3′ (reverse); VEGFR-3, 5′-GGT TCC TCC AGG ATG AAG AC-3′ (forward) and 5′-CAA GCA GTA ACG CCA GTG TC-3′ (reverse); and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), 5′-CAT CTT CCA GGA GCG AGA-3′ (forward) and 5′-CTG CTT CAC CAC CTT CTT GAT-3′ (reverse). The GAPDH gene was used as a control for calculation of ΔCt. The RT-PCR data were analyzed using the $2^{-(\Delta\Delta Ct)}$ method.

Immunoblotting

The lysates were resolved by SDS-PAGE. PVDF membranes with proteins were blocked and incubated for 2 h at room temperature, as described previously.[26,27] An E-cadherin antibody (610182) was purchased from BD Biosciences (San Jose, CA, USA). Antibodies for Rab25 (4314), Snail (3879), VEGFR-1 (2893) and p-EGFR (4407) were obtained from Cell Signaling Inc. (Danvers, MA, USA). Antibodies for Slug (15391), fascin (21743), Twist (15393), N-cadherin (7939), β1 integrin (53711), EGFR (03) and GAPDH (25778) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The immunoreactive bands were visualized via ECL (Thermo Fisher Scientific Inc., Rockford, IL, USA) using ImageQuant400 (GE Healthcare, Buckinghamshire, UK).

Immunofluorescence staining

After cell fixation with cold methanol for 15 min and permeabilization with 1% NP-40, immunofluorescence investigation was conducted

with E-cadherin (610182, 1:100, BD Bioscience), Snail (28199, 1:100, Santa Cruz Biotechnology Inc.) and fascin (21743, 1:100, Santa Cruz Biotechnology Inc.) antibodies overnight, as described previously.[28,29] The cells were washed with ice-cold phosphate-buffered saline and incubated with Cy2-conjugated goat anti-mouse IgG (111-223-003, green, 1:500; Jackson ImmunoResearch, West Grove, PA, USA) and Cy3-conjugated goat anti-rabbit IgG (111-156-003, red, 1:500; Jackson ImmunoResearch). The nuclei of the cells were counterstained with 4′,6′-diamidino-2-phenylindole dihydrochloride (DAPI; Molecular Probes, Carlsbad, CA, USA). The cells were examined by confocal microscopy (LSM710; Carl Zeiss, Jena, Germany).

In vitro invasion assay

The *in vitro* invasion assay was performed in triplicate using an invasion assay kit with Matrigel-coated inserts (BD Biosciences), as described previously.[30] A volume of 5×10^5 to 3×10^6 cells per ml was added to the upper compartment of the invasion chamber with or without pharmacologic inhibitors. To the lower compartment, we added serum-free conditioned medium (DMEM or RPMI, supplemented with 1% penicillin/streptomycin). After incubation for 16–48 h at 37 °C, the invaded cells were sequentially fixed, stained with Diff-Quik reagents (Dade Behring Inc., Newark, DE, USA) and quantified by counting the number of cells in five random high-power fields for each replicate (×200) under light microscopy.

Luciferase assay

Cells were co-transfected with 1 μg of promoter luciferase reporter constructs and 1 μg of β-galactosidase reporter plasmid using the Lipofectamine 2000 transfection reagent. Luciferase activities and β-galactosidase activity were assayed using the luciferase and β-galactosidase enzyme assay system (E1910, Promega). Luciferase activity was normalized to the β-galactosidase activity in the cell lysate and calculated as an average of three independent experiments.

Chromatin immunoprecipitation analysis

Chromatin immunoprecipitation (ChIP) analysis was performed using a kit purchased from Upstate Biotechnology (Charlottesville, VA, USA) according to the manufacturer's protocol. The primer sequences of Snail for the fascin promoter are 5′-TCA CAC AGC AAG TGA CCA CA-3′ (forward), 5′-AAT GTC CCC AAG AGA ACG TG-3′ (reverse). The PCR product was resolved on a 1.8% agarose gel and visualized by GelRed Nucleic Acid Gel Staining solution (Biotium, Hayward, CA, USA) and ultraviolet illumination.

Measurement of VEGF concentrations using enzyme-linked immunosorbent assay

Culture supernatants were collected and used in the determination of VEGF concentrations using a human VEGF-specific enzyme-linked immunosorbent assay (ELISA) kit (DVE00, R&D Systems) according to the manufacturer's instructions. Mean values were recorded in picograms per milliliter. The results represent triplicate experiments.

In vitro angiogenesis assay

The angiogenic activity of VEGF produced by the tested cells was analyzed using the *In vitro* Angiogenesis Assay Kit (Chemicon, Temecula, CA, USA) as described previously.[31] Human umbilical vein endothelial cells (HUVECs) were cultured in EGM-2 with 20% FCS, 50 U ml^{-1} penicillin, 50 μg ml^{-1} streptomycin sulfate, 25 μg ml^{-1} endothelial cell growth supplement, 100 μg ml^{-1} heparin, 2 mM sodium pyruvate and 1 mM HEPES at pH 7.4. HUVECs were serum starved in EGM-2 medium for 8 h at 37 °C. The supernatant of MCF-7 cells (conditioned medium of cultured cells) was collected and concentrated 10-fold using a 3 K Ultra centrifugal filter device (Millipore, Billerica, MA, USA). ECMatrix solution was mixed with ECMatrix diluent buffer, distributed to a 96-well plate and allowed to solidify at 37 °C for 1 h. The serum-starved HUVECs were resuspended with the concentrated supernatant (conditioned medium) and added to ECMatrix-coated 96-well plates, followed by incubation for 12 h. HUVEC capillary tube formation was inspected under an inverted light microscope and measured by counting the branch points in several random fields of view per well (averaged values). The results are taken from the experiment in triplicate of four independent experiments.

Tumor formation in nu/nu mice

For tumorigenesis analysis, stably transfected SKOV-3 cells with Rab25 or control vector (5×10^6 cells in 100 μl phosphate-buffered saline) were subcutaneously injected into the flanks of 4-week-old female nude mice (Nara Biotechnology Co. Ltd, Cheongwon, Korea). Tumor growth was monitored by measuring the tumor diameter with a caliper every 3 days until day 80. The tumor volume was calculated using the following equation: length × $width^2 \times 0.52$. To determine the survival time of the mice, the mice were examined for 18 weeks after cell inoculation, at which point the experiment was terminated. For experimental metastasis, the cells with Rab25 or control vector (2×10^6 cells in 200 μl phosphate-buffered saline) were injected into mice via the tail vein. After 14 weeks, the mice were killed, and the lungs were collected for analysis of tumor seeding and metastasis. All animal experiments were approved by the Animal Care and Use Committee of Konyang University of College of Medicine.

Immunohistochemical analysis

Samples from the largest tumors were fixed in formalin for 24 h at room temperature, embedded in paraffin and sliced for hematoxylin and eosin staining. Immunohistochemistry was performed with primary Rab25 (ab106175, 1:200, Abcam, Cambridge, MA, USA), VEGFR-1 (ab32152, 1:250, Abcam), Snail (ab180714, 1:100, Abcam) and fascin (ab126772, 1:250, Abcam) antibodies, as described previously.[25] The investigator was blinded during the experiments.

Gene expression data analysis

Gene expression data from TCGA ovarian cancer cohort were used to analysis for this study (https://tcga-data.nci.nih.gov/tcga/). TCGA breast and ovarian cancer gene expression data were used to calculate the correlation between *FSCN1* and *SNAI1* based on Pearson's correlation co-efficient value. The correlation value was used in plotting.

Kaplan–Meier (K–M) survival analysis

Patients in the indicated cohorts were dichotomized by expression of *FSCN1* with relatively high expression or relatively low expression and were considered for plotting. The log-rank test was applied to estimate the significance of difference.

Statistical analyses

Sample sizes were calculated to allow significance. All reported results are taken from three independent experiments performed in triplicate. All experiments were performed by an investigator aware of the experimental hypothesis and reproduced by a blinded investigator. All samples were used in statistical analysis. Data are shown as the

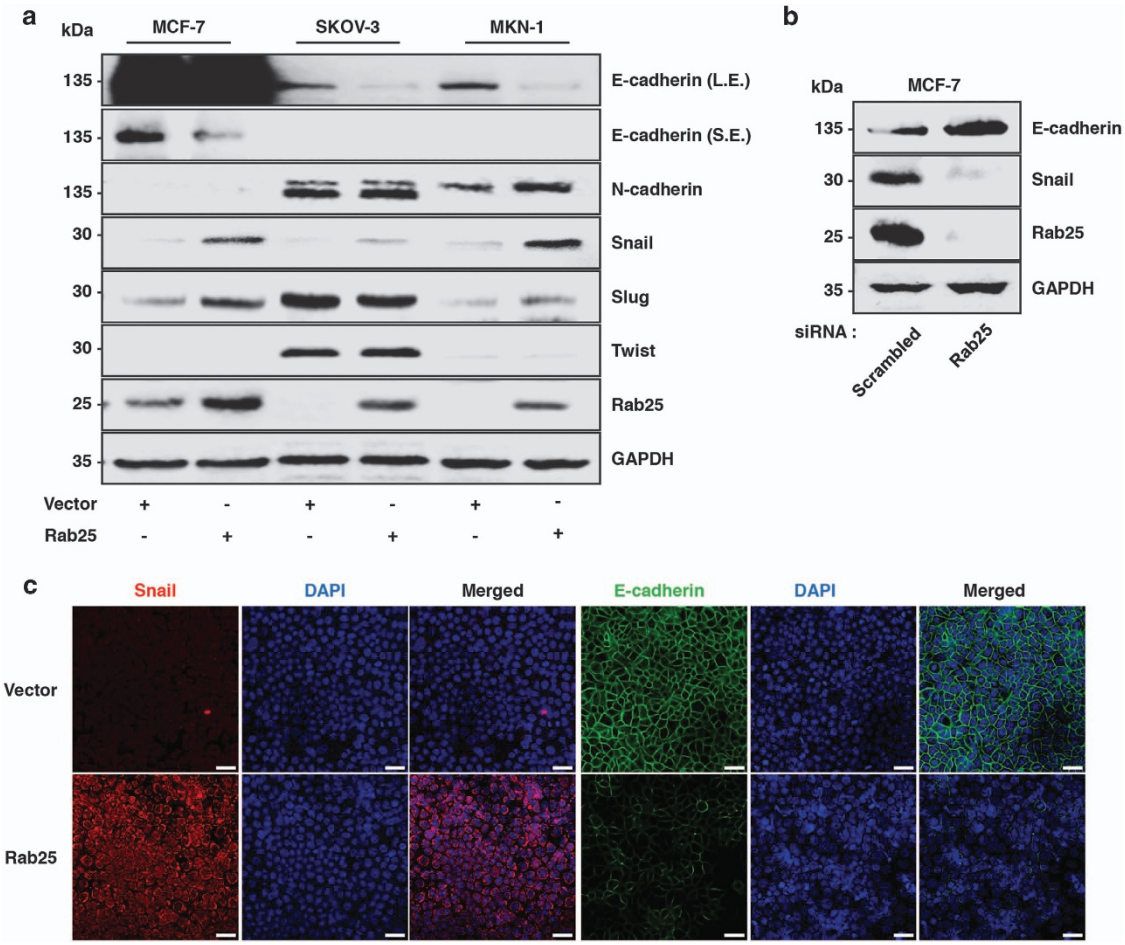

Figure 1 Rab25 modulates cancer cell EMT. (**a**, **b**) Cells were transfected with indicated vectors or siRNAs. LE, long-time exposure (60 s), SE, short-time exposure (10 s). Immunoblotting. (**c**) MCF-7 cells were transfected with indicated vectors, and the expression of E-cadherin and Snail was visualized by immunofluorescence. Original magnification, ×200; scale bar, 20 μm. Representative results are presented from at least three independent experiments with similar results.

means ± standard deviation (s.d.). Differences between two groups were assessed with SigmaPlot software (SYSTAT SOFTWARE, San Jose, CA, USA) using the unpaired two-tailed Student's *t*-test. Differences among three or more groups were evaluated by analysis of variance followed by Bonferroni multiple comparison tests.

RESULTS

Rab25 modulates cancer cell EMT

Given that EMT is one of the critical steps for cancer cell progression and that Rab25 functions as an enhancer of cancer aggressiveness depending on the type of cancer cells assessed, we first determined how Rab25 changes the expression of various EMT factors. Ectopic expression of Rab25 markedly reduces E-cadherin expression in luminal breast cancer MCF-7, ovarian SKOV-3 and gastric MKN-1 cells (Figure 1a).

In addition, silencing of Rab25 suppressed Snail expression with consequent increased E-cadherin expression in MCF-7 cells (Figure 1b). Further, immunofluorescence analysis confirmed that Rab25 induces Snail expression, whereas E-cadherin expression is reduced in MCF-7 cells (Figure 1c). In addition, transfection of the cells with Snail siRNA recovered Rab25-reduced E-cadherin expression (Supplementary Figure S1). Therefore, these data suggest that Rab25 functions as an inducer of EMT.

Rab25 induces β1 integrin expression

Since Rab25 has been shown to induce β1 integrin expression in polarized colonic epithelial cells,[32] we determined the effects of Rab25 on β1 integrin expression in the tested cancer cells. Rab25 significantly induced β1 integrin expression in MCF-7 cells (Figure 2a). However, silencing of β1 integrin expression dramatically reduced Rab25-induced MCF-7 and SKOV-3 cell invasion (Figure 2b). In addition, ectopic expression of β1 integrin induced cancer cell invasion (Figure 2c), confirming that β1 integrin plays a role in Rab25-induced cancer cell aggressiveness. Further, transfection of the cells with β1 integrin siRNA recovered Rab25-reduced E-cadherin expression (Figure 2d, Supplementary Figure S2). Given that Rab25 recycles β1 integrin with EGFR,[33] we subsequently determined whether Rab25 activates EGFR. Indeed, ectopic expression of Rab25 markedly induced EGFR phosphorylation (Figure 2e, Supplementary Figure S2). However, silencing of β1 integrin reduced Rab25-induced EGFR phosphorylation,

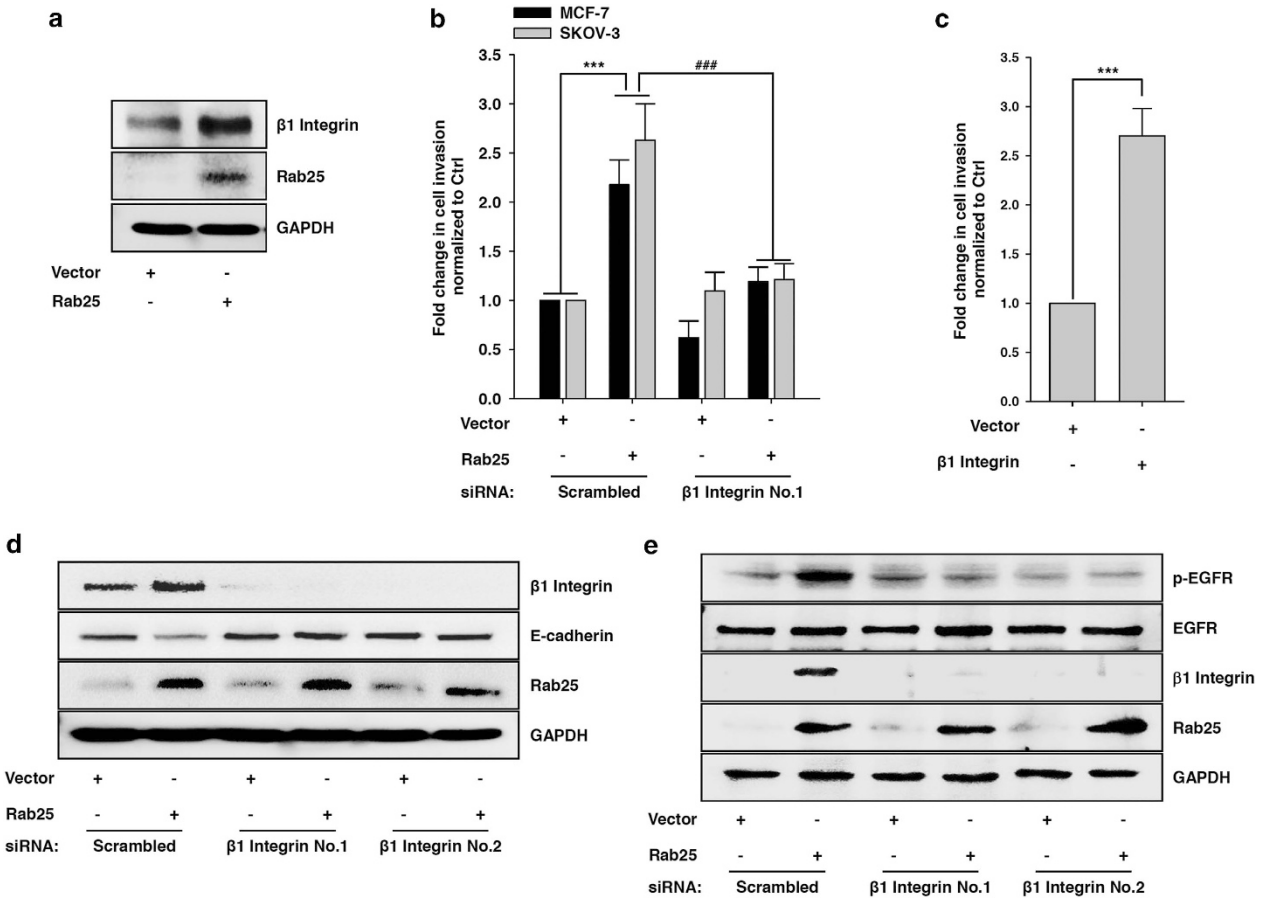

Figure 2 Rab25 induces β1 integrin expression. (**a**) MCF-7 cells were transfected with indicated vectors. Immunoblotting. (**b**) Cells were co-transfected with indicated vectors and siRNA prior to invasion assay (mean ± s.d. $^{***}P<0.001$ versus control vector with scrambled siRNA, $^{\#\#\#}P<0.001$ versus Rab25 overexpression with scrambled siRNA). (**c**) MCF-7 cells were transfected with indicated vector prior to invasion assay (mean ± s.d. $^{***}P<0.001$ versus control vector). (**d**, **e**) MCF-7 cells were co-transfected with indicated vectors and siRNAs. Immunoblotting. Representative results are presented from at least three independent experiments with similar results.

suggesting that β1 integrin is important for Rab25-induced EGFR activation. Therefore, these data strongly suggest that β1 integrin/EGFR signaling is important for Rab25-induced EMT and cancer cell invasion.

VEGF-A and VEGFR-1 are important for Snail expression

Rab25 was previously suggested to suppress VEGF-A secretion and consequent aggressiveness of MDA-MB-231 cells.[4] Therefore, we explore the role of VEGF and VEGFR in Rab25-induced Snail expression and cancer cell EMT. Rab25 induced VEGF-A transcript expression in MCF-7 and SKOV-3 cells (Figure 3a). ELISA analysis also showed that Rab25 induces VEGF-A secretion into the media of MCF-7 and SKOV-3 cells (Figure 3b). We detected little effect of Rab25 on transcript expression of other VEGFs (VEGF-B, VEGF-C and VEGF-D) (Supplementary Figure S3a). In addition, we observed that silencing of VEGF-A expression strongly inhibits Rab25-induced Snail expression (Figure 3c,Supplementary Figure S3b) and cancer cell invasion (Figure 3d, Supplementary Figure S3c). Further, Rab25-induced VEGFR-1 (Supplementary Figure S3a), and silencing VEGFR-1 abolished Rab25-induced Snail expression in MCF-7 cells (Figure 3e). Finally, we observed that conditioned medium of Rab25-transfected MCF-7 cells increases HUVEC capillary formation and that conditioned medium of MCF-7 cells transfected with siRNA of VEGF-A or VEGFR-1 abrogates Rab25-induced capillary formation (Figure 3f). Therefore, these data suggest that Rab25 induces Snail expression and angiogenesis through a VEGF-A/VEGFR-1 signaling axis.

Fascin is important for Rab25-induced cancer invasion

Given that fascin, an actin-bundling motility-associated protein, is implicated in cancer progression[34] and that a member of the Snail family of zinc finger transcription factors, Slug, has been reported to induce fascin expression in pancreatic adenocarcinoma cells,[21] we determined whether fascin contributes to Rab25-induced cancer cell invasion. Interestingly, ectopic expression of Rab25 induced fascin transcripts (Figure 4a), suggesting that Rab25 regulates fascin expression. In addition, we observed that Rab25 induces the promoter activity of fascin (Figure 4b) and that silencing of VEGF-A (Figure 4c) or VEGFR-1 (Figure 4d) significantly reduced Rab25-induced fascin expression. Consistently, stimulation of the cells with VEGF-A markedly upregulated Snail and fascin

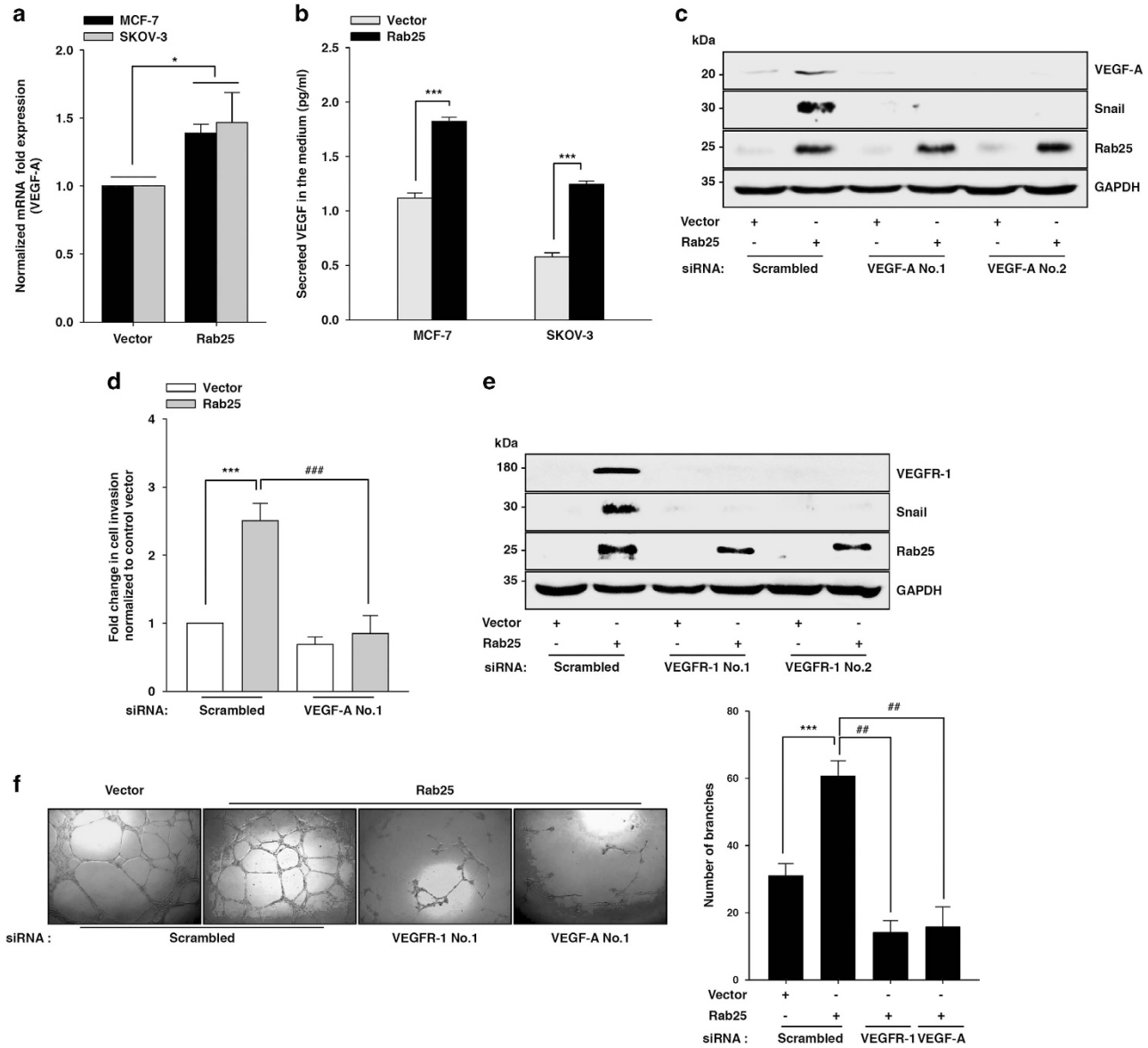

Figure 3 VEGF-A and VEGFR-1 are important for Snail expression. (**a**) Cells were transfected with indicated vectors. Quantitative RT-PCR (mean ± s.d. $^{*}P<0.05$ versus control vector). (**b**) Cells were transfected with indicated vectors. ELISA assay (mean ± s.d. $^{***}P<0.001$ versus control vector). (**c**) MCF-7 cells were co-transfected with indicated vectors and siRNAs. Immunoblotting. (**d**) MCF-7 cells were co-transfected with indicated vectors and siRNAs. Invasion assay (mean ± s.d. $^{***}P<0.001$ versus control vector with scrambled siRNA, $^{\#\#\#}P<0.001$ versus Rab25 overexpression with scrambled siRNA). (**e**) MCF-7 cells were co-transfected with indicated vectors and siRNAs. Immunoblotting. (**f**) MCF-7 cells were co-transfected with indicated vectors and siRNAs for 48 h. HUVEC capillary tube formation assay (mean ± s.d. $^{***}P<0.001$ versus control vector with scrambled siRNA, $^{\#\#}P<0.01$ versus Rab25 overexpression with scrambled siRNA). Representative results are presented from at least three independent experiments with similar results.

expression concomitant to reduced E-cadherin expression (Figure 4e,Supplementary Figure S4a). However, transfection of the cells with Slug siRNA did not inhibit VEGF-induced fascin expression (Figure 4f). More importantly, silencing of fascin expression significantly attenuated Rab25- and VEGF-induced cancer cell invasion (Figure 4g and h,Supplementary Figure S4b). Finally, blocking of VEGF by a VEGF neutralizing antibody (VEGF ab) completely inhibited Rab25-induced Snail and fascin expression (Figure 4i). Therefore, these data suggest that Rab25 induces fascin expression through a VEGF-A/VEGFR-1/Snail signaling axis and that fascin is important for Rab25-induced cancer aggressiveness.

Snail mediates Rab25-induced fascin expression

We next explored whether Snail contributes to Rab25-induced fascin expression. Ectopic expression of Rab25 induced fascin mRNA expression (Figure 5a). However, silencing of Snail expression significantly attenuated Rab25-induced fascin transcript expression. Consistently, transfection of cells with Snail siRNA strongly inhibited Rab25-induced fascin protein

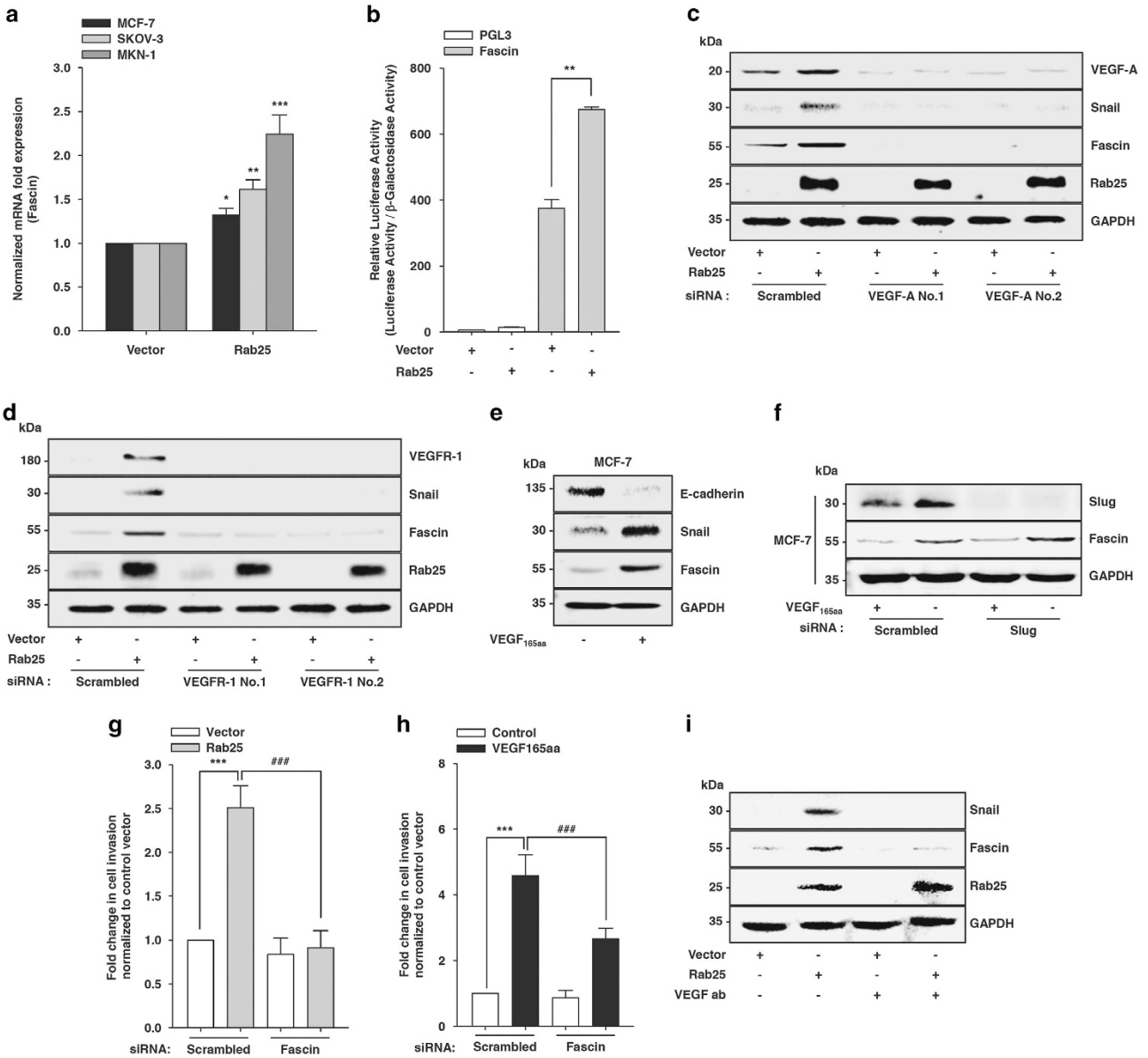

Figure 4 Fascin is important for Rab25-induced cancer invasion. (**a**) Cells were transfected with indicated vectors. Quantitative RT-PCR (mean ± s.d. $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ versus control vector). (**b**) SKOV-3 cells were co-transfected with indicated vectors. Luciferase activity (mean ± s.d. $^{**}P<0.01$ versus Rab25 with Fascin promoter overexpression). (**c**, **d**) MCF-7 cells were co-transfected with indicated vectors and siRNAs. Immunoblotting. (**e**) MCF-7 cells were stimulated with $VEGF_{165aa}$ (50 ng ml^{-1}) for 1 h. Immunoblotting. (**f**) MCF-7 cells were transfected with indicated siRNAs, serum starved and stimulated with $VEGF_{165aa}$ (50 ng ml^{-1}) for 1 h. Immunoblotting. (**g**) MCF-7 cells were co-transfected indicated vectors and siRNAs. Invasion assay (mean ± s.d. $^{***}P<0.001$ versus control vector with scrambled siRNA, $^{\#\#\#}P<0.001$ versus Rab25 overexpression with scrambled siRNA). (**h**) SKOV-3 cells were transfected with indicated siRNAs, serum-starved and treated with $VEGF_{165aa}$ (50 ng ml^{-1}). Invasion assay (mean ± SD $^{***}P<0.001$ versus scrambled siRNA, $^{\#\#\#}P<0.001$ versus scrambled siRNA with $VEGF_{165aa}$) (**i**) MCF-7 cells transfected with indicated vectors were treated with VEGF neutralizing antibody (VEGF ab, 5 μg ml^{-1}, 24 h). Immunoblotting. Representative results are presented from at least three independent experiments with similar results.

expression (Figure 5b), suggesting that Snail mediates Rab25-induced fascin expression. In addition, we observed that ectopic expression of Snail was sufficient to induce fascin expression (Supplementary Figure S5). We subsequently determined whether VEGF-A and VEGFR-1 contribute to Rab25-induced fascin expression. Immunofluorescence data show that silencing of Snail, VEGF-A and VEGFR-1 expression markedly reduced Rab25-induced fascin expression (Figure 5c), suggesting that the VEGF-A/VEGFR-1/Snail signaling axis is critical for Rab25-induced fascin expression. Further, VEGF upregulated fascin expression (Figure 5d) and binding of Snail to the fascin promoter (Figure 5e).

Gene expression data in the K–M plot shows that *FSCN1* (for fascin) expression correlates with breast, ovarian and gastric cancer patient outcomes (Figure 5f). Further, we used the TCGA data cohort to determine the clinical

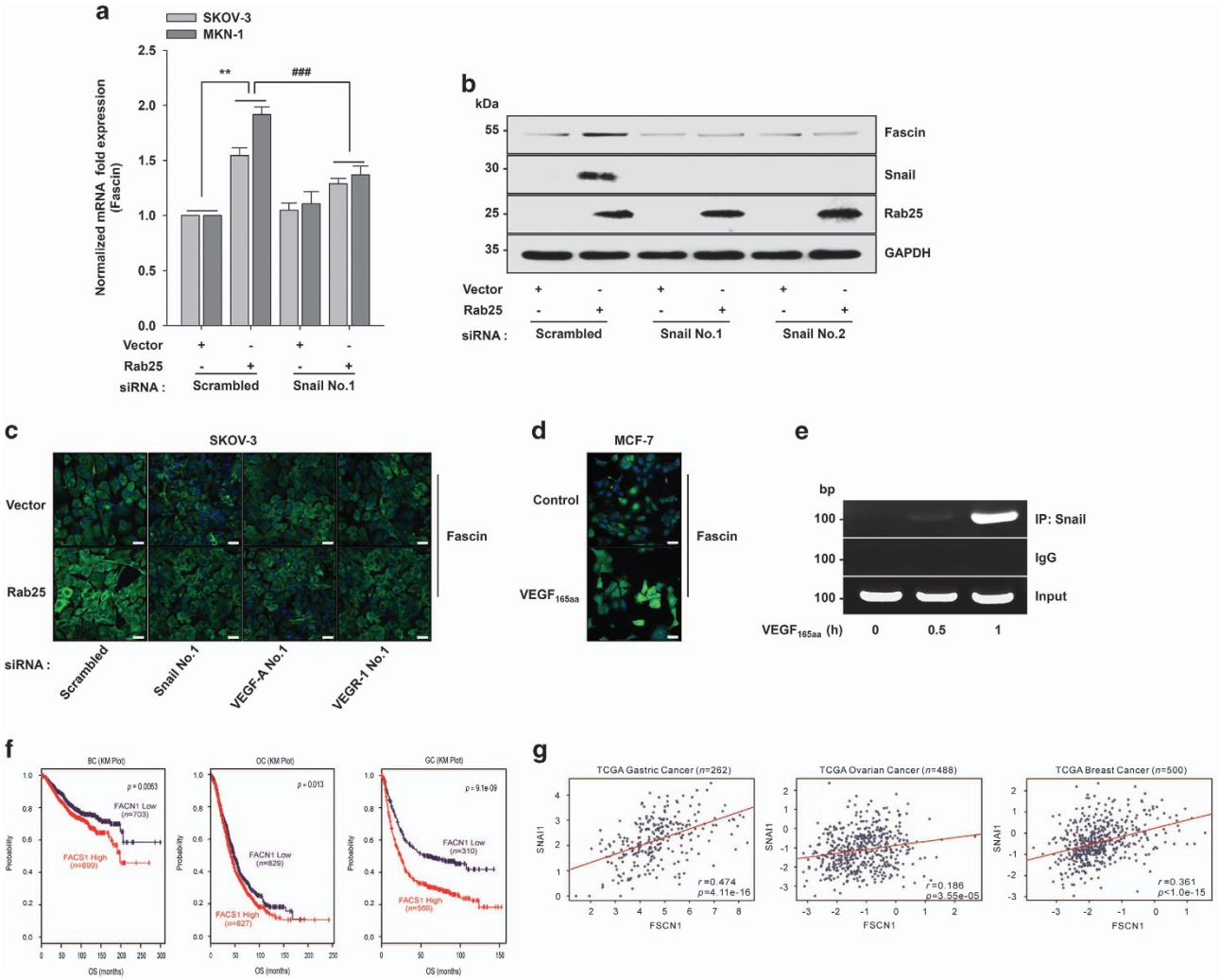

Figure 5 Snail mediates Rab25-induced fascin expression. (**a**) Cells were co-transfected with indicated vectors and siRNAs. Quantitative RT-PCR for fascin mRNA expression (mean ± s.d. $^{**}P<0.01$ versus control vector with scrambled siRNA, $^{\#\#\#}P<0.001$ versus Rab25 overexpression with scrambled siRNA). (**b**) MCF-7 cells were co-transfected with indicated vectors and siRNAs. Immunoblotting. (**c**, **d**) Cells were co-transfected with indicated vectors and siRNAs (**c**), or stimulated with $VEGF_{165aa}$ (**d**). Immunofluorescence with an anti-fascin antibody. Original magnification, ×200; scale bar, 20 μm. (**e**) SKOV-3 cells were stimulated with $VEGF_{165aa}$. ChIP analysis. (**f**) Correlation between *FSCN1* gene expression and overall survival in breast (BC), ovarian (OC) and gastric (GC) cancer patients, $P=0.01$. The K–M plot was drawn in 'kmplot.com'. (**g**) Correlation between *FSCN1* and *SANI1* gene expression in breast, ovarian and gastric cancer patients.

implication of *SNAI1* (for Snail) and *FSCN1* and observed that *SNAI1* expression is significantly correlated with *FSCN1* expression (Figure 5g). Therefore, these results suggest that Snail mediates Rab25-induced fascin expression and cancer invasion.

Rab25 increases tumorigenesis and metastasis

As EMT is a critical component of the metastatic cascade, we determined whether Rab25 could contribute to *in vivo* tumorigenesis and metastasis by injecting SKOV-3 cells stably transfected with Rab25 into nude mice. We observed that mice injected with Rab25-transfected cells had much larger tumors than those with vector-transfected cells (Figure 6a). Analysis of the tumor growth curves indicates that the difference was significant at 8 weeks after cell injection. We subsequently evaluated the lungs for tumor seeding. We observed much larger and higher numbers of metastatic colonies in the lungs of the mice implanted with SKOV-3 cells with ectopic expression of Rab25 compared with the control vector (Figure 6b and c). Further, RT–PCR analysis showed stronger VEGF-A, VEGFR-1, Snail and fascin mRNA expression in the lung tissues from mice bearing SKOV-3 cells with ectopic expression of Rab25 compared with cells expressing control vector (Figure 6d). Consistent with these findings, immunohistochemical analysis showed much higher VEGFR-1, Snail and fascin expression in lung tissues from mice bearing SKOV-3 cells with ectopic expression of Rab25 compared with mice bearing SKOV-3 cells transfected with control vector (Figure 6e). Therefore, these results strongly suggest that Rab25 increases cancer cell metastasis through a VEGF-A/VEGFR-1/Snail/fascin signaling axis.

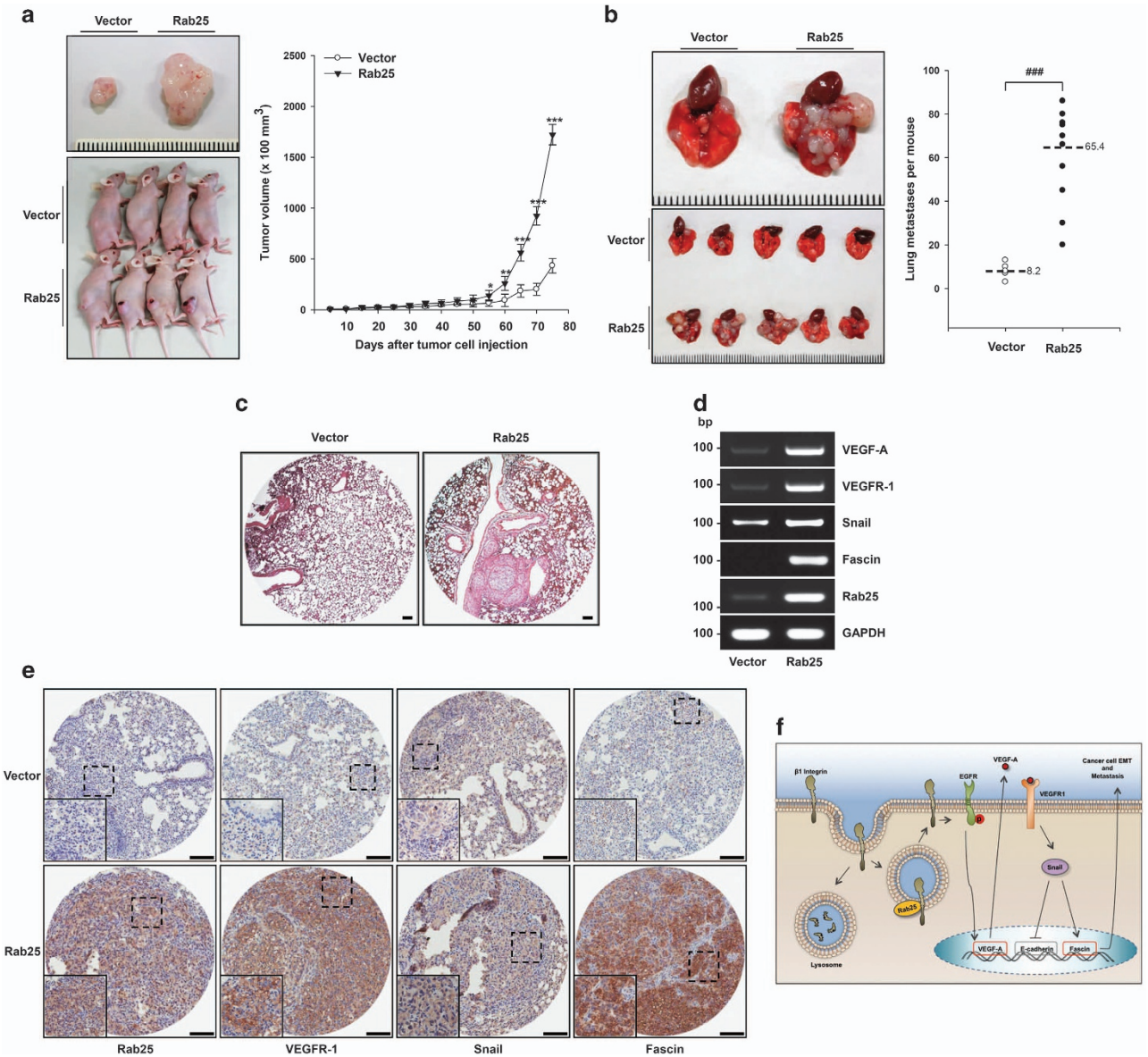

Figure 6 Rab25 increases tumorigenesis and metastasis. (**a**) Tumors from mice subcutaneously injected with stably transfected SKOV-3 cells with indicated vectors. Tumor dimensions were measured with calipers every 3 days (vector group number; $n=5$, Rab25 group number; $n=15$, mean ± s.d. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ versus vector). (**b**) Lungs from mice intravenously injected with stably transfected SKOV-3 cells with indicated vectors (vector group number; $n=5$, Rab25 group number; $n=10$). (**c**) Hematoxylin and eosin stain of lungs tissues. Original magnification, ×40. (**d**) Rab25, VEGFR-1, VEGF-A, Snail and fascin mRNA levels in tumors derived from lung tissue from mice. (**e**) Immunohistochemical staining for Rab25, VEGFR-1, Snail and fascin in the lung tissue of mice. Scale bar, 100 μm. Representative results are presented from the tissues of four lungs with similar results. (**f**) Working model of the Rab25-induced cancer cell aggressiveness. Rab25 activates a β1 integrin/EGFR/VEGFR-1/VEGF-A signaling axis and subsequently induces Snail and fascin expression, leading to cancer cell EMT and metastasis.

DISCUSSION

Cancer cell invasion and metastasis is the ultimate cause of death for most cancer patients. Rab25 has been shown to augment or suppress cancer cell progression depending on the cellular context. In the present study, we demonstrate the underlying mechanism by which Rab25 modulates cancer cell progression. Ectopic expression of Rab25-induced cancer cell EMT and invasion in luminal breast, ovarian and gastric cancer cells by modulating Snail and E-cadherin expression. In addition, we observed that Rab25 upregulates β1 integrin level and subsequent EGFR activation, leading to expression of VEGF-A and stabilization of Snail protein. Interestingly, we demonstrated that Rab25- and VEGF-A-induced Snail expression not only downregulates E-cadherin expression but also increases fascin expression, leading to a startling morphological change to a mesenchymal phenotype and a marked increase in cancer cell invasion and metastasis. Therefore, these findings strongly suggest a critical role for Rab25 in cancer cell invasion

and metastasis through a β1 integrin/EGFR/VEGF-A/VEGFR--1/Snail signaling axis and fascin expression.

Initially, the small GTPase protein Rab25 was reported to be overexpressed in approximately half of ovarian and breast cancers and to augment tumor aggressiveness of epithelial cancers.[3,7–9,35–37] However, subsequent studies suggest a tumor-suppressive role of Rab25 in several cancer lineages.[4–6,38] Consistent with these findings, our current study demonstrates a context-dependent role of Rab25 in cancer cell EMT and invasion.

Accumulating data show that Rab25 induces β1 integrin. Rab25-deficient mice decreased β1 integrin staining in the lateral membrane of villus cells.[5] In addition, Rab25 co-localized with α5β1 integrin, which is implicated in Caco2-BBE cell invasion.[32] However, previous studies suggest a tumor-suppressive role of β1 integrin. Loss of β1 integrin increases the invasive potential of Caco2 cells.[39] In addition, the overexpression of α5β1 integrin in HT29 colon carcinoma cells dramatically reduced tumorigenicity.[40] In contrast, our present studies show that Rab25 induces cancer cell invasiveness through β1 integrin expression. In addition, we demonstrated that β1 integrin mediates Rab25-induced EGFR activation. Consistent with these findings, we observed that RCP, known as Rab11 family-interacting protein 1 (Rab11FIP1), stabilizes β1 integrin protein to activate EGFR, leading to Slug expression and cancer cell metastasis.[41] These results suggest that Rab25 regulates cancer cell invasion in a context-dependent manner through β1 integrin expression.

Previously, Cheng *et al.*[4] claimed the important role of VEGF-A and VEGFR-1 in Rab25-induced suppression of angiogenesis and invasion of triple-negative breast cancer. In the current study, we offer detailed mechanistic evidence that Rab25 modulates VEGF-A and VEGFR-1 expression to govern cancer aggressiveness. We observed that Rab25 upregulates VEGF-A and VEGFR-1 expression in MCF-7, SKOV-3 and MKN-1 cells. Further, we demonstrated that VEGF-A is sufficient to induce Snail expression and cancer aggressiveness. Consistent with this notion, VEGF-A stimulates Snail expression in breast cancer cells,[42] suggesting that the level of VEGF-A and VEGFR-1 expression governs Rab25-induced cancer cell invasion.

Fascin has been associated with cancer progression. Fascin is overexpressed in malignant tumors compared with normal epithelial cells.[43,44] Recently, Li *et al.*[21] reported that Slug induces fascin expression to promote formation of filopodia and invasive activities of pancreatic adenocarcinoma cells. Our present study shows that Snail but not Slug mediates Rab25- and subsequent VEGF-A-induced fascin expression to induce cancer cell EMT and invasiveness. Given that Rab25 affects the organization of actin at the cell surface[37] and that fascin stabilizes actin in invadopodia to potentiate protrusive invasion, it is highly likely that Rab25 aggravates cancer cell invasiveness through a VEGF-A/VEGFR-1/Snail/fascin signaling cascade. Based on our current data, we present a working model (Figure 6f) in which a β1 integrin/EGFR/VEGF-A/VEGFR-1 signaling axis is required for Rab25-induced Snail and fascin expression.

Collectively, the present study shows that a β1 integrin/EGFR signaling axis governs Rab25-induced modulation of cancer invasion and that Rab25 induces Snail expression through the VEGF-A/VEGFR-1 signaling cascade to upregulate fascin expression, leading to cancer cell EMT and metastasis. Therefore, β1 integrin, EGFR, VEGF-A, VEGFR-1, Snail and fascin should be evaluated as potential therapeutic targets to reduce invasion and metastasis of Rab25-expressing cancer cells.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on Experimental & Molecular Medicine website (http://www.nature.com/emm)