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EGFR over-expression in non-small cell lung cancers harboring EGFR mutations is associated with marked down-regulation of CD82

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ABSTRACT

Epidermal growth factor receptor (EGFR) gene mutations are strongly associated with lung adenocarcinoma and favorable response to EGFR tyrosine kinase inhibitor. The mutated EGFR proteins (EGFRs) are hyper-phosphorylated and refractory to receptor down-regulation. To address the discrepancy between hyper-phosphorylation and lack of down-regulation of mutant EGFRs, we have examined the expression of EGFR negative regulators in non-small cell lung cancer (NSCLC) cell lines. We found that NSCLC cell lines expressing mutant EGFRs often had low expression of various negative regulators for EGFR. Among them, tumor suppressor CD82 was up-regulated by wild type (WT) EGFR but down-regulated by mutant EGFRs. Reconstitution of CD82 exerted stronger suppressive effects on mutant EGFRs than on WT EGFR. Active exportation of CD82 through the exosome was one of the mechanisms involved in achieving the overall CD82 down-regulation in mutant EGFR-expressing lung cancer cell lines. Over-expression of mutant EGFR protein frequently occurred in the lung cancer tissues of mutant EGFR-transgenic mice and also associated with CD82 down-regulation. Immunoblot analyses on the tumor tissues from 23 lung adenocarcinoma patients (12 with WT EGFR, and 11 with mutant EGFRs) also identified significantly stronger down-regulation of CD82 in tumors with mutant EGFRs than WT. Our data indicate that CD82 down-regulation could be a critical step involved in the EGFR over-expression and the stronger tumorigenic activity triggered by EGFR mutations. Up-regulation of the CD82 level may become a promising new treatment strategy for lung adenocarcinoma.

1. Introduction

Epidermal growth factor receptor (EGFR) participates in various cellular functions and abnormal EGFR signaling is involved in many malignant diseases [1,2]. EGFR mutations in the kinase domain frequently occur in lung adenocarcinoma (LAC) among East Asians [3,4]. The L858R mutation and deletions (Del) in exon 19 are the major EGFR mutations [3–7]. Mutant EGFR proteins (EGFRs) are strongly tyrosine-phosphorylated in the absence of ligands [8,9]. Phosphorylation of wild type (WT) EGFR leads to subsequent internalization, ubiquitination, and degradation [1]. In contrast, mutated EGFR is resistant to down-regulation albeit their hyper-phosphorylation status [8,10,11]. Agreeing with these observations, EGFR mutations are closely associated with EGFR over-expression in LAC tissues [3]. Thus, mutant EGFRs are supposed to exert stronger tumorigenic activity than WT EGFR [12]. However, the detailed molecular mechanism of mutant EGFRs' stronger tumorigenic ability remains to be elucidated.

To address the discrepancy between hyper-phosphorylation and lack of down-regulation of mutant EGFRs, we have examined the expression of various negative regulators for EGFR in lung cancer cell

Abbreviations: EGFR, epidermal growth factor receptor; LAC, lung adenocarcinoma; Del, deletion; WT, wild type; c-Cbl, casitas B-lineage lymphoma; GAK, cyclin G-associated kinase; NSCLC, non-small cell lung cancer; PKC, protein kinase C; SP-A, surfactant protein A; SP-C, surfactant protein C; BGH, bovine growth hormone; 5-aza-dC, 5-aza-deoxy-cytidine; TsA, trichostatin; Flot 1, Flotillin 1

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lines expressing mutant EGFRs, which include casitas B-lineage lymphoma (c-Cbl, the EGFR ubiquitin ligase) [13], cyclin G-associated kinase (GAK, an endocytosis regulator) [14], and CD82 [15–18]. We found that lung cancer cell lines expressing mutant EGFRs frequently had low expression of one or several of the above three proteins. Among them, the tumor suppressor CD82 was up-regulated by WT EGFR but down-regulated by mutant EGFRs, which is intriguing.

CD82, a member of the teraspanin family, is a cancer metastasis suppressor and is often down-regulated in cancers [15–18]. Moreover, CD82 expression is correlated with a good prognosis in non-small cell lung cancer (NSCLC) patients [19]. CD82's anti-metastatic ability is attributed to its interaction with integrin molecules [20–23]. CD82 interacts with Duffy antigen receptor for chemokines (DARC) on endothelial cells and causes the growth inhibition of the CD82-expressing tumor cells [24]. Additionally, CD82 interacts with EGFR and c-Met to suppress the receptor-induced lamellipodia formation [25]. CD82 enhances EGF-induced EGFR internalization, which is mediated through protein kinase C (PKC)α and ganglioside-dependent mechanisms [25–27]. A recent study shows that CD82 reduces the level of ubiquitylation of EGFR upon heparin-binding EGF (HB-EGF) or amphiregulin stimulation and alters the postendocytic trafficking of EGFR [28].

In this study, we found that EGFR over-expression was associated with a marked reduction of CD82 in (1) NSCLC cells expressing mutant EGFRs, (2) NSCLC cells with endogenous EGFR mutations, (3) lung cancer tissues derived from mutant EGFR-transgenic (Tg) mice, and (4) tumor tissues of LAC patients with EGFR mutation. Our data indicated that down-regulation of CD82 could be a mechanism involved in the mutant EGFR over-expression in LAC cells.

2. Material and methods

2.1. Cell cultures and transfection

Five human NSCLC cell lines (H1299, HCC1975, HCC1650, HCC827 and PE089) were used in this study. The H1299 cell line is established from a patient with lung carcinoma. The PE089, H1650, H1975, HCC827 cell lines (the latter three were provided by Dr. Tsu-An Hsu from the Institute of Biotechnology and Pharmaceutical Research, National Health Research Institutes, Taiwan) are derived from patients with LAC. H1299 cells express wild type (WT) EGFR; the H1975 cell line has L858R/T790M double mutations; and H1650, PE089, and HCC827 cells all have EGFR exon 19 deletion (Del). H1299 derivatives were established by transfection with an empty pcDNA4/TO/myc-His vector (Invitrogen; Carlsbad, CA) or with a vector containing WT-EGFR, EGFR-L858R, or EGFR-Del coding sequence. The transfected cells were subjected to 500 μg/ml of zeocin (Invitrogen) selection for one week to establish permanently transfected cells as previously described [8]. H1299-EGFR, H1299-L858R, and H1299-Del cells were combined in pools of drug-resistant colonies expressing the respective receptor, as confirmed by immunoblotting assays, to avoid clonal variations. H1299 and its derivatives were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS) plus penicillin and streptomycin [8]. H1650, H1975, and HCC827 cells were cultured in the same media as H1299 cells. PE089 cell line was cultured in DMEM basal medium supplemented with 10% FCS plus penicillin and streptomycin [29]. Transient transfections of cells were performed using the Lipofectamine-2000 reagent (Invitrogen) according to the manufacturer's instruction.

2.2. Construction of DNA vectors

CD82-coding sequence was PCR-amplified using a cDNA pool prepared from H1299 cells. The DNA fragment was enzyme-digested and inserted into a pCR3.1 vector (Invitrogen) between Hind III and Xho I sites with a carboxy-terminal Flag tag. To construct the vectors for Tg mice with mutated EGFR, the backbone plasmid used was the pcDNA4 mutant EGFR-expressing vector described previously [8]. Surfactant protein A (SP-A) promoter was PCR amplified using the genomic DNA of H1299 cells as the template. The bovine growth hormone (BGH) poly-adenylation sequence was PCR amplified using the pCR3.1 vector as a template. The chicken β-globin insulator sequence was kindly provided by Dr. Felsenfeld (National Institute of Diabetes and Digestive and Kidney Disease, MD, USA) [30]. All the PCR-derived DNA fragments inserted into the Tg vector were sequence-verified. The sequences of the oligonucleotides used in this study were listed in the Table 1.

2.3. Establishment of Tg mice with mutant EGFRs

The 8.9 kilo-based Tg cassette was released from the construction vector between Sca I and Pme I sites for mouse pronuclear injections. The micro-injections of C57BL/6 fertilized eggs were performed in the Level Biotechnology Inc. (Taipei, Taiwan). Successful injections were verified by positive identification of the human EGFR gene in the Tg mouse tail DNA through Southern blot analyses and PCR genome-typing. Mutant EGFR-Tg mice were sacrificed at indicated ages and were examined for the presence of any tumor-like lesions. Portions of the lung tissues, either with or without tumor formation, were snap frozen and stored at −80 °C deep freezer for future DNA, RNA and protein extractions. The remaining lung tissues were fixed in neutral formalin for paraffin section preparations. All experimental procedures using animals were approved by the Institutional Animal Care and Use Committees of National Health Research Institutes (NHRI).

2.4. Patient materials

Fresh frozen tumor and paired non-tumor lung tissue specimens of 23 LAC patients receiving surgical resection at Chang Gung Memorial Hospital (CGMH) and with signed informed consent were obtained from the tissue bank of CGMH. All of the specimens were snap frozen soon after resection and stored at −80 °C. Only tumor tissues with higher than 70% tumor content were included for this study. The specimens were used for protein extraction and immunoblot analysis to examine the expression of EGFR and CD82 in these specimens. The study protocol has been reviewed and approved by the Institutional Review Boards of CGMH and NHRI.

2.5. RNA extraction and complementary DNA (cDNA) synthesis for quantitative polymerase chain reaction (Q-PCR)

Fresh frozen tumor and paired non-tumor lung tissues from Tg mice, control non-Tg mice, or LAC patients were used as starting materials for

Table 1
DNA and RNA oligonucleotides used in this study.

No.	Name	Oligonucleotides sequences
1	CD82 cloning	5′-GCGAAGCTTACCATGGGCTCAGCCTGTATCAAAGTCAC & 5′-GCGCTCGAGATGTACTTGGGGACCTTGCTGTAG
2	SP-A promoter	5′-GCGCACGCGTGGACACTATTGGGGCATTGGGTAC & 5′-GCGAAGCTTCAGAGCCTCCAGCTGCTTGGGTCTC
3	BGH poly A seq	5′-GCCGCTAGCTCAGCCTCGACTGTGCC & 5′-GCGTCTAGACTCAGAAGCCATAGAGCC
4	Insulator I	5′-CGCGGGCCCGGGACAGCCCCCCCCCAAAG & 5′-GCGTCTAGAGACTCCGTCCTGGAGTTGG
5	Insulator II	5′-GGTCCGCGGGGGACAGCCCCCCCCCAAAG & 5′-CGCGGGCCCGACTCCGTCCTGGAGTTGG
6	CD82 qRT-PCR	5′-GTGAGGAAGGGCTTCTGCGAG & 5′-GTACTTGGGGACCTTGCTGTAG
7	GAPDH qRT-PCR	5′-CGGAGTCAACGGATTTGGTCGTAT-3′ & 5′-AGCCTTCTCCATGGTGGTGAAGAC-3′
8	Actin qRT-PCR	5′-CCTGGCACCCAGCACAAT & 5′-TCCTGCTTGCTGATCCACATC
9	CD82 siRNA	5′-GUGUAUCAAAGUCACCAAA & 5′-UUUGGUGACUUUGAUACAG

total RNA extraction and cDNA synthesis as previously described [31]. One microliter of the resulting cDNA was used for further PCR reactions. Q-PCR was performed using LightCycler FastStart DNA Masterplus SYBR Green I kit (Roche Molecular Biochemicals, Indianapolis, IN) in the LightCycler instrument with a total volume of 20 μl in the LightCycler glass capillaries. The reaction program was: initial heating to 95 °C for 10 min followed by 29 PCR cycles of heating to 95 °C for 10 s, incubation for 10 s at the annealing temperature specific for the use of primer, and incubation for 25 sections at 72 °C. A melting curve analysis was conducted after every Q-PCR to identify the PCR product and to detect the possible presence of contaminating products. Each sample was normalized on the basis of its β-actin mRNA content. The relative quantification was determined by using the comparative CT method.

2.6. Tissue protein extraction

The tissue proteins were prepared by homogenizing the tissues in lysis buffer (50 mM Tris [pH 8.0], 150 mM NaCl, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS, 2 μg/ml leupeptin, 5 μg/ml aprotinin, 1 mM PMSF, 1 mM dithiothreitol, and 1 mM Na_3VO_4) using the MagNA Lyser Green Beads protocol (Roche Diagnostics, Indianapolis, IN) and stored in −80 °C for further characterization.

2.7. Antibodies and reagents

The anti-EGFR, EGFR-L858R, EGFR-Del, and phospho-EGFR (pEGFR; Y1068 and Y1045) antibodies were purchased from Cell Signaling Technology (Beverly, MA). The anti-CD82, anti-GAPDH, and anti-GAK antibodies were purchased from AbCam (Cambridge, UK). The anti-c-Cbl and peroxidase-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-Flotillin 1 and gp78 antibodies were purchased from GeneTex, Inc. (Irvine, CA). EGF was purchased from Sigma-RBI (Natick, MA). The anti-β-actin antibody, small interference RNA (siRNA) against CD82, and other chemical inhibitors were purchased from Sigma-Aldrich (St. Louis, MO). Gefitinib was provided by the Institute of Biotechnology and Pharmaceutical Research, NHRI, Taiwan.

2.8. Cell extract, exosome fraction preparation, and immunoblot analyses

Cell extract was prepared as described previously [32]. Exosome preparation was performed by differential ultra-centrifugation as previously described [33]. Twenty-four hours before harvesting the samples (cells and conditioned media), the cells were fed with fresh culture medium. The exosome-enriched fractions from the conditioned media were recovered with 70,000 *g* centrifugation and were re-suspended in the lysis buffer. Immunoblot analysis was performed as described previously [8]. For the paired comparison of cellular and exosome proteins, 50 μg of any individual cellular extract was subjected to analyses. The amount of exosome proteins to be examined was adjusted according to the proportion of the corresponding cellular extract used in the same analysis. The relative ratio (in term of recovered protein) of paired exosome/cell samples subjected to immunoblot analyses is 3:1 to reveal the less abundant proteins in the exosome fractions.

2.9. Immuno-histochemical (IHC) stain

IHC stains were performed for EGFR, EGFR-L858R and EGFR-Del and SP-C on the Tg mice lung tumor tissue on a formalin fixed paraffin section. The detailed procedures were the same as previously described [31]. The dilutions for the antibodies were: 1:150 for EGFR, EGFR-L858R and EGFR-Del, respectively and 1:1000 for SP-C.

3. Results

3.1. Wild type and mutated EGFRs have opposite effects on CD82 expression in H1299 cells

Previously we have established NSCLC H1299 cell lines permanently expressing WT EGFR (H1299-EGFR), L858R mutant (H1299-L858R), or exon 19 deletion mutant (H1299-Del) [8]. We found that c-Cbl and GAK were slightly increased in H1299-L858R and H1299-Del cells in comparison with H1299-EGFR cells and H1299-Vec (vector control). Interestingly, the expression of CD82 was markedly increased in H1299-EGFR cells, but decreased in H1299-L858R and H1299-Del cells in comparison with H1299-Vec (Fig. 1a). CD82 mRNA expression levels varied but were comparable among H1299 cell derivatives (Fig. 1b). The results indicated that the differential CD82 expression could be controlled at the post-transcriptional level. Gefitinib, an EGFR kinase inhibitor, did not reverse the CD82 expression patterns (Fig. 1c), indicating that the up- and down-regulations of CD82 in H1299 derivatives were not directly caused by aberrant signaling immediately downstream of mutant EGFRs.

3.2. NSCLC cells harboring EGFR mutation also show low levels of CD82

CD82 expression was examined in NSCLC cells with endogenous EGFR mutations. In comparison to H1299 cells (WT EGFR), H1975 (L858R/T790M double mutations), H1650 (exon 19 Del), PE089 (exon 19 Del), and HCC827 (exon 19 Del) all showed lower levels of CD82 expression (Fig. 1d). H1975, PE089, and HCC827 cells also had lower CD82 mRNA expression, but H1650 cells had high CD82 mRNA expression (Fig. 1e). Since CD82 expression is subjected to epigenetic regulations in myeloma cell lines [34], we have treated NSCLC cells with 5-aza-deoxy-cytidine (5-aza-dC), a DNA demethylation agent, which did not affect CD82 protein expression (Fig. 1f). Trichostatin (TsA), a histone deacetylase inhibitor, was also used to treat NSCLC cells. TsA caused slightly increased CD82 expression in H1975 cells but not in other cells (Fig. 1f). The above data indicated that CD82 expression in these NSCLC cells was mainly controlled by mechanisms other than epigenetic regulation.

3.3. Suppression of CD82 promotes EGFR expression in H1299 cells

The up-regulation of CD82 by WT EGFR was intriguing (Fig. 1a). H1299-vector and H1299-EGFR cells were both treated with or without a small interference RNA against CD82 (siCD82). CD82 knockdown increased the expression of EGFR in H1299-vector cells and partially prevented the down-regulation of EGFR upon EGF stimulation (Fig. 2a). Because of the over-expression of EGFR, ligand-induced EGFR down-regulation was less evident in H1299-EGFR cells. But CD82 knockdown in H1299-EGFR cells also alleviated ligand-induced EGFR down-regulation and retained stronger EGFR phosphorylation (Fig. 2a). This result indicated that CD82 was functional in down-regulation of EGFR signaling in both H1299-vector and H1299-EGFR cells.

3.4. Reconstitution of CD82 reduces mutant EGFR expression in H1299 cells

Low CD82 levels in NSCLC cells expressing mutant EGFR suggested that lacking CD82 might be beneficial to mutant EGFR-induced cell growth. We reconstituted CD82 expression in H1299-L858R or H1299-Del cells and found that CD82 expression significantly decreased the protein and phosphorylation levels of mutant EGFRs (Fig. 2b). In addition, CD82 greatly decreased the expression of mutant EGFRs, but the suppressive effect was less evident on the WT receptor (Fig. 2c). These results indicated that mutant EGFRs were more sensitive to the CD82-mediated negative regulation. Gefitinib treatment reduced the phosphorylation of EGFRs but did not reverse the down-regulation of WT

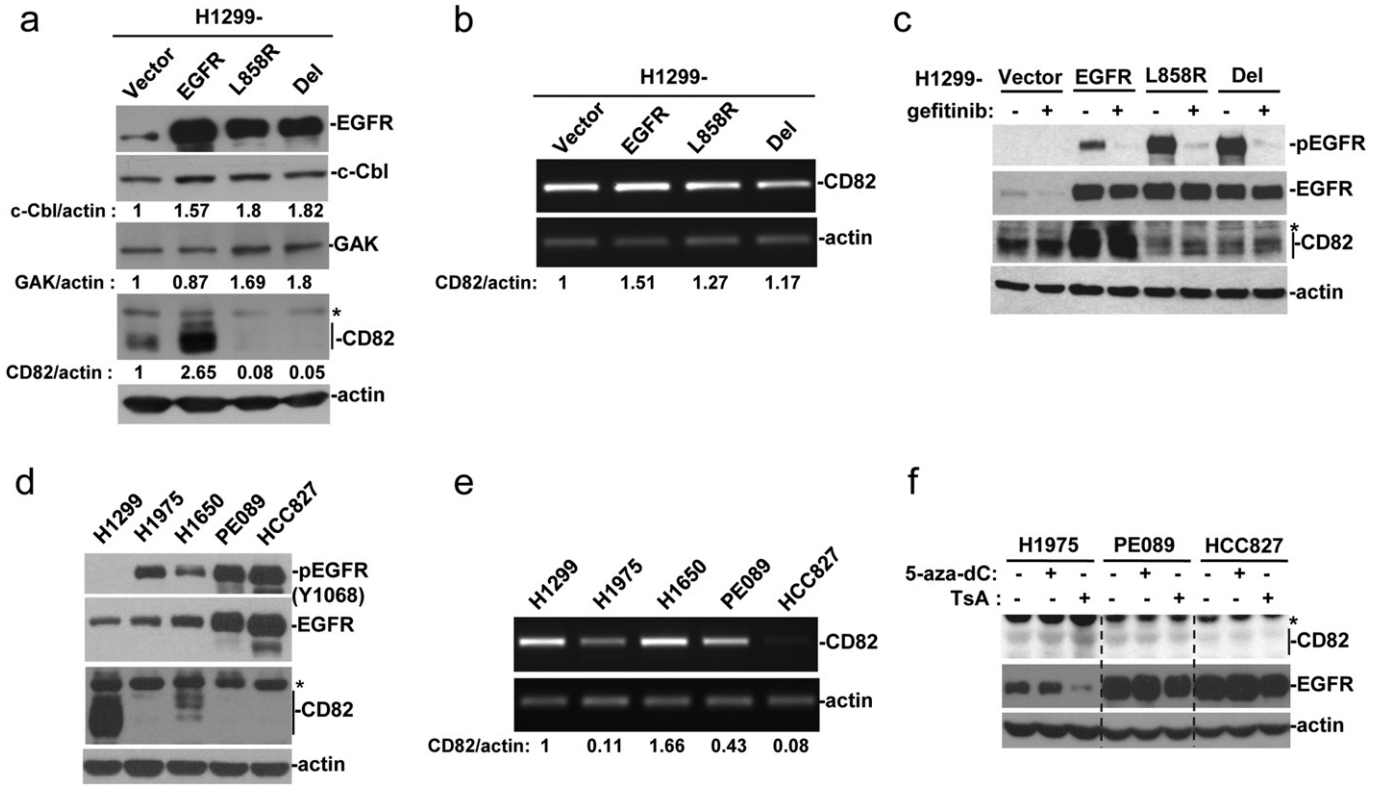

Fig. 1. CD82 expression levels are decreased in NSCLC cells harboring mutant EGFRs. (a and d) Immunoblot analyses for c-Cbl, GAK and CD82 expression levels in the cell extracts from the regularly cultured H1299 derivatives (panel a), and from the 5 NSCLC cells (panel d). (b and e) The gel electrophoresis assays presented the CD82 and actin mRNA levels by RT-PCR in the H1299 derivatives (panel b) and the 5 NSCLC cells (panel e). The CD82/actin mRNA ratios were calculated from the results of quantitative RT-PCR. (c) Immunoblot analyses for the expression levels of phosphorylated EGFR (pEGFR), EGFR and CD82 in the cell extracts of regularly cultured H1299 derivatives (with or without gefitinib treatment for 24 h). (f) Immunoblot analyses for the expression levels of EGFR and CD82 in the cell extracts prepared from the 5 NSCLC cells treated with 5-aza-dC (10 μM, 72 h) or TsA (100 nM, 24 h). The asterisks indicated a cross-reactive protein recognized by the anti-CD82 antibody.

or mutant EGFR by CD82, suggesting that the CD82-mediated negative regulation is independent of the kinase phosphorylation (Fig. 2c).

3.5. Gp78/proteasome and lysosome-mediated degradation pathways do not participate in the CD82 down-regulation in mutant EGFR-expressing NSCLC cells

We then examined the potential mechanisms for CD82 down-regulation in mutant EGFR-expressing NSCLC cells. E3 ubiquitin ligase gp78 mediates CD82 ubiquitination and degradation [35]. We found that gp78 expression was not increased in NSCLC cells expressing mutant EGFR in comparison with H1299 cells (Fig. 3a). Moreover, treatment of MG132, a proteasome inhibitor, did not increase CD82 expression in H1650 cells and H1299 derivatives (Fig. 3b–c). Inhibiting protein transport from the endoplasmic reticulum to the Golgi apparatus with brefeldin A apparently reduced the levels of both EGFR and CD82. The addition of MG132 restored EGFR expression but had little effect on CD82 expression (Fig. 3d). Additionally, we found that chloroquine, a

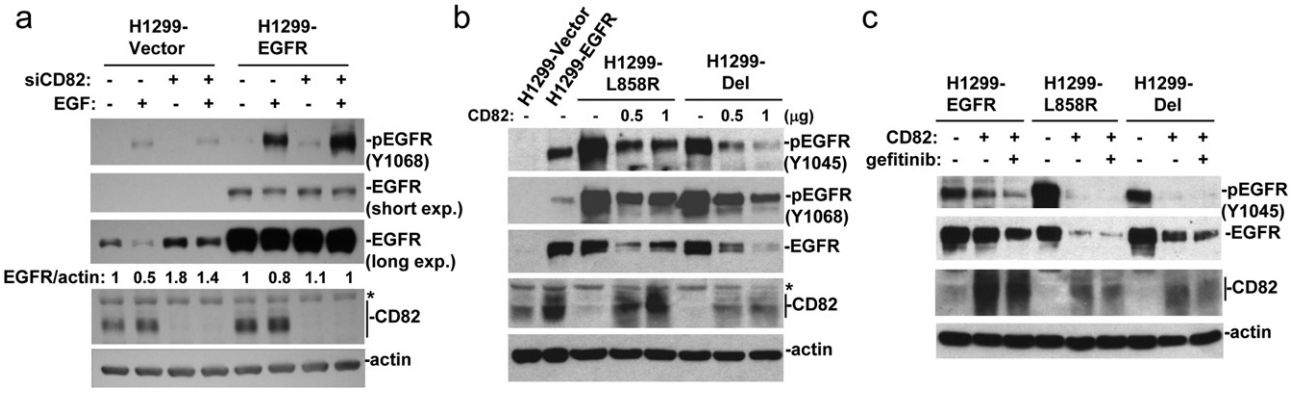

Fig. 2. Suppression of CD82 increases EGFR expression and reconstitution of CD82 efficiently suppresses mutant EGFR expression. (a) H1299-vector and H1299-EGFR cells were transfected with control siRNA or CD82 siRNA (sicontrol and siCD82). The transfected cells were then treated with or without EGF (10 ng/ml) for 10 min. The levels of indicated proteins (or phosphorylation) of these samples were determined by immunoblot analyses. (b) H1299-L858R and H1299-Del cells were transfected with a CD82-expressing vector, and the levels of the indicated proteins in the transfected cells and control H1299 derivatives were examined by immunoblot analyses. (c) H1299-EGFR, H1299-L858R, and H1299-Del cells were transfected with the CD82 expressing vector. The transfected cells were treated with or without gefitinib (2 μM) 8 h before the cell harvests. The levels of indicated proteins (or phosphorylation) in these samples were determined by immunoblot analyses.

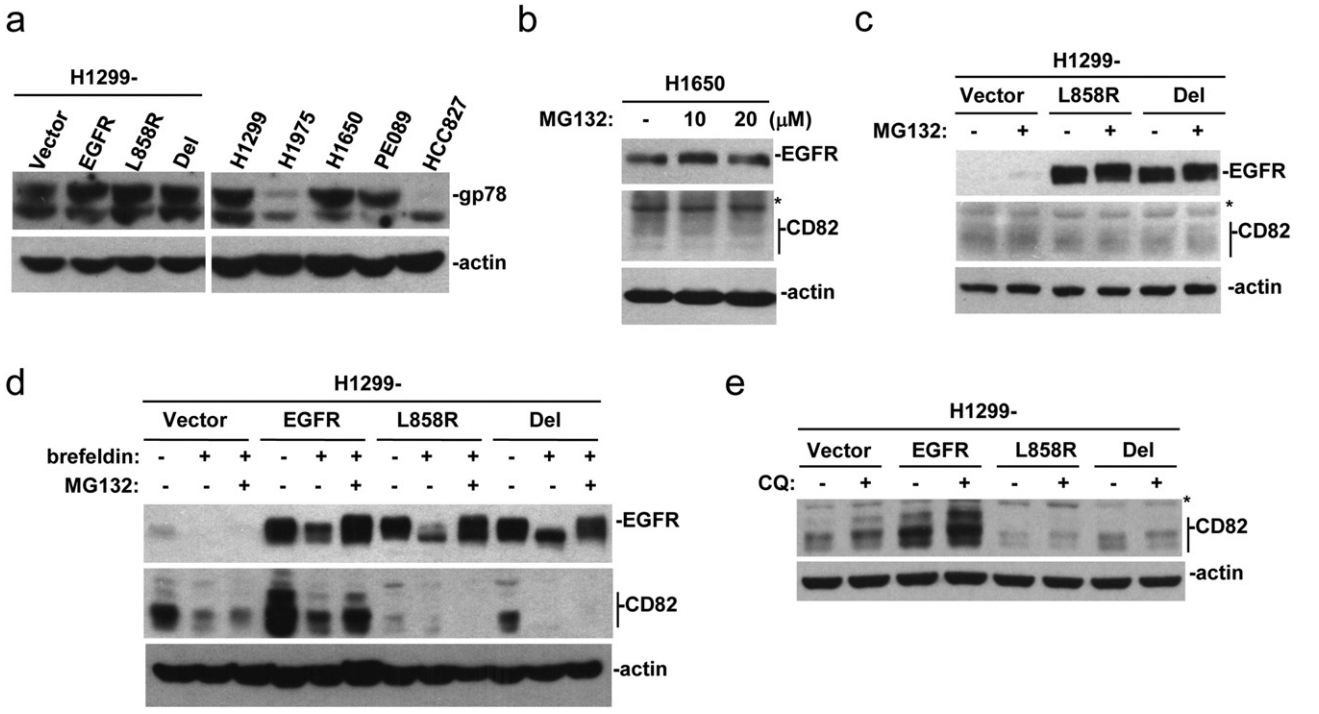

Fig. 3. Gp78/proteasome and lysosome-mediated protein degradation are not involved in the differential regulation of CD82 in NSCLC. (a) Expression levels of gp78 and actin were examined in cell extracts prepared from regularly cultured H1299 derivatives or from NSCLC cell lines by immunoblot analyses. (b and c) Expression levels of CD82 and actin were examined in cell extracts prepared from regularly cultured H1299 derivatives or from NSCLC cell lines treated with or without MG132 (20 M, 16 h). (d) Expression levels of EGFR, CD82, and actin were examined in cell extracts prepared from H1299 derivatives treated with or without brefeldin A (10 M) and MG132 (20 M) for 16 h. (e) Expression levels of CD82 and actin were examined in cell extracts prepared from H1299 derivatives treated with or without chloroquine (CQ, 100 M) for 16 h.

lysosome activity inhibitor, increased the levels of CD82 in H1299-Vec and H1299-EGFR cells but failed to increase the expression of CD82 in H1299-L858R and H1299-Del cells (Fig. 3e). These data indicated that both gp78/proteasome and lysosome-mediated degradation pathways were not participated in the CD82 down-regulation in mutant EGFR-expressing NSCLC cells.

3.6. NSCLC cells expressing mutant EGFR show increased CD82 exportation through the exosome pathway

CD82 is present in the exosomes [33]. We found that secreted exosome CD82 levels in H1299-EGFR, H1299-L858R, and H1299-Del cell cultures were higher than that of H1299-vector cells (Fig. 4a). The high exosome/cell CD82 ratios in H1299-L858R and H1299-Del cells indicated that increased exosome exportation might have caused the low levels of cellular CD82. EGFR, actin, and Flotillin 1 (Flot 1, a protein exists in exosomes) [36] levels were all increased in the secreted exosome fractions prepared from H1299-EGFR, H1299-L858R, and H1299-Del cell cultures, when compared with H1299-vector cells. Interestingly, heat shock protein 90 (HSP90, a cytoplasmic chaperon molecule) was nearly undetectable in the exosome fractions, indicating that the exportation of proteins through the exosome is a selective process (Fig. 4b). Based on the exosome/cell protein ratios, CD82 was exported most effectively among all the examined proteins.

We also examined CD82 exportation in NSCLC cell lines with endogenous EGFR mutation and low cellular CD82 expression. The presence of CD82 in exosomes secreted from H1650 cells indicated the active exportation of this protein (Fig. 4c). Additionally, CD82 was better detected in the exosomes but not in the cellular fraction of H1975 cells. This phenomenon was more evident in the TsA-treated H1975 cells (Fig. 4d). These data indicated that despite the low expression of CD82 protein in H1650 and H1975 cells, the residual CD82 was actively exported through the exosome pathway.

3.7. Transgenic (Tg) mice with surfactant protein A (SP-A) promoter-driven mutant EGFR expression develop LAC

Tg mice preferentially expressing EGFR-L858R or exon 19 deletion mutant in lung tissues through the SP-A promoter were generated (Fig. 5a–b). Four lines of Tg mice (LR-D and LR-E from L858R mutant, Del-A and Del-C from exon 19 deletion mutant, respectively) with different mutant EGFR expression levels were selected for further characterization (Fig. 5c). Mouse lines (LR-D and Del-A) with higher Tg EGFR expression levels also had higher incidence of tumor development (Table 2). Histopathology examination confirmed that all tumors were adenocarcinomas (Fig. 5d). Tumors developed in the lungs of mutant EGFR-Tg mice were positive in SP-C expression, indicating their type II lung alveolar cell origin (Fig. 5d). The correlation between tumor incidence and EGFR mRNA levels suggested that a threshold level of mutant EGFR expression might be essential for the development of lung cancers in Tg mice.

3.8. EGFR over-expression associated with CD82 down-regulation in the tumors of mutant EGFR-Tg mice

We found that the mutant EGFRs in Tg mice were expressed initially (6-week of age) at levels similar to, or lower than the endogenous mouse EGFR in age/gender-matched non-Tg control mice (Fig. 6a). The levels of CD82 and pEGFR were also not evidently different between mutant EGFR-Tg and non-Tg control mice (Fig. 6a). Then we examined the EGFR expression in tumor tissues from 18-month old Tg mice and normal lung tissues from age/gender-matched non-Tg control mice, respectively. We found that tumor tissues from Del-A and LR-D all expressed much higher levels of EGFR in comparison with the lung tissues of the control mice (Fig. 6b–c). To confirm this observation, we examined the expression of mutant EGFRs by immuno-histochemical (IHC) staining using the EGFR-L858R or exon 19 Del mutant-specific

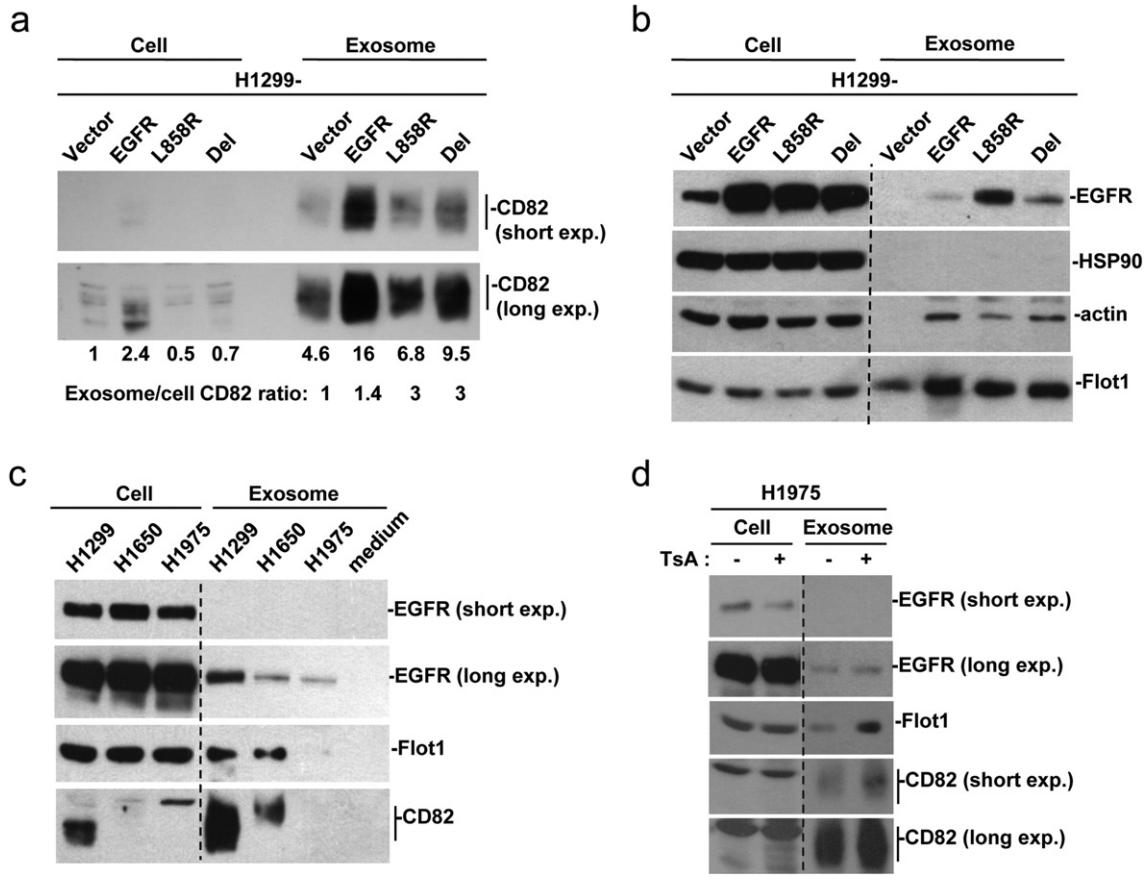

Fig. 4. Mutant EGFR-harboring NSCLC cells export CD82 through exosomes excessively. (a and b) Cellular and exosome fractions of H1299 derivatives were prepared as described in the Material and methods. The proteins of interest were examined by immunoblot analyses. The intensity of protein signals was determined and presented as numerical numbers, setting the level of H1299-vector as 1. The relative exosome/cell (E/C) CD82 ratios were calculated, taking the loading proportions of exosome/cell fractions into account. (c) The proteins of interest in the cellular extracts and exosome samples of H1299, H1650, and H1975 cells were examined by immunoblot analyses. (d) H1975 cells were treated with or without TsA (100 nM) for 24 h. The proteins of interest in the cellular and exosome fractions of treated cells were examined by immunoblot analyses. The ratio of corresponding exosome/cell samples subjected to the analyses is 3:1.

antibody. The IHC studies revealed that majority of the tumors developed in Tg mice, 85% (34/40) in Del-A (Fig. 6d) and 89% (58/65) in LR-D mice (Fig. 6e), showed over-expression of mutant EGFR when compared with the adjacent lung tissues which also harbored the mutant EGFR gene. The specificity of the EGFR-L858R or exon 19 Del mutant-specific antibody had been verified by immunoblotting (Fig. 7a–b).

In addition, tumor tissues from mutant EGFR-Tg mice also had lower levels of CD82 in comparison with the normal lung tissues (Fig. 6b–c). Moreover, we found that c-Cbl, but not GAK, expression levels were decreased in tumor tissues of Tg mice (Fig. 8a–b). Consistent with this observation, c-Cbl expression levels were also lower in NSCLC cell lines with EGFR mutation (Fig. 8c). The collective data showed that mutant EGFR over-expression, associated with the loss of EGFR negative regulators such as CD82 and c-Cbl, frequently occurred during the development of mutant EGFR-driven LAC.

3.9. EGFR over-expression associates with CD82 down-regulation in human LAC

Immunoblot analyses for EGFR and CD82 on the paired tumor and non-tumor lung tissues from 23 LAC patients (12 with WT EGFR, and 11 with mutant EGFRs) were performed. Representative cases are shown for patients with WT EGFR (Fig. 9a) and mutant EGFRs (Fig. 9b). The expression levels of EGFR and CD82 in the tumor tissue (T) compared with the paired non-tumor lung tissue (N) were counted as T/N ratio according to the immunoblot study. The T/N ratio data is shown in Table 3. Similar to the tumors of the mutant Tg mice, for the 11 human LAC tumors with mutant EGFRs, all of them (100%) had EGFR over-expression and marked down-regulation of CD82. Only 3 tumors had CD82 T/N ratios higher than 0.3. For the 12 LAC tumors with WT EGFR, although most of the tumors also had EGFR over-expression (10/12, 83.3%) and CD82 down-regulation (9/12, 75%), the extent of EGFR over-expression and CD82 down-regulation was much weaker, with no tumor having a CD82 T/N ratio lower than 0.3. This difference in CD82 down-regulation was statistically significant ($p = 0.0003$) between tumors with WT and mutated EGFR. When combining the T/N ratio data of EGFR and CD82 in a scatter plot figure (Fig. 9c), it clearly demonstrates the separation of these two groups of patients, with only a small number of overlapping cases.

4. Discussion

Mutations at the EGFR kinase domain promote EGFR tyrosine phosphorylation in the absence of ligand stimulation [8,9]. Phosphorylation of WT EGFR leads to receptor internalization, ubiquitination, and degradation [1]. However, hyper-phosphorylated mutant EGFRs are equally or more stable in comparison with the WT receptor [8,10,11]. CD82, c-Cbl, and GAK are molecules involved in EGFR down-regulation [13,14,25,27]. Our results showed that c-Cbl and CD82, but not GAK, were expressed at lower levels in NSCLC cells harboring EGFR mutations and in lung cancer tissues of mutant EGFR Tg mice. Many other molecules also participate in EGFR down-regulation. Breast tumor kinase (BRK, also named PTK6) inhibits EGFR down-regulation [37], partially

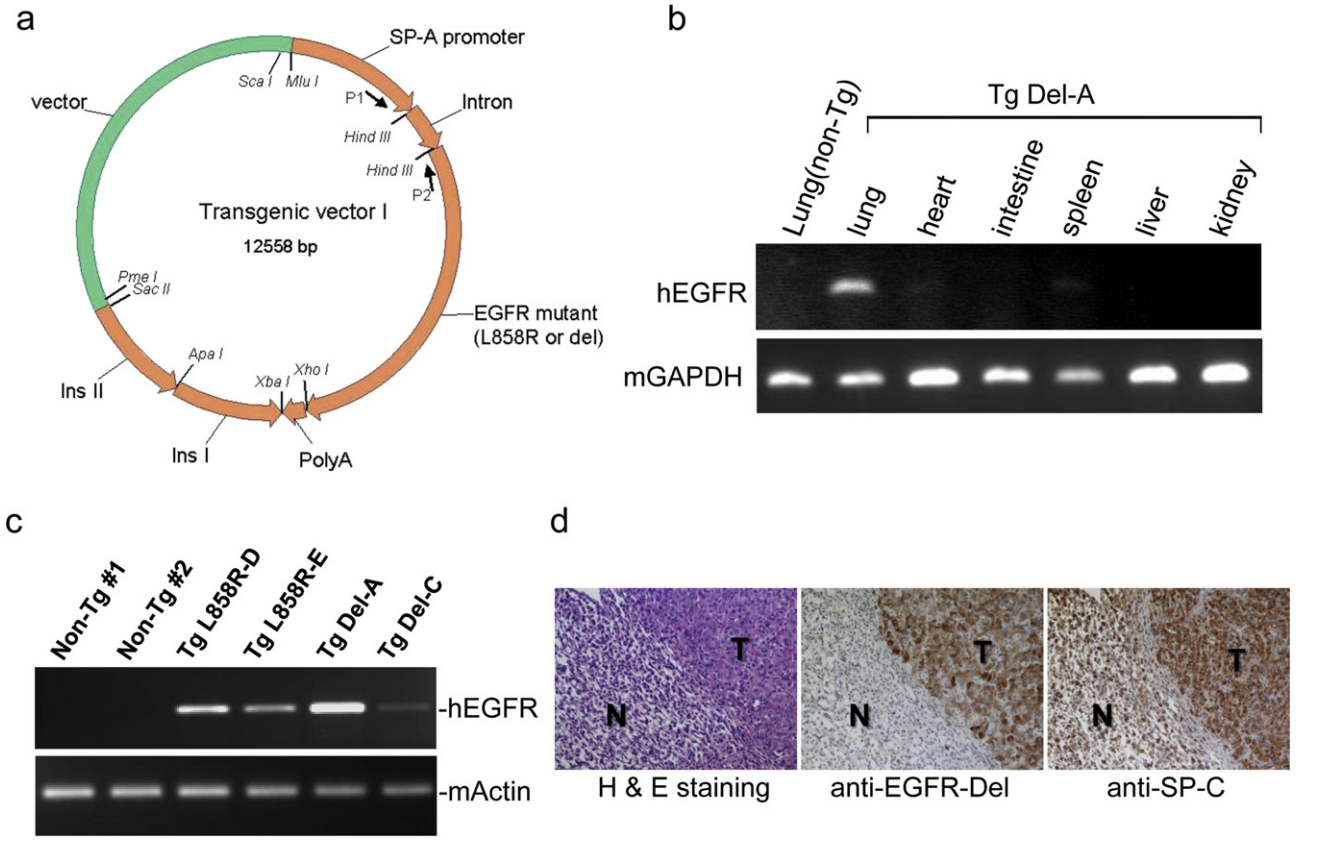

Fig. 5. SP-A promoter-driven mutant EGFR expression induces lung cancer in Tg mice. (a) A schematic illustration of the construction of the SP-A-driven mutant EGFR Tg vectors. (b) The expression of Tg EGFR mRNA was examined by RT-PCR in RNA samples isolated from major visceral organs of Tg and from control mice. (c) The expression of Tg EGFR mRNA was examined by RT-PCR in RNA samples isolated from the lung tissues of indicated mouse lines. (d) The expression of surfactant protein C (SP-C) or EGFR-Del in tumor (T) and adjacent normal tissue (N) was examined by IHC in the consecutive lung sections from a Del-A Tg mouse.

through phosphorylating ARAP1 (Arf-GAP, Rho-GAP, ankyrin repeat, and pleckstrin homology domain-containing protein), a molecule that prevents EGFR degradation [38,39]. Caveolin-1 has also been shown to affect EGFR down-regulation [27]. Nevertheless, we did not observed a significant difference in BRK and caveolin expression among NSCLC cell lines with or without EGFR mutations (data not shown). Therefore, we did not include these 2 molecules in the current study.

In previous studies, the stability of mutant EGFRs is attributed to their resistance to c-Cbl-mediated ubiquitination and degradation [10,11]. Our results suggested that reduction of CD82 and/or c-Cbl may have also contributed to the stabilization of mutant EGFRs. Over-expression of WT EGFR increased CD82 level and over-expression of mutated EGFRs decreased it. This feature may contribute to the reported different oncogenicity between WT and mutant EGFRs [12]. In the 23 human LAC tumors we have examined, majority of tumors with WT EGFR still had CD82 expression levels lower than those in adjacent non-tumor lung tissues; however, the T/N ratio of CD82 was significantly higher in tumors with WT EGFR than those with EGFR mutation ($p = 0.0003$). This result is consistent with our data from NSCLC cell lines and Tg mice. Since human LAC tumors have a heterogeneous genetic background and involved multiple oncogenic pathways, it could explain why not all human LAC tumors with WT EGFR showed CD82 over-expression.

Table 2
Lung adenocarcinoma in non-transgenic and EGFR mutant-transgenic mice.

Age	6 months	12 months	18 months	24 months
Mouse line	Tumor incidence (%)			
Non-Tg	1/14	0/16	1/18	2/16
L858R-D	nd	6/22	8/15	13/14
L858R-E	nd	1/21	2/25	3/16
Del-A	5/14	13/18	10/10	nd
Del-C	nd	1/22	5/22	8/21

Abbreviations: non-Tg, non-transgenic; nd, not determined.

CD82 level is decreased in various types of cancer and its expression is regulated at multiple levels [15,17,18]. Three NSCLC cell lines (H1975, PE089, and HCC827) with low CD82 protein expression also showed low CD82 mRNA levels. TsA increased CD82 expression in H1975 cells, suggesting that histone acetylation-mediated chromatin remodeling participated in regulating CD82 gene expression in this cell line. H1650 cells showed low CD82 protein expression with an mRNA level higher than that of H1299 cells, suggesting the existence of post-transcriptional regulatory mechanisms. We found that H1299-L858R and H1299-Del cells exported higher proportions of CD82 through exosomes than did H1299-vector and H1299-EGFR cells. Additionally, H1650 and H1975 cells exported CD82 excessively through exosomes despite their low cellular CD82 levels. These results indicated that the ability to actively export CD82 protein is a common mechanism in cells with mutated EGFR regardless of their overall CD82 expression levels. However, increasing exportation of CD82 may also facilitate the exportation of CD82-interacting molecules, such as receptor tyrosine kinases and integrin-associated molecules. The exosome has been suggested to play a role in eliciting immune responses against tumors [40]. Reduction of potential tumor antigen exportation through silencing CD82 gene expression could be the ultimately selected mechanism during cancer development.

In this study, Tg mice at 6 weeks of age expressed mutant EGFR at levels similar to, or even lower than, the age/gender matched control mice (the endogenous mouse EGFR). But by the time of tumor

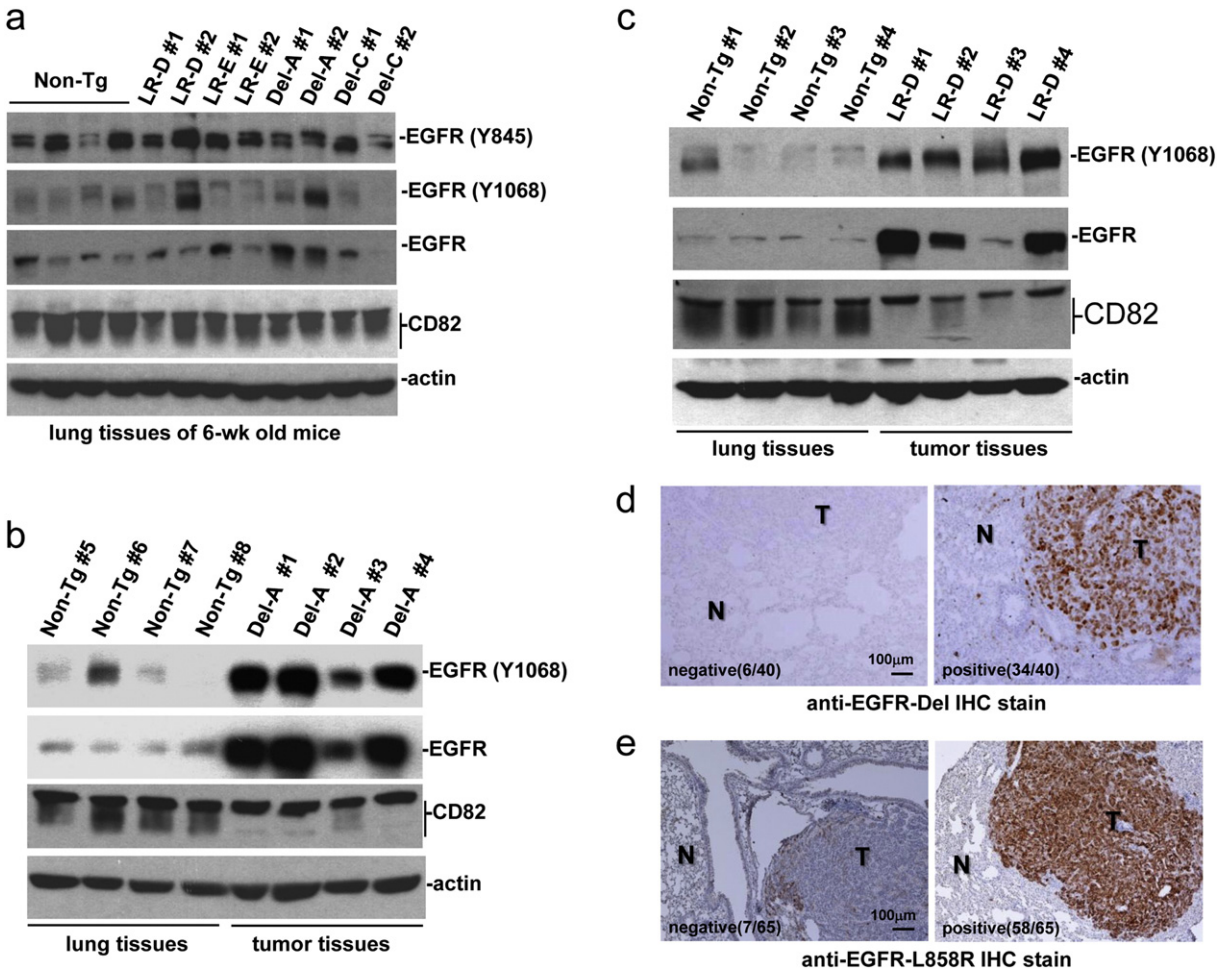

Fig. 6. Over-expression of EGFR is associated with down-regulation of CD82 in lung cancer tissues in mutant EGFR Tg mice. (a) Lung tissue extracts from 6-wk old control (non-Tg) mice or mutant EGFR-Tg mice were analyzed for EGFR, CD82 and pEGFR expression by immunoblot analyses. (b and c) The same immunoblot analyses were also performed for lung tumor tissues from 1.5-year old, male Del-A Tg mice and 1.5-year old, male LR-D Tg mice and compared with the lung tissues from age- and gender-matched control mice. (d) Immunohistochemical stain demonstrated that 85% (34/40) of the lung tumor tissues from Del-A Tg mice had over-expression of mutant EGFR-Del protein (right), and only 6 Tg mice were negative (left) (200×). (e) Immunohistochemical stain demonstrated that 89% (58/65) of the lung tumor tissues from LR-D Tg mice had over-expression of mutant EGFR-L858R protein (right), and only 7 Tg mice were negative (left) (200×).

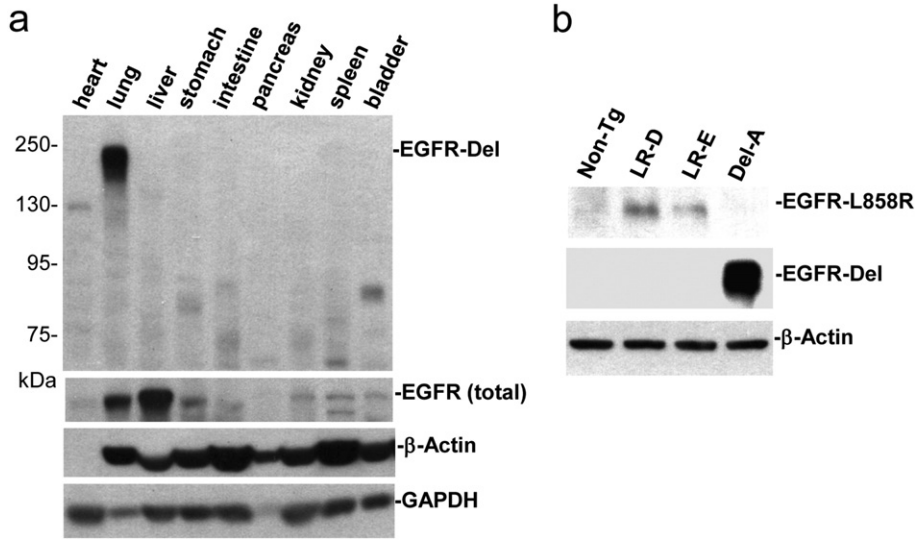

Fig. 7. Tg EGFR is over-expressed in tumor tissues. (a) The expression of Tg EGFR-Del was examined by immunoblotting using a EGFR-Del-specific antibody. The protein samples were isolated from the major visceral organs of a Del-A Tg mouse. (b) The lung tissue extracts from control, L858R-D, L858R-E, and Del-A mice were examined by immunoblotting using an EGFR-L858R- or EGFR-Del-specific antibody.

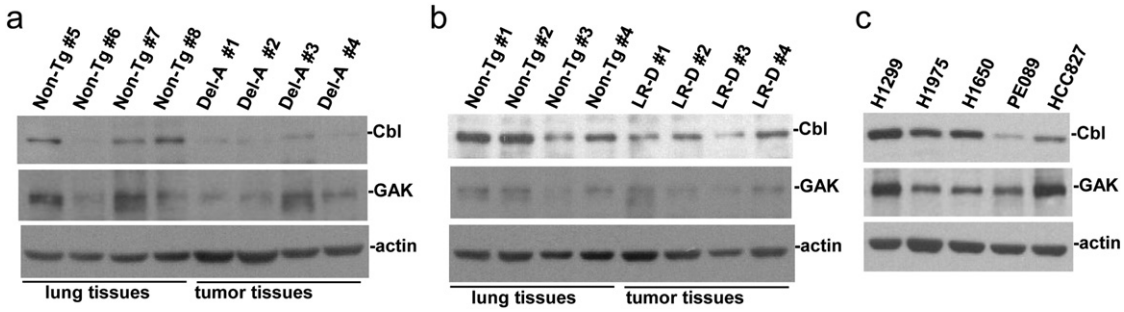

Fig. 8. Cbl expression is decreased in mutant EGFR-containing cell lines and lung cancers. (a and b) The same panels of tissue extracts used in Fig. 4b and c were subjected to immunoblot analyses using the indicated antibodies. (c) Expression levels of c-Cbl, GAK, and actin were examined in cell extracts, which were prepared from NSCLC cell lines, by immunoblot analyses.

development in Tg mice, majority of the tumors showed over-expression of mutant EGFRs. The late development of LAC in these animals (after 6 months of age) suggested that other synergistic mechanisms were involved in the tumorigenic process. Decreasing CD82 expression could be one of the mechanisms that promoted mutant EGFR over-expression in Tg mice. We also found that CD82 showed better suppressive effects on mutant EGFR expression than on WT EGFR. Therefore, down-regulation of CD82 could be a critical step in EGFR mutation-induced lung cancer. The study on human LAC tumor tissues from patients with or without EGFR mutation also demonstrated a stronger down-regulation of CD82 in patients with mutant EGFRs. The above data is supportive for our assumption that reduction of CD82 was associated with over-expression of EGFR protein in lung cancer triggered by mutant EGFR. We have demonstrated that despite the low expression of CD82 protein in H1650 and H1975 cells, the residual CD82 was still actively exported through the exosome pathway. Thus, for assessment of CD82 expression, the level of CD82 in serum exosomes may not be similar to the LAC tumor tissue.

5. Conclusion

The present study revealed that down-regulation of EGFR negative regulators, such as CD82, could be the mechanism facilitating the over-expression of EGFR in LAC with EGFR mutation. Since CD82 is also involved in regulating other receptor (or non-receptor) tyrosine kinases [15,17,18], the loss of CD82 may lead to the activation of multiple oncogenic pathways. Therefore, the down-regulation of CD82 could be a critical step involved in the stronger tumorigenic activity triggered by EGFR mutations. Up-regulation of the CD82 level may become a promising new treatment strategy for LAC. Whether CD82 down-regulation is involved in the drug resistance to EGFR inhibitors is also worthy of further study.

Conflict of interest

The authors declared no conflict of interest.

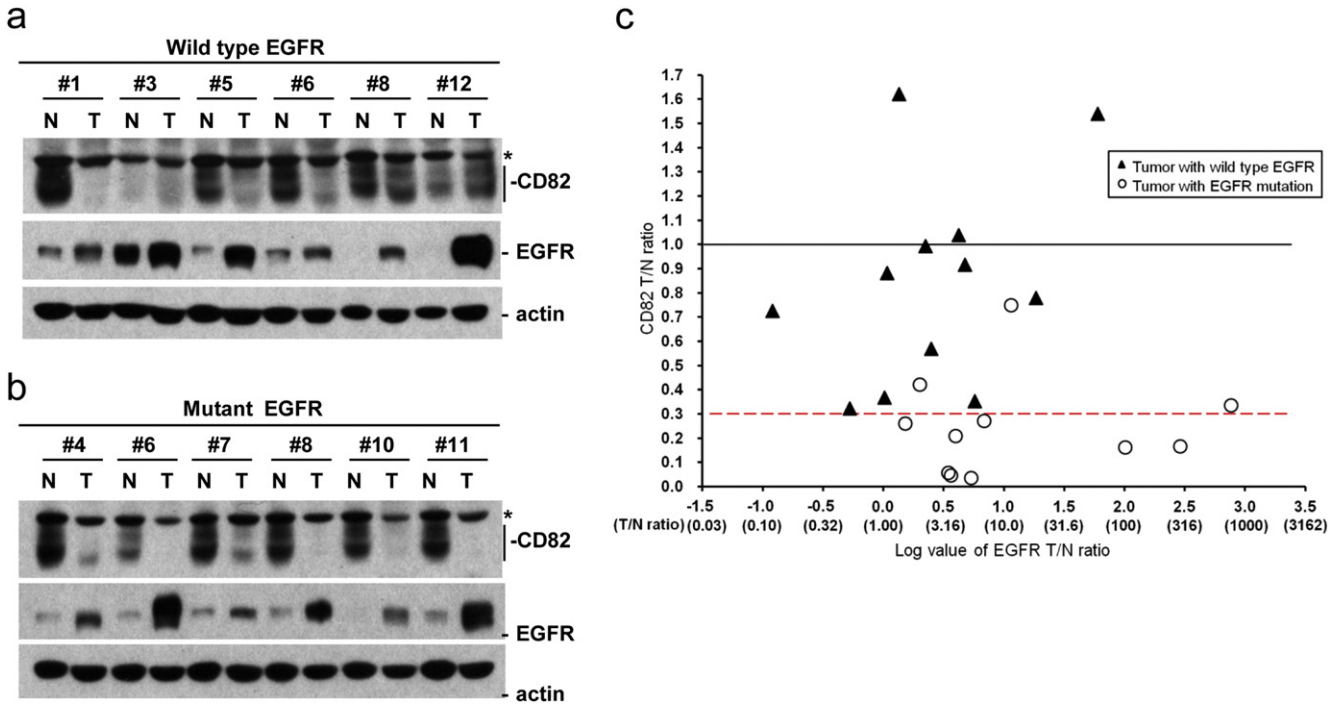

Fig. 9. EGFR over-expression associates with CD82 down-regulation in human lung adenocarcinomas. The tumor tissues (T) from 23 lung adenocarcinoma patients (12 with WT EGFR, and 11 with mutant EGFRs) and paired non-tumor lung tissues (N) were examined for EGFR and CD82 expression by immunoblot analyses. The expression levels of EGFR or CD82 was normalized by the actin levels of the individual samples. (a) Paired tumor and non-tumor tissue samples with WT EGFR are shown. (b) Paired tumor and non-tumor tissue samples with mutant EGFRs are shown. (c) The expression levels of EGFR and CD82 in the tumor tissue (T) compared with the paired non-tumor lung tissue (N) were counted as T/N ratio according to the immunoblot study and shown in the scatter plot figure.

Table 3
Expression of EGFR and CD82 in the 23 human lung adenocarcinoma tumor and paired non-tumor tissues.

WT EGFR case no.	CD82	EGFR	MT EGFR case no.	CD82	EGFR
	T/N ratio	T/N ratio		T/N ratio	T/N ratio
1	0.353	5.664	1	0.168	287.758
2	0.323	0.524	2	0.273	6.805
3	1.622	1.333	3	0.211	3.924
4	0.570	2.476	4	0.423	1.995
5	0.781	18.335	5	0.337	759.870
6	0.917	4.716	6	0.037	5.285
7	0.726	0.120	7	0.262	1.510
8	1.040	4.165	8	0.058	3.421
9	0.883	1.062	9	0.750	11.358
10	0.368	1.018	10	0.163	100.645
11	0.994	2.222	11	0.048	3.602
12	1.541	59.612			

T/N ratio: the ratio of the protein expression (determined by immunoblot) between tumor tissue and paired non-tumor tissue. WT, wild type; MT, mutant.

Transparency document

The Transparency document associated with this article can be found, in the online version.

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