博士論文

The role of a cell adhesion molecule, CADM1, in the internalization and degradation of EGFR

（細胞接着分子**CADM1**による**EGF**受容体の分解制御機構の解明）

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Abstract

Non-small cell lung cancer, NSCLC, is one of the most common human malignancies. Cell adhesion molecule 1, CADM1, has been identified as a tumor suppressor in NSCLC. Loss or reduced expression of CADM1 protein is often observed in advanced NSCLC similarly as overexpressed EGFR. Here, we investigated whether expression of CADM1 affected the degradation of EGFR in NSCLC cells. First, overexpression of CADM1 reduced the amount of EGFR in NSCLC cell lines, NCI-H1838, NCI-H1993, and A549 cells. Moreover, CADM1 and EGFR were accumulated in lysosome by the treatment with EGF and a lysosomal inhibitor, chloroquine. It indicates that CADM1 and EGFR are degraded through the same endocytosis pathway by EGF stimulation. Furthermore, we observed that CADM1 did not only facilitate internalization of EGFR by EGF stimulation but regulated the degradation of EGFR by EGF stimulation through a clathrin-dependent manner as well. These results suggest that CADM1 regulates the internalization and degradation of EGFR and contributes to tumor suppression.

Introduction

Lung cancer is one of the most common human malignancies in the world with more than 1.61 million new cases by the year 2008. It is also the major cause of cancer mortality, with 1.38 million deaths around the world [1]. Lung cancer can be typically classified into small cell lung cancer (SCLC, commonly associated with smoking) and non-small cell lung cancer (NSCLC) by distinctive biological features [2].NSCLC is further divided into adenocarcinoma, squamous cell carcinoma, and large-cell carcinoma [2-4], representing the majority group of lung cancers (85~90%). Treatments for patients with early phase NSCLC include surgery, chemotherapy, radiation therapy, and a combined modality approach. However, even after treatment there is a high rate of relapse [5]. In the past few decades, the understanding of the molecular mechanism behind lung cancers has progressed, which has provided novel aspects of potential therapy for lung cancer patients.

The activation of oncogene and/or inactivation of tumor suppressor genes often cause tumorigenesis. In NSCLC, genetic alterations of proto-oncogenes, such as *KRAS* mutations, *EGFR* mutations, *ALK* rearrangements, *HER2* mutation and *BRAF* mutation have been reported to play roles in tumor progression [6]. In addition, inactivation of tumor suppressors, such as *TP53* [7-9], *RB* [10], *p16*, and *PPP2R1B* [11], have also been reported in NSCLC. Among those genetic alterations,

dysregulation of EGFR represents a major group that includes gene mutation, gene amplification and protein overexpression. EGFR tyrosine kinase mutations have been described in 5-15% of NSCLC patients [12]. In addition to genetic mutations of EGFR, overexpression of EGFR is found in 62% of NSCLC cases and is correlated with poor prognosis [13-15].

In previous studies, we have identified a tumor suppressor gene, namely Cell adhesion molecule 1 (CADM1, also named TSLC1, SynCAM1, Necl2) in NSCLC. CADM1 expresses in the brain, testis, lung and various epithelial tissues. It has been reported that loss of CADM1 expression is related to 20%-60% of various cancers of lung, liver, esophagus, stomach, pancreas, nasopharynx, breast, and uterine cervix [16] (Table 1). The main reason for depressed expression of CADM1 is hypermethylation of *CADM1* promoter and this has been observed in various cancers (Table 1). In NSCLC, it has been reported that hypermethylation of *CADM1* promoter is more likely to be observed in relatively advanced tumors than in primary ones, which suggests that the regulation of CADM1 expression may be involved in the progression of human NSCLC [17]. Furthermore, in bladder cancers, experimental results show that the expression of CADM1 is down-regulated or lost as tumor progresses (unpublished data). Moreover, *in vitro* experimental results demonstrated the

relationship between CADM1 expression and tumorigenic ability in 12 NSCLC cell lines. Accordingly, six cell lines loss of CADM1 expression have been reported of higher tumorigenic ability and CADM1-expressing cell lines have lower tumorigenic ability [18]. These findings support that CADM1 plays a critical role in the suppression of lung cancer progression and metastasis.

Table 1. Inactivation of the CADM1 gene expression in human cancers

Tumors	Promoter methylation (%) (primary tumor)	Loss of expression (%) (primary tumor)	Reference
NSCLC	21/48 (44)	60/93 (65)	[17,19]
Nasopharyngeal cancer	33/38 (87)	43/67 (64)	[20]
Breast cancer		47/67 (70)	[21]
Bladder cancer		92/147 (63)	[submitted]
Prostate	7/22 (31)		[22]
Esophageal	28/56 (50)		[23]
Ovarian carcinoma		94/160 (59)	[24]

CADM1 belongs to immunoglobulin superfamily and functions as a cell adhesion molecule. It contains 442 amino acids and comprises three immunoglobulin-like type loops (V-, C2-, C2-type), a single hydrophobic membrane-spanning alpha-helix, and a cytoplasmic domain containing a protein 4.1-binding motif, and a type II PSD95/Dlg/ZO-1 (PDZ) binding motif (Fig. 1A) [16]. It is expressed at the lateral membrane in epithelial cell and forms a cis-homodimer on plasma membrane and trans-interacts with another CADM1 in adjacent cells [25].

Since expression of CADM1 is lost or reduced in various cancers, it is important to study the mechanism for regulating CADM1. Several experimental evidences demonstrated that the alternation of CADM1 expression occurs at the transcriptional level. Methylation at CpG sites of the *CADM1* promoter was demonstrated in 44% of NSCLC, 27% of pancreatic cancers, 29% of hepatocellular carcinomas, and 32% of prostate cancers. In addition, promoter methylation and/or loss of expression of CADM1 were reported in 20-60% of cancers in the esophagus, stomach, pancreas, nasopharynx, breast, and uterine cervix. These findings have shown that the altered expression of CADM1 is deeply involved in various cancers at the transcriptional level.

Moreover, post-transcriptional regulation of CADM1 expression has been reported by our group and others. In our previous study, a luciferase reporter assay

demonstrated that miR-214 and miR-375 reduced the expression of the reporter gene through 3'UTR of *CADM1*, while introduction of miR-214 and miR-375 into NSCLC cell line reduced the expression of CADM1 protein [26]. In addition, a recent study indicates that treatment a mouse germ cell line with transforming growth factor-β1 (TGF-β1) down-regulates CADM1 through clathrin-dependent pathway [27]. However, the post-transcriptional regulation of CADM1 in tumor cells remains to be elucidated.

As disruption of cell adhesion in the primary tumor is an initial step of cancer metastasis, CADM1 plays a critical role in metastasis of cancer cells. In fact, loss of CADM1 expression is associated with the metastasis of lung adenocarcinoma [28] and bladder cancer [submitted] by clinical studies. In addition, there have been reported about restoration of CADM1 expression in A549 cell line strongly suppressed the metastasis from spleen to liver in nude mice [18,29]. However, the tumor suppressor activity of CADM1 was abrogated by truncating the cytoplasmic domain, although the cell-cell adhesion activity was partially retained in vitro [28]. These data suggested that the cytoplasmic domain is necessary for CADM1 tumor suppressor activity.

The cytoplasmic domain of CADM1 (46 amino acids long) contains a protein 4.1-binding motif and a PDZ binding motif, which are important for protein-protein

interaction. Through PDZ binding motif, CADM1 interacts with a group of proteins belonging to the membrane-associated guanylate kinase homologus (MAGuKs) including MPP1, MPP2, MPP3, CASK and Pals2 [30], which function as molecular scaffold. In addition, CADM1, DAL-1/4.1B, and MAGuk appear to form a ternary complex and maintain the epithelial cell structures associated with cell adhesion (Fig. 1B).

CADM1 interacts with an actin-binding protein, DAL-1/4.1B, through protein 4.1-binding motif at the cell-cell attached site, and this complex is involved in cell motility [29]. Notably, loss of DAL-1/4.1B is an early event in tumorigenesis [31], and methylation on *DAL-1/4.1B* promoter site predicts poor prognosis in NSCLC [32] suggesting that *DAL-1/4.1B* is a lung tumor suppressor gene. Taken together, these data suggest that DAL-1/4.1B might be the downstream target of CADM1. However, the precise molecular mechanism of CADM1 tumor suppressor activity has not been well clarified.

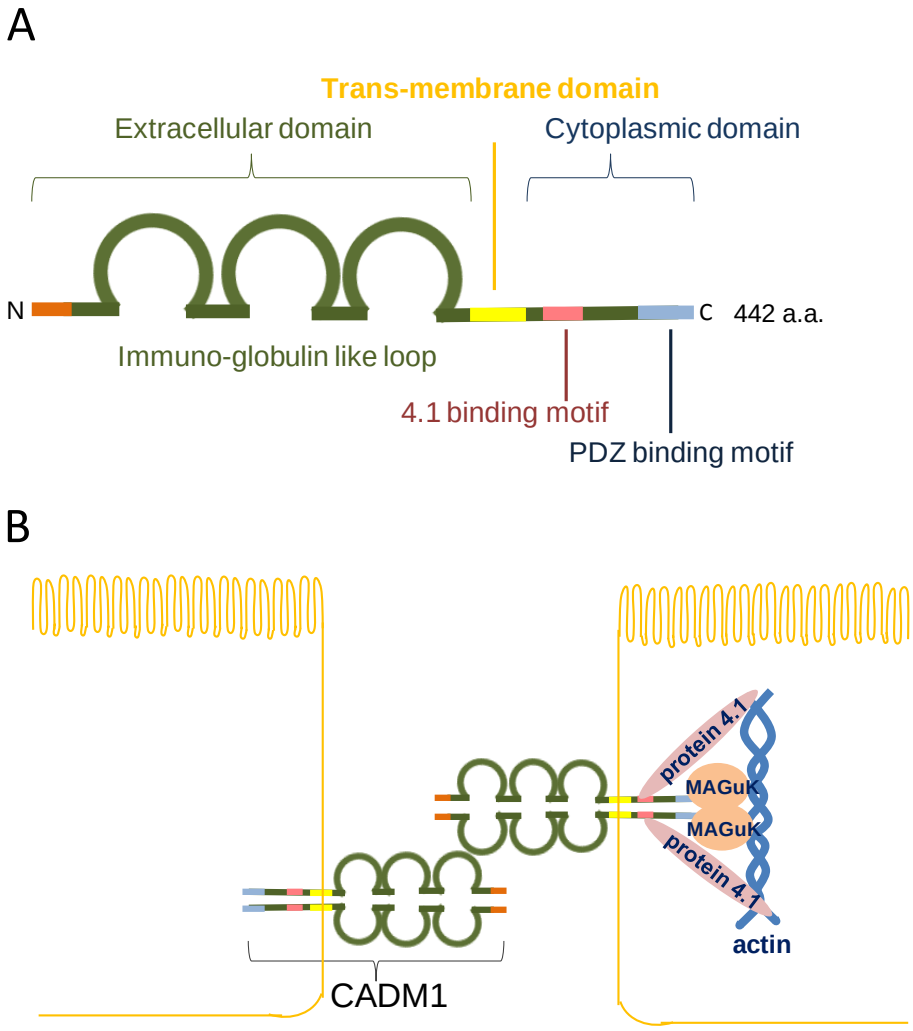


Figure 1. Schematic representation of the CADM1 protein and its cascade.

(A) The model of structure of CADM1. CAMD1 belongs to immunoglobulin superfamily. The extracellular domain consists of three immuno-globulin like loops and the cytoplasmic domain contains the protein 4.1 binding motif and the PSD95/Dlg/ZO-1 (PDZ) binding motif.

(B) A cascade of CADM1, DAL-1/4.1B, and membrane-associated guanylate kinase homologue (MAGuK) in polarized epithelial. CADM1 forms *cis*-homodimers at the lateral membrane. It interacts with actin through protein 4.1 by its 4.1 binding motif and participates in cell mobility. Furthermore, CADM1 has cell adhesion activity through interaction with another CADM1 in adjacent cells.

The role of growth factors-driven signaling in the pathogenesis of human cancer has been studied these past decades. Different families of growth factors and growth factor receptors have been shown to involve in the autonomous growth of cancer cells. Among these, the epidermal growth factor receptor (EGFR, also named as HER1/ErbB-1) is the well studied growth factor receptor that contributes to carcinogenesis.

Dysregulation of EGFR including overexpression, gene amplification, and genetic mutation has been reported in various cancers. Overexpression of EGFR has been reported in cancers of the head and neck, cervix, ovary, esophagus, stomach, brain, breast, colon, bladder and lung, and confer an adverse prognosis [14,33]. In addition, amplification of the *EGFR* gene has been reported in gliomas [34-36], squamous cell carcinomas [37,38], breast carcinomas [39] and bladder tumors [40]. In NSCLC, 12 of 32 (37.5%) primary NSCLC have wild-type *EGFR* amplification, and 5 (16%) of these cases have *EGFR* phospho-mutation, resulted in continuous signaling transduction [41]. The overexpression of EGFR has been observed in >60% of metastatic NSCLC tumor and correlates with poor prognosis [42]. Furthermore, clinical studies showed increased EGFR protein expression in stage III NCSLC compared to that of stage I or II, suggesting the role of EGFR in tumorigenesis [43,44]. These reports imply that the up-regulated expression level of EGFR plays an

important role in tumorigenesis.

EGFR is a receptor tyrosine kinase and belongs to the ErbB family. It is involved in signal transduction which regulates cellular pathogenesis and cellular differentiation [45,46]. The ErbB family encompasses three additional proteins, ErbB2 (neu, HER2), ErbB3 (HER3), and ErbB4 (HER4). Binding of ligands to the extracellular domain of ErbB receptors induces the receptors to form homo- or hetero-dimer.

EGFR consists of a extracellular ligand-binding domain, a single hydrophobic trans-membrane domain and a cytoplasmic tyrosine kinase-containing domain [47]. EGF binding leads to receptor dimerization and trans-autophosphrylation on specific tyrosine residues within cytoplasmic domain. These phosphorylated tyrosine residues serve as a docking site for protein binding, such as Src-homology-2 (SH2) or phosphotyrosine-binding-domain-containing downstream effector proteins which then leads to intracellular signal transduction [48]. It involves the mitogen-activated protein kinase (MAPK)/ERK pathway [49,50], phosphatidylinositol 3-kinase (PI3K)/Akt pathway [51] and Jak/Stat pathway [52]. Through these pathways, EGFR regulates cell proliferation and differentiation and involves in metastasis and tumor invasion (Fig. 2).

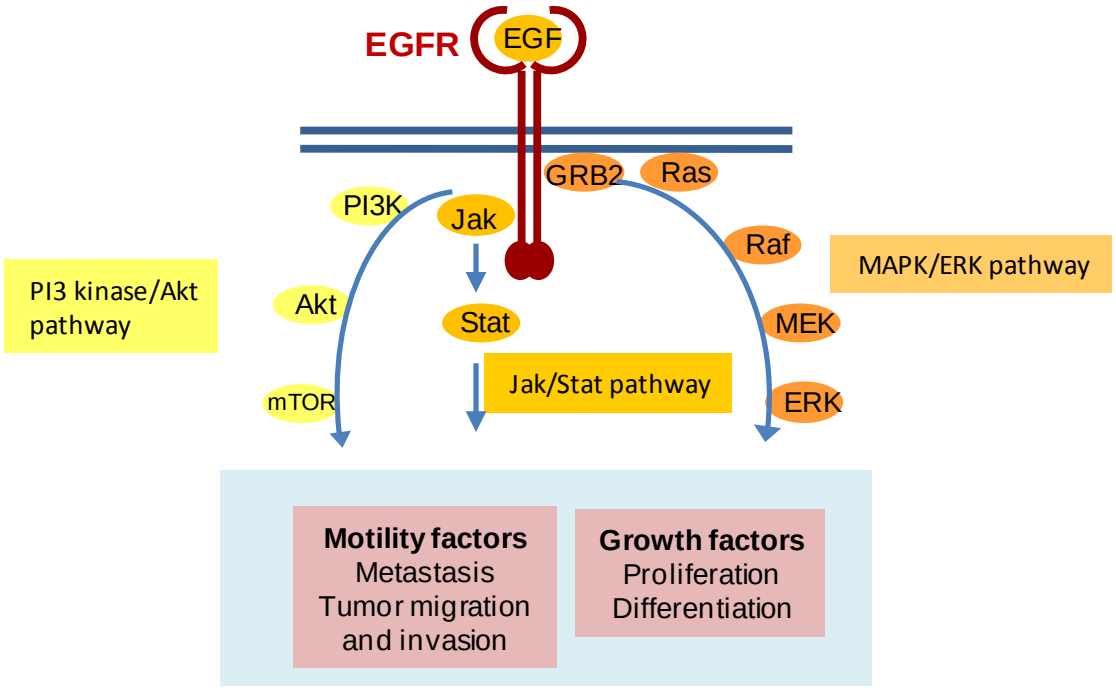


Figure 2. Simplified schematic illustration of the EGFR signaling pathways.

Ligand-binding results in EGFR dimerization leading to phosphorylation of specific tyrosin residues within the cytoplasmic domain. The phosphorylated EGFR activates the downstream signal transduction, including PI3 kinase/Akt, Jak/Stat, and MAPK/ERK pathway. These pathways activate transcription factors and modulate of cell growth and cell mobility.

Ligand binding induces not only downstream signal transduction but also EGFR internalization followed by degradation (Fig. 3). Experimental evidences support that EGFR might undergo two types of internalization pathways including clathrin-mediated and clathrin-independent endocytosis. However, the clarified mechanism is still a riddle. Some reporters showed that clathrin-mediated endocytosis of the EGFR occurs with lower concentrations (1.5 ng/mL) of EGF [53,54], while clathrin-independent endocytosis, such as caveolae is associated with higher EGF concentrations (20 ng/mL) [53,55]. However, some reporters showed that EGFR go through clathrin-dependent endocytosis with stimulation by high concentration of EGF [56]. Furthermore, several studies have reported that EGFR can be internalized by macropinocytosis in certain cell types, for example A431 cell line [55,57,58]. Although there are different explanations of EGFR endocytosis, it has reached in consensus that functional clathrin-coated pits are the general portals for efficient EGFR endocytosis.

After being activated, the ligand-bound EGFR dimerizes, and is translocated to clathrin-coated pit followed by endocytosis. This process is mediated by Cbl, which is a RING finger containing E3 ubiquitin ligase. Ubiquitination of EGFR by Cbl controls the fate of internalized EGFR by promoting the fusion of EEA1-positive endosomes to undergo lysosomal-dependent degradation [59].

The ingested intermediate vesicle fuses to early endosome, and is sorted to its final cellular destination. Depending on the cell lines, EGFR may be trafficked to the late endosome, leading to the lysosomal-dependent degradation followed by signal termination, or be recycled to the plasma membrane which might be re-stimulated by extracellular ligands [60]. In conclusion, endocytotic pathway of EGFR has a crucial function in degradation of EGFR and in terminating the signal transduction.

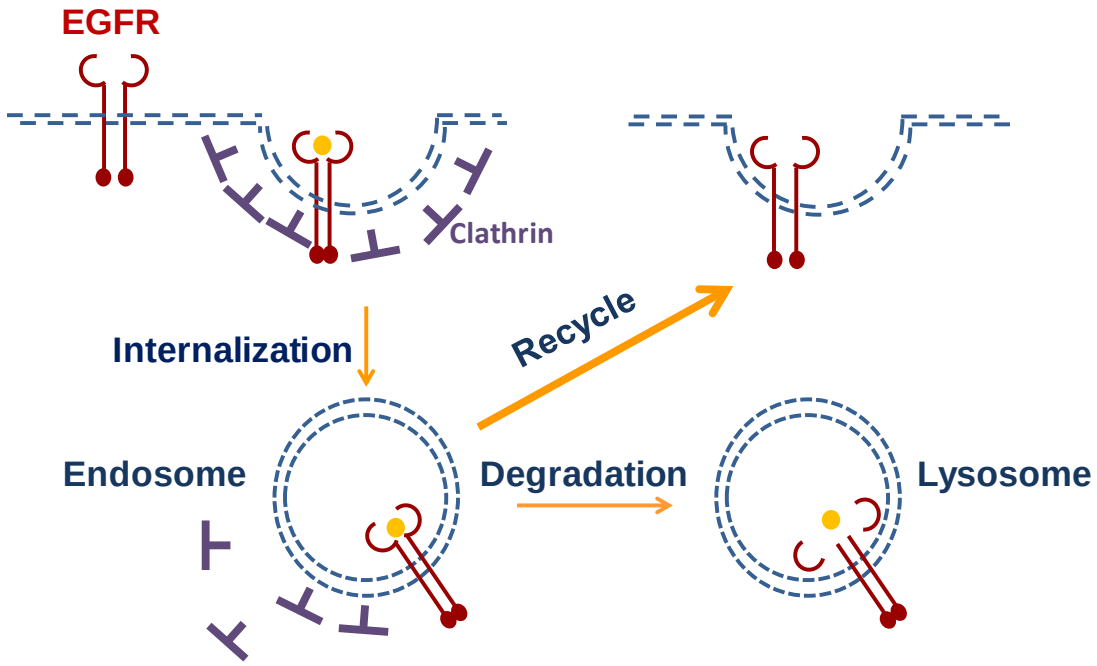


Figure 3. Epidermal growth factor receptor (EGFR) trafficking pathways.

Ligand-binging induces the dimerization of EGFR, resulting in auto-phosphorylation, activation, and internalization. From the cell surface, activated EGFR are internalized into clathrin-coated pits then be endocytosed. Newly formed clathrin-coated vesicles rapidly shed their coat and fuse with early endosome. From the early endosome, EGFR are recycled back to the cell surface or are sorted toward the late endosome/ lysosome then be degraded. The fate of the receptor has important consequences for biological outputs. The recycle pathway favors cell proliferation, while the degradation pathway results in normal cellular homeostasis.

In previous work, we have identified Cell adhesion molecule 1 (CADM1) as a tumor suppressor in NSCLC. Mao et al has reported that truncating the cytoplasmic domain of CADM1, with retained function of adhesion resulted in abrogation of tumor suppressor activity [28]. These data imply that the cytoplasmic domain of CADM1 is important for tumor suppressor activity. Previously, we have demonstrated that the cytoplasmic domain of CADM1 interacts with protein 4.1B and MPP2 and was involved in the maintenance of epithelial cell structure [30]. However, the precise molecular mechanism of CADM1 in tumor suppression has not been well clarified.

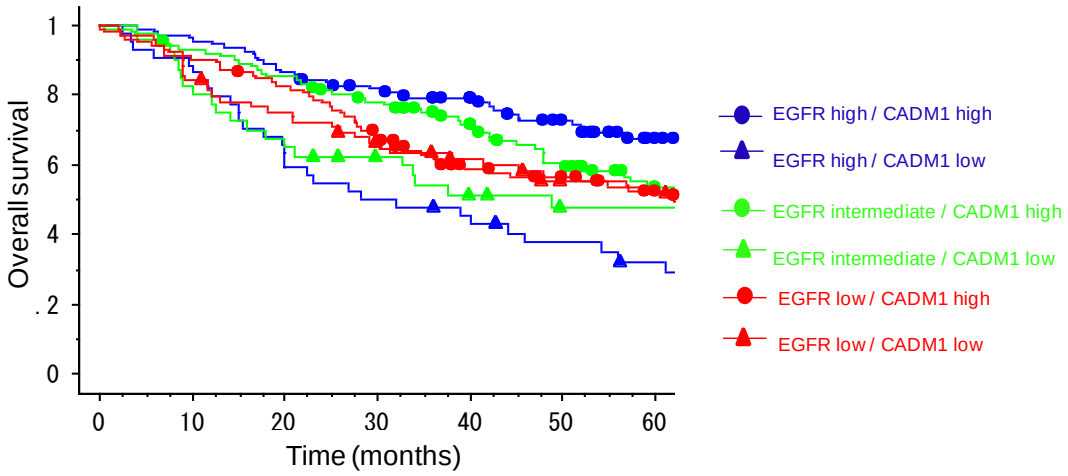
In these years, it has been reported that re-expression of CADM1 decreased cell viability in various carcinoma cell lines, such as hepatocellular carcinoma, esophageal carcimona [61], pancreatic Cancer [62], and cutaneous squamous cell carcinoma [63,64]. These results implied that the tumor suppression function of CADM1 might function through reduction of cell proliferation. Based on previous studies in NSCLC, lost expression of CADM1 as tumor progresses has been reported [17,65]. In addition, increased expression of EGFR has been shown in advanced tumors more than in primary ones [43,44], suggesting that overexpression of EGFR might be involved in tumorigenesis. Furthermore, increased expression of EGFR and loss expression of CADM1 were not only reported in NSCLC but also in urinary bladder cancers [65], breast cancers [21], and nasopharyngeal carcinoma [20]. Notably, the small cell lung

cancer with overexpression CADM1, on the other hand, shows conspicuously negative EGFR expression [66]. These reports showed the inverse expression pattern of CADM1 and EGFR in the process of tumor progression.

In a total of 469 lung adenocarcinoma with high-quality gene-expression publicly available data [67], the Kaplan-Meier plots illustrate the survival dependent on CADM1 and EGFR mRNA expression (Fig. 4). In the low EGFR mRNA expression groups, the prognosis between the CADM1 high and low groups had no significant difference. However, in the high EGFR mRNA expression groups, loss of co-expression with CADM1 showed a poor prognosis compare to high expression of CADM1.This data showed that the prognostic ability of CADM1 is associated with EGFR mRNA expression level, and implied that CADM1 may have functional interaction with EGFR. Moreover, clinical studies in NSCLC showed that CADM1 expression was preserved in a non-invasive, bronchiole-alveolar histological pattern of tumor cells, while EGFR expression was increased in metastatic NSCLC tumor [19,43,44] (Fig. 5). In addition, it has been shown that CADM1 bind with erbB3 and erbB4 to down-regulate the phosphorylation of erbB3 [68]. It has been reported that ligand activated EGFR forms homo- and hetero-dimarization with membranes of the HER kinase family, such as EGFR/erbB3, and EGFR/erbB4. These studies suggest that CADM1 may directly or indirectly interact with EGFR.

First, the mRNA microarray data of 469 lung adenocarcinoma showed that CADM1 is a prognostic indicator significantly in high EGFR mRNA expression group. Second, loss expression of CADM1 and increased expression of EGFR have been reported in various cancers. Third, CADM1 and EGFR have the same interaction factor, such as erbB3 and erbB4. Based on these findings, I hypothesized that CADM1 might contribute to the down-regulation of EGFR.

In this study, I investigated the possible functional interaction between tumor suppressor protein, CADM1, and the oncoprotein, EGFR, as well as its underlying molecular mechanism. Exogenously expression of CADM1 in NSCLC cell lines NCI-H1838, NCI-H1993, and A549 resulted in down-regulation of EGFR expression without effect on mRNA expression. Furthermore, CADM1 facilitates the internalization and degradation of EGFR by EGF stimulation through a clathrin-dependent pathway. These results implied that CADM1 participates in the internalization and degradation of EGFR.



p=0.7 (EGFR low/ CADM1 high) vs (EGFR low/ CADM1 low)
p=0.07 (EGFR intermediate/ CADM1 high) vs (EGFR intermediate/ CADM1 low)
p<0.0001 (EGFR high/ CADM1 high) vs (EGFR high/ CADM1 low)

Figure 4. Analysis of the publicly available data of 469 primary lung adenocarcinoma cases.

Correlation of CADM1 and EGFR mRNA expression with disease free survival of lung adenocarcinoma patients. The 469 high-quality gene-expression cases of lung adenocarcinoma were obtained from publicly available data [67]. The Kaplan-Meier plots illustrate the survival dependent on CADM1 and EGFR mRNA expression. Patients were separated into six groups according to the expression pattern of CADM1 and EGFR as follows: cases with high expression level of EGFR and CADM1 (EGFR high/ CADM1 high), cases with high expression level of EGFR and low expression level of CADM1 (EGFR high/ CADM1 low), cases with intermediate expression level of EGFR and high expression level of CADM1 (EGFR intermediate/ CADM1 high), cases with intermediate expression level of EGFR and low expression level of CADM1 (EGFR intermediate/ CADM1 low), cases with low expression level of EGFR and high expression level of CADM1. (EGFR low/ CADM1 high), cases with high expression level of EGFR and CADM1 (EGFR low/ CADM1 low). p value was calculated by Breslow-Gehan-Wilcoxon test.

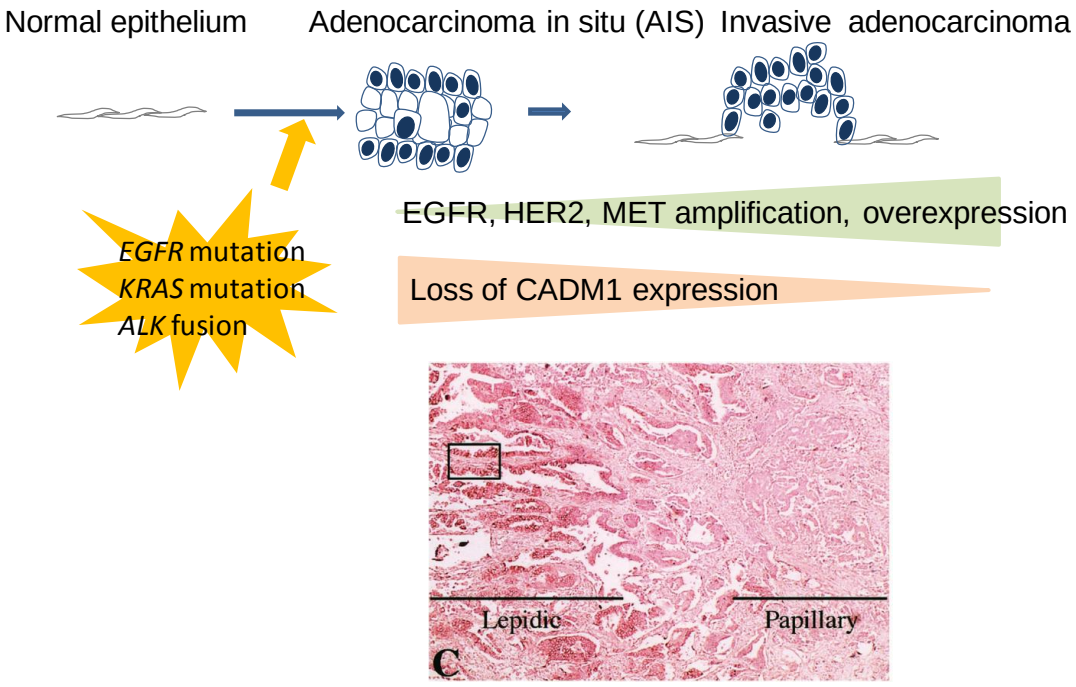


Figure 5. The genetic and protein alteration in tumorigenesis of NSCLC.

In NSCLC, genetic alterations of proto-oncogenes, such as *KRAS* mutations, *EGFR* mutations, and *ALK* fusion have been reported to play roles in tumor progression. In addition, clinical studies in NSCLC showed that CADM1 expression was preserved in a non-invasive, bronchiole-alveolar histological pattern of tumor cells, while EGFR, HER2, and MET expression was increased in metastatic NSCLC tumor. Immunohistochemistry analysis showed that CADM1 expression was lost in the metastasis region (Papillary) [69].

Materials and Methods

Plasmid constructs and siRNA

To obtain HA-CADM1 expression vectors, HA and CADM1 cDNA of full length (Full, amino acids 45-442), ΔCT (amino acids 45-396) containing the extracellular and the trans-membrane domain, ΔEC (amino acid 360-442) containing the trans-membrane domain and cytoplasmic domain, and ΔPDZ (amino acids 45-398) only deletion the PDZ binding motif at C-terminal were amplified by PCR and cloned into BamHI and XhoI sites of pSecTag2/Hygro B (Invitrogen Corporation, CA, USA). Control siRNA (ON-TARGETplus siCONTROL Non-targeting pool, D-001810-10) and siRNA against CADM1 (siCADM1#5: 5'->3' CGAAAGACGUGACAGUGAU, siCADM1#8: 5'->3' GCGCUUGAGUUAACAUGUG) were obtained from Thermo scientific (Thermo Fisher Scientific, MA, USA). siRNA against clathrin heavy chain siClathrin#1: 5'->3' GCAAUGAG CUGUUUGAAGATT was obtained from ambion, and siClathrin#2:5'->3' CCAAGUAAUCCAAUUCGAATT was from Sigma (Sigma-Aldrich, MO, USA).

Antibodies and reagents

The chicken monoclonal anti-CADM1 extracellular domain antibody (3E1) was described previously [Ito A, 2003, Nagata M, 2011]. The rabbit polyclonal anti-EGFR

antibody (1005) and mouse monoclonal anti-LAMP1 (H4A3) antibody were purchased from Santa Cruz Biotechnology (TX, USA). A mouse monoclonal antibody against GAPDH and a rat monoclonal antibody against HA (clone 3F10) were purchased from Millipore (Billerica, MA, USA), and Roche (Indianapolis, IN, USA), respectively. A mouse monoclonal anti-Clathrin (X22) antibody was purchased from Abcam. Secondary antibodies conjugated to HRP were purchased from Millipore. Cy3- and Alexa Fluor 488-, and 633-labeled secondary antibodies were purchased from Molecular Probes (Eugene, OR). EGF and chloroquine were purchased from PeproTech (Lodon) and Wako (Osaka, Japan), respectively.

Cell culture

NCI-H1838, an adenocarcinoma from non-small cell lung cancer (NSCLC), was obtained from The American Type Culture Collection (ATCC). We obtained A549 from RIKEN cell bank, Japan, A431 cells from the Health Science Research Resources Bank, Japan. NCI-H1993, and NCI-H358, both are NSCLC cell lines with wild-type form of EGFR were from ATCC. HCC827, HCC4006 and H1650, which are NSCLC cell lines with mutant form of EGFR were from ATCC. Cells were cultured according to the supplier's recommendation. NCI-H1838, HCC827, NCI-H358, NCI-H1993, HCC4006, and H1650 were maintained in RPMI 1640

medium (Nacalai Tesque) with 10% FBS, 5% Sodium Pyruvate (Gibco), 5% MEM Non-Essential Amino Acids (Gibco), and 5% Penicillin Streptomycin (Gibco). A431 and A549 were maintained in low glucose Dulbecco's Modified Eagle's medium (DMEM) (Nacalai Tesque) with 10% FBS, 5% MEM Non-Essential Amino Acids (Gibco), and 5% Penicillin Streptomycin (Gibco). Cell were cultured at 37℃ in a humidified incubator in an atmosphere of 5% CO_2.

Transfection

Plasmid transfection of NCI-H1838, HCC827, NCI-H358, NCI-H1993, HCC4006, and H1650 cells were carried out using Lipofectamine LTX (Invitrogen) according to the supplier's recommendation. siRNA transfection of NCI-H1838 cells was performed by using Lipofectamine LTX (Invitrogen) as suggested by the manufacturer. Plasmid transfection of A549 cells was carried out using Lipofectamine 2000 (Invitrogen) as manufacture's suggestions. Plasmid transfection of A431 cells was preformed by electroporation. A431 cells were trypsinized than suspended in Nucleofactor solution T (Lonza). Electroporation was performed by Nucleofactor (Amaxa biosystems) (program X-01).

Immunoblot analysis

For exogenously express experiments, cell lysate were harvested after transfection for 24 hrs. For knockdown experiments, cell lysate were prepared after transfection for 72 hrs. Cells were lysed in lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, and 1 mM EDTA plus phosphatase inhibitor 10 mM NaF, 0.1 mM Na_3VO_4) with protease inhibitors (200 μM AEBSF, 10 μM leupeptin, 1 μM pepstatin A) on ice for 30 mins. The lysates were cleared by centrifugation at 15,000 r.p.m for 10 mins at 4℃. The concentrations of lysates were determined by Protein Assay Reagent (Bio-Rad, Hercules, CA, USA). Then 3X SDS sample buffer (0.2 M Tris-HCl (pH 6.8), 30% glycerol, 6% SDS, 15% β-ME, 0.03% BPB) was applied and boiled at 98℃ for 5 mins.

The proteins were resolved on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) within SDS-PAGE electrode buffer (25 mM Tris, 0.2 M Glycine, 0.1% SDS) and transferred to polyvinylidene difluoride (PVDF) membrane. Transfer was performed by using transblot SD cell (Bio-Rad, CA, USA) within Transfer buffer (50 mM Tris, 40 mM Glycine, 20% Methanol, 0.1% SDS). After transfer, the PVDF membrane was blocked with 10% skim milk in PBS-T for 20 mins followed by blotting with first antibody for 2 hrs at room temperature. The dilution conditions of antibodies were showed as follow: rabbit anti-EGFR polyclone antibody

(1:1000), rat anti-HA polyclone antibody (1:1000), anti-clathrin polyclone antibody (1:500), and anti-GAPDH monoclone antibody (1:5000). PVDF membrane was washed with PBS-T for 3 times followed by blotting with secondary antibody conjugated with HRP for 1 hr at room temperature. The signal was detected by ECL™ Western Blotting Detection Reagent (GE Healthcare, USA). Quantification of signal intensities was performed using Image J software Ver. 1.44 from three independent experiments.

Immunofluorescence Microscopy

To detect the expression of CADM1 deletion mutants, cells were seeded on coverslips and maintained for 24 hrs. Then transfection with CADM1 deletion mutants was carried out, and cells were fixed followed by immunoflouresence staining 24 hrs after transfection. For chloroquine analysis, cells were seeded on coverslips and maintained for 24 hrs. Cells were pre-treated with chloroquine for 18 hrs. After treatment with EGF for 0 or 3 hrs, cells were fixed followed by immunoflouresence staining. In detail, cells were fixed in 4% paraformaldehyde for 20 mins and then permeabilized with 0.1% Triton-X-100 in PBS for 5 mins. After blocking with 5% (w/v) FBS in PBS, cells were incubated with primary antibody for 1 hr at room temperature. The dilutions of antibodies were shown as follow: rat

anti-HA polyclone antibody (1:50), rabbit anti-EGFR polyclone antibody (1:100), chicken anti-CADM1 polyclone antibody (1:300), and anti-LAMP1 antiboody (1:100). Cells were washed with PBS and then blotted with secondary antibodies for 30 mins at room temperature. Coverslips were then mounted with ProLong Gold (Invitrogen). Cells were imaged with Nikon A1Rsi confocal microscope equipped with a laser at the excitation of 405, 488, 543, and 628nm.

Quantitative Real-time polymerase chain reaction (qRT-PCR)

RNA was extracted from cells using RNeasy kit (QIAGEN Science, German-town, MD) and reverse transcribed using a Transcriptor first-strand cDNA synthesis kit (Roche Diagnostics, Basel, Switzerland). For Real-time PCR, the mRNA level of EGFR was detected using Light Cycler® 1.5 with LightCycler FastStart DNA Master SYBR Green (Roche Diagnostics). Oligonucleotide primer sequences for real-time PCR are shown in Table 2. The GAPDH was used as an internal control for normalization. The expression level of the target gene was determined using $2^{-\Delta\Delta C_T}$ method.

Table 2. Nucleotide sequence of primers used in qRT-PCR

Target gene	Direction	Sequence (5'-3')
GAPDH	S	ACCACAGTCCATGCCATCAC
GAPDH	AS	TCCACCACCCTGTTGCTGTA
EGFR	S	AGCTTGTGGAGCCTCTTACACC
EGFR	AS	CACCTTCTGGGATCCAGAGT

S, sense; AS, antisense

Statistical Analysis

The statistical significance of microarray analysis was calculated by Breslow-Gehan-Wilcoxon test. The statistical significance of the other experimental results was calculated by paired t-test. In quantitative RT-PCR experiments, paired t-test was used to compare the log fold-changes of the mRNA expression for the two treatments. All data are expressed as mean ± standard deviation (SD) values. Results were considered statistically significant at $p<0.05$.

Results

1. Reduction of EGFR is induced by exogenously expressed CADM1

First, I investigated whether CADM1 is involved in the regulation of expression of EGFR. For this purpose, a non-small cell lung cancer (NSCLC) cell line, NCI-H1838, expressing both CADM1 and wild type EGFR was transfected with control vector or the expression vectors of HA-CADM1-full. After transfection, the expression of EGFR was examined by immunoblot analysis. As shown in Figure 6A, EGFR protein was significantly reduced after transfected with HA-CADM1-full. Quantification data showed that expression of EGFR at HA-CADM1-full transfected cells were reduced to 75±10% (Fig. 6B). To further confirm CADM1 involved in the transcriptional regulation of EGFR, the quantitative RT-PCR of EGFR mRNA was performed. As Figure 6G shown, the mRNA expression of EGFR did not decrease by transfection of HA-CADM1-full.

To further confirm this phenomenon, the same experiments were performed in other NSCLC cell lines, NCI-H1993 and A549, which both endogenously expresses wild type EGFR and lose of CADM1 expression. The quantitative protein expression of EGFR at HA-CADM1-full transfected NCI-H1993 and A549 cells were reduced to 70±11% and 75±11%, respectively (Fig. 6C, D, E, F). However, quantitative RT-PCR showed the mRNA of EGFR was unaltered (Fig. 6H, I). These data indicated that

CADM1 reduces EGFR protein expression possibly through post-translational regulation but not at transcriptional level.

It is well known that mutation of EGFR occurs in 5-15% of NSCLC patients. To evaluate CADM1 reduces the protein expression of mutant EGFR, the NSCLC cell lines HCC827 and PC9 are used in this experiment. HCC827 and PC9 cell lines have an acquired mutation in EGFR tyrosine kinase domain (E746-A750 deletion) that results in constitutively phosphorylation of EGFR. As figure 7 indicates, with exogenously overexpression CADM1 in HCC827 and PC9 cells, there is no significant change of protein expression of EGFR. These findings imply that CADM1 did not affect mutant form of EGFR.

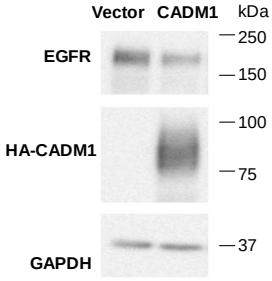
The same experiments were also performed in other NSCLC cell lines NCI-H358 (wild type EGFR), H1650 (mutant EGFR) and HCC4006 (mutant EGFR), but the protein expression of EGFR was difficult to detect (Fig. 8).

Finally, to confirm CADM1 reduce EGFR protein expression specifically occurs in NSCLC cell lines, the same experiment was performed in the epidermoid carcinoma cell line A431 with expression of wild type EGFR. As shown in figure 9, overexpressed CADM1 resulted in reduction of EGFR expression to 75±10%. This indicated that CADM1 reduced the expression of EGFR not only in NSCLC cell lines but also in epidermoid carcinoma cell line.

In conclusion, exogenously overexpression of CADM1 in three NSCLC cell lines (NCI-H1838, NCI-H1993, and A549) and one epidermoid carcinoma cell line (A431) induced down-regulation of EGFR expression without effect on EGFR mRNA expression. On the other hand, overexpression of CADM1 in two NSCLC cell lines with mutant form of EGFR did not reduce the expression of EGFR (Table 3). These data imply that CADM1 might participate in the down-regulation of expression of wild type EGFR.

NCI-H1838

A



B

NCI-H1838

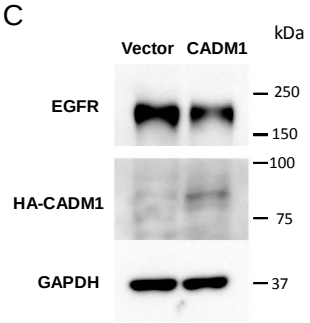
	vector	CADM1	ratio
1	1.29	1.14	0.88
2	2.45	1.56	0.64
3	6.31	4.67	0.74
4	4.08	3.03	0.74

Ave 0.75±0.10

p=0.028

NCI-H1993

C



D

NCI-H1993

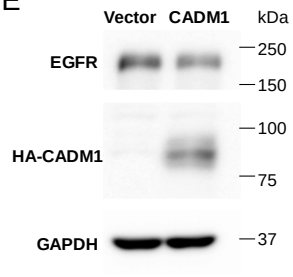
	vector	CADM1	ratio
1	0.13	0.11	0.83
2	0.19	0.12	0.62
3	0.18	0.12	0.66

Ave 0.70±0.11

p=0.011

A549

E



F

A549

	vector	CADM1	ratio
1	0.22	0.19	0.86
2	0.38	0.24	0.62
3	0.69	0.56	0.80
4	0.18	0.13	0.70

Ave 0.75±0.11

p=0.024

G

NCI-H1838	dCt		$2^{-\Delta\Delta C_T}$	
	vector	CADM1	vector	CADM1
1	3.34	3.07	1	1.21
2	2.63	2.51	1	1.09
3	2.61	2.31	1	1.24

p=0.059

H

NCI-H1993	dCt		$2^{-\Delta\Delta C_T}$	
	vector	CADM1	vector	CADM1
1	4.46	4.61	1	0.9
2	4.52	4.65	1	0.91
3	3.89	3.6	1	1.22
4	4.03	4.23	1	0.87

p=0.78

I

A549	dCt		$2^{-\Delta\Delta C_T}$	
	vector	CADM1	vector	CADM1
1	5.44	5.57	1	0.91
2	6.38	6.69	1	0.81
3	6.44	6.37	1	1.05
4	6.35	6.54	1	0.89

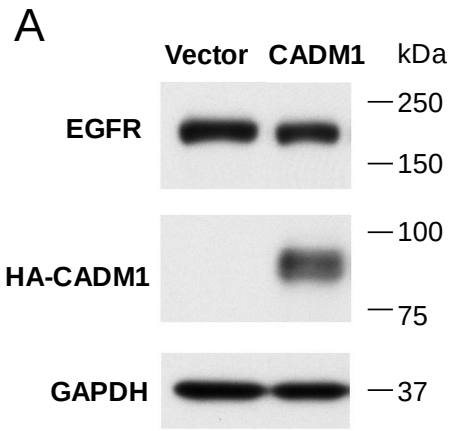
p=0.19

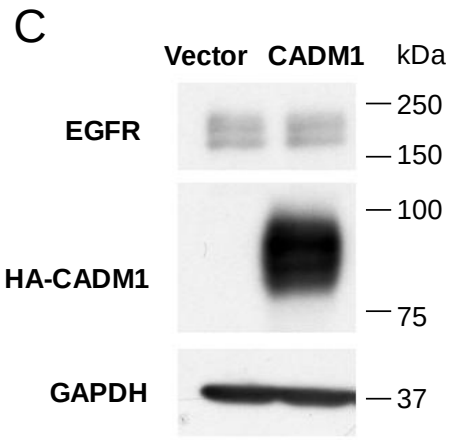
Figure 6. Exogenous expression of CADM1 reduced EGFR protein in NCI-H1838, NCI-H1993 and A549 cells.

(A, C, E) NCI-H1838, NCI-H1993, and A549 cells were transfected with vector control or HA-CADM1-full expression plasmids. The cells were grown under normal condition for 24 hrs and then cell lysate were harvested. Immunoblot analysis of NCI-H1838 (A), NCI-H1993 (D), and A549 (E) were performed. Anti-EGFR (upper), anti-HA (middle), and anti-GAPDH (lower) antibodies were used for detection.

(B, D, F) Intensities of EGFR in A, C, and E were normalized to those of GAPDH, respectively. Data shows mean ±SD of 3(NCI-H1993) or 4 (NCI-H1838, A549) independent experiments. p value was calculated by paired t test.

(G, H, I) Quantitative Real-time PCR analysis of EGFR in mRNA from NCI-H1838 (G), NCI-H1993 (H), and A549 (I) cells. NCI-H1838, NCI-H1993, and A549 cells were transfected with vector control or HA-CADM1-full expression plasmids. Cells were maintained in normal condition for 24 hrs. The mRNA was extracted and reverse transcription was performed. Subsequently, mRNA expression level of EGFR was determined by quantitative real-time PCR, and GAPDH was co-amplified as an internal control for normalization. Data shows mean ±SD of 3(NCI-H1838) or 4 (NCI-H1993, A549) independent experiments. The expression level of the target gene was determined using $2^{-\Delta\Delta C_T}$ method. p value was calculated by paired t test.





B

HCC827

	vector	CADM1	ratio
1	1.78	2.00	1.12
2	1.00	0.99	0.99
3	0.90	0.78	0.87

p=0.396

Ave 0.99±0.13

D

PC9

	vector	CADM1	ratio
1	0.37	0.37	1.00
2	0.08	0.13	1.63
3	0.17	0.24	1.41

p=0.097

Ave 1.34±0.32

Figure 7. Exogenous expression of CADM1 did not reduced EGFR expression in HCC827 and PC9 cells.

(A, C) HCC827 (A) and PC9 (C) cells were transfected with vector or HA-CDM1-full expressing plasmids. The cells were grown under normal condition for 24 hrs and then cell lysate were harvested. Western blot analysis of cell lysate was carried out by using anti-EGFR (upper), anti-HA (middle), and anti-GAPDH (lower) antibodies.

(B, D) Intensities of EGFR in A and C were normalized to those of GAPDH. Data shows mean ±SD of 3 independent experiments. p value was calculated by paired t test.

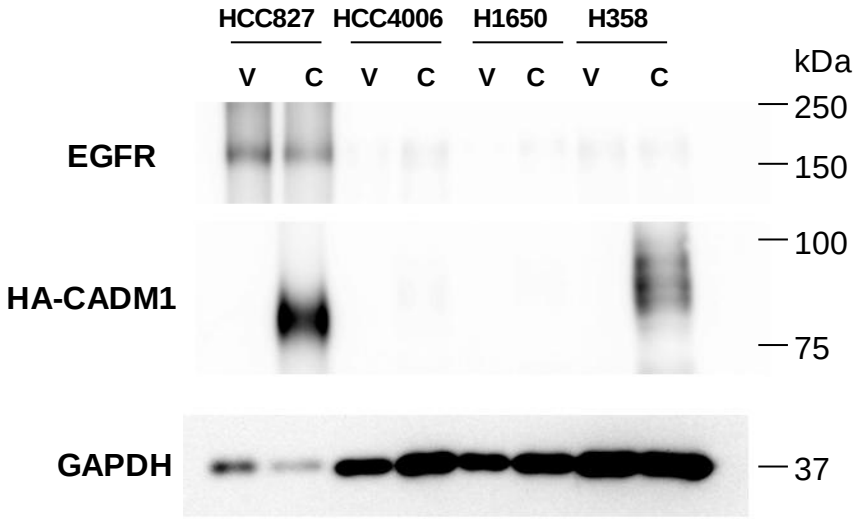
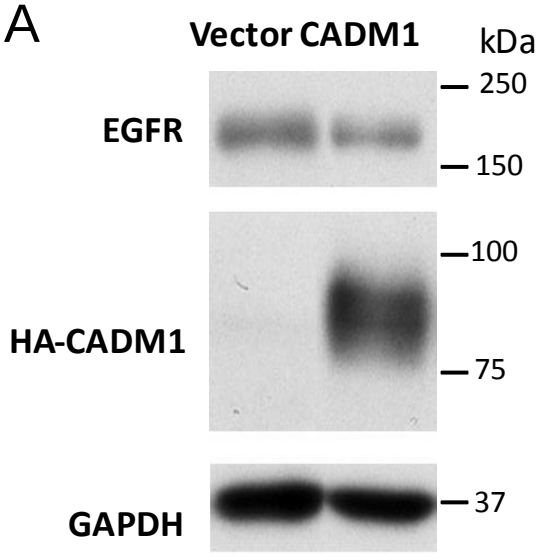


Figure 8. The relative expression of EGFR in NSCLC cell lines.
HCC827, HCC4006, H1650, and H358 cells were transfected with vector (V) or HA-CADM1-full (C) expressing plasmids. After 24 hrs, cell lysate were prepared and immune-blot analysis was performed. Anti-EGFR (upper), anti-HA (middle), and anti-GAPDH (lower) antibodies were used for detection.

A



B

A431

	vector	CADM1	ratio
1	1.81	1.38	0.76
2	0.46	0.28	0.61
3	0.88	0.75	0.85
4	0.65	0.51	0.78

Ave 0.751±0.103

p=0.027

Figure 9. Exogenous expression of CADM1 reduced EGFR expression in A431 cells.

(A)A431 cells were transfected with vector or HA-CDM1-full expressing plasmids. Cells were grown under normal condition for 24 hrs, and cell lysate was prepared. Immunoblot analysis was used to detect the expression of EGFR, CADM1, and GAPDH. Anti-EGFR (upper), anti-HA (middle), and anti-GAPDH (lower) antibodies were used for detection.

(B)Intensities of EGFR in A were normalized to those of GAPDH. Data shows mean ±SD of 4 independent experiments. p value was calculated by paired t-test.

Table 3. Change of EGFR expression by exogenous CADM1 in NSCLC and epidermoid carcinoma cell lines

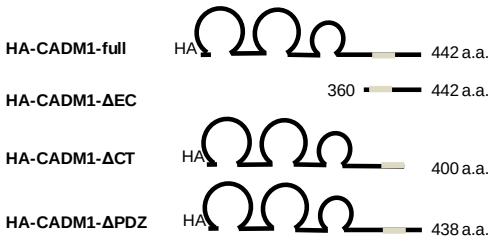
			exogenously expression of CADM1	
	cell type	genotype of EGFR	protein expression of EGFR	mRNA expression of EGFR
NCI-H1838	NSCLC	WT	down-regulated (75±10%)	no difference
NCI-H1993	NSCLC	WT	down-regulated (70±11%)	no difference
A549	NSCLC	WT	down-regulated (75±11%)	no difference
NCI-H358	NSCLC	WT	not detectable	nd
HCC827	NSCLC	mut, amp	no difference	nd
PC9	NSCLC	mut	no difference	nd
HCC4006	NSCLC	mut	not detectable	nd
H1650	NSCLC	mut	not detectable	nd
A431	epidermoid carcinoma	WT, amp	down-regulated (75±10%)	nd

NSCLC, non-small cell lung cancer; WT, wild type; mut, mutant; amp, amplification; nd, not done

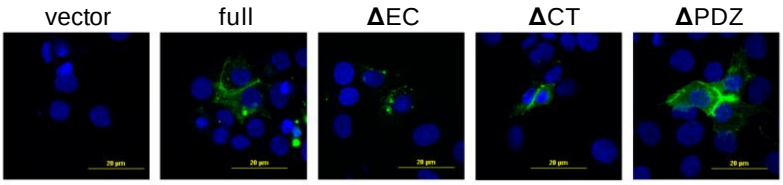
2. Reduction of EGFR is induced by exogenously expressed CADM1 depending on its cytoplasmic domain

Then I investigated which domain of CADM1 is responsible for EGFR degradation. To examine this, the deletion mutations of CADM1 were constructed (Fig. 10A). NCI-H1838 cells were transfected with CADM1 deletion mutants, including deletion of extracellular domain, HA-CADM1-ΔEC, deletion of cytoplasmic domain, HA-CADM1-ΔCT, and deletion of PDZ binding motif, -ΔPDZ, and immunofluorescence analysis was performed. Confocal photomicrographs indicated that HA-CADM1-full, -ΔCT, and –ΔPDZ localized at cell membrane, but -ΔEC localized at cytoplasm (Fig. 10B). Since CADM1 is a membrane protein, the mutants of membrane localization were used for the subsequent experiments.

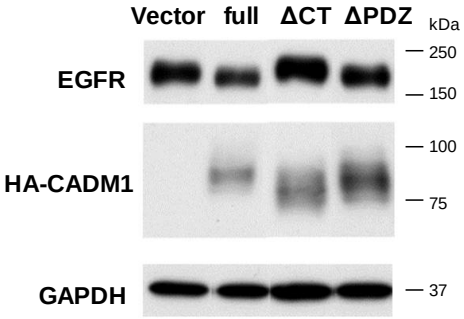
NCI-H1838 cells were transfected with CADM1 deletion mutants, including HA-CADM1-ΔCT, -ΔPDZ, and immunoblot analysis was performed. EGFR protein was significantly reduced after transfected with HA-CADM1-full (Fig. 10C, D), and this is consistent with the result shown in Figure 7. However, expression of EGFR was not reduced in HA-CADM1-ΔCT and -ΔPDZ transfected cells (Fig. 10C, D). This suggests that overexpression of CADM1 induced the degradation of EGFR through its cytoplasmic domain.



B



C



D

NCI-H1838

	vector	CADM1	Δ CT	Δ PDZ
1	0.71	0.55	0.90	0.90
2	0.95	0.66	0.97	0.83
3	1.48	1.01	2.24	1.99

p=0.038

Ratio

	vector	CADM1	Δ CT	Δ PDZ
1	1.00	0.77	1.27	1.27
2	1.00	0.69	1.02	0.87
3	1.00	0.68	1.51	1.34
Ave	1 ±0	0.72± 0.5	1.27 ±0.24	1.16 ±0.25

Figure 10. Exogenous expression of CADM1 reduced EGFR protein in NCI-H1838 cells.

(A) A scheme of HA-CADM1 construction, including full length, deletion mutant of extracellular domain, -ΔEC, deletion mutant of cytoplasmid domain, -ΔCT, and deletion mutant of PDZ binding motif, -ΔPDZ.

(B) Confocal imaging of NCI-H1838 cells transfected with control or CADM1 deletion mutants. Cells were plated on coverslips and transfected with vector or CADM1 deletion mutants expressing plasmids. Cells were then grown in normal condition for 24 hrs. The cells were fixed followed by immunofluorescence staining. The coverslips were mounted on slides and analyzed using confocal microscopy. Green color indicates CADM, and blue color represents DAPI. Bar, 20μM.

(C) Imunoblot analysis of NCI-H1838 cells transfected with control or CADM1 deletion mutants. Cells were transfected with vector or CADM1 deletion mutants expressing plasmids. Cells were then grown in normal condition for 24 hr. Cell lysate was prepared and immunoblot analysis was performed. Anti-EGFR (upper), anti-HA (middle), and anti-GAPDH (lower) antibodies were used for detection.

(D) Intensities of EGFR in C were normalized to those of GAPDH. Relative intensities to vector control (ratio) were shown. Data shows mean ±SD of 3 independent experiments. p value was calculated by paired t-test.

3. Reduction of EGFR expression induced by exogenously expressed CADM1 is through lysosomal-degradation pathway

I have shown that exogenously expressed CADM1 reduced EGFR protein expression but not EGFR mRNA expression. It has been reported that degradation of EGFR is through lysosomal pathway after EGF stimulation. Subsequently, I examined whether CADM1 induce the degradation of EGFR by using chloroquine, an inhibitor of lysosomal protein degradation. Thus I have examined the localization of both CADM1 and EGFR in NCI-H1838 cells treated with chloroquine followed by EGF stimulation. NCI-H1838 cells were pretreated with chloroquine for 18 hr and then stimulated with 100 ng/ml of EGF for 3 hr followed by immunofluorescence staining and confocal microscopy. In unstimulated cells, diffused membrane staining of EGFR and plasma membrane staining of CADM1 were observed. Treatment with EGF resulted in punctuate cytoplasmic localization for EGFR and CADM1. The image showed that CADM1 and EGFR were co-localized in endocytic compartments displaying bubble-like patterns after EGF stimulation (Fig. 11). Triple-staining with LAMP1, a lysosome marker, further confirmed that these bubble-like organelles were lysosome. These data indicate that CADM1 and EGFR were accumulated at lysosome by the treatment with chloroquine and EGF, suggesting that CADM1 and EGFR were internalized and degraded together through lysosomal pathway by the EGF

stimulation.

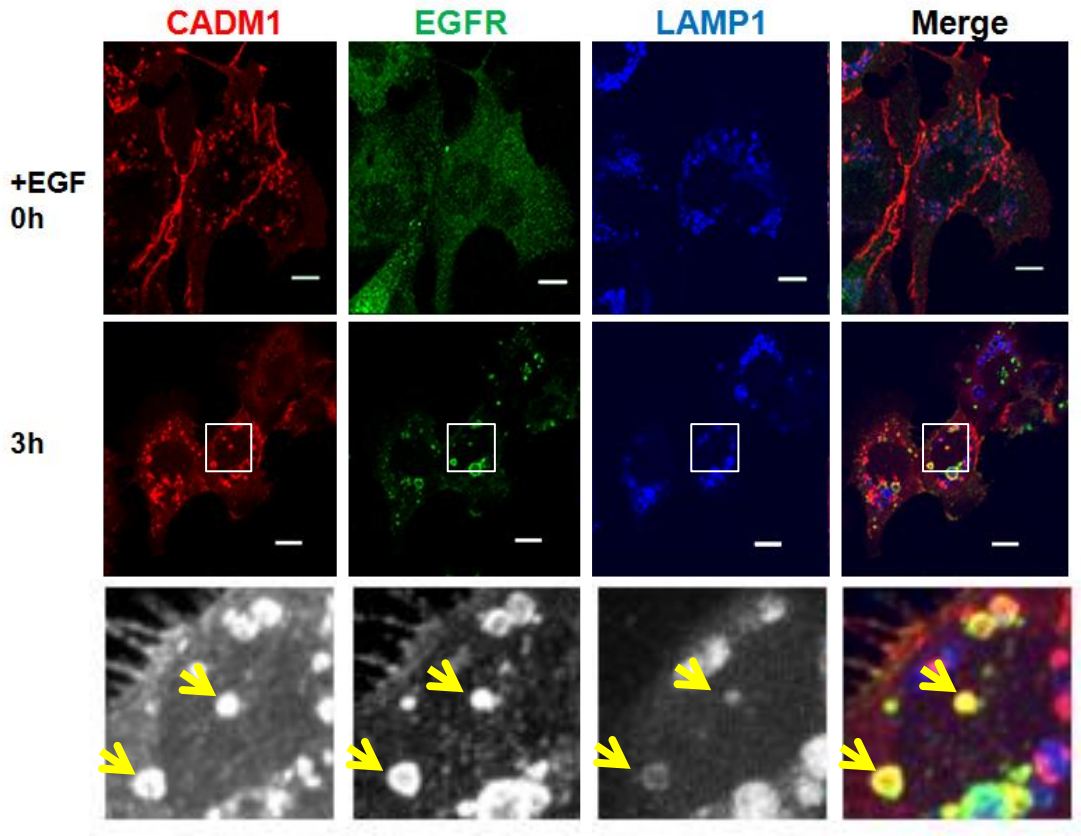


Figure 11. Reduction of EGFR expression induced by exogenously expressed CADM1 is through lysosomal-degradation pathway.

NCI-H1838 cells were plated on coverslips and maintained in normal condition for 24 hrs. Cells were pre-treated with chloroquine (200 μM) for 18 hrs followed by stimulated with EGF (100 ng/ml) for 0 hr (upper) or 3 hrs (middle). The cells were fixed followed by immunostaining for CADM1, EGFR and LAMP1. The coverslips were mounted on slides and analyzed using confocal microscopy. Red color indicates CADM1, green color represents EGFR, and blue color represents LAMP1. Merged image was shown in the right. The lower panel shows the enlarged images as indicated by the white squares respectively. Bar, 10μM.

4. CADM1 facilitates EGFR degradation through Clathrin-dependent pathway

It is well studied that EGFR was endocytosed through clathrin-dependent pathway. However, some studies performed on A431 cell line showed that EGFR was endocytosed through a clathrin-independent pathway, micropinocytosis [70,71]. These reports pointed out the possibility that the degradation pathway of EGFR might vary due to cell line differences.

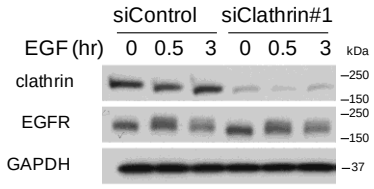
Here, I intended to identify the molecular mechanism of CADM1-facilitated EGFR degradation. First, to investigate whether the endocytosis of EGFR in NCI-H1838 cells is through clathrin-dependent pathway, knockdown of clathrin was achieved by siRNA (siClathrin#1, #2). The immunoblot analysis of clathrin showed that clathrin expression was down-regulated efficiently (Fig. 12A, C). After EGF treatment for 0.5 hr, EGFR expression was down-regulated in control cells. Conversely, EGFR expression in clathrin knockdown cells was up-regulated compared with unstimulated cells (Fig. 12A, B, C, D). These results indicated that EGFR is degraded through clathrin-dependent pathway in NCI-H1838 cell line.

To further investigate the role of CADM1 in EGFR reduction, expression of EGFR was examined in NCI-H1838 cells when CADM1 is knockdown by siCADM1#5 or #8. The immunoblot analysis indicated that CADM1 expression was down-regulated (Fig. 12E). When these cells were treated with EGF for 0.5 hr, the expression of

EGFR in siCADM1-treated cells were higher as compared with that of unstimulated cells (Fig. 12F). This result confirms that CADM1 facilitates the degradation of EGFR by EGF stimulation.

Next, NCI-H1838 cells were transfected with siClathrin or siCADM1 or both. After EGF treatment for 0.5 hr, EGFR expression in clathrin knockdown cells and CADM1 knockdown cells was up-regulated compared to unstimulated cells. These results show that both clathrin and CADM1 participate in the internalization of EGFR. However, there has no synergistic effect in EGFR expression in cells co-transfected with siClathrin and siCADM1 compare to cells transfected with only siClathrin or siCADM1 (Fig. 12G). Here I propose that CADM1 facilitates EGFR degradation through clathrin-dependent pathway.

A



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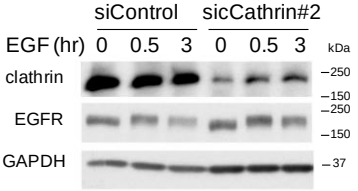


B

	(EGF)	1	2	3	
siControl	0h	0.72	0.39	0.74	
	0.5h	0.72	0.36	0.61	
	3h	0.49	0.24	0.64	
siClathrin#1	0h	0.63	0.37	0.65	p=0.007
	0.5h	0.84	0.51	0.85	
	3h	0.69	0.22	0.84	

Ratio

	(EGF)	1	2	3	ave
siControl	0h	1	1	1	1±0
	0.5h	1	0.92	0.82	0.92±0.09
	3h	0.68	0.62	0.86	0.72±0.13
siClathrin#1	0h	1	1	1	1±0
	0.5h	1.33	1.38	1.31	1.34±0.04
	3h	1.10	0.59	1.29	0.99±0.36

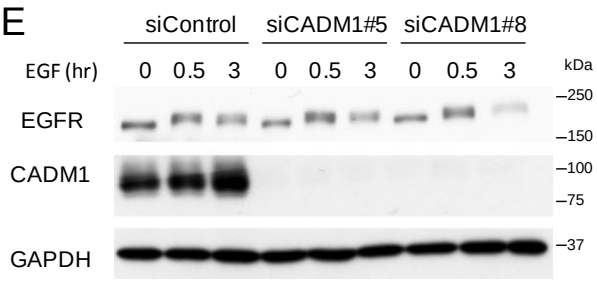
D

	(EGF)	1	2	3	
siControl	0h	0.86	0.65	0.77	
	0.5h	0.69	0.71	0.80	
	3h	0.45	0.34	0.51	
siClathrin#2	0h	0.62	0.60	0.77	p=0.009
	0.5h	0.77	0.83	0.98	
	3h	0.55	0.39	0.60	

Ratio

	(EGF)	1	2	3	ave
siControl	0h	1	1	1	1±0
	0.5h	0.81	1.10	1.04	0.98±0.15
	3h	0.52	0.52	0.66	0.57±0.08
siClathrin#2	0h	1	1	1	1±0
	0.5h	1.2	1.4	1.3	1.3±0.09
	3h	0.9	0.7	0.8	0.78±0.12

E



F

	(EGF)	1	2	3	4	
siControl	0h	0.91	0.49	1.07	2.61	
	0.5h	0.93	0.45	1.13	2.46	
	3h	0.54	0.31	0.99	1.29	
siCADM1#5	0h	0.97	0.34	1.17	0.93	p=0.039
	0.5h	1.19	0.49	1.36	1.56	
	3h	0.65	0.33	0.84	0.80	
siCADM1#8	0h	0.69	0.41	1.16	1.02	p=0.027
	0.5h	1.16	0.45	1.60	1.29	
	3h	0.27	0.19	1.10	0.52	

Ratio

	(EGF)	1	2	3	4	ave
siControl	0h	1.00	1.00	1.00	1.00	1±0
	0.5h	1.03	0.92	1.05	0.94	0.98±0.06
	3h	0.60	0.63	0.92	0.50	0.66±0.18
siCADM1#5	0h	1.00	1.00	1.00	1.00	1±0
	0.5h	1.23	1.44	1.16	1.68	1.38±0.24
	3h	0.67	0.97	0.71	0.87	0.8±0.14
siCADM1#8	0h	1.00	1.00	1.00	1.00	1±0
	0.5h	1.67	1.09	1.38	1.27	1.35±0.24
	3h	0.38	0.46	0.95	0.51	0.58±0.26

G

	(EGF)	1	2	3	
siControl	0h	0.72	0.74	0.58	
	0.5h	0.7	0.64	0.51	
siCADM1	0h	0.36	0.67	0.65	p=0.037
	0.5h	0.42	0.83	0.74	
siClathrin	0h	0.69	0.63	0.7	p=0.005
	0.5h	0.93	0.86	1.01	
siCADM1+siClathrin	0h	0.36	1.68	0.74	
	0.5h	0.52	1.92	0.77	

Ratio

	(EGF)	1	2	3	ave
siControl	0h	1	1	1	1±0
	0.5h	0.97	0.86	0.88	0.9±0.06
siCADM1	0h	1	1	1	1±0
	0.5h	1.17	1.24	1.14	1.18±0.05
siClathrin	0h	1	1	1	1±0
	0.5h	1.35	1.37	1.44	1.39±0.05
siCADM1+siClathrin	0h	1	1	1	1±0
	0.5h	1.44	1.14	1.04	1.21±0.21

Figure 12. CADM1 facilitates EGFR degradation through clathrin-dependent pathway in NCI-H1838 cells.

(A, C) Depletion of clathrin was performed in NCI-H1838 cells. Cells were transfected with control or anti-clathrin siRNA (siClathrin#1,A; siClathrin#2, C). Cells were grown for 54 hrs under normal conditions and then serum starved for 18 hrs. Subsequently, cells were treated with EGF (100 ng/mL) for different time as described, cell lysate was prepared and immunoblot was performed. anti-clathrin (upper), anti-EGFR (middle), anti-GAPDH (lower) antibodies were used for detection.

(B, D) Quantification of EGFR in A and C. Signal intensities of immunoblotting of EGFR were normalized to those of GAPDH. Relative intensities to 0 hr (ratio) were shown. Data shows mean ±SD of 3 independent experiments. p value was calculated by paired t-test.

(E) Imunoblot analysis of cells knockdown of CADM1.Cells were transfected with control or anti-CADM1 siRNA (siCADM1#5, #8). Cells were grown for 54 hrs under normal conditions and then serum starved for 18 hrs. Subsequently, cells were treated with EGF (100 ng/mL) for different time as described. Cell lysate was prepared and immunoblot was performed. anti-EGFR, anti-CADM1, and anti-GAPDH antibodies were used for detection.

(F) Intensities of EGFR were normalized to those of GAPDH. Relative intensities to 0 hr (ratio) were shown. Data shows mean ±SD of 4 independent experiments. p value was calculated by paired t-test.

(G) Depletion of clathrin or CADM1 or both were performed in NCI-H1838 cells. After transfected with siCADM1#5, or siClathrin#1, or both, cells were grown for 54 hrs under normal conditions and then serum starved for 18 hrs. Subsequently, cells were treated with EGF for 0.5 hr, and cell lysate was prepared and immunoblot was performed. Here showed the quantification of EGFR intensity. Intensities of EGFR were normalized to those of GAPDH. Relative intensities to 0 hr (ratio) were shown. Data shows mean ±SD of 3 independent experiments. p value was calculated by paired t-test.

Discussion

In the present study, I have demonstrated that exogenous expression of CADM1 in NSCLC cell lines with wild-type EGFR, NCI-H1838, NCI-H1993, and A549, induced down-regulation of EGFR without affecting the expression level of EGFR mRNA. This finding suggests that CADM1 down-regulated EGFR protein expression is a common phenomenon in NSCLC cell lines. Furthermore, CADM1 also down-regulated the EGFR expression in an epidermoid carcinoma cell line, A431 with wild-type EGFR. These data further indicate that CADM1 down-regulates wild-type EGFR protein expression in carcinoma cell lines. In addition, when cells were treated with EGF, CADM1 facilitated degradation of EGFR through clathrin-dependent pathway. Overall, CADM1 down-regulates EGFR expression in NSCLC cell lines expressing the wild-type EGFR by facilitating the degradation of EGFR. Taken together, I propose a model that CADM1 facilitates the degradation of EGFR by EGF treatment (Fig. 13) and keeps the homeostasis in terms of the amount of EGFR.

The findings presented in this study would be the first demonstration that the tumor suppressor, CADM1, and the oncoprotein, EGFR, functionally interact with each other. Exogenous expression of CADM1 induced down-regulation of EGFR and facilitated EGFR internalization.

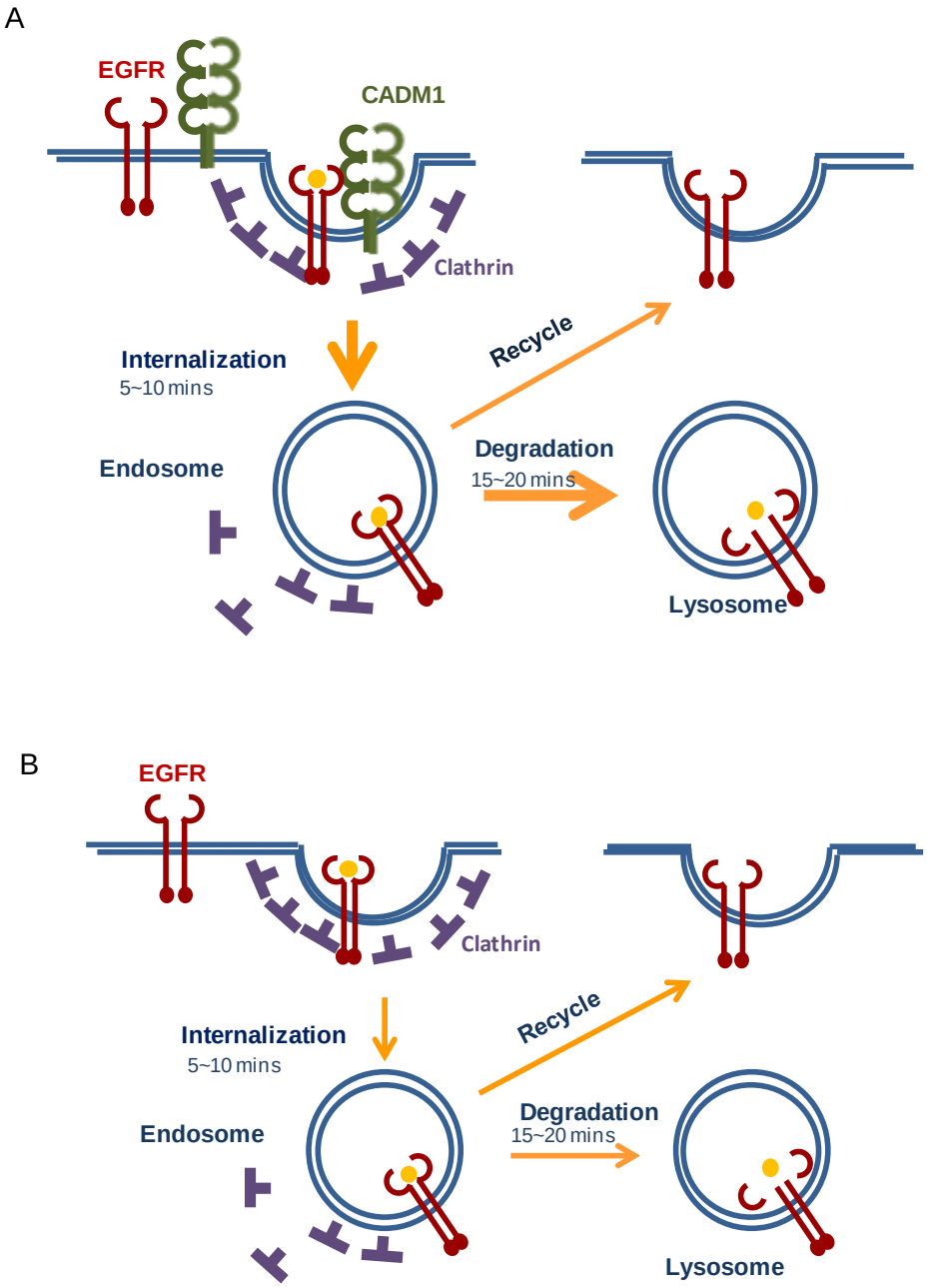


Figure 13. The hypothesis that CADM1 effects on EGFR internalization.

(A) In normal cells, EGF-stimulated EGFR is auto-phospharylated and transported into endosome through clathrin-dependent endocytosis pathway. At this stage, EGFR might sort into two different pathways, recycling or degradation pathways. In cells expressing CADM1, CADM1 facilitates EGFR go through the degradation pathway. Thus, the total amount of EGFR keeps in homeostasis.

(B) In cells lost CADM1 expression, the degradation of EGFR was retarded, and results in accumulation of EGFR for continuous activation of signaling pathway.

CADM1 has been identified as a tumor suppressor gene in various cancers. Loss of CADM1 expression has been observed in metastasis region of NSCLC [69], and is correlated with poor prognosis [72]. However, the precise molecular mechanism of CADM1 in decreasing tumorigenecity is still unclarified. Tumorigenesis is a multistage process which includes uncontrolled cell proliferation and metastasis. Since CADM1 functions as an adhesion protein, and disruption of cell adhesion is an initial step of cancer metastasis. These suggest that CADM1 might suppress tumorigenesis through inhibit metastasis. Indeed, we have indicated that re-expressed CADM1 in A549 cell line reduced the metastasis from spleen to liver [18,29]. Furthermore, CADM1 inhibited the Epithelial-mesenchymal transition (EMT), which was considered as the initiating process of metastasis induced by HGF stimulation [73]. These studies indicated that CADM1 suppresses tumor metastasis through inhibition of EMT. However, re-expressing CADM1 in A549 cells reduced the volume of xenografted tumors [28]. This study implies that the tumor suppressor activity of CADM1 is not only in inhibition of metastasis but also in regulation of cell proliferation. Indeed, it has been reported that re expressing CADM1 decreased cell viability in various carcinoma cell lines [61-64]. In this study, exogenous expression of CADM1 induced down-regulation of EGFR proteins. This phenomenon was observed not only in the NSCLC cell lines, NCI-H1838, NCI-H1993, and A549 (Fig.

6), carrying wild-type EGFR, but also in an epidermoid carcinoma cell line A431 overexpressing the EGFR (Fig. 9). These data indicated that down-regulation of EGFR protein expression by CADM1 overexpression is a common phenomenon in carcinoma cell lines. Since EGFR triggers signals for cell proliferation and promotes tumorigenesis, these results imply that CADM1 functions as a tumor suppressor passively through down-regulation of EGFR expression. Furthermore, the results of deletion mutants of CADM1 showed that the cytoplasmic domain plays an essential role in the degradation of EGFR. This was consistent with the previous study that CADM1 lacking its cytoplasmic domain within the adhesion function lost the tumor suppressor activity [28]. Taken together, this study indicated that CADM1 down regulated EGFR protein expression and the cytoplasmic domain of CADM1 is essential for this regulation.

Moreover, confocal images indicated that CADM1 and EGFR co-localized at lysosome with EGF stimulation (Fig. 11). These data indicated that CADM1 and EGFR undergo lysosomal dependent degradation. Ligand binding induce internalization and degradation of EGFR. Although clathrin-dependent and -independent pathways both have been reported, it is well considered that clathrin-dependent pathway is the main mechanism of endocytosis for EGFR. Simultaneous knockdown in protein expression of clathrin and CADM1 showed no

synergistic effect in EGFR down-regulation (Fig. 12G). These data indicated that CADM1 down-regulates EGFR protein expression, partly through clathrin-dependent endocytosis and lysosomal degradation.

Noteworthy, exogenous expression of CADM1 in NSCLC cell lines, HCC827 and PC9, carrying a mutant form of EGFR did not reduce the protein expression level of EGFR. Both HCC827 and PC9 cells express a mutant EGFR lacking a fragment corresponding to exon 19 and show constitutive activation of EGFR. I have shown that CADM1 facilitated the degradation of the wild-type EGFR through clathrin-dependent pathway. This implies that CADM1 might participate in the endocytosis of EGFR. However, several studies have reported that endocytosis induced by ligand binding was impaired in NSCLC cells expressing mutants EGFR and that mutant EGFR expressed in PC9 cells was not degraded but sorted for recycle in comparison with wild-type EGFR expressed in H1666 cells [74,75] . These data also support the hypothesis that CADM1 down-regulates EGFR protein expression by modulating the process of endocytosis.

EGFR is a well-known oncogene product in various cancers, and overexpression of EGFR correlates with tumor progression and poor prognosis. Since EGFR is a key molecule to regulate cell proliferation and tumorigenesis, the signal transduction of EGFR should be elaborately controlled. These complex cascades controlling EGFR

signaling include various stimuli, the amount of the ligand, the duration of ligand binding, and the cytoplasmic interacting proteins [76]. In recent years, the roles of dysregulated internalization of EGFR in EGFR signaling have been investigated intensively. Dysregulated intracellular trafficking of EGFR results in mislocation and poor down-regulation of EGFR are associated with enhanced signaling [77], which can lead to the development of cancer [78]. Indeed, several oncoproteins have been shown to affect EGFR trafficking. For example, Vav2, the Rho GTPase guanine nucleotide exchange factor, regulates cytoskeletal dynamics, leading to modulation of cell adhesion, motility, and proliferation. Exogenous expression of Vav2 delayed EGFR internalization and degradation, and enhanced EGFR, ERK, and Akt phosphorylation [79]. In addition, Sprouty2 and MIG6 regulates EGFR signaling through regulating EGFR trafficking in PC9 cells and H1666 cells [75]. Furthermore, a recent study in a NSCLC cell line, A549, has indicated that β1 integrin regulates EGFR signaling by sustaining the endocytosis mechanism required for normal EGFR signaling [80]. These papers indicate that trafficking of EGFR (endocytosis) is an important mechanism to regulate EGFR signaling. In the present study, I indicated that CADM1 facilitated EGFR degradation through clathrin-dependent pathway. Therefore, the internalization of EGFR mediated by CADM1 appeared to be one of the important mechanisms to down-regulate EGFR signaling for tumor suppression.

Based on the understanding of the molecular biology of lung cancer, new targeted therapy has been established for the clinical treatment. Targeting EGFR is an important treatment modality for NSCLC, and the tyrosine kinase inhibitors, such as gefitinib and erlotinib, were approved and clinically used widely. However, these inhibitors are only effective in tumors with specific types of EGFR mutations. This means that more than 50% of the patients who harbor the tumors showing overexpression of wild-type EGFR are still treated with classic chemotherapy that is generally more toxic with limited benefits. In this study, I have identified that overexpression of CADM1 reduced wild-type EGFR protein expression. This study implies that re-expressing CADM1 is a potential therapy for NSCLC patients with wild-type EGFR. A recent study has indicated that overexpression of CADM1 by adenovirus-mediated gene transfer resulted in inhibition of hepatocellular carcinoma growth in nude mice xenografted with Huh7 liver cancer [63]. These reports suggest that restored expression of CADM1 could be a potential new therapy for treatment of NSCLC.

In conclusion, my study indicates that expression of CADM1 in NSCLC cell lines with wild-type EGFR down-regulated EGFR expression. This phenomenon is also observed in A431 cells with EGFR amplification. This would shed lights on the possibility that the restoration of CADM1 expression could provide a novel approach

to anti-EGFR therapy. Taken together, this paper proposes a new viewpoint of a cell adhesion protein, CADM1, in which CADM1 may participate in the process of endocytosis and regulate protein degradation.

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