

## The Cell to Cell Interaction of Breast Cancer Cells Regulates Cancer Invasion Via ADAM15

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### ABSTRACT

Increasing evidence suggests that a disintegrin and metalloproteinase 15 (ADAM15) have essential roles in the process of cancer metastasis via degradation of the extracellular matrix and binding to integrins. Among them, ADAM15 possesses an Arg-Gly-Asp (RGD) sequence within its disintegrin domain (d.d., hereafter) and binds to RGD recognizing-integrins such as  $\alpha v \beta 3$  and  $\alpha 5 \beta 1$  and also interacts with integrin  $\alpha 9 \beta 1$  in RGD-independent manner. Although these integrins play important roles in the process of cancer metastasis, the role of the interactions between ADAM15 and integrins during processes of cancer metastasis remains to be elucidated. We produced the specific antibody (8F7) that interferes with the interaction of human ADAM15 and integrin receptors and performed in vitro aggregation assay, invasion assay, proliferation assay, proteinase activity assay and cell-cell adhesion assay. 8F7 inhibited tumor cell aggregation, invasion and migration, but not proliferation of breast cancer cells and proteinase activity of ADAM15. Furthermore, the interactions between ADAM15 and integrin receptors induced collective cell migration, phosphorylation of Akt, which was known to promote invasion of breast cancer cells. These data suggested that the binding of ADAM15 to  $\alpha v \beta 3$  or  $\alpha 9 \beta 1$  integrins through its d.d. Induces cell aggregation, migration and invasion of human breast cancer cells with concomitant activation of Akt signaling pathway.

**Keywords:** ADAM15, Integrins, Cell to Cell Interaction, Cell Motility, Phosphorylation of Akt

### 1. INTRODUCTION

In western countries, breast cancer is developed in the 10 % of women and 20% of these breast cancer patients died by metastasis (Weigelt *et al.*, 2005). The process of metastasis consists of various steps, in which tumor cells escape from primary tumor sites by the degradation of the basement membrane which consists of Extracellular Matrix (ECM) including collagen type IV and laminin and invade into blood or lymphatic vessels

and reach to target organs and establish metastatic foci. Therefore, cell motility and proteolytic activity are necessary for tumor cells to metastasize. Increasing evidence suggests that Matrix Metalloproteinases (MMPs) have essential roles in invasion. For instance, tumor cells control their proteolytic activity at the cell surface by inducing binding of tumor-derived MMP-2 and MMP-9 to  $\alpha v \beta 3$  and  $\alpha v \beta 5$  integrin, expressed on tumor cell surface, respectively (Bjorklund and Koivunen, 2005; Deryugina and Quigley, 2006).

Integrins are a family of heterodimeric cell surface receptors, consisting of  $\alpha$  and  $\beta$  subunit. At least, eighteen  $\alpha$  subunits can associate with eight  $\beta$  subunits to form 24 distinct integrins. They bind to various ECM proteins and contribute to the cell adhesion, proliferation, survival and migration (Hynes, 2002). Recently, many reports suggested that not only MMPs, but also a Disintegrin and Metalloproteinases (ADAMs) are integrin-binding proteinases. ADAMs are transmembrane glycoproteins, which consist of a metalloproteinase domain with catalytic activity, a disintegrin domain (d.d., hereafter) that interacts with several integrins, a cysteine-rich domain, an Epidermal Growth Factor (EGF)-like domain and a cytoplasmic tail (Seals and Courtneidge, 2003). Among the ADAM family, only human ADAM15 possesses an Arg-Gly-Asp (RGD) sequence within its d.d. (Herren *et al.*, 1997; Kratzschmar *et al.*, 1996; Lum *et al.*, 1998) and binds to RGD recognizing-integrins such as  $\alpha v \beta 3$  and  $\alpha 5 \beta 1$  (Nath *et al.*, 1999; Zhang *et al.*, 1998) and also interacts with other integrin,  $\alpha 9 \beta 1$  in RGD-independent manner (Eto *et al.*, 2000; 2002; Lu *et al.*, 2006). The expression of ADAM15 has been shown to be up-regulated in breast tumors relative to normal tissue (Kuefer *et al.*, 2006). Furthermore, ADAM15 has a potential activity as a proteinase whose substrates are collagen typeIV, gelatin (Martin *et al.*, 2002), heparin-binding EGF (Hart *et al.*, 2005; Ohtsu *et al.*, 2006) and E-cadherin (Najy *et al.*, 2008b). Thus, ADAM15 seems to be involved in the tumor cell invasion (Zhong *et al.*, 2008). On the other hand, integrins that interact with ADAM15 also play important roles in the process of cancer metastasis.

Alphavbeta3 integrin is involved in anchorage independent survival of cancer cells through Src activation (Desgrosellier *et al.*, 2009) and tumor angiogenesis through up-regulation of Vascular Endothelial cell Growth Factor (VEGF) (Chakraborty *et al.*, 2008). Alpha9beta1 integrin also promotes the invasion of breast cancer cells *in vitro* (Allen *et al.*, 2011) and is expressed by lymphatic and vascular endothelial cells and interacts with VEGF-A, C and D, thereby involved in angiogenesis, lymphangiogenesis and metastasis (Vlahakis *et al.*, 2005; 2007). However, it should be pointed out that many reports demonstrated that ADAM15 mediates cell to cell interactions via binding to integrins (Charrier *et al.*, 2007; Eto *et al.*, 2000; Herren *et al.*, 2001), which suggest that tumor cell migration or invasion from primary tumor sites into surrounding tissues might be inhibited by those ADAM15-mediated cell to cell interactions. In sharp contrast, the concept of collective cell migration

(invasion of cancer cells as a mass rather than single cell) was introduced for the explanation of tumor invasion and metastasis (Friedl and Gilmour, 2009; Friedl and Wolf, 2008). Nevertheless, those single cell invasion and collective cell migration may occur at the same time and may not be mutually exclusive. Thus, to clarify the functional roles of cell to cell interactions between ADAM15 and integrins in human breast cancer cells, we generated a monoclonal antibody specifically recognizing the d.d. of human ADAM15 where binding domains for various integrins reside. We demonstrate here that cell to cell interactions between human breast cancer cells via their own ADAM15 and RGD-recognizing and non RGD-recognizing integrins favor human breast cancer cell invasion and induce activation of Akt kinase.

## 2. MATERIALS AND METHODS

### 2.1. Cell Lines and Cell Culture

Human breast cancer cell line MDA-MB-231 luc-D3H2LN (D3H2LN, hereafter) was purchased from Caliper Life sciences (Hopkinson, MA) and cultured in MEM (GIBCO, Carlsbad, CA) supplemented with 10% fetal bovine serum, 1% non-essential amino acid (GIBCO) and 1% sodium pyruvate (Wako, Osaka, Japan). Human breast cancer cell line with potent lymphatic metastatic ability, MDA-MB-468LN (468LN, hereafter) (Vantyghem *et al.*, 2005) was cultured in MEM-Alpha (GIBCO) supplemented 10% fetal bovine serum. These cells were maintained in a 5% CO<sub>2</sub> atmosphere at 37°C and passage 12-14 were used in this study. Human  $\alpha 9$  integrin over-expressing CHO cells ( $\alpha 9$ /CHO, hereafter) were established as previously described (Ito *et al.*, 2009) and human  $\beta 3$  integrin over-expressing CHO cells ( $\beta 3$ /CHO, hereafter) were established by Dr. Yoshikazu Takada (Eto *et al.*, 2000). We confirmed that both D3H2LN and 468LN cells express full-length, but not truncated form of ADAM15 (data not shown). Human ADAM15 over-expressing CHO cells (ADAM15/CHO) was established as described below. These CHO cells were cultured in DMEM F-12 Ham's (Wako) containing 5% fetal bovine serum.

### 2.2. Construction of a Human ADAM15 d.d. and Preparation of Recombinant Protein

A cDNA fragment encoding an ADAM15 d.d. was amplified by PCR with cDNA library of HUVEC using 5'-CCTATGGCTGCTTTCTGC-3' and 5'-CATGCACA CAGCTTGCCC-3' as primers. The fragment was ligated into pCRII-TOPO vector (Invitogen, Carlsbad, CA) and amplified by PCR using 5'-AAGGATCCGCTTT

CTGCGGA-3' and 5'-ATTCTCGAGATCCCCTAGGCTGACAT-3' to add the restriction sites for BamHI and XhoI. The fragment was ligated into pGEX-6P-1 vector (GE Healthcare Biosciences, Uppsala, Sweden) which was digested with BamHI and XhoI (designated as d.d./pGEX-6P-1, hereafter). For construction of d.d.-RAA, in which the RGD sequence is mutated into RAA, PCR was performed using d.d./pGEX-6P-1 as a template with two primers listed below:

- Forward:  
5'-CAGTGTCTACCAGAGCTGCTGTGACTTGCTG-3'
- Reverse:  
5'-CAGCAAGTCACAAGCAGCTCTGGTAGGACGACACTG-3'.

The fragment was digested with *DpnI* according to the manufacturer's instruction of QuickChange Site-Directed Mutagenesis Kit (Invitrogen) and ligated into pGEX-6P-1 vector (designated as d.d.-RAA/pGEX-6P-1, hereafter). The d.d./pGEX-6P-1 or d.d.-RAA/pGEX-6P-1 was transformed into *E. Coli*, JM609 and expressed as GST-fusion protein as described (Ito *et al.*, 2009). For adhesion assay, GST was removed with PreScission Protease (GE Healthcare Biosciences) according to the manufacturer's instruction.

### 2.3. Production of an Anti-Human ADAM15 d.d. Monoclonal Antibody (8F7)

BALB/c mice were purchased from Japan SLC (Hamamatsu, Japan). All mice were housed in a specific pathogen-free barrier facility and screened regularly for pathogens. All studies and procedures were approved by the committee on animal experimentation of Institute for Genetic Medicine, Hokkaido University. These mice were immunized with GST-fusion protein of human ADAM15 d.d. and splenocytes were fused with X63-Ag8-653 mouse myeloma cells using PEG (IBL, Gunma, Japan). Hybridoma cells were selected by ELISA using GST-fusion protein of human ADAM15 d.d. and a clone of hybridoma cells, 8F7 was established due to its binding specificity to ADAM15 d.d., but not to other portions of ADAM15.

### 2.4. Establishment of Stable Transfectants of Human ADAM15

A cDNA of full-length form of human ADAM15 was amplified by PCR with human ADAM15/pOTB7 vector (Invitrogen) using 5'GGGGACAAGTTTGTACAAAAA GCAGGCTATATGCGGTTGGCGCTGCTCT-3' and 5'GGGGACCACTTTGTACAAGAAAGCTGGGTGCTAG AGGTAGAGCGAGGACAC-3. The PCR product was

inserted to pDONR 221 vector (Invitrogen) with BP Clonase II (Invitrogen). A human ADAM15 cDNA fragment in pDONR 221 vector was recombined to pEF5/FRT-DEST vector (Invitrogen) with LR Clonase II (Invitrogen) and transfected to Flp-in/CHO cells (Invitrogen) and stably expressing cells were selected by the culture in DMEM F-12 Ham's containing 500µg m<sup>-1</sup> of hygromycin (Invitrogen) and 5% fetal bovine serum. The expression of ADAM15 was examined by flow-cytometry analysis.

### 2.5. Flow-Cytometry Analysis

Cells were trypsinized, washed with PBS and blocked with fetal bovine serum in FACS buffer for 30 min on ice. Then, these cells (5×10<sup>5</sup> cells) were incubated with a mouse monoclonal antibody against human integrin α9β1 (clone: Y9A2, Millipore, Tokyo, Japan), αvβ3 (clone: LM609, Millipore), ADAM15 (clone: 23G9, R&D systems, Minneapolis, MN USA) or control IgG for 20min on ice. After washing with FACS buffer twice, these cells were treated with PE conjugated-goat anti-mouse IgG (Jackson ImmunoResearch Laboratories Inc. WestGrove, PA) and incubated for 20min on ice. These labeled cells were washed with FACS buffer twice and treated with 7-amino-actinomycin D (7-AAD) (Sigma-Aldrich, St Louis, MO, USA) for 15min on ice. After incubation, cells were washed with FACS buffer three times and were subjected to flow-cytometry analysis by FACS Caliber (Becton Dickinson, Tokyo, Japan).

### 2.6. Cell Migration and Invasion Assay

To evaluate cell invasion, Matrigel invasion chamber (Becton Dickinson) were used according to the manufacturer's instruction. Cultured D3H2LN cells were harvested and suspended in serum free culture media. These cells were pretreated with 8F7, Y9A2, LM609 or control IgG (20 µg mL<sup>-1</sup>) for 20min at 37°C-0.5-50µM of LY294002, a PI3K inhibitor (Sigma-Aldrich) or U0126, a MEK1/2 inhibitor (Merck, Tokyo, Japan) for 1h at 37°C. Then these cells (5×10<sup>4</sup> cells) were seeded onto the insert. As the chemoattractant, the conditioned culture media of HT-1080 cells was used (Johnson *et al.*, 1993). After 22h at 37°C, cells remained in the upper surface of the insert were removed using swab and cells in the under surface of the insert were fixed with methanol and stained with Giemsa's staining solution (MUTO PURE CHEMICALS CO. LTD. Tokyo, Japan). Six fields were selected at random and the number of the invading cells was counted and averaged for each study. To evaluate the collective cell migration, cell invasion into Matrigel was assessed according to the

previous report (Hotary *et al.*, 2000). 200 $\mu$ L of Matrigel (Becton Dickinson) was coated to the Millicell culture insert (Millipore) and  $5 \times 10^4$  cells, which were treated with antibodies ( $50 \mu\text{g mL}^{-1}$ ) were seeded to upper chamber. After 7 days, Matrigel cultures were fixed with 4% paraformaldehyde in PBS and embedded in paraffin. Sections ( $5 \mu\text{m}$  thick) were cut and stained with hematoxylin and eosin. To evaluate cell migration, cell migration assay with Millicell culture insert (Millipore) was performed. Cells were treated with antibodies and seeded as described above. In this assay 10% fetal bovine serum containing MEM was used as chemoattractant. After incubation for 4h at  $37^\circ\text{C}$ , migrated cells were counted by the same method, described in invasion assay.

### 2.7. Cell Proliferation Assay

Either D3H2LN cells or D3H2LN cells, transfected with siRNA (50pmol) for ADAM15 (SiRNA) (sense: aaccagcugucaccucgaaTT, antisense: uucgagggugacagcugguuTT, B-Bridge International Inc., Tokyo, Japan) or scramble siRNA as a control (ScrRNA) were suspended in MEM containing 10% fetal bovine serum and were seeded to the 96 well titer plate at the density of  $2.5 \times 10^3$  cells/well and culture for 24h at 5%  $\text{CO}_2$ ,  $37^\circ\text{C}$ . Then, culture media was changed to MEM containing 5% fetal bovine serum and antibody ( $20 \mu\text{g mL}^{-1}$  of 8F7 or control IgG). After 72h culture,  $10 \mu\text{L}$  of Cell counting kit-8 (Dojindo, Tokyo, Japan) was added to each well followed by the incubation for 2h at  $37^\circ\text{C}$ . The resulting supernatants from each well were analyzed by the immunoreader (Nalge Nunc, Tokyo, Japan) and absorbance at  $450\text{nm}$  was measured to determine the number of cells proliferated.

### 2.8. Cell Adhesion Assay and Cell-Cell Adhesion Assay

Cell adhesion to human ADAM15 d.d. was examined as previously described (Ito *et al.*, 2009). 96-well flat-bottom microtiter plates (Nalge Nunc) were coated with human ADAM15 d.d. or d.d.-RAA ( $5 \mu\text{g mL}^{-1}$ ) at  $37^\circ\text{C}$  for 1h and blocked with 0.5% BSA in PBS at room temperature for 1h. After washing with PBS, these wells were treated with 8F7 or control IgG ( $0-10 \mu\text{g mL}^{-1}$ ) at  $37^\circ\text{C}$  for 20min. Cells were trypsinized and suspended in 0.25% BSA containing DMEM F-12 Ham's at the density of  $2 \times 10^5$  cells/mL. Then,  $100 \mu\text{L}$  of cell suspension was added to each well and incubated at  $37^\circ\text{C}$  for 1h. After discarding non-adherent cells by washing with PBS, adherent cells were fixed and stained by 0.5% crystal violet in 20% methanol for 30min. These wells were gently washed with water and  $100 \mu\text{L}$  of 20% acetic acid was added to each well to lyse adherent cells and supernatants of each well is analyzed by the

immunoreader (Nalge Nunc) and absorbance at  $590\text{nm}$  was measured to determine the number of adherent cells.

Cell-cell adhesion assay was examined as previously described (Charrier *et al.*, 2007; Eto *et al.*, 2000).  $\alpha 9/\text{CHO}$ ,  $\beta 3/\text{CHO}$  or ADAM15/CHO cells ( $5 \times 10^4$  cells/well) were seeded to 96-well Black w/Lid flat-bottom microtiter plates (Nalge Nunc) and incubated at  $37^\circ\text{C}$  for 24h and blocked with 0.5% BSA in PBS. A single cell suspension of ADAM15/CHO or D3H2LN cells was labeled with Carboxy Fluorescein Succinimidyl Ester (CFSE) (Sigma-Aldrich) and suspended in 0.25% FCS containing media at the density of  $2 \times 10^5$  cells  $\text{mL}^{-1}$  and  $100 \mu\text{L}$  of cell suspension was added to each cell-coated well in the presence of antibody ( $20 \mu\text{g mL}^{-1}$  of 8F7, Y9A2, LM609 or control IgG). After incubation at  $37^\circ\text{C}$  for 1h, non-adherent cells were removed by the washing with PBS and  $100 \mu\text{L}$  of lysis solution (10mM Tris, 150 mM NaCl, 3mM EDTA, 1% Triton X-100) was added to each well. After 30min, supernatants of each well were analyzed using Biotek Synergy4 microplate reader (DS PHARMA BIOMEDICAL, Osaka, Japan) and absorbance at ex:  $492\text{nm}$ , em:  $520\text{nm}$  was measured to determine the number of adherent cells.

### 2.9. Cell Aggregation Assay

Cell aggregation assay was performed as previously reported (Zhang *et al.*, 2010). D3H2LN cells were suspended in serum free media and pretreated with 8F7, Y9A2, LM609 or control IgG ( $50 \mu\text{g mL}^{-1}$ ) at  $37^\circ\text{C}$  for 20min. These cells ( $3 \times 10^4$  cells/well) were seeded onto poly-HEMA coated plates and incubated  $37^\circ\text{C}$  for 24h. Four fields were selected at random, photographed and the number of the single cells was counted and averaged for each study.

### 2.10. Proteinase Activity Assay and Gelatin Zymography

The proteinase activity assay was performed according to the manufacturer's instruction of EnzChek Gelatinase/Collagenase Assay Kit (Molecular Probes). The cell lysates of Flp-in/CHO and ADAM15/CHO cells ( $100 \mu\text{g}$ ) were preincubated with 2mM of 4-Aminophenylmercuric Acetate (APMA) (Sigma-Aldrich), which is known as a MMP activator, at  $37^\circ\text{C}$  for 30min and then further incubated with 8F7 or control IgG ( $20 \mu\text{g mL}^{-1}$ ) or 1.10-Phenanthroline ( $0.5\text{mM}$ ) at  $37^\circ\text{C}$  for 20min. These samples were mixed with DQ gelatin ( $12.5 \mu\text{g mL}^{-1}$ ) and incubated at room temperature for 6h. To evaluate the proteinase activity, supernatants of each well were analyzed by Biotek Synergy4 microplate reader (DS PHARMA BIOMEDICAL) and absorbance at ex:  $485\text{nm}$ , em:  $515\text{nm}$  was measured to determine the amount of the proteinase-cleaved gelatin substrates. For gelatin zymography, D3H2LN cells were cultured on

Matrigel (Becton Dickinson) for 48h with 8F7 or control IgG ( $25\mu\text{g mL}^{-1}$ ) and supernatants were collected. These samples were concentrated using Amicon Ultra concentrators (Millipore). Then, equal volume of samples were loaded on gelatin zymogram gel and developed according to the manufacturer's instruction (Invitrogen).

### 2.11. Phosphorylation of Akt by Cell-Cell Interaction

D3H2LN cells ( $1.5\times 10^5$  cells/well) were seeded to 12-well culture plates (Becton Dickinson, Tokyo, Japan) and cultured at  $37^\circ\text{C}$  for 48h. Flp-in/CHO or ADAM15/CHO cells ( $2.0\times 10^5$  cells/well) were added to the monolayer of D3H2LN cells with or without  $10\mu\text{g}$  of inhibitor (antibodies, recombinant protein) and incubated at  $37^\circ\text{C}$  for 30min. After removal of the media, each well was washed with PBS and immediately lysed with  $100\mu\text{L}$  of RIPA buffer containing protease inhibitor (Roche, Tokyo, Japan). Cell lysates were centrifuged and supernatants were collected. The protein concentration was measured using Pierce BCA protein assay kit (Thermo SCIENTIFIC, Yokohama, Japan) and western blotting was performed as previously described (Nakayama *et al.*, 2010). To detect phosphorylation of Akt and Erk, rabbit monoclonal antibody against phospho Akt (193H2) or total Akt (C67E7) and mouse monoclonal antibody against phospho Erk (E10) or total Erk (3A7) (Cell Signaling Technology, Danvers, MA, USA) were used as primary antibody. After incubation with primary antibody, goat anti-rabbit or mouse IgG HRP conjugated (Jackson ImmunoResearch Laboratories Inc. WestGrove, PA) was used as secondary antibody.

### 2.12. The Statistical Evaluation of Data

Experimental data were analyzed by using ANOVA with post test analysis (PLSD). N.S. denotes no significant difference between two groups.

## 3. RESULTS

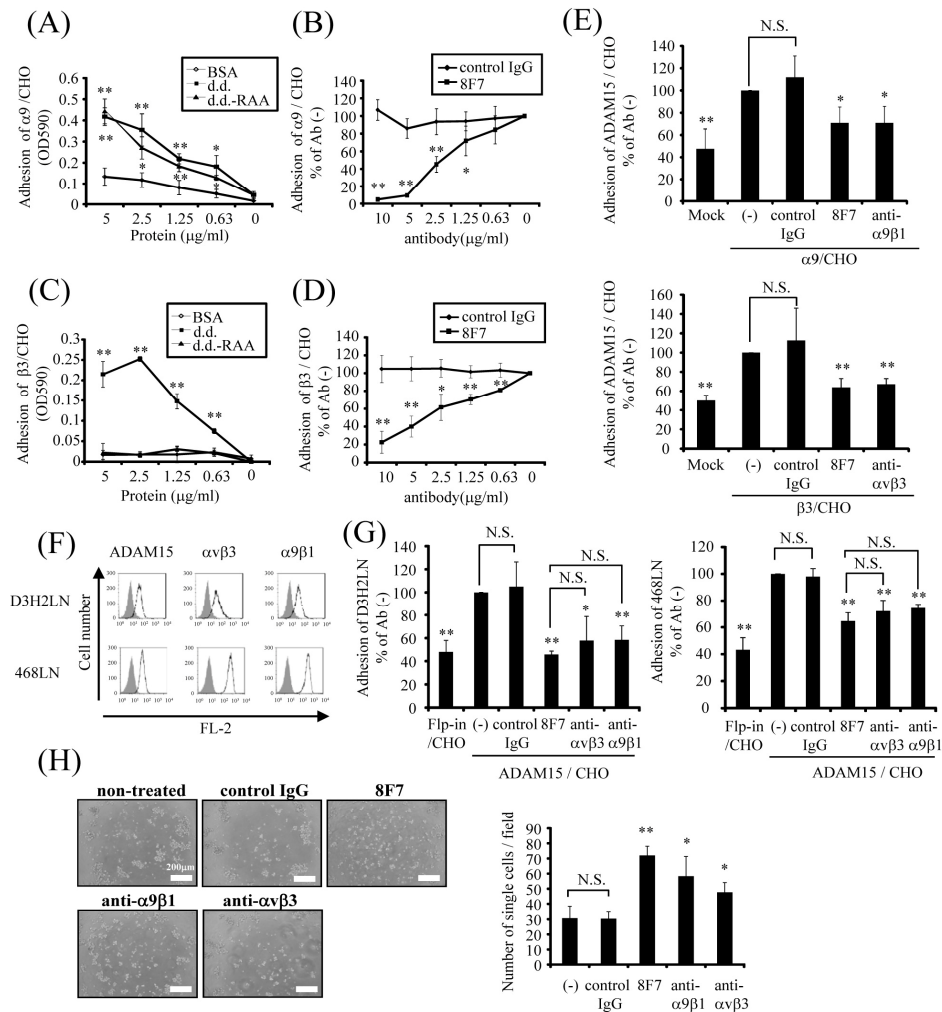
### 3.1. Both Exogenous and Endogenous Integrin $\alpha 9\beta 1$ and $\alpha v\beta 3$ Interact with ADAM15 Via its d.d

We first examined whether ADAM15 proteins could interact with integrin  $\alpha 9\beta 1$  and  $\alpha v\beta 3$  via d.d.. Thus, cell adhesion assay was performed using recombinant protein of ADAM15 d.d. or mutant d.d.-RAA (in which the RGD sequence is mutated to RAA to rule out the involvement of RGD recognizing integrins) and CHO cells that express  $\alpha 9$  integrin, one of the receptors for

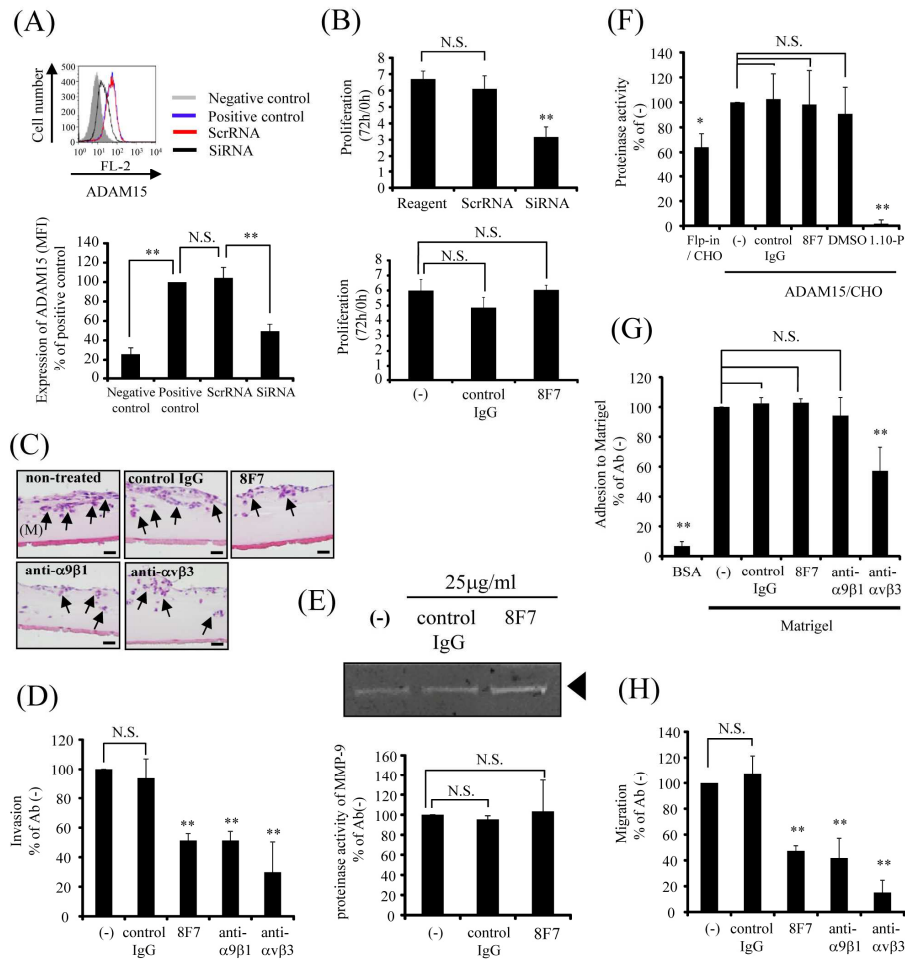
ADAM15 (**Fig. 1A**).  $\alpha 9/\text{CHO}$  (which express  $\alpha 9\beta 1$  integrin) adhered both to d.d. and d.d.-RAA (**Fig. 1A**), confirming that the RGD sequence is dispensable for the binding of ADAM15 to  $\alpha 9\beta 1$  integrin. We also found that  $\alpha 9/\text{CHO}$  cell adhesion to d.d.-RAA was inhibited by 8F7 in dose dependent manner (**Fig. 1B**). Furthermore,  $\beta 3/\text{CHO}$  (which express  $\alpha v\beta 3$  integrin) adhered to d.d., but not to d.d.-RAA (**Fig. 1C**), demonstrating that the binding of ADAM15 d.d. to  $\alpha v\beta 3$  integrin is RGD-dependent and this adhesion was also inhibited by 8F7 in dose dependent manner (**Fig. 1D**). Thus our results demonstrated that ADAM15 can interact with both non-RGD recognizing  $\alpha 9\beta 1$  integrin and RGD-recognizing  $\alpha v\beta 3$  integrin via its d.d..

Next we examined whether ADAM15,  $\alpha 9\beta 1$  and  $\alpha v\beta 3$  are involved in cell to cell interaction. Therefore, cell to cell adhesion assay was performed using ADAM15/CHO and  $\alpha 9/\text{CHO}$  or  $\beta 3/\text{CHO}$  cells (**Fig. 1E**). The adhesion of  $\alpha 9/\text{CHO}$  or  $\beta 3/\text{CHO}$  to ADAM15/CHO was significantly higher as compared to CHO which lacks  $\alpha 9$  and  $\beta 3$  integrins (Mock CHO). Cell bindings were significantly inhibited by antibody specific for  $\alpha 9\beta 1$  or  $\alpha v\beta 3$  integrin and importantly the adhesion of  $\alpha 9/\text{CHO}$  or  $\beta 3/\text{CHO}$  to ADAM15/CHO was similarly inhibited by 8F7. Results demonstrated that interaction of ADAM15 and integrins mediates cell to cell adhesion via d.d..

Those results led us to examine whether cell to cell adhesion via ADAM15 and integrins also operates in human breast cancer cells. First, the expression of ADAM15,  $\alpha 9\beta 1$  integrin and  $\alpha v\beta 3$  integrin in human breast cancer cell lines, D3H2LN and 468LN were analyzed by flow-cytometry. All molecules of interest were similarly expressed in two distinct human breast cancer cells (**Fig. 1F**). We then tested whether endogenously expressed integrins bind to ADAM15. The adhesion of D3H2LN to ADAM15/CHO was significantly increased as compared to control Flp-in/CHO (**Fig. 1G** left panel). These adhesions were inhibited by 8F7 and antibodies specific for  $\alpha 9\beta 1$  or  $\alpha v\beta 3$  integrin. In the same assay using another human breast cancer cell line, 468LN, similar results were obtained (**Fig. 1G** right panel). To test whether ADAM15 and integrins were involved in homotypic cell adhesion of breast cancer cells, cell aggregation assay using D3H2LN cells was performed. 8F7, anti- $\alpha 9\beta 1$  or anti- $\alpha v\beta 3$  integrin antibody significantly inhibited cell aggregation (**Fig. 1H**). These results suggested that the interactions between  $\alpha 9\beta 1$  or  $\alpha v\beta 3$  integrin and ADAM15 d.d. mediate cell to cell adhesion of breast cancer cells. The results led us to further test whether the cell to cell interactions between ADAM15 and integrins may regulate cancer cell functions.



**Fig. 1.** Alpha9beta1 and  $\alpha v\beta 3$  integrins expressed in tumor cells interact with ADAM15 and mediate cell-cell interaction. The adhesion of  $\alpha 9$ /CHO (A) or  $\beta 3$ /CHO (C) to ADAM15 d.d., d.d.-RAA or BSA-coated well ( $0-5\mu\text{g mL}^{-1}$ ). Bars indicate  $\pm$ S.D. from triplicate experiments. Experiments were performed three times independently. \*:  $p<0.05$  of d.d vs BSA, \*\*:  $p<0.01$  vs BSA. The adhesion of  $\alpha 9$ /CHO (B) or  $\beta 3$ /CHO (D) to ADAM15 d.d.-RAA (B) or d.d. (D) -coated well ( $2.5\mu\text{g mL}^{-1}$ ) in the presence of 8F7 at indicated concentrations. Bars indicate  $\pm$ S.D. from triplicate experiments. Experiments were performed three times independently. \*:  $p<0.05$  vs no antibody, \*\*:  $p<0.01$  vs no antibody. (E) Adhesion of ADAM15/CHO to monolayer of  $\alpha 9$ /CHO (upper panel) or  $\beta 3$ /CHO (lower panel) was examined in the presence of no antibody, control IgG, 8F7, anti- $\alpha 9\beta 1$  or  $\alpha v\beta 3$  integrin antibody ( $50\mu\text{g mL}^{-1}$ ). Bars indicate  $\pm$ S.D. from three independent experiments. \*:  $p<0.05$  vs no antibody, \*\*:  $p<0.01$  vs no antibody. (F) Flow-cytometry analysis of the expression of ADAM15,  $\alpha 9\beta 1$  and  $\alpha v\beta 3$  integrin in D3H2LN (upper panel) and in 468LN (lower panel). Shaded histogram indicates the staining of cells without primary antibody. Solid lines indicate the positive staining. (G) The adhesion of D3H2LN cells (left panel) or 468LN (right panel) to the monolayer of ADAM15/CHO in the presence of no antibody, control IgG, 8F7, anti- $\alpha 9\beta 1$  or  $\alpha v\beta 3$  integrin antibody ( $50\mu\text{g mL}^{-1}$ ). Bars indicate  $\pm$ S.D. from three independent experiments. \*:  $p<0.05$  vs no antibody, \*\*:  $p<0.01$  vs no antibody. (H) The aggregation of D3H2LN cells in the presence of no antibody, control IgG, 8F7, anti- $\alpha 9\beta 1$  or  $\alpha v\beta 3$  integrin antibody ( $50\mu\text{g mL}^{-1}$ ). Representative micrograph of aggregated cells was depicted (left panel) and the number of single cells was counted (right panel). Bars indicate  $\pm$ S.D. from three independent experiments. \*:  $p<0.05$  vs no antibody, \*\*:  $p<0.01$  vs no antibody



**Fig. 2.** ADAM15 d.d. enhanced invasion and migration, but not proliferation, adhesion and proteolytic activity of tumor cells *in vitro*. (A) Knock down of ADAM15 on D3H2LN cells by SiRNA. The effect of SiRNA was examined by flow-cytometry analysis. Shaded histogram (gray) indicates that non-treated cells were stained with control IgG (Negative control). Blue, red, or black lines indicated that non-treated (Positive control), ScrRNA-treated, or SiRNA-treated cells, respectively were stained with anti-human ADAM15 antibody, followed by secondary antibody (lower panel). The mean fluorescence intensity (MFI) was measured (right panel). \*\*: p<0.01. (B) The proliferation of D3H2LN cells treated with SiRNA for ADAM15 (upper panel) and 8F7 (lower panel) for 72h. Column indicates the fold increase of OD.450nm at 72h as compared to that at the time of culture initiation (0h). Bars indicate ±S.D. from three independent experiments. \*\*: p<0.01 vs non-treated cells. (C) Cell invasion into the Matrigel (M) was represented. Cell masses were indicated by arrows. Bar: 30µm (D) Matrigel invasion assay using D3H2LN cells. Bars indicate ±S.D. from three independent experiments. \*\*: p<0.01 vs no antibody. (E) Proteolytic activity of conditioned media derived from D3H2LN cells was examined by gelatin zymography (upper panel). Arrowhead indicates the band of pro-MMP-9. This intensity of band in the absence of any inhibitor was expressed 100 and other bands in the presence of inhibitors were expressed as relative to 100 (lower panel). Experiments were performed three times independently. (F) Proteinase assays using cell lysates (100µg) obtained from Flp-in/CHO and ADAM15/CHO in the absence or presence of inhibitors indicated. Gelatin (12.5µg mL<sup>-1</sup>) was used as a substrate. Bars indicate ±S.D. from three independent experiments. \*\*: p<0.01 vs no antibody. (G) Adhesion of D3H2LN cells to Matrigel in the presence of various antibodies indicated (20µg mL<sup>-1</sup>). Bars indicate ±S.D. from three independent experiments. \*\*: p<0.01 vs no antibody. (H) Migration of D3H2LN cells in the presence of various antibodies indicated (20µg mL<sup>-1</sup>). 10% FCS containing media was used as chemoattractant. Bars indicate ±S.D. from three independent experiments. \*\*: p<0.01 vs no antibody.

### 3.2. ADAM15 is Involved in Collective Cell Migration and Cell Invasion of Breast Cancer Cells

We first designed small interference RNA for human ADAM15 (SiRNA) to knock down the expression of endogenous ADAM15 molecules. As shown **Fig. 2A**, designed SiRNA could reduce the expression of ADAM15 significantly as compared to control scramble RNA (ScrRNA). The knock down of ADAM15 molecules by SiRNA significantly inhibited cell proliferation (2.1 fold reduction as compared to ScrRNA treatment) (**Fig. 2B** upper panel), indicating that ADAM15 molecule is involved in cell proliferation. However, 8F7 did not affect cell proliferation (**Fig. 2B** lower panel). These results suggested that ADAM15 molecule is involved in the proliferation of tumor cells in d.d.-independent manner. Next, the involvement of ADAM15 and its integrin receptors on single cell invasion and collective cell migration was assessed using human breast cancer cells. As shown **Fig. 2C**, it was shown that breast cancer cells invaded as a single cell or aggregates. However, the size of invading cells masses were smaller in 8F7 and anti- $\alpha 9\beta 1$  integrin or  $\alpha v\beta 3$  integrin antibody treated groups compared to non- or control IgG treated group. Next, the *in vitro* invasion assay was performed. 8F7 significantly reduced the invasion of D3H2LN (49% reduction as compared to non-treated group). Similarly, anti- $\alpha 9\beta 1$  integrin or  $\alpha v\beta 3$  integrin antibody reduced the invasion (49% and 30% reduction as compared to non-treated group, respectively) (**Fig. 2D**). Please be reminded that all 8F7, anti- $\alpha 9\beta 1$  integrin or anti- $\alpha v\beta 3$  integrin antibody inhibited cell aggregation (**Fig. 1H**) and collective cell migration (**Fig. 2C**) *in vitro*. Taken together, these result suggested that the cell to cell interaction between ADAM15 and its integrin receptors mediated collective cell migration and cell invasion.

### 3.3. ADAM15 Promote Breast Cancer Cell Migration Via d.d.

Matrigel, which contains collagen type IV, laminin, entactin and heparan sulfate proteoglycan was used to examine the cancer cell invasion. It has been shown that ADAM15 cleaves collagen type IV (Martin *et al.*, 2002) and regulates the expression of MMP-9 (Najy *et al.*, 2008a). Therefore, we tested whether ADAM15 regulates the expression of MMP-9 and catalytic activity of metalloproteinase domain of ADAM15 via d.d.. Thus, gelatin-zymography was performed using culture

supernatants obtained from D3H2LN cultured in the presence or absence of 8F7 antibody. D3H2LN-derived MMP-9 secretion was not affected by 8F7 (**Fig. 2E**). It should be noted that MMP-2 was not detected in this assay (data not shown). Next, the catalytic activity of ADAM15 was examined using DQ-gelatin, which releases luminescence upon cleavage by gelatinases such as MMP-2, 9, or ADAM15 (**Fig. 2F**). The lysate of ADAM15/CHO showed higher catalytic activity as compared to that of Flp-in/CHO, demonstrating that ADAM15 is involved in the cleavage of gelatin. Note that catalytic activity was inhibited by 1.10-phenanthroline, which is a broad spectrum inhibitor of metalloproteinases.

However, 8F7 did not affect the catalytic activity. These results suggested that catalytic activity of ADAM15 is not mediated by the d.d..

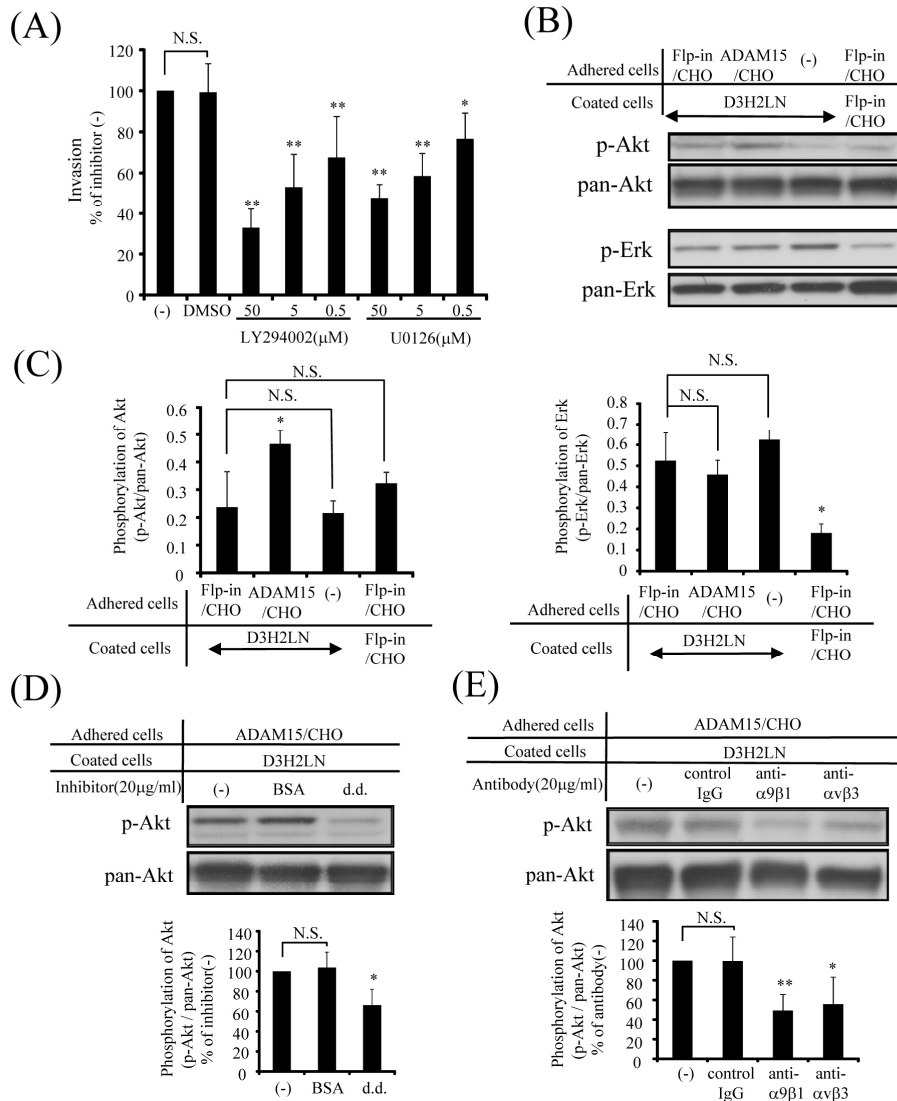
Adhesive and migratory abilities are also known to be involved in the processes of cell invasion. Thus, the involvement of d.d. in adhesion to Matrigel was examined using D3H2LN (**Fig. 2G**). D3H2LN showed significant adhesion to Matrigel as compared to BSA. However, 8F7 and anti- $\alpha 9\beta 1$  integrin antibody did not inhibit adhesion to Matrigel. On the other hand, anti- $\alpha v\beta 3$  integrin antibody significantly reduced adhesion to Matrigel (43% reduction as compared to non-treated group). Thus, it is unlikely that the interaction between ADAM15 d.d. and  $\alpha 9\beta 1$  or/and  $\alpha v\beta 3$  integrins on D3H2LN is involved in cell adhesion to Matrigel.

Next, the involvement of ADAM15 d.d. in migration was examined (**Fig. 2H**). 8F7 and anti- $\alpha 9\beta 1$  or  $\alpha v\beta 3$  integrin antibody reduced migration of D3H2LN (53, 59 and 85% reduction as compared to non-treated group, respectively). Therefore, it seems that cell to cell interaction of D3H2LN cells using its own ADAM15 d.d. and  $\alpha 9\beta 1$  or/and  $\alpha v\beta 3$  integrins may increase the cell motility.

### 3.4. Cell to Cell Interaction Via ADAM15 and $\alpha 9\beta 1$ or/and $\alpha v\beta 3$ Integrins Induces Akt Signaling Pathway

To investigate whether cell to cell interaction via ADAM15 and integrins, expressed on D3H2LN cells induces any intracellular signaling, we examined the Akt signaling pathway. First, the involvements of Akt and Erk signaling pathways in D3H2LN cell invasion were examined (**Fig. 3A**), because Akt and Erk are the downstream to PI3K and MEK1/2, respectively and were reported to be involved in cell migration (Guo and Giaccotti, 2004).





**Fig. 3.** Cell to cell interaction mediated by ADAM15 and integrins activates Akt signaling pathway. (A) Invasion of D3H2LN cells in the presence of DMSO, PI3K inhibitor (0.5-50μM of LY294002) or MEK1/2 inhibitor (0.5-50μM of U0126). Bars indicate ±S.D. from three independent experiments. \*\*\*: p<0.01 vs no inhibitor, \*: p<0.05 vs no inhibitor. (B) Phosphorylation of Akt and Erk by cell to cell interaction. Flp-in/CHO or ADAM15/CHO cells was added to the monolayer of D3H2LN or Flp-in/CHO cells for 30min. The cell lysates were obtained and phosphorylated Akt (p-Akt) and total Akt (pan-Akt) or phosphorylated Erk (p-Erk) and total Erk (pan-Erk) were detected by western blot analysis. (C) The density of p-Akt/pan-Akt (left panel) and p-Erk/ pan-Erk (right panel) were indicated. Bars indicate ±S.D. from three independent experiments. \*: p<0.05 vs Flp-in/CHO+D3H2LN. (D)The phosphorylation of Akt by cell to cell interaction was inhibited by 20μg mL<sup>-1</sup> of d.d.. The Phosphorylated Akt (p-Akt) and total Akt were detected by western blot analysis (upper panel). The density of p-Akt/pan-Akt was indicated (lower panel). Bars indicate ±S.D. from three independent experiments. \*\*\*: p<0.01 vs no inhibitor, \*: p<0.05 vs no inhibitor. (E) phosphorylation of Akt by cell to cell interaction in the presence of antibodies indicated (20μg mL<sup>-1</sup>). The Phosphorylated Akt (p-Akt) and total Akt were detected by western blot analysis (upper panel). The density of p-Akt/pan-Akt was indicated (lower panel). Bars indicate ±S.D. from three independent experiments. \*\*\*: p<0.01 vs no antibody, \*: p<0.05 vs no antibody. The density of p-Akt/pan-Akt in cell lysate obtained from no inhibitor was expressed as 100 (control) and others are expressed relative to control.

In invasion assay, LY294002, which is a highly selective inhibitor of PI3K and U0126, which is a MEK1/2 inhibitor significantly reduced the invasion of D3H2LN in dose dependent manner. Next, we examined whether phosphorylation of Akt and Erk can be induced by the cell to cell interaction via ADAM15 and integrins (**Fig. 3B and C**). The Flp-in/CHO or ADAM15/CHO cells were added to the confluent monolayer of D3H2LN cells or Flp-in/CHO and incubated for 30min. The weak phosphorylation of Akt was detected by the interaction of Flp-in/CHO and D3H2LN, Flp-in/CHO and Flp-in/CHO or D3H2LN only. Importantly, the phosphorylation of Akt was 2 fold increased by the interaction between ADAM15/CHO and D3H2LN as compared to that between Flp-in/CHO and D3H2LN. In contrast, the phosphorylation of Erk was not increased by the interaction between ADAM15/CHO and D3H2LN. The phosphorylation induced by the cell to cell interaction between ADAM15/CHO and D3H2LN was significantly inhibited by d.d. recombinant protein (31% reduction as compared to non-treated group) (**Fig. 3D**) and anti- $\alpha$ 9 $\beta$ 1 or  $\alpha$ v $\beta$ 3 integrin antibody (51% and 45% reduction as compared to non-treated group, respectively) (**Fig. 3E**). These results demonstrated that cell to cell interaction via ADAM15 and  $\alpha$ 9 $\beta$ 1 or  $\alpha$ v $\beta$ 3 integrin, expressed on cancer cells induces invasion and activation of Akt signaling pathway.

#### 4. DISCUSSION

The ADAM15 molecule consists of various domains with proteinase and integrin binding activities (Daugimont *et al.*, 2011; Hart *et al.*, 2005; Marezky *et al.*, 2009; Najy *et al.*, 2008b; Ohtsu *et al.*, 2006; Trochon-Joseph *et al.*, 2004; Wu *et al.*, 2008; Zhong *et al.*, 2008). One of the reason for exhibiting complex functions of ADAM15 might be due to its ability to interact with various integrins, leading to the distinct intracellular signaling. It is known that only human ADAM15 binds to both RGD-recognizing and non-RGD recognizing integrins (Eto *et al.*, 2002; 2002; Lu *et al.*, 2006; Nath *et al.*, 1999; Zhang *et al.*, 1998). In this regard, we confirmed that recombinant protein of ADAM15 d.d. or exogenously expressed cell surface ADAM15 can interact with integrins in both RGD-dependent and RGD-independent manner (**Fig. 1**). In addition, our specific antibody against ADAM15 d.d. could interfere with the interaction between ADAM15 and  $\alpha$ v $\beta$ 3 (RGD-recognizing) and/or  $\alpha$ 9 $\beta$ 1 (non-RGD-recognizing) integrins. In this study, the knock down of ADAM15 by SiRNA resulted in the inhibition of cancer cell proliferation in vitro, while blocking of interactions between ADAM15 d.d. and integrins by 8F7 antibody

failed to inhibit cell proliferation, indicating that ADAM15 is involved in cell proliferation in integrin-independent manner (**Fig. 2B**). Therefore, ADAM15 can transduce intracellular signaling without involvement of binding to integrins and regulate cell proliferation. Indeed, there are some reports that metalloproteinase domain transduces intracellular signaling for proliferation and migration (Hart *et al.*, 2005; Najy *et al.*, 2008b; Ohtsu *et al.*, 2006). On the other hand, previous reports demonstrated that addition of recombinant ADAM15 d.d. inhibited the tumor growth and metastasis of B16F10 murine melanoma cells and MDA-MB-231 human breast cancer cells *in vivo* (Daugimont *et al.*, 2011; Trochon-Joseph *et al.*, 2004), indicating that interaction of ADAM15 and integrins via d.d. plays a critical role in tumor cell growth and metastasis. The reason for the discrepancy between our results and previous reports is not clear. However, this point should be clarified by future studies.

In contrary to cell proliferation, cell invasion was significantly inhibited by not only 8F7 antibody, but also anti- $\alpha$ 9 $\beta$ 1 or anti- $\alpha$ v $\beta$ 3 antibody, indicating that cell to cell interaction via integrins and ADAM15 d.d. is involved in cell invasion (**Fig. 2D**). It should be pointed out that this cell to cell interaction via ADAM15 d.d. does not modulate expression of MMP-9 from cancer cells or proteinase activity of ADAM15 (**Fig. 2E and F**). Our data is in good agreement with previous report in which ADAM15 d.d. does not regulate the catalytic activity of metalloproteinase domain (Hart *et al.*, 2005; Najy *et al.*, 2008b; Ohtsu *et al.*, 2006).

The molecular basis for cell invasion is further examined by two assays such as adhesion to Matrigel, the first step for invasion and migration, other step for invasion (**Fig. 2G and H**). Neither ADAM15 d.d. nor  $\alpha$ 9 $\beta$ 1 integrin are involved in adhesion to Matrigel (**Fig. 2G**). Alphavbeta3 integrin on D3H2LN cells might bind to heparin sulfate proteoglycan within Matrigel (Faye *et al.*, 2009), thus contributing to the adhesion to Matrigel. In sharp contrast, cell migration was significantly inhibited by 8F7, anti- $\alpha$ 9 $\beta$ 1 or anti- $\alpha$ v $\beta$ 3 antibody (**Fig. 2H**), suggesting that cell to cell interactions via ADAM15 d.d. and integrins are important for cell migration. During the course of tumor cell invasion, cell to cell interaction induces 'collective cell migration'. Collective cell migration differ from single cell migration in that cells remain connected as they move (Friedl and Gilmour, 2009; Friedl and Wolf, 2008). The key molecule in the collective migration was reported to be cadherins, which mediate the cell to cell junction. ADAM15 is known to co-localize with VE-cadherin and the over-expression of ADAM15 strengthens cell to cell interaction (Herren *et al.*, 2001). 8F7 inhibited not only invasion and migration but also

collective cell migration of breast cancer cells (**Fig. 2C, 2D and 2H** respectively). In addition, 8F7, anti- $\alpha 9\beta 1$  integrin or anti- $\alpha v\beta 3$  integrin antibody inhibited cell aggregation (**Fig. 1H**). Thus, it is possible that cell to cell interactions between ADAM15 and integrins act cooperatively with VE-cadherin to enhance collective cell migration and invasion. We further analyzed the basis for ADAM15-mediated cell invasion. We demonstrated that both Akt and Erk are involved in cell invasion (**Fig. 3A**). Those two kinases are down-stream to PI3K and MEK1/2, respectively (Guo and Giancotti, 2004). Importantly, Akt was specifically activated by cell to cell interaction and ADAM15 was critically involved in this process (**Fig. 3B and C**). Furthermore, ADAM15 d.d.,  $\alpha 9\beta 1$  or  $\alpha v\beta 3$  integrin is involved in the activation of Akt (**Fig. 3D and E**). It is known that cytoplasmic tail of phosphorylated ADAM15 interacts with Src (Maretzky *et al.*, 2009; Poghosyan *et al.*, 2002), which is up-stream to MEK1/2 (Guo and Giancotti, 2004), leading to Erk1/2 signaling pathway (Sun *et al.*, 2010). In this study, phosphorylation of Erk was not affected by the cell to cell interaction via ADAM15 and integrins (**Fig. 3B and C**). Src is known to be up-stream to PI3K and required for activation of Akt (Basson, 2008; Guo and Giancotti, 2004). Thus, it seems likely that the stimulation through ADAM15 d.d. may activate Akt signaling pathway via Src without affecting Erk signaling pathway. On the other hand, many reports showed that integrin mediated-cell to ECM adhesion induce two independent pathways. One is that through Integrin-Linked Kinase (ILK) (Delcomenne *et al.*, 1998; Hannigan *et al.*, 1996), which binds directly to the cytoplasmic region of  $\beta 1$  and  $\beta 3$  integrin and phosphorylate Akt (Legate *et al.*, 2006). Another is that through the complex of Focal Adhesion Kinase (FAK) and Src, which binds directly to integrin  $\beta$  subunit and activates PI3K and MEK1/2, respectively (Guo and Giancotti, 2004). Thus, it is also possible that both integrins and ADAM15 may induce the Akt signaling. However, further study is needed to determine whether Akt signaling pathway is involved in collective cell migration.

## 5. CONCLUSION

In conclusion, our data collectively suggested that ADAM15 which is expressed in D3H2LN cells is involved in cell invasion by inducing cell to cell interaction with  $\alpha 9\beta 1$  and  $\alpha v\beta 3$  integrins.

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