The Ectodomain Shedding of E-cadherin by ADAM15 Supports ErbB Receptor Activation*

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The zinc-dependent disintegrin metalloproteinases (a disintegrin and metalloproteinases (ADAMs) have been implicated in several disease processes, including human cancer. Previously, we demonstrated that the expression of a catalytically active member of the ADAM family, ADAM15, is associated with the progression of prostate and breast cancer. The accumulation of the soluble ectodomain of E-cadherin in human serum has also been associated with the progression of prostate and breast cancer and is thought to be mediated by metalloproteinase shedding. Utilizing two complementary models, overexpression and stable short hairpin RNA-mediated knockdown of ADAM15 in breast cancer cells, we demonstrated that ADAM15 cleaves E-cadherin in response to growth factor deprivation. We also demonstrated that the extracellular shedding of E-cadherin was abrogated by a metalloproteinase inhibitor and through the introduction of a catalytically inactive mutation in ADAM15. We have made the novel observation that this soluble E-cadherin fragment was found in complex with the HER2 and HER3 receptors in breast cancer cells. These interactions appeared to stabilize HER2 heterodimerization with HER3 and induced receptor activation and signaling through the Erk pathway, supporting both cell migration and proliferation. In this study, we provide evidence that ADAM15 catalyzes the cleavage of E-cadherin to generate a soluble fragment that in turn binds to and stimulates ErbB receptor signaling.

The classic cadherins, epidermal cadherin (E-cadherin), neuronal cadherin (N-cadherin), and placental cadherin (P-cadherin), are type I transmembrane glycoproteins (1). The epidermal specific cadherin, E-cadherin, has five extracellular domain repeats that are involved in cell binding mediated by E-cadherin homotypic interaction (2). The intracellular domain consists of a conserved sequence that associates with β-, γ-, and p120-catenins. The interaction of β- or γ-catenin with α-catenin links E-cadherin to the cytoskeletal matrix to stabilize the adherens junction mediated by the homotypic E-cadherin complex (3). The involvement of E-cadherin in cell-cell interaction is well established in embryonic development, organ morphogenesis, tissue integrity, and wound healing (4). The disruption of E-cadherin by genetic mutation, promoter hypermethylation, or proteolytic cleavage leads to the loss of cell contact integrity as a consequence of adherens junction dissolution. E-cadherin disruption has been observed in multiple pathophysiological conditions, including inflammation and cancer (5). In fact, E-cadherin is considered to function as a metastasis suppressor due to its inhibition of cancer cell migration and invasion (6).

Several proteases have been implicated in the extracellular cleavage of E-cadherin, including MMP3, MMP7, MT1-MMP, plasmin, kallikrein 7, and ADAM10. In addition, the cytoplasmic domain of E-cadherin is cleaved by caspase-3 and calpain (7, 8). The ectodomain shedding of a stable 80-kDa soluble E-cadherin (sE-cad)2 fragment has been shown to increase in the urine and serum of patients with cancers of the bladder, breast, prostate, ovarian, gastric, and melanoma and is a marker of poor prognosis (5). At the molecular level, sE-cad is disruptive to cell contact, inducing cell scattering and eroding the adherens junction by antagonizing full-length E-cadherin (9).

The a disintegrin and metalloproteinase (ADAM) family is composed of 40 members, of which 13 are catalytically active. These zinc-dependent proteases are transmembrane glycoproteins composed of five extracellular domains: prodomain, metalloproteinase, disintegrin, cysteine-rich, and EGF-like domains, respectively. The ADAMs also possess a cytoplasmic C-terminal tail containing Src homology 2 and 3 recognition sequences that have been shown to interact with different adapter proteins, such as Grb2, SH3PX1, and endophilin I, which may play a role in protein localization and signal transduction (10, 11). The catalytic metalloproteinase domain of the ADAM family has a consensus HEXXGXXH sequence and is known to mediate extracellular matrix protein degradation as well as ectodomain shedding of growth factors, growth factor receptors, and adhesion molecules (12). Complementing the metalloproteinase domain is the disintegrin domain, which has been shown to bind different integrins that may support cell migration, adhesion, and ectodomain shedding (13). The presence of these functional domains suggests multiple functional roles for the ADAMs in a variety of normal and pathophysiological conditions, including cancer progression. To this end, ADAM9 has been demonstrated to support lung cancer inva

2 The abbreviations used are: sE-cad, soluble E-cadherin; ADAM, a disintegrin and metalloproteinase; EGF, epidermal growth factor; GFP, green fluorescent protein; shRNA, short hairpin RNA; shScrm, scrambled shRNA; shADAM15, short hairpin ADAM15; EGFR, epidermal growth factor receptor; Fc-Ecad, Fc/E-cadherin fusion protein.

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Potential ligand for the HER2 receptor and a role for soluble To our knowledge, this is the first report demonstrating a HER3 heterodimerization, leading to Erk-dependent signaling. 

in turn, binds to and stabilizes the ErbB receptor HER2 and is thought to support the metastatic progression of cancer cells by promoting tumor angiogenesis and angiogenesis (16–20). ADAM15 also plays a role in cell migration, neovascularization, and chondrocyte survival (21, 22), possibly through its role in EGFR transactivation, by cleaving the pro-forms of the EGFR ligands transforming growth factor-α, HB-EGF, and amphiregulin (23, 24).

The ErbB family of receptors is composed of four members: epidermal growth factor receptor (EGFR, ErbB1, or HER1), HER2 (ErbB2), HER3 (ErbB3), and HER4 (ErbB4) (25). When bound by their respective ligands, these receptors undergo homo- or heterodimerization that activates their inherent receptor kinase domain, leading to receptor auto- and transphosphorylation and downstream signaling (26). ERB receptor signaling has been demonstrated to support cancer cell migration, proliferation, and invasion (27). The dysregulation of this family of receptor-tyrosine kinases is found in a myriad of pathophysiological conditions, including cancer (28). EGFR overexpression and hyperactivity have been implicated in several human cancers, including non-small cell lung cancer, ovarian cancer, and breast cancer (29). Similarly, the HER2 receptor is found to be overexpressed in 20–30% of breast cancer and is a marker of poor prognosis (4). Interplay between the ErbB family members and their ligands is necessary to induce a cell response. EGFR has been shown to interact with all of the seven EGFR ligands, whereas HER3 and HER4 favor binding to the heregulin (also known as neuregulins) family of ligands (30). In contrast to the other ErbB family members, HER2 has not yet been demonstrated to bind to a specific ligand.

The ADAM family members, including ADAM15, play an important role in the transactivation of ErbB family members by releasing the latent transmembrane EGFR ligands from their pro-form on the cell surface (12, 31). Previously, ADAM15 was shown to be up-regulated during breast cancer progression using cDNA and tumor microarrays (18). The role of ADAM15 as a membrane sheddase that is up-regulated during breast cancer progression, coupled with the fact that increased E-cad levels also correlate with breast cancer progression, led us to assess the role of ADAM15 in E-cadherin shedding in breast cancer cells. We report here that ADAM15 is capable of cleaving full-length E-cadherin into a soluble, extracellular fragment. We also show that the solubilized E-cadherin fragment, in turn, binds to and stabilizes the ErbB receptor HER2 and HER3 heterodimerization, leading to Erk-dependent signaling. To our knowledge, this is the first report demonstrating a potential ligand for the HER2 receptor and a role for soluble E-cadherin in stabilizing ErbB receptor dimerization and signaling.

**Experimental Procedures**

Cell Lines and Culture—LNCaP and SKBr3 cells were maintained in RPMI (Bio Whittaker, Walkersville, MD) with 8% fetal bovine serum (HyClone, Logan, UT). MCF-7GFP cells (which were a kind gift from Dr. Jacques Nör, University of Michigan Dental School) were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) with 10% fetal bovine serum. All culture media were supplemented with 2 mmol/liter l-glutamine (Invitrogen), 100 units/ml penicillin (Invitrogen), 100 μg/ml streptomycin (Invitrogen), and 0.25 μg/ml Fungizone (Invitrogen). ADAM15-overexpressing cells were grown under selection with 800 μg/ml of G418 (Cellgro, Manassas, VA). Cells were incubated at 37°C and subcultured weekly.

Generation of ADAM15 Cell Lines—MCF-7GFP cells were infected with ADAM15-specific knockdown oligonucleotides (shADAM15) or vector control oligonucleotides consisting of a scrambled sequence designed to control for off-target effects (scrambled shRNA; shScr). The forward and complementary targeting sequences for ADAM15 were 5′-AACCAGCTGTCA-CCCTCGAA-3′ and 5′-TTCCAGGTTGACAGCTGGT-3′. The shRNA cassette also featured a TTCAGAGA loop situated between the sense and reverse complementary targeting sequences and a TTTTT terminator at the 3′-end. ADAM15-overexpressing MCF-7 cells were generated as described by Kueker et al. (18).

To generate ADAM15 mutants, first the ADAM15 cDNA was tagged with HA (hemagglutinin) at the C terminus and transfected into LNCaP cells, as described previously (18), to establish wild-type ADAM15. Catalytically dead ADAM15 was generated by mutating the glutamic acid residue into an alanine at position 350 (E350A) using the QuikChange II site-directed mutagenesis kit (Stratagene, La Jolla, CA). DNA was sequenced to confirm mutations (University of Michigan Sequencing Core).

Microarray Analysis—Expression levels of ADAM15 and HER2 in published breast cancer cDNA arrays were derived using the Oncomine data base available on the World Wide Web. The terms “ADAM15” or “HER2” were used to search the data base for differential expression of both of these markers in different breast cancer arrays.

Protein Isolation, Immunoprecipitation, and Western Blotting—Cells were harvested by mechanical disruption with cell scrapers, followed by gentle centrifugation at 6000 rpm for 3 min. Cell pellets were then lysed in appropriate volumes of lysis buffer (50 mmol/liter Tris (pH 7.6), 120 mmol/liter NaCl, 0.5% Nonidet P-40, 1 mmol/liter EDTA, 100 μg/ml phenylmethylsulfonyl fluoride, 50 μg/ml aprotinin, 50 μg/ml leupeptin, and 1.0 mmol/liter sodium orthovanadate) for 1 h on ice. Cellular debris was then pelleted by centrifugation at 12,000 rpm for 8 min, and supernatants were collected and quantitated using a microtiter Bradford protein assay (Bio-Rad) with experimental and standard samples run in triplicate. Equal amounts of protein were then separated on precast Tris-glycine SDS-polyacrylamide gels (Novex, Carlsbad, CA) and transferred to reinforced 0.2-μm nitrocellulose membrane (Millipore, Temecula, CA). Membranes were then blocked, probed, and developed. Primary antibodies were obtained as follows: actin (Sigma), phospho-Erk and Erk (Cell Signaling Technology, Danver, MA), ADAM15, tubulin, phosphotyrosine clone 4G10 (Millipore), E-cadherin clone HEDC-1 (Zymed Laboratories Inc, San Francisco, CA), HER2 clone e2-4001 + 3B5, and HER3 clone 2B5 (Lab Vision Corp., Fremont, CA). All appropriate

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secondary antibodies conjugated with horseradish peroxidase were purchased from Bio-Rad.

For immunoprecipitation, 1 mg of cell lysate was precleared with an equal amount of a mixture of 2.5% dry milk in TBST-Sepharose Protein A beads (Zymed Laboratories Inc.) for 30 min at room temperature with end-over-end rotation, and then beads were spun out. Precleared protein was then immunoprecipitated with 2 μg of HER2 and HER3 (Lab Vision Corp.) antibody or an equal amount of isotype IgG (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for 3 h at 4 °C with end-over-end rotation. This was followed by incubating the immunoprecipitation mixture with 75 μl of blocked Sepharose Protein A beads for 90 min at 4 °C with rotation. The complex was then centrifuged and washed three times with ice-cold phosphate-buffered saline. The complex was dissociated from the beads with the addition of 5× sample-reducing loading buffer and heated for 5 min at 100 °C. Samples were then loaded on SDS-PAGE for protein analysis. An ECL system was used to visualize proteins (Millipore).

**Immunocytochemistry**—ADAM15-GFP-overexpressing cells were grown to subconfluence in 2-well chambers (BD Falcon, Bedford, MA) and then cross-linked with 2% formaldehyde in phosphate-buffered saline and fixed with 100% ethanol for 5 min on ice. Fixed cells were washed three times with ice-cold phosphate-buffered saline and blocked with 0.1% milk, fetal bovine serum solution for 30 min. The E-cadherin antibody clone 1702-1 (Epitomic, Burlingame, CA) or an isotype-matched IgG (Zymed Laboratories Inc.) from MCF-7 whole cell lysate expressing either wild-type or catalytically dead ADAM15 mutant. E-cadherin was also isolated from LNCaP whole cell lysate expressing either wild-type or catalytically dead ADAM15 mutant. E-cadherin was also isolated from LNCaP whole cell lysate expressing either wild-type or catalytically dead ADAM15 mutant. Isolated ADAM15 and E-cadherin were mixed together at the designated ratio and incubated for the designated time point. The cells were washed once with warm growth medium and then washed on ice. Cells were then incubated with either vehicle or a 1.5 μg/ml concentration of the Fc/E-cadherin recombinant protein per manufacturer’s recommendation (R&D Systems) in serum-free medium for 5 min with anti-rabbit ALEXA Fluorochrome 555 secondary antibody (Invitrogen). Slides were mounted with a 1-mm coverslip (Fisher), and photomicrographs were taken utilizing confocal microscopy at the University of Michigan Imaging Core.

**E-cadherin Proteolysis**—**In vitro** proteolysis of E-cadherin by ADAM15 was performed as described by Noe et al. (32). Briefly, ADAM15 was isolated through immunoprecipitation using a hemagglutinin-specific antibody clone DW2 (Millipore) from LNCaP whole cell lysate expressing either wild-type or catalytically dead ADAM15 mutant. E-cadherin was also isolated through immunoprecipitation using an E-cadherin-specific HEDC-1 antibody (Zymed Laboratories Inc.) from MCF-7 whole cell lysate. Isolated ADAM15 and E-cadherin were mixed together at the designated ratio and incubated for the chosen time points in a 37 °C water bath. For the inhibitor assays, 0.05 μM of 1,10-phenanthroline (Sigma) was added to each tube. At the end of each time point, the reactions were stopped by the addition of 5× sample-reducing loading buffer and heating to 100 °C for 5 min. Samples were then loaded on SDS-PAGE for protein separation and analysis via Western blot.

**Conditioned Medium Analysis**—MCF-7 cells were grown up to subconfluence (~65–70%) and then serum-starved in serum-depleted medium for 24 h. Conditioned medium was collected by centrifugation at 2,000 rpm for 15 min to pellet any cell debris. Levels of soluble E-cadherin in conditioned media were monitored via E-cadherin immunoprecipitation using the HECED-1 monoclonal antibody (Zymed Laboratories Inc.).

Cell Migration Assays—To assess the effects of endogenous soluble E-cadherin on MCF-7 cell migration, cells were plated in 6-well tissue culture dishes until confluence. Cells were then serum-starved for 16 h and then abraded with a 10-μl pipette tip. The cells were washed once with warm growth medium and incubated in normal growth medium. Cell migration was monitored through microscopic imaging at the designated time points. Migration was quantitated as the percentage of remaining cleared area by dividing the cleared area at each time point by the original 0 h time point. Each experiment contained four separate samples and performed three times.

**Cell Proliferation Assays**—To assess the effects of endogenous soluble E-cadherin on MCF-7 cell proliferation, 1×10⁴ cells were plated in 96-well plates for 24 h. Cells were then washed once with warm serum-free medium and then incubated for the appropriate amount of time under serum-free medium. Cell proliferation was assessed using the 3-(4,5-di-methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay and quantified as -fold change over time by dividing the OD readings of each time point by the original time point at 0 h. Each experiment contained eight separate samples and was performed three times.

To analyze the exogenous effects of E-cadherin, the E-cadherin null cell line SKBr3 was plated at 3×10⁶ cells for 4 days and then washed once with warm serum-free medium. Cells were then incubated with either vehicle or a 1.5 μg/ml concentration of the Fc/E-cadherin recombinant protein per manufacturer’s recommendation (R&D Systems) in serum-free medium for the designated time points. Cell proliferation was measured using the trypan blue exclusion assay (Invitrogen) and quantified as -fold change over time by dividing the number of cells at each time point by the cell number at the original time point at 0 h. Each experiment was run in triplicates and performed three times.

**Statistical Analysis**—All statistical work was performed using Student’s unpaired t test with a one-tailed distribution. p < 0.05 was considered statistically significant. Densitometry was performed using NIH Image J software.

**RESULTS**

**Up-regulation of ADAM15 and HER2 during Breast Cancer Progression**—The ADAM15 chromosomal locus, 1q21.3, is amplified during the metastatic progression of multiple adenocarcinomas and melanoma (33, 34). We utilized the oncomine data base to comprehensively examine ADAM15 expression in published human cDNA microarrays of breast cancer. We observed that ADAM15 was significantly up-regulated in eight different cDNA microarray studies. Seven of the aforementioned arrays also demonstrated significant up-regulation of the HER2 receptor, which is known to be a marker of poor prognosis (Fig. 1A). One of these studies is graphically presented in Fig. 1B to demonstrate the correlative increase in both ADAM15 and HER2 expression in breast cancer tumors over normal tissues (35). Interestingly, ADAM15 expression was down-regulated in estrogen receptor-positive breast cancer tumors (data not shown). Seven of the eight data sets show that ADAM15 and HER2 transcripts are simultaneously and signif-
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**A** Table 1. ADAM15 and HER2 expression in breast cancer cDNA microarray *

| Author       | ADAM15 Status | p-Value | HER2 Status | p-Value |
|--------------|---------------|---------|-------------|---------|
| Bild_Breast  | ↑             | 0.007   | ↑           | 2.20E-11|
| Ginestier_Breast | ↑             | 2.00E-03| ↑           | 3.10E-02|
| Hess_Breast  | ↑             | 0.015   | ↑           | 1.50E-14|
| Minn_Breast_2| ↑             | 0.044   | ↑           | 6.00E-04|
| Perou_Breast | ↑             | 0.019   | ↑           | 0.002   |
| Richardson_Breast_2 | ↑             | 2.00E-03| ↑           | 2.10E-02|
| Sotiriou_Breast_3 | ↑             | 3.30E-02| ↑           | 8.00E-03|

*All studies were obtained from the Oncomine database at www.oncomine.org

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**B**

**FIGURE 1.** ADAM15 and HER2 expression in breast cancer. A, ADAM15 and HER2 are simultaneously overexpressed in seven different breast cancer cDNA microarrays. B, a graphical representation of ADAM15 and HER2 expression in normal and tumor breast tissues using the published Richardson_Breast_2 array available on the World Wide Web.

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**FIGURE 2.** ADAM15 cleaves E-cadherin in breast cancer cells. A, GFP-tagged and endogenous ADAM15 in MCF-7 cells are indicated (Lysate). Due to the intense banding pattern observed in ADAM15-overexpressing (ADAM15-GFP) cells, a lower exposure of the GFP fusion protein was cropped into the ADAM15 panel. Shown is an analysis of sE-cad in the conditioned media (CM) of ADAM15-overexpressing cells. B, ADAM15 expression was down-regulated in MCF-7 cells using a stable shRNA against ADAM15 (Lysate), and sE-cad was assessed in the conditioned media. Full-length E-cadherin (FL/E-cad) remained unchanged in these experiments.

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**In Vitro E-cadherin Proteolysis by ADAM15**—Immunohistochemistry revealed prominent co-localization of ADAM15 and E-cadherin at the junctional cell membrane (Fig. 3A). To directly implicate ADAM15 in E-cadherin cleavage, we isolated ADAM15 and E-cadherin and performed an in vitro cleavage analysis. When ADAM15 and E-cadherin were co-incubated at equal ratios, ADAM15 cleaved E-cadherin into the sE-cad fragment in a time-dependent manner (Fig. 3B). This fragment migrated at 80 kDa on reducing gels and showed immunoreactivity with extracellular domain-specific E-cadherin antibodies. E-cadherin alone was not cleaved, and ADAM15 isolation significantly up-regulated during breast cancer progression, suggesting a role of ADAM15 in breast cancer development (Fig. 1B).

**ADAM15 Cleaves E-cadherin in Breast Cancer Cells**—Since ADAM15 was found to be overexpressed in breast cancer and sE-cad levels have been demonstrated to be up-regulated during the progression of this disease, we hypothesized that ADAM15 may play a critical role in E-cadherin shedding. To evaluate whether ADAM15 mediates E-cadherin pro-teolysis, we stably overexpressed an ADAM15-GFP fusion protein in MCF-7 breast cancer cells (ADAM15-GFP cells; Fig. 2A). ADAM15-GFP cells exhibited both endogenous and recombinant ADAM15. Two species of endogenous ADAM15 were detected by an ADAM15-specific antibody at 110 kDa (inactive precursor) and 90 kDa (catalytically active). Two species of recombinant ADAM15-GFP were detected at 136 and 116 kDa, representing the recombinant precursor and active forms, respectively. ADAM15 overexpression exhibited no effects on ADAM15 family relatives, ADAM10 or ADAM17, or other assessed targets (data not shown).

Previously, Damsky et al. (36) demonstrated that serum deprivation of MCF-7 cells for 24 h led to the release of sE-cad into the conditioned media of these cells. Vector and ADAM15-GFP MCF-7 cells were serum-starved for 24 h, and the presence of sE-cad was analyzed in the conditioned media. We found that sE-cad was elevated in the ADAM15-overexpressing cells in comparison with vector control (Fig. 2A).

To substantiate the overexpression findings, we stably down-regulated ADAM15 in our breast cancer cells using an shRNA construct against ADAM15. Both the precursor and mature forms of ADAM15 were reduced in response to the shADAM15 construct in comparison with the scrambled sequence (shScrm) control cells (Fig. 2B). Analysis of the ADAM15 RNA message also demonstrated significant down-regulation in response to the shADAM15-inhibitory construct (data not shown). We observed that the ADAM15 shRNA construct was specific to ADAM15 and did not affect ADAM15 relatives, ADAM10 and ADAM17, or other targets (data not shown). Serum starvation of shScrm control and shADAM15 MCF-7 cells resulted in decreased shedding of sE-cad into the conditioned media in response to ADAM15 down-regulation (Fig. 2B).
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E-cadherin is a substrate for ADAM15. A, immunocytochemistry analysis of ADAM15 (green) and E-cadherin (red) in MCF-7 cells. Shown is confocal microscopy at ×400 concentration. Isolated ADAM15 and E-cadherin were co-incubated. ADAM15 cleaves full-length E-cadherin (FL/E-cad) into its soluble fragment (sE-cad) in a time-dependent (B) and concentration-dependent (C) manner. D, E-cadherin and ADAM15 were co-incubated with vehicle (veh) or 1,10-phenanthroline (1,10P). E, wild-type (WT) or catalytically dead (CD) ADAM15 were co-incubated with isolated E-cadherin. Isolated E-cadherin and ADAM15 alone were loaded as controls on the end lanes. Whole cell lysate (WCL) was used to demarcate the E-cadherin banding pattern.

FIGURE 3. E-cadherin is a substrate for ADAM15. A, immunocytochemistry analysis of ADAM15 (green) and E-cadherin (red) in MCF-7 cells. Shown is confocal microscopy at ×400 concentration. Isolated ADAM15 and E-cadherin were co-incubated. ADAM15 cleaves full-length E-cadherin (FL/E-cad) into its soluble fragment (sE-cad) in a time-dependent (B) and concentration-dependent (C) manner. D, E-cadherin and ADAM15 were co-incubated with vehicle (veh) or 1,10-phenanthroline (1,10P). E, wild-type (WT) or catalytically dead (CD) ADAM15 were co-incubated with isolated E-cadherin. Isolated E-cadherin and ADAM15 alone were loaded as controls on the end lanes. Whole cell lysate (WCL) was used to demarcate the E-cadherin banding pattern.

FIGURE 4. Soluble E-cadherin mediates HER2-HER3 heterodimerization through ErbB receptor binding. ADAM15 knockdown MCF-7 whole cell lysates were immunoprecipitated (IP) with HER2 (A), HER3 (B), or isotype IgG. Control shScr control MCF-7 cells were treated with either vehicle (veh) or trastuzumab (TZM) for 24 h prior to immunoprecipitation either with HER2 (C), HER3 (D), or isotype IgG under growth factor-depleted conditions. Immunoblotting with E-cadherin (E-cad), HER2, or HER3 antibodies were used to assess E-cadherin binding to ErbB receptors and receptor dimerization. The input lane (2% of the amount of protein used for the immunoprecipitation) was used for the E-cadherin banding pattern.
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The sE-cad fragment preferentially bound HER2 over HER3, and the HER2 receptor was observed to bind to a sE-cad doublet, whereas HER3 only bound the lower band of this sE-cad doublet.

To confirm that sE-cad interaction with HER2 is HER2-specific, we treated MCF-7 shScrm control cells with the humanized HER2 antibody, trastuzumab (Herceptin). We found that this extracellular domain-specific antibody to HER2 completely eliminated the interaction of sE-cad with HER2 in MCF-7 cells in comparison with vehicle treatment (Fig. 4C). In addition, the trastuzumab treatment also abrogated the sE-cad/HER3 complex and the HER2-HER3 heterodimerization mediated by sE-cad within the MCF-7 cells (Fig. 4D).

Soluble E-cadherin Mediates HER2-dependent Signaling—The interaction of EGFR ligands with their complementary receptors leads to receptor phosphorylation on C-terminal tyrosine residues and concomitant receptor activation, resulting in downstream signaling (27). To assess whether endogenous sE-cad interaction with HER2 induces receptor phosphorylation, we stimulated E-cadherin shedding by serum-starving shScrm control or shADAM15 MCF-7 cells. We observed increased phosphorylation of HER2 in shScrm control cells; however, tyrosine phosphorylation of HER2 in shADAM15 cells was less (Fig. 5A). We also monitored HER3 phosphorylation in our MCF7 cells and found that, like HER2, shScrm control cells demonstrated more receptor phosphorylation compared with shADAM15 cells in response to serum deprivation (Fig. 5B).

The HER2-HER3 dimer has been shown to signal through both the Erk and Akt pathway when activated (28). We assessed Erk signaling in the MCF-7 cells at time points where we observed soluble E-cadherin-mediated HER2 activation. The shScrm control cells exhibited increased phosphorylation of Erk in response to serum starvation. In contrast, Erk phospho-

FIGURE 5. Soluble E-cadherin mediates HER2-HER3 phosphorylation and induces ErbB-mediated cell signaling. Phosphorysine (pTyr) status was assessed in control shScrm or shADAM15 (shA15) MCF-7 cells in response to serum starvation. Whole cell lysates were collected and immunoprecipitated (IP) with HER2 (A), HER3 (B), or isotype IgG prior to immunoblotting with antibodies specific to Tyr(P) (pTyr), HER2, or HER3 (C). An increase in Erk activation (pErk) in MCF-7 cells was observed at 24 and 26 h after growth factor withdrawal.

FIGURE 6. ADAM15 supports cell migration and proliferation. A, control shScrm or shADAM15 MCF-7 cells were abraded with a 10-μl pipette tip, and wound closure was monitored over time. The columns represent the mean of three separate experiments quantitated in four different samples. Bars, S.D. * , p < 0.04. B, control shScrm or shADAM15 MCF-7 cells were grown under serum-depleted conditions, and cell proliferation was analyzed as a function of time. The columns represent the mean of three separate experiments quantitated in eight different samples. Bars, S.D. * , p < 4.6E-05; ** , p < 5.5E-04.

ADAM15 Mediates Soluble E-cadherin-dependent Cell Migration and Proliferation—Because Erk signaling is known to mediate cell migration and proliferation (28), we assessed whether the MCF-7 shScrm control cells possessed a migratory advantage over the shADAM15 cells in response to serum starvation. In a wound channel migration assay, the MCF-7 shScrm control cells exhibited more rapid migration than the shADAM15 cells over time (Fig. 6A). To analyze if ADAM15 down-regulation compromised the proliferative potential of MCF-7 cells, we performed proliferation assays on these cells and found that shScrm control MCF-7 cells proliferated more than the shADAM15 cells during serum deprivation (Fig. 6B).

Exogenous Stimulation of HER2 with Soluble E-cadherin—Within this study, we demonstrated that ADAM15 mediates endogenous generation of sE-cad, which interacts with ErbB receptors and induces their transactivation. To verify that sE-cad is responsible for HER2 binding and activation, we utilized an extracellular domain of Fc/E-cadherin fusion protein (Fc-Ecad). Experiments with this fusion protein were performed in an E-cadherin-negative, HER2-positive cell line to eliminate endogenous soluble E-cadherin background. The breast cancer cell line, SKBr3, which is E-cadherin-negative and expresses copious amounts of HER2, was treated with vehicle or the Fc-Ecad fusion protein under serum-free conditions, and complex formation between sE-cad and HER2 was assessed. These experiments demonstrated clear Fc-Ecad interaction with HER2 as compared with vehicle control (Fig. 7A), but no Fc-Ecad binding to HER3 was observed. Although the HER3/Fc-Ecad complex was not detected in our experiments, we did observe that Fc-Ecad treatment mediated HER2-HER3 heterodimerization and HER3 receptor phosphorylation (Fig. 7B). HER2 phosphorylation was unaffected in response to Fc-Ecad treatment, potentially due to constitutive activation as a result of HER2 receptor overexpression (data not shown). We monitored Erk phosphorylation in these cells and observed an increase in its phosphorylation status in response to Fc-Ecad treatment (Fig. 7C). Furthermore, densitometry revealed a significant up-regulation in Erk phosphorylation in Fc-Ecad treated cells (data not shown). Vehicle-treated cells exhibited...
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basal Erk phosphorylation, which could be due to HER2 hyperactivation resulting from HER2 overexpression in these cells. In confirmatory experiments, we utilized a human E-cadherin peptide and found that Erk phosphorylation was stimulated in response to E-cadherin peptide addition (data not shown). To assess whether Erk activation mediated by exogenous stimuli affected cell growth, we performed proliferation assays using Fc-Ecad and examined the proliferative response in comparison with vehicle control over time (Fig. 7D). We demonstrated that the Fc-Ecad fusion peptide induced a significant increase in SKBr3 cell proliferation as compared with vehicle treatment.

**DISCUSSION**

The cell adhesion molecule, E-cadherin, serves a crucial role in inhibiting tumor cell migration and invasion by maintaining the cell-cell adhesion complex, and the inactivation of E-cadherin by gene deletion, promoter hypermethylation or proteolytic cleavage, renders tumor cells prone to a migratory and invasive phenotype due to the loss of cellular contact and polarity (41, 42). Ectodomain cleavage of E-cadherin by several different proteases has been reported to yield an 80-kDa fragment known as sE-cad. Soluble E-cadherin accumulates in the serum or urine of patients suffering from multiple types of cancers, including prostate, breast, bladder, and lung cancer (5). Using published cDNA arrays, we report here that a catalytically active member of the ADAM family, ADAM15, is up-regulated during the progression of breast adenocarcinoma. Furthermore, ADAM15 expression was elevated in HER2-positive breast cancer tumors and was found to be down-regulated in estrogen receptor-positive breast cancer, correlating ADAM15 levels with disease progression. To assess the role of ADAM15 in sE-cad shedding, we overexpressed or knocked down ADAM15 in the MCF-7 breast cancer cell lines and observed an elevation of sE-cad shedding in response to ADAM15 overexpression and a reduction of the sE-cad in ADAM15 knockdown cells. Previously, ADAM10 has been demonstrated to cleave E-cadherin in keratinocytes (43, 44), but in our models, ADAM10 levels were unaffected by ADAM15 protein modulation and were constant throughout the analyses. Based on the data presented here, we believe that growth factor deprivation may activate ADAM15 at the cell surface, which in turn sheds the ectodomain of E-cadherin into the extracellular milieu (Fig. 7).

MT1-MMP has been shown to activate MMP2 and -9 to support cell invasion through extracellular matrix degradation (45, 46). To ascertain that ADAM15 is cleaving E-cadherin directly and not activating another protease, we isolated both ADAM15 and E-cadherin and then co-incubated them together to induce ADAM15-directed proteolysis. We demonstrated that ADAM15 cleaves E-cadherin in a time- and concentration-dependent manner. ADAM15 proteolysis was inhibited by introducing an inactivating mutation in the catalytic domain, thus implicating ADAM15 as a direct sheddase of E-cadherin.

Soluble E-cadherin is known to inhibit cell aggregation and induce cell invasion through a yet uncharacterized signaling mechanism (47, 48). These same events have also been shown to be initiated by ligand interaction to the ErbB family, which is composed of four members, EGFR (HER1), HER2, HER3, and HER4. When bound to their cognate ligands, these receptors mediate cell proliferation, migration, invasion, and differentiation (4). The EGFR ligands are synthesized as inactive transmembrane precursors, which are liberated from their inactive state by metalloproteinases, including ADAM family members (12). The activation of the ADAM proteases by a G-protein-coupled receptor signal leads to the shedding of EGFR ligands, which in turn bind and transactivate their complementary receptors to mediate downstream signaling (31). All of the ErbB family members have a specific ligand except HER2, which functions by forming heterodimers with the other family mem-

**FIGURE 7. HER2 stimulation by exogenous soluble E-cadherin.** A, E-cadherin-negative SKBr3 cells were treated with either vehicle (veh) or the extracellular Fc-Ecad, and lysates were then immunoprecipitated (IP) with HER2 or isotype IgG. Immunoblotting with E-cadherin (E-cad) or HER2 was performed to detect Fc/E-cadherin binding. B–D, Fc/E-cadherin fusion protein mediated HER2-HER3 heterodimerization and HER3 phosphorylation as well as increased Erk activation (pErk) and cell proliferation in SKBr3 cells. Columns represent the mean of three separate experiments quantitated in eight different samples. Bars, S.D. *p < 0.05; **p < 0.001.
expression in our experimental models and found that they are
regulated in response to growth factor deprivation, we wanted
to assess the interaction of E-cadherin with these receptors as a
potential ligand. We observed that HER2 bound a sE-cad dou-
bled in response to growth factor deprivation in an ADAM15-
dependent manner. In addition, HER3 interacted with the
lower molecular weight sE-cad, and this complex mediated
HER2-HER3 heterodimerization. Since we used whole cell
lysates for these experiments, the difference in sE-cad banding
observed bound to the ErbB receptors may be due to differential
phosphorylation of this fragment by HER2 as a result of recep-
tor internalization. In addition, sE-cad was found to complex
preferentially with HER2 rather than with HER3 in our assays.
The order of sE-cad binding to HER2 and HER3 is yet to be
elucidated and is a focus for future work. HER2 is known to be
indirectly activated by members of the neuregulin family of
ligands through their binding with HER3 (49). To rule out neu-
regulin-mediated HER2-HER3 activation, we assessed ligand
expression in our experimental models and found that they are
not expressed (data not shown). Furthermore, Horiuchi et al.
(50) performed an exhaustive study demonstrating that neu-
regulin shedding is ADAM17-dependent and probably not
mediated by any other ADAM family member, including
ADAM15. Our findings here showed that serum deprivation of
MCF-7 cells induced ADAM15-dependent phosphorylation of
HER2 and the kinase-inactive HER3 potentially through the
HER2-HER3 heterodimer is known to signal through the Erk
signaling pathway, which supports cell survival, proliferation,
and migration (51, 52). In our models, we found that either
endogenous shedding of E-cadherin or the addition of exog-
eneous soluble E-cadherin fusion proteins or peptides supported
cancer cell migration and proliferation, possibly through Erk
signaling. Previous studies demonstrated that full-length cad-
herin ligation and activation of growth factor receptors activ-
ated only Erk signaling (53, 54). In our experimental models,
Akt activation was not detected, which may be a consequence
of E-cadherin-specific receptor activation.

In this study, we demonstrated that ADAM15 and HER2 are
simultaneously up-regulated during breast cancer progression.
Additionally, overexpression of the ErbB receptor, HER2, and
loss of E-cadherin expression are frequently observed in breast
cancer and are considered indicators of poor prognosis (4, 55).
Although these findings are suggestive of an interactive mecha-
nism, the functional association between these molecules has
not been investigated. To this end, we have shown that
ADAM15 catalyzed sE-cad shedding, which in turn bound to
and transactivated both HER2 and HER3. This sE-cad fragment
also enhanced Erk activation to support breast cancer cell
migration and proliferation. In conclusion, this study has iden-
tified the functional interaction between sE-cad and ErbB
receptors, although the precise structural requirements for
these interactions have yet to be elucidated. Further character-
ization of this signaling axis is warranted and may ultimately
lead to novel therapeutic strategies targeting ADAM15, E-cad-
herin, and HER2 in breast cancer.
ADAM15 Signaling through E-cadherin Shedding

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