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Induction of E-cadherin endocytosis by loss of protein phosphatase 2A expression in human breast cancers ☆

Katsuo Suzuki, Kazuhide Takahashi *

Molecular Cell Biology Division, Kanagawa Cancer Center Research Institute, 1-1-2 Nakao, Asahi-ku, Yokohama 241-0815, Japan

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Abstract

The cell–cell adhesion molecule E-cadherin is stabilized by linking intracellularly with the actin cytoskeleton through PP2A-mediated recruitment of IQGAP1 to Rac1-bound E-cadherin–catenins complex in nonmalignant HME cells. However, little is known about the dysfunction of E-cadherin by loss or reduced expression of PP2A in human breast cancer cells. We report here that both human breast cancer MDA-MB-231 and MCF-7 cells were deficient in expression of the PP2A-A protein and lost the IQGAP1 recruitment to Rac1-bound catenins. In MDA-MB-231 cells, E-cadherin was also deficient. Immunohistochemical analysis of the normal–carcinoma matched human breast tissue arrays revealed that PP2A-A was expressed in 96% of normal tissue specimens but not in 57% of carcinoma specimens. Expression of E-cadherin in MCF-7 cells was 1.5-fold higher than that in HME cells, however, 80% of E-cadherin was endocytosed and incompletely anchored to F-actin. Therefore, we propose that the dysfunction of E-cadherin due to its endocytosis may occur in some proportion of human breast carcinomas in which the PP2A-A protein is lost or significantly reduced.

Keywords: Cell–cell adhesion; E-Cadherin endocytosis; Protein phosphatase 2A; IQGAP1 recruitment

E-Cadherin is one of the family of calcium-regulated cell–cell adhesion molecules called cadherins and plays a crucial role in embryonic morphogenesis and maintenance of epithelial layers, such as those lining organ surfaces [1,2]. E-Cadherin interacts intracellularly with a group of proteins, collectively termed catenins [3,4] and is stabilized by anchoring to the actin cytoskeleton through catenins. In many epithelial cancers, loss or significant reduction in E-cadherin expression has been noted [5–8]. β-Catenin links E-cadherin to α-catenin and is tyrosine phosphorylated by several receptor- or nonreceptor-type tyrosine kinases, concomitant with a decline in the cell adhesion function of cadherins [9–13] through the loss of α-catenin linking [14]. α-Catenin plays a role in anchoring E-cadherin–β-catenin to the actin cytoskeleton [14–17], and the α-catenin gene is sometimes lost [18,19] or mutated [8] in human cancer cell lines.

In addition, induction of the endocytosis of E-cadherin is reported as one of the ways by which the cadherin function is diminished, with no significant alteration in the protein expression of E-cadherin or catenins [20–25]. Recent findings suggest that E-cadherin–catenins complex involves PP2A along with the small GTPase Rac1 and its downstream effector IQGAP1 and that PP2A plays a role in recruitment of IQGAP1 to Rac-bound E-cadherin–catenins complex in nonmalignant HME cells [25].

In this study, we examined whether the cell–cell adhesive function of E-cadherin is diminished by loss or reduced expression of PP2A in human breast cancers. We report here that expression of PP2A-A was lost or significantly reduced in human breast cancer cell lines MDA-MB-231

☆ *Abbreviations:* PP, serine/threonine protein phosphatase; HME, nonmalignant human mammary epithelial; PP2A-A, regulatory subunit A of PP2A; F-actin, actin filaments; FBS, fetal bovine serum; EGF, epidermal growth factor; SDS, sodium dodecyl sulfate; PMSF, phenylmethylsulfonyl fluoride; BSA, bovine serum albumin; PAGE, polyacrylamide gel electrophoresis; PP2A-C, catalytic subunit C of PP2A; PBS, phosphate-buffered saline; siRNA, small interfering RNA.

* Corresponding author. Fax: +81 45 366 3157.
E-mail address: ktaka@gancen.asahi.yokohama.jp (K. Takahashi).

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and MCF-7 cells and in 57% (16 out of 28) of human breast carcinomas. In MDA-MB-231 cells, E-cadherin was deficient and either α-catenin or β-catenin was reduced to 50% of that in HME cells. Concomitantly, the recruitment of IQGAP1 to Rac1-bound catenins was lost in both cancer cell lines and 80% of E-cadherin in MCF-7 cells was endocytosed. Therefore, we propose that the PP2A-A protein that is sometimes lost or significantly reduced in breast carcinomas plays a crucial role in suppression of the E-cadherin endocytosis by recruitment of IQGAP1.

Materials and methods

Cell culture. Human breast cancer MDA-MB-231 cells (European Cell Culture Collection) and MCF-7 were cultured in 10% or 5% FBS-containing RPMI 1640 medium, respectively. HME cells (Cambrex Bio Science) were cultured in MCDB 170 medium supplemented with EGF, insulin, hydrocortisone, ethanolamine, phosphoethanolamine, and prostaglandin E2 [26].

Immunoblot analysis. For the preparation of whole cell lysates, cells were lysed in 1% SDS, 20 mM Tris–HCl, pH 7.4, 1 mM PMSF, and 1 mM sodium orthovanadate, boiled for 2 min, and sonicated on ice at 5 W, using a cell disrupter. The protein content was determined using a protein reagent kit with BSA as the standard (Pierce). For immunoprecipitation, cells were lysed in RIPA buffer (10 mM Tris–HCl, pH 7.4, 0.15 M NaCl, 1% sodium deoxycholate, 1% Triton X-100, 0.1% SDS, 1 mM PMSF, and 0.1 mM sodium orthovanadate) containing 5 mM $CaCl_2$ by sonication of the cells on ice. The cell lysates were incubated with the specific antibody to E-cadherin (Takara) or β-catenin (BD Biosciences), and the protein A–Sepharose beads (Amersham). The immunoprecipitates or whole cell lysates were resolved by SDS–PAGE, transferred onto membranes (Millipore), and immunoblotted with the primary antibodies followed by peroxidase-conjugated secondary antibodies (Amersham). The primary antibodies used were raised against E-cadherin, β-catenin, α-catenin (Zymed), PP2A-A (Upstate Biotechnology), PP2A-C (BD Bioscience), Rac1 (Upstate Biotechnology), and IQGAP1 (Santa Cruz Biotechnology). The reactivity was visualized using an ECL kit (Amersham). The band intensity was quantified using an Edas 290 system equipped with a digital camera (Eastman Kodak).

Tissue array analysis. Formalin-fixed and paraffin-embedded human breast carcinoma tissue specimens and matched normal breast tissue specimens on a glass slide (Super Bio Chips) were deparaffinized and immunohistochemically stained with antibody to PP2A-A (Santa Cruz Biotechnology), and then counter-stained with hematoxylin for the nuclei.

Biotinylation of cell surface proteins. Confluent cultures of cells were incubated with 0.5 mg/ml sulfosuccinimidyl 2-(biotinamido)ethyl-1,3′-dithiopropionate (Pierce) in PBS for 30 min at room temperature, followed by 50 mM NH_4Cl and 1 mM $CaCl_2$ in PBS. For the preparation of total or biotinylated E-cadherin, cells were lysed in RIPA buffer containing 5 mM $CaCl_2$, then incubated with antibody to E-cadherin or the streptavidin-immobilized agarose beads (Sigma), respectively.

Immunofluorescence. Cells grown on glass slides were fixed with 3.7% buffered-formaldehyde, permeabilized with 0.2% Triton X-100, blocked with 3% BSA [26], and stained with Alexa Fluor 568-conjugated phalloidin (Molecular Probes) and antibody to E-cadherin, followed by the secondary antibody conjugated with Alexa Fluor 488 (Molecular Probes). After staining, cells were examined under a confocal laser-scanning microscope (Carl Zeiss).

Results

Cell morphology and expression of proteins involved in E-cadherin–catenins complex

Nonmalignant HME cells formed a monolayer characteristic of epithelial cells in culture at confluent (Fig. 1). Similarly, human breast cancer MCF-7 cells appeared to form a monolayer, however, the precise observation under a phase contrast microscope revealed the occurrence of partial stratification (Fig. 1). Successive cultivation of such cultures of MCF-7 cells resulted in the extensive stratification beyond the saturation density (data not shown). Contrary to these, human breast cancer MDA-MB-231 cells exhibited the fibroblastic morphology and tended to stratify each others even at subconfluence and hardly formed a monolayer after confluence (Fig. 1).

To examine the expression levels of proteins that are associated with E-cadherin–catenins complex, whole cell lysates were prepared from the cells. Immunoblot analysis revealed that each member of the core E-cadherin–catenins complex, consisting of E-cadherin, β-catenin, and α-catenin, was detected in HME cells (Fig. 2). By contrast, E-cadherin was not detected in MDA-MB-231 cells and the relative amount of either β-catenin or α-catenin was reduced to 50% of that in HME cells, respectively (Fig. 2). Contrary to MDA-MB-231 cells, E-cadherin, and α- and β-catenins, were detected in MCF-7 cells and the relative amount of each protein was 1.5-fold above that in HME cells, respectively (Fig. 2). The cadherin–catenins complex binding proteins, that include PP2A-A, PP2A-C, IQGAP1, and Rac1 [25], were detected in whole cell lysates of HME cells (Fig. 2). However, PP2A-A was not detected in either MDA-MB-231 or MCF-7 cells (Fig. 2). The relative amounts of PP2A-C and IQGAP1 in the two cancer cell lines were comparable to those in HME cells (Fig. 2), but Rac1 expression in MDA-MB-231 cells or MCF-7 cells was more than 2- or 4-fold higher than that in HME cells, respectively (Fig. 2).

Loss or reduced expression of PP2A-A in human breast carcinomas

To examine the frequency of loss of PP2A-A expression in human breast carcinomas, immunohistochemical analysis was carried out for tissue arrays that included 28 informative normal–carcinoma matched human breast tissue specimens, using the specific antibody to PP2A-A (Figs. 3A and B). Whereas almost all (96%, 27 out of 28) of normal breast tissue specimens were immunostained with anti-PP2A-A antibody and only 4% (one out of 28) of those were not, 57% (16 out of 28) of the matched breast carcinoma specimens were not immunostained with the antibody (Table 1).

Formation of multiprotein complex with E-cadherin and catenins

In HME cells at confluence, the core E-cadherin–catenins complex involves PP2A-A, PP2A-C, Rac1, and IQGAP1 [25]. As expression of E-cadherin was deficient in MDA-MB-231 cells (Fig. 2), β-catenin was immunoprecipitated from the cells to examine the complex formation of the core cadherin–catenins complex with the binding

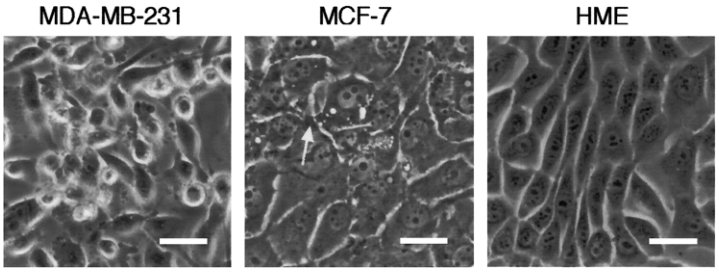

Fig. 1. Phase contrast micrographs of MDA-MB-231, MCF-7, and HME cells, showing the differential cell morphology and cell–cell adhesion. An arrow indicates the cell stratification in confluent culture of MCF-7 cells. Bar indicates 50 μm.

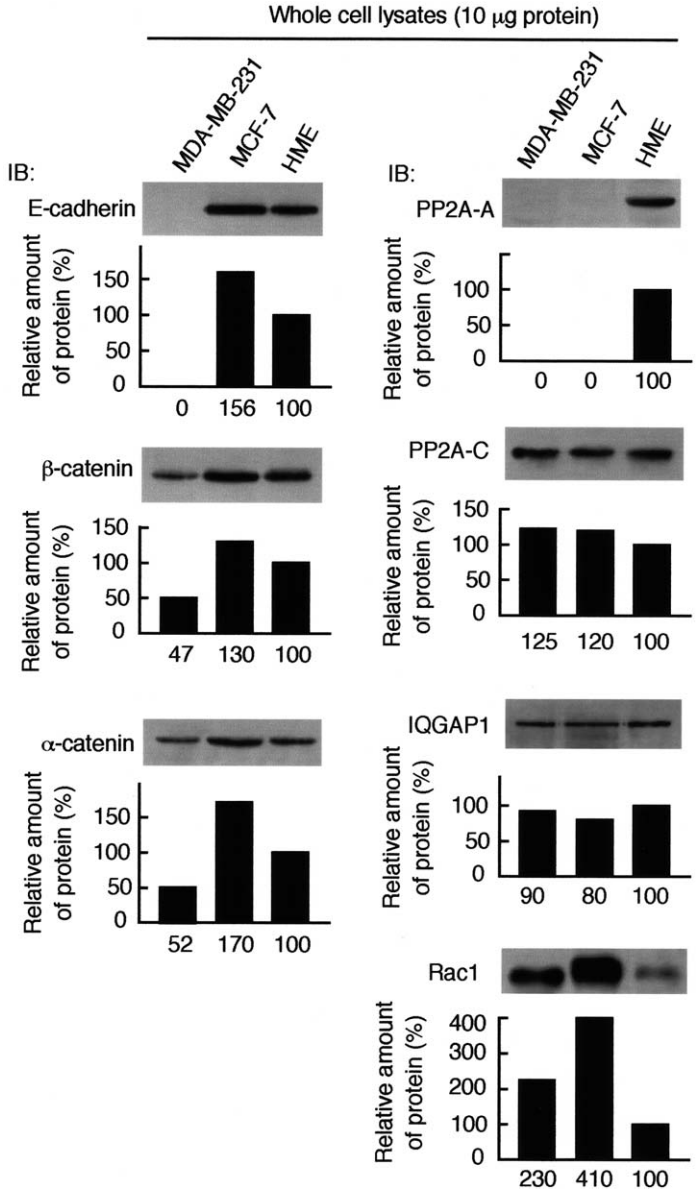

Fig. 2. Expression of the proteins involved in E-cadherin–catenins complex. Whole cell lysates (10 μg protein) prepared from MDA-MB-231, MCF-7, or HME cells were resolved on SDS–PAGE and immunoblotted with antibody to E-cadherin, β-catenin, α-catenin, PP2A-A, PP2A-C, Rac1, or IQGAP1. Relative amount of each protein was determined by measurement of the band intensity and normalized to that in HME cells, respectively.

proteins. Immunoblot analysis revealed that α-catenin and Rac1, but not E-cadherin, PP2A-A, PP2A-C, or IQGAP1, were coprecipitated with β-catenin in MDA-MB-231 cells (Fig. 4A). Similarly, E-cadherin, α-catenin, and Rac1, but not PP2A-A, PP2A-C, or IQGAP1, were coprecipitated with β-catenin in MCF-7 cells (Fig. 4A).

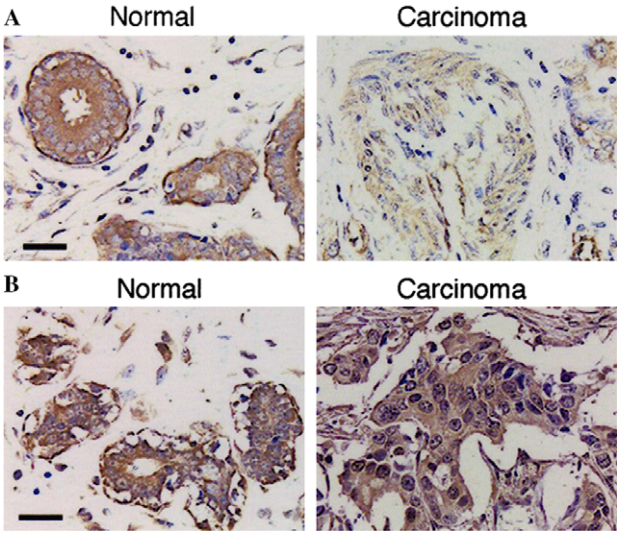

Fig. 3. Expression of PP2A-A in human breast carcinomas. The normal–carcinoma matched human breast tissue arrays on a glass slide were immunostained with anti-PP2A-A antibody and counter-stained with hematoxylin. Representative micrographs of the matched positively stained normal and negatively stained carcinoma tissue specimens (A) and those of positively stained normal and carcinoma tissue specimens (B) are shown. Bar indicates 50 μm.

Table 1
Immunohistochemical detection of PP2A-A expression in normal and carcinoma human breast tissues

Breast tissue	Total number of specimen	PP2A staining[a]	
		Positive (%)	Negative (%)
Normal	28	27 (96)	1 (4)
Carcinoma	28	12 (43)	16 (57)

[a] Tissue arrays consisting of the normal and carcinoma matched human breast tissue specimens were immunostained with antibody to PP2A-A and counter-stained with hematoxylin.

Induction of endocytosis of E-cadherin in MCF-7 cells

Inhibition of PP2A activity by an inhibitor of PP2A or downregulation of PP2A-A by siRNA causes the endocytosis of E-cadherin in HME cells, concomitant with dissociation of IQGAP1 from Rac-bound E-cadherin–catenins complex [25]. To examine whether the absence of PP2A-C and IQGAP1 in E-cadherin–catenins complex in MCF-7 (Fig. 4A) is accompanied by the E-cadherin endocytosis, the cell surface proteins were biotinylated and precipitated with the streptavidin–agarose beads. Immunoblot analysis using anti-E-cadherin antibody revealed that more than 50% of E-cadherin present in HME cells were biotinylated (Fig. 4B). By contrast, only 20% of E-cadherin was biotinylated in MCF-7 cells (Fig. 4B).

Colocalization of E-cadherin and F-actin in cell–cell adhesion sites

To examine whether the absence of coprecipitation of IQGAP1 with β-catenin (Fig. 4A) and decrease in cell surface expression of E-cadherin in MCF-7 cells (Fig. 4B) are accompanied by loss of E-cadherin anchoring to the actin cytoskeleton, cells were doubly stained with anti-E-cadherin antibody and phalloidin for actin. Confocal microscopic observations revealed that the merged images of E-cadherin and actin stainings indicated the colocalization of E-cadherin with F-actin in cell–cell adhesion sites between adjacent HME cells (Fig. 5). Contrary to this, E-cadherin staining was completely absent in MDA-MB-231 cells (Fig. 5). In MCF-7 cells, colocalization of E-cadherin staining with actin staining was hardly observed in cell–cell adhesion sites and E-cadherin staining appeared discontinuously between the adjacent cells (Fig. 5).

Discussion

Morphological observations suggest that cell–cell adhesion in MDA-MB-231 cells is completely lost. This assumption was confirmed by quantification of the amounts of proteins that are thought to participate in the formation of E-cadherin-mediated cell–cell adhesion. Immunoblot analysis revealed a complete loss of E-cadherin expression and reduced expression of both α- and β-catenins in MDA-MB-231 cells compared to those in HME cells. Since loss or reduced expression of E-cadherin or catenins has been previously reported in many human cancer cells [5–8,18,19], loss of cell–cell adhesion in MDA-MB-231 cells is suggested to be mainly due to the loss or reduced expression of E-cadherin, or α- and β-catenins. By contrast, MCF-7 cells appeared to form a monolayer at confluence, with partial stratification. Such morphological feature is suggested to be due to the fact that at least three major components of E-cadherin–catenins complex, consisting of E-cadherin, β-catenin, and α-catenin, were expressed in MCF-7 cells at higher levels than those in HME cells.

With respect of other proteins that were associated with E-cadherin–catenins complex, PP2A-A was commonly deficient in MDA-MB-231 and MCF-7 cells. However, coprecipitation analysis revealed that IQGAP1 was not coprecipitated with Rac1-bound β-catenin in the two cancer cell lines. Since downregulation of PP2A-A by siRNA causes the loss of IQGAP1, but not Rac1, in E-cadherin–catenins complex of HME cells [25], the present results suggest that recruitment of IQGAP1 to Rac1-bound E-cadherin–catenins complex requires the presence of PP2A-A. Coprecipitation of Rac1 and α-catenin with β-catenin in E-cadherin-deficient MDA-MB-231 cells suggests that Rac1 does not interact with E-cadherin but with catenins complex, consisting of α- and β-catenins.

Immunohistochemical analysis of 28 informative normal–carcinoma matched human breast tissue specimens revealed that 57% (16 out of 28) of breast carcinoma specimens were not immunostained with anti-PP2A-A antibody whereas almost all (96%) of the matched normal breast tissue specimens were immunostained with the antibody. The results suggest that PP2A-A expression is sometimes lost or significantly reduced in human breast carcinomas. The

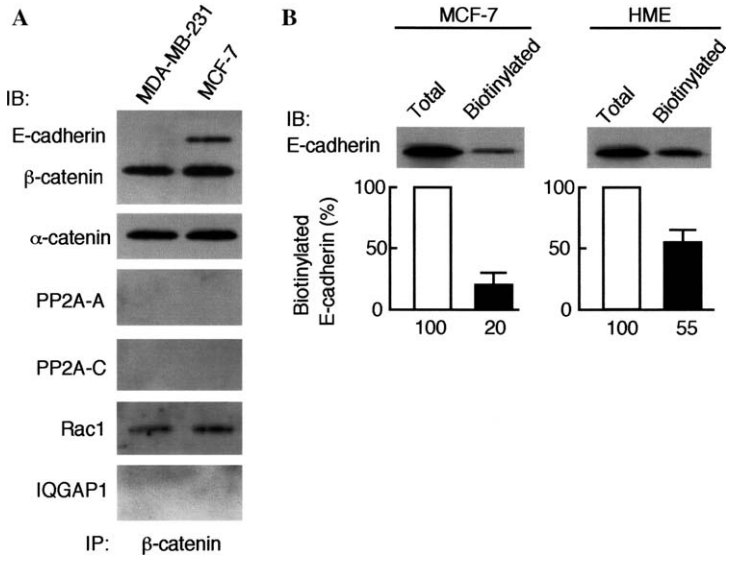

Fig. 4. Coimmunoprecipitation of the proteins with β-catenin and E-cadherin expression on the cell surface. (A) β-Catenin was immunoprecipitated (IP) from MDA-MB-231 and MCF-7 cells. After resolving on SDS–PAGE, the immunoprecipitates were immunoblotted (IB) with antibody to β-catenin, E-cadherin, α-catenin, PP2A-A, PP2A-C, Rac1, or IQGAP1, respectively. (B) MCF-7 or HME cells were lysed directly (Total) or after biotinylation (Biotinylated) in RIPA buffer and precipitated with anti-E-cadherin antibody followed by the protein A–Sepharose beads or the streptavidin-immobilized agarose beads, respectively. After resolving on SDS–PAGE, E-cadherin was detected with anti-E-cadherin antibody. The relative amount of biotinylated E-cadherin to total E-cadherin was quantified by measurement of the band intensity of E-cadherin. Results represent the means ± SD of triplicate assays.

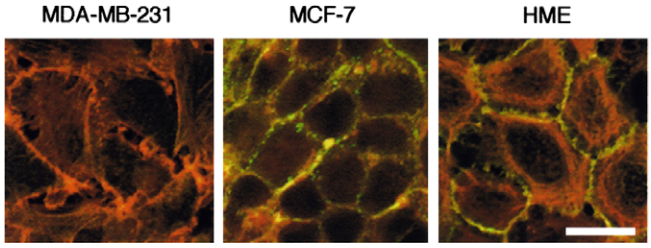

Fig. 5. Colocalization of E-cadherin and F-actin. Cells grown on glass slides were fixed, permeabilized, and stained with Alexa Fluor 568-conjugated phalloidin (red) and anti-E-cadherin antibody followed by Alexa Fluor 488-conjugated secondary antibody (green). After staining, cells were examined under a confocal laser-scanning microscope. Bar indicates 20 μm.

PP2A-A gene alterations are reported in some human carcinomas that include lung and colon carcinomas [27,28], and in melanomas and breast carcinomas [29]. However, the frequency of gene alterations in these carcinomas is low [27,28]. Contrary to these, the present result suggests that loss or significant reduction in the PP2A-A protein is more frequently observed in human breast carcinomas. The reason for the differential frequencies between gene alterations and protein expression remains to be elucidated in the present study.

Since either inhibition of PP2A activity by an inhibitor of PP2A or downregulation of PP2A-A expression by siRNA induces the endocytosis of E-cadherin concomitant with the dissociation of IQGAP1 from Rac1-bound E-cadherin–catenins complex in HME cells [25], the absence of IQGAP1 in Rac1-bound E-cadherin–catenins complex in PP2A-A-deficient MCF-7 cells would be accompanied by the E-cadherin endocytosis. Biotinylation of the cell surface proteins indicated that approximately half of E-cadherin present in HME cells was biotinylated. This agrees with the previous result [25]. By contrast, only 20% of E-cadherin in MCF-7 cells was biotinylated, suggesting that most of E-cadherin in MCF-7 cells is endocytosed.

The previous findings suggest that the E-cadherin endocytosis is suppressed by PP2A through recruitment of IQGAP1 and anchoring E-cadherin–catenins complex to the actin cytoskeleton [25]. Double staining of E-cadherin and actin demonstrating the continuous colocalization of E-cadherin with F-actin along the cell–cell adhesion sites between adjacent HME cells indicates the intracellular anchoring of E-cadherin–catenins complex to F-actin. By contrast, discontinuous colocalization of E-cadherin with F-actin in MCF-7 cells suggests the incomplete anchoring of E-cadherin–catenins complex to F-actin. Since induction of the endocytosis of E-cadherin, with no significant alteration in the protein expression of E-cadherin or catenins, is reported as one of the ways by which the cadherin function is diminished [20–25], weakened cell–cell adhesion that allows cell stratification in confluent culture of MCF-7 cells may be induced by the PP2A-A deficiency through the loss of IQGAP1 recruitment to Rac1-bound E-cadherin–catenins complex and the E-cadherin endocytosis. Therefore, the dysfunction of E-cadherin due to its endocytosis may occur in some proportion of human breast carcinomas in which PP2A-A is lost or significantly reduced.

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