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Stabilin-2 mediates homophilic cell–cell interactions via its FAS1 domains

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ABSTRACT

Stabilin-2 was recently shown to mediate a heterophilic interaction with integrin alphaMbeta2 via its FAS1 domain. Here, we demonstrate that stabilin-2 also mediates homophilic cell–cell interactions. L cells expressing stabilin-2 mediate a significant level of cell aggregation, and this aggregation is significantly inhibited by anti-stabilin-2 antibody. Stabilin-2-mediated aggregation is mediated by homophilic interactions and enhanced in the presence of Ca^{2+} and Mg^{2+}. Interestingly, exogenous addition of FAS1 domains but not EGF-like domains enhances stabilin-2-mediated cell aggregation, suggesting that exogenous FAS1 domains may form polymeric structure with FAS1 domains of stabilin-2. Together, these data show the participation of stabilin-2 in homophilic cell adhesion and role of FAS1 domains.

1. Introduction

Stabilin-2 is a large multi-functional glycoprotein that contains a large, extracellular region encompassing seven FAS1 domains, 23 EGF-like domains (4 EGF-like domain repeats), and a Link domain. Stabilin-2 (also known as FEEL-2 and HARE) was originally reported as an endocytic hyaluronan receptor of hepatic sinusoidal endothelial cells [1]. Stabilin-2 has been independently characterized as FEEL-2 and HARE. FEEL-2 was reported to be a scavenger receptor that endocytoses acetylated LDL and advanced glycation end products [2,3]. HARE has been shown to act as the endocytic receptor for hyaluronan, chondroitin sulfates, and heparin [4–7]. Recently, we demonstrated that stabilin-2 mediates lymphocyte adhesion to hepatic sinusoidal endothelial cells [8] and is a phosphatidylserine receptor that is involved in apoptotic cell clearance [9].

FAS1 domain consists of about 140 amino acid residues and is originally found in fasciclin I, an insect cell adhesion molecule (CAM) [10]. In humans, FAS1 domains are shared by four molecules, including TGFBI/βig-h3 [11,12], periostin/OSF-2 [13], stabilin-1, and stabilin-2 [14]. Accumulating data suggest that the FAS1 domain could act as a functional domain for adhesive activity in cell adhesion molecules. βig-h3 and periostin have been shown to function as cell adhesion substrates via the interaction with integrins [11,15–17]. Recently, we showed that FAS1 domains of stabilin-2 also mediate a heterophilic interaction with integrin alphaMbeta2 [8]. In this study, we demonstrate that stabilin-2 facilitates cell aggregation via homophilic trans-interactions. In addition, the potential for FAS1 domains to serve as a domain for homophilic interaction is addressed and supported by the evidence reported herein.

2. Materials and methods

2.1. Antibodies

To produce polyclonal antibody (Stab2-N) directed against human stabilin-2, His-tagged recombinant proteins corresponding to amino acids 554–655 were expressed in *Escherichia coli* and purified using Ni-NTA resin (Qiagen), in accordance with the manufacturer's instructions. Antibodies were raised in rabbits by subcutaneous injection of the recombinant protein (200 μg). The antiserum was further purified by Protein A affinity chromatography (Amersham Pharmacia).

2.2. Cell culture

L cells that stably expressed stabilin-2 (L/Stab-2) or empty vector (L/Mock) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) as previously described [8,9].

2.3. Cell aggregation assays

Cell aggregation assays were performed as previously described with slight modification [18]. Briefly, to obtain a single-cell

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suspension, cells were washed with PBS, incubated with 5 mM EDTA/PBS at 37 °C for 5 min, and dispersed by gentle pipetting. After wash twice with Hank's balanced salt solution (HBSS) without divalent cations, the cells were resuspended in HBSS containing Ca^{2+} and Mg^{2+} (Invitrogen). 1×10^6 cells per well were reseeded in 24-well plates precoated with bovine serum albumin fraction V (Sigma) and rotated on a gyratory shaker at 37 °C for 15, 30, 45, or 60 min. Aggregation was terminated by the addition of 2% glutaraldehyde. The degree of aggregation (D) was calculated as $D = (N^0 - N^t)/N^0$, where N^t represents the number of remaining particles at time-point 't' and N^0 is the initial number of particles corresponding to the total number of cells [19]. To determine the divalent cation dependence of cell–cell interactions, Ca^{2+}- and Mg^{2+}-free HBSS supplemented with 2 mM Ca^{2+}, 2 mM Mg^{2+}, 2 mM Ca^{2+} and 5 mM EGTA or 2 mM Mg^{2+} and 5 mM EDTA was used instead of HBSS containing Ca^{2+} and Mg^{2+}.

For aggregation inhibition studies, aggregation assays were performed using Ca^{2+}- and Mg^{2+}-free HBSS. A single-cell suspension was seeded in 24-well plate precoated with BSA. The cells were incubated for 30 min in the presence of recombinant proteins. The degree of aggregation was calculated as above.

2.4. Mixed cell aggregation assays

For a mixed cell aggregation assay between two L cell lines, one cell line was prelabeled with DiI (Molecular Probes), as previously described [20]. The labeled cell line was mixed with an equal number of other cell lines to a final concentration of 1×10^6 cells/ml, and incubated at 37 °C for 30 min. Following the addition of 4% formaldehyde, aggregates were subjected to light and fluorescence microscopy.

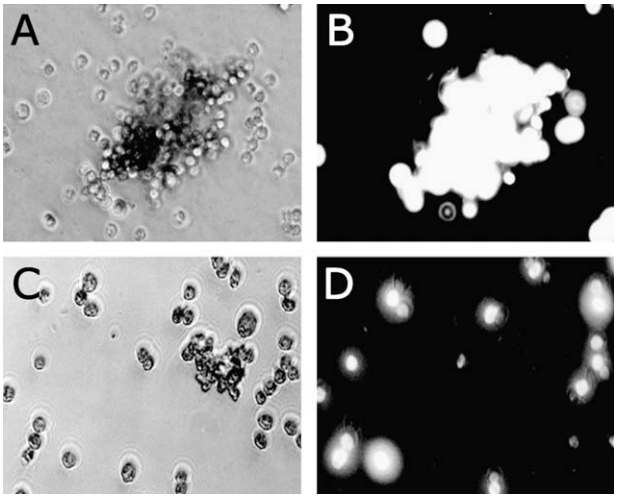

Fig. 2. Stabilin-2-mediated cell aggregation is homophilic interaction. Mixed aggregation assays. Single-cell suspensions of labeled L/Stab-2 cells were allowed to aggregate in the presence of an equal number of unlabeled L/Mock cells (A and B). Conversely, labeled L/Mock cells were incubated in the presence of an equal number of unlabeled L/Stab-2 cells (C and D). After 60 min, the cells were observed under a microscope. The same fields are depicted in phase-contrast (A and C) and fluorescence (B and D) microscopy. In mixed aggregation assays, aggregates were almost entirely composed of L/Stab-2 cells.

2.5. Preparation of recombinant proteins

A Nus tag was fused to each recombinant protein to enhance the solubility of proteins corresponding to the repeated unit

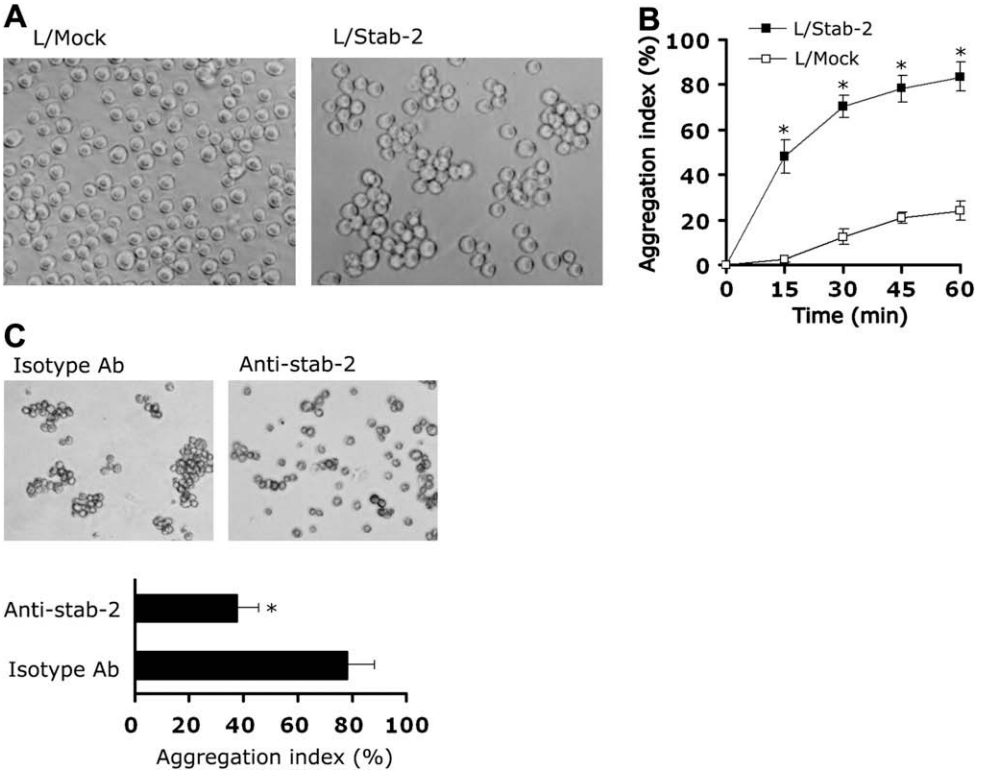

Fig. 1. Stabilin-2 mediates aggregation in suspension. (A) Cell aggregation activity. Cell aggregation assays were performed with L cells expressing stabilin-2 (L/Stab-2) or empty vector (L/Mock) as described in Section 2. After 60 min, the aggregation index was determined. A representative result of three independent experiments is shown. (B) A single-cell suspension was incubated for the indicated periods of time. □, L/Mock cells; ■, L/Stab-2 cells. (C) Aggregation assays were performed in the presence of anti-stabilin-2 antibody (Stab-N) or isotype-matched antibody. Results are expressed as the means ± S.D. of three independent experiments. $^*P < 0.01$.

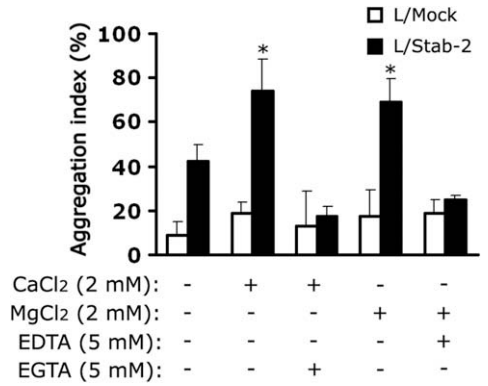

Fig. 3. Stabilin-2-mediated cell aggregation is enhanced by divalent cations. Aggregation assays using L/Mock or L/Stab-2 cells were performed in HBSS containing the indicated concentrations of $CaCl_2$, $MgCl_2$, EGTA, and EDTA. After 60 min, the aggregation index was determined. Results are expressed as the means ± S.D. of three independent experiments. $^{*}P < 0.01$.

(Fig. 4A). Nus-tagged recombinant proteins corresponding to repeated units or EGFrps were expressed and purified as described previously [8]. To produce recombinant proteins for each FAS1 domain of stabilin-2, each fragment of stabilin-2 cDNA encoding amino acids 406–508, 554–655, 1030–1130, 1173–1268, 1631–1727, 1778–1883, and 2356–2449 was generated by PCR, cloned into the BamHI and XhoI sites of pET-43.1a (Novagen). These recombinant proteins were expressed in BL-21cells, harvested, and purified using Ni-NTA resin (Qiagen) in accordance with the manufacturer's instructions. Contaminating endotoxins were removed with Detox-i-gel (Pierce).

2.6. Statistical analysis

The statistical significance was assessed by the *t*-test. A $P < 0.05$ was considered to be statistically significant.

3. Results and discussion

3.1. Stabilin-2 mediates cell aggregation in suspension

Stabilin-2 is a multi-functional glycoprotein with multi-domains. Recently, we showed that FAS1 domains of stabilin-2 mediate lymphocyte adhesion to the hepatic sinusoidal endothelium via the interaction with integrin alphaMbeta2 [8]. However, previous study in *Drosophila* fasciclin I, an insect neural cell adhesion molecule consisting of four FAS1 domains [21] indicate that the FAS1 domain also mediates homophilic cell adhesion. Furthermore, we observed that TGFBI/βig-h3 consisting of four FAS1 domains self-assembles to form a fibrillar structure [22], suggesting that the FAS1 domain of stabilin-2 could also mediate homophilic interaction. To investigate whether stabilin-2 is involved in homophilic cell–cell interaction, we performed an aggregation assay using L cells which were stably transfected with human stabilin-2 (re-

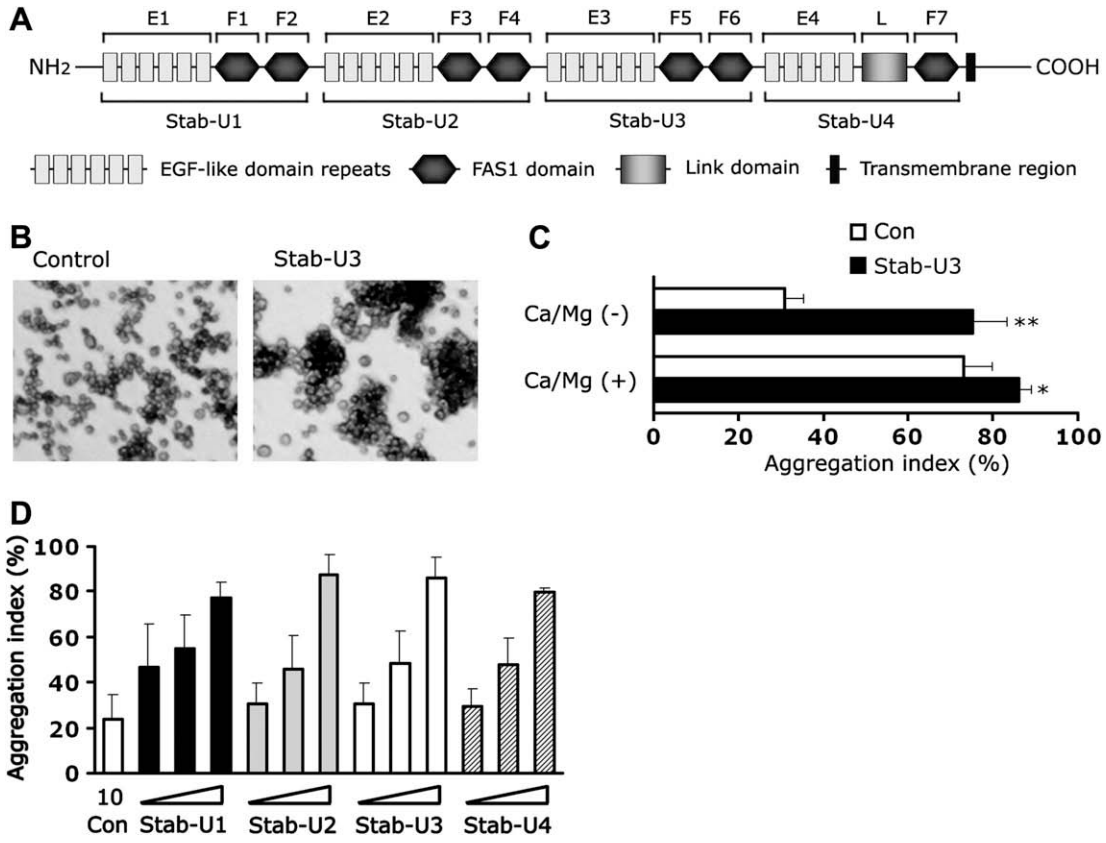

Fig. 4. Four repeated unit proteins facilitate stabilin-2-mediated cell aggregation. (A) Schematic diagram of stabilin-2 protein and deletion mutant proteins. A Nus tag was fused to each recombinant protein to enhance the solubility of the proteins. Stab-U1, -U2, and -U3 are composed of one EGFrp and two FAS1 domains. Stab-U4 is composed of an EGFrp, a FAS1 domain, and a Link domain. (B) Aggregation assay were performed in the presence of repeated unit proteins or control Nus protein. A representative result of three independent experiments is shown. (C) Aggregation assays were performed in the presence of Stab-U3 or Nus (control) protein using HBSS with or without Ca^{2+} and Mg^{2+}. $^{**}P < 0.01$; $^{*}P < 0.05$. (D) The effect of four repeated units on the aggregation of L/Stab-2 cells. Aggregation assay were performed using Ca^{2+}- and Mg^{2+}-free HBSS. The cells were incubated for 30 min in the presence of three different concentrations (0.1, 1, and 10 μM) of the indicated proteins. Results are expressed as the means ± S.D. of three independent experiments.

ferred to as L/Stab-2 cells) or empty vector (referred to as L/Mock cells) [9]. Mouse fibroblast L cells do not express any cadherins and have been widely used in cell adhesion studies [23]. L/Mock cells were scattered as a single-cell suspension, whereas L/Stab-2 cells formed clusters in a time-dependent manner (Fig. 1A). Over 15 min of incubation, more than 50% of the cells formed clusters. More than 80% cells formed clusters after 60 min of incubation (Fig. 1B). Aggregation of L/Stab-2 cells was performed in four stable L cell clones, and similar results were obtained (data not shown). To confirm stabilin-2-mediated aggregation, we performed aggregation experiments in the presence of anti-stabilin-2 antibody (Stab2-N) or isotype-matched control antibody. L/Stab-2 cells in the presence of control antibody yielded large clusters similar to untreated experiment, accounting for ~80% of aggregation index. In contrast, treatment of anti-stabilin-2 antibody significantly inhibited cell aggregation, accounting for ~40% of aggregation index (Fig. 1C). These data show that stabilin-2 can mediate cell–cell aggregation.

3.2. Stabilin-2-mediated cell aggregation is induced by homophilic interaction

To determine whether cell aggregation is mediated by homophilic or heterophilic interaction, we performed a mixed aggregation experiment between L/Mock and L/Stab-2 cells. In the first series of experiments, L/Stab-2 cells were labeled with the DiI red fluorescent dye and allowed to aggregate in the presence of an identical number of unlabeled L/Mock cells. Under these conditions, the main body of aggregates was composed of labeled cells only, while occasional unlabeled cells were observed at the periphery (Fig. 2A and B). Conversely, when labeled L/Mock cells were mixed with unlabeled L/Stab-2 cells, aggregates were devoid of labeled cells (Fig. 2C and D). These results indicate that L/Stab-2 cells selectively aggregate with each other, but not with L/Mock cells and also suggest that aggregation is mediated by homophilic interaction of stabilin-2, rather than heterophilic interaction with an endogenous membrane component of L cells. In addition, the divalent cation requirement of stabilin-2-mediated homophilic interaction was investigated. Minimal aggregation of L/Stab-2 cells was observed when experiments were performed in Ca^{2+}- and Mg^{2+}-free HBSS. Aggregation was enhanced by the addition of 2 mM $CaCl_2$ or $MgCl_2$ and completely inhibited by 5 mM EDTA or EGTA (Fig. 3). Our data indicate that stabilin-2-mediated homophilic interaction requires divalent cations.

3.3. Repeated unit proteins enhance cell aggregation

Next, we investigated which regions are involved in cell aggregation. Because stabilin-2 consists of interrupted repeats of seven FAS1 and four EGF-like domain repeats (EGFrps), we dissected the extracellular region into four repeated units (Stab-U1, -U2, -U3, and -U4) (Fig. 4A), and then examined the effect of each unit on cellular aggregation activity. We expected that each unit would inhibit cell aggregation by interfering homophilic interaction of stabilin-2. However, each unit protein rather enhanced cell aggregation (Fig. 4B). Empty vector transfected L cells (L/Mock) do not form cellular clusters in the presence of each unit protein (data not shown). To confirm further the enhancement of the cell aggregation, we performed a cell aggregation assay in divalent cation free medium which shows minimal aggregation. In this condition, L/Stab-2 cells in the presence of control Nus protein yielded small clusters, accounting for ~35% of aggregation index, whereas aggregation of these cells in the presence of repeated unit proteins was increased to ~80% of aggregation index (Fig. 4C). Enhancement of aggregation by each unit protein was found to be concentration-dependent, and ~80% of aggregation index was achieved at 10 μM of the proteins (Fig. 4D). These results suggest that each unit could possibly bridge

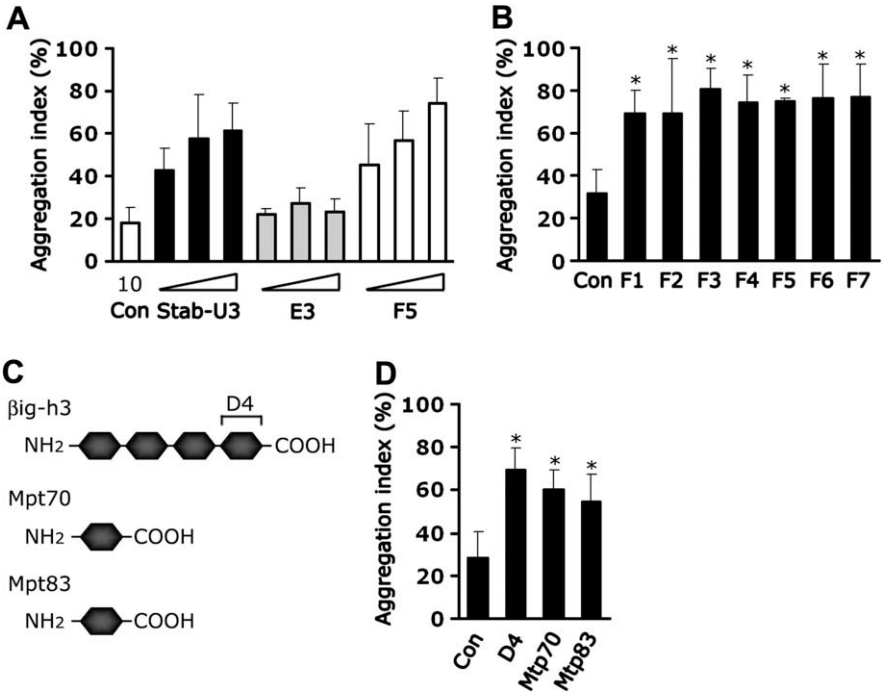

Fig. 5. FAS1 domain is responsible for stabilin-2-mediated cell aggregation. (A) Aggregation assay were performed in Ca^{2+}- and Mg^{2+}-free HBSS in the presence of three different concentrations (0.1, 1, and 10 μM) of the indicated proteins. (B) Aggregation assay were performed in Ca^{2+}- and Mg^{2+}-free HBSS in the presence of 10 μM of FAS1 proteins. Results are expressed as the means ± S.D. of three independent experiments. $^{*}P < 0.01$. (C) The schematic diagram of βig-h3, MPB70, and MPB83. (D) Aggregation assay were performed in Ca^{2+}- and Mg^{2+}-free HBSS in the presence of 10 μM of recombinant FAS1 domain of TGFBI/βig-h3, MPB70, or MPB83. Results are expressed as the means ± S.D. of three independent experiments.

two or more ectodomains of stabilin-2 and facilitate stabilin-2-mediated cell–cell interaction.

3.4. FAS1 domains are responsible for stabilin-2-mediated cell aggregation

In order to further evaluate the domain which is involved in cell aggregation, we produced Nus-tagged recombinant proteins for the third EGF-like domain repeat (E3) and fifth FAS1 domain (F5), and tested their effects on cell aggregation in L/Stab-2 cells. When L/Stab-2 cells were incubated with F5 protein, cell aggregation was enhanced in a dose-dependent manner similar to what was observed with Stab-U3 (Fig. 5A). On the other hand, the E3 and Nus protein had no effect. Next, we produced recombinant proteins for other FAS1 domains of stabilin-2 and performed aggregation assays in the presence of each FAS1 domain protein. L/Stab-2 cells in the presence of each FAS1 protein yielded larger aggregates than control (Fig. 5B). We also examine the effect of FAS1 domains of TGFBI/βig-h3 and two *Mycobacterium tuberculosis* proteins, MPB70 and MPB83 on cell aggregation. All FAS1 domains showed an increase of cell aggregation, comparable with those of stabilin-2 (Fig. 5C and D). Considering that FAS1 domains of TGFBI/βig-h3 which self-assembles to form a fibrillar structure [22] also enhanced cell aggregation, exogenous FAS1 domains may form polymeric structure with FAS1 domains of stabilin-2, thereby enhancing stabilin-2-mediated aggregation. Thus, these results indicate that FAS1 domains play an important role in stabilin-2 mediated homophilic cell–cell interaction.

In the present study, we found that stabilin-2-mediated aggregation was enhanced by divalent cations, indicating that stabilin-2 retains one of the characteristics of cell adhesion molecules, which mediate homophilic interaction, including cadherin [24] and nijurin [25]. The FAS1 domain, however, has not been reported to bind calcium and does not seem to include calcium-binding motif based on sequence analysis. On the other hand, sequence analysis reveals that several EGF-like domains possess the motif for calcium binding (data not shown). Nevertheless, EGF-like domain repeats neither inhibit nor enhance cell aggregation. Although our observations using recombinant proteins do not support a role for EGF-like domain in the homophilic interaction of stabilin-2, it was previously reported that EGF-like domains are likely to support ligand-binding activity [26]. EGF-like repeats mediate lateral and reciprocal interaction of Ep-CAM molecules in homophilic adhesions [27]. Thus, it is possible that EGF-like domain repeats, not directly involved in trans-interaction, may be required for adhesion formation or stabilization by recruiting additional molecules to the adhesion site.

Although our results provide evidence that stabilin-2 mediates homophilic cell–cell interaction, its physiological and pathological function is largely unknown. To examine whether stabilin-2 was functionally involved in cellular integrity, L/Stab-2 cells were cultured in the presence of anti-stabilin-2 antibody for 48 h. Isotype-matched control antibody did not affect the cell clustering, while anti-stabilin-2 antibody resulted in dissociation of the cell clustering (Fig. 6). Considering stabilin-2 is highly expressed in sinusoidal endothelial cells of spleen, lymph node, and liver [5,28], this result suggests that stabilin-2 may be important for the maintenance of vascular integrity in sinusoids. Recently, we demonstrated that stabilin-2 is expressed in monocyte-derived macrophages (HMDMs) and functions as a phosphatidylserine (PS) receptor which is involved in clearance of apoptotic cells [9]. Thus, it is possible that the homophilic interaction of stabilin-2 plays a role in the interaction between macrophages and endothelial cells under some physiological or pathological conditions. In conclusion, we have demonstrated that stabilin-2 mediates cell–cell adhesion via homophilic interaction mainly through FAS1 domains. Further characterization of stabilin-2 should facilitate elucidation of the physiological functions of stabilin-2-mediated cell–cell interactions.

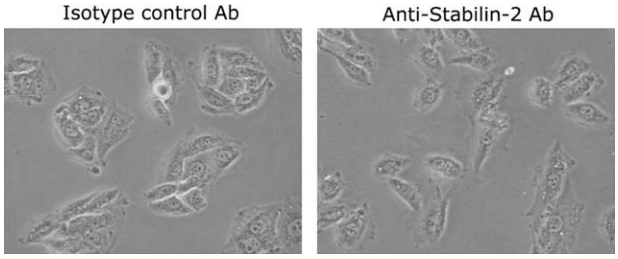

Fig. 6. Distruption of cell clustering in stabilin-2 expressing cells by anti-stabilin-2 antibody. L/Stab-2 cells were seeded at a density of 5×10^4 cells/well in 12-well plates and grown in the presence of anti-stabilin-2 antibody (20 μg/ml) or isotype-matched antibody (20 μg/ml). After 48 h, the cells were photographed under a phase-contrast microscope.

Acknowledgments

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