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CEACAM1 inhibits cell-matrix adhesion and promotes cell migration through regulating the expression of N-cadherin

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ABSTRACT

Carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1) is a member of the immunoglobulin super family and has been observed to have two paradoxical functions: tumor suppression and the promotion of tumor invasion. In the present study, we discovered that CEACAM1 functions as an adhesion inhibitor and a migration promoter. The CEACAM1 transfected cells, either 293-CEACAM1 or LOVO/trans-CEACAM1, was proved to have lower adhesion rate. Furthermore, HT29/siRNA-CEACAM1 cells had a higher adhesion rate than HT29 cells. These results indicated that CEACAM1 was an inhibitor of cell-matrix adhesion. Additionally, 293-CEACAM1 LOVO/trans-CEACAM1 cells exhibited better motility in a trans-well migration assay. N-cadherin expression levels were positively correlated with CEACAM1 in 293-CEACAM1, LOVO/trans-CEACAM1 and HT29/siRNA-CEACAM1 cells. When blocked by a GC-4 antibody, the adhesive capacities of 293-CEACAM1 and LOVO/trans-CEACAM1 were recovered and the motilities of them were suppressed, which suggested that CEACAM1 functioned through N-cadherin.

1. Introduction

Carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1), which is also called biliary glycoprotein (BGP), C-CAM and CD66a, is a member of the immunoglobulin superfamily. It is a glycoprotein that is expressed on the surface of certain epithelial, endothelial, lymphoid and myeloid cells [1]. Human CEACAM1 has been shown to have 11 isoforms with different numbers of extracellular immunoglobulin-like domains, of which four isoforms have a long cytoplasmic tail (CEACAM1-L) while the others have a short cytoplasmic tail (CEACAM1-S) [1]. The long cytoplasmic domain contains two immunoreceptor tyrosine-based inhibitory motifs (ITIMs) at position 488 and 515, which can be phosphorylated by Src kinases [2,3].

It has been reported that CEACAM1 is a tumor suppressor and has a lower level of expression in prostate, breast, colorectal cancer and hepatocellular carcinoma [4,5]. However, CEACAM1 is overexpressed in lung cancer and malignant melanoma [6,7].

Despite the controversial finding of tumor-suppressive activity by CEACAM1, several studies have shown that the high expression of CEACAM1-L was positively correlated with tumor invasion, migration and stage [4,8,9]. Immunohistochemical analyses have been performed on clinical samples from 164 colorectal cancer patients, which showed that CEACAM1-L, but not CEACAM1-S, was overexpressed at the invasion front, suggesting that CEACAM1-L contributed to colorectal cancer invasion [3]. Similarly, CEACAM-1 expression was immunohistochemically evaluated in 96 patients with metastatic pulmonary adenocarcinoma, and the results showed a positive correlation between CEACAM-1 expression on cells of the primary tumor, lymph node metastases and hematogenous metastases [10]. It has also been reported CEACAM1$^{-/-}$ mice exhibited a reduced metastatic burden when injected with metastatic mouse colorectal cancer cells, which made CEACAM1 a novel metastatic colorectal carcinoma therapeutic target [11]. Furthermore, CEACAM1-L dominance was associated with a significantly shorter patient survival time [3].

However, the molecular mechanism of CEACAM1-L on cancer invasion and migration remains unknown. Notably, N-cadherin, a

Abbreviations: CEA, carcinoembryonic antigen; CEACAM1, carcinoembryonic antigen related cell adhesion molecule 1; EMT, epithelial–mesenchymal transition; ITIMs, immunoreceptor tyrosine-based inhibitory motifs; siRNA, small interfering

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calcium dependent cell–cell adhesion glycoprotein and widely regarded as a marker of epithelial–mesenchymal transition (EMT), also stimulates tumor invasion and migration. It was in a balance with E-cadherin between suppression and promotion of invasion [12].

In this study, we analyzed the role of CEACAM1 in cell-matrix adhesion and cell migration studies as a model of tumor metastasis, and found that CEACAM1 functioned through regulating the expression of N-cadherin.

2. Materials and methods

2.1. Cell lines and cell culture

The human embryonic kidney (HEK) 293 cell line (American Type Culture Collection, Rockville, MD, USA) and the stable-transfected cell line, 293-CEACAM1, were both cultured in Dulbecco's modified Eagle's medium (Gibco, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS) at 37 °C under a humidified atmosphere of 5% CO_2. Three human colon cancer cell lines, LOVO and HT29, were cultured in RPMI 1640 medium (Gibco) supplemented with 10% fetal bovine serum at 37 °C under a humidified atmosphere of 5% CO_2.

2.2. Antibodies and reagents

N-cadherin blocking mouse monoclonal antibody (GC-4) was purchased from Sigma–Aldrich (St. Louis, MO, USA). The rabbit anti-N-cadherin antibody and rabbit anti-CEA antibody for Western blotting were from Epitomics (Burlingame, CA, USA). Mouse anti-CEACAM1 antibody was from R&D (Minneapolis, MN, USA). Mouse anti-GAPDH antibody was from GeneSci (Beijing, China).

2.3. Transfection and RNA interference

Plasmids containing the CEACAM1 gene (refseq ID: BC014473) and green fluorescent protein (GFP) reporter gene were purchased from Genechem Co. Ltd. (Shanghai, China). Transfection was performed with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Forty-eight hours after transfection, G418 solution was added to a final concentration of 800 μg/ml. Ten days later, the surviving cells were digested, resuspended in medium at a concentration of 100 cells/10 mL and plated in a 96-well plate with 100 μL cell suspension per well. The stably transfected cells were maintained from wells with only one green fluorescent clone.

The siRNA of human CEACAM1 was synthesized by Sigma–Aldrich as follows: CCA CAA ATG ACA CTG. The siRNA was transfected by Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Cells were harvested for the following experiments 48 h after transfection.

2.4. Western blot assay

Cell samples were lysed in ice-cold cell lysis buffer (Beyotime, China) with 1 mM PMSF (phenylmethylsulfonyl fluoride) for 45 min, and then centrifuged at 12,000 rpm for 20 min at 4 °C. The protein concentration of the resulting supernatant was determined by the bicinchoninic acid (BCA) protein assay kit (YuanPingHao Bio, China). Proteins (80 μg) were separated by 10% SDS–PAGE electrophoresis and subsequently transferred to a PVDF membrane. The membrane was then blocked with 5% nonfat dry milk in TBS/Tween 20 (0.1%, v/v) for 2 h at room temperature and incubated overnight at 4 °C with primary antibodies. The blots were washed with TBST, incubated with the horseradish peroxidase-conjugated secondary antibody and developed with a chemiluminescent substrate, ECL (enhanced chemiluminescence) Plus. An autoradiograph was then obtained. Assays were performed in triplicate for each experiment.

2.5. Adhesion assay

Briefly, 96-well plates were coated with collagen I at 4 °C overnight and then blocked with 3% bovine serum albumin (BSA) at 37 °C for 1 h. After wells were washed with PBS, 5×10^4 cells were plated into each well without FBS, incubated at 37 °C for 1 h and washed with PBS. The remaining cells were fixed in 4% paraformaldehyde solution for 40 min at room temperature, washed with 0.1 M borate buffer and stained using 1% methylene blue for 10 min. After cells were washed three times with borate buffer, 0.1 M HCl was added and adherent cells were quantified by absorbance at 600 nm. Assays were performed in quadruplicate for each experiment.

2.6. Trans-well migration assay

Trans-well migration assays were carried out using 24-well polycarbonate trans-well inserts (8 μm, Corning, NY, USA). Complete medium containing 10% FBS was added to the lower compartment (600 μL/well). Cells were harvested in serum-free medium and added (1.0×10^5 cells/well) into the upper compartment (200 μL/well). After being cultured for 48 h at 37 °C, cells on the microporous membrane were fixed by 4% paraformaldehyde solution and stained using crystal violet staining solution (0.1% crystal violet in 10% methanol solution). Non-migratory cells on the upper surface of the membrane were wiped with a cotton-tip applicator, and cells that migrated to the lower side of the membrane were quantified using phase contrast microscopy.

2.7. Flow cytometric assays

To confirm that the plasmid containing CEACAM1 and the GFP reporter gene were stably transfected into HEK293 cells, the transfected cells and HEK293 cells were harvested respectively and green fluorescence was detected by flow cytometry. To detect the expression of CEACAM1 in different color cancer cell lines, cells were digested, resuspended and washed with PBS with 2% FBS, and then incubated with 200 μL mouse anti-CEACAM1 antibody solution at a recommended concentration at 4 °C for 1 h. After cells were washed three times with PBS and 2% FBS, cells were incubated with 200 μL fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse antibody (1:500) at 4 °C for 45 min. Another wash was carried out with PBS and 2% FBS, and green fluorescence was then detected by flow cytometry.

2.8. Statistical analysis

All experiments were repeated at least three times. Statistical significance was assessed by two-tailed unpaired Student's *t*-test.

3. Results

3.1. Establishing the stable transfection of cells with CEACAM1

After the transfection of HEK293 cells with a plasmid containing the CEACAM1 gene and GFP reporter gene, a stably transfected clone, 293-CEACAM1, which expressed CEACAM1 was gained. This line could be cultured for more than five passages without G418 in culture medium. The results of fluorescent microscopy and Western blot assays proved that CEACAM1 was overexpressed by

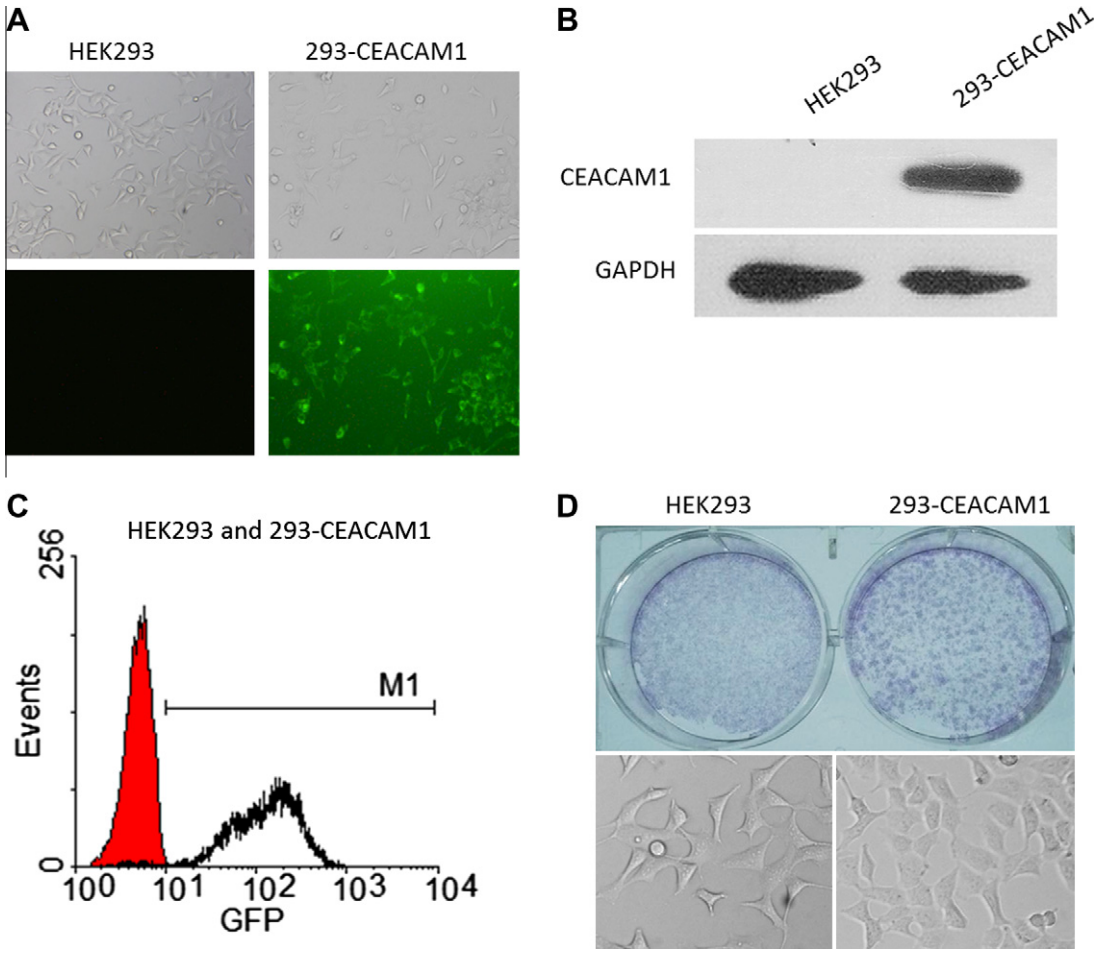

Fig. 1. Establishing CEACAM1 stably transfected cells. HEK293 cells were transfected with a plasmid containing CEACAM1 and a GFP reporter gene. By culturing them with G418 and isolating in 96-well plate, a stably transfected clone, 293-CEACAM1, was gained. (A) Light (upper) and fluorescent (lower) microscopy of HEK293 and 293-CEACAM1 cells, respectively (×400). (B) Western blot analysis of the expression of CEACAM1 in HEK293 and 293-CEACAM1 cells. (C) Flow cytometric assay of CEACAM1 expression in HEK293 (red curve) and 293-CEACAM1 (black curve) cells. (D) Clones (upper) and light microscopy (lower) of HEK293 and 293-CEACAM1 cells (×400).

293-CEACAM1 cells, whereas there was no expression of CEACAM1 in HEK293 cells (Fig. 1A and B). The results of the flow cytometric assay indicated that 97.53% of 293-CEACAM1 cells expressed CEACAM1 (Fig. 1C). Therefore, 293-CEACAM1 was established as a stably-transfected cell line that could be used in the following experiments.

Being cultured in 6-well plates and stained by crystal violet solution, HEK293 cells grew uniformly on the plate whereas 293-CEACAM1 cells formed cell clusters (Fig. 1D). Microscopy illustrated that 293-CEACAM1 cells appeared to have better cell–cell adhesion properties whereas HEK293 were stretched over the plate, implying it had a better adhesive capacity to the plate than to other HEK293 cells. The differences in cellular morphology between HEK293 and 293-CEACAM1 cells prompted us to ascertain the role of CEACAM1 in cell-matrix adhesion.

3.2. CEACAM1 expression inhibits cell-matrix adhesion

To understand the function of CEACAM1 in cell-matrix, two colorectal carcinoma cell lines, HT29 and LOVO, were involved in the experiments. Flow cytometric assay results demonstrated that HT29 cells expressed CEACAM1 positively and LOVO cells did not express CEACAM1 (Fig. 2A). However, in the cell adhesion assay, HT29 cells showed a lower adhesion rate than LOVO (Fig. 2B, 0.36-fold, $p < 0.001$). Similarly, compared with HEK293 cells, 293-CEACAM1 cells showed a lower adhesion rate (0.4-fold, $p < 0.001$, Fig. 2C). To further confirm the association between CEACAM1 expression and the capacity for cell adhesion, RNA interference was performed. The expression of CEACAM1 protein was efficiently suppressed by a targeted siRNA against CEACAM1 in HT29 cells, and the expression of CEA (carcinoembryonic antigen) was not disrupted (Fig. 2D). As a result, compared with HT29 and HT29/siRNA-con, the relative number of adherent cells of HT29/siRNA-CEACAM1 was increased (Fig. 2E, 2.6-fold, $p < 0.001$). Thus, it appeared that CEACAM1 could inhibit cell-matrix adhesion.

3.3. N-cadherin is involved in the cell-matrix adhesion inhibition induced by CEACAM1 overexpression

N-cadherin, a calcium dependent cell–cell adhesion glycoprotein, is widely regarded as a marker of EMT that stimulates cell invasion. CEACAM1 decreases the cell-matrix adhesive capacity, which might increase cell motility. We wanted to understand the association between N-cadherin and the CEACAM1-induced cell-matrix adhesion inhibition. In our study, we found that N-cadherin was expressed at significantly higher levels in CEACAM1-transfecting cells, 293-CEACAM1 and LOVO/trans-CEACAM1. Meanwhile, when the expression of CEACAM1 was markedly down-regulated in HT29 by a targeted siRNA, the level of N-cadherin in HT29 was correspondingly down-regulated (Fig. 3A, $p < 0.001$).

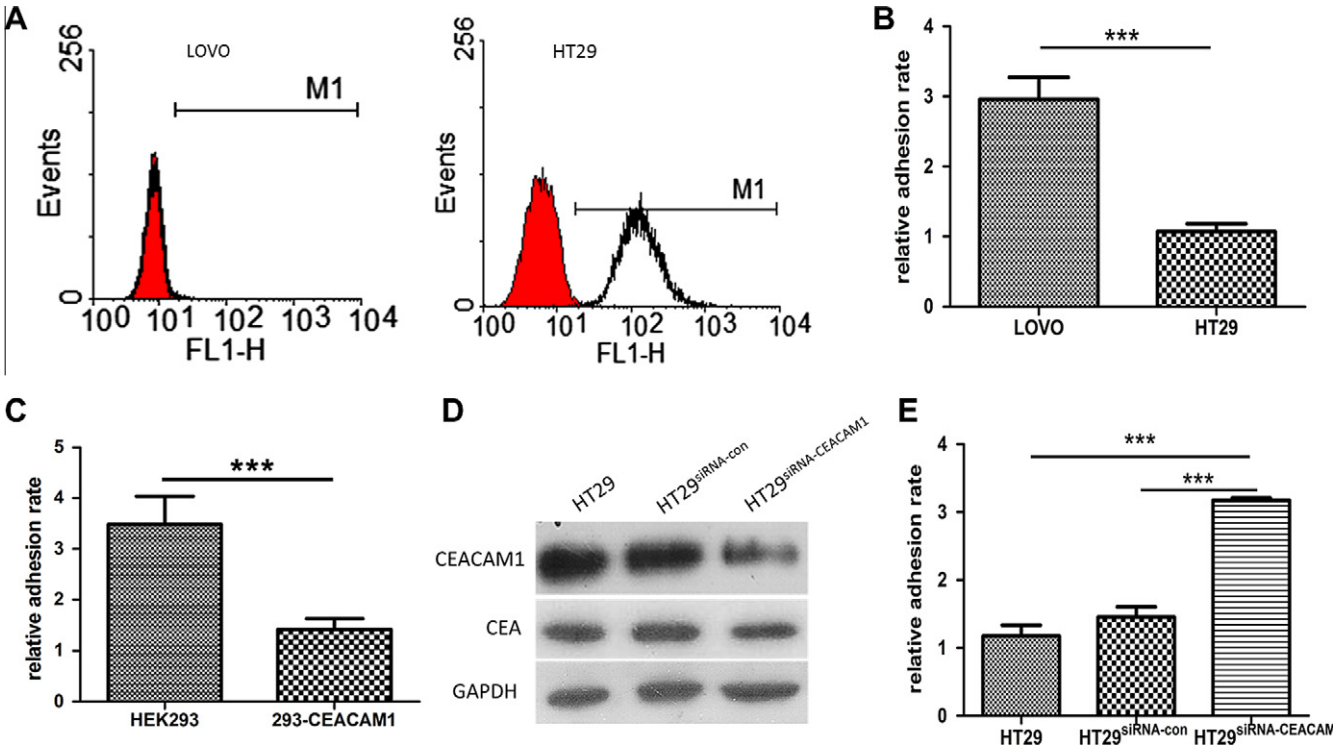

Fig. 2. CEACAM1 inhibits cell-matrix adhesion. (A) Flow cytometric assay of CEACAM1 expression in LOVO and HT29 cells (red curves represent controls in which cells were treated with no primary antibody and black curves represent the detection of CEACAM1). (B) Adhesion assay showed a lower adhesion rate of HT29 than LOVO ($p < 0.001$). (C) Adhesion assay showed a lower adhesion rate of 293-CEACAM1 than HEK293 ($p < 0.001$). (D) Western blot assay of CEACAM1 expression in HT29 cells treated with siRNA-con or siRNA-CEACAM1; treatment with siRNA did not affect CEA expression. (E) Adhesion assay showed an enhancement in the adhesion rate of HT29/siRNA-CEACAM1 cells ($p < 0.001$).

To investigate whether N-cadherin was involved in the inhibition of cell adhesion induced by CEACAM1, an anti-N-cadherin antibody, GC-4 (Sigma), was used. GC-4 binds to the extracellular domain of N-cadherin and has been confirmed as an effective blocking antibody of N-cadherin [13]. When 293-CEACAM1 cells were treated with GC-4 at 37 °C for 40 min before adhering to collagen I, they appeared to have an enhanced rate of adhesion (Fig. 3B, 2.2-fold, $p < 0.05$). Similarly, when treated with GC-4, LOVO/trans-CEACAM1 cells also showed a better adhesive capacity (Fig. 3C, 1.3-fold, $p < 0.001$). Based on the above results, N-cadherin is likely to mediate the inhibition of cell-matrix adhesion induced by CEACAM1 overexpression.

3.4. N-cadherin mediates the cell motility promotion induced by CEACAM1 overexpression

As the high expression of CEACAM1 has been detected in metastatic cancer by immunohistochemistry [3,10], we performed a trans-well migration assay to investigate the motility of CEACAM1-transfected cells. Significantly higher numbers of 293-CEACAM1 cells transferred compared to control HEK293 cells (2.3-fold, $p < 0.001$) which implied they had a better motility (Fig. 4a). The motility of 293-CEACAM1 cells was suppressed by treatment with a blocking antibody of N-cadherin (0.7-fold, $p < 0.001$). Similar results were occurred on LOVO cells. More LOVO/trans-CEACAM1 cells transferred through the membrane than LOVO cells (1.7-fold, $p < 0.001$, Fig. 4B). However, when N-cadherin was blocked, the motility of LOVO/trans-CEACAM1 cells was decreased (0.6-fold, $p < 0.001$). Given the results of the adhesion and motility assays, CEACAM1 appears to inhibit cell-matrix adhesion and promote cell motility and N-cadherin is a crucial protein involved in the processes.

4. Discussion

CEACAM1, a member of the immunoglobulin superfamily, has been observed to have two paradoxical functions: tumor suppression and the promotion of metastasis. The loss of CEACAM1 expression has been significantly associated with large tumor sizes [5]. When the mRNA levels of CEACAM1 were detected by Northern blots, two CEACAM1 transcripts sized 3.9 kb and 1.5 kb were demonstrated to be decreased by more than 50% in 20 out of 22 human colorectal adenomas mucosa specimens compared with 18 out of 22 normal specimens [14]. CEACAM1 shows lower expression levels in prostate, breast and colorectal cancer and hepatocellular carcinoma [4,5]. In this study, we investigated the impact of CEACAM1 on tumor cell proliferation *in vitro*. The growth curves of HEK293 cells showed a higher growth rate than 293-CEACAM1 cells (data not shown), which implied that CEACAM1 was an inhibitor of cell proliferation.

However, CEACAM1 is overexpressed in lung cancer and malignant melanoma [6,7], and it acts as a stimulator of tumor migration and invasion. In this study, we examined the role of CEACAM1 in tumor metastasis. In our opinion, CEACAM1 is a potential marker of metastatic carcinoma and advanced carcinoma, and may contribute to tumor cell EMT.

The expression of CEACAM1 was assayed by flow cytometry in various cell lines, including color cancer cell lines, HT29, LS180, SW620, HCT116 and LOVO, a lung cancer cell line, A549, and a prostatic carcinoma cell line, PC3 (data not shown). Among these cell lines, CEACAM1 was expressed at its highest levels in HT29, LS180 and SW620 cells, but not expressed in LOVO and HCT116 cells.

To investigate the functional activity of CEACAM1, we constructed a stably transfected clone, 293-CEACAM1, which

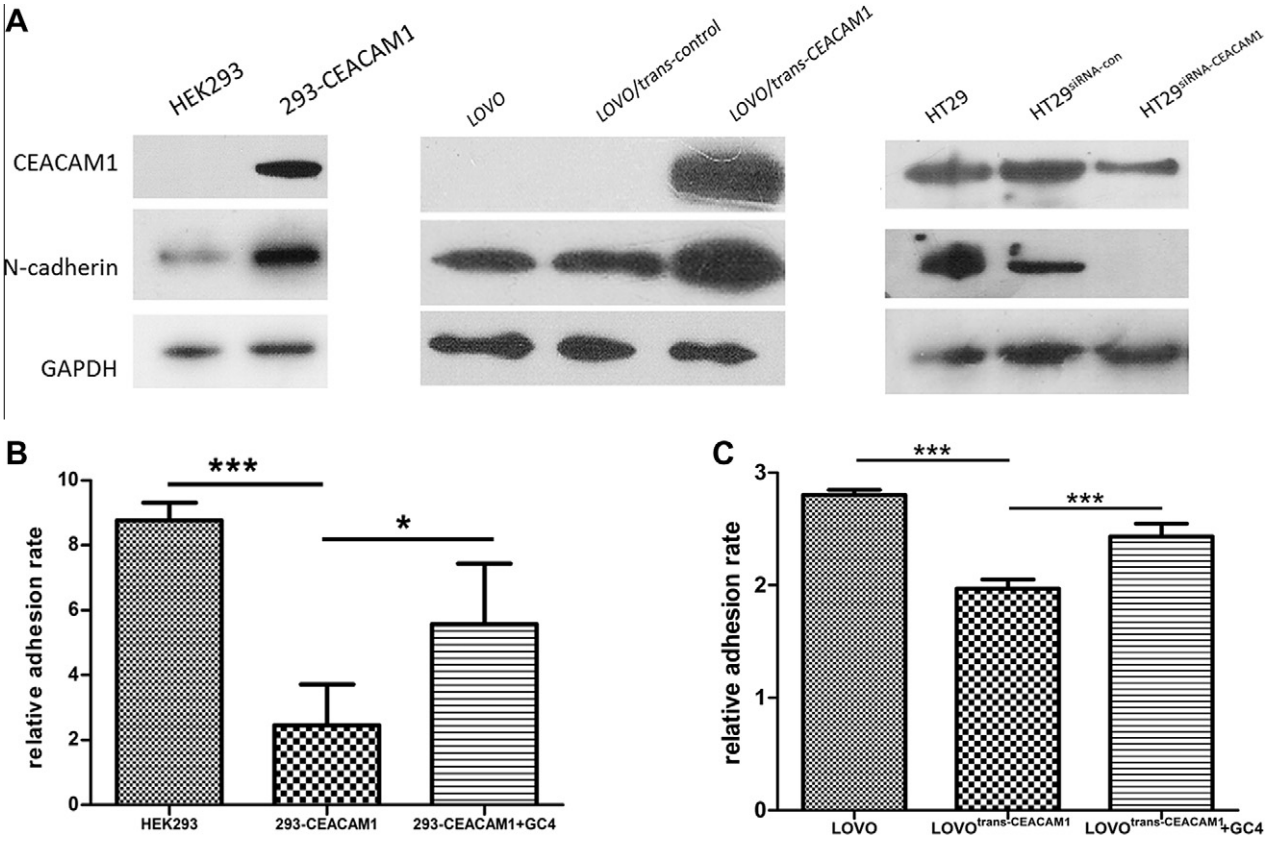

Fig. 3. N-cadherin is involved in the CEACAM1-mediated inhibition of cell-matrix adhesion. (A) Western blot results showed a positive correlation in expression level between N-cadherin and CEACAM1. (B, C) An adhesion assay showed that the adhesive capacity of either 293-CEACAM1 or LOVO/trans-CEACAM1 cells recovered when N-cadherin in cells was blocked with a GC-4 antibody.

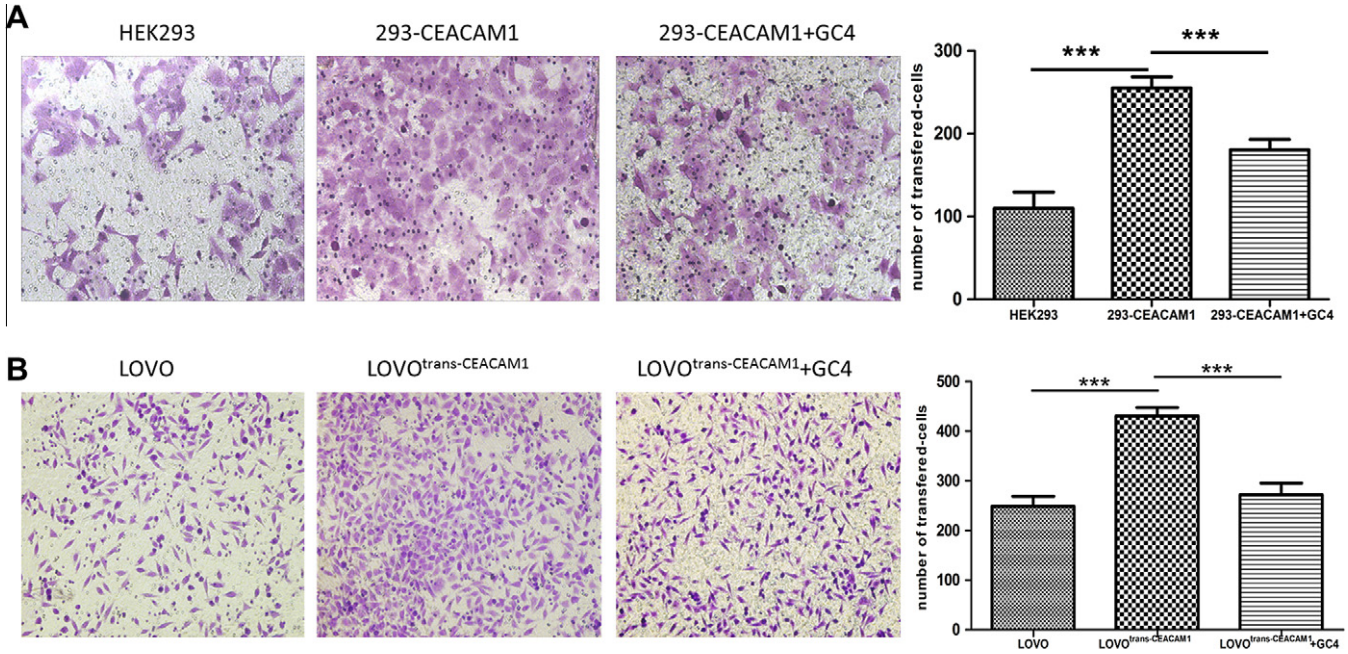

Fig. 4. N-cadherin mediates the CEACAM1-mediated promotion of cell motility. Cell motility was analyzed by trans-well inserts. (A, B) Microscopy and counting of cells that transferred to the lower surface of the porous membrane (×400).

overexpressed CEACAM1 (Fig. 1A, B and C). HEK293 cells grew uniformly whereas 293-CEACAM1 cells formed cell clusters and appeared to have better cell–cell adhesion properties (Fig. 1D). The results of the adhesion assay showed that LOVO cells which did not express CEACAM1 had a better adhesive capacity to collagen I than HT29 cells which expressed CEACAM1 (Fig. 2A and B). Besides, 293-CEACAM1 cells showed a higher adhesion rate than HEK293 cells (Fig. 2C). When CEACAM1 was efficiently

down-regulated by siRNA in HT29 (Fig. 2D), the relative adhesion rate of HT29/siRNA-CEACAM1 increased by almost 2.6-fold (Fig. 2E). These results implied a potential function of CEACAM1 in the inhibition of cell-matrix adhesion.

We hypothesized that the reported CEACAM1-mediated tumor metastasis may result from its inhibition of cell-matrix adhesion. N-cadherin is widely regarded as a marker of EMT in cancer, and it also stimulates tumor metastasis [12]. Therefore, experiments were performed to understand the association between N-cadherin and CEACAM1, and whether N-cadherin was involved in CEACAM1-induced cell-matrix adhesion inhibition. Western blotting demonstrated that N-cadherin was expressed at significantly higher level in 293-CEACAM1 cells and LOVO/trans-CEACAM1 cells compared to HEK293 cells or LOVO cells respectively, and the expression was reduced in HT29/siRNA-CEACAM1 cells compared to HT29 and HT29/siRNA-con (Fig. 3A), revealing a positive correlation between N-cadherin and CEACAM1 expression. When 293-CEACAM1 and LOVO/trans-CEACAM1 cells were treated with GC-4 (a blocking antibody against N-cadherin) before adhering to collagen I, their adhesion rates recovered (Fig. 3B and C). These findings suggest that N-cadherin plays a role in the CEACAM1-mediated inhibition of cell adhesion.

Taking the high expression of CEACAM1 in metastatic cancer [10] into consideration, trans-well migration assays were performed. As expected, 293-CEACAM1 cells had better levels of motility than HEK293 (2.3-fold higher numbers of transferred cells). After cells were treated with GC-4, the motility of 293-CEACAM1 was suppressed (Fig. 4A). In addition, the enhanced motility of LOVO/trans-CEACAM1 cells was also suppressed by GC-4 treating (Fig. 4B), indicating an N-cadherin-mediated active function of CEACAM1 in cell migration.

Immunoprecipitation was also performed. Anti-GFP antibody was used to pull down GFP-CEACAM1 protein in 293-CEACAM1 cells, and then Protein A/G Plus Agarose was added to catch the antibody-CEACAM1-complex. However, no specific band was detected by the anti-N-cadherin antibody (data not shown), which implied that there might not be a direct interaction between CEACAM1 and N-cadherin.

Cell adhesion is crucial for tumor invasion and metastasis. In case of alterations of cell–cell and cell-matrix interactions, tumor cells may break away from primary cancer site and migrate to distant sites [10]. In most cells, N-cadherin, a marker of EMT, is in a balance with E-cadherin. Cells that express more N-cadherin than E-cadherin appear to have better migration and invasion capacities. We propose that it promotes cell migration by preventing cells from adhering to the extracellular matrix. CEACAM1 had also been reported to contribute to tumor migration and invasion. Taking the above results in consideration, CEACAM1 might disrupt cell-matrix adhesion through up-regulating N-cadherin, therefore promoting cell migration. However, other molecules may be involved in this process as no complex was detected between CEACAM1 and N-cadherin.

EMT is a process in which cells lose their epithelial features, such as cell–cell and cell-matrix contacts, and acquire mesenchymal characteristics including migration and invasion [15]. The EMT process of cancer cells promotes tumor invasion and metastasis. In this current research, we discovered that CEACAM1 inhibited cell-matrix adhesion and enhanced cell motility through regulating the EMT marker, N-cadherin. This process was consistent with the features of EMT. We hypothesize that CEACAM1 might play a part in the EMT process of tumor cells.

Acknowledgments

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