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c-Src-dependent cross-talk between CEACAM6 and $\alpha_v\beta_3$ integrin enhances pancreatic adenocarcinoma cell adhesion to extracellular matrix components

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Abstract

Carcinoembryonic antigen-related cell adhesion molecule 6 (CEACAM6) is an immunoglobulin superfamily member with a diversity of extracellular ligands that is implicated in the initiation and progression of a variety of malignancies. We sought to characterize the effects of CEACAM6 crosslinking on pancreatic adenocarcinoma cellular interaction with the extracellular matrix (ECM) components fibronectin and vitronectin. Antibody-mediated CEACAM6 crosslinking was performed and the ability of BxPC3 cells, which inherently overexpress CEACAM6, to adhere to fibronectin and vitronectin was quantified. The roles of the archetypal fibronectin ($\alpha_5\beta_1$ integrin) and vitronectin ($\alpha_v\beta_3$ integrin) receptors were determined. The effects of c-Src inhibition were investigated using the Src family kinase inhibitor 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-d]pyrimidine (PP2) and c-Src specific RNA interference. CEACAM6 crosslinking initiates c-Src-dependent cross-talk between CEACAM6 and $\alpha_v\beta_3$ integrin, leading to increased ECM component adhesion. CEACAM6-mediated signaling events may contribute to the invasive and metastatic potential of pancreatic adenocarcinoma cells by promoting their interaction with ECM components.

Keywords: Pancreatic; Adenocarcinoma; Cancer; CEACAM6; Integrin; Fibronectin; Vitronectin; Src; Metastasis

Metastatic spread is a major cause of death among cancer patients; however, the mechanisms underlying metastasis remain poorly understood. Metastasis of tumor cells can be viewed as a complex series of sequentially linked interrelated steps [1,2]. Tumor cell adhesion to extracellular matrix (ECM) components constitutes a critical step in this model of cancer cell metastasis and involves cell surface molecules including integrins, selectins, immunoglobulin superfamily members, and cadherins [3].

The glycosylphosphatidylinositol (GPI)-anchored membrane protein carcinoembryonic antigen-related cell adhesion molecule 6 (CEACAM6) is a member of the immunoglobulin superfamily [4]. CEACAM6 is a determinant of malignant cellular behavior in a variety of human cancers [5,6]. Previously, we have shown that suppression of CEACAM6 expression inhibits the metastatic ability of human pancreatic adenocarcinoma cells in a nude mouse xenograft model [7]. Although GPI-anchored proteins do not span the cell membrane, they are capable of influencing intracellular signaling events. Antibody-mediated CEACAM6 crosslinking induces activation of neutrophils and increases their adhesion to ECM components [8], a response reportedly abolished by tyrosine kinase inhibition [9]. We sought to determine whether a comparable mechanism might promote pancreatic adenocarcinoma adhesion to ECM components, thereby modulating metastatic cellular behavior.

Integrins are heterodimeric integral membrane glycoproteins comprising non-covalently bound α and β subunits. Integrin family members mediate cellular adhesion between adjacent cells as well as between cells and ECM components and soluble proteins. Over 20 heterodimeric $\alpha\beta$ combinations are possible, and specific adhesion to ECM components such as fibronectin, vitronectin, and collagen is determined by integrin subunit composition [10]. Adhesive interactions between

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integrins and ECM components are reported to play important roles in tumorigenesis, invasion, and metastasis [11–13]. We hypothesized that CEACAM6 may influence the metastatic ability of tumor cells by modulating integrin-dependent adhesion to ECM components. Here, we show that antibody-mediated crosslinking of CEACAM6 results in c-Src-dependent augmentation of adhesion to fibronectin and vitronectin via $\alpha_v\beta_3$ integrin. Cross-talk between CEACAM6 and other cell surface adhesion molecules such as integrin receptors, leading to altered affinity for ECM components, may account for the change in metastatic potential of tumor cells observed following modulation of CEACAM6 expression.

Materials and methods

Cell culture. BxPC3 pancreatic ductal adenocarcinoma cells were obtained from ATCC (Rockville, MD). BxPC3 was specifically chosen as these cells inherently overexpress CEACAM6 [14]. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS, Gibco-BRL, Gaithersburg, MD). Cells were incubated in a humidified (37 °C, 5% CO_2) incubator and passaged upon reaching 80% confluence.

CEACAM6 crosslinking. The By114 mouse monoclonal antibody (Innogenex, San Ramon, CA) was used as the primary crosslinking antibody. This antibody is highly specific for CEACAM6 and is non-crossreactive with closely related CEACAM family molecules [15,16]. Irrelevant isotype-matched mouse IgG served as a control. Crosslinking was performed in a manner similar to that previously described with some modifications [17]. BxPC3 cell cultures, 3 days post-seeding in 35 mm well plates, were trypsinized and then incubated with 50 μg/ml By114 or control IgG on ice for 10 min in BSA medium (DMEM without fetal bovine serum containing 1% BSA and 20 mM Hepes (pH 7.4)). After washing with cold BSA medium, the cells were incubated with 50 μg/ml anti-mouse IgG affinity-purified polyclonal antibody $F(ab')_2$ in BSA medium at 37 °C for a further 10 min. Cells exposed to primary antibodies only served as additional controls. To disrupt GPI anchor assembly, cells were cultured for 18 h in medium containing 10 mM mannosamine (2-amino-2-deoxy-D-mannose, Sigma) [18], prior to performing antibody crosslinking. Where required, cell lysates were prepared using lysis buffer (1% Triton X-100, 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1mM EGTA, and 1 mM phenylmethylsulfonyl fluoride) at 4 °C. Following centrifugation at 14,000 rpm for 3 min, the supernatants were used for further analysis.

Cell adhesion assay. Cellular adhesion to fibronectin and vitronectin (Sigma, St. Louis, MO) was quantified using a crystal violet-based adhesion assay. Ninety-six well plates were coated with fibronectin (1 μg/well) or vitronectin (0.5 μg/well) for 2 h at room temperature. Untreated sites were blocked with 200 μl of 0.1% heat-treated bovine serum albumin (BSA) to minimize non-specific adhesion. Cells were suspended in Hanks' balanced salt solution (HBSS) containing 0.1% BSA. Following appropriate antibody treatment, 5×10^4 cells in 100 μl HBSS were added to each well of the ligand-coated microtiter plates and incubated for 30 min at 37 °C. The plate was washed three times with 200 μl HBSS to remove unattached cells. Adherent cells were quantified by fixing, crystal violet staining followed by lysis using 0.1% SDS. Absorbance was measured using a V_{max} microplate spectrophotometer (Molecular Devices, Sunnyvale, CA) at a wavelength of 560 nm, corrected to 650 nm, and normalized to respective controls. Cellular adhesion to BSA-coated wells and poly-L-lysine (Sigma) was used as a control for non-specific adhesion. Each independent experiment was performed three times, with 10 determinations for each condition tested. The contribution of $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrins was determined by performing the adhesion assay in the presence of specific inhibitory antibodies (2.5 μg/ml, Chemicon, Temecula, CA). In parallel triplicate experiments, the number of spread adherent cells was counted in three random fields from three wells and expressed as a mean percentage of the total number of cells counted, following incubation and washing in HBSS as described.

Quantification of cellular integrin expression. Cell surface integrin expression was quantified using a commercially available immunoadhesion assay (Chemicon, Temecula, CA). Nintey-six well trays coated with goat anti-mouse IgG were coated with anti-$\alpha_v\beta_3$ integrin or anti-$\alpha_5\beta_1$ integrin mouse monoclonal antibodies. Goat anti-mouse IgG-treated wells blocked with irrelevant mouse IgG were used as negative controls. 5×10^4 cells in single cell suspension were seeded onto the coated wells and incubated for 1 h at 37 °C. Following three gentle washes, adherent cells were fixed and stained with crystal violet. Following lysis, cell attachment was quantified by measuring absorbance readings at 560 nm.

siRNA. CEACAM6-specific (sense 5′-CCGGACAGUUCCAUGUAUA-dTT-3′, antisense (5′-UAUACAUGGAACUGUCCGG-dTT-3′) and control (sense 5′-AGGAGAUAUUUCGAGGCUU-dTT-3′, antisense 5′-AAGCCUCGAAAUAUCUCCU-dTT-3′) siRNAs were purchased from Qiagen (Qiagen–Xeragon, Germantown, MD, USA). c-Src SMARTPool and respective control siRNAs were obtained from Dharmacon (Lafayette, CO). In vitro transfection was performed using siPORT*Amine* transfection reagent (Ambion, Austin, TX) as recommended by the manufacturer. One microgram of siRNA was used for 10^6 cells. Following transfection, cells were incubated in DMEM containing 10% FBS. All further studies were performed 48 h following transfection.

PP2. 4-Amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-d]pyrimidine (PP2) was obtained from Chemicon (Temecula, CA). Cells were exposed to 10 μM PP2, which we have shown to potently inhibit c-Src activity in BxPC3 cells [19,20], for 2 h prior to performing further assays. Dimethyl sulfoxide (DMSO) served as a vehicle control.

Western blotting. Total protein concentrations of lysates prepared as described were measured using the BCA assay kit (Sigma, St. Louis, MO) with BSA as a standard. Cell lysates containing 30 μg total protein were analyzed by immunoblotting using anti-CEACAM6 anti-CEA (Innogenex), anti-pp60(c-Src), (Santa Cruz Biotechnology), and anti-actin antibodies (LabVision, Freemont, CA). Chemoluminescent detection (Upstate, Lake Placid, NY) was performed in accordance with the manufacturer's instructions. Signals were quantified densitometrically using ImagePro Plus software version 4.0 and normalized to those of β-actin.

In vitro c-Src tyrosine kinase assay. c-Src tyrosine kinase activity was determined in triplicate using a commercially available kinase assay kit (Sigma, St. Louis, MO), according to the manufacturer's instructions. c-Src immunoprecipitates (20 g total protein) were prepared from cell lysates using anti-c-Src monoclonal antibody immobilized onto protein G–Sepharose beads (Zymed Laboratories, San Francisco, CA). Immunoprecipitates were washed and dissolved in tyrosine kinase buffer (final solution containing 0.3 mM ATP) and incubated for 30 min in 96-well plates coated with tyrosine kinase substrate solution (poly-Glu-Tyr). Phosphorylated substrate was quantified by chromogenic detection using horseradish peroxidase-conjugated anti-phosphotyrosine antibody. Optical densities were determined at 492 nm using a V_{max} microplate spectrophotometer. c-Src kinase activity was compared to an epidermal growth factor receptor (EGFR) standard.

Statistical analysis. Differences between groups were analyzed using Student's *t* test, multifactorial ANOVA of initial measurements, and Mann–Whitney *U* test, for non-parametric data, as appropriate, using GraphPad InStat (GraphPad software, San Diego, CA). In cases where averages were normalized to controls, the standard deviations of each nominator and denominator were taken into account in

calculating the final standard deviation. A value of $P < 0.05$ was considered statistically significant.

Results

CEACAM6 crosslinking promotes pancreatic adenocarcinoma cellular adhesion to ECM components

Antibody-mediated crosslinking of CEACAM6 was performed by exposing BxPC3 cells to anti-CEACAM6 primary antibody, followed by anti-mouse IgG secondary antibody $F(ab')_2$. $F(ab')_2$ was used to exclude Fc-mediated effects, although use of intact secondary antibody produced identical results (data not shown). CEACAM6 crosslinking induced a marked increase in cellular adhesion to both fibronectin and vitronectin. The effect of anti-CEACAM6 antibody alone was minimal and secondary antibody $F(ab')_2$ alone had no effect on cellular adhesion (Figs. 1B and C). Non-specific adhesion to bovine serum albumin (BSA) or poly-L-lysine was unaffected by any of the antibody treatments (data not shown). Disruption of GPI assembly by prior treatment with mannosamine abolished the increased ECM component adhesion induced by CEACAM6 crosslinking, indicating a requirement for the GPI anchor for CEACAM6 crosslinking to promote this aspect of ECM component interaction.

Downregulation of CEACAM6 expression suppresses CEACAM6 crosslinking-induced adhesion to extracellular matrix components

We confirmed that CEACAM6 expression is required for enhanced adhesion to fibronectin and vitronectin induced by CEACAM6 crosslinking by suppressing CEACAM6 expression using RNA interference (RNAi). Forty-eight hours following transfection of CEACAM6-specific small interfering RNA (siRNA), downregulation of CEACAM6 expression was confirmed by Western blot analysis (Fig. 2A). Expression of the related CEACAM family member CEA was unaffected by CEACAM6-specific siRNA. Control mismatched siRNA had no effect on CEACAM6 or CEA expression. Forty-eight hours following CEACAM6 siRNA transfection, crosslinking antibody treatment was performed and adhesion to fibronectin and vitronectin was quantified as before. Suppression of CEACAM6 expression abolished the increase in ECM component adhesion induced by crosslinking antibody treatment. ECM component adhesion was unaffected by transfection with control siRNA (Figs. 2B and C). These findings confirm that CEACAM6 expression is required for the increased ECM component adhesion induced by CEACAM6 antibody-mediated crosslinking, confirming the specificity of the antibody-mediated crosslinking.

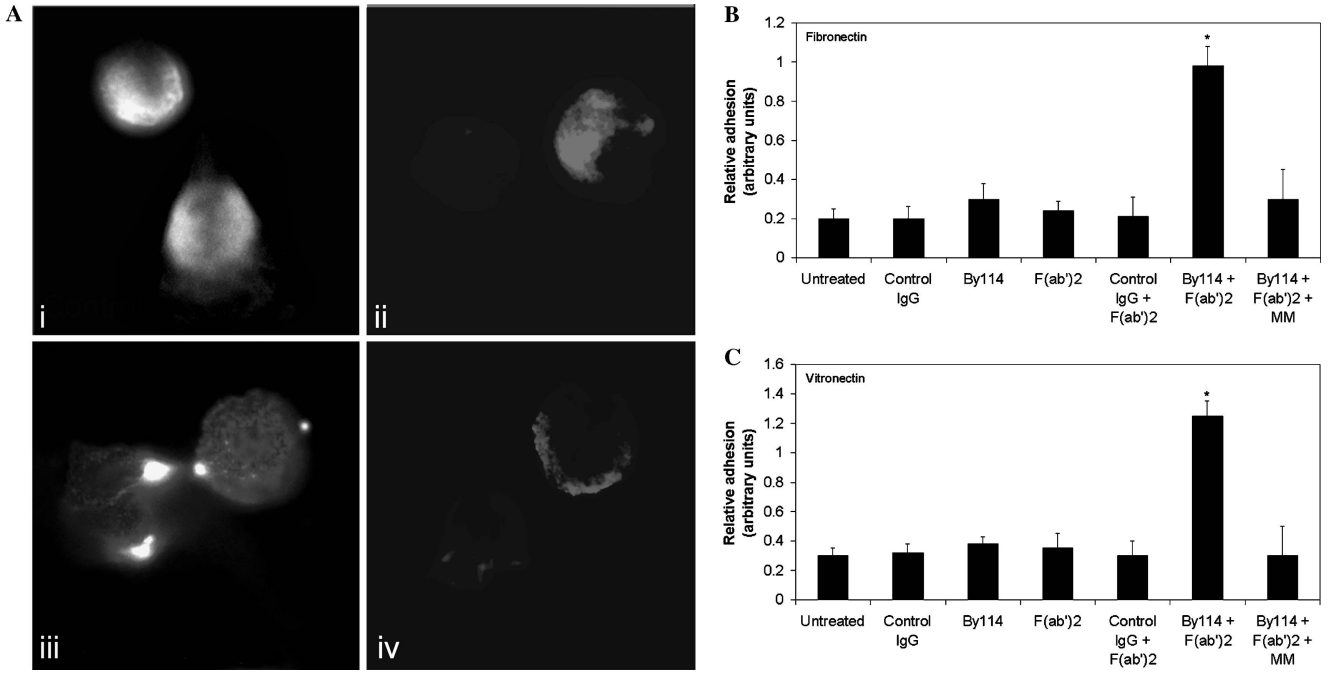

Fig. 1. (A) Cells were labeled with FITC-labeled By114 (i), FITC labeled irrelevant IgG (ii), unlabeled By114 followed by FITC-labeled $F(ab')_2$ (iii) or unlabeled irrelevant IgG followed by FITC-labeled $F(ab')_2$ (iv). Significant aggregation was seen only with By114 in combination with $F(ab')_2$. CEACAM6 crosslinking significantly increased cellular adhesion to the extracellular matrix components fibronectin (B) and vitronectin (C). This effect was almost completely abolished by prior exposure of cells to the GPI-disrupting agent mannosamine (MM). Mean values from triplicate experiments. $^*P < 0.05$ versus control IgG + $F(ab')_2$.

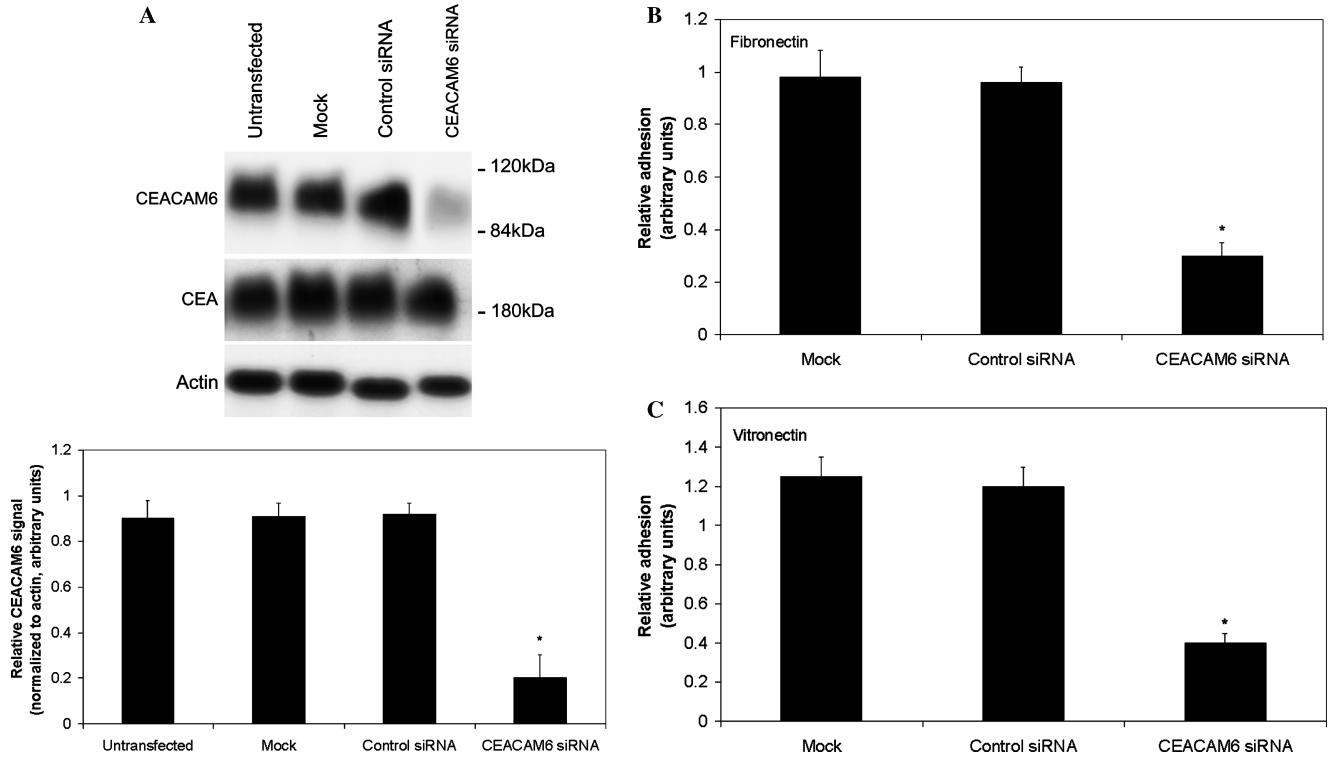

Fig. 2. (A) Suppression of CEACAM6 expression was confirmed 48 h post-siRNA transfection by Western blot analysis. Control siRNA has no effect on CEACAM6 expression. CEA expression was unaffected by either siRNA. Representative example of triplicate blots. Densitometric values are means (± SD). *$P < 0.05$ versus control siRNA. Knockdown of CEACAM6 expression significantly attenuated the effects of crosslinking antibody treatment on cellular adhesion to fibronectin (B) and vitronectin (C). Mean values (± SD) from triplicate experiments. *$P < 0.05$ versus control siRNA.

$\alpha_v\beta_3$ integrin mediates the enhanced ECM component adhesion induced by CEACAM6 crosslinking

It has previously been established that pancreatic adenocarcinoma cells including BxPC3 adhere to fibronectin via the archetypal fibronectin receptor, $\alpha_5\beta_1$ integrin [21]. We therefore determined the effect of inhibitory anti-$\alpha_5\beta_1$ integrin antibody on fibronectin adhesion following CEACAM6 antibody crosslinking. Surprisingly, anti-$\alpha_5\beta_1$ integrin antibody had minimal effect on the increase in fibronectin adhesion induced by CEACAM6 crosslinking (Fig. 3). Next, we tested the effect of an inhibitory antibody specific for the vitronectin receptor, $\alpha_v\beta_3$ integrin. Treatment with anti-$\alpha_v\beta_3$ integrin almost completely abolished the increase in vitronectin adhesion following CEACAM6 crosslinking and, interestingly, markedly suppressed the enhanced adhesion to fibronectin induced by CEACAM6 crosslinking. Neither anti-$\alpha_v\beta_3$ integrin nor anti-$\alpha_5\beta_1$ integrin inhibitory antibody affected adhesion to poly-L-lysine or BSA. These observations suggest

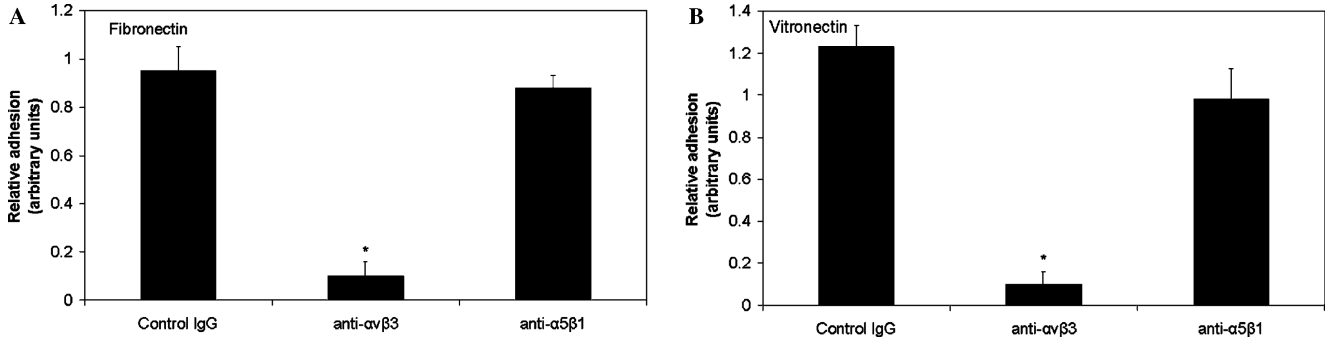

Fig. 3. Cellular adhesion assays were performed, following CEACAM6 crosslinking, in the presence of blocking anti-$\alpha_v\beta_3$ integrin or anti-$\alpha_5\beta_1$-integrin antibodies. While anti-$\alpha_5\beta_1$-integrin blocking antibody had minimal effect on cellular adhesion to either fibronectin (A) or vitronectin (B), anti-$\alpha_v\beta_3$ integrin antibody suppressed adhesion to fibronectin by 90% and vitronectin by 92%. Mean values (± SD) from triplicate experiments. *$P < 0.05$ versus control IgG.

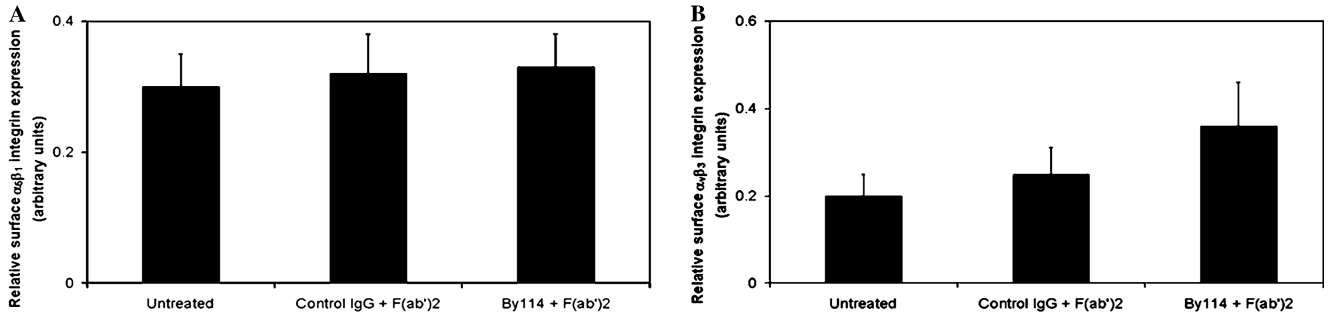

Fig. 4. Surface levels of integrin expression were quantified by immunoadhesion assay. CEACAM6 crosslinking had minimal effect on surface expression of either $\alpha_v\beta_3$ integrin (A) or $\alpha_5\beta_1$-integrin (B), suggesting that the $\alpha_v\beta_3$ integrin-dependent increases in cellular adhesion result from changes in integrin affinity, rather than changes in cell surface integrin expression.

that CEACAM6 crosslinking enhances adhesion to both fibronectin and vitronectin via upregulation of $\alpha_v\beta_3$ integrin-mediated adhesion.

Cell surface integrin expression following CEACAM6 antibody crosslinking

Increased integrin-mediated adhesion can result from increased cell surface integrin expression or altered integrin affinity. Following CEACAM6 antibody cross-linking, cell surface expression of $\alpha_5\beta_1$ and $\alpha_v\beta_3$ integrin was quantified by integrin subtype-specific immunoadhesion assay. CEACAM6 antibody cross-linking induced only a marginal increase in cell surface expression of $\alpha_v\beta_3$ integrin. Levels of $\alpha_5\beta_1$ integrin were unaffected (Fig. 4). The absence of a marked increase in membrane expression of $\alpha_v\beta_3$ integrin suggests that modulation of integrin affinity may contribute to enhanced ECM component adhesion.

c-Src is required for increased fibronectin adhesion resulting from CEACAM6 antibody-induced crosslinking

In neutrophils, CEACAM family molecules associate with Src family kinases [8]. In view of this observation and the central role of Src family kinases in the adhesion

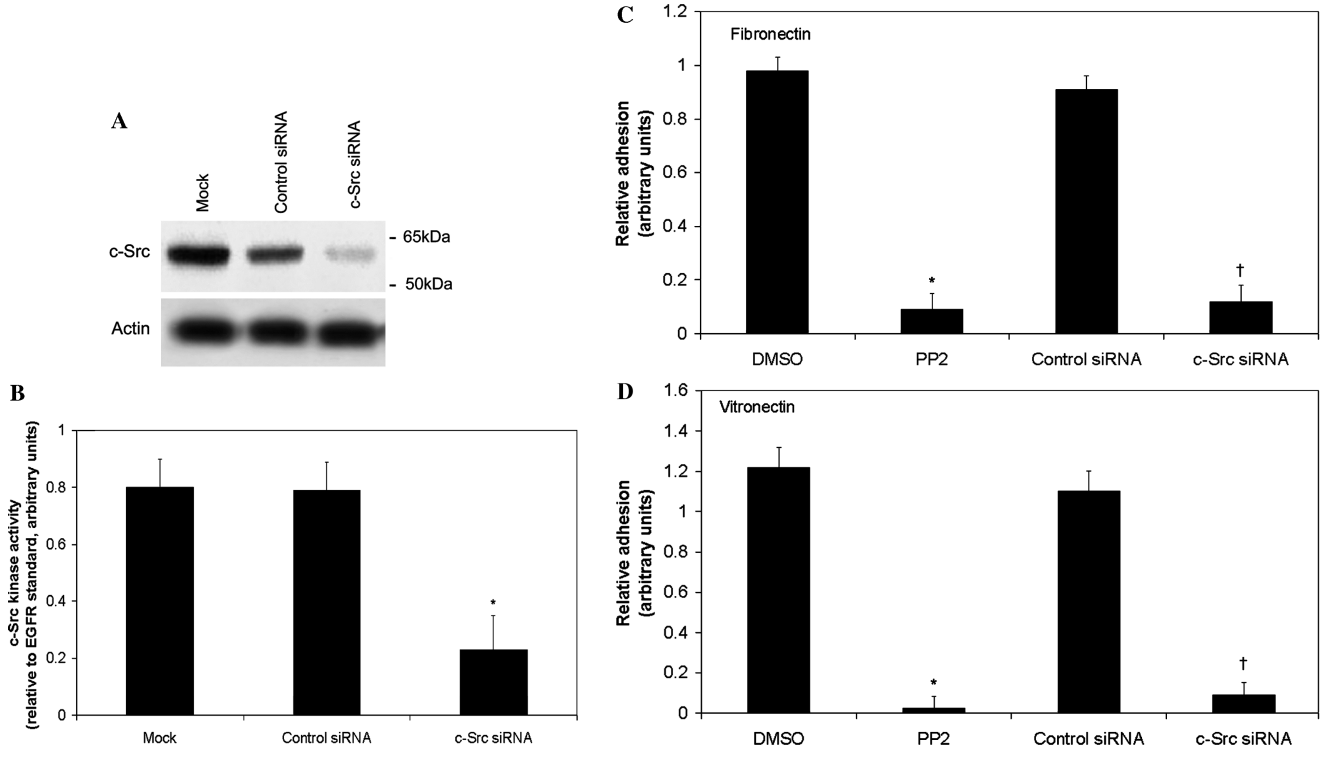

Fig. 5. Suppression of c-Src expression by RNAi was quantified by Western blot analysis (A) and by in vitro kinase assay (B). Mean values (± SD) from triplicate experiments. *$P < 0.05$ versus control siRNA. Knockdown of c-Src expression almost completely abolished the increases in cellular adhesion to fibronectin (C) and vitronectin (D) induced by CEACAM6 crosslinking. Control siRNA treatment had no effect on cellular adhesion. Mean values (± SD) from triplicate experiments. *$P < 0.05$ versus DMSO. †$P < 0.05$ versus control siRNA.

of human gastrointestinal adenocarcinoma cells to ECM components [23], we determined the role of the prototype Src kinase c-Src in mediating cross-talk between CEACAM6 and the $\alpha_v\beta_3$ integrin receptor. Following CEACAM6 crosslinking c-Src kinase activity, quantified by in vitro kinase assay, was significantly increased. Next, we inhibited c-Src using two strategies: (1) by exposing cells to the Src family kinase inhibitor PP2 and (2) transfection of c-Src-specific siRNA. Knockdown of c-Src expression by c-Src siRNA was confirmed by Western blotting and in vitro kinase assay (Figs. 5A and B). Transfection of c-Src siRNA almost completely abolished the increase in adhesion to both fibronectin and vitronectin induced by subsequent CEACAM6 antibody-mediated crosslinking (Figs. 5C and D), indicating that c-Src is required for CEACAM6 crosslinking-induced $\alpha_v\beta_3$ integrin-mediated enhancement of ECM component adhesion.

Because c-Src appeared to be necessary for enhanced $\alpha_v\beta_3$ integrin-mediated ECM component adhesion following CEACAM6 crosslinking, we next assessed whether constitutively activated c-Src would be sufficient to increase $\alpha_v\beta_3$ integrin-mediated adhesion. Cells were transiently transfected with a constitutively active c-Src expression construct (SrcY529F). Overexpression of constitutively active c-Src, but not control vector (pUSE) transfection, significantly increased cellular adhesion to both fibronectin and vitronectin (Fig. 6).

CEACAM6 crosslinking promotes cell spreading on ECM components

The increased adhesion to ECM components induced by CEACAM6 crosslinking is associated with changes in the morphology of the adherent cells. In the presence of control antibodies, over 90% of cells exhibited

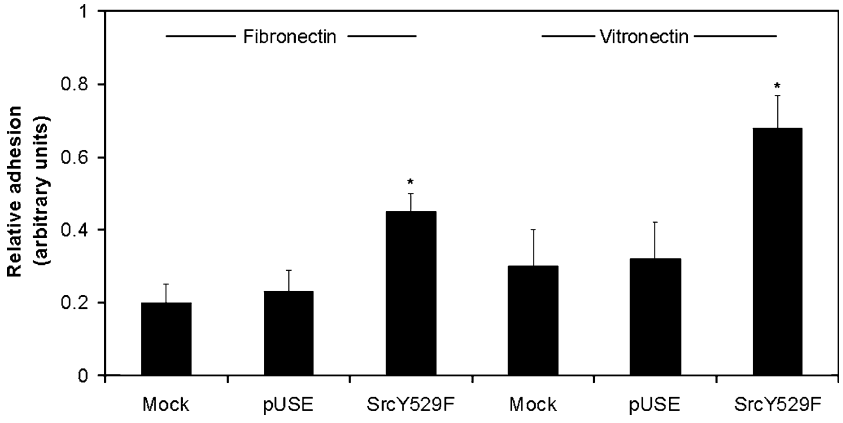

Fig. 6. Overexpression of constitutively active Src (SrcY529F) significantly increased cellular adhesion of BxPC3 cells to both fibronectin and vitronectin. Control empty vector had no effect on cellular adhesion to these ECM components. Mean values (±SD) from triplicate experiments. *$P < 0.05$ versus pUSE.

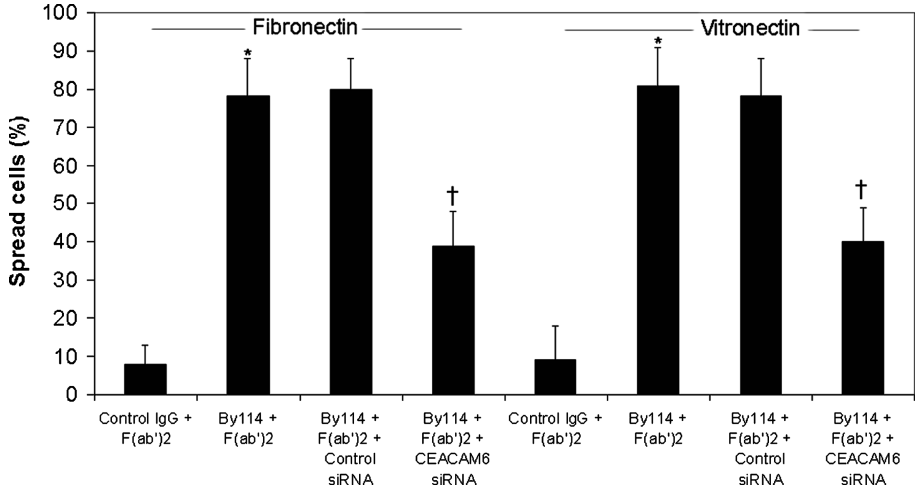

Fig. 7. CEACAM6 crosslinking induces changes in the cellular morphology of adherent cells. In the presence of control antibodies, over 90% of cells exhibited rounded morphology. Following CEACAM6 crosslinking, 70–80% of cells adopted a spread morphology. The proportion of spread cells following CEACAM6 crosslinking was significantly reduced by prior transfection of c-Src-specific, but not control, siRNA. Mean values (±SD) from triplicate experiments. *$P < 0.05$ versus control IgG + F(ab′)$_2$. †$P < 0.05$ versus control siRNA.

a rounded morphology. Following CEACAM6 crosslinking, approximately 80% of adherent cells adopted spread morphology. The proportion of spread cells was reduced by 60% by treatment with c-Src-specific, but not control, siRNA, prior to performing the adhesion assay (Fig. 7). Taken together, these observations indicate that c-Src kinase is both necessary and sufficient to increase $\alpha_v\beta_3$ integrin-mediated ECM component adhesion.

Discussion

In this study, we have shown that antibody-induced crosslinking of CEACAM6 enhances $\alpha_v\beta_3$ integrin-mediated adhesion to ECM components. We have also shown that c-Src is required for this cross-talk between CEACAM6 and $\alpha_v\beta_3$ integrin to occur. Despite lacking an intracellular domain, CEACAM6 is capable of independently activating neutrophil adhesion to human vascular endothelial cells (HUVECs) [23] and crosslinking of the GPI-linked CEACAM family members has been reported to induce a respiratory burst in neutrophils [24]. Clear parallels can be drawn between neutrophil margination, extravasation, and migration and tumor cell invasion and metastasis. In neutrophils, CEACAM6 has a well-recognized role in regulating cell activation and adhesion to ECM components. Our observations indicate that comparable mechanisms operate in pancreatic adenocarcinoma cells, which may contribute to oncogenic activity of CEACAM6.

Surface expression of integrins alone is insufficient to mediate ligand binding and must be accompanied by integrin activation [25]. The process of integrin activation is incompletely understood but involves integrin conformational changes, leading to increased substrate affinity [26]. Integrin binding specificity depends upon integrin subunit composition and integrin binding activities can be regulated by factors inside the cell as well as from the cell exterior [27–29]. In our study, CEACAM6 crosslinking increased adhesion to both vitronectin and fibronectin. The archetypal fibronectin receptor, $\alpha_5\beta_1$ integrin, binds selectively to fibronectin [30] and is the principal mediator of adhesion to fibronectin in human pancreatic adenocarcinoma cells [21]. However, we observed that anti-$\alpha_5\beta_1$ integrin antibody had minimal effect on the enhanced adhesion to fibronectin that occurred following CEACAM6 crosslinking, suggesting the involvement of other adhesion molecules. As expected, $\alpha_5\beta_1$ integrin antibody had no significant effect on the enhanced vitronectin adhesion following CEACAM6 crosslinking, which was abolished by anti-$\alpha_v\beta_3$ integrin antibody. Integrin heterodimers other than $\alpha_5\beta_1$ integrin function as fibronectin receptors in pancreatic adenocarcinoma cells [31]. In addition to mediating adhesion to vitronectin, $\alpha_v\beta_3$ integrin also mediates adhesion to fibronectin and a number of other RGD (Arg-Gly-Asp)-containing ECM components [15,32]. $\alpha_v\beta_3$ integrin does not mediate adhesion to BSA, which is consistent with the findings of the present study. Using antibodies directed against $\alpha_v\beta_3$ integrin, we determined that this integrin contributes significantly to the enhanced adhesion to both fibronectin and vitronectin induced by CEACAM6 crosslinking. Following CEACAM6 crosslinking, membrane levels of $\alpha_v\beta_3$ integrin showed only a marginal increase and $\alpha_5\beta_1$ integrin levels were unaffected. It therefore appears that enhanced ECM matrix adhesion following CEACAM6 crosslinking is mediated primarily through $\alpha_v\beta_3$ integrin, rather than the classical fibronectin receptor, $\alpha_5\beta_1$ integrin and that modulation of $\alpha_v\beta_3$ integrin affinity for ECM components, rather than overall cell surface levels of $\alpha_v\beta_3$ integrin membrane expression, may be responsible for the increased ECM component adhesion induced by CEACAM6 crosslinking.

Increased c-Src expression and kinase activity are common in epithelial malignancies including pancreatic adenocarcinoma [33,34]. Many groups have reported an association of GPI-linked proteins with Src family kinases [35–38]. We therefore examined the effect of CEACAM6 crosslinking on activation of the prototype Src kinase, c-Src, which is functionally important in pancreatic adenocarcinoma [20,33,39]. Src family kinases are known to play important roles in integrin signaling [40–43] and c-Src may directly regulate integrin function [44]. Previously, Src inhibition has been reported to suppress tumor cell adhesion to fibronectin [22] while overexpression of constitutively active c-Src reportedly enhances tumor cell adhesion to fibronectin, without affecting non-specific adhesion to poly-L-lysine [22]. As PP2 is reported to inhibit Src family kinases other than c-Src [45], some of which have been reported to associate with CEACAM family molecules [46], we used c-Src-directed siRNA to inhibit c-Src expression more specifically. Both PP2 and c-Src knockdown markedly attenuated the enhanced cellular adhesion to vitronectin following CEACAM6 crosslinking. Enhanced fibronectin adhesion was also markedly suppressed by these strategies of c-Src inhibition. Although c-Src does not co-precipitate with $\alpha_5\beta_1$ integrin in whole cell lysates, it does co-precipitate with the α_v component of the vitronectin receptor [47]. We hypothesize that CEACAM6 crosslinking induces a c-Src-mediated increase in $\alpha_v\beta_3$ integrin binding activity, which enhances cellular adhesion to its classical ligand, vitronectin, as well as to fibronectin.

Modulation of ECM component adhesion following CEACAM6 crosslinking may contribute to the effects of altering tumor cell CEACAM6 expression on in vivo metastatic ability [7]. Integrins, in particular $\alpha_v\beta_3$, also mediate the binding of tumor cells to the ECM, thus affecting cell adhesion, migration, proliferation, and survival. Interestingly, $\alpha_v\beta_3$ integrin is implicated in

tumor angiogenesis [48,49]. In a murine model of colorectal cancer, treatment with an $\alpha_v\beta_3$ integrin antagonists suppressed tumor growth and angiogenesis, increased tumor apoptosis, inhibited liver metastasis, and improved survival [50,51]. The role of CEACAM6 in tumor angiogenesis warrants investigation.

In conclusion, this is the first report of cross-talk between CEACAM6 and integrin receptors in human pancreatic adenocarcinoma cells. Altered tyrosine kinase-mediated cross-talk between CEACAM6 and other cell adhesion molecules, such as $\alpha_v\beta_3$ integrin, may contribute to the inhibitory effects of CEACAM6 gene silencing on the in vivo metastatic ability of pancreatic adenocarcinoma cells. CEACAM6 may play a role in tumor metastasis through altered interactions between tumor cells and ECM components. It will be important to investigate the role other GPI-linked proteins have in pancreatic adenocarcinoma progression. CEACAM6 warrants further evaluation as a potential target for novel anti-metastatic strategies for the treatment of pancreatic adenocarcinoma.

Acknowledgments

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