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Metastasis Suppressor KAI1/CD82 Attenuates the Matrix Adhesion of Human Prostate Cancer Cells by Suppressing Fibronectin Expression and β_1 Integrin Activation

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Key Words
KAI1 • CD82 • Metastasis • Adhesion • Integrin • Extracellular matrix • Fibronectin

Abstract
KAI1/CD82, a tetraspanin membrane protein functions as a metastasis suppressor in many types of human cancers and has been shown to regulate cell adhesion properties. In the present study, we investigated the underlying mechanism of KAI1/CD82-mediated changes in cell adhesion to the extracellular matrix using human prostate cancer cells. We found that high KAI1/CD82 expression attenuated short-term cell adhesion to uncoated- or fibronectin-coated plates. Moreover, high KAI1/CD82 expression generated an extracellular environment unfavorable for cell adhesion as compared to low KAI1/CD82 expression, suggesting KAI1/CD82-dependent regulation of extracellular matrix (ECM) molecule(s) expression and/or secretion. Among ECM components examined, fibronectin exhibited decreased expression and secretion in high KAI1/CD82-expressing cells. Furthermore, high KAI1/CD82 expression interfered with the activation of β_1 integrin at the cell surface while total β_1 integrin levels remained unchanged, concomitant with reduced formation of focal adhesion complex and decreased bundling of actin filaments. Finally, high KAI1/CD82 expression significantly retarded cell motility in a scratch wound assay. Taken together, our results strongly suggest that KAI1/CD82 attenuates the activation of β_1 integrin, and thereby down-regulates outside-in signaling of β_1 integrin, leading to the reduction of focal adhesion formation and fibronectin expression/secretion, which subsequently interferes with cell adhesion properties and motility.

Introduction

Tetraspanin KAI1/CD82 possesses cancer metastasis-suppressing function, which was first identified by genetic screening in prostate cancer cells [1]. A number of xenograft studies using various metastatic cell lines demonstrated that KAI1/CD82 can suppress tumor metastasis [2-5]. Moreover, in a variety of malignancies, including prostate [6-8], gastric [9], colon [10, 11], cervix [12, 13], breast [14, 15], skin [16], bladder [16-19], lung [20], pancreas [21], liver [22, 23], and thyroid cancers

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[24], the metastatic potential of cancer was inversely correlated with the expression level of KAI1/CD82.

KAI1/CD82 is a member of the tetraspanin superfamily, which consists of 33 integral membrane proteins harboring four transmembrane and two extracellular domains [25, 26]. Although the mechanism remains obscure, tetraspanins regulate cell adhesion, migration, fusion, and proliferation. Since tetraspanins have no intrinsic catalytic activity, it is assumed that tetraspanins exert their function by regulating membrane organization through interactions with cell surface molecules, such as cell adhesion molecules, growth factor receptors, and other tetraspanins. Indeed, KAI1/CD82 has been shown to interact with integrins such as $\alpha_3\beta_1$, $\alpha_4\beta_1$, $\alpha_5\beta_1$, $\alpha_6\beta_1$, and $\alpha_L\beta_2$, E-cadherin, EWI-2, epidermal growth factor receptor, tetraspanins such as CD9 and CD81, and intracellular signaling molecules such as protein kinase C in tetraspanin-enriched microdomains [27, 28]. Since many KAI1/CD82-associated proteins are involved in cell adhesion and motility, KAI1/CD82 is suggested to regulate cell adhesion and migration by modulating the activity of its associated proteins. Since the metastasis suppressor function of KAI1/CD82 is primarily dependent on the inhibition of cancer cell motility and invasiveness, understanding the underlying mechanism of KAI1/CD82-mediated regulation of these interacting proteins is of great importance.

Integrins are a family of α/β heterodimeric cell surface adhesion glycoprotein receptors that bind to specific ECM components. The integrin ectodomain interacts with extracellular ligands in a metal-dependent manner, which induces the conformational changes of integrins to an active form. Ligand binding to integrins triggers the formation of organized adhesive contacts, called focal complexes or focal adhesions, which induces signal transduction cascades that regulate many aspects of cell behavior including cell adhesion, migration, proliferation, survival, and differentiation [29-31]. This process is called integrin outside-in signaling. Alternatively, integrin conformation and its interaction with ligands are regulated from inside the cells through the cytoplasmic domains, an inside-out signaling, which also regulates major changes in cell shape, behavior and fate [29]. Specifically, integrin-mediated adhesion to the ECM plays a critical role in cell migration of many cell types, including fibroblasts and carcinoma cells [30]. Increasing attention has been paid to the molecular mechanism of KAI1/CD82-mediated integrin regulation since integrin-mediated adhesion to the ECM is reduced upon overexpression of KAI1/CD82 [32, 33]. Particularly, KAI1/CD82 has been implicated in the internalization/endocytosis and post-translational modification of integrins through their association with integrins [34, 35]. A KAI1/CD82 re-expressing metastatic prostate cancer cell line has been shown to exhibit reduced cell adhesion presumably due to reduced cell surface α_6 integrin expression following enhanced α_6 internalization [34]. Furthermore, re-expression of KAI1/CD82 in tumor cells negatively affects pre-β_1 integrin processing, resulting in reduced β_1 integrin on the cell surface [35].

In an effort to reveal the role of KAI1/CD82 in cell adhesion to the ECM, we here examined KAI1/CD82-mediated modulation of integrin binding activity to the matrix using stable transfectant clones of human prostate cancer cell lines, DU145 and LNCaP transfectant cells with increased and decreased expression of KAI1/CD82, respectively. We also examined whether KAI1/CD82 can modulate the ECM environment to regulate the adhesion properties of cells. In the present study, we show that KAI1/CD82 expression attenuated the adhesion of prostate cancer cells to the matrix by interfering with β_1 integrin activation and reducing fibronectin expression/secretion.

Materials and Methods

Cell culture

Human prostate cancer cell lines, PC3, DU145, LNCaP, and TSU-Pr1, were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 μg/ml streptomycin in 5% CO_2 at 37°C. Stable transfectant clones of DU145 and LNCaP cells were maintained with the addition of neomycin G418 to a final concentration of 0.4 mg/ml and 0.3 mg/ml, respectively.

RT-PCR

Total cellular RNA was purified from the cultured cells using Trizol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's protocol. cDNA synthesis was performed with 1 μg of total RNA using a cDNA synthesis kit (Promega, Madison, WI, USA). cDNAs were subjected to PCR amplification using the following primer pairs: 5'-CTA CAA CAG CAG TCG CGA GG-3' (forward) and 5'-CAG CTG CCT CAG TAC TTG GG-3' (reverse) for *KAI1/CD82*; 5'-CAC CAT CCA ACC TGC GTT TC-3' (forward) and 5'-TGT CCT ACAT TCG GCG GGT-3' (reverse) for fibronectin 1 (*FN1*); 5'-CCA ATA TCA TGC CCT GGT GAG CTA-3' (forward) and 5'-TGC AGA ACA GTA GCT GAG TCT GTG-3' (reverse) for laminin β3 (*LAMB3*); 5'-ATG TCA ATG GCA CCC ATC AC-3' (forward) and 5'-CTT CAA GGT GGA CGG CGT AG-3' (reverse) for collagen type IV α1 (*COL4A1*); 5'-GAT ATC GCC GCG CTC GTC GTC GAC-3' (forward) and 5'- CAG GAA GGA AGG CTG GAA GAG TGC-3' (reverse) for β-actin (*ACTB*).

Stable transfection of DU145 and LNCaP cells with KAI1/CD82 sense and antisense cDNAs, respectively

Full-length KAI1/CD82 cDNA, generously provided by Dr. J. Carl Barrett (National Cancer Institute, MD, USA), was transfected into DU145 cells using lipofectAMINE/PLUS reagent (Invitrogen) according to the manufacturer's instructions. KAI1/CD82 transfectant clones of DU145 cells were isolated by growing the cells in RPMI-1640 medium containing 10% FBS and 450 μg/ml G418. Neomycin-resistant clones were characterized by RT-PCR and immunoblotting analyses for KAI1/CD82 expression and clones with high KAI1/CD82 expression were selected. For antisense expression of KAI1/CD82 gene, human KAI1/CD82 mRNA was isolated from LNCaP human prostate cancer cell line and cDNA fragment, which corresponds to human KAI1/CD82 cDNA (upper:1-20 bp; lower: 86-105 bp; GenBank NM 002231), was amplified by RT-PCR using primers (sense primer-5'-CCG ctc gag ATG GGC TCA GCC TGT ATC AA-3' and antisense primer-5'-GCT TCG GGG TGT gga tcc TG-3'; lower-case letters represent *Xho*I and *BamH*I restriction sequences, respectively). The 109 bp PCR product was cloned into pcDNA3 vector (Invitrogen) in the antisense orientation using *BamH*I and *Xho*I restriction enzymes. KAI1/CD82 antisense construct was transfected into LNCaP human prostate cancer cell lines using lipofectAMINE/PLUS reagent. Neomycin-resistant clones were isolated by growing cells in RPMI-1640 medium containing 10% FBS and 325 μg/ml G418. Stable transfectant clones with low KAI1/CD82 expression were identified by immunoblotting analysis.

Immunoblotting

Cells were washed, harvested, and lysed in lysis buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 3 mM $MgCl_2$, 0.5% Nonidet P-40, 2 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 20 μg/ml leupeptin, and 2 mM benzimidine) on ice for 10 min. After centrifugation at 15,000 x *g* for 10 min, the supernatants were collected and quantified for protein concentration by BCA assay. Equal amounts of protein per lane were separated onto 10-12% SDS-polyacrylamide gel and transferred to an Immobilon-P membrane (Millipore Corp., Bedford, MA, USA). The membrane was blocked in 3% bovine serum albumin (BSA) in TBST (25 mM Tris, pH 7.4, 3.0 mM KCl, 140 mM NaCl and 0.1% Tween 20) for 2 h and then incubated with primary antibodies for 2 h. Primary antibodies against KAI1/CD82 (G-2, 1:500), α_3 integrin (I-19, 1:400), α_5 integrin (P-19, 1:400), α_6 integrin (N-19, 1:750), β_1 integrin (N-20, 1:400), focal adhesion kinase (FAK, A-17, 1:750), phospho-FAK^{Tyr925} (1:750), and phospho-Src^{Tyr416} (9A6, 1:750) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies to Src (36D10, 1:750) and β-actin (1:5000) were from Cell signaling Technology (Danvers, MA, USA) and Sigma (St. Louis, MO, USA), respectively. After washing, the membrane was incubated with a secondary antibodies conjugated with horseradish peroxidase (Sigma, 1:2000). After final washes, the proteins were visualized using enhanced chemiluminescence reagents (Amersham Biosciences/GE Healthcare, Piscataway, NJ, USA).

Adhesion assay

Adhesion assay was either performed on uncoated 24-well plates or plates coated with ECM components. To prepare ECM-coated plates, 24-well plates were incubated with human fibronectin (Roche, Mannheim, Germany), type IV collagen (MP Biomedicals, Aurora, OH, USA), laminin (BD Biosciences, Bedford, MA, USA) or Matrigel (BD Biosciences) to final 10 μg/cm^2 at 4°C overnight, washed three times with phosphate-buffered saline (PBS) and then nonspecific binding sites were blocked with PBS containing 10 mg/ml heated BSA for 30 minutes. The cells were collected, washed twice with PBS and washed once with serum-free RPMI-1640 medium. DU145 stable transfectant cells (2x10^5 cells) or LNCaP stable transfectant cells (1x10^5 cells) resuspended in serum-free RPMI-1640 medium were allowed to adhere to the wells for 30 min. Non adherent cells were removed by washing and the remaining adherent cells were fixed and stained with crystal violet for evaluation. Cells were then photographed and counted. Values were obtained in triplicate independent experiments. Adhesion assay on cell-secreted ECM was performed as follows. Stable transfectant cells were seeded into 24-well plates and incubated for 48 h to 100% confluency and then completely removed from the plates using 0.1% deoxycholate or 0.0125% trypsin-EDTA solution. After washing with PBS, the plates were blocked with 0.2% BSA for 5 min to avoid nonspecific cell adhesion to the plates. Then, freshly prepared DU145 (1x10^5 cells) and LNCaP (5x10^4 cells) stable transfectant cells were seeded into the prepared 24-well plates to perform the adhesion assay. Cells were allowed to attach to the wells for 30 min and non-adherent cells were removed. After washing, the remaining cells were fixed and stained with crystal violet for measurement.

Detachment assay

Transfectant cells were allowed to grow up to 70% confluent monolayer on uncoated 24-well plates. The cells were washed once with PBS and treated with trypsin-EDTA for the indicated time periods. After removal of unattached cells, remaining cells were further incubated in growth medium for 15 min. Following removal of unattached cells from wells, cells were fixed and stained with crystal violet. The cells were then photographed and counted. Results are expressed as the percentage of cells remaining on the surface of each wells referenced to the number of total cells. Each point represents the mean of four samples.

ELISA

For the measurement of ECM molecules secreted from the transfectant cells, ELISA was performed as follows: DU145 and LNCaP stable transfectant cells were seeded into 96-well plates and incubated for 48 h to 100% confluency. After removal of cells using 0.0125% trypsin-EDTA solution, the wells were blocked with 1% BSA in PBS and incubated with primary antibodies against fibronectin (BD Biosciences, 1:200), laminin β3 (H-300, Santa Cruz Biotechnology, 1:250) or type IV collagen (COL-94, Santa Cruz Biotechnology, 1:200) for 2 h. The wells were then incubated with secondary antibodies conjugated with HRP. *o*-Phenylenediamine (OPD) substrate solution (50

mM citrate-phosphate buffer, 0.03% H_2O_2, 0.4 mg/mL OPD) was added to each well and incubated for 10 min for color development and the reaction was stopped by adding 1N HCl to each well. Absorbance at 450 nm was measured.

Flow cytometry

Cell surface expression of active β_1 integrins was analyzed by flow cytometry. DU145 and LNCaP stable transfectant cells grown on non-coated 60 mm dish were harvested using a cell scraper, washed with PBS, dispersed into single cell suspension by pipetting in PBS. Cells were then incubated with antibodies against integrin β_1 (1:500) or active integrin β_1 (HUTS-4, Chemicon, Billerica, MA, USA, 1:100) for 30 min on ice, washed with PBS, and labeled with Alexa Fluor 488-conjugated secondary antibody (Molecular Probes, Eugene, OR, USA) for 30 min on ice. After washing with PBS, cells were fixed by adding an equal volume of 2% formaldehyde. Fluorescence labeled cells were measured with FACS Calibur and analyzed with CellQuestPro software (Becton Dickinson, San Jose CA, USA).

Confocal microscopy

DU145 ($1x10^5$ cells) and LNCaP ($5x10^4$ cells) stable transfectant cells were seeded onto glass coverslips in 24-well plates and incubated for 24 h. Cells were fixed with 4% paraformaldehyde for 10 min and then permeabilized with 0.4% triton X-100 for 10 min. Nonspecific antibody binding sites were blocked by incubation with 1% BSA in TBST for 30 min. Cells were then incubated with primary antibodies specific to paxillin (BD Biosciences, 1:200), FAK (1:200), integrin β_1 (1:150), active integrin β_1 (1:200) or β-actin (1:100) for 2 h, followed by washing with TBST three times. Alexa Fluor 488- or Alexa Fluor 555-conjugated secondary antibodies (Molecular Probes) were added to cells and incubated for 1 h. Coverslips were then washed and mounted by applying Mount solution (Biomeda, Foster City, CA, USA). Fluorescence images were acquired using a confocal laser-scanning microscope and software (Fluoview ver. 2.0) with a 60X objective (Olympus FV300, Tokyo, Japan).

Scratch wound assay

For the measurement of cell motility during wound healing, transfectant cells were allowed to grow up to 100% confluent monolayer on uncoated 24-well plates. Cell layers were then wounded with a plastic micropipette tip having a large orifice. The medium and debris were aspirated away and replaced by 2 ml of fresh serum-free medium. 3 days after wounding, cells were photographed by phase contrast microscopy. For evaluation of 'wound closure', five randomly selected points along each wound were marked and the horizontal distance of migrating cells from the initial wound was measured.

Statistical analysis

Student's *t*-test was used for the comparison of two different groups. All data are represented as mean ± SD and the differences between groups were considered significant at $P<0.05$.

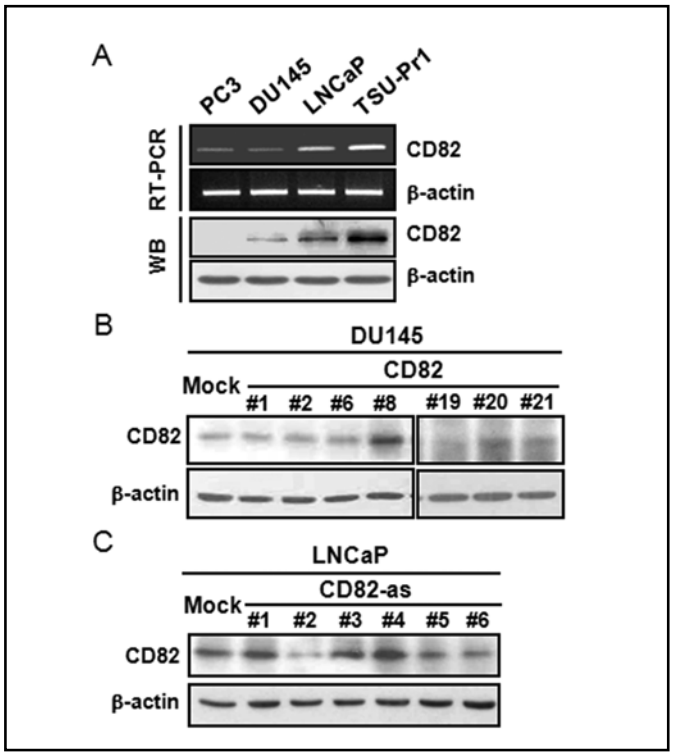

Fig. 1. KAI1/CD82 expression in various prostate cancer cell lines and generation of DU145 and LNCaP cells stably transfected with KAI1/CD82 sense and antisense cDNAs, respectively. (A) Both mRNA and protein levels of KAI1/CD82 were analyzed by RT-PCR and immunoblotting (WB) from four human prostate cancer cells lines, PC3, DU145, LNCaP and TSU-Pr1. (B, C) Stable KAI1/CD82 sense cDNA transfectant clones of DU145 (CD82) (B) and KAI1/CD82 antisense cDNA transfectant clones of LNCaP cells (CD82-as) (C) were generated and the KAI1/CD82 expression level was analyzed by immunoblotting. Empty vector-transfected clones of DU145 or LNCaP were used as controls (Mock).

Results

Generation of stable KAI1/CD82 sense and antisense cDNAs transfectant clones

The expression level of KAI1/CD82 in several human prostate cancer cell lines, including PC3, DU145, LNCaP, and TSU-Pr1, was assessed by RT-PCR and immunoblotting. While the protein level was low or undetectable in DU145 and PC3 cells, LNCaP and TSU-Pr1 showed moderate to high expression of KAI1/CD82 (Fig. 1A). We next generated stable KAI1/CD82 sense cDNA transfectant clones of DU145 cells and stable KAI1/CD82 antisense cDNA tranfectant clones of LNCaP cells (Fig. 1B and 1C). DU145 transfectant clones which overexpressed KAI1/CD82, #8 and #20, and LNCaP transfectant clones demonstrating KAI1/CD82 knockdown, #2 and #5, were chosen to analyze the role of KAI1/CD82 in cell adhesion.

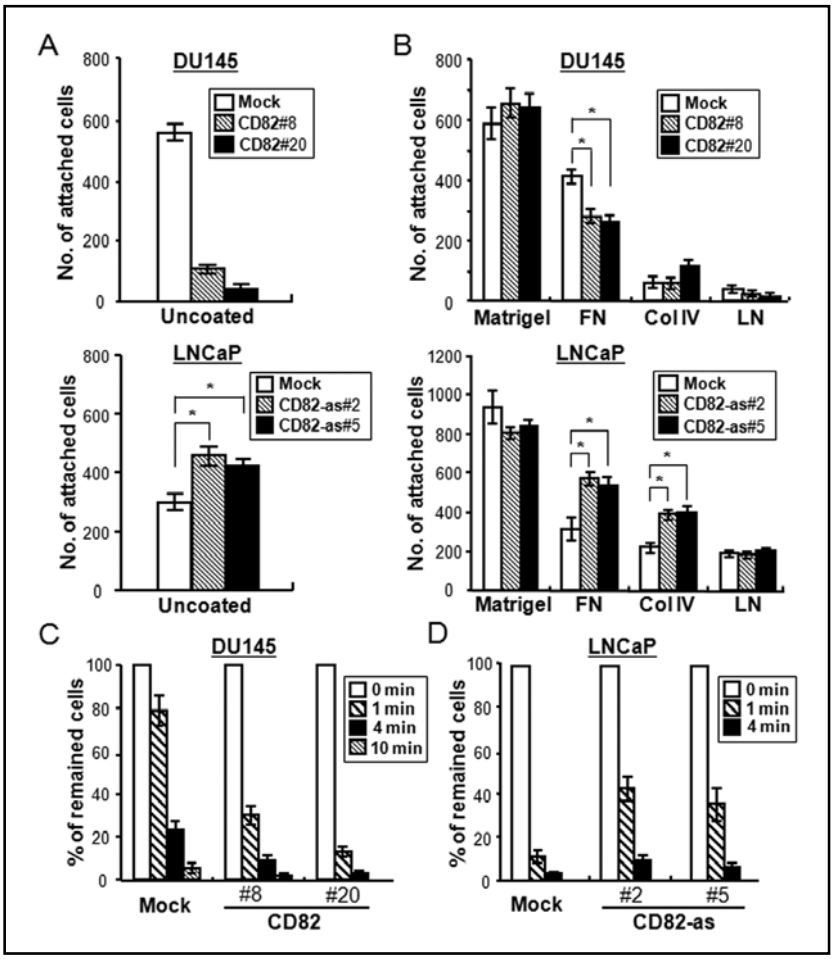

Fig. 2. KAI1/CD82 expression modulates cell adhesion and detachment from the matrix. (A, B) KAI1/CD82 sense cDNA-transfected DU145 cells (CD82) and KAI1/CD82 antisense cDNA-transfected LNCaP cells (CD82-as) were allowed to attach to uncoated plates (A) and to plates coated with type IV collagen (Col IV), fibronectin (FN), laminin (LN) or Matrigel (B). After 30 min of incubation, attached cells were counted. Data represent the mean ± SD of three independent experiments (*$P < 0.05$). (C, D) KAI1/CD82 transfectants of DU145 (C) and LNCaP cells (D) were treated with 0.0125% trypsin for the indicated time periods and the remaining adherent cells were counted. The results are presented as a percentage of the total (untreated) adherent cells.

KAI1/CD82 expression reduces cell attachment to the matrix, particularly to fibronectin

To analyze the effect of KAI1/CD82 on adhesion properties of prostate cancer cells, transfectant cells were allowed to attach to uncoated plates or to plates coated with ECM components. Attachment of KAI1/CD82 sense cDNA-transfected DU145 cells to uncoated plates was significantly reduced as compared to mock transfectant DU145 cells (Fig. 2A). Consistently, KAI1/CD82 antisense cDNA-transfected LNCaP cells showed increased attachment to uncoated plates as compared to mock transfectant LNCaP cells (Fig. 2A). We also carried out cell adhesion assay with plates coated with Matrigel, fibronectin, collagen type IV, or laminin. Interestingly, KAI1/CD82-dependent reduction of cell adhesion was observed only with fibronectin-coated plates (Fig. 2B). These results indicate that high KAI1/CD82 expression reduces cell adhesion to the matrix, particularly to fibronectin. Moreover, prostate cancer cell adhesion was largely dependent on the presence of fibronectin as compared to other ECM components such as collagen type IV and laminin, which implicates that fibronectin is a major ECM component to which prostate cancer cells adhere. Next, to determine the effect of KAI1/CD82 on cell detachment, transfectant cells grown on uncoated plates were treated with trypsin-EDTA for varying time points and the remaining cells on the culture plates were counted. KAI1/CD82-overexpressing DU145 transfectant cells were detached from the plates with faster kinetics than were mock transfectant cells (Fig. 2C). Conversely, decreased expression of KAI1/CD82 rendered LNCaP cells more resistant to trypsin treatment for detachment from the culture plates (Fig. 2D). These results suggest that KAI1/CD82 decreases cellular adhesion strength to the matrix. Taken together, it thus appears that KAI1/CD82 affects adhesion between prostate cancer cells and the matrix in a negative manner.

KAI1/CD82 expression modulate extracellular environment for cell adhesion

Cell detachment from culture plates is largely affected by two major factors, the activity/expression of cell adhesion proteins and the amount/composition of deposited ECM molecules. In an attempt to discriminate the effect of KAI1/CD82 on these two factors, an alternative adhesion assay was performed employing cell

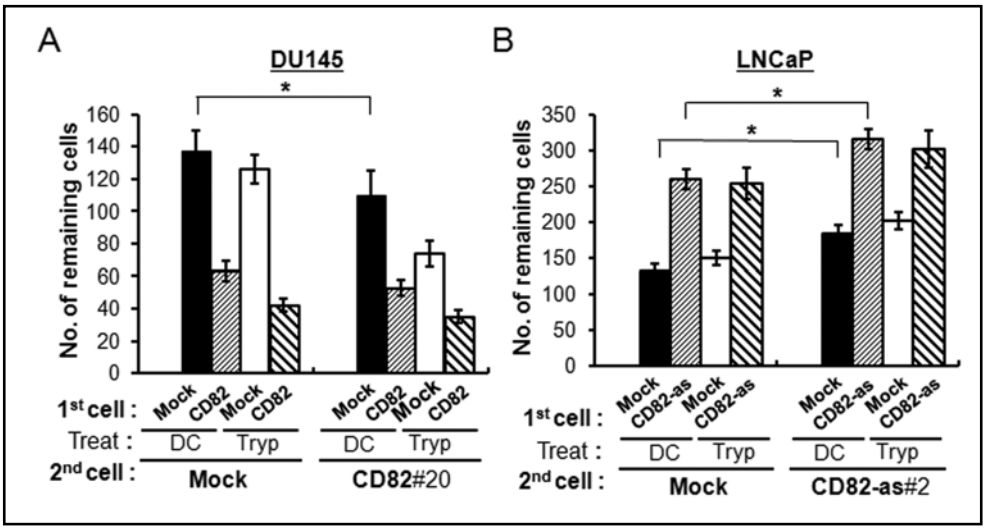

Fig. 3. High KAI1/CD82–expressing cells provide an extracellular environment unfavorable for cell adhesion. (A, B) KAI1/CD82 sense cDNA-transfected DU145 cells (clone#20, CD82) and KAI1/CD82 antisense cDNA-transfected LNCaP cells (clone #2, CD82-as) were incubated for 48 h (1st cell) and cells were then removed from the plates by treatment with either 0.1% deoxycholate (DC) or 0.0125% trypsin (Tryp). Upon complete removal of cells, the plates were blocked with 0.2% BSA to avoid nonspecific cell adhesion to the plates, and then fresh transfectant cells (2nd cell) were seeded to the plates for adhesion assay. After 30 min of incubation, adherent cells were counted. Data represent the mean ± SD of three independent experiments (*$P < 0.05$).

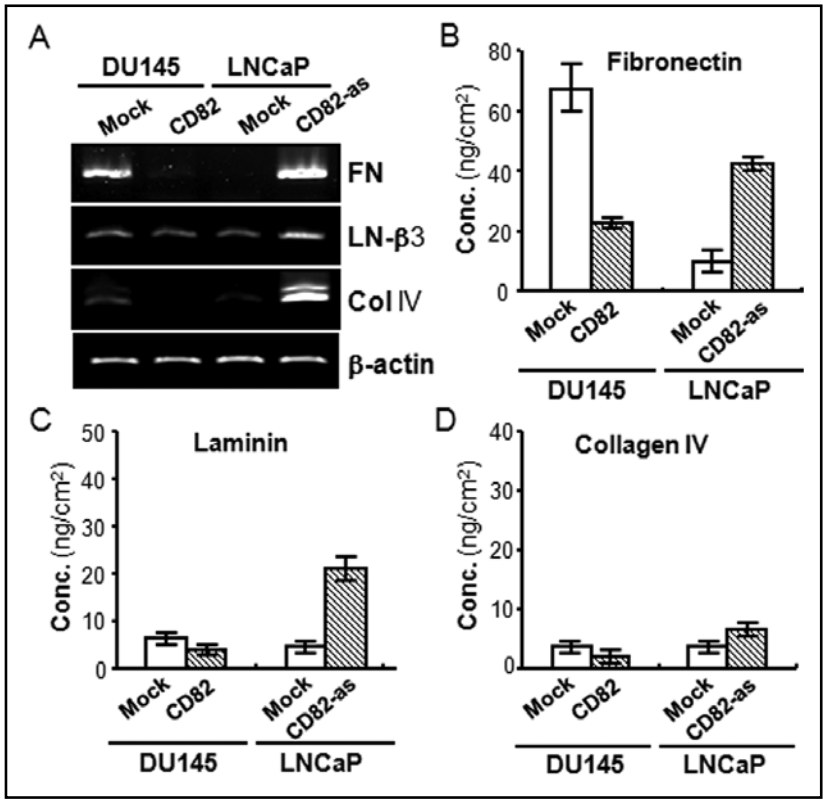

Fig. 4. KAI1/CD82 expression down-regulates the synthesis and secretion of fibronectin. (A) Expression levels of ECM molecules including fibronectin (FN), laminin-β3 (LN), and type IV collagen (ColIV) were determined in KAI1/CD82 sense cDNA-transfected DU145 cells (clone#20, CD82) and KAI1/CD82 antisense cDNA-transfected LNCaP cells (clone#2, CD82-as) by RT-PCR analysis. (B-D) Transfectant cells were seeded on 96-well plates and incubated for 48 h. After the removal of cells and blocking with BSA, specific ECM molecules remaining on the culture plates were quantitatively measured by ELISA using antibodies specific to each ECM protein. Standard curves for each ECM molecule were created after coating 96-wells with varying amounts of ECM proteins. Data represent the mean ± SD of three independent experiments.

attachment on plates previously coated with cell-secreted ECM. Stable transfectant cells grown on culture plates were completely removed by either deoxycholate or trypsin-EDTA, and then fresh transfectant cells were allowed to adhere to the plates after blocking with BSA. As shown in Fig. 3A, mock or KAI1/CD82 transfectant DU145 cells adhered more strongly to plates pre-coated with mock transfectant DU145 cell-secreted ECM as compared to those of KAI1/CD82 transfectant-secreted ECM regardless of the detachment method. These results imply that KAI1/CD82 transfectant cell-secreted ECM provides a less favorable environment for cell adhesion than mock transfectant cell-secreted ECM. Similar to the data in Fig. 2, KAI1/CD82 transfectant DU145 cells were less adhesive than mock transfectant cells to plates pre-coated with either mock or KAI1/CD82 transfectant cell-secreted ECM. Conversely, ECM secreted by KAI1/CD82 antisense cDNA-transfected LNCaP cells provided a more favorable environment for cell adhesion than mock transfectant LNCaP cell-secreted ECM (Fig.

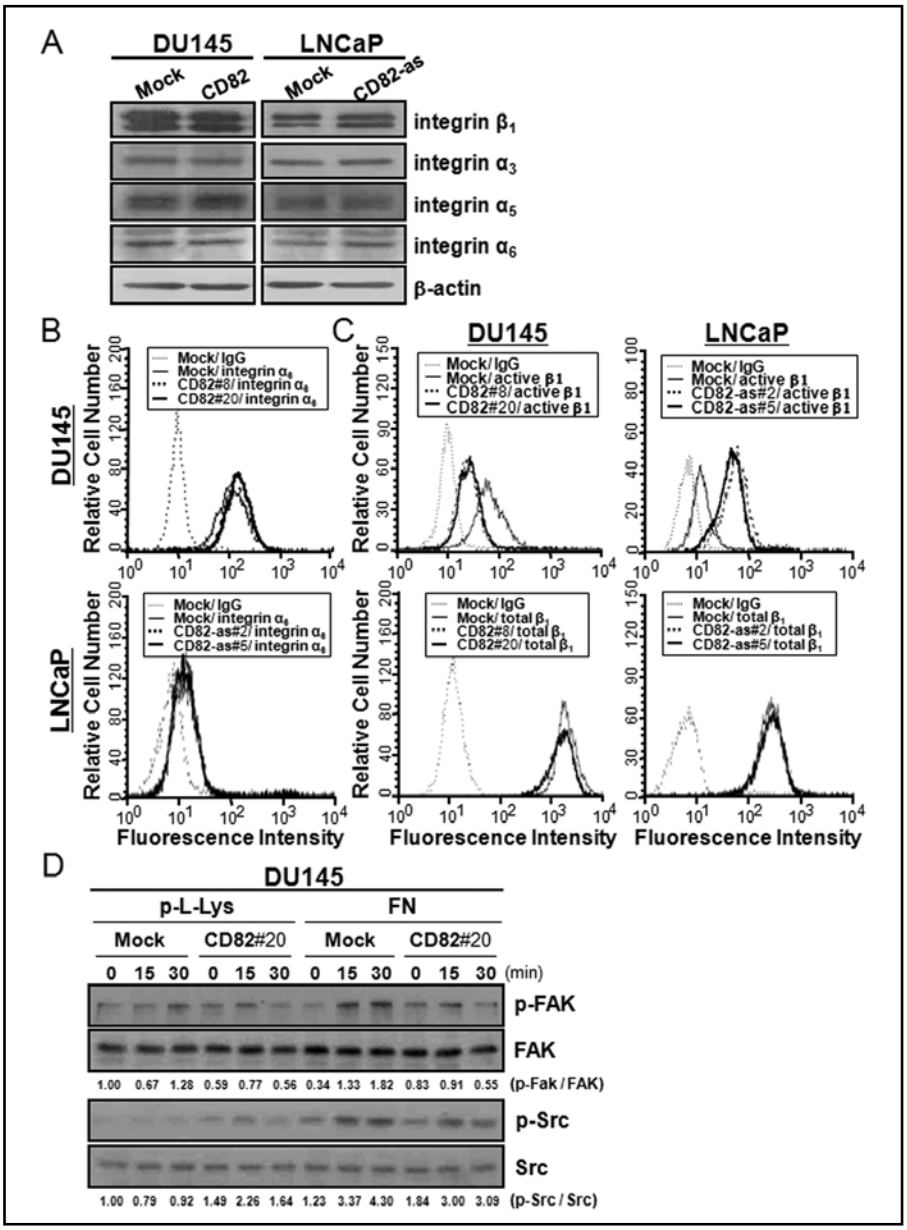

Fig. 5. KAI1/CD82 expression attenuates the activation of β_1 integrin. (A) Protein levels of integrins β_1, α_3, α_5, and α_6 in KAI1/CD82 sense cDNA-transfected DU145 cells (clone#20, CD82) and KAI1/CD82 antisense cDNA-transfected LNCaP cells (clone#2, CD82-as) were examined by immunoblotting analysis. (B) α_6 integrin level at the cell surface was analyzed by flow cytometry after immunostaining. (C) The level of the active form of β_1 integrin (upper panel, active β_1) and total β_1 integrin (lower panel, total β_1) at the cell surface was analyzed by flow cytometry after immunostaining. (D) Serum-starved KAI1/CD82-transfectant cells were plated onto poly-L-lysine- and fibronectin-coated plates for the indicated time periods. Phosphorylation and levels of FAK and Src in cell lysates were compared by immunoblotting analyses using specific antibodies for phospho-FAK$^{Tyr-925}$ and phsopho-Src$^{Tyr-416}$, respectively. Numbers under the immunoblot indicate relative ratio of phospho-FAK/ total FAK and phospho-Src/total Src and are the mean of three immunoblots obtained from separate experiments.

3B). In addition, KAI1/CD82 antisense cDNA transfectant cells exhibited stronger adherence to these plates than mock transfectant cells. Altogether, these results indicate that high KAI1/CD82-expressing prostate cancer cells are less adhesive to cell-secreted ECM and secrete ECM to which cells are less adhesive as compared to low KAI1/CD82-expressing cells. Therefore, it seems likely that KAI1/CD82 expression in prostate cancer cells alters cell-to-matrix adhesion by two different mechanisms, modulation of ECM secretion and regulation of cell adhesion protein function.

KAI1/CD82 expression decreases the expression/ secretion of fibronectin

Next, we attempted to identify ECM component(s) in prostate cancer cells, which are regulated by KAI1/CD82. mRNA levels of fibronectin (*FN1*) and type IV collagen (*COL4A1*) were significantly reduced by *KAI1/CD82* expression in both DU145 and LNCaP cells, while the mRNA level of laminin β3 (*LAMB3*) was minimally altered by KAI1/CD82 (Fig. 4A). These results strongly suggest that KAI1/CD82 down-regulates the expression of fibronectin and type IV collagen in prostate cancer cells. To evaluate the amount of ECM proteins secreted from cells, ELISA was performed using the culture plates upon removal of confluent transfectant cells. Consistent with RT-PCR results, high expression of KAI1/CD82 significantly diminished the amount of fibronectin secreted and deposited by both DU145 and LNCaP cells (Fig. 4B). However, the level of laminin secreted by DU145 cells was minimally altered by KAI1/CD82 expression, although KAI1/CD82 knockdown in LNCaP cells enhanced laminin

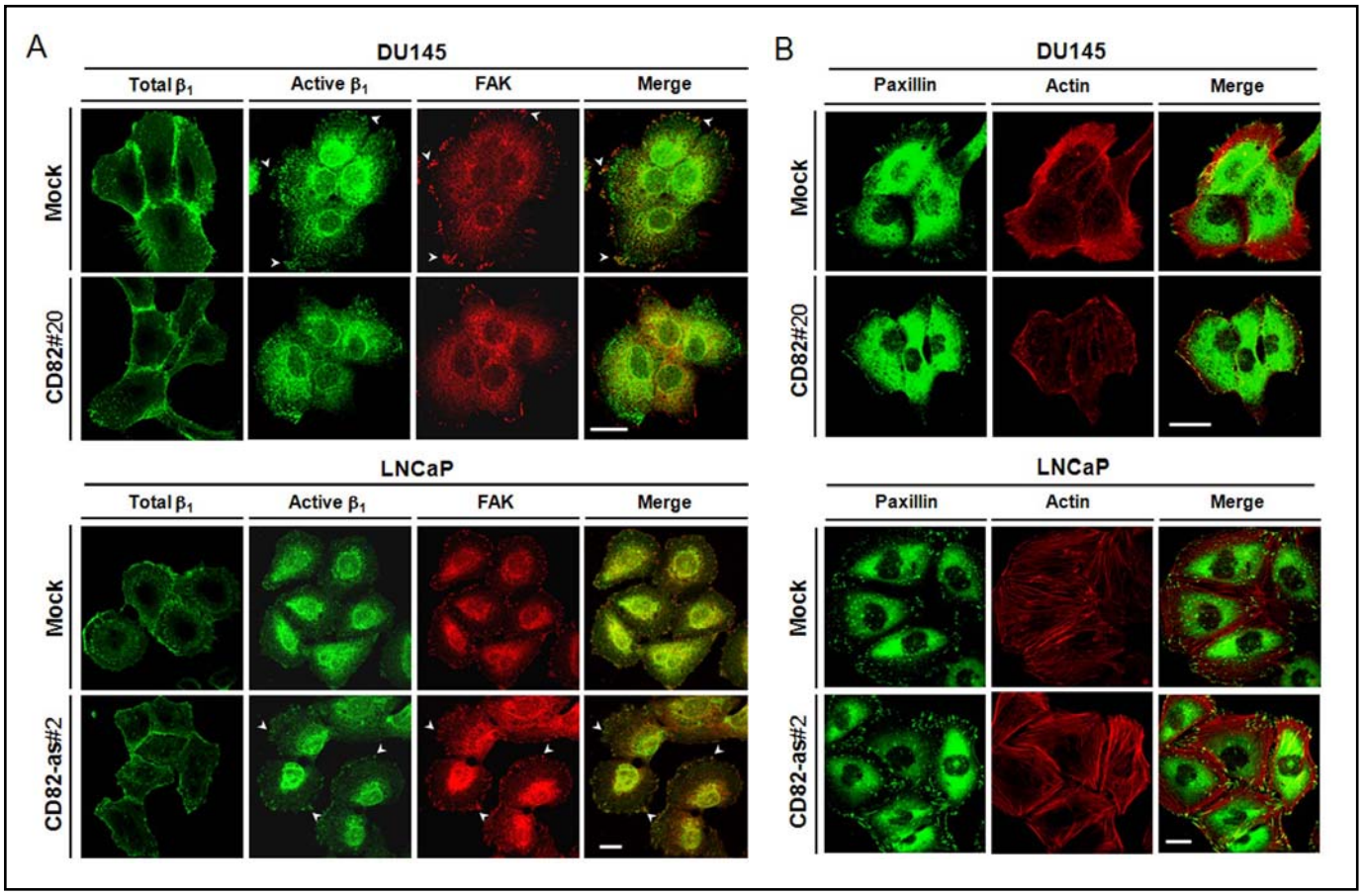

Fig. 6. KAI1/CD82 interferes with the formation of the focal adhesion complex and actin stress fibers. (A) KAI1/CD82 sense cDNA-transfected DU145 cells (clone#20, CD82) and KAI1/CD82 antisense cDNA-transfected LNCaP cells (clone#2, CD82-as) were immunostained for total β_1 integrin (green) and double-stained for the active form of β_1 integrin (green) and FAK (red). Arrowheads indicate colocalization of active integrin β_1 with FAK at focal contacts. (B) DU145 and LNCaP transfectant cells were double-stained for paxillin (green) and actin (red). Distribution of total β_1 integrin, active form of β_1 integrin, FAK, paxillin, and actin was analyzed by confocal microscopy. Bars indicate 20 μg.

secretion from cells (Fig. 4C). Although the extracellular level of type IV collagen was affected by KAI1/CD82 in both DU145 and LNCaP cells, its extracellular level was much lower as compared to fibronectin level (Fig. 4D). Altogether, these data demonstrate that KAI1/CD82 expression significantly reduces the expression and secretion of fibronectin in prostate cancer cells.

Activation of β_1 integrin and its downstream signaling is attenuated by KAI1/CD82 expression

Since integrins are the primary adhesion molecules responsible for cell adhesion to the matrix, we analyzed the level of integrins in KAI1/CD82 sense and antisense cDNAs transfectant cells by immunoblotting. The protein level of β_1, α_3, α_5, and α_6 integrins, subunits of $\alpha_3\beta_1$, $\alpha_5\beta_1$, and $\alpha_6\beta_1$ integrins that interact with fibronectin and laminin, were not changed by KAI1/CD82 expression (Fig. 5A). Next, we determined the cell surface level of α_6 integrin by FACS since KAI1/CD82 has been suggested to regulate the internalization of α_6 integrin [34]. However, as shown in Fig. 5B, the cell surface level of α_6 integrin was not altered by KAI1/CD82 expression in our experimental setup. Although the total protein level (Fig. 5A) and total cell surface level of β_1 integrin was unaltered, the level of active β_1 integrin on the cell surface was significantly diminished by KAI1/CD82 expression in both DU145 and LNCaP cells (Fig. 5C), implying the involvement of KAI1/CD82 in the activation of β_1 integrin rather than its expression. We next examined the effect of KAI1/CD82 expression on downstream signaling following β_1 integrin-mediated adhesion. As expected, the phosphorylation levels of FAK and Src were decreased by KAI1/CD82 expression, which was more prominent on fibronectin-coated plates than poly-L-lysine-coated plates (Fig. 5D), supporting the role of KAI1/CD82 in the down-regulation of β_1 integrin signaling.

Activation of β_1 integrin and concomitant focal adhesion complex formation are impaired by KAI1/CD82 expression

Inhibition of β_1 integrin activation by KAI1/CD82 was further confirmed by immunostaining of active β_1 integrin and FAK. Focal adhesions containing active β_1 integrin and FAK were more abundant in cells with low KAI1/CD82 expression, while total β_1 integrin level at the cell surface remained unchanged regardless of KAI1/CD82 expression level (Fig. 6A). Furthermore, immunostaining of paxillin and actin showed that KAI1/CD82 expression reduced not only the number of focal contacts but also the formation of actin stress fibers (Fig. 6B), implying reduced cell adhesion to the ECM. Thus, KAI1/CD82-mediated retardation of β_1 integrin activation and concomitant reduction of focal contacts could, in part, account for the reduced matrix adhesion of prostate cancer cells by KAI1/CD82 expression.

KAI1/CD82 expression retards cell motility

Next, the scratch wound assay was performed to analyze whether KAI1/CD82-mediated cell adhesion reduction to the matrix could affect cell motility. As shown in Fig. 7, high KAI1/CD82 expression significantly retarded the migration of prostate cancer cells, indicating that KAI1/CD82-mediated alterations in cell adhesion properties indeed reduce cell motility.

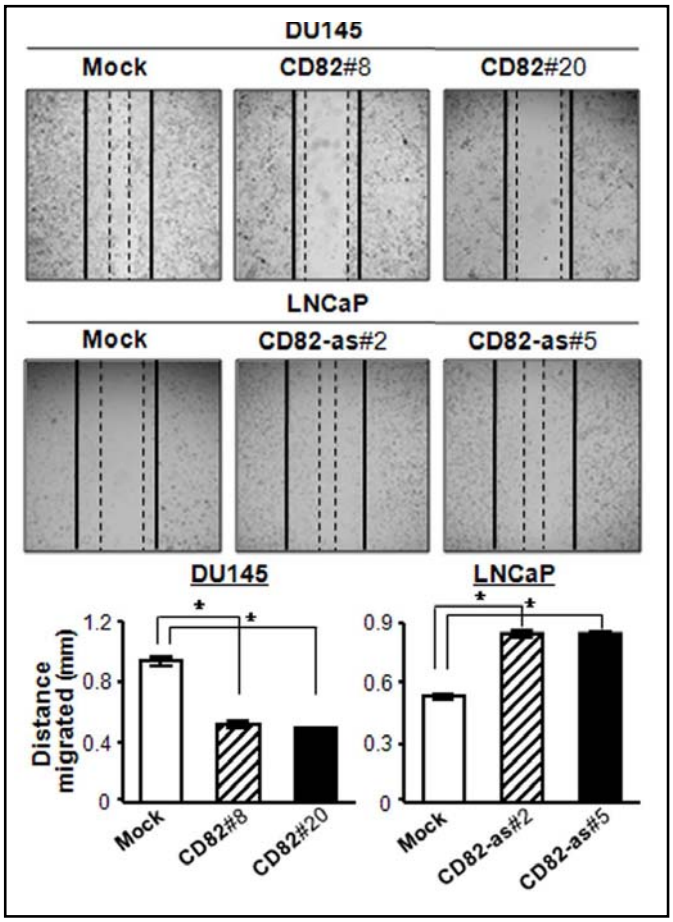

Fig. 7. KAI1/CD82 expression reduces cell motility. Confluent KAI1/CD82 sense cDNA-transfected DU145 cells (CD82) and KAI1/CD82 antisense cDNA-transfected LNCaP cells (CD82-as) were subjected to *in vitro* scratch wounding. Images were captured at 72 h after wounding using phase-contrast microscope. The rate of migration was measured by quantifying the total distance that the cells moved from the edge of the scratch (solid lines) toward the center of the scratch (dotted lines). Data represent the mean ± SD of three independent experiments (*$P < 0.05$).

Discussion

The metastasis suppressive function of KAI1/CD82 has been identified by numerous studies including a xenograft animal model and the evaluation of KAI1/CD82 expression level in invasive and metastatic human cancers. However, despite extensive research, the mechanism by which KAI1/CD82 functions as a metastasis suppressor and its role in normal biology remains obscure. The most well characterized aspect of KAI1/CD82 as a metastasis suppressor is its involvement in cell motility and invasion. Cell adhesion to the ECM is the first step of metastasis, required for cell migration and invasion of primary tumors. Moreover, adhesion of cancer cells to the ECM in microvessels and migration into the other organs are critical in the multistep metastatic process [36]. Therefore, in an attempt to understand the underlying molecular mechanism of KAI1/CD82 as a metastasis suppressor, we investigated the role of KAI1/CD82 in cell adhesion, specifically the modulation of integrin cell adhesion proteins and ECM.

Cell adhesion to uncoated plates or fibronectin-coated plates was altered by KAI1/CD82 expression, specifically higher levels of KAI1/CD82 was correlated with lower cell adhesion to plates (Fig. 2A and 2B). Consistent with these observations, previous reports have shown that KAI1/CD82 expression inhibits adhesion of DLD-1 and CT-26 colon cancer cells to fibronectin, while KAI1/CD82 silencing enhanced the adhesion of BM314 colon cancer cells [10, 37]. In addition to cell adhesion, KAI1/CD82 expression level also affected cell detachment from plates (Fig. 2C and 2D), further supporting the role of KAI1/CD82 in cell-to-ECM interactions. KAI1/CD82 expression was inversely correlated with fibronectin expression/secretion (Fig. 4)

and extracellular fibronectin was shown to be critical for prostate cancer cell adhesion to the matrix (Fig. 2B). The level of secreted and deposited type IV collagen was also regulated by KAI1/CD82 expression (Fig. 4A and 4D). However, this effect with type IV collagen was only significant in LNCaP cells, as also shown in the cell adhesion assay (Fig. 2B). Moreover, the extracellular level of type IV collagen deposited by prostate cancer cells was very low as compared to the fibronectin level. Taken together, these results suggest that KAI1/CD82-mediated changes in cell adhesion properties are intimately related to KAI1/CD82-dependent down-regulation of fibronectin.

The expression levels of α_3, α_5, α_6, and β_1 integrins, adhesion receptor subunits responsible for binding to fibronectin and laminin, were not shown to be altered by KAI1/CD82 expression (Fig. 5A). However, FACS analysis and immunostaining using antibodies against the active conformation of β_1 integrin revealed that the level of the active form at the cell surface was significantly reduced by KAI1/CD82 expression (Fig. 5C and 6A), which correlates with KAI1/CD82-mediated reduction of cell adhesion to fibronectin (Fig. 2B). Activation of FAK and Src, downstream signal transducers of β_1 integrin, was attenuated and focal adhesion assembly was diminished by high KAI1/CD82 expression. Moreover, reduced co-localization of the active form of β_1 integrin with FAK by KAI1/CD82 expression was observed (Fig. 5D, 6A and 6B), indicating KAI1/CD82-mediated down-regulation of β_1 integrin signaling. Consistent with the reduction in cell adhesion to the matrix, cell motility was also reduced by high KAI1/CD82 expression (Fig. 7). Therefore, it seems likely that KAI1/CD82-mediated reduction of β_1 integrin signaling results in decreased formation of the focal adhesion complex, thereby diminishing cell adhesion and motility.

Growing evidence suggests that KAI1/CD82 suppresses cancer metastasis by modulating the activities of associated proteins including integrins through lateral interactions at tetraspanin-enriched microdomains [33, 38]. Direct interaction between KAI1/CD82 and integrins $\alpha_3\beta_1$, $\alpha_4\beta_1$, $\alpha_5\beta_1$, and $\alpha_6\beta_1$ has been observed by co-immunoprecipitation in various adherent cells [39-41]. Two mechanisms underlying the KAI1/CD82-mediated regulation of integrin activity have been previously suggested. First, KAI1/CD82 decreases the surface level of integrin subunits. Upon KAI1/CD82 expression, the migration of DU145 human prostate cancer cell was substantially inhibited through diminished α_6 integrin-dependent cell adhesion, which resulted from enhanced internalization of α_6 integrins and subsequently decreased α_6 integrins at the cell surface [34]. Furthermore, in dendritic cells, surface expression of β_1 and β_2 integrins was decreased when cells were treated with anti-KAI1/CD82 antibodies [42]. Secondly, KAI1/CD82 is involved in maturation of β_1 integrin. β_1 integrin polypeptide is glycosylated in the ER and then in the Golgi complex, converting immature β_1 to mature β_1 integrin which can only form complexes with integrin α subunits [43]. KAI1/CD82 attenuated the conversion of pre-integrin β_1 to the mature form in the H1299 human lung carcinoma cell line [35]. However, we observed that the total protein level and cell surface level of α_6 integrin was not changed by KAI1/CD82 expression (Fig. 5A and 5B). Furthermore, KAI1/CD82 expression did not affect the ratio of immature (faster-migrating form) versus mature β_1 integrin (slower-migrating form) levels (Fig. 5A), implicating that the maturation of β_1 integrin was not regulated by KAI1/CD82 expression in prostate cancer cell lines. Altogether, these results indicate that KAI1/CD82 expression affect neither the internalization of α_6 integrin nor the maturation process of β_1 integrin in DU145 and LNCaP human prostate cancer cells. This discrepancy may in part derive from the different cellular context depending on the origin of cell lines used. Taken together, our results provide another mechanism of KAI1/CD82-mediated integrin regulation in addition to these two modes, which involves KAI1/CD82 as a modulator of conformational changes of β_1 integrin through molecular interactions.

Mature integrins may exist in various conformations with bent or extended forms. Extended conformation can exist in either high ligand affinity forms with the headpiece open for ligands or low ligand affinity forms bearing a closed conformation [29, 44]. Integrin binding to ECM ligands results in conformational changes of integrins and subsequently induces the formation of the focal adhesion complex, leading to actin cytoskeletal reorganization. This integrin outside-in signaling also influences gene expression, cell survival and proliferation. CD151, another member of the tetraspanin superfamily, has been shown to regulate platelet function by modulating outside-in signaling events of $\alpha_{IIb}\beta_3$ integrin [45]. Direct association of tetraspanin CD151 with integrins, such as $\alpha_3\beta_1$, $\alpha_6\beta_1$, and $\alpha_4\beta_1$, has been observed in many cell types [46-48]. Importantly, CD151 association with integrin was shown to increase the binding of $\alpha_3\beta_1$ integrin to laminin by stabilizing its active conformation [39, 49]. Direct interaction between KAI1/CD82 and integrins $\alpha_3\beta_1$, $\alpha_4\beta_1$, $\alpha_5\beta_1$, and $\alpha_6\beta_1$ has also been observed by co-

immunoprecipitation in various adherent cells [39-41]. Altogether, it seems that KAI1/CD82 has higher affinity towards integrins of either bent forms or extended forms with low ligand affinity as compared to high ligand affinity forms. As such, their interaction may interfere with the conformational change of β_1 integrin into high ligand affinity form upon ECM protein binding, which, in turn, regulates integrin outside-in signaling.

Transcription factor Egr1 is known to transactivate the fibronectin gene, which harbors two closely spaced Egr1-binding elements in its promoter region [50, 51]. Moreover, integrin-dependent phosphoinositide 3-kinase/Akt activation is required for Egr1 expression [52], suggesting that integrin outside-in signaling modulates the activity of Egr1. Therefore, it is presumable that KAI1/CD82-mediated down-regulation of integrin outside-in signaling interferes with Egr1 expression, which subsequently reduces fibronectin expression.

In summary, we have demonstrated that KAI1/CD82 expression inhibits the activation of β_1 integrin into its active conformation upon cell binding to the matrix, and subsequently reduces β_1 integrin signaling and focal adhesion complex formation. Also, KAI1/CD82 expression diminished the expression/secretion of fibronectin, which is crucial for prostate cell adhesion to the matrix, presumably due to attenuated β_1 integrin signaling. Therefore, KAI1/CD82 expression in human prostate cancer cells seems to alter both the ECM environment and cell surface adhesion proteins, which is unfavorable for prostate cancer cell adhesion to the matrix. Thus, KAI1/CD82-mediated modification of adhesion properties could be one of the key events that affect cell motility, and possibly invasion and metastasis of prostate cancer cells.

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