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Downregulation of CD44 regulates extracellular matrix degradation in human pancreatic ductal adenocarcinoma cells

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Abstract

Aim: Pancreatic ductal adenocarcinoma (PDAC) has high mortality and early stage metastatic potential. Thus, the developing new clinical approach and metastasis blocking strategy-based drugs are essential for cure of PDAC. In this study we aimed to investigate the effects of cell surface transmembrane glycoprotein CD44 on the regulation of key ECM proteins, integrin β1, fibronectin and collagen IV, in Panc-1 and MiaPaCa-2 cells.
Materials and Methods: Followed by cell viability assay using MTS, fibronectin and collagen IV protein expression levels and integrin β1 mRNA level were analyzed in Panc-1 and MiaPaCa-2 cells treated with 50 nM negative siRNA and CD44 siRNA for 72 h using western blot and RT-PCR, respectively.
Results: Based on our findings, the downregulation of CD44 using specific siRNA led to decrease fibronectin and collagen IV proteins expressions, and also Integrin β1 mRNA expression in both Panc-1 and MiaPaCa-2 cell lines.
Conclusion: CD44 siRNA based therapies have effective role to inhibit ECM degradation in PDAC progression. CD44 is also promising target for against PDAC through inhibiting migration, invasion and metastasis steps.

Introduction

Pancreatic ductal adenocarcinoma (PDAC), which is caused by the neoplasm, invasion and damage of molecular mechanisms in the duct cells of the pancreas, constitutes 95% of all pancreatic cancers. After diagnosis, these patients have unfortunately very short survival rate by 2-3% within 5 years (1). Because of these, it is thought to be most lethal second cancer types in 2030 at the U.S. and Europe (2). The lethality of pancreatic ductal adenocarcinoma was considered to be responsible of the absence of early-stage diagnostic markers, resistance to chemotherapy and apoptosis, high metastatic potential, and the difficulty of performing a surgical operation due to the location of the pancreas in the body (3). Under the normal conditions extracellular matrix (ECM) regulates cell proliferation, survival, biochemical and biophysical process such as delivery of hormones and immune system cells, mechanical fluidic pressure and tissue homeostasis. However, ECM undergoes structural changes resulted from the modification in content and distribution of collagen and further coordinate cellular biological properties in cancer cells (4). PDAC characterized dense fibrotic stroma playing a role as a barrier for drug delivery, chemotherapy and radiotherapy. Significant hallmark of desmoplastic reaction in the ECM is aberrant expression fibronectin and dysregulated collagen network such as collagen I, collagen III, collagen IV and other collagen types (5). In the PDAC progression tumor microenvironment (TME) contains abundant collagens, proteoglycan, glycoprotein, fibronectin, glycosaminoglycan such as hyaluronan (6). These components create desmoplastic structure. Hyaluronan, a vital component of ECM, is primer ligand for CD44 cell surface receptor. Both hyaluronan and CD44 expressions increase in PDAC (7). CD44 binds also other ligands such as osteopontin, Matrix Metalloproteinase (MMP), serglycin, fibroblast growth factor (FGF), heparin-binding fibroblast growth factor (HB-FGF). CD44 receptors consist of extracellular domain, intracellular domain and cytosolic domain. Cytosolic domain of CD44 mainly interacts ankyrin and Ezrin-Radexin-Moesin (ERM) proteins and regulates cytoskeletal conformation and migration (8). The CD44 gene contains 19 exons. CD44 variants are formed in the extracellular domain as a result of alternative splicing of 6 to 15 exons. The fixed part of the extracellular domain encoded by 1 to 5 exons is attached to hyaluronan binding (9). In PDAC, it was observed that the expression of the CD44 standard isoform increased significantly (10). The role and molecular mechanisms of CD44 and CD44 variants in tumor metastasis are not fully understood (11). In this study, we aimed to investigate the relationship between ECM proteins and CD44 for developing of the possible CD44 and ECM degradation targeted therapeutic approaches. In the near future, ECM targeted therapies

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will help improving effective drug delivery systems and break chemoresistance due to desmoplastic stroma. CD44 targeted therapies are also important for blocking metastasis and PDAC molecular characterization.

Materials and Methods

Cell Culture

PDAC cells, Panc-1 and MiaPaCa-2, were cultured in Dulbecco's Modified Eagle Medium (DMEM)/F12 supplemented with %10 Fetal Bovine Serum (FBS) and penicillin (100 U/mL)-streptomycin (100 µg/mL) antibiotic cocktail, and incubated at 37 °C in a water-saturated 95% air and 5% CO_2 atmosphere. Cell culture reagents were purchased from Gibco (Thermo Fisher Sci, MA, USA).

Cell ProliferationAssay

Cell proliferation was analyzed using MTS assay [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] as previously described (12). Briefly, Panc-1 and MiaPaCa-2 were seeded into 96 well-plate as 5×10^3 cells/well and then transfected with negative siRNA or CD44 siRNA in doses of 25, 50, 100 and 150 nM (Life Technologies, CA, USA) for 72 h using Hiperfect Transfect Reagent (Qiagen-Hilden, Germany) according to the manufacturer's protocol. Based on the total amount of formazan produced upon MTT reduction, cell proliferation was determined at 490 nm wavelength in an enzyme-linked immunosorbent assay microplate reader (Thermo Multiskan Go).

RNA Isolation and Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

Trizol reagent was used for total RNA isolation in PDAC cells transfected with 50 nM negative siRNA or CD44 siRNA for 72 h. Based on the manufacturer's recommended protocol, cDNA was synthesized from 1 µg RNA with High Capacity cDNA Reverse Transcription Synthesis Kit (Applied Biosystems, MA, USA) under the following conditions: 25°C for 10 min, 37 °C for 120 min and 85°C for 5 min. CD44 and Integrin β1 gene expressions were analyzed with Platinium Taq DNA Polymerase (Invitrogen, CA, USA) and the sequence-specific primers. Briefly, cDNA samples was reacted with PCR reaction mixture containing PCR buffer, 1.5 mM $MgCl_2$, 200 μM deoxynucleotide triphosphates (dNTPs), 1 unit of Platinum Taq polymerase, and 0.2 M of forward and reverse primers for each gene (Integrated DNA Technologies, IDT) under reaction condition as 94 °C 2 min to denature the template and activate the enzyme and followed by 35 cycles of PCR amplification (in each cycle, the samples were incubated at 94 0C for 30 s., 55 0C for 30 s. and 72 0C for 60 s.) with an additional cycle at 72 °C for 5 min. The following sense and anti-sense primers were used: CD44, 5' GTA CAT CCT CAC ATC CAA CAC CTC 3' and 5' TGC TCC ACC TTC TTG ACT CCC 3'; Integrin β1, 5' CCT ACT TCT GCA CGA TGT GAT G 3' and 5' CCT TTG CTA CGG TTG GTT ACA TT 3', respectively. The amplificated PCR products were visualized on a 1.2% agarose gel containing SyberGreen under LED transilluminator. GAPDH was used as an internal control.

Protein Extraction and Western Blot Analysis

Following by 72 h incubation period with 50 nM negative siRNA or CD44 siRNA, Panc-1 and MiaPaCa-2 cells were homogenized in cell lysis buffer (Promega Madison, WI, USA) containing protease/phophatase inhibitor cocktail (Thermo Fisher Sci, MA, USA). In supernatant samples obtained by centrifugation at 13,000 × g and 4 °C for 20 min, protein quantities were obtained using Bradford Protein Assay Kit (BioRad, Hercules, CA, USA). 40 g of total protein from each sample were first loaded into a 4-15% Tris-HCI gel for SDS-PAGE and then transferred to polyvinylidene difluoride (PVDF) membranes. The Collagen IV and fibronectin protein expressions were detected by specific primary antibodies and the respective AP-linked seconder antibodies. Immunoblots were visualized using BCIP/NBT Substrate Detection Reagent (Sigma) in colorimetric imager and quantified with a densitometer using Image J software (NIH). All experiments were independently repeated three times. β-actin antibodies were used as an internal control.

Statistical Analysis

In this study, SPSS Windows Version 20.0 (Armonk, NY, IBM Corp.) was used to determine significant differences between control, negative siRNA and CD44 siRNA groups with Kruskal-Wallis Test. Pairwise comparison of the control group with negative siRNA and CD44 siRNA, respectively, was evaluated with the Mann-Whitney U test. $p < 0.05$ value was evaluated considering statistically significant. The means of ± SEM (Standart Error) were explained differences between three independent experiment.

Results

CD44 suppresses the proliferation of Panc-1 and MiaPaCa-2 cells

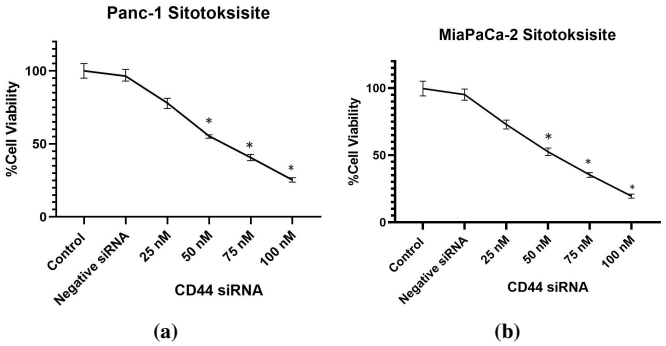

Figure 1. CD44 suppresses the proliferation of Panc-1 and MiaPaCa-2 cells. In order to determine the proliferative effect of CD44 siRNA on Panc-1(a) and MiaPaCa-2(b) cells, cell viability analysis was performed mainly in 3 groups as control, negative siRNA and CD44 siRNA groups. (Three independent experiments). $p<0.05$ * means statistically significant when it was compared to control groups.

The effects of CD44 on cell viability was performed using MTS analysis in PDAC cells transfected with 25, 50, 75, 100 nM CD44 siRNA or negative siRNA for 72 h. Compared to negative conditions, CD44 suppressed the cell viability depending on enhanced siRNA doses in both Panc1 (Fig 1A) and

MiaPaCa-2 (Fig 1B) cells. This inhibition was statistically observed from 50 nM to 100 nM doses of CD44 siRNA compared with negative siRNA. Since 50 nM dose of negative and CD44 siRNAs caused to kill the Panc-1 and MiaPaCa-2 cells as 46.6% and 47.5%, respectively, compared to non-treated conditions in cells, it was chosen for our further experiments.

CD44 siRNA confirms to silence CD44 mRNA expression in Panc-1 and MiaPaCa-2 cells

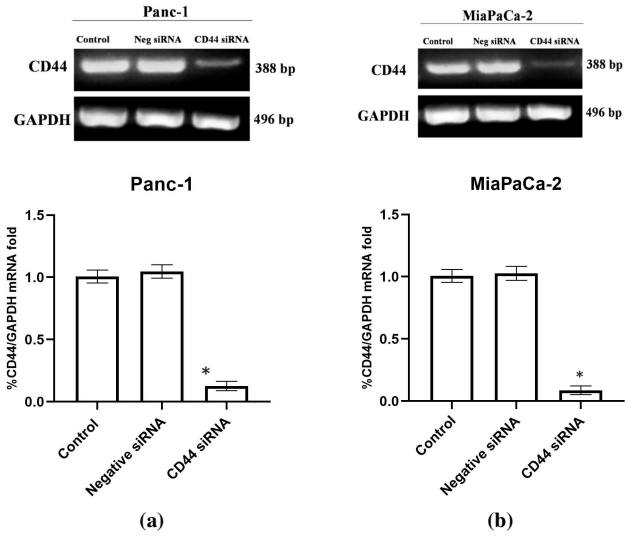

Figure 2. CD44 siRNA confirms to silence CD44 mRNA expressions in Panc-1 and MiaPaCa-2 cells. In the Panc-1(a) and MiaPaCa-2(b) CD44/GAPDH mRNA expression ratios obtained by quantification of both CD44 and GAPDH gene expression bands in Image J (NIH, Maryland, USA) densitometer screening program, and this ratio of control cells was considered to be 1 negative and/or CD44 siRNA as multiples of 1 it was evaluated by giving. $p < 0.05$ * means statistically significant when it was compared to control groups.

To prove CD44 siRNA caused CD44 gene expression silences, PDAC cells were transfected with 50 nM negative siRNA or CD44 siRNA for 72 h. Based on RT-PCR analysis, CD44 siRNA led to inhibition CD44 mRNA expression as 0.87 fold and 0.92 fold lower than negative siRNA treatment in Panc-1 (Fig 2A) and MiaPaCa-2 (Fig 2B) cells, respectively. This prove in silencing of the CD44 gene expression allowed us to use CD44 siRNA for our further experiments related CD44 mediated ECM degradation in PDAC. We also observed that CD44 siRNA had a slightly higher effect in MiaPaCa-2 than Panc-1.

Downregulation of CD44 decreases Integrin-1 mRNA expression in PDAC cells

Integrin β1 was considered as a marker in invasion and migration in many cancers, including pancreatic cancer (13). Due to metastatic importance of ECM degradation, we analyzed if CD44 mediates integrin β1 mRNA expression in PDAC cells. Based on RT-PCR bands, CD44 directly regulated integrin β1 gene and also downregulation of CD44 using specific siRNA inhibited integrin β1 gene expression as 0.65 fold and 0.8 fold

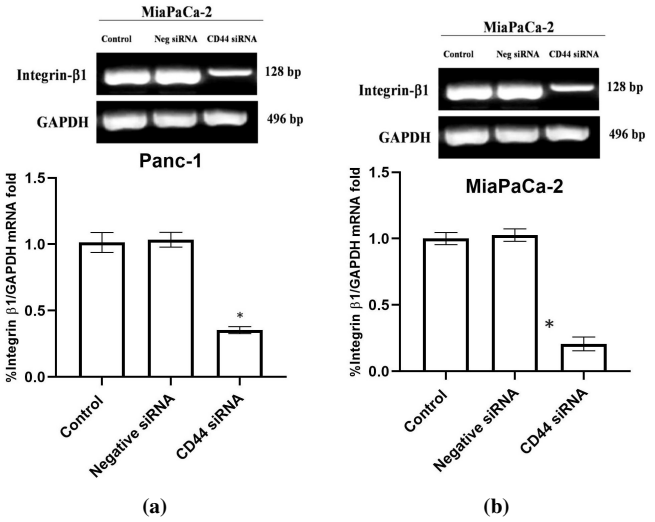

Figure 3. Downregulation of CD44 decreases Integrin-β1 mRNA expression in PDAC cells. In the Panc-1(a) and MiaPaCa-2(b) Integrin β1/GAPDH mRNA expression ratios obtained by quantification of both Integrin β1 and GAPDH gene expression bands in Image J (NIH, Maryland, USA) densitometer screening program, and this ratio of control cells was considered to be 1 negative and/or CD44 siRNA as multiples of 1 it was evaluated by giving. $p<0.05$ * means statistically significant when it was compared to control groups.

in Panc-1 (Fig 3A) and MiaPaCa-2 (Fig 3B), respectively, compared to negative condition. Our findings clearly demonstrated that CD44 targeted gene silencing might serve as an effective therapeutic approach for PDAC via inhibition of metastasis.

CD44 targeting inhibition degrades ECM proteins in PDAC cells

To evaluate the CD44 targeting gene downregulation on ECM protein degradation, we performed to analyze the protein expressions of two major ECM proteins, fibronectin and collagen-IV, in PDAC cells. We observed 0.19 and 0.13 fold lower protein expressions of fibronectin and collagen IV, respectively, in Panc-1 cells treated with CD44 siRNA than negative condition (Fig 4A, C). The same inhibitory effects of CD44 siRNA on both ECM protein expressions were also obtained in MiaPaCa-2 cells (Fig 4B, D), as 0.5 and 0.48 fold, respectively. When the effects of CD44 gene silencing on fibronectin and collagen IV protein expressions were compared in Panc-1 and MiaPaCa-2 cells, we observed two-fold greater loss of expression of both proteins in MiaPaCa-2 cells than Panc-1.

Discussion

In this study, we investigated the potential effect of CD44 gene targeted therapy using specific siRNA on ECM degradation via integrin β1 and two major ECM proteins, fibronectin and collagen IV, in PDAC cells. Our findings suggest that CD44 is closely linked to metastatic and invasive properties of PDAC cells due to integrin β1 gene expression. When treated with CD44 siRNA as a therapeutic approach, PDAC cells expressed

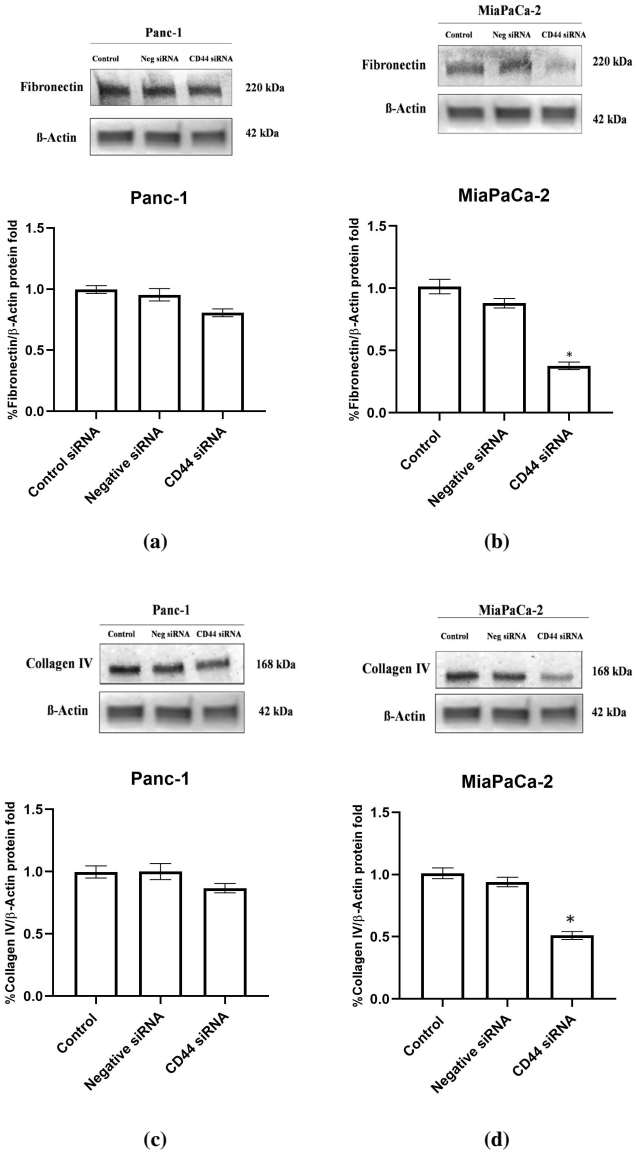

Figure 4. CD44 targeting inhibition degrades ECM proteins in PDAC cells. In the Panc-1(a) and MiaPaCa-2(b) cell lines Fibronectin / β-Actin protein expression ratios obtained by quantification of both Fibronectin and β-Actin protein expression bands in Image J (NIH, Maryland, USA) densitometer screening program and when this ratio of control cells is accepted as 1, negative and/or CD44 siRNA group It was evaluated in multiples of. Same way Collagen IV/ β-Actin evaluated Panc-1(c) and MiaPaCa-2 (d). $p<0.05$ * means statistically significant when it was compared to control groups.

less integrin β1 mRNA expression even in aggressive pancreatic cancer cell line, Panc-1, compared to negative condition. In addition to this, we also found that CD44 regulates the expressions of ECM protein in both PDAC cells. The silencing of CD44 resulted in decreased expression of fibronectin and collagen IV, which is higher in MiaPaCa-2 cells than Panc-1. The aggressive and high metastatic potential cells, Panc-1, had resistance against CD44 targeting therapies.

PDAC mass contains abundant fibronectin compared to normal tissues (14). Fibronectin binds integrin containing β1 and β3 subunit. At least 18 α subunit and 8 β subunit combines different variants of integrin receptor family (15). RGD tripeptide residues (Arg-Gly-Asp) of fibronectin are able to recognize β1 integrin subunits and αvβ3 integrin. This receptor - ligand interaction leads to integrin β1 conformational changes due to talin mediated Src-FAK pathway and cytoskeleton activation of cells (16). Collagen IV is also one of the main components in basement membrane and highly expressed in PDAC (17). In the normal tissue collagen IV regulates mechanic stability, basement membrane attachment the cells and interact with type I collagen and integrins (18). Collagen IV expression is stimulated by pancreatic stellate cells, cancer cells and cancer associated fibroblasts (CAFs) (19). We observed an inhibition of both fibronectin and collagen IV expressions depending on CD44 downregulation in PDAC cells. CD44 targeted therapeutic strategies might be helpful to reduce PDAC progression and also drug resistance.

The finding of cell viability assay confirmed that CD44 is necessary for human PDAC proliferation. Li et al. (2015) demonstrated that pancreatic cancer samples of 67 patients, lymph node metastasis positive samples were shown strong CD44 expression by immunohistochemistry compared to lymph node metastasis negative samples (20). CD44- PDAC patients's was reported to have shorter survival time, 25.3 months, than CD44+ patients, 16.9 months (21). The data obtained in 192 PDAC patients showed that increased CD44 expression was associated with shortened survival period of less than 10 months (22). Based on other study conducted in the biopsy tissue samples of 155 pancreatic cancer patients, CD44+ patients showed positive correlation with cancer recurrence and high mortality ratio (23). Molejon et al. (2015) described CD44+, CD44+/EpCAM and CD44+/CD133+ molecular phenotype cells gained chemo resistance and increased the tumor recurrence possibility (24). Durko et al. (2017) shows that CD44 expression was observed much higher in 23 pancreatic cancer samples than in chronic pancreatitis compared to CD133 and CD24 (25). As a directly evidence for CD44's metastatic edge for PDAC, transfection of viral vector containing CD44v6 region resulted in the conversion of non-metastatic PDAC to metastatic PDAC in has spontaneous metastasis BDX rat model study (26). The binding of CD44 and CD44 isoforms to different ligands regulates cell cytoskeleton morphology, proliferation, growth, migration and cell motility (27). Hyaluronan is able to bind all common to CD44 and isoforms, because extracellular domain of CD44 has N-and O-glycosylation (28). In the PDAC development CAF, PSC and cancer cells produce abundant ECM protein such as collagen, fibronectin, hyaluronan (29). Unlike Tian et al. (2019), the proteomic investigation concerning PDAC stromal proteins revealed that there is no significant difference between under the normal condition and PDAC progression in terms of proteoglycans and glycoproteins expression (30). Different cytokines and chemokines expressed from PSC, tumor associated macrophages, CAF stimulate tumor microenvironmenal activity, migration, invasion and metastatic properties (31). Integrin β1-fibronectin interaction leads also to induce migration through Integrin/Src/FAK signaling (32). It was observed that there was no correlation between parameters such as age, gender, tumor location and fibronectin expression among the patients. However, a direct proportion was found between tumor size and fibronectin protein expression (33). This can be explained by cancer phenotype and /

or the use of various signal transduction mechanisms of different types of cancer driver genes. A study regarding to effect of collagen IV on PDAC in pancreatic cancer tissues, collagen IV is overexpressed in cells close proximity to tumor stroma (34). Additionally, protein expressions of integrin $\alpha1\beta1$ and integrin $\alpha2\beta1$ binding collagen IV were detected in both normal and pancreatic cancer tissues. It was observed that collagen IV released from pancreatic cancer cells was joined the basement membrane structure surrounding pancreatic cancer cells (35). Collagen IV protein expression might not be directly related with CD44 and Integrin $\beta1$ expression. Alternatively, collagen IV binds many types integrin subunits for survival. In our study, we demonstrated similar results that CD44 appears the upstream regulator of Integrin $\beta1$ expression in gene level. It is possible CD44 dependent Integrin $\beta1$ regulation has similar molecular mechanisms different cancer types. Finally, it is essential to elucidate the molecular mechanisms of cell-cell and cell-extracellular matrix interactions of the CD44 protein to develop new therapeutic targets for PDAC. Nowadays, siRNA and miRNA based therapeutic approaches are developing against PDAC (36). Hyaluronan-conjugated, nanoliposomal, or hyaluronidase treatment strategies used in improve siRNA and miRNA-based therapeutics (37). CD44 receptor targeted siRNA delivery is promising target for cancer biology and nanomedicine (38). In the ovarian cancer models serum-stabil, propylene imine modified siRNA shows decreased cancer cell proliferation successfully (39). Other study about CD44 siRNA gene therapy on the nude mice models, CD44 siRNA inhibits hyaluronan adhesion to CD44 and reduced colon cancer cells viability (40).

In conclusion, CD44 expression level data is helpful for early predict of recurrence. CD44 siRNA based therapies have effective role to inhibit ECM degradation in PDAC progression. CD44 is also promising target for against PDAC through inhibiting migration, invasion and metastasis steps.

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