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Divalent cations modulate $\alpha_2\beta_1$ integrin-mediated malignancy in a novel 3D in vitro model of pancreatic cancer

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Abstract

Objectives—We previously showed that divalent cations regulate $\alpha_2\beta_1$ integrin-mediated pancreatic cancer cell interactions with type I collagen in two-dimensions (2D), including cell adhesion, migration, and proliferation. Presently, we examined divalent cation-dependent $\alpha_2\beta_1$ integrin-mediated pancreatic cancer cell adhesion and proliferation on type I collagen in a novel three-dimensional (3D) in vitro model.

Methods—Cell attachment, proliferation, and antibody inhibition assays on type I collagen in both 2D and 3D, as well as microscopy and immunoblotting were used for these studies.

Results—As in 2D, cell attachment on type I collagen in 3D is Mg^{2+} -dependent and inhibited Ca^{2+} . Proliferation in 3D is also Mg^{2+} -dependent, but maximal when Mg^{2+} is present at concentrations that promote maximal cell adhesion and Ca^{2+} is present at concentrations less than Mg^{2+} . Immunoblotting studies demonstrate that the divalent cation-dependent changes in cell-cell adhesion observed on type I collagen in both 2D and 3D are associated with changes in E-cadherin and β -catenin expression. Antibody inhibition assays indicate further that the $\alpha_2\beta_1$ integrin specifically mediates proliferation on type I collagen in 3D under altered divalent cation conditions.

Conclusions—Divalent cation shifts could activate $\alpha_2\beta_1$ integrin-mediated malignancy in the type I collagen-rich 3D tumor microenvironment of pancreatic cancer.

Keywords

cell adhesion; cell proliferation; type I collagen; pancreatic adenocarcinoma

Introduction

Integrins are a family of heterodimeric transmembrane receptor proteins that mediate the binding of cells to the extracellular matrix (ECM) as well as, in some cases, other cells.^{1–6} A common characteristic of all integrins is their absolute requirement for divalent cations, such as Mg^{2+} and Ca^{2+} , to function.^{3,4,6–11} These cations exert their effects by binding to the three to five putative cation-binding domains located on all integrin α subunits¹², and possibly by direct interactions with β subunits.¹³

We have previously demonstrated that $\alpha_2\beta_1$ integrin-mediated pancreatic cancer cell adhesion to type I collagen, an ECM protein shown to be highly up-regulated in pancreatic cancer^{14–18} and to promote the malignant phenotype in vitro and in vivo^{18–25}, is promoted

by Mg^{2+} but not by Ca^{2+} ²², and that the $\alpha_2\beta_1$ integrin from pancreatic cancer cell lysates bound specifically to type I collagen by affinity chromatography in Mg^{2+} , can be eluted with Ca^{2+} .²⁶ And while Mg^{2+} supports and Ca^{2+} inhibits pancreatic cancer cell migration and proliferation on type I collagen, when Mg^{2+} is present at concentrations that promote maximal cell adhesion and Ca^{2+} is present at concentrations less than Mg^{2+} , both cell migration and proliferation can be significantly increased by as much as two-fold.^{27,28} These data are consistent with our previous observations regarding $\alpha_2\beta_1$ integrin-mediated cellular interactions with type I collagen, including the various cell types involved in cutaneous wound repair²⁹⁻³¹, of which strong parallels to the pancreatic cancer paradigm have been described.^{32,33}

During normal cutaneous wound healing in vivo, shifts in the concentrations of extracellular Mg^{2+} and Ca^{2+} occur early in the process, activating the $\alpha_2\beta_1$ integrin-mediated migration of various wound healing cell types on type I collagen, including epithelial keratinocytes.³¹ This shift in extracellular divalent cation concentration is characterized by increased Mg^{2+} and decreased Ca^{2+} . Similar shifts probably occur in pancreatic cancer as well, as pancreas juice contains Mg^{2+} in the range of 137-244 μM and Ca^{2+} in the range of 0.111 – 0.197 μM .³⁴ This represents a more than 1200-fold difference between extracellular Mg^{2+} and Ca^{2+} . As pancreatic ductal epithelial basement membranes are discontinuous or absent in pancreatic cancer^{15,16,35}, pancreas juice could be expected to leak into the tumor microenvironment. Additionally, and similar to cutaneous wound healing³⁶, solid tumors are distinguished by increased Mg^{2+} load, even at the expense of healthy adjacent tissue.^{37,38} Collectively, these data suggest that pathophysiological divalent cation shifts like those found in cutaneous wound repair could also occur in the local pancreatic cancer tumor microenvironment, activating the $\alpha_2\beta_1$ integrin-mediated malignant phenotype on type I collagen.

In the present study, we examined the regulatory role of divalent cations on the adhesion and proliferation of pancreatic cancer cells on type I collagen in a novel 3D in vitro model.²⁶ Our results indicate that cells attach to 3D type I collagen scaffolds maximally when Mg^{2+} is greater than about 1 mM, and that addition of increasing concentrations of Ca^{2+} reduces this Mg^{2+} -dependent adhesion. We also demonstrate that cells proliferate on 3D type I collagen scaffolds through 72 hours in Mg^{2+} alone, but maximally when Mg^{2+} is present at concentrations that promote maximal cell adhesion and Ca^{2+} is present at concentrations less than Mg^{2+} . Immunoblotting studies indicate further that the divalent cation-dependent phenotypic changes in cell-cell adhesion observed in 3D are associated with altered expression levels of E-cadherin and β -catenin. Inhibition of cell proliferation assays on 3D type I collagen scaffolds using anti-integrin function-blocking monoclonal antibodies demonstrate that the $\alpha_2\beta_1$ integrin specifically mediates cell interactions in 3D under these different divalent cation conditions. These results are consistent with our 2D data, indicating a potential role for extracellular divalent cation shifts in the activation of the $\alpha_2\beta_1$ integrin-mediated malignant phenotype in the type I collagen-rich 3D tumor microenvironment of pancreatic cancer.

Materials and methods

Cells

BxPC-3 and Panc-1 cells were from ATCC (Rockville, MD). FG cells are a fast-growing (FG), metastatic variant of the pancreatic adenocarcinoma cell line, Colo-357.³⁹ Cells were cultured in DMEM (high-glucose) supplemented with 10% FBS in a humidified atmosphere containing 5% CO_2 at 37°C.

Three-dimensional type I collagen/glycosaminoglycan scaffolds

FDA-approved and commercially available dermal regeneration templates, composed of type I collagen/glycosaminoglycan (GAG) were prepared as described (Integra Life Sciences, Plainsboro, NJ).⁴⁰⁻⁴³ Briefly, type I collagen is partially solubilized from bovine tendon in 50 mM acetic acid and co-precipitated with the glycosaminoglycan chondroitin-6-sulphate in a refrigerated homogenizer. Chondroitin-6-sulfate proteoglycan has been shown to be upregulated in pancreatic cancer.^{44,45} The co-precipitate is cast into sheets and frozen on the refrigerated shelves of a lyophilizer. The frozen co-precipitate is lyophilized overnight and subject to vacuum dehydration for 24 hours. Dry membranes are rehydrated in 50 mM acetic acid for 24 hours, cross-linked in 0.25% glutaraldehyde for 24 hours, washed extensively in purified water and stored in phosphate-buffered saline. The GAG content of these matrices is $7.8\% \pm 1.5\%$ by weight.

3D cell adhesion assays

3D adhesion assays were conducted as previously described.^{26,46} Briefly, 4 mm punch biopsies (~125 μ g per punch) of collagen/GAG matrices, about 3 mm thick, were washed for 5 min in TBS with moderate agitation and non-specific binding sites were blocked in TBS containing 1 mg/ml BSA for 1 hour at 37°C on a rotating platform at 90 rpm. Pancreatic cancer cell lines, serum-starved 24 hours prior to assay, were harvested at approximately 50-70% confluency from tissue culture flasks, and resuspended in serum- and divalent cation-free DMEM at 5×10^4 cells/ml, supplemented with $MgCl_2$ and $CaCl_2$ at the indicated concentrations. One ml of pancreatic cancer cell suspension was then added to each collagen/GAG matrix inside the well of a 24 well tissue culture plate, and the plates were incubated at 37°C for 1 hour on a rotating platform at 90 rpm. This allows the cells to penetrate and slow down within the 3D matrix, thus increasing the possibility for receptor-ligand interactions. After the incubation period, 3D matrices were vigorously washed with TBS on a rotating platform at 180 rpm for 5 minutes, and attached cells were stained with 1% Neutral Red (a viable cell stain; Sigma-Aldrich, St. Louis, MO) in TBS for 10 minutes at room temperature followed by 0.0008% Crystal Violet in 10% formalin-buffered phosphate (Fisher Scientific, Fair Lawn, NJ) for 1 hour at room temperature. This gives the necessary contrast between non-viable materials (collagen fibers and dead cells) and viable cells, so that they can be visualized and quantified. Samples were photographed using a phase contrast Nikon Eclipse TE300 inverted microscope (Nikon Inc., Melville, NY) equipped with a SPOT Digital Imaging package (Diagnostic Instruments, Inc., Burlingame, CA), and quantified by counting five high powered fields ($\times 100$ magnification) per template. When scoring adherent cells in these 3D scaffolds, we first focus at the top and then progressively deeper within the scaffold, counting all cells within each clear field of focus.

3D proliferation assays

Twenty-four hour, serum-starved pancreatic cancer cells were subjected to the 3D cell adhesion assay described above with $MgCl_2$ at 3.5 mM. After washing in TBS, collagen/GAG matrices with adherent cells were transferred to 96 well non-tissue culture plates that were previously blocked with BSA (1 mg/ml in TBS for 1 hour at 37°C), and cultured under serum-free conditions for three days in the same media described above for adhesion assays supplemented with the indicated concentrations of $MgCl_2$ and $CaCl_2$. After 72 hours, triplicate proliferation determinations were quantified by measuring the absorbance at 450 nm and subtracting the value obtained for each cell line after initial attachment using CellTiter 96 Aqueous One Solution Cell Proliferation AssayTM reagent according to manufacturer's instructions (Promega, Madison, WI). This reagent is composed of a novel tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, innersalt; MTS], and an electron coupling reagent (phenazine ethosulfate; PES). Pancreatic cancer cell proliferation was determined after 72 hours as

described above in the presence or absence of the indicated function-blocking anti-integrin monoclonal antibodies (P1E6, anti- α_2 ; P1D6, anti- α_5 ; P5D2, anti- β_1)⁴⁷⁻⁴⁹ at 25 $\mu\text{g/ml}$ (Millipore Corp., Temecula, CA). Antibody concentrations used are based on previous inhibition of adhesion, migration, and proliferation assays with these pancreatic cancer cell lines as well as fibroblasts, keratinocytes and endothelial cells in both 2D and 3D in vitro assays.^{21,26,27,29,46,50} Antibodies were added 24 hours after initial cell adhesion as previously described.²⁷

Immunoblotting

After 72h on type I collagen in 2D and 3D, BxPC-3 and FG cultures were harvested. Cell extracts were prepared by adding 1 ml lysis buffer (250 mM Tris, pH 7.4, 0.25% Nonidet P-40 and 2 mM EDTA)/10 scaffolds or 3 wells of a 6-well plate/divalent cation condition, gentle pipetting to collect the lysate, centrifugation for 15 minutes at $16,000 \times g$, and transfer of the supernatant to a fresh tube. Total cell protein was then measured using the DC protein assay with BSA as the standard according to manufacturer's instructions (Bio-Rad Laboratories, Inc., Hercules, CA). Five to ten μg total protein was separated for each lysate under each divalent cation condition in 2D and 3D, and transferred to nitrocellulose using standard electrophoretic techniques. Membranes were subsequently exposed to monoclonal or polyclonal antibodies directed against E-cadherin at 1:1000 or a polyclonal antibody directed against β -catenin (Millipore Corp.) at 1:500 overnight at 4°C. After washing in PBS/0.1% Tween, the membranes were subsequently incubated with HRP-conjugated goat anti-mouse or anti-rabbit IgG secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) at 1:10,000 for 1 hour at RT. Peroxidase activity was detected with chemiluminescence (GE Healthcare, Buckinghamshire, England).

Densitometry

Immunoblotting results were quantitatively analyzed with NIH Image J software. The intensities of the bands were assigned integrated density values, which represent the sum of all pixels in the box. All bands were quantified in equal area boxes and compared with β -actin controls to generate a ratio. Relative expression values were generated by comparing these ratios to the maximum value obtained, which was arbitrarily assigned a value of 1.

Statistical analysis

Statistical significance was determined using two-tailed Student *t* tests.

Results

$\alpha_2\beta_1$ -integrin-mediated attachment of pancreatic cancer cells to 3D type I collagen/GAG scaffolds is Mg^{2+} -dependent and inhibited by Ca^{2+}

We have previously demonstrated that the $\alpha_2\beta_1$ integrin specifically mediates pancreatic cancer cell attachment to 3D type I collagen/GAG scaffolds.²⁶ We have also shown in 2D cell adhesion assays on type I collagen, that $\alpha_2\beta_1$ integrin-mediated pancreatic cancer cell attachment is Mg^{2+} -dependent and inhibited by Ca^{2+} .^{22,27,28} In the present study, we examined the divalent cation-dependency of pancreatic cancer cell adhesion on 3D type I collagen in titration experiments. As shown in Figure 1A, maximal cell adhesion occurs at concentrations of Mg^{2+} greater than about 1 mM for BxPC-3, FG, and Panc-1 cells, and Ca^{2+} does not support cell adhesion on 3D type I collagen/GAG scaffolds. Additionally, the attachment of BxPC-3 and FG pancreatic cancer cells to 3D type I collagen substrates in the presence of optimal Mg^{2+} concentrations (3.5 mM) decreased with increasing Ca^{2+} concentration (Figure 1B). With the more undifferentiated cell line, Panc-1, cell attachment was maximal with extracellular Ca^{2+} concentrations between 0.94 and 3.75 mM, after which

increasing Ca^{2+} concentrations decreased adhesion. These data generally indicate that, as in 2D, $\alpha_2\beta_1$ integrin-mediated pancreatic cancer cell attachment to type I collagen in 3D is Mg^{2+} -dependent and inhibited by Ca^{2+} .

Divalent cation shifts promote maximal $\alpha_2\beta_1$ integrin-mediated pancreatic cancer cell proliferation on 3D type I collagen/GAG scaffolds

We next examined the effect of divalent cations on the proliferation of pancreatic cancer cells on 3D type I collagen/GAG scaffolds. As shown in Figure 2, Mg^{2+} alone supports BxPC-3, FG, and Panc-1 cell proliferation after 72 hours. Remarkably, the addition of Ca^{2+} increased cell proliferation in BxPC-3 and FG cells, as long as Ca^{2+} was present at concentrations less than Mg^{2+} (3.5 mM). As the Ca^{2+} concentration exceeded that of Mg^{2+} , cell proliferation declined. In Panc-1 cells, however, increasing Ca^{2+} concentrations through 3.75 mM had no effect on cell proliferation. Only at 7.5 mM Ca^{2+} and above did Panc-1 cell proliferation decrease. Figure 3A-C shows the morphologic differences between BxPC-3, FG, and Panc-1 cell proliferation after 72 hours in the presence of these different divalent cation conditions. It is clear that, in the absence of Ca^{2+} , cells are predominantly single for all three cell lines. As the Ca^{2+} concentration increases, cell-cell interactions increase dramatically in BxPC-3 and FG cells. In agreement with our previous results on these 3D type I collagen scaffolds in normal media,²⁶ however, undifferentiated Panc-1 cells remain single with essentially no cell-cell interactions, regardless of Ca^{2+} concentration.

It is well-established that E-cadherin-mediated cell-cell adhesion is Ca^{2+} -dependent.⁵¹ Consistent with our previous immunofluorescence studies,²⁸ immunoblotting analyses of these three cell lines indicate that BxPC-3 and FG cells express E-cadherin as well as its cytoplasmic anchor protein, β -catenin,⁵² while Panc-1 cells express essentially no E-cadherin and relatively low levels of β -catenin (Figure 4).

Divalent cation shifts regulate E-cadherin and β -catenin expression in BxPC-3 and FG cells after 72h in culture on 3D type I collagen/GAG scaffolds

We have previously shown by immunoblotting analyses with extracts from FG pancreatic cancer cells²² and HaCaT keratinocytes³⁰ cultured on type I collagen in 2D that increasing the concentration of extracellular Ca^{2+} increases E-cadherin expression. We therefore examined immunoblotting and densitometry analyses of E-cadherin and β -catenin expression from BxPC-3 and FG cell lysates after 72 hours on type I collagen in both 2D and 3D, and under various divalent cation conditions. Figure 5 demonstrates that maximal E-cadherin and β -catenin expression in BxPC-3 and FG cells occurs in both 2D and 3D type I collagen cultures at approximately 1.0 mM Ca^{2+} and decreases with decreasing Ca^{2+} .

The $\alpha_2\beta_1$ integrin specifically mediates the divalent cation-dependent effects of pancreatic cancer cells on 3D type I collagen/GAG scaffolds

We have previously demonstrated with function-blocking monoclonal antibodies directed against particular integrins and integrin subunits, that the $\alpha_2\beta_1$ integrin specifically mediates pancreatic cancer cell adhesion on type I collagen in both 2D and 3D, as well as cell migration in media containing normal levels of extracellular Mg^{2+} (1.0 mM) and Ca^{2+} (1.8 mM).^{21,26} Additionally, we demonstrated further that the $\alpha_2\beta_1$ integrin specifically mediates pancreatic cancer cell proliferation on type I collagen in 2D in the presence of altered divalent cations.^{27,28} We therefore examined the specificity of divalent cation-dependent, $\alpha_2\beta_1$ integrin-mediated interactions of pancreatic cancer cells with 3D type I collagen/GAG scaffolds using inhibition of proliferation assays and anti-integrin function-blocking monoclonal antibodies. Figure 6 demonstrates that addition of the anti- α_2 and anti- β_1 integrin subunit antibodies 24 hours after initial cell seeding inhibited BxPC-3 cell proliferation on type I collagen under the indicated divalent cation conditions to levels

similar to those observed on type I collagen in 2D. By contrast, the anti- α_5 antibody had no inhibitory effect. These data indicate that the $\alpha_2\beta_1$ integrin specifically mediates the divalent cation-dependent effects on pancreatic cancer cell proliferation in 3D type I collagen/GAG scaffolds.

Discussion

In the present study, we demonstrate that pancreatic cancer cell adhesion to 3D type I collagen/GAG scaffolds is Mg^{2+} -dependent, and inhibited by Ca^{2+} . We also demonstrate that, while pancreatic cancer cells proliferate on 3D type I collagen scaffolds in the presence of Mg^{2+} alone, the inclusion of both divalent cations with extracellular Mg^{2+} at concentrations that promote maximal cell adhesion (3.5 mM), and Ca^{2+} present at concentrations less than Mg^{2+} , resulted in maximal cell proliferation. These divalent cation-dependent proliferation data are also associated with Ca^{2+} -dependent phenotypic changes in E-cadherin-mediated cell-cell adhesion as well as β -catenin expression. Antibody inhibition studies demonstrate further that the $\alpha_2\beta_1$ integrin specifically mediates proliferation of pancreatic cancer cells on 3D type I collagen/GAG scaffolds under various divalent cation conditions. Together, these results indicate that changes in the concentrations of extracellular Mg^{2+} and Ca^{2+} regulate the competitive interplay between $\alpha_2\beta_1$ integrin-mediated interactions with type I collagen and E-cadherin-mediated cell-cell adhesion in 3D, and suggest that divalent cation shifts could potentially influence the malignant phenotype of pancreatic cancer cells in the type I collagen-rich 3D local tumor microenvironment in vivo.^{14-17,53,54}

Under normal physiological conditions, the extracellular levels of total magnesium and calcium are about 1.0 and 2.5 mM, respectively.²⁹⁻³¹ We have previously shown in porcine, and rat partial- and full-thickness models of cutaneous wound repair, however, that the local concentrations of extracellular magnesium and calcium shift early in the wound healing response, with decreased calcium and increased magnesium.²⁹⁻³¹ This pathophysiological shift activates the $\alpha_2\beta_1$ integrin-mediated migratory phenotype of keratinocytes, fibroblasts, endothelial cells, and macrophages on type I collagen. Our recent results indicate that, similar to the wound healing cell types mentioned above, pancreatic cancer cells respond similarly to such divalent cation shifts, including increased $\alpha_2\beta_1$ integrin-mediated adhesion, migration, and proliferation on type I collagen in 2D assays.^{22,27,28} As previously shown with HaCaT keratinocytes³⁰, changes in the concentration of extracellular Ca^{2+} directly correlates with E-cadherin expression. Our present results extend these findings to pancreatic cancer cells on type I collagen in both 2D to 3D adhesion and proliferation assays, and include a previously unappreciated and novel observation that divalent cations regulate β -catenin expression as well. Furthermore, these data suggest that in the 3D tumor microenvironment, such divalent cation shifts could play a significant role in the progression of the malignant phenotype by shifting the adhesive properties of the cancer cells themselves from E-cadherin-mediated cell-cell interactions to integrin-mediated cell-ECM interactions.

It is not obvious whether such pathophysiological divalent cation shifts occur in pancreatic cancer. As mentioned earlier, pancreas juice contains Mg^{2+} at a 1200-fold excess relative to Ca^{2+} .³⁴ Additionally, solid tumors are characterized by increased Mg^{2+} load, even at the expense of healthy adjacent tissue.^{37,38} While it is not yet clear to what extent the extracellular divalent cation concentrations are altered in the tumor microenvironment in pancreatic cancer in vivo, these data collectively suggest that changes in the extracellular concentrations of Mg^{2+} and Ca^{2+} similar to that seen during cutaneous wound repair probably occur, at least to some extent. Our present data indicate that these shifts would be capable of activating the $\alpha_2\beta_1$ integrin-mediated malignant phenotype in the type I collagen-rich 3D tumor microenvironment.

Most of the previous in vivo studies of the effects of divalent cations are related to cutaneous wound repair. Recently however, studies of the influence of extracellular divalent cations on colon cancer cell adhesion in a murine transplantable tumor model demonstrated that Mg^{2+} and Mn^{2+} stimulated tumor formation to 96% and 92%, respectively, but Ca^{2+} reduced tumor formation to 56% with no apparent affect on serum Ca^{2+} .^{55,56} Together with our 3D results, these data collectively suggest that during pancreatic cancer progression, a resulting shift in divalent cation concentrations in the local 3D tumor microenvironment, derived from pancreas juice or other physiological factors, and sharing many similarities to the wound healing microenvironment^{32,33}, could activate the $\alpha_2\beta_1$ integrin-mediated malignant phenotype on type I collagen.

A common problem encountered during surgical resection of pancreatic cancer tumors is a high incidence of postoperative liver metastasis, local recurrence, and peritoneal tumor dissemination (38% to 86%, depending on the diagnostic methodology).^{57,58} Our present data suggest that, as with colon cancer⁵⁵, irrigation of the peritoneum with low concentrations of $CaCl_2$ during pancreatic cancer tumor resection may potentially and significantly reduce $\alpha_2\beta_1$ integrin-mediated tumor cell dissemination. Future studies will examine this hypothesis in our fluorescent orthotopic murine models of pancreatic cancer.⁵⁹⁻⁶²

In conclusion, we demonstrate that even subtle changes in the concentrations of extracellular Mg^{2+} and Ca^{2+} can dramatically shift the competitive interplay between $\alpha_2\beta_1$ integrin-mediated pancreatic cancer cell interactions with type I collagen and E-cadherin-mediated cell-cell interactions in 3D. It may be important to consider these pathophysiological divalent cation conditions when designing therapeutics targeting the $\alpha_2\beta_1$ integrin-mediated malignant phenotype in pancreatic cancer.

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Figure 1. Divalent cations regulate $\alpha_2\beta_1$ integrin-mediated pancreatic cancer cell adhesion on 3D type I collagen/GAG scaffolds

(A) Pancreatic cancer cells were added to each well of 24-well plates containing 4 mm punch biopsies of 3D type I collagen/GAG scaffolds in the presence of increasing concentrations of Mg^{2+} or Ca^{2+} in cation- and serum-free DMEM supplemented with 1 mg/ml BSA for 1 hour at 37°C as described in Materials and Methods. Attached cells were fixed, stained, and quantitated by counting five high-power fields per scaffold. Data are expressed as % of maximum, and represent the mean $\pm$ SEM from three independent experiments conducted in duplicate. ■ – Mg^{2+} (mM); ▲ – Ca^{2+} (mM). For BxPC-3 cells, 100% = 339 cell/high-power field; for FG cells, 100% = 190 cells/high-power field; for Panc-1 cells, 100% = 141.6 cells/high-power field. (B) Adhesion assays on 3D type I collagen/GAG scaffolds were conducted as described in (A) above in the presence of 3.5 mM Mg^{2+} with a titration of Ca^{2+} at the indicated concentrations. Data are expressed as % of maximum, and represent the mean $\pm$ SEM from five independent experiments conducted in duplicate. For BxPC-3 cells, 100% = 216 cells/high-power field; for FG cells, 100% = 141 cells/high-power field; for Panc-1 cells, 100% = 166.1 cells/high-power field.

Figures 2. Divalent cations regulate pancreatic cancer cell proliferation on 3D type I collagen/GAG scaffolds

Pancreatic cancer cells were subjected to the 1 hour 3D cell adhesion assay as described in Materials and Methods in the presence of 3.5 mM MgCl₂. After washing in TBS, collagen-GAG matrices with adherent cells were transferred to 96 well non-tissue culture plates that were previously blocked with BSA, and cultured under serum-free conditions for 72 hours in the presence of the indicated concentrations of MgCl₂ and CaCl₂. Duplicate proliferation determinations were quantified by measuring the absorbance at 450 nm after 72 hours and subtracting the value obtained for each cell line after initial attachment using CellTiter 96 Aqueous One Solution Cell Proliferation Assay™ reagent according to manufacturer's instructions (Promega, Madison, WI). Data are expressed as % of maximum, and represent the mean ± SEM from at least 5 independent experiments conducted in duplicate.

Figure 3. Divalent cation shifts alter pancreatic cancer cell morphology on 3D type I collagen/GAG scaffolds

Light photomicrographs of BxPC-3 (A), FG (B), and Panc-1 (C) cells shown are representative examples of proliferation assays conducted on 3D type I collagen/GAG scaffolds under the indicated divalent cation conditions after 72 hours. Photomicrographs were obtained using an Olympus BX-60 microscope equipped with a Spot Digital Imaging package (Diagnostic Instruments, Inc., Burlington, CA). Bar, 50 μm.

Figure 4. Expression of E-cadherin and β -catenin in FG, BxPC-3, and Panc-1 cells

FG, BxPC-3 and Panc-1 cells were cultured on tissue culture plastic in serum-containing media. Cell lysates were subsequently prepared and analyzed for E-cadherin and β -catenin expression by electrophoresis under reducing conditions and immunoblotting as described in Materials and Methods. Membranes were also probed for β -actin to control for protein loading. Representative examples from two separate experiments with essentially identical results are shown.

Figure 5. Divalent cation shifts regulate E-cadherin and β -catenin expression in BxPC-3 and FG cells after 72h in culture on type I collagen in both 2D and 3D cultures

BxPC-3 and FG cells were cultured on type I collagen in both 2D and 3D for 72 hours under the indicated divalent cation conditions as described in Materials and Methods. Cell lysates were subsequently prepared and analyzed for E-cadherin and β -catenin expression by immunoblotting and densitometry. Membranes were also probed for β -actin to control for protein loading and for subsequent densitometric analyses. Relative expression data represent the mean from 2 experiments.

A

Figure 6. The $\alpha_2\beta_1$ integrin specifically mediates pancreatic cancer cell proliferation on 3D type I collagen/GAG scaffolds

(A) Inhibition of cell proliferation assays conducted as described in Materials and Methods demonstrate that addition of the anti- α_2 and anti- β_1 integrin subunit antibodies (25 μ g/ml) 24 hours after initial cell adhesion inhibited BxPC-3 cell proliferation on type I collagen under the indicated divalent cation conditions. By contrast, the anti- α_5 antibody had no inhibitory effect. The results, expressed as % Control (in the absence of antibody), represent the mean absorbance at 450 nm minus the value obtained at initial seeding $\pm$ SEM from four independent experiments conducted in triplicate. Two-tailed Student's t-tests were used to identify significant differences (* $p < 0.05$) compared to control. (B) Light photomicrographs of BxPC-3 cells shown are representative examples of inhibition of proliferation assays conducted on 3D type I collagen/GAG scaffolds under the indicated divalent cation conditions after 72 hours. Photomicrographs were obtained using an Olympus BX-60 microscope equipped with a Spot Digital Imaging package (Diagnostic Instruments, Inc., Burlington, CA).