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Adhesion and protease activity in cell lines from human salivary gland tumors are regulated by the laminin-derived peptide AG73, syndecan-1 and β1 integrin ☆

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Abstract

We studied the induction of protease activity by the laminin α1-derived peptide AG73 in cells from adenoid cystic carcinoma (CAC2) and myoepithelioma (M1), respectively a malignant and a benign salivary gland tumors. Laminin α1 chain and MMP9 were immunolocalized in adenoid cystic carcinoma and myoepithelioma *in vivo* and *in vitro*. Cells grown inside AG73-enriched laminin-111 exhibited large spaces in the extracellular matrix, suggestive of remodeling. The broad spectrum MMP inhibitor GM6001 decreased spaces induced by AG73 in CAC2 and M1 cells. This result strongly suggests that AG73-mediated matrix remodeling involves matrix metalloproteinases. CAC2 and M1 cells cultured on AG73 showed a dose-dependent increase of MMP9 secretion, as detected by zymography. Furthermore, siRNA silencing of MMP9 decreased remodeling in 3D cultures. We searched for AG73 receptors regulating MMP9 activity in our cell lines. CAC2 and M1 cells grown on AG73 exhibited colocalization of syndecan-1 and β1 integrin. siRNA knockdown of syndecan-1 expression in these cells resulted in decreased adhesion to AG73 and reduced protease and remodeling activity. We investigated syndecan-1 co-receptors in both cell lines. Silencing β1 integrin inhibited adhesion to AG73, matrix remodeling and protease activity. Double-knockdown experiments were carried out to further explore syndecan-1 and β1 integrin cooperation. CAC2 cells transfected with both syndecan-1 and β1 integrin siRNA oligos showed significant decrease in adhesion to AG73. Simultaneous silencing of receptors also induced a decrease in protease activity. Our results suggest that syndecan-1 and β1 integrin signaling downstream of AG73 regulate adhesion and MMP production by CAC2 and M1 cells.

Keywords: Salivary gland tumors; Adenoid cystic carcinoma; Myoepithelioma; Extracellular matrix; Laminin; Matrix metalloproteinases; Syndecan; Integrins

1. Introduction

Adenoid cystic carcinoma is a frequently occurring malignant salivary gland neoplasm with recurrence and metastasis (Seifert and Sobin, 1992). This tumor exhibits solid, tubular and pseudocystic subtypes (Dardick, 1996; Seifert and Sobin, 1992). A prominent feature of adenoid cystic carcinoma is the perineural spreading, probably caused by the affinity of the neoplasm for basement membrane rich tissues, such as nerves and blood vessels (Dardick, 1996; Seifert and Sobin, 1992).

Myoepithelioma is a rare benign tumor of myoepithelial cell differentiation (Seifert and Sobin, 1992). Growth patterns may be solid, myxoid and reticular, with two main cytological subtypes: spindle-shaped and plasmacytoid (Dardick, 1995; Sciubba and Brannon, 1982; Takai et al., 1995, 1994). Myoepithelioma and adenoid cystic carcinoma share the same origin, cells from the intercalated duct of salivary gland (Batsakis, 1980).

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Both adenoid cystic carcinoma and myoepithelioma express prominent extracellular matrix (Cheng et al., 1995, 1992). It has been suggested that this matrix plays an important role as regulatory factor of phenotypic differences among salivary gland neoplasms (Capuano and Jaeger, 2004; de Oliveira et al., 2001; Franca et al., 2000, 2001; Freitas and Jaeger, 2002; Freitas et al., 2004, 2007; Jaeger et al., 1997a,b; Morais Freitas et al., 2007). We have already demonstrated that laminin regulates the phenotype of an adenoid cystic carcinoma cell line (CAC2) and myoepithelioma cell line (M1) (Capuano and Jaeger, 2004; Freitas and Jaeger, 2002; Freitas et al., 2004, 2007; Morais Freitas et al., 2007). Growth of CAC2 cells within laminin-111 created duct-like and pseudocystic structures, similar to that occur in the neoplasm *in vivo* (Freitas and Jaeger, 2002; Freitas et al., 2004). We have also reproduced myoepithelioma architecture by growing a cell line (M1) derived from this tumor inside Matrigel (de Oliveira et al., 2001).

We are currently studying the effect of particular domains of laminin on CAC2 and M1 cells. We have started this approach using the laminin-derived peptide SIKVAV (Freitas and Jaeger, 2002; Freitas et al., 2004, 2007). This peptide regulates morphology and protease activity of CAC2 cells (Freitas and Jaeger, 2002; Freitas et al., 2004, 2007). Among the laminin-derived bioactive peptides the RKRLQVQLSIRT sequence (designated as AG73 and located at the LG4 globular domain of α1 chain) is the most effective in many biological assays (Nomizu et al., 1995; Suzuki et al., 2003a). AG73 has been tested both *in vitro* and *in vivo* in different cells and systems (Engbring et al., 2002, in press; Hoffman et al., 2001, 1998). It promotes attachment of numerous cell types (Nomizu et al., 1995, 1998), induces salivary acinar cell differentiation (Hoffman et al., 1998), inhibits branching morphogenesis of embryonic salivary glands (Kadoya et al., 2003, 1998; Kadoya and Yamashina, 2005), stimulates neurite outgrowth (Richard et al., 1996), stimulates matrix metalloproteinase secretion by PC12 cells (Weeks et al., 1998).

AG73 has a striking relevance in tumor biology (Engbring et al., 2002, in press; Kim et al., 1998; Mochizuki et al., 2007; Song et al., 1997; Suzuki et al., 2003b, 2005). It caused liver metastasis and increased lung colonization of B16F10 melanoma cells (Kim et al., 1998; Song et al., 1997). Ovarian cancer cells increased metastasis in the presence of AG73 (Yoshida et al., 2001). This peptide is also related to angiogenesis in different systems, such as aortic ring sprouting, endothelial tube formation, and chick chorioallantoic membrane assays (Mochizuki et al., 2007). This evidence strongly suggests that AG73 may have different roles in promoting tumor growth and metastasis. Because of its potential AG73 has been proposed as therapeutic material for tissue regeneration and engineering (Ikemoto et al., 2006; Mochizuki et al., 2003). Despite its biological significance, the mechanisms regulating the bioactivity of AG73 are not fully understood.

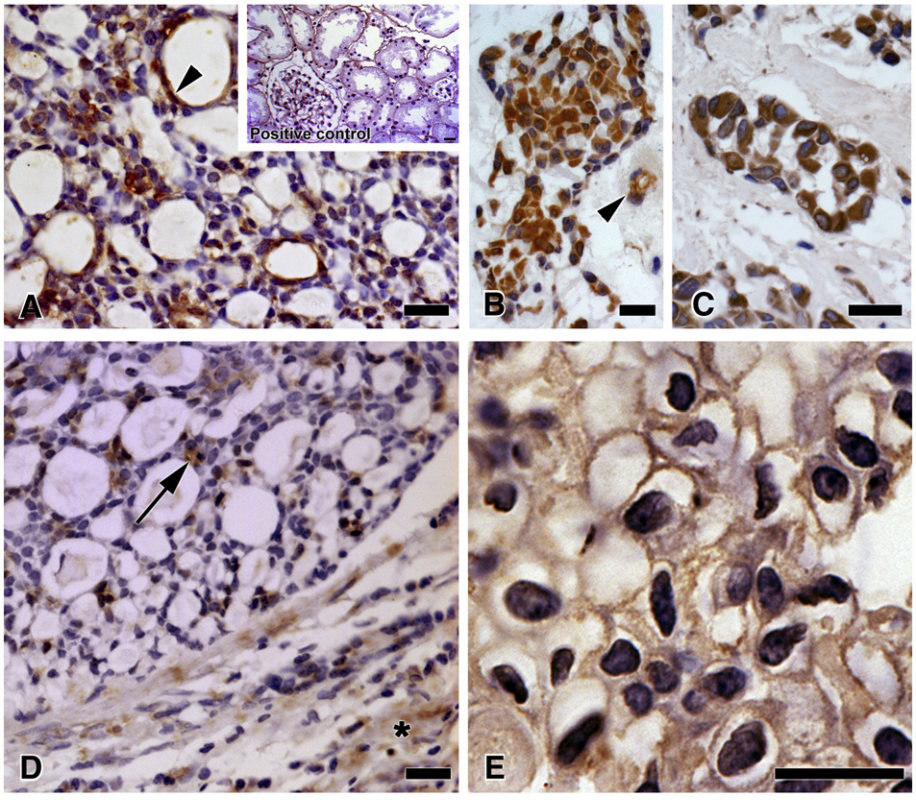

Fig. 1. Laminin α1 and MMP9 are detected in adenoid cystic carcinoma and myoepithelioma. In adenoid cystic carcinoma this protein is observed either as a diffuse pattern or a basement membrane-like linear structure (A, arrowhead). In myoepithelioma laminin α1 chain shows cytoplasmic localization (B, C). Laminin is also found staining blood vessels (B, arrowhead). Human kidney positive control exhibits laminin α1 chain in proximal tubules (A, inset). MMP9 is observed in adenoid cystic carcinoma (D) and myoepithelioma (E). In adenoid cystic carcinoma this enzyme is present in the cribriform subtype (D, arrow) and in stromal regions (D, asterisk). Myoepithelioma expresses MMP9 at cell edges (E). Bars: 20 μm.

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In this paper we studied the regulatory mechanisms underlying protease activity induced by AG73 in CAC2 and M1 cells. Our results showed that AG73 regulate morphology, adhesion and protease activity in these cell lines. The signal generated by AG73 is probably transduced by syndecan-1 and β1 integrin.

2. Results

2.1. Laminin α1 and MMP9 are present in adenoid cystic carcinoma and myoepithelioma in vivo and in vitro

Laminin α1 chain (antibody HK-175) was detected in both adenoid cystic carcinoma and myoepithelioma *in vivo* (Fig. 1A–C). Labeling specificity was confirmed by human kidney staining, showing laminin α1 chain in proximal tubules (Fig. 1A, inset). In adenoid cystic carcinoma this protein was observed either as a diffuse pattern or a basement membrane-like linear structure (Fig. 1A). In myoepithelioma, laminin α1 chain showed a diffuse cytoplasmic localization in plasmacytoid cells (Fig. 1B, C). Laminin α1 chain was also found in blood vessels (Fig. 1B, arrowhead). Cytoplasmic localization of laminin α1 in pathological situations has already been reported (Christie et al., 1994). It probably represents laminin synthesis and accumulation, to be released afterwards in the extracellular matrix. It is known that myoepithelial tumors are intriguing low-grade neoplasms that exhibit the property of accumulating an abundant extracellular matrix (Sternlicht et al., 1996).

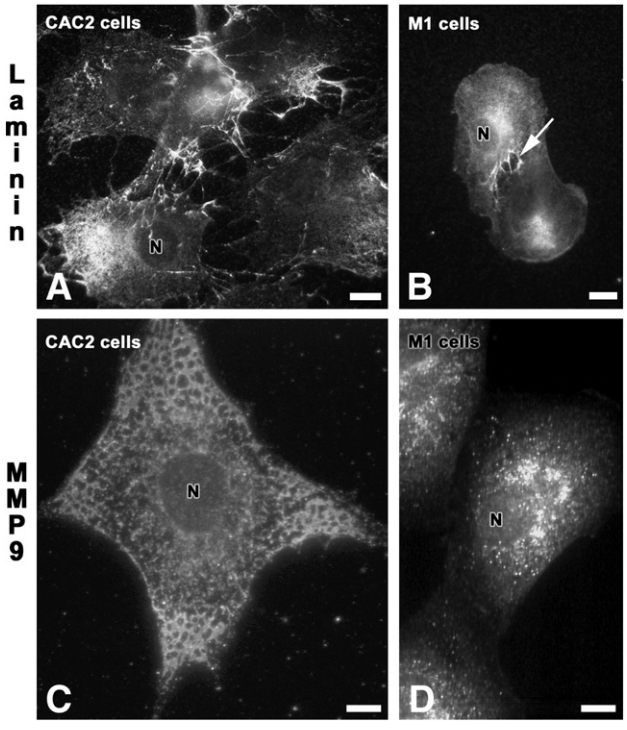

Fig. 2. CAC2 and M1 cells express laminin α1 and MMP9. Laminin α1 forms a linear network in both cell lines (A, B, arrow). Laminin staining represents protein distribution on the cell membrane, since samples were not permeabilized. MMP9 is observed in the cytoplasm as a delicate network in CAC2 cells (C), and as dots in M1 cells (D). N: Nuclei. Bars: 10 μm.

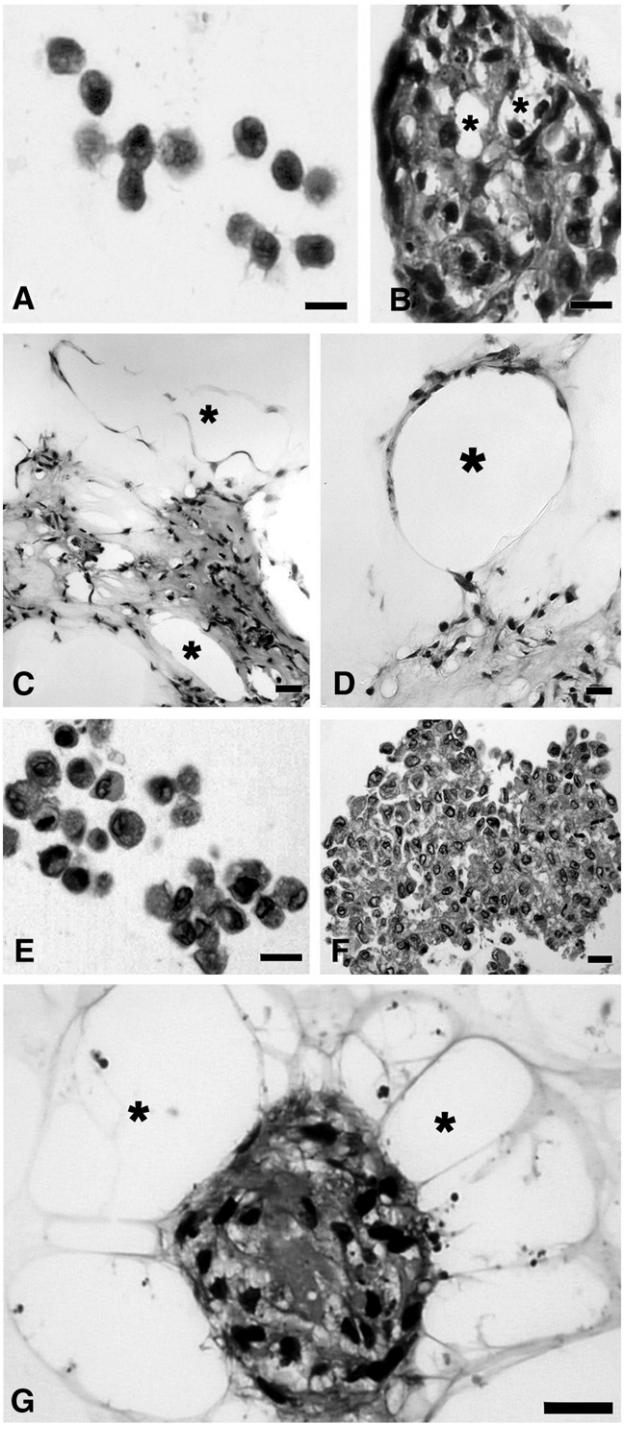

Fig. 3. AG73 increases remodeling in CAC2 (A–D) and M1 (E–G) cells grown in 3D matrices. Control CAC2 cells, grown inside agarose, are round and non-cohesive (A). CAC2 cells grown inside laminin-111 gel form aggregates of polyhedral cells delineating pseudocystic spaces (B, asterisks). CAC2 cells grown inside AG73-enriched laminin-111 exhibit large spaces underlined by flat cells (asterisks in C and D). M1 cells are round in agarose (E), and plasmacytoid when grown inside laminin-111 gel (F). Three-dimensional culture of M1 cells inside AG73-enriched laminin-111 show large spaces in the matrix (asterisks in G). Bars: 20 μm.

The antibody HK-175 (kindly provided by Dr Hynda Kleinman, NIDCR, NIH, USA) was raised against the peptide CSRARKQAASIKVAVSADR from laminin α1 chain. To

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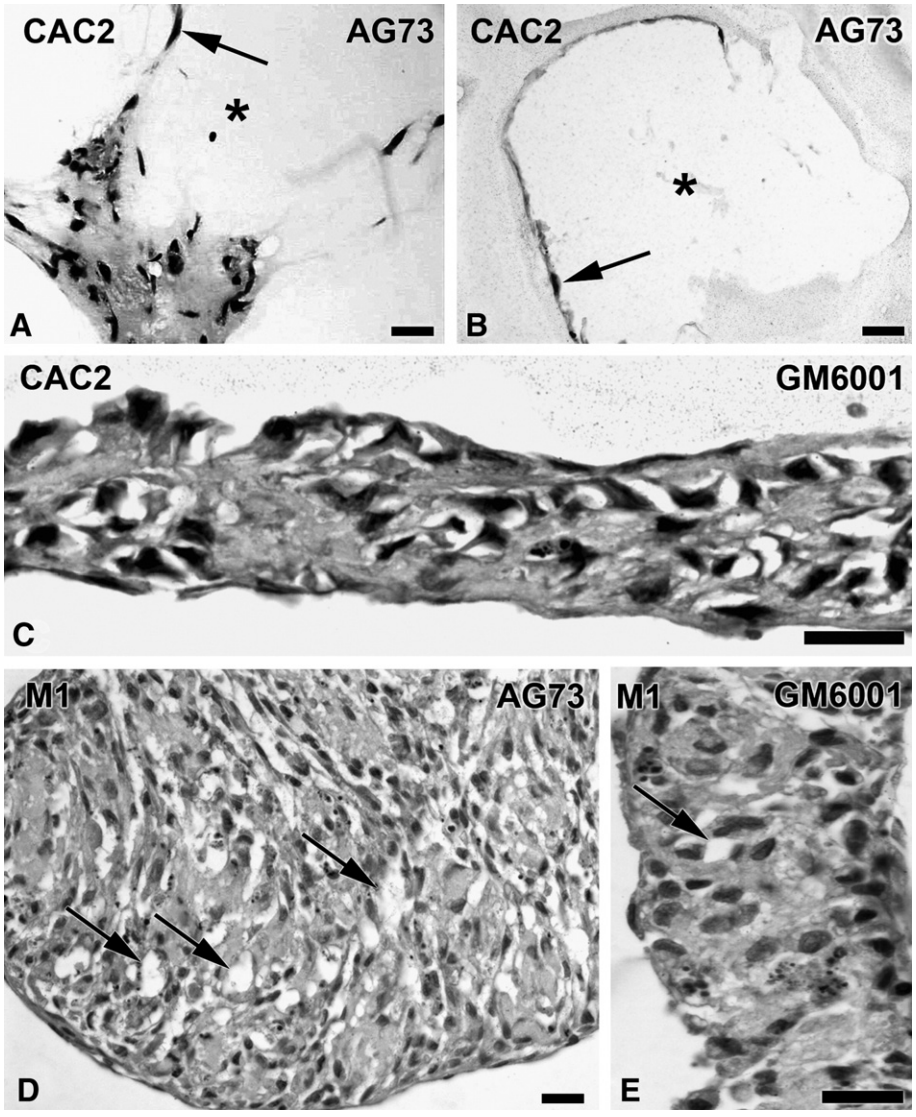

Fig. 4. Protease activity is essential for matrix remodeling regulated by AG73 in both CAC2 and M1 cells. Controls and GM6001-treated cells were cultured within AG73-enriched laminin-111. Control CAC2 cells form large spaces in the extracellular matrix (asterisks in A and B). The spaces are underlined by flat cells (A and B, arrows). MMP inhibitor GM6001 dramatically decreases the effect of AG73 in CAC2 cells (C). GM6001 also affects the effect of AG73 in M1 cells. Control cells exhibit spaces throughout the three-dimensional matrix (D, arrows), while M1 cells treated with GM6001 show less spaces with smaller diameters (E, arrow). Bars: 20 μm.

address the specificity of α1 laminin staining two other antibodies were used: a rabbit polyclonal antibody against human laminin α1 chain (H-300, Santa Cruz) (Faisal Khan et al., 2002) and a goat polyclonal antibody against mouse laminin α1 chain (M-20, Santa Cruz). We obtained similar results observed with the antibody HK-175.

MMP9 was detected in adenoid cystic carcinoma and myoepithelioma (Fig. 1D, E). This enzyme was mostly located in the cytoplasm of adenoid cystic carcinoma cells (Fig. 1D). In myoepithelioma, MMP9 was found at the cell edges (Fig. 1E). Negative controls showed no staining in all samples observed (not illustrated).

Cell lines derived from adenoid cystic carcinoma (CAC2) and from myoepithelioma (M1) expressed laminin α1 chain (Fig. 2A, B) and MMP9 (Fig. 2C, D). Laminin α1 (antibody HK-175) formed a linear network connecting adjacent cells (Fig. 2A, B). This protein was also found as dots distributed throughout the cell membrane. Laminin staining represents protein distribution on the cell membrane, since samples were not permeabilized. CAC2 cells exhibited MMP9 as a prominent network in the cytoplasm (Fig. 2C). M1 cells showed this enzyme as a punctate staining (Fig. 2D).

2.2. AG73 increases remodeling in 3D matrices

We examined by light microscopy the phenotypic alterations induced by either laminin-111 gel or AG73-enriched laminin-111 in our cell lines. CAC2 cells grown inside agarose (controls) showed a round and non-cohesive morphology (Fig. 3A). Cells cultured within laminin-111 delineated small pseudocystic spaces (Fig. 3B). Cells grown inside AG73-enriched laminin-111 presented conspicuous extracellular spaces, suggesting different

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degrees of extracellular matrix remodeling (Fig. 3C, D). In many areas AG73 peptide induced CAC2 cells to form large spaces underlined by flat cells (Fig. 3C, D). Control M1 cells, grown inside agarose, were round and loose (Fig. 3E) while cells cultured within laminin-111 gel presented a plasmacytoid morphology (Fig. 3F), resembling the tumor *in vivo*. M1 cells grown inside AG73-enriched laminin-111 presented large spaces (Fig. 3G), suggestive of matrix remodeling.

2.3. AG73-mediated remodeling in 3D matrices depends on MMPs

The large spaces observed in both cell lines treated by AG73 could represent matrix remodeling. We addressed whether these spaces would depend on protease activity. CAC2 and M1 cells were pretreated with the broad spectrum MMP inhibitor GM6001, followed by culture in AG73-enriched laminin-111. Control cells were grown inside AG73-enriched laminin-111. GM6001 clearly diminished AG73-mediated matrix remodeling in both cell lines (Fig. 4C, E), compared to controls (Fig. 4A, B, D).

We measured the diameter of spaces observed in CAC2 and M1 cells grown inside AG73-enriched laminin-111, compared to cells pretreated with GM6001 followed by culture in AG73-enriched laminin-111.

CAC2 cells grown in AG73-enriched laminin-111 exhibited spaces with mean diameter of 115.8±7.553 μm (n=20). Cells pretreated with GM6001 showed spaces with a mean diameter of 26.56±1.439 μm (n=20). Measurements were taken with the Axiovision software (Carl Zeiss). Differences were statistically significant (p<0.0001).

GM6001 also reduced AG73-mediated matrix remodeling in M1 cells. Cells grown in AG73-enriched laminin-111 presented spaces with mean diameter of 53.62±4.426 μm (n=20). Cells pretreated with GM6001 showed spaces with a mean diameter of 18.19±1.421 μm (n=20). Differences were statistically significant (p<0.0001).

These results indicate that protease activity is essential for AG73-regulated matrix remodeling in both CAC2 and M1 cells. We then decided to further investigate AG73-mediated MMP secretion in these cell lines.

2.4. AG73 induces MMPs in CAC2 and M1 cells

Zymography of the conditioned medium of CAC2 and M1 cells grown on different concentrations of AG73 showed gelatinolytic bands corresponding to the molecular weights of MMP2 and MMP9 (Fig. 5A). MMP2 and MMP9 positive controls were analyzed on the same gel to confirm the result.

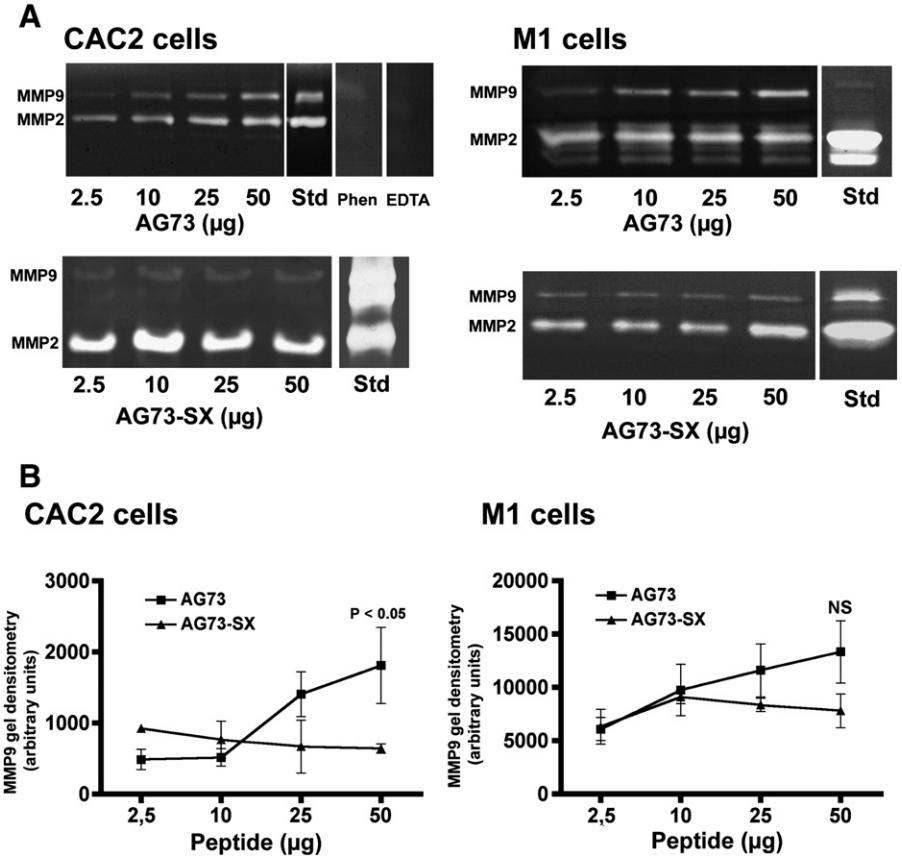

Fig. 5. AG73 increases MMP9 secretion. The conditioned media of CAC2 and M1 cells cultured on AG73 or a scrambled (AG73-SX) control peptide (2.5, 10, 25, and 50 μg/well) were analyzed by zymography (A). MMP2 and MMP9 are detected by the zymograms (A). Positive controls for MMPs (Std) are included in all zymograms (A). Treatments with either 1,10-phenantroline or EDTA (negative controls) demonstrate that the bands are MMPs (A, upper left panel). Gel densitometry of the zymograms shows the dose-dependent increase of MMP9 in CAC2 cells (B. left). M1 cells show a trend (not statistically significant) of MMP9 increase induced by AG73 (B. right). Results represent a mean±SEM of six experiments.

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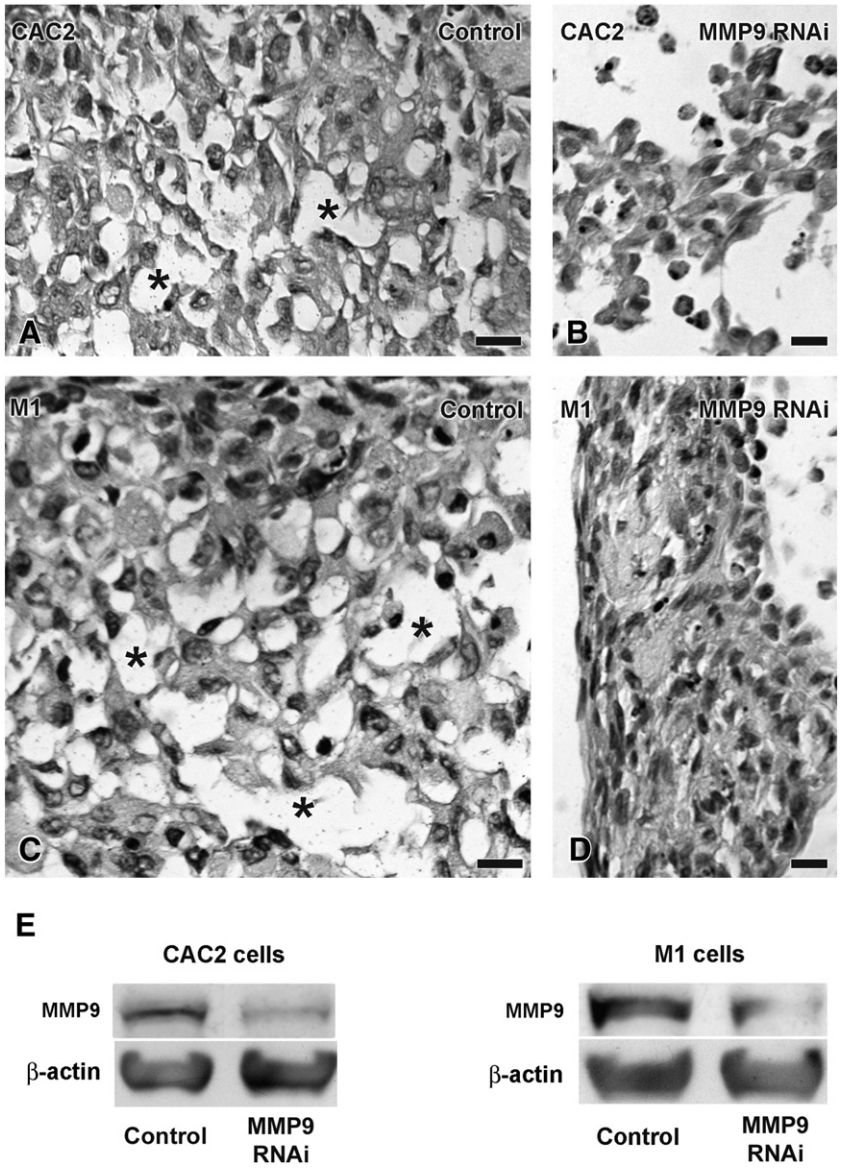

Fig. 6. Silencing of MMP9 inhibited remodeling in CAC2 and M1 cells grown in 3D matrices. Controls and siRNA-transfected cells were cultured within AG73-enriched laminin-111. Control CAC2 cells exhibit areas of matrix remodeling (A, asterisks). Cells with MMP9 silenced are spindle-shaped or round (B). Control M1 cells show spaces in the matrix, suggestive of remodeling (C, asterisks). Cells with receptors knocked down by siRNA exhibit a clear decrease in these spaces (D). Immunoblot (E) confirms the efficiency of siRNA transfection. The control is transfected with the non-silencing siRNA. Bars: 20 μm.

The zymogram resolved only one MMP9 band but both the latent and the active form of MMP2 were observed. Cells grown on different concentrations of AG73-SX (scrambled peptide control) also produced MMP2 and MMP9 (Fig. 5A). To determine whether these bands were MMPs zymograms of conditioned media from AG73-treated CAC2 cells were incubated in the presence of the calcium chelator EDTA and the heavy metal chelator 1,10-phenanthroline. Both of these treatments resulted in the loss of gelatinase activity demonstrating that the gelatinolytic bands were MMPs (Fig. 5A). Gel densitometry (Image J software) of gelatinolytic bands showed that AG73 induced in CAC2 cells a dose-dependent increase of MMP9 compared to the scrambled peptide control (Fig. 5B). M1 cells treated by AG73 showed a trend (not statistically significant) of MMP9 increase (Fig. 5B). The volume of conditioned media loaded on the zymogram gel was normalized to the amount of protein in the CAC2 and M1 cell lysates. Western blot analysis of β-actin in the cell lysates confirmed equal amounts of lysate were used to estimate the volume of conditioned media (data not shown). Zymographic experiments were carried out at least six times with consistent results.

2.5. MMP9 silencing by siRNA decreases AG73-induced matrix remodeling in 3D cultures

Results obtained with the broad spectrum MMP inhibitor GM6001 suggested that different MMPs could be involved in AG73-regulated matrix remodeling in both CAC2 and M1 cells. On the other hand, zymography showed that AG73 enhances MMP9 secretion. We then decided to use siRNA to reduce

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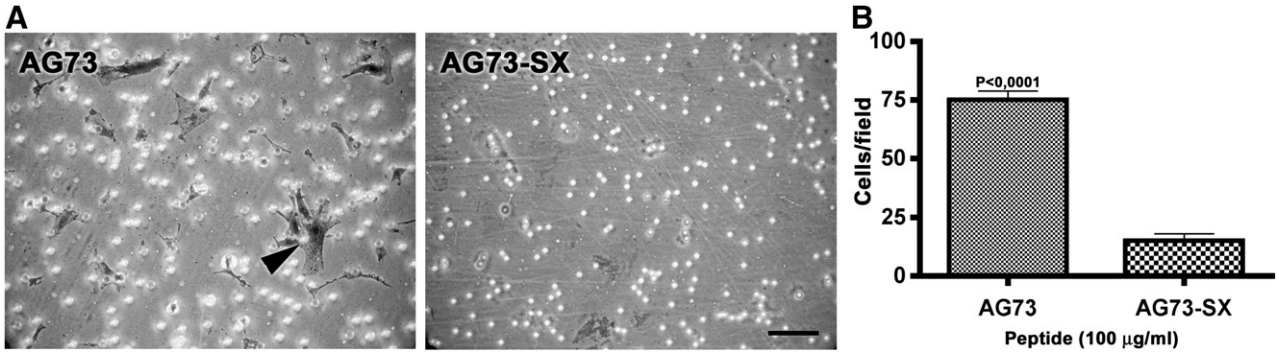

Fig. 7. AG73 increases invasion of CAC2 cells. We carried out invasion assays in CAC2 cells treated either with AG73 or with a scrambled control peptide (AG73-SX). Arrowhead indicates invaded cells on the lower side of the filter (A). The peptide AG73 induces a 5-fold increase in cell invasion (B). The results (±SEM) are triplicate experiments carried out at least three times. Scale bar in A: 50 μm.

MMP9 expression in CAC2 and M1 cells, followed by growth inside AG73-enriched laminin-111. Cells transfected with scrambled siRNA followed by growth within AG73-enriched laminin-111 served as controls. Light microscopy showed that silencing of MMP9 by siRNA dramatically reduced remodeling in CAC2 and M1 cells (Fig. 6B, D) compared to controls (Fig. 6A, C).

2.6. AG73 increases invasion of CAC2 cells

To analyze the importance of AG73-induced MMP9 activity and to relate this event to cancer cell invasiveness we carried out invasion assays in CAC2 cells treated either with AG73 or with a scrambled control peptide (AG73-SX). The peptide AG73 induced a 5-fold increase in cell invasion compared to controls (Fig. 7). M1 cells showed no AG73-mediated invasive activity (not shown). This result was expected since M1 cells are derived from a benign tumor.

2.7. Syndecan-1 and β1 integrin mediate the effect of AG73 in CAC2 and M1 cells

Adhesion assays demonstrated that AG73 is an adhesive peptide for both CAC2 and M1 cells (Fig. 8A). Cells exhibited strong cell attachment activity to both laminin-111 and AG73, while cells grown on the scrambled peptide AG73-SX show negligible adhesion. We also evaluated the effects of heparin on CAC2 and M1 cell attachment to AG73 (Fig. 8B). Cell attachment to AG73 was inhibited by heparin, suggesting that AG73 binds negatively charged surface molecules. Furthermore CAC2 and M1 cells treated with calcium chelators (EDTA or EGTA) exhibited decreased adhesion to AG73 (Fig. 8B), indicating that the adhesion to this peptide is mediated via cation-dependent cellular receptors such as integrins.

Adhesion experiments suggested that proteoglycans and integrins would mediate AG73 effect in our cell lines. Therefore we decided to study the role played by syndecan-1 and β1

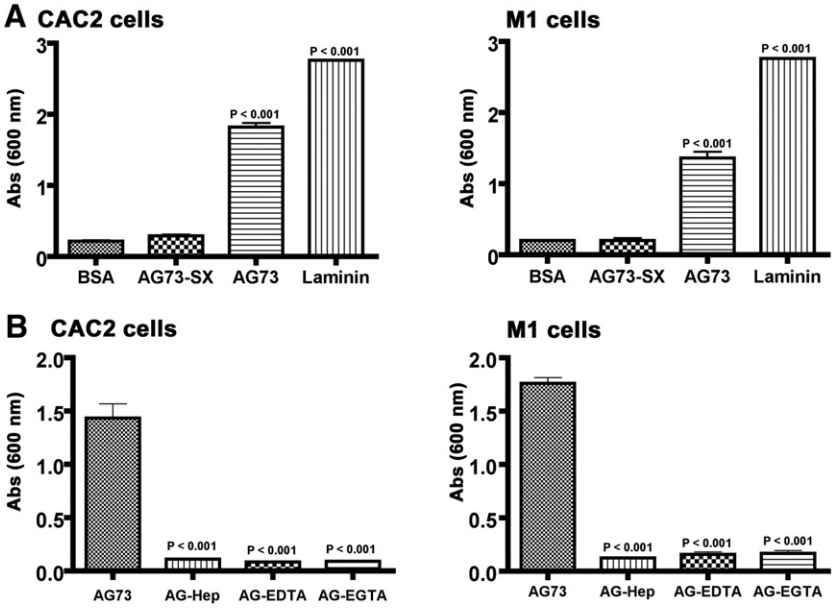

Fig. 8. Adhesion of CAC2 and M1 cells to AG73 is sensitive to heparin, EDTA, and EGTA. Cell lines adhere to laminin and AG73 but not to BSA and a scrambled peptide (AG73-SX) in a cell adhesion assay (A). Heparin, EDTA and EGTA decrease adhesion of both cell lines to AG73 (B). The results (±SEM) are triplicate experiments carried out at least three times.

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integrin in protease activity induced by AG73 in CAC2 and M1 cell lines.

Immunofluorescence showed that CAC2 and M1 cells cultured on AG73 for 6 h cluster and colocalize syndecan-1 and β1 integrin subunit on the cell surface (Fig. 9A–C, G–I). This result was not observed in cells plated on scrambled peptide AG73-SX (Fig. 9D–F, J–L). Statistical analysis showed that AG73 induced a significant colocalization ($p<0.0001$) compared to scrambled peptide control (Fig. 9M). Syndecan-1 and β1 integrin staining represent protein distribution on the cell membrane, since samples were not permeabilized.

Small interfering RNA (siRNA) provided evidence that syndecan-1 and β1 integrin interact with AG73. CAC2 and M1 cells with decreased syndecan-1 levels were submitted to adhesion assays and showed reduced adhesion to AG73 compared to controls (Fig. 10A). CAC2 cells with silenced syndecan-1 were treated with AG73 and the presence of MMPs in the conditioned medium was assessed by zymography.

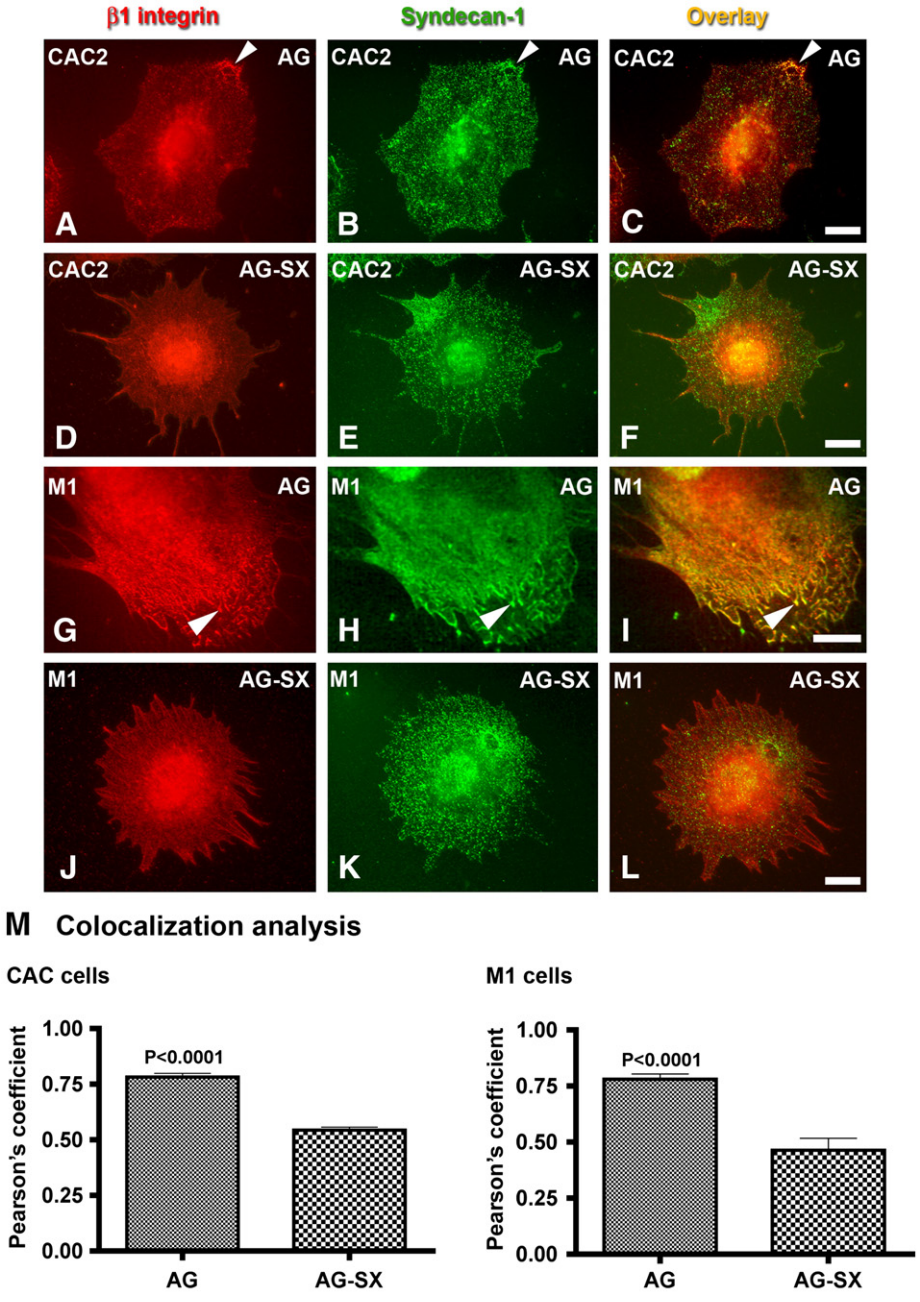

Fig. 9. Syndecan-1 and β1 integrin colocalize in CAC2 and M1 cells cultured on AG73. Immunofluorescence staining of cells cultured on AG73 and AG73-SX shows β1 integrin (red in A, D, G, J) and syndecan-1 (green in B, E, H, K). Colocalization appears yellow in the overlay images (C, F, I, L). Staining represents protein distribution on the cell membrane, since samples were not permeabilized. Cells grown on AG73 exhibit areas of colocalization (C and I, arrowheads). Statistical analysis shows that AG73 induced a significant colocalization ($p<0.0001$) compared to scrambled peptide control (M). Bars: 10 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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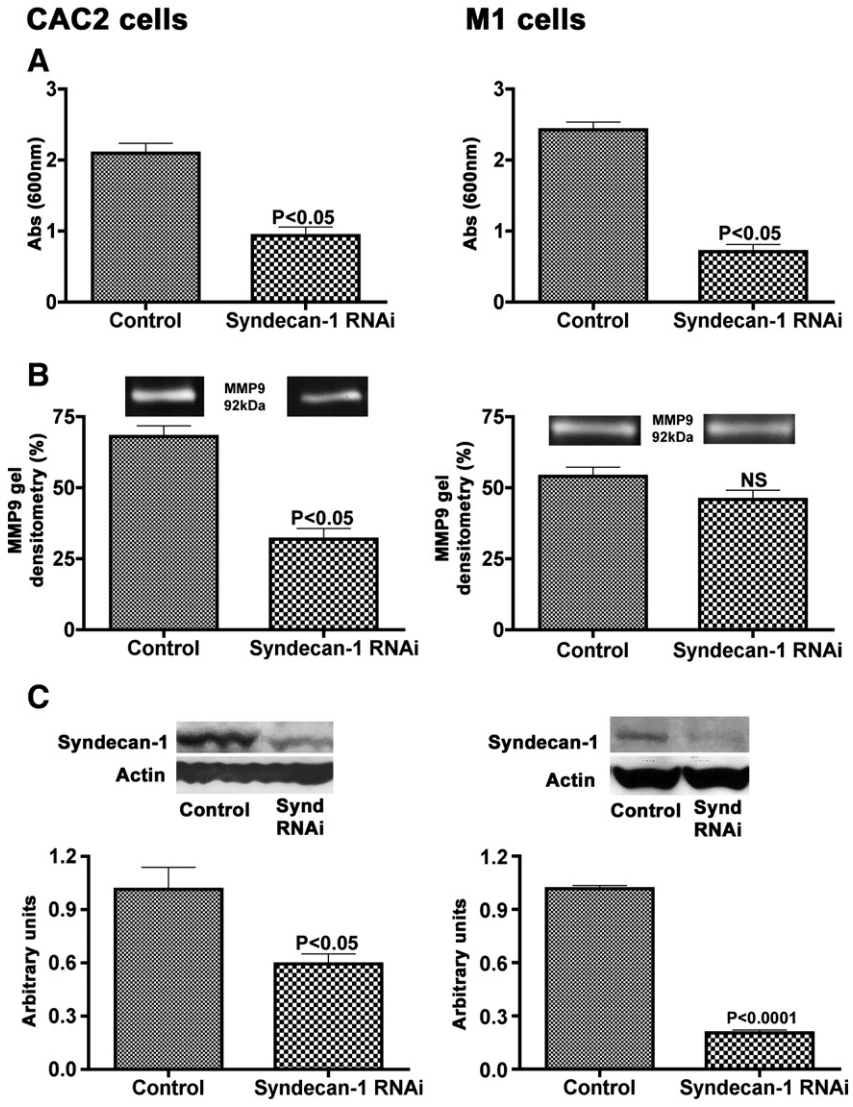

Fig. 10. Silencing syndecan-1 by siRNA inhibits both adhesion to AG73 and protease activity. CAC2 and M1 cells with reduced expression of syndecan-1 show decrease in adhesion to AG73 (A). CAC2 cells treated with siRNA were cultured with AG73 and the MMP9 activity measured by zymography. Syndecan-1 knockdown diminish MMP9 activity in these cells (B, left panel). M1 cells also show a decrease in protease activity, but not statistically significant (B, right panel). Immunoblot (C, upper panels) confirms the efficiency of siRNA transfection. Inspection of β-actin demonstrates equal loading conditions (C, upper panels). Efficiency of siRNA transfection is corroborated by qRT-PCR (C, lower graphs). The control is transfected with the non-silencing siRNA. Adhesion results (±SEM) are triplicate experiments carried out at least three times. Densitometric results (±SEM) are six lanes of zymography combined and repeated at least three times.

Silencing of syndecan-1 expression induced a significant decrease in protease activity in CAC2 cells compared to controls (Fig. 10B).

The same results were observed in cells with β1 integrin knocked down. Silencing of β1 integrin by siRNA reduced adhesion of both CAC2 and M1 cells to AG73 (Fig. 11A). CAC2 cells with reduced β1 expression showed significant decrease in protease activity (Fig. 11B).

We also addressed whether silencing of either syndecan-1 or β1 integrin would induce phenotypic changes in CAC2 and M1 cells cultured in 3D environment. Controls and siRNA-transfected cells were cultured within AG73-enriched laminin-111. Control CAC2 cells, transfected with the non-silencing siRNA, showed areas suggestive of matrix remodeling (Fig. 12A). Cells with either syndecan-1 (Fig. 12B) or β1 integrin (Fig. 12C) silenced exhibited spindle-shaped or round morphology, with no particular organization. The same features were observed in M1 cells. Control cells presented areas of matrix remodeling (Fig. 12D) while cells with receptors knocked down were round and non-cohesive (Fig. 12E, F).

CAC2 cells with silenced expression of either syndecan-1 or β1 integrin presented a significant decrease in AG73-induced protease activity. Furthermore, siRNA receptor knockdown changed the phenotype of CAC2 cells cultured in AG73-enriched laminin-111. Thus, we decided to study whether both receptors may cooperate to regulate AG73 effect in CAC2 cells.

Double-knockdown experiments suggested that syndecan-1 and β1 integrin cooperate to regulate adhesion of CAC2 cells to AG73. Cells transfected with both syndecan-1 and β1 integrin siRNA oligos showed a decrease of 80% in adhesion to AG73

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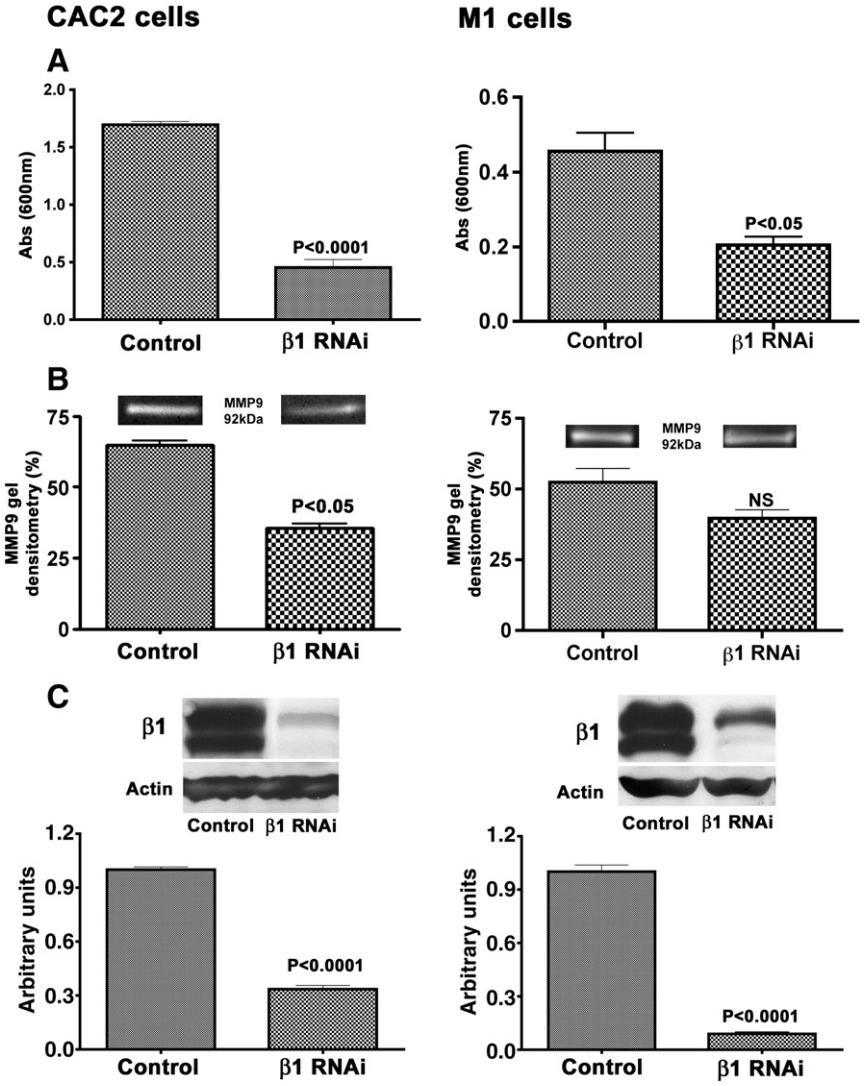

Fig. 11. Silencing β1 integrin decreases adhesion of both CAC2 and M1 cells to AG73 (A) and reduces AG73-related protease activity in CAC2 cells (B, left panel). M1 cells also show a decrease in protease activity, but not statistically significant (B, right panel). Immunoblot (C, upper panels) confirms the efficiency of siRNA transfection. Inspection of β-actin demonstrates equal loading conditions (C, upper panels). Efficiency of siRNA transfection is corroborated by qRT-PCR (C, lower graphs). The control is transfected with the non-silencing siRNA. Adhesion results (±SEM) are triplicate experiments carried out at least three times. Densitometric results (±SEM) are six lanes of zymography combined and repeated at least three times.

(Fig. 13A). Transfection with either syndecan-1 or β1 integrin siRNA oligos alone resulted in a 45–50% decrease in CAC2 cells adhesion to the peptide (Fig. 13A). Furthermore CAC2 cells with double-silenced syndecan-1 and β1 integrin were treated with AG73 and the presence of MMPs in the conditioned medium was assessed by zymography. Simultaneous silencing of receptors induced a decrease in protease activity (Fig. 13B). These results suggest that syndecan-1 cooperate with β1 integrin to regulate adhesion and protease activity of CAC2 cells.

3. Discussion

We have demonstrated that AG73 regulates the morphology, adhesion and protease activity of cell lines derived from malignant and benign salivary gland tumors. We also investigated the mechanisms regulating MMP9 production in CAC2 and M1 cells. Syndecan-1 and β1 integrin signaling downstream of AG73 regulate MMP production by CAC2 and M1 cells. To our knowledge, this is the first report establishing a relationship among AG73, integrins, syndecan-1 and secretion/activity of MMP9 in tumor cells.

Salivary gland neoplasms express prominent basement membrane. This phenomenon is particularly interesting in adenoid cystic carcinoma. This neoplasm presents a massive basement membrane enlargement compared to basement membrane of dermal–epidermal junction (Supplementary Fig. 1A, B). Morphometric measurements indicated that basement membrane of adenoid cystic carcinoma cells has a mean thickness of 4.734± 0.1961 μm, whereas basement membrane at the dermal–epidermal junction has an average thickness of 0.1187± 0.012 μm (Supplementary Fig. 1C). Thus, basement membrane of adenoid cystic carcinoma is at least 40-fold thicker compared to basement membrane at dermal–epidermal junction. Our results are supported by findings published elsewhere (Tunggal et al.,

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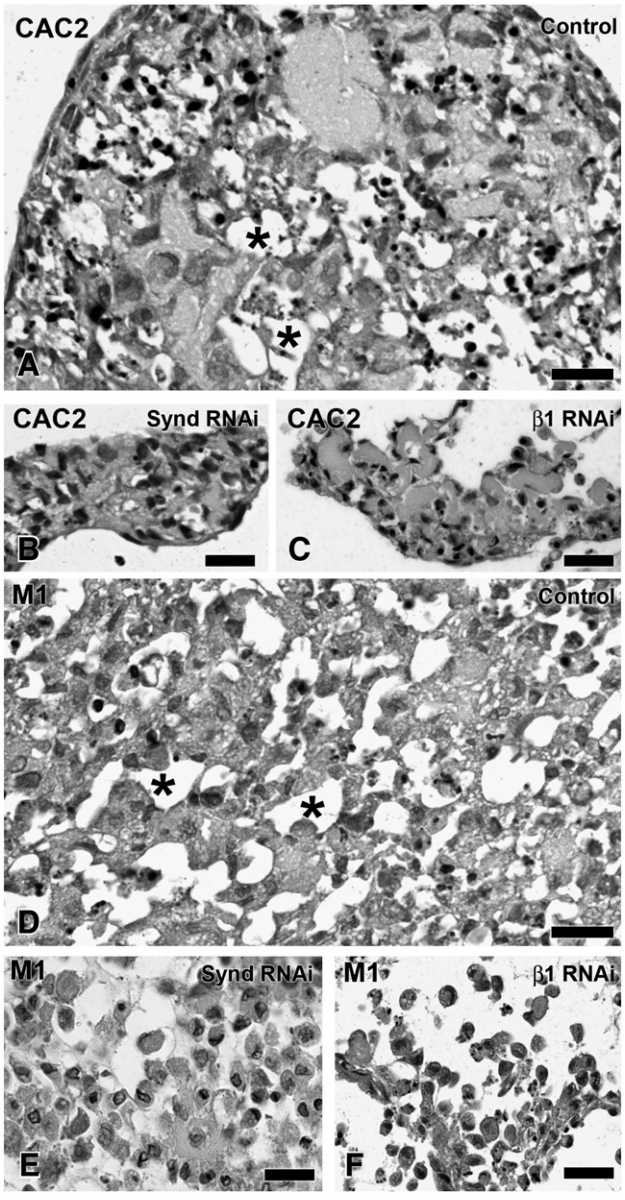

Fig. 12. Silencing of receptors induce phenotypic changes in CAC2 and M1 cells cultured in 3D environment. Controls and siRNA-transfected cells were cultured within AG73-enriched laminin-111. Control CAC2 cells show areas of matrix remodeling (A, asterisks). Cells with either syndecan-1 (B) or β1 integrin (C) silenced exhibit spindle-shaped or round morphology. Control M1 cells present areas of matrix remodeling (D, asterisks) while cells with receptors knocked down by siRNA are round and non-cohesive (E, F). The control is transfected with the non-silencing siRNA. Bars: 20 μm.

2002). It is obvious from the basement membrane thickness that an overproduction of laminin occurs *in vivo* in adenoid cystic carcinoma cells. Laminin expression in this tumor undoubtedly surpasses the occurrence of laminin in normal tissues. Due to this high expression we may assume that the effects observed in culture with laminin-111 and its derived peptide AG73 in CAC2 and M1 cells are likely to be at physiological range.

Laminin-111 (formerly laminin-1) (Aumailley et al., 2005) is composed by α1, β1 and γ1 chains and the α1 chain contains the AG73 peptide. Laminin α1 is expressed *in vivo* in both adenoid cystic carcinoma and in myoepithelioma. This expression was also observed *in vitro* in our cell lines.

Laminin α1 chain is prominently expressed *in vivo* during early embryogenesis but exhibits a restrict expression in adult tissues (Scheele et al., 2007; Sorokin et al., 1997; Virtanen et al., 2000). Presence of laminin α1 chain is often detected in adult kidney (Scheele et al., 2007; Sorokin et al., 1997; Virtanen et al., 2000). Our antibodies recognized α1 chain in human kidney positive controls. This result strongly suggests that detection of laminin α1 chain in both adenoid cystic carcinoma and myoepithelioma was specific. Furthermore tumor cell populations are considered caricatures of normal development (Dirks, 2006; Lind et al., 2006). Thus, we may assume that a protein expressed during early embryogenesis in normal tissues could be found in different neoplasms. Blood vessels from tumor stroma exhibited laminin α1 chain. Blood vessels are generally negative for laminin α1 chain except for capillaries within the central nervous system (Hallmann et al., 2005; Virtanen et al., 2000). Since we have positive controls we considered this staining specific. These blood vessels stained by laminin α1 may represent angiogenic features of both adenoid cystic carcinoma and myoepithelioma. It is important to emphasize that laminin α1 chain and its derived peptide AG73 are related to angiogenesis in different systems, such as aortic ring sprouting, endothelial tube formation, and chick chorioallantoic membrane assays (Mochizuki et al., 2007).

Immunolocalization of the laminin α1 chain in adenoid cystic carcinoma appears in both the basement membrane (described as a linear pattern) and as diffuse staining distributed throughout the tumor. This result confirms our previous observations (Freitas et al., 2007). The diffuse non-linear distribution may suggest a breakdown of the basement membrane present in the neoplasm. Disruption of the basement membrane in the invading area of adenoid cystic carcinoma has been reported (Shintani et al., 1997). We also observed expression of MMP9 at similar locations to laminin α1 in adenoid cystic carcinoma *in vivo*. Laminins are substrates for MMPs, and adenoid cystic carcinoma cells secrete laminin, which could be processed by MMPs resulting in the formation of laminin fragments containing bioactive peptides and cryptic sites (Davis et al., 2000; Faisal Khan et al., 2002; Mott and Werb, 2004; Schenk and Quaranta, 2003). Many bioactive peptides have been shown to regulate cell behavior (Suzuki et al., 2005) and cryptic domains contained in the ECM are exposed by proteolysis and stimulate biological responses (Davis et al., 2000; Faisal Khan et al., 2002; Mott and Werb, 2004; Schenk and Quaranta, 2003). Myoepithelioma also expressed MMP9 *in vivo* and *in vitro*. The appearance of such enzyme in a benign tumor is probably related to remodeling activity rather than aggressiveness.

Our results showed that laminin-111 and the laminin-derived peptide AG73 regulate the morphology and protease activity of cells derived from human adenoid cystic carcinoma (CAC2 cells) and myoepithelioma (M1 cells). Cells grown inside AG73-enriched laminin-111 exhibited large spaces in the extracellular matrix, suggestive of remodeling. The broad spectrum MMP inhibitor GM6001 decreased the number and the size of spaces induced by AG73 in CAC2 and M1 cells. The

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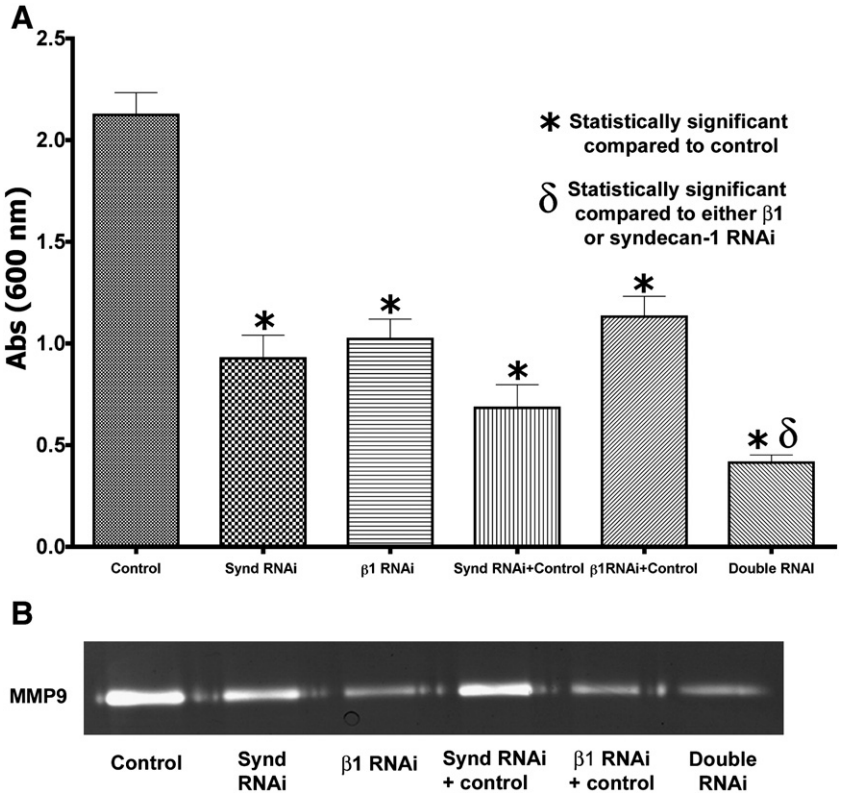

Fig. 13. Simultaneous knockdown of syndecan-1 and beta1 integrin regulate adhesion and protease activity in CAC2 cells. Double-transfected cells were submitted to adhesion assays to AG73, showing decrease in adhesion compared to either siRNA oligos alone or the non-silencing siRNA control (A). CAC2 cells with both receptors silenced were treated by AG73 exhibiting a decrease in protease activity compared to different controls (B). Adhesion results (±SEM) are triplicate experiments carried out at least three times. Zymograms were carried out at least three times with similar results. Control: scrambled siRNA sequence; Synd RNAi: syndecan-1 siRNA alone; beta1 RNAi: beta1 siRNA alone; synd RNAi+control: syndecan-1 siRNA+scrambled siRNA sequence; beta1 RNAi+control: beta1 siRNA+scrambled siRNA sequence; double-RNAi: syndecan-1 siRNA+beta1 siRNA.

same result was observed by silencing siRNA of MMP9. These results strongly suggest that AG73-mediated matrix remodeling involves matrix metalloproteinases, or more specifically MMP9. Furthermore, CAC2 and M1 cells cultured on AG73 showed a dose-dependent increase of MMP9 secretion.

The biological relevance of AG73 activity regulating protease activity was also demonstrated by invasion assays using a cell line (CAC2) derived from a malignant human salivary tumor. The peptide promoted a 5-fold increase in invasion of CAC2 cells. Thus, we may infer that the AG73 would play a part regulating invasiveness of adenoid cystic carcinoma *in vivo*. Invasive tumor cells that are grown on an extracellular matrix substrate extend invadopodia into the surrounding matrix, which results in MMP-mediated degradation of the substrate, a crucial event in cell-invasive behavior (Buccione et al., 2004).

To further explore the function of AG73 regulating protease activity and invasiveness of adenoid cystic carcinoma we decided to study putative receptors for this peptide. It is known that AG73 binds syndecan-1 (Hoffman et al., 1998; Weeks et al., 1998). This prompted us to study whether syndecan-1 and other receptors would play a role in AG73-induced protease activity in CAC2 and M1 cells.

Silencing syndecan-1 by siRNA decreased adhesion to AG73 and protease activity. The decrease in adhesion was expected, since this proteoglycan has been identified as an AG73 ligand. We revealed further effects of syndecan-1 and AG73 by showing that this complex regulates matrix remodeling and protease activity in our cell lines. Syndecans consist of a family of highly conserved type I transmembrane heparan sulfate proteoglycans that are expressed in developmental and cell type-specific pattern, and bind a wide range of components through their heparan sulfate side chains (Bernfield et al., 1992; Cattaruzza and Perris, 2006; Engbring et al., 2002; Hoffman et al., 1998; Iozzo, 1998). It has been proposed that syndecans function as co-receptors for components in the extracellular microenvironment, probably by their GAG side chains (Beauvais et al., 2004; Humphries et al., 2005).

Integrins are likely candidates to interact with syndecan-1 in our cell lines. Different reports have shown direct interaction between these two molecules (Beauvais et al., 2004; Humphries et al., 2005; McQuade et al., 2006; Ogawa et al., 2007; Peterson et al., 2005; Yokoyama et al., 2005). CAC2 and M1 cells exhibited AG73-induced colocalization of syndecan-1 and β1 integrin. Moreover, adhesion of CAC2 and M1 cells to AG73 is inhibited by heparin, EDTA and EGTA, suggesting that proteoglycans and integrins mediate this process. Silencing either syndecan-1 or β1 integrin decreased adhesion, protease activity, and extracellular matrix remodeling in our cell lines. We further investigated the interaction between syndecan-1, β1 integrin and AG73 using double siRNA knockout followed by

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adhesion and protease activity assays in CAC2 cells. Results strongly suggested that syndecan-1 and β1 integrin combine forces to regulate MMP9 secretion in CAC2 cells.

Extracellular proteolytic modifications of laminin play a critical role in determining important domains of this molecule (Ghosh and Stack, 2000). Proteolytic fragments of laminin have been widely utilized to study the influence of individual domains on cellular behavior (Ghosh and Stack, 2000). We may attempt to establish a relationship between laminin peptides and laminin fragments underlying regulation of matrix metalloproteinases in CAC2 and M1 cells. Binding of AG73 to cellular receptors would trigger signal transduction pathways modulating matrix metalloproteinase expression. Secretion of MMPs may participate in the cleavage of laminin present in the pericellular matrix. Release of laminin-derived bioactive peptides has important effects on cellular interactions that control adhesion, motility and invasion of cell lines (Ghosh and Stack, 2000).

Spreading of malignant tumors involves a large number of molecules including proteolytic enzymes, adhesion molecules and other membrane receptors, cytokines and growth factors (Davidson et al., 2003). A complex system of signal transduction pathways relays messages from the extracellular matrix via membrane receptors and intracellular molecules to the nucleus, resulting in the activation of transcription factors and synthesis of different genes (Davidson et al., 2003). It is evident that understanding the interactions between host and cancer cells is crucial for the study of cancer pathogenesis. Based on our experimental findings we propose that cells from salivary gland tumors bind AG73 and signal through syndecan-1 and integrins resulting in increased secretion/activity of MMP9. The knowledge on biological effects of laminin-derived peptides such as AG73 is fragmented without a clear sequence and flow of information. It is known that this peptide induces protease activity and binds syndecan. On the other hand, it has also been demonstrated that syndecan cooperate with integrins. However these facts and ideas are not within a clear conceptual framework. Our results have attempted to provide an initial framework for the effect of AG73 on tumor biology. We have tried to link biological events, to better understand the role played by this peptide on salivary gland neoplasms.

4. Experimental procedures

4.1. Peptides

AG73 (RKRLQVQLSIRT) and the scrambled peptide AG73-SX (RTLRIKQSVRLQ) were synthesized by EZ Biolab, Westfield, IN, USA; and by Molecula Research Laboratories, Herndon, VA, USA. Peptides purity was 98% (RP-HPLC), with molecular weight confirmed by mass spectrometry.

4.2. Immunolocalization of laminin α1 chain and MMP9 in adenoid cystic carcinoma and myoepithelioma in vivo

Five cases of adenoid cystic carcinoma and two of myoepithelioma were retrieved from our files. Formalin-fixed paraffin-embedded tissues were studied by immunohistochemistry. Sections (3 μm) were obtained and subjected to the EnVision method (EnVision; Dako Corp., Carpinteria, CA, USA). Sections mounted on 3-aminopropyltriethoxy-silane coated slides (Sigma Chemical Corp, St Louis MO, USA) were dewaxed in xylene and hydrated in graded ethanol. Endogenous peroxidase activity was inhibited by incubation of the sections in 3% H_2O_2 in methanol for 20 min. Sections were then blocked with 1% bovine serum albumin (BSA, Sigma) in Tris–HCl. We used three antibodies to detect laminin α1 chain: 1) the rabbit antiserum HK-175 (kindly provided by Dr Hynda Kleinman, NIDCR, NIH, USA), raised against the peptide CSRARKQAASIKVAVSADR from laminin α1 chain; 2) a rabbit polyclonal antibody against human laminin α1 chain (H-300, Santa Cruz Biotechnology, Santa Cruz, CA, USA) (Faisal Khan et al., 2002), and 3) a goat polyclonal antibody against mouse laminin α1 chain (M-20, Santa Cruz, kindly provided by Dr. Vilma R. Martins, Ludwig Institute for Cancer Research, São Paulo Branch, Brazil). MMP9 was labeled by a mouse monoclonal antibody (Calbiochem–Novabiochem Co, La Jolla, CA, USA). Both antibodies were diluted 1:20 in Tris–HCl. For laminin α1 staining antigen retrieval was carried out by treating sections with 1% pepsin in 10 mM HCl for 1 h at 37 °C. Diaminobenzidine (Sigma) was used as the chromogen and the sections were counterstained with Mayer's haematoxylin (Sigma). Non-specific sera served as negative controls. Human kidney was used as positive control for laminin α1 chain immunostaining.

4.3. Cell culture

CAC2 and M1 cells were derived from a human adenoid cystic carcinoma and myoepithelioma. The characterization of CAC2 and M1 cells was published elsewhere (de Oliveira et al., 2001; Freitas and Jaeger, 2002). These cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Sigma) supplemented by 10% fetal bovine serum (Cultilab, Campinas, SP, Brazil) and 1% antibiotic–antimycotic solution (Sigma). The cells were maintained in 25 cm^2 flasks in a humidified atmosphere of 5% CO_2 at 37 °C.

4.4. Immunofluorescence

Cells grown on glass coverslips were fixed in 1% paraformaldehyde in phosphate-buffered saline (PBS) for 10 min, blocked with 1% BSA in PBS, and stained with the same rabbit antibodies against laminin α1 chain described above. Primary antibodies were revealed by anti-rabbit Alexa-568 conjugated secondary antibody (Invitrogen Co, Carlsbad, CA, USA). To stain for MMPs, cells were fixed and permeabilized with 0.5% Triton X-100 (Sigma) in PBS for 15 min, followed by incubation with mouse monoclonal antibody to MMP9 (Calbiochem) diluted in PBS 1:50. Primary antibody was revealed by an anti-mouse Cy3 conjugated secondary antibody (Zymed-Invitrogen). All incubations were carried out for 60 min at room temperature and Pro Long (Invitrogen) was used as a mounting medium. Non-specific sera served as negative controls.

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For immunofluorescence analysis of syndecan-1 and β1 integrin CAC2 and M1 cells were cultured on AG73 and AG73SX (scrambled control peptide), and allowed to spread for 6 h. After this time has elapsed CAC2 and M1 cells adhere and spread to either AG73 or to AG73-SX. Cells were fixed in 1% paraformaldehyde in PBS for 10 min, rinsed and blocked with 1% BSA for 30 min. Samples were then double-labeled with antibodies against syndecan-1 (mouse monoclonal, clone B-B4, Chemicon, Temecula, CA, USA) and β1 integrin (mouse monoclonal antibody, Chemicon) diluted 1:100 in PBS for 1 h. To avoid non-specific staining the mouse antibody to β1 integrin was biotinylated (EZ link sulfo-NHS-LC-biotinylation kit, Pierce Biotechnology Inc., Rockford IL, USA) following manufacturer's instructions. CAC2 and M1 cells were then incubated first with biotinylated antibody to β1 integrin, followed by detection with streptavidin-Texas Red (Vector Laboratories Inc Burlingame, CA, USA). After that, cells were overlaid with anti-syndecan-1 mouse antibody, followed by labeling with anti-mouse Alexa 488 (Invitrogen). Samples were mounted in Pro Long (Invitrogen).

We quantified the colocalization between syndecan-1 and β1 integrin in CAC2 and M1 cells grown either on AG73 or on AG73SX. Treated and control samples double-labeled with antibodies against syndecan-1 and β1 integrin were analyzed by the software ImageJ, through the plugin "Manders' Coefficient" (developed by Tony Collins, Wright Cell Imaging Facility, Toronto, Canada; and by Wayne Rasband, NIMH, NIH). This plugin analyzes the overlap of red and green pixels and generates a colocalization coefficient (Pearson's). Twenty cells from either control or treated samples were analyzed.

4.5. Growth of CAC2 and M1 cells in three-dimensional preparation of laminin-111 gel and AG73-enriched laminin-111

We used a laminin-111 gel in DMEM (1 mg/ml, Trevigen Inc., Gaithesburg, MD, USA). This preparation is referred throughout the text as "laminin-111 gel". Three-dimensional (3D) cultures were prepared by growing either CAC2 or M1 cells to confluence as monolayers, followed by trypsinization and embedment into laminin-111 gel as single cells (2×10^5 cells/ml). The laminin-111 gel containing CAC2 and M1 cells was then dispensed into cryovials and maintained at 37 °C with 5% CO_2. Cells were grown within this three-dimensional preparation of laminin-111 for 48 h. Three-dimensional cultures followed the protocol described by Weaver et al. (1997) with some modifications (Freitas and Jaeger, 2002; Freitas et al., 2004, 2007; Morais Freitas et al., 2007). The placement of the laminin-111 gel containing cells into cryovials was made due to the nature of the gel, too soft to be dispensed into culture plates.

We also prepared a mixture of laminin-111 gel and AG73. The peptide was diluted in ultrapure water (1 mg/ml). After that, AG73 was mixed with laminin-111, to reach a final concentration of 50 μg/ml. This preparation is referred throughout the text as "AG73-enriched laminin-111". Three-dimensional (3D) cultures were prepared by growing cells to confluence as monolayers, followed by trypsinization and embedment into AG73-enriched laminin-111 as single cells (2×10^5 cells/ml). The AG73-enriched laminin-111 containing either CAC2 or M1 cells was then dispensed into cryovials and maintained at 37 °C with 5% CO_2. Cells were grown within this three-dimensional preparation for 48 h. Cells grown inside laminin-111 gel served as controls.

We studied by light microscopy the effect of either laminin-111 gel or AG73-enriched laminin-111. Treated and control samples were fixed in 10% formalin for 24 h. Even after fixation, the laminin-111 gel used in our preparation was too soft to be directly embedded in paraffin. To circumvent this problem, we dehydrated and embedded the samples in Histogel (Perk Scientific Inc., Devon, PA, USA). Heating the Histogel to 50 °C converts the gel into a liquid state, which allows infiltration of the samples. After the infiltration, the Histogel converts back into a solid as it cools. Final hardening is achieved at room temperature. Since Histogel is in aqueous media, the samples were then dehydrated again, paraffin-embedded and stained by hematoxylin–eosin (H&E).

CAC2 and M1 cells were treated with the broad spectrum MMP inhibitor GM6001 (25 μM, Sigma), followed by growth inside AG73-enriched laminin-111. This experiment was designed to study whether AG73-mediated remodeling in 3D matrices would be the result of protease activity. Samples were study by light microscopy as described before.

CAC2 and M1 cells had MMP9 silenced by siRNA, followed by growth inside AG73-enriched laminin-111. Cells transfected with scrambled siRNA followed by growth within AG73-enriched laminin-111 served as controls. Samples were study by light microscopy.

CAC2 and M1 cells with either syndecan-1 or β1 integrin silenced by siRNA were cultured inside AG73-enriched laminin-111. The rationale to carry out this experiment was to address whether the knock down of putative AG73 receptors would induce phenotypic changes in CAC2 and M1 cells cultured in 3D environment. Samples were study by light microscopy.

4.6. Invasion assay

Invasion assays were carried out in a modified Boyden chamber with filter inserts (8-μm pores) for 6-well plates (BD Biosciences San Jose, CA, USA). Filters were coated with 50 μl of Matrigel (13 mg/ml). CAC2 and M1 cells (2×10^5) were plated into the upper chamber in 1 ml of DMEM without serum. The lower chamber was filled with 1.5 ml of medium containing AG73 (100 μg/ml). Samples with the lower chamber filled with DMEM containing scrambled peptide (100 μg/ml) served as controls. CAC2 cells were cultured in these conditions for 48 h while M1 cells were grown for 96 h. Cells were then fixed with 4% paraformaldehyde. Cells on the upper side of the filter, including those in the Matrigel, were removed with a cotton swab. Invaded cells, on the lower side of the filter, were stained with crystal violet, photographed and counted at a final magnification of 160×. On each filter 7 random fields were evaluated. Each experiment was carried out in triplicates, and repeated at least three times.

4.7. Zymography of AG73 treated cells

CAC2 and M1 cells were cultured in six multiwell plates were coated with 100 μl of either AG73 or the scrambled

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control AG73-SX (2.5, 10, 25 and 50 μg/well) diluted in Milli-Q water and allowed to evaporate overnight in a hood. Cells (10^4) were plated and allowed to adhere and spread for at least 8 h. The adherent cells were washed three times with PBS, and the culture media replaced by serum-free media for 24 h. The presence of MMPs in the conditioned medium was assessed by zymography. The conditioned medium was collected, concentrated (Microcon 30 K, Millipore Co, Bedford, MA, USA) and resuspended in SDS-PAGE sample buffer (without β-mercaptoethanol). The remaining cells were lysed and the amount of protein estimated by BCA assay (Pierce). In zymograms, the volume of conditioned medium loaded per lane was standardized on the basis of protein content in the cell lysate. Samples were separated on 10% polyacrylamide gels containing 0.2% gelatin (Novex-Invitrogen). After electrophoresis, the gels were washed in 2.5% Triton X-100 for 30 min, equilibrated in 10 mM Tris (pH 8.0), and incubated at 37^0 C in a development buffer containing 50 mM Tris pH 8.0, 5 mM $CaCl_2$, 0.02% NaN_3 for 16-24 h. The gels were stained with 0.2% Coomassie blue R250 (Amersham Co Arlington Heights IL, USA) and destained with acetic acid/methanol.

The MMP activity was also inhibited with either 10 mM EDTA (calcium chelator, Sigma) or 5 mM 1,10-phenanthroline (heavy metal chelator, Sigma) in the zymogram development buffer to confirm the bands were MMPs. We also ran parallel polyacrylamide gels with M1 or CAC2 cell lysates and did Western blots for β-actin to confirm equal loading conditions.

CAC2 and M1 cells treated with siRNA to syndecan-1 and β1 integrin were cultured in six multiwell plates coated with AG73 (50 μg/well). The MMP activity in the conditioned medium was analyzed by zymography as described above.

We also conducted double siRNA experiments for silencing both syndecan-1 and β1-integrin in CAC2 cells. Cells were transfected with syndecan-1 siRNA alone, β1 integrin siRNA alone, both syndecan-1 and β1-integrin siRNA, or with control siRNA oligos. For double siRNA experiments, the total siRNA oligos were kept the same among different wells by addition of scrambled siRNA oligos. Cells were cultured in six multiwell plates coated with AG73 (50 μg/well). The MMP activity in the conditioned medium was analyzed by zymography.

4.8. Cell adhesion assays

Cell adhesion to AG73 was compared to laminin-111 and to AG73-SX. Adhesion assays were performed in 96-well round-bottomed plates. Wells were coated overnight at 4 °C with AG73 (1 μg/well), laminin-111 (1 μg/well), or AG73SX (1 μg/well). Wells were blocked with 3% BSA for 30 min at 37 °C, rinsed in PBS with 0.1%BSA, and cells incubated for 20 min at 37 °C. Attached cells were fixed/stained for 10 min with 0.2% (w/v) crystal violet in 20% (v/v) methanol. After three washes with H_2O, cells were dissolved in 10% SDS (w/v), and the absorbance at 600 nm was measured. We analyzed the effects of heparin (50 μg/ml in PBS), EDTA (2 mM in PBS) and EGTA (2 mM in PBS) on the adhesion of CAC2 and M1 cells to AG73. Cells were preincubated with these reagents for 10 min before being added to cell adhesion assays.

CAC2 and M1 cells treated with siRNA to either syndecan-1 or β1 integrin were used in adhesion assays to AG73. Adhesion assays were performed in 96-well round-bottomed plates. Wells were coated overnight at 4 °C with 100 μl of AG73 (1 μg/well). Adhesion assays were carried out as described above.

Cells with simultaneous knockdown of syndecan-1 and β1 integrin were also submitted to the adhesion assays to AG73.

4.9. Small interfering RNA (siRNA)

CAC2 cells were transfected with commercially available siRNA targeting MMP9, syndecan-1 and β1 integrin (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) following the manufacturer's instructions. One day before transfection, sub confluent CAC2 and M1 cells were cultured in DMEM, supplemented by 10% fetal bovine serum without antibiotic–antimycotic solution. Cells were incubated with a complex formed by the siRNA (50 nM), transfection reagent (lipofectamine 2000, Invitrogen), and transfection medium (Opti-MEM I, Invitrogen) for 30 h at 37 °C. The following oligonucleotide sequences for siRNA were used: MMP9: CAUCACCUAUUGGAUCCAATT, syndecan-1: a pool of 3 mRNA strands: CGCAAAUUGUGGCUACUAA, AGCAGGACUUCACCUUUGA, CGUGGGGCUCAUCUUUGCU; β1 integrin: a pool of 3 mRNA strands: GAGAUGAGGUUCAAUUUGA, GAUGAGGUUCAAUUUGAAA, GUACAGAUCCGAAGUUUCA. A siRNA scrambled sequence (Santa Cruz proprietary target sequence) was used as negative controls. For syndecan-1 and β1 integrin double siRNA experiments, the total siRNA oligos (50 nM) were kept the same among different wells by addition of scrambled siRNA oligos. Transfection efficiency was confirmed by quantitative polymerase chain reaction (Real time PCR) and Western blot.

4.10. Real time PCR

RNA was collected after siRNA treatment using the Trizol reagent (Invitrogen). Reverse transcription of total RNA (1 μg) with oligo dT (500 μg/ml), 10 mM of each dNTP, 5X First-Strand Buffer, 0.1 M of DTT and 200U of reverse transcriptase (M-MLV-Promega, Madison, WI, USA) was performed at 70 °C for 10 min followed by 42 °C for 60 min and 10 min at 95 °C. qRT-PCR was quantified with the ABI Prism 5700 sequence detector (Applied Biosystems, Foster City, CA, USA). The PCR reactions contained 40–160 ng/μl of cDNA in 25 μl of SYBR® Green PCR master mix (Invitrogen), and 50–900 nM primers (forward and reverse). Cycling conditions were: 50 °C for 2 min and 95 °C for 10 min, followed by 50 cycles: 15 s at 95 °C and 60 s at 60 °C. The primers were synthesized by Integrated DNA Technologies, Inc., USA, with the following sequences: Human Syndecan-1 (Sun et al., 2005): GGAGCAGGACTTCACCTTTG (forward) and CTCCCAGCACCTCTTTCCT (reverse); Human β1 Integrin (designed by Primer Express Software, Applied Biosystems,) TGCAGTTTGTGGATCACTGATTG (forward) and CCTGTGGACTGTCGAGGCATAAC (reverse); 18S protein: GTAACCCGTTGAACCCCATT (forward) and CCATCCAATCGGTAGTAGCG (reverse). Accession numbers for human sequences

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to Syndecan-1, β1 integrin, and 18S rRNA are J05392, X07979, and M11188 respectively. Results were expressed as the ratio of the mRNA level of each gene of interest normalized to 18S.

4.11. Western blots

Immunoblots for MMP9, syndecan-1 and β1 integrin were used to test the efficiency of RNA silencing in CAC2 and M1 cells. Samples were lysed in RIPA buffer (150 mM NaCl, 1.0% NP-40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris pH 8.0) with protease inhibitor cocktail (Sigma). Samples were centrifuged (10,000 g) for 10 min at 4 °C, the supernatants recovered and quantified (BCA kit, Pierce). Lysates to be immunoblotted with syndecan-1 antibody were treated with chondroitinase ABC (1.0 units/ml) and heparitinase I, II, III (0.1 units/ml) at 37 °C for 30 min. Samples were resuspended in Laemmli buffer containing 62,5 mM Tris–HCl pH 6.8, 2% sodium dodecyl sulphate (SDS), 10% glycerol, 5% mercaptoethanol, 0.001% bromophenol blue. Equal amounts (20 μg) of cell lysates were electrophoresed in 4–12% polyacrylamide gradient gels. Proteins were transferred to a Hybond ECL nitrocellulose membrane (Amersham), blocked in TBS with 2.5% non-fat milk overnight at 4 °C. Following one wash in TBS with 0.05% Tween 20 (TBST), the membrane was probed with antibodies against MMP9 (rabbit polyclonal from Chemicon, 1:250), syndecan-1 (mouse Mab from Chemicon, clone B-B4, 1:100) and β1 integrin (rabbit polyclonal from Chemicon, 1:1,000). Primary antibodies were detected by HRP-conjugated secondary antibodies (1:10,000), and developed using an ECL chemiluminescent substrate (Amersham). To confirm equal loading conditions membranes were stripped and reprobed with β-actin antibody (Sigma).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.matbio.2008.02.007.

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