# Laminin-1-derived scrambled peptide AG73T disaggregates laminin-1-induced ovarian cancer cell spheroids and improves the efficacy of cisplatin

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**Abstract.** We found previously that the laminin-1-derived synthetic peptide AG73 (LQVQLSIR) promoted ovarian cancer cell metastasis *in vivo*. We have now studied the role of this metastasis-promoting peptide *in vitro* using TAC3 ovarian cancer cells, which display anchorage-independent growth and form multicellular spheroids. Our goal is to better understand how this peptide can regulate metastasis *in vivo*. We found that the exogenous addition of either laminin-1 or peptide AG73 stimulated the formation and growth of the spheroids. Western blot analysis indicated that laminin-1 enhanced the expression of integrin ß1, and that AG73 peptide enhanced expression of syndecan-1 and downstream effectors, including mitogen-activated protein kinase (MAPK) and extracellular signal-related kinase (ERK), and also phosphatidylinositol (PI)-3 kinase/AKT activity signaling. The soluble peptide AG73T, which is a scramble peptide of AG73, was able to disaggregate the laminin-1-induced spheroids. Furthermore, the disaggregated cells were twice as sensitive to cisplatin as the intact spheroids. The AG73T peptide in the presence of laminin-1 suppressed expression of integrin ß1 and its downstream effectors, including MAPK/ERK and PI3/AKT activity signaling. The MEK inhibitor U0126 reduced TAC3 cell growth more effectively in the presence of both laminin-1 and AG73T than in the presence of laminin-1 alone. Inhibition of the PI3-K cascade with LY294002 was also more effective in the presence of laminin-1 and AG73T. The increased sensitivity to cisplatin in the presence of AG73T may be due to the greater bioavailability of the drug to the free-floating cells over the spheroids. These findings suggest a novel function of AG73T in ovarian cancer and help to define mechanisms important in ovarian cancer spheroid formation and spread.

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## Introduction

The most common mode of dissemination of ovarian cancer cells is via the peritoneal fluid (1), where free-floating ovarian cancer cells are found as multicellular spheroids. Cancer cells grown as spheroids are known to exhibit enhanced survival and increased resistance to anticancer drugs (2,3). To improve the efficacy of anticancer drugs, it is important to understand the mechanism of spheroid formation and to inhibit cells growing in this way.

During the progression of epithelial ovarian cancer, cancer cells invade the basement membrane to reach the underlying ovarian stroma. The sloughing of ovarian cancer cells from the ovarian surface results in altered production or enzymatic destruction of laminin and leads to the formation of ascites (4). In fact, loss of laminins in the basement membrane is a common early event associated with morphologic alteration and tumorigenicity of ovarian cancer cells (5). In addition, ascites fluids from patients with ovarian cancer have significantly higher levels of laminin than normal peritoneal fluid (6). Thus, altered production and/or enzymatic destruction of laminin by the aggressive ovarian cancer cells might play an important role in the formation of ovarian cancer spheroids.

Laminins are trimeric proteins that consist of α, ß and γ chains; tissue-specific sequence variations and processing of these chains have resulted in 15 identified isoforms. Laminin-1, composed of α1, ß1 and γ1 chains, is the most extensively characterized isoform. It promotes various biological activities including cell migration, adhesion and tumor growth. Using proteolytic laminin-1 fragments and laminin-1-derived synthetic peptides, several of the domains on laminin-1 capable of stimulating these cellular activities

have been identified (7). A 12-mer synthetic peptide, AG73, derived from the laminin α1 chain carboxyl-terminal globular domain (G domain), has been observed to promote cell adhesion, migration, invasion and gelatinase production (8). Furthermore, heparan sulfate/chondroitin sulfate-containing proteoglycans, such as syndecan, bind to AG73 as their cellular receptor (9-12). Syndecan-binding peptide AG73 plays an important role in the promotion of tumor metastasis in several tumor cell lines, including B16F10 murine melanoma cells and MDA-MB-231 breast cancer cells (13,14).

We have previously demonstrated that when ovarian cancerous cells were injected into the murine ovary with laminin-1 and peptide AG73 there was an increase in the volume of ascites and in the number of peritoneal and liver metastases (15). The effects of laminin-1 and this peptide have not been studied *in vitro* and the mechanism of its activity in ovarian metastasis promotion is not known. Here, we have studied the role of AG73 in regulating ovarian cancer spheroid formation using TAC3 cells (16).

We found that the individual addition of exogenous laminin-1 and AG73 stimulated the formation and growth of multicellular spheroids. The laminin-1-stimulated spheroids were disaggregated by the addition of AG73T peptide, which is a scrambled peptide of AG73. These disaggregated floating cells had increased sensitivity to cisplatin. Western blot analysis indicated that the added AG73 peptide suppressed expression of integrin ß1 in the presence of laminin-1, and also suppressed its downstream effectors, including Akt and extracellular signal-regulated kinase. These data suggest a novel function for AG73 in ovarian cancer.

## Materials and methods

*Synthetic peptides*. AG73 peptide (RKRLQVQLSIRT) from the C-terminal globular domain of the murine laminin α1 chain (residues 2719-2730) and the scrambled AG73T peptide (LQQRRSVLRTKI) were synthesized by the CBER Facility for Biotechnology Resources, FDA, Bethesda, MD. Matrigel and laminin-1 (11,12) were purchased from Trevigen, Inc. (Gaitherburg, MD).

*Cells and cell culture*. TAC3 cells were cultured in RPMI-1640 medium supplemented with 0.1 mg/ml gentamycin and 1% fungizone (Life Technologies, Inc., Rockville, MD). On uncoated dishes, the cells survived in medium and formed spheroids (16).

*Cell growth*. Growth curves were estimated by two methods: counting cells, and a cell viability assay based on quantification of ATP (16) for both adherent and non-adherent cells. For cell counting, $1.5x10^5$ cells were seeded into 35-mm dishes with 2 ml of RPMI-1640 medium. The number of viable cells in each dish was counted by the dye exclusion method every 24 h for 3 days. For the cell viability assay, we used a CellTiter-Glo cell viability assay kit (Promega Corp., Madison, WI) in accordance with the instruction manual. Briefly, $1.5x10^4$ cells were incubated in 100 µl of medium in 96-well micro-plates (BD BioCoat; BD Biosciences, Franklin Lakes, NJ), to which 100 µl of CellTiter-Glo reagent was added. After 2 min of mixing and 10 min of stabilization, luminescence was measured using a luminometer (Fusion; Packerd Instruments, Downers Grove, IL) every 24 h for 3 days.

*Attachment assays*. Cells were observed morphologically using a phase-contrast microscope. Attachment assays were performed using $1.5x10^5$ TAC3 cells in RPMI-1640 medium. Plates were blocked with 1% bovine serum albumin (BSA). Dose-response experiments used 0-200 µg of AG73T on laminin-1-coated dishes. Cells were incubated for 4 h at 37˚C. Unbound cells were decanted and attached cells were fixed, and the number of viable cells in each dish was counted by the dye exclusion method.

*Formation of TAC3 cell spheroids*. Multicellular aggregates of TAC3 cells were observed morphologically using a phase-contrast microscope. Multicellular aggregates measuring >250 µm in diameter were considered to be countable spheroids.

*Fluorescence-activated cell sorting (FACS) analysis*. To quantify the chemosensitivity of the TAC3 cells to cisplatin, FACS analysis was used to measure apoptosis with 0.5 µg of soluble laminin-1, or 5 µg of soluble AG73T peptides plus 0.5 µg of soluble laminin-1, for 24 h. Cells were plated and incubated overnight. The next day, the cells were treated with various peptides for 24 h in the presence of 10 µM cisplatin and then analyzed in the fluorescence-activated cell sorter (FACSort; Becton-Dickinson). In brief, floating cells and trypsinized cells were collected, washed with cold phosphate-buffered saline (PBS), and fixed in cold methanol (-20˚C) for 1 h. Subsequently, cells were centrifuged, resuspended in 0.5 ml cold PBS, and stained for at least 15 min with 50 µg/ml propidium iodide in the presence of RNase A (100 µg/ml). Cells were then analyzed in the FACSort. Five thousand events from the gated sub-population were recorded separately. FACSort was performed at least three times in triplicate.

*Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL)*. Cultures were fixed with 3% paraformaldehyde in PBS (pH 7.4) and permeabilized for 2 min with 0.1% Triton V-100 in 0.1% sodium citrate at 0˚C. TUNEL staining was performed with the In-situ Cell Death Detection Kit (Apotag) using fluorescein-dUTP for 24 h.

*Growth-inhibitory assay*. The anti-proliferative effects of the MEK inhibitor U0126 (2.5, 10, 50 µM) (Promega Corp.,) and the PI3-K inhibitor LY294002 (5 µM) for TAC3 were assayed. The concentrations of U0126 and LY294002 were chosen based upon published values demonstrating 50% inhibition for each of the kinases effectively without causing non-specific toxicity (16,17). Cells were inoculated on uncoated 96-well plates (for non-adherent conditions) in a volume of 50 µl at density of $1.5x10^4$ cells per well. U0126 and LY294002, dissolved in 50 µl of medium, were added immediately, and cells were cultured for 24 h. Luminescent cell viability assays (described above) were performed for assessment of growth rates.

*Western blot analysis*. Signal transduction by free-floating TAC3 cells was examined by Western blotting. Cells on 60-mm dishes were incubated with 30 $\mu$g of soluble AG73 peptide, AG73T peptide, or laminin-1, or 5 $\mu$g of soluble AG73T peptide plus 0.5 $\mu$g of soluble laminin-1. Cells were lysed in a buffer containing Tris-hydrochloric acid (50 mM, pH 7.4), sodium chloride (150 mM), Triton X-100 (1%), sodium deoxycholate (0.25%), EDTA (1 mM), phenylmethylsulfonyl fluoride (1 mM), aprotinin (10 $\mu$g/ml), leupeptin (10 $\mu$g/ml), sodium fluoride (25 mM), and sodium orthovanadate (1 mM). After the insoluble material was removed, the lysates were boiled in Laemmli sample buffer. Protein concentration was determined using a Bio-Rad RC-DC Protein Assay Kit (Bio-Rad, Richmond, CA). Lysates containing 20 $\mu$g of total protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane (Bio-Rad). Blots were blocked by either 1% BSA or 1% non-fat dry milk in PBS and were then probed with primary antibody. Protein bands were detected by a chemiluminescence system (ECL or ECL Plus, Amersham Pharmacia Biotech, Buckinghamshire, UK). The relative intensities of hybridization signals were quantified using a chemiluminescence imaging analyzer (Chemi Imager, Alpha Inotech Corp., San Leandro, CA).

*Antibodies*. Primary antibodies and dilution ratios were as follows: anti-ERK 1 (K-23, Santa Cruz), 1:200; anti-phospho-ERK (E-4, Santa Cruz), 1:200; anti-MEK-1 (12-B, Santa Cruz), 1:300; anti-phospho-MEK1/2 (Cell Siganaling Technology, Inc., Beverly, MA), 1:500; anti-integrin ß1 (E-10, Santa Cruz), 1:200; anti-syndecan-1,2 (S338) (Santa Cruz), 1:100; anti-PI3K (PharMingen, BD Bioscience, San Jose, CA), 1:1000; and anti-AKT(Y397) (Transduction Laboratories, BD Bioscience), 1:1000.

*Statistical analysis*. For quantitative analysis, the sum of the density of bands corresponding to the protein stained with the antibody under study was calculated and normalized to α-tubulin. Differences in cell number and protein levels were analyzed by the Kruskal-Wallis test. Differences were considered statistically significant at $P<0.01$.

## Results

*Free-floating TAC3 cell proliferation in the presence of various concentrations of AG73 peptide*. As shown in Fig. 1A, free-floating TAC3 cells showed stable growth and formed multicellular spheroids for 72 h. In the presence of 30 $\mu$g/ml of soluble AG73 peptide and laminin-1, the population-doubling times of TAC3 cells were approximately 21 and 27 h, respectively. On uncoated dishes, the population-doubling time of TAC3 cells was approximately 36 h.

These results indicate that the addition of exogenous laminin-1 and AG73 significantly promotes the growth of TAC3 cells, making spheroids. Interestingly, the addition of exogenous scrambled peptide (AG73T) inhibited the growth of TAC3 cells (result not significant) for 72-h incubation. These results suggest that AG73T has a biological effect on the growth of TAC3 cells.

*AG73 peptide enhanced activation of free-floating TAC3 cell signaling pathways*. Following the finding that the addition of exogenous laminin-1 and AG73 can stimulate the formation and growth of multicellular spheroids, we next determined whether cell surface receptors, involving syndecan-1, might be important for mediating cell attachment (9-12). Other cell-adhesive surface proteins, such as integrins, might play a role in this interaction and were also evaluated. Thus, Western blot analysis using sydecan-1 and -2 antibodies and different integrin antibodies (α1, αV, ß1 and ß3) was performed on TAC3 cell extracts. Syndecan-1 and integrin ß1 were enhanced in the TAC3 cells exposed to soluble AG73 peptide. With AG73, expression of syndecan-1 was more enhanced than integrin ß1. With laminin-1, expression of integrin ß1 was more enhanced than syndecan-1 (Fig. 1B). Other cell-adhesive surface receptors, such as integrin α1, αV, ß3 and syndecan-2, were not significantly affected (data not shown).

We studied changes in the activities of the PI3-kinase-Akt and the MEK-ERK pathways. The active forms of ERK and MEK were increased in the presence of soluble AG73 (~4-fold for p-ERK and ~5-fold for p-MEK) compared with TAC3 cells alone, and soluble laminin-1 (~2-fold for p-ERK and ~3-fold for p-MEK). There were significantly ($P<0.01$) higher amounts of active forms of ERK and MEK in the presence of soluble AG73 than with soluble laminin-1 (Fig. 1C). The PI3K-AKT survival signaling pathway was enhanced by either soluble laminin-1 (~3-fold for PI3K and ~5-fold for p-AKT) or soluble AG73 (~1.5-fold for PI3K and ~2-fold for p-AKT). There was significantly ($P<0.01$) higher expression of PI3K (~2-fold) and of active forms of AKT (~2.5-fold) in the presence of soluble laminin-1 than with soluble AG73 (Fig. 1D). Expression of the tumor suppressor PTEN was not changed by either laminin-1 or AG73 peptide. These results demonstrate that AG73 enhanced free-floating TAC3 cell activation of MAPK signaling via syndecan-1, and also had an effect on the PI3K-AKT survival signaling pathway.

*The effect of added soluble AG73T peptide on free-floating TAC3 cells in the presence of laminin-1*. As AG73T was shown to have a biological effect, we investigated the role of biological AG73T in TAC3 cells. We found that AG73T scrambled the peptide bound to free-floating TAC3 cells (Fig. 2A). To determine whether AG73T and TAC3 cells might be acting through similar binding sites to laminin-1, we investigated whether AG73T peptides could reciprocally inhibit attachment to laminin-1 and found that TAC3 cell attachment to laminin-1 was inhibited by ~30% by exogenously added soluble AG73T peptide at a concentration of 100 $\mu$g/ml (Fig. 2B).

As shown in Fig. 3, free-floating TAC3 cells showed stable growth for 72 h even in the presence of soluble laminin-1 (0.5 $\mu$g/ml) alone or with AG73T (5 $\mu$g/ml). Although the difference was not statistically significant, the growth rate of TAC3 cells was different in the two cases (soluble laminin-1 plus soluble AG73T, or laminin-1 alone).

We found that soluble AG73T peptide partially inhibited free-floating TAC3 cell attachment to laminin-1 and inhibited laminin-1 from promoting TAC3 cell growth, suggesting that

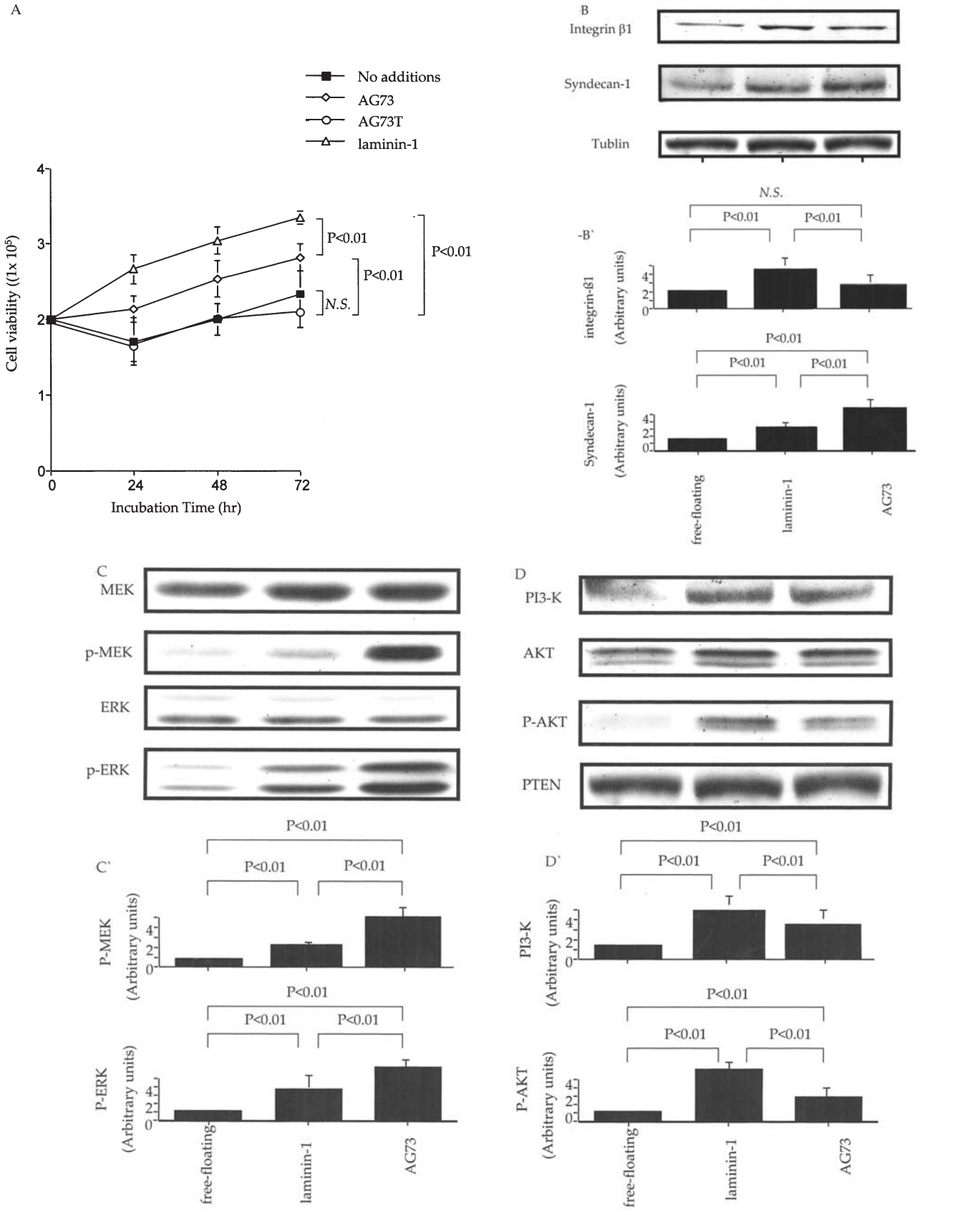


Figure 1. (A) Time course of the growth effects of AG73 and laminin-1 on free-floating cells. TAC3 cells were incubated with soluble laminin-1 (open triangles), soluble AG73T (open circles), and soluble AG73 (open squares) on 35-mm uncoated dishes. Non-adherent TAC3 cells were fixed and stained. Values are expressed as the mean of SD number of free-floating cells. (B-D) The expression of adhesion molecules and activation of signaling pathways involved in TAC3 cell proliferation and in survival. Expression of integrin ß1 and syndecan-1 (B), MEK, p-MEK, ERK, and pERK (C), and PI3-K, AKT, p-AKT, and PTEN (D) in TAC3 cells with soluble laminin-1, soluble AG73 peptide, and soluble AG73T peptide on uncoated dishes. Cells were incubated for 24 h in RPMI-1640 medium without serum. Cell lysates were prepared, and Western blot analysis was performed using specific antibodies. Densitometric tracing of integrin ß1 and syndecan-1 (B`), PI3-K and p-AKT (C`), and p-MEK and pERK (D`) are shown. Data are mean ± standard deviation of three independent measurements. The deviation of individual measurements from the mean did not exceed 5%.

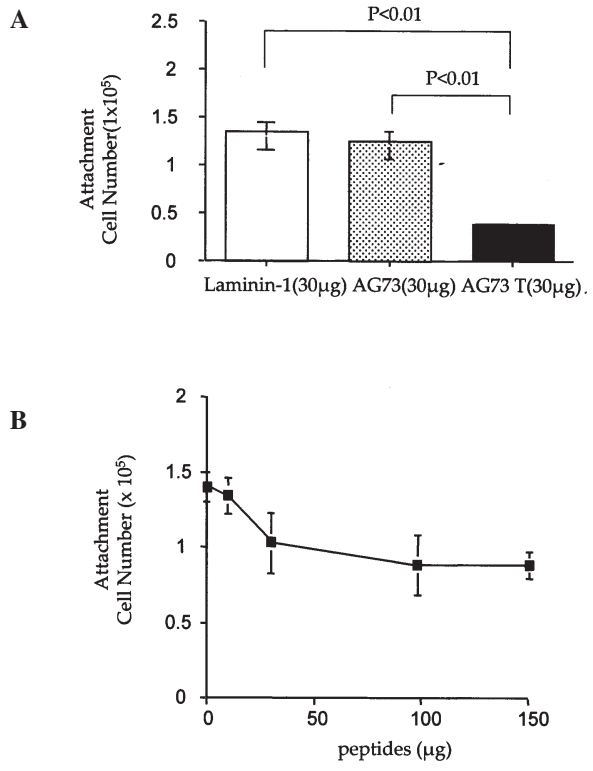


Figure 2. (A) TAC3 cells adhere to laminin-1, AG73, and AG73T. TAC3 cells were added to 35-mm dishes coated with laminin-1 (open), AG73 (shaded), or AG73T (closed) and allowed to adhere for 4 h. Non-adherent cells were rinsed away, and the remaining cells were fixed and stained. Values are expressed as the percentage of TAC3 cells that adhered to each substrate. Data are expressed as mean ± standard deviation for triplicate wells. (B) Inhibition of TAC3 cells adherent to laminin-1 by exogenously added soluble AG73T peptide. TAC3 cells were incubated for 4 h with 0, 10, 30, 100, or 150 $\mu$g/ml AG73 before being added to 35-mm dishes coated with 1 mg/ml laminin-1. Assays were performed in the continued presence of the exogenously added soluble AG73T peptide for 4 h. Non-adherent cells were rinsed away, and the remaining cells were fixed and stained. Values are expressed as the number of free-floating cells. Data from a representative experiment are shown as mean ± standard deviation for triplicate wells.

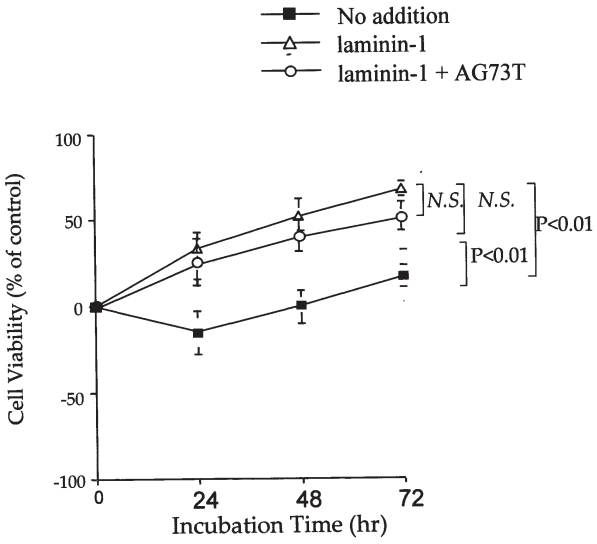


Figure 3. Growth promotion effects of laminin-1 and AG73 on free-floating cells. TAC3 cells were incubated without any peptides (closed squares) or with soluble laminin-1 (open triangles), soluble AG73 plus laminin-1 (open diamonds), or soluble AG73T plus laminin-1 (open circles) on 35-mm uncoated dishes every 24 h for 72 h.

the AG73T site functions as a TAC3 cell attachment site in laminin-1.

*AG73T disaggregated laminin-1 stimulated spheroid formation*. The addition of exogenous laminin-1 enhanced spheroid formation (Fig. 4A2). The laminin-1-stimulated TAC3 spheroid formation was disaggregated by the addition of AG73T peptide (Fig. 4A3). The number of laminin-1-stimulated TAC3 spheroids was significantly decreased by the addition of AG73T peptide (3 and 5 $\mu$g) for 48 h (Fig. 4B).

To quantify the sensitivity of TAC3 cells to cisplatin, FACS analysis was used to measure apoptosis. When cells were incubated in the presence of 10 $\mu$M cisplatin for 24 h, FACS analysis revealed that the incidence of apoptosis of TAC3 cells was elevated 2-fold in cells incubated in the presence of laminin-1 and AG73T compared with those incubated in the presence of laminin-1 alone (Fig. 4C). This pattern suggests that AG73T acts mainly by induction of apoptosis in laminin-1-treated TAC3 cells rather than suppression of growth in G2/M (DNA synthesis) (Fig. 4D1, 3). When TAC3 cells were fixed and stained by the TUNEL method, cells incubated in the presence of 10 $\mu$M cisplatin with AG73T plus laminin-1 showed an elevation in the number of apoptotic nuclei compared with laminin-1 alone. In general, the *in situ* labeling of cleaved DNA by laminin-1 plus AG73T or laminin-1 alone correlated well with the FACS analysis (Fig. 4D2, 4). We conclude that AG73T increases apoptosis of laminin-1-treated cells.

*AG73T inhibited activation of free-floating TAC3 cells in the presence of laminin-1 signaling pathways*. AG73T can affect growth and induce apoptosis in laminin-1-treated TAC3 cells, so we determined whether the addition of soluble AG73T changed the expression of cell surface proteins, such as syndecan-1 and integrin ß1 and signaling pathways that involved the PI3-kinase-Akt- and the MEK-ERK pathways. Western blot analysis indicated that addition of AG73T peptide in the presence of laminin-1 enhanced expression of syndecan-1 but suppressed expression of integrin ß1, and suppressed its downstream effectors including Akt and extracellular signal-regulated kinase (Fig. 5). The active forms of ERK were also changed in the free-floating TAC3 cells with any soluble peptides. However, there were significantly higher (>2-fold; P<0.01) amounts of active forms of PI3K in the presence of soluble laminin-1 alone than with soluble laminin-1 plus AG73T (Fig. 5). These results demonstrate that AG73T partially inhibits activation of the PI3K-AKT survival signaling pathway through decreased integrin ß1 on laminin-1-treated ovarian cancer free-floating cells.

*Signaling activity*. The pharmacologic MEK inhibitor U0126 reduced free-floating TAC3 cell growth and ERK phosphorylation (P<0.01) in the presence of laminin-1 plus AG73T in a concentration-dependent manner. U0126 was more effective with laminin-1 plus AG73T peptide

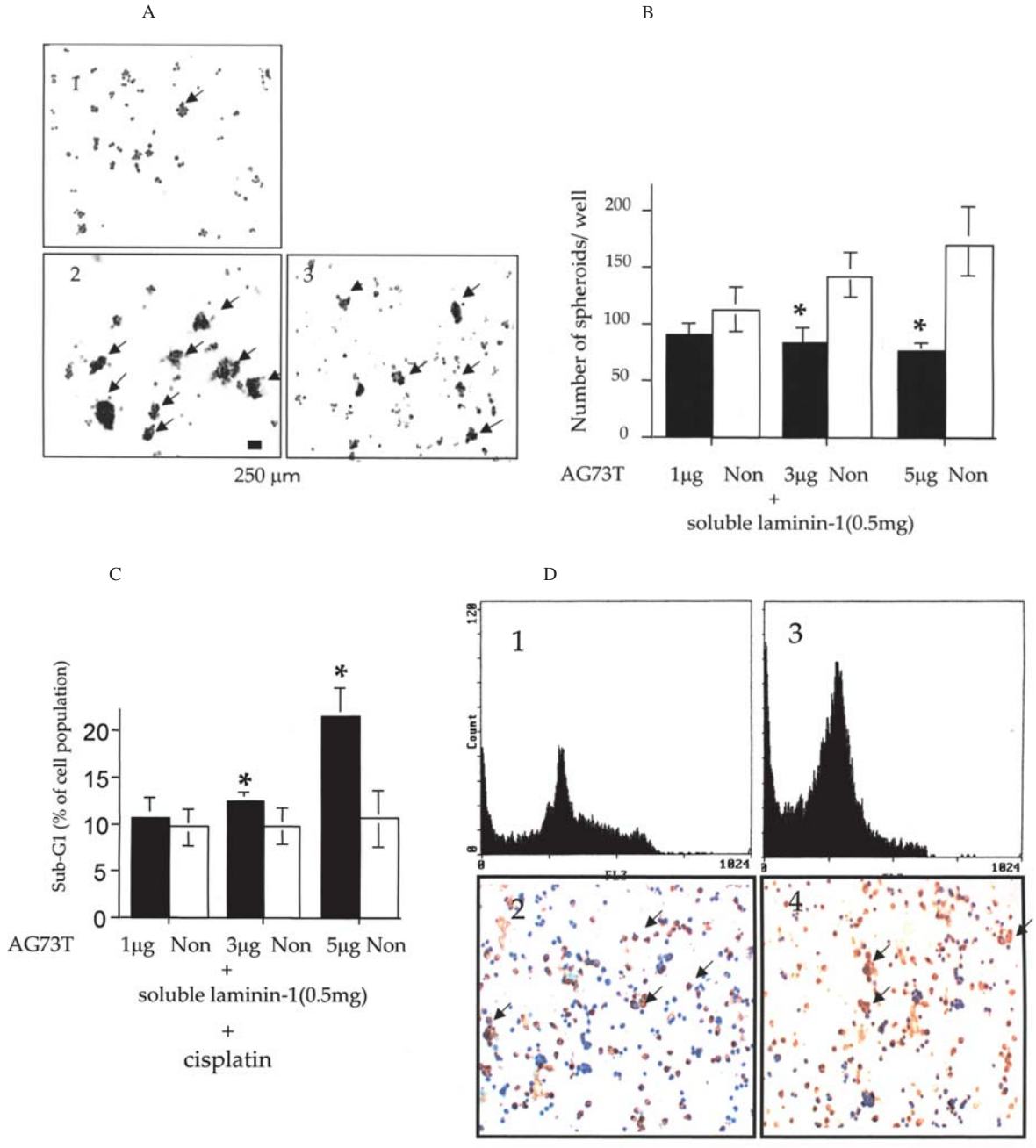


Figure 4. (A) TAC3 cells spheroid formation. TAC3 cells were cultured on plane 24-well dishes at a density of 20,000 cells/well for 48 h then photographed. On plain culture dishes, TAC3 cells formed spheroids (1). TAC3 cell spheroid formation was augmented by soluble AG73 (2), and soluble laminin-1 plus AG73T (3). Arrows indicate spheroids. Scale bar, 250 $\mu$m. (B) TAC3 cells were cultured on plane 24-well dishes at a density of 20,000 cells/well for 48 h then photographed and the number of spheroids counted. TAC3 cells were incubated with laminin-1 (open bar), and soluble AG73T plus laminin-1 (closed bar) on 35-mm uncoated dishes up for 48 h. Values are expressed as the number of free-floating cells. *P<0.01 (C) Induction of TAC3 cell apoptosis. TAC3 cells were incubated in the presence of cisplatin with soluble laminin-1 (open bar) and soluble AG73T plus laminin-1 (closed bar) on 35-mm dishes for 24 h. Cells were fixed with methanol, stained with propidium iodide, and examined by FACS. Data from a representative experiment are expressed as mean ± standard deviation for triplicate wells. *P<0.01. (D) The data in (D) 1,3 are expressed as the sub-G1 population, which serves as an index for the incidence of apoptosis. TAC3 cells were incubated in the presence of cisplatin with soluble laminin-1 (1,2) and soluble AG73T plus laminin-1 (3,4) on 35-mm dishes for 24 h. Note the low incidence of apoptotic nuclei treated with laminin-1 and high incidence of apoptotic nuclei (brown stained, arrows) in cell cultures with laminin-1 plus AG73T peptides. Cells were fixed with 3% paraformaldehyde and stained for apoptotic nuclei using the TUNEL method (2,4).

than with laminin-1 alone (Fig. 6A). There was a significant inhibition of PI3 kinase with LY294002 when TAC3 cells were treated in the presence of laminin-1 alone; in the presence of laminin-1 with AG73T, the inhibition of PI3-kinase and AKT phosphorylation was more dramatic (P<0.01) (Fig. 6B). Our data show that when TAC3 cells are treated with AG73T, the PI3-kinase-AKT pathway is sufficient by itself to support survival of TAC3 cells, and the MEK-ERK pathway is not required.

## Discussion

Our data demonstrate that the addition of exogenous laminin-1 and AG73 individually stimulated the formation and growth

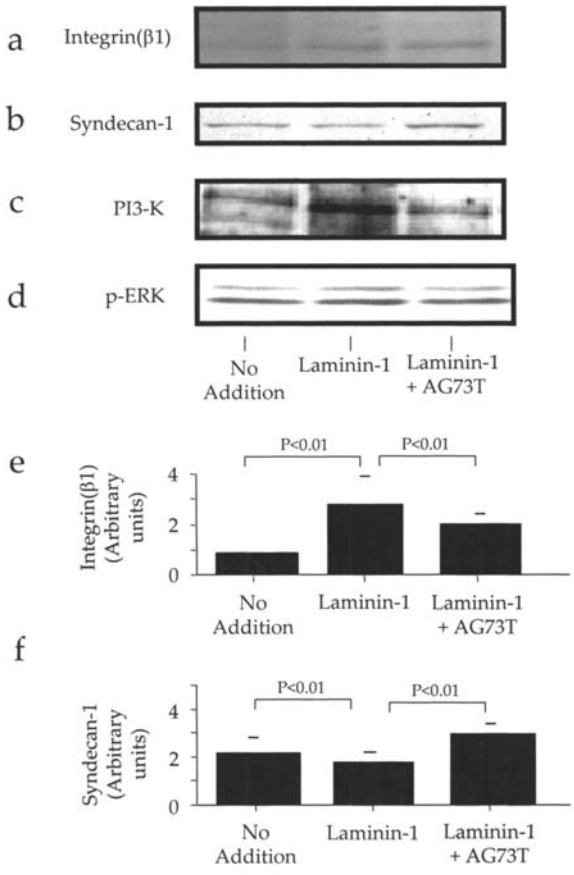


Figure 5. Expression of adhesion molecules and activation of signaling pathways involved in the control of cell proliferation and survival in TAC3 cells. Expression of integrin ß1 and syndecan-1 (a), MEK, p-MEK, ERK, and pERK (b) effect of AG73 on ERK phosphorylation status (c), and PI3-K (d) in TAC3 cells without any peptide, soluble laminin-1, soluble AG73 plus laminin-1, and soluble AG73T plus laminin-1. Cells were incubated in each case for 24 h in RPMI-1640 medium without serum. Cell lysates were prepared, and Western blot analysis was performed using specific antibodies to integrin ß1, syndecan-1, and phosphorylated ERK and PI3-K. Densitometric tracing of PI3-K (e) and pERK (f) are shown. Data are mean ± standard deviation of three independent measurements. The deviation from the mean of individual measurements did not exceed 5%.

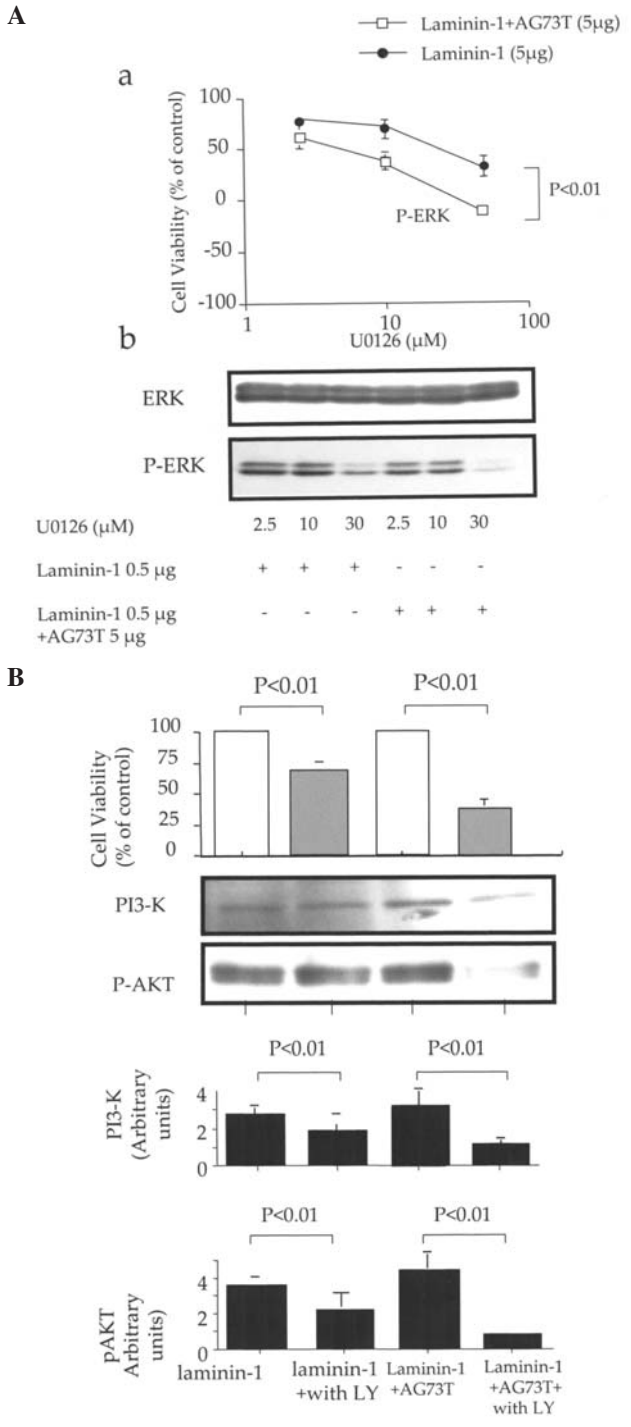


Figure 6. (A) (a) Anti-proliferative effect of U0126 on TAC3 with soluble laminin-1 (solid circles) and with soluble laminin-1 plus AG73T (open squares). U0126 was added at the indicated concentrations. The DMSO (solvent) concentration was adjusted to 0.2%. Cell proliferation was assessed by measurement of the increase in viability. One hundred percent represents the growth rate under U0126-free conditions (0.2% DMSO), and 0% indicates that cell viability did not change during incubation. Values represent the mean of three separate experiments; bars indicate standard deviation. (b) The phosphorylated and total levels of ERK, assessed using a phosphospecific and general antibody and Western blot analysis on lysates from cells treated with 2.5, 10, or 50 $\mu$M U0126 in the presence or absence of AG73T peptides with laminin-1. (B) Effect of AG73T peptide on PI3-K and AKT phosphorylation levels. PI3-K and phosphorylation of AKT were determined by Western blot analysis using a phosphospecific antibody on TAC3 cell extracts after treatment with soluble laminin-1 with and without AG73T in the presence (solid bars) or absence (open bars) of LY294002 (LY). Densitometric tracing of PI3-K and p-AKT are shown. Data are mean ± standard deviation of three independent measurements. The deviation from the mean of individual measurements did not exceed 5%.

of multicellular spheroids. The TAC3 cell spheroid formation was augmented by soluble laminin-1, but the laminin-1-stimulated spheroids were disaggregated by the addition of scrambled AG73T peptide. These disaggregated cells have increased susceptibility to cisplatin, and suppressed integrin ß1 downstream effectors, including Akt and extracellular signal-regulated kinase. These results suggest that AG73T might improve the efficacy of chemotherapeutic drugs by disaggregating spheroids of ovarian cancer cells.

Free-floating ovarian cancer cells are found in malignant ascites as multicellular spheroids. Although Casey *et al* have shown that the addition of exogenous laminin enhanced spheroid formation, and ß1 integrins play an important role in ovarian cancer spheroid formation (18), the biological mechanisms by which the spheroids form have not been defined. The integrin family is the most well-documented family of adhesion molecules for cell growth, differentiation, and survival. ß1 integrin activation favors cell survival, and adhesion via ß1 integrins might protect cancer cells from

apoptosis (19,20). The direct downstream targets of integrin activation that have been implicated in mediating an anti-apoptotic phenotype include the activation of AKT and ERK (19,20). Aoudjit and Vuori demonstrated that adhesion of breast cancer cells to laminin results in resistance to anti-cancer drugs, which correlates with a 2-fold increase in AKT activation (20). Furthermore, treatment of cells with the PI-3 kinase inhibitor reversed resistance.

In this study, the addition of exogenous laminin-1 stimulated spheroid formation and cell proliferation via enhanced integrin ß1, and activated its downstream effectors, including the PI3-K/Akt and MEK/ERK pathways, which has been associated with resistance to anticancer chemotherapeutic drugs.

Laminin-1 contains three chains (α1, ß1 and γ1). The laminin α1 chain interacts with several integrins through the N terminus and C-terminal triple-helical region. Peptide AG73 (RKRLQVQLSIRT, residues 2719-2730) from laminin α1 LG4 binds to syndecans, membrane-associated heparan sulfate proteoglycans. Syndecans have multiple functions and are involved in cell-ECM interactions, cell motility, and focal adhesion assembly (21,22). The syndecan-binding peptide AG73 plays an important role in the promotion of tumor metastasis in several tumor cell lines, including B16F10 murine melanoma cells, MDA-MB-231 breast cancer cells, and ovarian cancer (13-15). Recently, Davies *et al* indicated that stromal cell syndecan-1 may play a role in mediating migration and invasion of host stromal cells in ovarian adenocarcinoma and, thus, support and maintain tumor growth (23-25). AG73-containing proteolytic fragments of laminin may be generated by the proteases from tumor cells, and such fragments may influence spheroid formation via syndecan-1. AG73 enhances syndecan-1 and causes more activation of the MEK/ERK pathway than the PI3-K/Akt pathway.

Ascitic spheroids display resistance to anoikis, a form of apoptotic cell death that occurs upon loss of matrix attachment. Although there are many pathways that contribute to provide protection from anoikis, the ERK pathway is important. There is a growing consensus that an absence of signaling through the ERK pathway, through loss of integrin engagement, contributes to anoikis. This is consistent with ERK promoting survival against a variety of apoptotic stimuli, such as anti-cancer drugs (26,27). Danilkovitch *et al* reported a partial role for the ERK pathway in providing protection from anoikis (28). In epithelial cells, both the ERK and PI3-kinase pathways have an anti-apoptotic effect; these are independent of one other (29).

In this study, ovarian cancer cell spheroid adhesion to laminin-1 was inhibited by ~30% on the addition of exogenous scrambled AG73T peptide. Furthermore, ovarian cancer cell spheroid formation was increased by soluble laminin-1, but laminin-1-stimulated spheroids were disaggregated by the addition of scrambled AG73T peptide. Induction of apoptosis by cisplatin was increased in the disaggregated spheroids (2-fold compared with laminin-1-stimulated spheroids). Multicellular ovarian cancer spheroids resist anticancer drugs better than monolayers (2,18). Burleson *et al* suggested that the decreased proliferation of ovarian cancer cells in spheroids compared with monolayers might explain this different response to anticancer drugs (2). In this study, there was no significant difference in cell growth in the presence of laminin-1 with or without scrambled AG73T peptide. Cells incubated with cisplatin and soluble AG73T plus laminin-1 were disaggregated and showed an elevation in the number of apoptotic nuclei compared with those incubated with laminin-1 alone.

We suggest that the scrambled AG73T peptide might play a dominant-negative inhibitory role in laminin-1-stimulated spheroid formation and growth. Scrambled AG73T peptide can bind directly to syndecan-1 and suppress its downstream effectors, including Akt and extracellular signal-regulated kinase, via suppression of integrin ß1 in laminin-1-stimulated multicellular spheroids. In the case of ovarian cancer cells, scrambled AG73T peptide in combination with cisplatin or with other anticancer drugs may function as an improved therapeutic agent.

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