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RESEARCH PAPER

Versican induces a pro-metastatic ovarian cancer cell behavior which can be inhibited by small hyaluronan oligosaccharides

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Abstract The assembly of pericellular matrix containing hyaluronan (HA) and versican has been shown to be a pre-requisite for proliferation and migration of mesenchymal cells. In this study, we investigated whether treatment with recombinant versican could induce the formation of a pericellular matrix by ovarian cancer cells (OVCAR-3, OVCAR-5, and SKOV-3) and promote their motility, invasion, and adhesion to peritoneal cells in vitro. We also determined whether versican-induced pericellular matrix formation and metastatic cancer cell behavior could be blocked by small HA oligosaccharides. Only combined treatment with recombinant versican and HA resulted in pericellular matrix formation by OVCAR-5 and SKOV-3 but not by OVCAR-3 cells, which lack the HA receptor, CD44. The motility of OVCAR-5 and SKOV-3 cells was significantly increased in scratch wound and chemotaxis assays following treatment with recombinant versican and HA. Versican and HA also promoted invasion of SKOV-3 and OVCAR-5 cells but had no effect on OVCAR-3 cells. We have demonstrated that exogenous HA significantly increased OVCAR-5 and SKOV-3 adhesion to peritoneal cells but adhesion was not further increased by versican treatment. Small HA oligomers (6–10 disaccharides) were able to significantly block formation of pericellular matrix by OVCAR-5 cells, as well as the increased motility and invasion induced by recombinant versican. HA oligomers also significantly blocked OVCAR-5 adhesion to peritoneal cells both in the presence and absence of exogenous HA. The dependence of CD44 for the versican and HA mediated effects were demonstrated by the inhibition of pericellular matrix formation as well as motility and invasion of OVCAR-5 cells following treatment with CD44 neutralizing antibody in the presence of versican and HA. We conclude that the acquisition of a HA/versican pericellular matrix by ovarian cancer cells increases their metastatic potential. HA oligomers can block this mechanism and are promising inhibitors of ovarian cancer dissemination.

Keywords Extracellular matrix · Versican · Hyaluronan · CD44 · Motility · Invasion

Abbreviations

BSA	Bovine serum albumin
CM	Conditioned media
ECM	Extracellular matrix
EHS	Engelbreth-Holm-Swarm
FBS	Fetal bovine serum
FIGO	Federation of Gynecologist and Obstetricians
ERK	Extracellular signal-regulated kinase
HA	Hyaluronan
Hase	Hyaluronidase
MAPK	Mitogen-activated protein kinase
PI 3	Phosphatidylinositol 3

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Introduction

Ovarian cancer is the most lethal gynecological cancer and ranks as the fifth most common cause of cancer-related death in women in the Western world [1, 2]. It has been estimated that there will be 21,550 new cases of ovarian cancer and 14,600 deaths due to ovarian cancer in the United States in 2010 [2]. Furthermore, the mortality rate due to ovarian cancer has been reduced by only 4% between 1991 and 2005 [2]. Significant improvements in ovarian cancer survival will therefore require the development of more effective molecularly targeted diagnostics and/or therapeutics.

Ovarian cancer is believed to spread by detaching from the surface of the ovary and attaching to and invading the peritoneum which lines the organs of the abdominal cavity [3]. Once ovarian cancer cells adhere to the peritoneum, they can migrate through the peritoneal cell layer and invade local organs. There is increasing evidence to suggest that extracellular matrix (ECM) components play an active role in tumor progression and are an important determinant for the growth and progression of solid tumors [4, 5]. Tumor cells are known to interfere with the normal programming of ECM biosynthesis and can extensively modify the structure and composition of the matrix [6]. Alterations in the extracellular environment are critical for tumor initiation and progression and intra-peritoneal dissemination [7–16].

Hyaluronan (HA) is a large polysaccharide which is assembled into pericellular and ECM matrices in many tissues [17]. HA plays a role in various cell functions such as adhesion, motility, and differentiation and has also been implicated to play a key role in cancer metastasis [17, 18]. Many human tumors, including ovarian cancer, are surrounded by a connective tissue matrix enriched with HA [11, 19, 20]. Increased HA has been shown to be an independent predictor of ovarian cancer survival. Its levels significantly correlate with the degree of invasiveness and metastatic potential in ovarian cancer tumors [11, 21] and it promotes attachment of cancer cells to peritoneal cells via interactions with the HA receptor, CD44 [22–24].

Increased expression of the HA-binding proteoglycan, versican, has been identified to be significantly involved in ovarian cancer metastasis [25, 26]. Versican is also a candidate marker for the early detection of ovarian cancer [27]. Versican is a member of the lectican family of proteoglycans also known as large aggregating proteoglycans and hyalectans which also includes aggrecan, neurocan, and brevican [28, 29]. Elevated versican levels are associated with cancer relapse and poor patient outcome in breast, prostate, and many other cancer types including ovarian cancer [12, 29–38]. Elevated levels of versican have been observed in primary ovarian tumors and secondary metastases when compared with normal ovaries [39] and increased by three fold in ovarian cancer tissues when compared with normal ovarian surface epithelial cells [40]. In ovarian cancer, increased versican stroma staining was associated with advanced FIGO (International Federation of Gynaecologists and Obstetricians) stage, large residual tumor, serous histologic type, and reduced patient survival [12].

The assembly of pericellular matrix rich in HA and versican is a prerequisite for proliferation and migration of mesenchymal cells [41] and has recently been shown to promote the motility of prostate cancer cells [42]. In this study, we investigated whether treatment with recombinant versican and HA could induce the formation of a pericellular matrix around ovarian cancer cells (OVCAR-3, OVCAR-5, and SKOV-3) and promote their motility, invasion and adhesion to peritoneal cells, key processes in ovarian cancer metastasis. Furthermore, we investigated whether small HA oligomers, previously shown to inhibit tumor growth [43–45], could be used block pericellular matrix formation and versican effects on ovarian cancer cell metastatic behavior.

Materials and methods

Cell culture

The human ovarian cancer cell lines OVCAR-3 and SKOV-3 were obtained from American Type Culture Collection (ATCC, Manassas, VA). OVCAR-5 cells were obtained from Dr. Stephen Williams (Fox Chase Cancer Center, Philadelphia, PA, USA). All ovarian cancer cell lines were maintained in RPMI 1640 medium supplemented with 4 mM L-glutamine, antibiotics (100 U penicillin G, 100 μg/ml streptomycin sulfate and 0.25 μg/ml amphotericin B, Sigma–Aldrich, St Louis, MO, USA). OVCAR-3 and SKOV-3 cells were supplemented with 5% FBS (Sigma–Aldrich) whilst OVCAR-5 cells were supplemented with 10% FBS and 7.5 μg/ml insulin. All cell lines were maintained at 37°C in an environment of 5% CO_2.

Versican purification

Versican was isolated from the CM from CHO cells transfected to overexpress V1 versican (CHO V1) kindly provided by Prof R LeBaron (University of Texas at San Antonio) [46] using a combination of anion exchange and gel filtration chromatography as described previously [42]. Versican-containing fractions detected by dot blot using the 12C5 mouse monoclonal versican antibody (Developed by Dr Richard Asher and obtained from by the Developmental

Studies Hybridoma Bank, NICHD, University of Iowa, USA) were then pooled and concentrated 10–30 fold using Centriprep centrifugal filters (Amicon Bioseparations, Bedford, MA, USA) and Nanosep™ microconcentrators (Pall Gelman Laboratory, Ann Arbor, MI, USA) with M_r 50,000 and 300,000 cut-offs, respectively. The molecular integrity of the purified versican samples was determined by immunoblotting with 12C5 versican antibody using non-reducing conditions. Visualization was achieved by anti-mouse IgG peroxidase-conjugated secondary antibodies (Chemicon Australia, Sydney, Australia) with enhanced chemiluminescence (ECL, Amersham Biosciences, Little Chalfont, Buckinghamshire, UK) (Supplemental Fig 1a). Identical gels were stained using silver stain (BioRad, Hercules, CA, USA) to assess the purity of the versican fractions (Supplemental Fig 1b). Versican concentration was determined using an in-house ELISA assay as described previously [42].

Motility and invasion assay

Ovarian cancer cells (OVCAR-5, OVCAR-3, and SKOV-3) were trypsinized, washed, and resuspended at a concentration of 1×10^6 cells/ml in RPMI media containing 0.1% BSA and antibiotics, and labeled with calcein-AM (1 μg/ml, Invitrogen, Carlsbad, CA, USA) as described previously [47]. Cells were centrifuged and washed three times with 0.1% BSA in RPMI and subsequently resuspended at a concentration of 1×10^6 cells/ml with 0.1% BSA RPMI or rV1 versican (0–10 U/ml) and 20 μg/ml HA for 3 h at room temperature on a Nutator. Additional experiments were performed in the presence or absence of HA oligomers (HYA-OLIGO6-10, 250 μg/ml, North Star Bioproducts, Associates of Cape Cod, Inc, East Falmouth, MA, USA) or rat anti-CD44 (20 μg/ml, clone A020, Calbiochem, Merck, Darmsyadt, Germany) or control IgG immunoglobulins (Sigma–Aldrich). An aliquot (10 μl) of these cells (3×10^3 cells) from each treatment were mixed with 200 μl of a suspension of human red blood cells (10^7 cells/ml, 0.1% BSA in PBS) and allowed to attach in 48-well plates for 2 h at 37°C for assessment of pericellular matrix formation. For motility and invasion assays the cell suspension (50,000 cells/well in 50 μl) was added to either the top of uncoated 12-μm filter inserts (96-well plate, ChemoTx, Neuro Probe Inc, Gaitherburg, MD, USA) or to 12-μm filters coated with Geltrex (ECM matrix derived from Engelbroth-Holm-Swarm (EHS) tumors similar to Matrigel, 0.6 μl/well, 9 mg/ml, Invitrogen) containing 30 μl of 10% FBS RPMI as a chemoattractant in the lower chamber. Cells were allowed to migrate for 6 h at 37°C in an environment of 5% CO_2 in air. Non-migratory cells on the top side of the filter were gently removed with a moistened cloth and the fluorescence was measured as described previously [47].

Red blood cell exclusion assay

OVCAR-3, OVCAR-5, or SKOV-3 cells were plated into 48-well plates at low density (3000 cells/well) in RPMI medium containing 0.1% BSA (serum-free RPMI) and treated with CHO V1 CM, CHO K1 CM or purified rV1 versican (0–10 U/ml) and HA (20 μg/ml, high MW from human umbilical cord, Sigma Ulrich) for 3–24 h. To demonstrate the necessity for CD44 for pericellular matrix formation, cells were additionally treated with rat anti-CD44 antibody (20 μg/ml, clone A020, Calbiochem) or control IgG (20 μg/ml Sigma–Aldrich). Pericellular matrix formation was visualised by a red blood cell exclusion assay [42] using an inverted microscope with Hoffman interference optics (BX50, Olympus Australia, Sydney, Australia). The proportions of cells with a surrounding zone excluded by red blood cells were counted in 10 random fields. To demonstrate the dependence on HA for the formation of the pericellular matrix, cells were additionally treated with *Streptomyces* hyaluronidase (Hase, 10 U/ml, Sigma–Aldrich) for 30 min at room temperature.

Wound migration assays and time-lapse photography

Wound migration assays were performed using OVCAR-5 and SKOV-3 cells (2×10^4 cells/well) cultured in 8-well chamber slides (Nuclon™ Lab-Tek II Chamber slide, RS Glass Slide, Roskilde, Denmark) in 500 μl of 5% FBS RPMI for 3–4 days. The resulting confluent cell monolayers were wounded and washed to remove floating cells prior to treating with CHO K1 CM (control medium) or CHO V1 CM (~5 U/ml versican). Cell migration was monitored over a 24 h treatment period using an IX 81 microscope (Olympus, Australia) equipped with a 37°C incubator (Solent Scientific, Segensworth, UK) and aerated with 5% CO_2 in oxygen. Pericellular matrix formation and directional movement were assessed by particle exclusion assays (10^7 red blood cells/ml) and time-lapse photography over a 3 h period, respectively, using Analysis™ software (Soft Imaging System, Munster, Germany). The number of migrating cells, the direction of motion and the proportion of cells forming a pericellular matrix were estimated. CD44 and HA abundance and localization in motile ovarian cancer cells were determined as described previously after 24 h treatment with CHO V1 or CHO K1 CM [42].

Ovarian cancer cell adhesion assays

LP-9 peritoneal cells were plated at 16,000 cells/well in 96-well plates for 48 h. Confluent monolayers were washed with RPMI media containing 0.1% BSA and antibiotics for 30 min prior to the adhesion assays. Ovarian cancer cell lines (OVCAR-5 and SKOV-3) were trypsinized, washed, and

resuspended at a concentration of 1×10^6 cells/ml in RPMI medium containing 0.1% BSA and antibiotics, and labeled with calcein-AM (1 μg/ml) as described previously [47]. Cells were washed and mixed at a concentration of 100,000 cells/ml with PBS or HA (20 μg/ml) in the presence (CHO-V1) or absence (CHO-K1) of versican containing CM for 3 h at room temperature on a Nutator. Additional experiments were performed in the presence of HAse (10 units/ml) or increasing concentrations of HA oligomers (0–250 μg/ml). The cell suspension (80 μl) was added to each well of LP-9 cell monolayers, and cells were allowed to adhere for 8 min. LP-9 cell monolayers were subsequently washed three times with RPMI medium containing 0.1% BSA and antibiotics. Fluorescence of adhering cells was measured with excitation and emission filters at 485 and 520 nm, respectively using a fluorescent plate reader (Fluostar Galaxy, BMG Labtech Offenburg, Germany) as described previously [47].

Flow cytometry

Ovarian cancer cells (OVCAR-3, SKOV-3, and OVCAR-5) were trypsinized and blocked with 10% normal donkey serum for 30 min prior to incubating with rat anti-CD44 antibody (0.75 μg/ 5×10^5 cells, clone A020, Calbiochem) in 500 μl of 0.1% BSA RPMI for 1 h on a Nutator. Cells were washed 3 times with 0.1% BSA RPMI and subsequently incubated with 5 μg of donkey anti-rat-FITC in 500 μl 0.1% BSA RPMI for 30 min. Following extensive washes with 0.1% BSA PBS, cells were resuspended in 500 μl of 0.1% BSA PBS and 10,000 events were analyzed using a FACScan (BioRad) and FACSDiva v6.0 software (BioRad).

Statistical analyses

All analyses were performed using the SPSS 15.0 for Windows Software (SPSS Inc., Chicago, IL, USA). The one way ANOVA test, Student's *t*-test, and the Dunnett C post-hoc test were used to determine statistical significance between control and treatment groups. Statistical significance was accepted at $P < 0.05$.

Results

Versican induces pericellular matrix formation by ovarian cancer cells

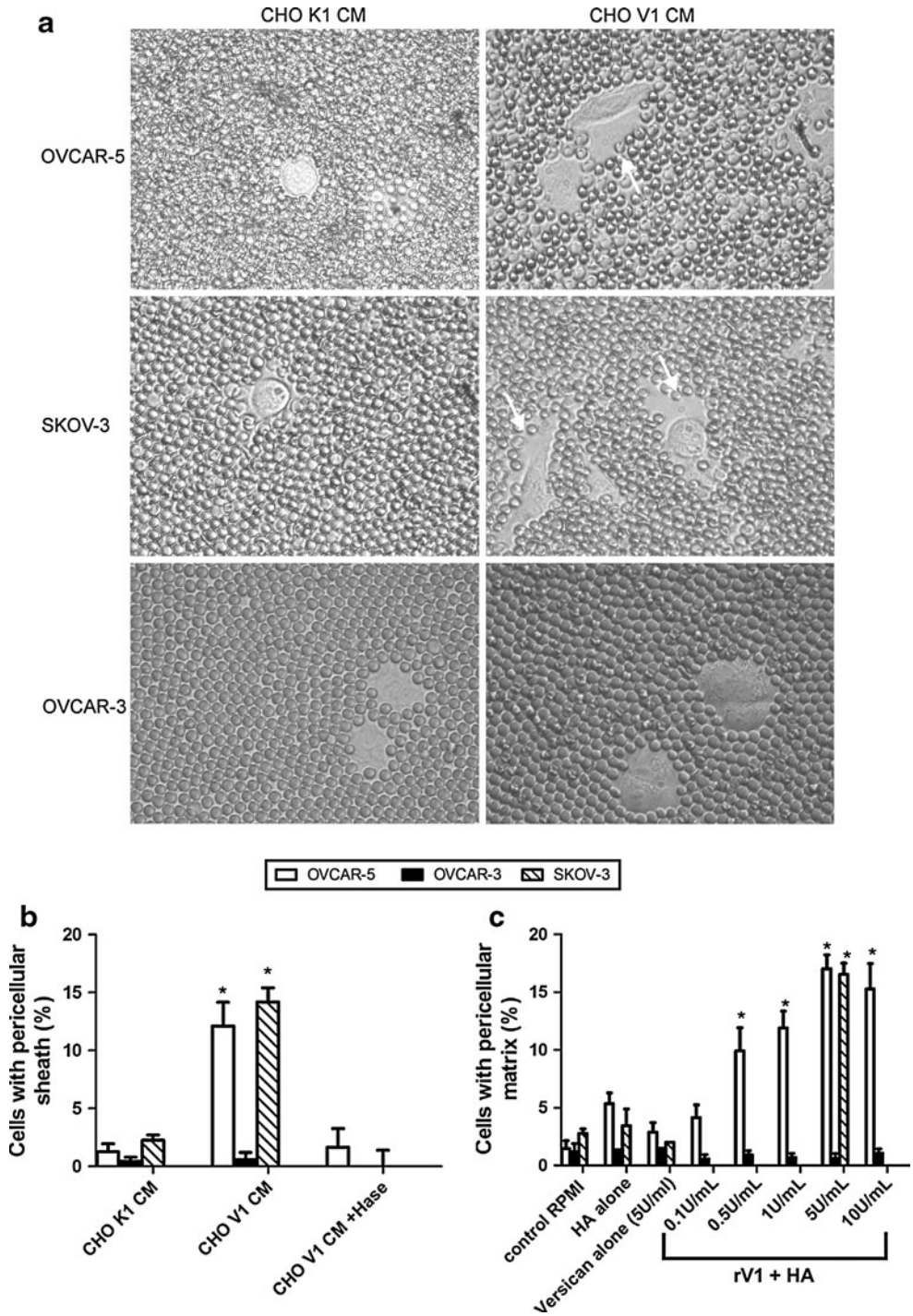
Negligible pericellular matrix formation was detected around ovarian cancer cells (OVCAR-3, OVCAR-5, and SKOV-3) under control conditions (CHO K1, control media) in the absence of versican (Fig. 1a, b, c). Treatment with either conditioned medium (CM) containing versican, CHO V1 (Fig. 1a, b) or purified recombinant versican (rV1)

Fig. 1 Versican promotes the formation of a pericellular matrix by ovarian cancer cells. **a** Pericellular matrix formation by ovarian cancer cells following 24 h treatment with versican was assessed using a particle exclusion assay. Ovarian cancer cells (3×10^3) were plated in 48-well tissue, cultured and treated with versican containing CHO V1 CM (~5 U/ml) or CHO K1 from parental cells not expressing versican. Red blood cell diameter = 7 μm. The *white arrows* illustrate OVCAR-5 cells and SKOV-3 cells with a prominent polar pericellular matrix. **b** Pericellular matrix formation by ovarian cancer cells following treatment with CHO V1 and CHO K1 CM. Structural necessity for HA within the pericellular matrix was confirmed by treatment with hyaluronidase (Hase) (30 min at room temperature, 10 U/ml). Data represent percentage (mean ± SD) of cells ($n = 100$) with pericellular matrix from triplicate determinations in three separate experiments. **c** Effect of purified rV1 versican on pericellular matrix formation by ovarian cancer cells. Ovarian cancer cells (OVCAR-5, OVCAR-3, and SKOV-3, 5×10^5 cells/ml) were treated with recombinant versican (0.1–10.0 U/ml) + HA (20 μg/ml) or PBS + HA (20 μg/ml) in 0.1% BSA RPMI on a rotating platform for 3 h at room temperature and plated in 48-well plates (3×10^3 cells) with red blood cells for 2 h prior to assessing pericellular matrix formation. Data represent percentage (mean ± SD) of cells ($n = 100$) with pericellular matrix from at least three determinations in three independent experiments. *Significantly different from control at $P < 0.05$ (Student *t* test)

(Fig. 1c) in the presence of exogenous HA resulted in increased pericellular matrix formation by OVCAR-5 and SKOV-3 cells but not OVCAR-3 cells. A 3 h treatment with increasing concentrations of purified rV1 versican and exogenous HA resulted in pericellular matrix formation by OVCAR-5 in a dose-dependent manner (Fig. 1c). Pericellular matrix formation was, however, not induced in the ovarian cancer cells following treatment with HA or versican alone (Fig. 1c). Maximal pericellular matrix was observed (18% of OVCAR-5 cells) following treatment with 5 U/ml rV1 versican. Treatment with 5 U/ml rV1 versican resulted in pericellular matrix formation in 17% of SKOV-3 ovarian cancer cells (Fig. 1c). Pericellular matrix formation was dependent on the presence of HA as treatment with hyaluronidase (Hase 10 U/ml) almost completely removed the pericellular matrix around OVCAR-5 and SKOV-3 cells (Figs. 1b and 2a). The ability of ovarian cancer cells to form a pericellular matrix correlated with expression of the HA receptor, CD44. Membranous CD44 was detected in 27% of OVCAR-5 and 13.4% SKOV-3 but undetectable in OVCAR-3 cells by immunocytochemistry when plated at low density (Fig. 2b). By FACS analysis, up to 92% of OVCAR-5 and 82% of SKOV-3 cells were positively labeled with the CD44 antibody, whilst only 3.6% of OVCAR-3 cells were CD44 positive (Supplementary Fig. 2).

Versican promotes ovarian cancer cell motility and invasion

We investigated the effects of rV1 versican on ovarian cancer motility and invasion using a modified chemotaxis assay. rV1 versican treatment for 9 h significantly increased the motility



of ovarian cancer OVCAR-5 and SKOV-3 but not OVCAR-3 cells. rV1 versican treatment significantly increased OVCAR-5 and SKOV-3 motility (Fig. 3a). Maximal effects of rV1 on OVCAR-5 cell motility were observed at 1 U/ml rV1 versican (125% of control) (Fig. 3a). rV1 versican also significantly increased invasion of OVCAR-5 and SKOV-3 cells through EHS basement membrane proteins (Geltrex) by up to 20% (Fig. 3b, $P < 0.0001$). Maximal effect on OVCAR-5 cell invasion (119% of control) was observed with 5 U/ml rV1 versican. rV1 versican treatment did not, however, affect OVCAR-3 cell motility (Fig. 3a) or invasion (Fig. 3b).

Pericellular matrix formation in migrating ovarian cancer cells

A wound migration assay was used to examine effects on directional ovarian cancer cell motility following versican

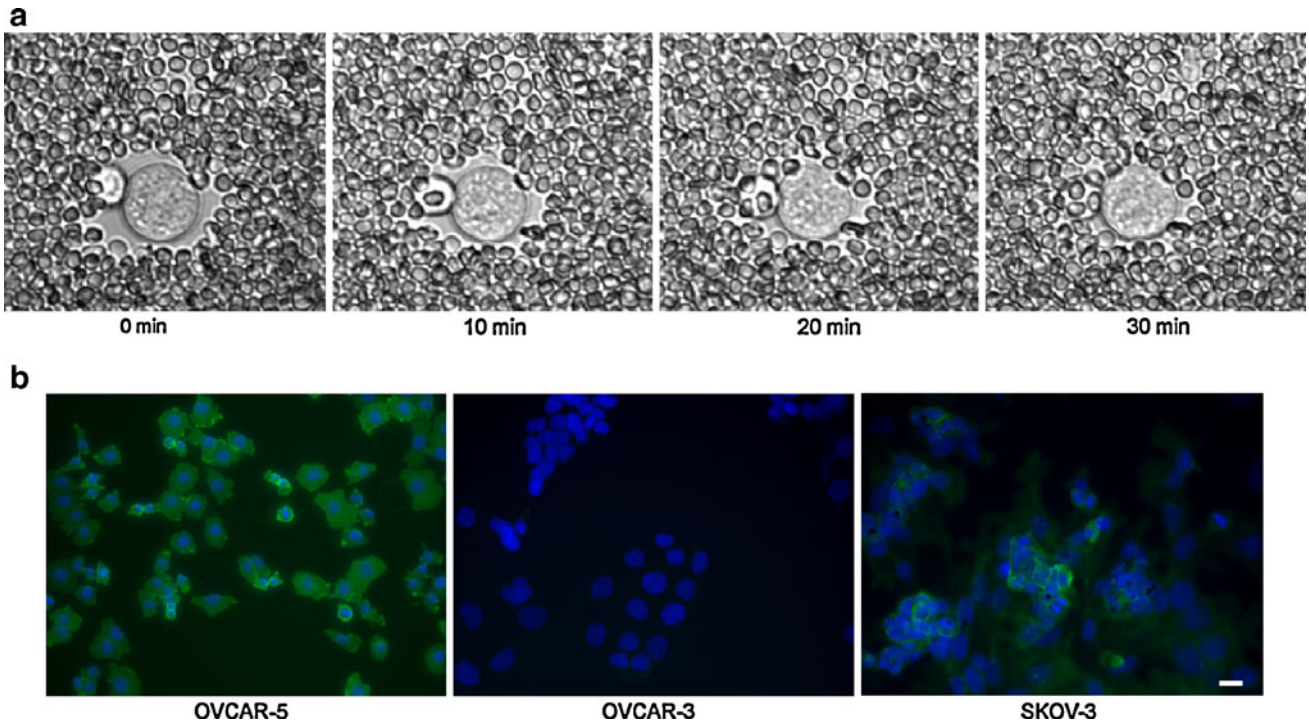


Fig. 2 Pericellular matrix formation by ovarian cancer cells is HA dependent and parallels CD44 expression. **a** Versican induced pericellular matrix formation was reduced following treatment with hyaluronidase (0–30 min at room temperature, 10 U/ml). **b** CD44 expression by ovarian cancer cell lines. Ovarian cancer cells (OVCAR-3, OVCAR-5, and SKOV-3) were plated at a low density in 8-well chamber slides. Cells were fixed and incubated with CD44 monoclonal antibody. Immunostaining was detected by immunofluorescence and nuclei are counterstained with Hoescht dye. Magnification *bar* = 20 μm

treatment. A 24 h treatment with CHO-V1 CM (5 U/ml versican) and HA (20 μg/ml) significantly increased the number of SKOV-3 and OVCAR-5 cells entering the wounded area by two fold compared with treatment with CHO K1 CM and HA (20 μg/ml) in the absence of any versican (Fig. 4a, b). Time lapse photography combined with a particle exclusion assay enabled simultaneous observation of directional cell movement and pericellular matrix formation. A polar pericellular matrix was consistently observed by motile SKOV-3 cells migrating into the scratch wound over a 2 h treatment period (Fig. 5a, white asterisks and Supplementary material 3). No pericellular matrix was observed in non-motile cells (Fig. 5a, black asterisks and Supplementary material 3). Interestingly, immunohistochemical localization of CD44 demonstrated polar expression of CD44 in SKOV-3 and OVCAR-5 cells following treatment with versican-containing media (Fig. 5b,c). Polarized CD44 was not observed in SKOV-3 cells treated with control media (Fig. 5b). Polarized CD44 and HA staining is illustrated in a motile OVCAR-5 cell following treatment with versican containing media but was not present in non-motile OVCAR-5 cells (Fig. 5c).

HA-oligomers and anti-CD44 can block pericellular matrix formation by OVCAR-5 and their motility and invasion induced by versican treatment

We investigated whether small HA oligomers (6–10 disaccharides), previously shown to block pericellular matrix formation around chondrocytes [48, 49], could inhibit pericellular matrix formation by OVCAR-5 cells and their motility and invasion induced by versican. Treatment with HA oligomers (250 μg/ml) significantly inhibited pericellular matrix formation by OVCAR-5 cells (Fig. 6a, $P < 0.0001$) and significantly reversed OVCAR-5 motility (Fig. 6b, $P = 0.007$) and invasion (Fig. 6c, $P = 0.013$) induced by versican treatment.

Additionally, we investigated whether direct blocking of CD44 with a neutralizing antibody would inhibit the formation of pericellular matrix formation as well as the motility and invasion induced by versican and HA treatment. We observed that the addition of 20 μg/ml of the neutralizing CD44 antibody completely blocked the formation of pericellular matrix by OVCAR-5 cells compared with control antibody treatment (Fig. 7a, $P < 0.0001$). The increased motility and invasion observed following

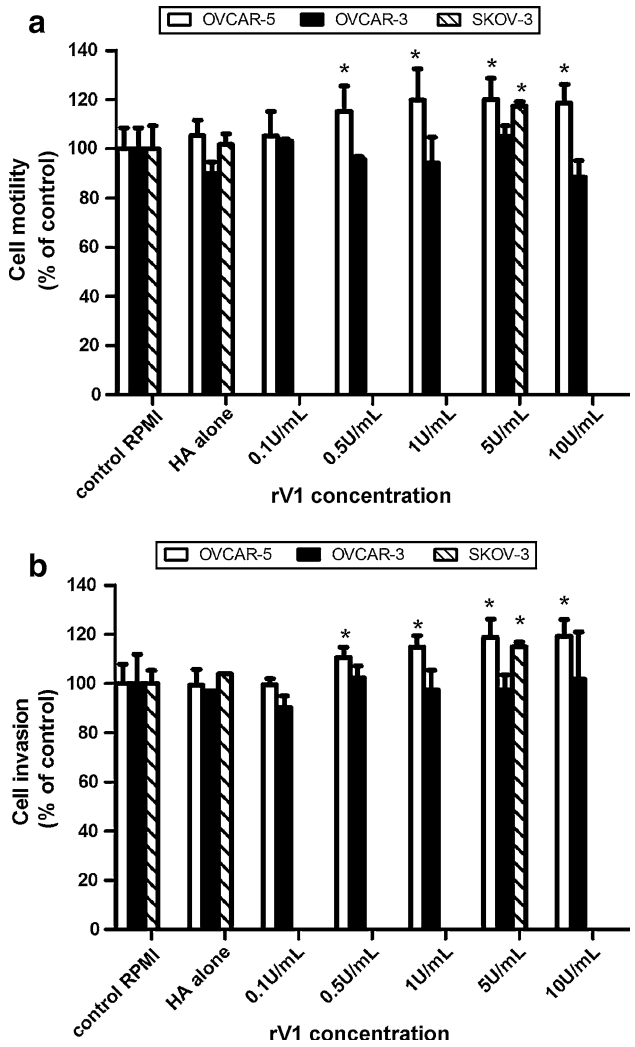


Fig. 3 Versican promotes ovarian cancer motility and invasion. Ovarian cancer cells (OVCAR-5, OVCAR-3, and SKOV-3) were treated as in Fig. 1c. A cell suspension (50,000 cells/well in 50 µl) was added to the top of uncoated 12-µm filter inserts for motility assays (**a**) or to 12-µm filters coated with Geltrex (0.6 µl/well, 9 mg/ml) for invasion assays (**b**). Cells were allowed to migrate for 6 h at 37°C in an environment of 5% CO_2 in air using 10% FBS in RPMI as a chemoattractant in the lower chamber. The fluorescence of migratory cells was measured at 485–520 nm. Data are expressed as percentage of control ± SD of three determinations from three independent experiments. *Significantly different from control at $P < 0.05$ (Student t test)

treatment of OVCAR-5 cells with versican and HA was also completely abrogated by the addition of 20 µg/ml of CD44 antibody (Fig. 7b; $P < 0.0001$ and 7c; $P = 0.029$) compared with treatment with control antibody.

HA oligomers inhibit ovarian cancer adhesion to peritoneal cells

Cell adhesion experiments were conducted to determine whether versican treatment could augment ovarian cancer cell adhesion to peritoneal cells (LP-9). Whilst HA significantly increased adhesion of OVCAR-5 and SKOV-3 cell to LP-9 cells (Fig. 6d, $P < 0.0001$), treatment with versican containing media (CHO V1) in the presence of HA did not have any additional effect on ovarian cancer cell adhesion (Fig. 6d). Treatment with Hase (10 U/ml) and HA oligomers (250 µg/ml) significantly reduced OVCAR-5 cell adhesion to LP-9 cells to below that of the control level in the presence and absence of exogenous HA (Fig. 6e, $P < 0.0001$).

Discussion

Our study demonstrates for the first time that ovarian cancer cells can assemble a pericellular matrix utilizing the ECM components HA and versican which promotes their motility and invasion in vitro. These findings indicate a role for versican and HA in ovarian cancer metastasis. Furthermore, we show that small HA oligomers (6–10 disaccharides) inhibit versican/HA pericellular matrix formation around CD44-expressing ovarian cancer cells. They also block the motility and invasion induced by versican and ovarian cancer adhesion to peritoneal cells. Our results therefore encourage further investigations into utilizing HA oligomers as a therapeutic to block ovarian cancer metastasis.

The formation of a HA pericellular matrix has been shown to be essential for breast and prostate carcinoma cells for specific adhesion to bone marrow endothelial cells and contributes to the common bone metastasis by these malignancies [50, 51]. We demonstrate the formation of a polar HA/versican pericellular matrix in motile ovarian cancer cells in the presence of both HA and versican but not HA or versican alone. These findings indicate that both versican and HA are required for pericellular matrix formation. Pericellular matrix formation is only observed in CD44 positive ovarian cancer cells and is particularly visible at the trailing edge of motile ovarian cancer cells. These findings are in agreement with our previous work demonstrating the formation of a polar HA/versican matrix in motile prostate cancer cells [42]. Furthermore, we demonstrate that versican treatment can induce ovarian cancer cell invasion through an ECM barrier. These findings are also supported by a recent study demonstrating that versican treated ovarian cancer cells have increased invasion potential [52]. Our results suggest that formation of a CD44/HA/versican macromolecular complex promotes the motility and invasion of ovarian cancer cells. The dependence of CD44 for the versican and HA mediated effects was confirmed by the inhibition of pericellular matrix formation as well as motility and invasion of OVCAR-5 cells following treatment with CD44 neutralizing antibody in the presence of versican and HA.

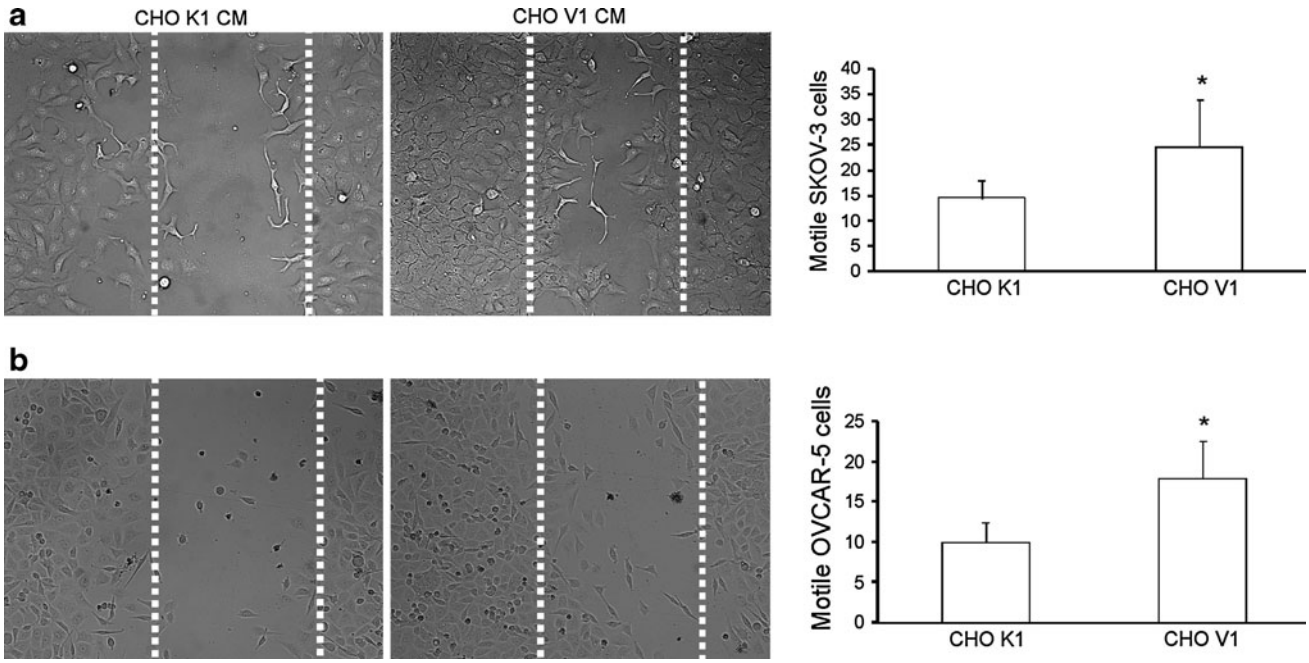


Fig. 4 Versican promotes ovarian cancer cell motility in a wound migration assay. Confluent monolayers of SKOV-3 cells (**a**) and OVCAR-5 (**b**) were wounded and treated with CHO K1 CM or CHO V1 CM for 24 h in presence of HA (20 μg/ml). Data represent number of cells which migrated into the wounded area (mean ± SD, eight determinations from two independent experiments). *Significantly different from CHO K1 at $P < 0.05$ (Student t test)

Our study verifies the observations of previous work which demonstrated that HA plays an important role in promoting cell attachment to peritoneal cells [22–24, 53]. However, versican treatment in the presence of HA did not further increase ovarian cancer cell adhesion to peritoneal cells. Our findings support the notion that adhesion of ovarian cancer cells to peritoneal cells is mediated by HA binding CD44 found on both cell types which allows a strong anchoring and interaction between ovarian cancer and peritoneal cells. Our working model proposes that versican from the peritumoral stroma binds HA in the ECM (Fig. 8). The formation of a stabilized HA/versican pericellular matrix surrounding ovarian cancer cells, protects the ovarian cancer cells against the mechanical forces in the peritoneal cavity and enables strong ovarian cancer cell adhesion to CD44 expressed by peritoneal cells. This provides the basis for subsequent ovarian cancer dissemination throughout the abdominal cavity.

The molecular mechanisms whereby a HA/versican pericellular matrix promotes the motility and invasion of cancer cells have not been elucidated in this study but are likely to involve signaling pathways activated by HA-CD44 interactions. HA-CD44 interactions have been shown to regulate several oncogenic pathways important for mediating motility and invasion of ovarian cancer cells [54–61]. The assembly of HA pericellular matrices by articular chondrocytes during joint formation has been shown to involve the activation of the MEK-ERK cascade [62]. More recently, p38 MAPK inhibitors have been shown to block HA pericellular matrix assembly in embryonic chicken joints [63]. It is conceivable that activation of p38 MAPK and ERK leading to the accumulation of HA-rich ECM may also play a role in cancer cell metastatic behaviour.

It is known that HA oligomers compete with larger HA polymers for CD44 binding [44, 64]. Whilst HA fragments in the 30–50 disaccharide range have been shown to be angiogenic [65], smaller HA fragments have been shown to inhibit tumor growth [43–45] and block the formation of a pericellular matrix formation around chondrocytes [48, 49]. Small HA oligomers in the 6–10 disaccharides range appear to have unique biological properties and can also inhibit a variety of tumors in vitro and in vivo [43, 44, 66–69]. Ghatak et al. [44] have demonstrated that HA oligomers can inhibit PI 3-kinase activity and phosphorylation of Akt and stimulate the expression of PTEN, a phosphatase which degrades the major signaling product of PI 3-kinase action, phosphoinositide 3,4,5-trisphosphate. It is therefore likely that HA oligomers inhibit tumor growth by suppressing the PI 3-kinase/Akt cell survival pathway [44, 66]. In our study, we have demonstrated that small HA oligomers (6–10 disaccharides) can inhibit the formation of HA-versican pericellular matrix around CD44 expressing ovarian cancer cells and the motility and invasion induced by versican treatment. Furthermore, these small HA oligomers also inhibited the increase in peritoneal ovarian

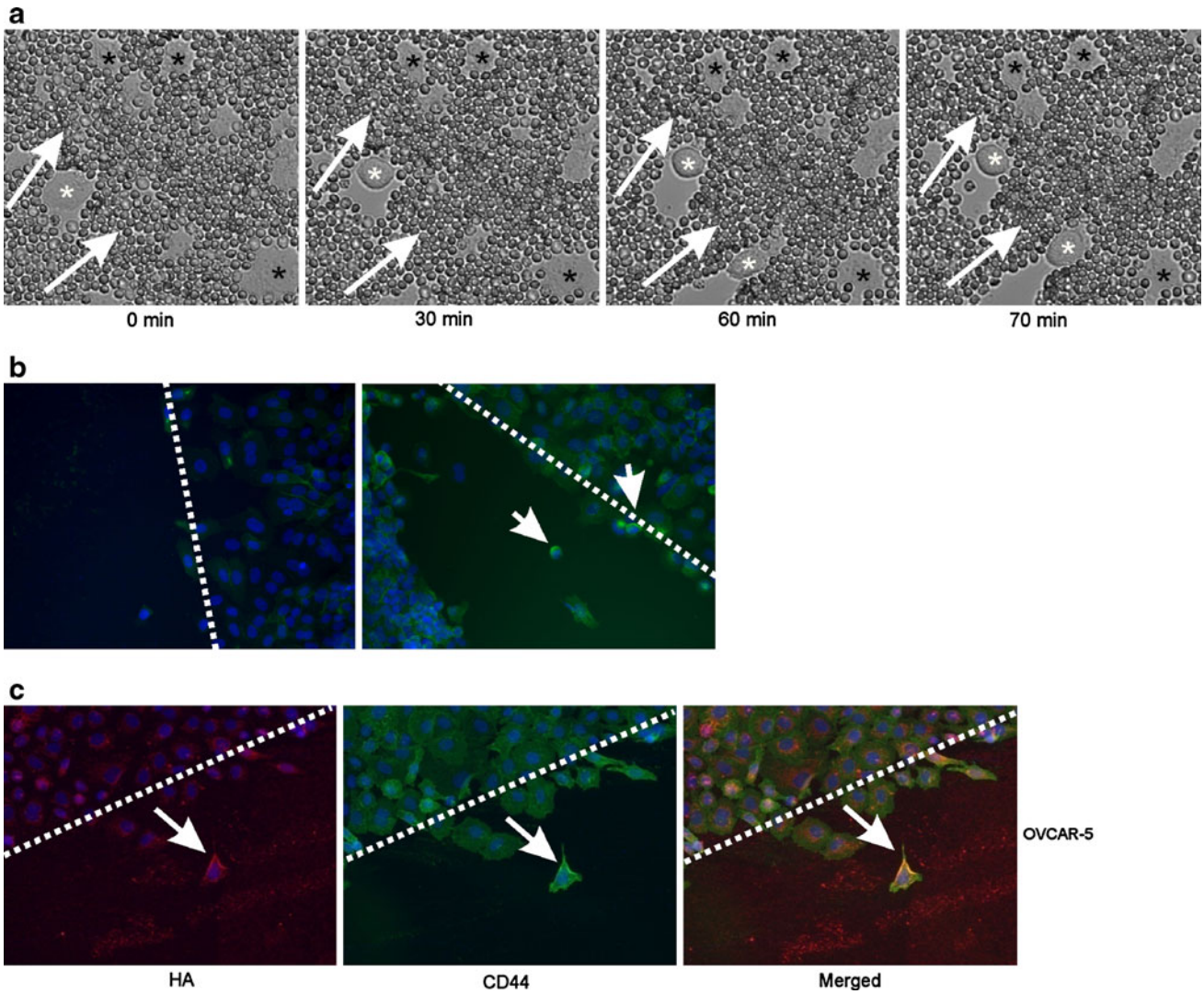


Fig. 5 Versican promotes formation of a polarized pericellular matrix by OVCAR-5 cells. **a** The confluent SKOV-3 monolayer was wounded and treated with CHO V1 CM (5 U/ml) for 16 h in the presence of HA (20 μg/ml). The *white asterisks* indicate motile SKOV-3 cell with a polar pericellular matrix observed over a 2 h time period. The *black asterisks* indicate non-motile SKOV-3 cell lacking a pericellular matrix. The *white arrows* indicate the direction of cell movement. Red blood cells diameter = 7 μm. **b** CD44 expression in SKOV-3 cells following 24 h treatment with HA (20 μg/ml) and CHO K1 or CHO V1 CM (~5 U/ml). *White dashed line* indicates edge of wound. *White arrows* indicate polar CD44 expression. **c** Co-localization of polar CD44 and HA in a motile OVCAR-5 cell following treatment with CHO V1 CM (~5 U/ml) in the presence of HA (20 μg/ml). *White dashed line* indicates the edge of the wound. *White arrows* indicate polar CD44 and HA expression

cancer adhesion induced by HA treatment and peritoneal ovarian cancer adhesion in the absence of HA. These findings suggest that HA oligomers act by blocking HA binding to CD44 on the peritoneal cells. A recent study demonstrated that HA octasaccharides inhibited the formation of pericellular matrix by osteosarcoma cells and reduced HA accumulation in local tumors, tumor growth, motility, invasion, and the formation of distant lung metastases [67].

More recently HA oligomers have also been shown to reverse chemotherapy resistance of myeloid leukemia [70] and lymphoma cell lines [71] and to increase chemotherapy sensitivity in multi-drug resistant malignant peripheral nerve matrix tumor [68] and ovarian cancer cells [72]. Our results support further investigations into the use of HA oligomers either alone or in conjunction with chemotherapeutic agents to block adhesion and metastasis of CD44 positive ovarian cancers. A weekly intra-peritoneal administration of HA oligomers (500 μg) appears to have been well tolerated by SCID mice xenografted with ovarian cancer cells [72]. Intra peritoneal administration of a bioconjugate of HA and paclitaxel was well tolerated and significantly decreased growth human ovarian cancer xenografts in SCID mice compared

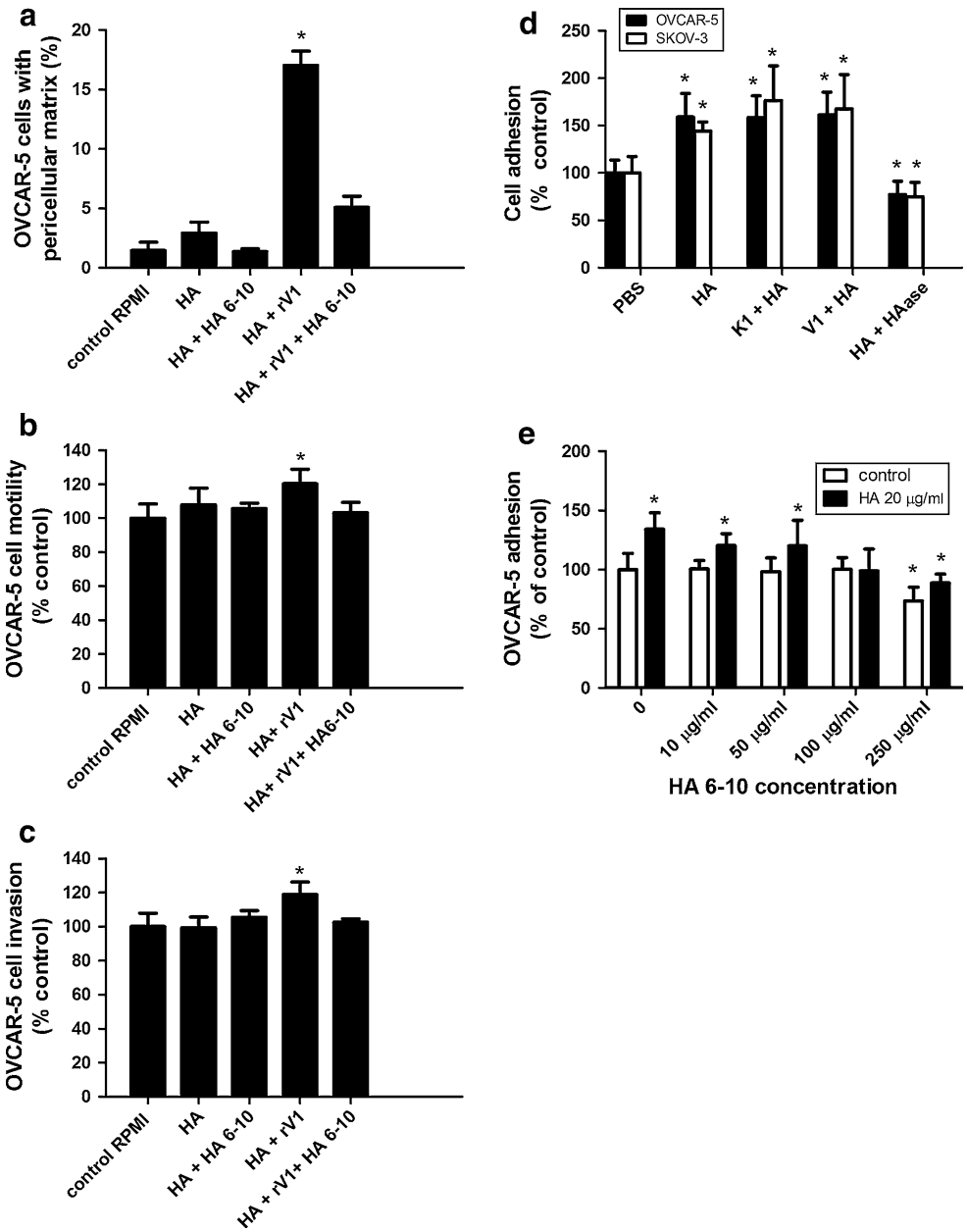
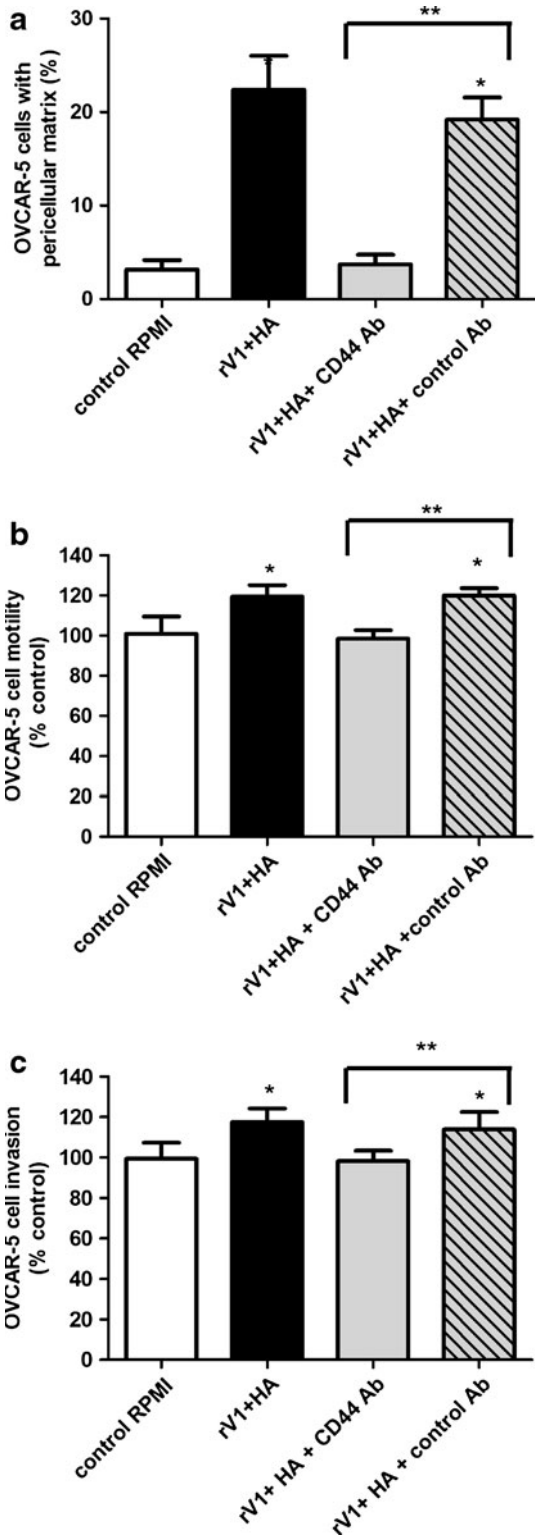


Fig. 6 HA oligosaccharides block the formation of pericellular matrix and OVCAR-5 cell motility, invasion induced by versican and adhesion to peritoneal cells. OVCAR-5 cells were treated as in Fig. 1b in the presence or absence of HA oligosaccharides (250 µg/ml). **a** Pericellular matrix formation was assessed as described in Fig. 1. Data represents percentage (mean ± SD) of cells ($n = 100$). *Significantly different from control at $P < 0.05$, one-way ANOVA. Motility (**b**) and invasion (**c**) of OVCAR-5 were performed as described for Fig. 3. The fluorescence of migratory cells was measured at 485–520 nm. Data are expressed as percentage of control media, mean ± SD from at least three independent experiments performed in triplicate. *Significantly different from control at $P < 0.05$, one-way ANOVA. **d** Adhesion assays were performed as described in "Material and methods" section. Ovarian cancer cells (OVCAR-5 & SKOV-3) were incubated with control media (0.1% BSA in RPMI), HA (20 µg/ml) alone or in the presence of CM containing versican (CHO V1) or no versican containing (CHO K1) for 3 h. Additional cells were also treated with Hase (10 U/ml). *Significantly different from control at $P < 0.05$, one-way ANOVA. **e** Ovarian cancer cells (OVCAR-5) were treated with HA (20 µg/ml) or PBS with increasing concentrations of HA oligosaccharides (5–250 µg/ml) for 3 h. *Significant differences from PBS control at $P < 0.05$, one-way ANOVA

with paclitaxel alone [73, 74]. More recently phase I and phase II clinical trials have demonstrated that HA and the chemotherapy drug irinotecan can be safely administered together to patients with metastatic colon cancer and improve progression free survival [75, 76]. We envisage that HA oligomers could be administered intra-peritoneally



◀ **Fig. 7** CD44 neutralizing antibody blocks formation of pericellular matrix and OVCAR-5 cell motility, invasion induced by versican and HA. OVCAR-5 cells were treated as in Fig. 1b with control medium or versican + HA in the presence of CD44 antibody (20 μg/ml) or control IgG (20 μg/ml). **a** Pericellular matrix formation was assessed as described in Fig. 1. Data represents percentage (mean ± SD) of cells ($n = 100$) from three independent experiments. Motility (**b**) and invasion (**c**) of OVCAR-5 were performed as described for Fig. 3. The fluorescence of migratory cells was measured at 485–520 nm. Data are expressed as percentage of control media, mean ± SD from at least three independent experiments performed in triplicate. *Significantly different from control media at $P < 0.05$, Student t test. **Significantly different from IgG control at $P < 0.05$, Student t test

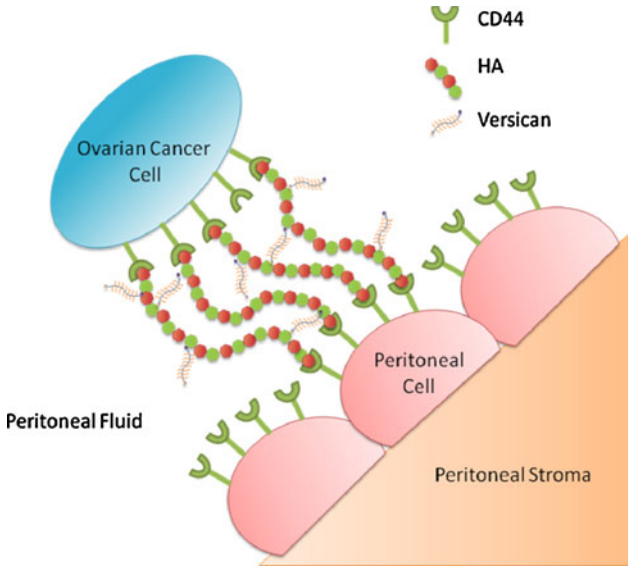


Fig. 8 Proposed model of HA, CD44 and versican interactions between ovarian cancer and peritoneal cells. The formation of a stabilized HA/versican pericellular matrix surrounding ovarian cancer cells increases motility and protects the ovarian cancer cells against the mechanical forces in the peritoneal cavity and enable ovarian cancer cells to strongly adhere to CD44 expressed on peritoneal cells. This allows subsequent ovarian cancer invasion and peritoneal dissemination

together with chemotherapy drugs to patients following debulking surgery to inhibit residual CD44 positive ovarian cancer cells from repopulating and invading the peritoneal surfaces.

In conclusion, our results show that ovarian cancer cells have the ability to recruit stromal ECM components to form a pericellular matrix which in turn promotes ovarian cancer cell motility and invasion. HA oligomers can block this mechanism and are therefore a potential therapeutic against ovarian cancer progression.

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