# Role of versican V0/V1 and CD44 in the regulation of human melanoma cell behavior

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**Abstract.** Versican is a hyaluronan-binding, large extracellular matrix chondroitin sulfate proteoglycan whose expression is increased in malignant melanoma. Binding to hyaluronan allows versican to indirectly interact with the hyaluronan cell surface receptor CD44. The aim of this work was to study the effect of silencing the large versican isoforms (V0 and V1) and CD44 in the SK-mel-131 human melanoma cell line. Versican V0/V1 or CD44 silencing caused a decrease in cell proliferation and migration, both in wound healing assays and in Transwell chambers. Versican V0/V1 silencing also caused an increased adhesion to type I collagen, laminin and fibronectin. These results support the proposed role of versican as a proliferative, anti-adhesive and pro-migratory molecule. On the other hand, CD44 silencing caused a decrease in cell adhesion to vitronectin, fibronectin and hyaluronan. CD44 silencing inhibited the binding of a FITC-hyaluronan complex to the cell surface and its internalization into the cytoplasm. Our results indicate that both versican and CD44 play an important role regulating the behavior of malignant melanoma cells.

## Introduction

Melanoma is a skin cancer with poor prognosis that fails to respond to currently available therapies and whose incidence is rising in Western populations. It arises from melanocytes, pigmented cells residing in the epidermis. During melanoma progression, a large number of changes occur in the extracellular microenvironment where tumor cells reside (1-3). Amongst these changes, we have previously described the appearance of versican in human and canine melanocytic lesions (4,5).

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Versican is a large chondroitin sulfate proteoglycan, which belongs to the hyalectan family, as it is able to bind hyaluronan (HA). The versican core protein is divided into three clearly differentiated domains (6): the HA binding domain (G1), the central domain where chondroitin sulfate chains are linked (G2), and the C-terminal globular region (G3) consisting of two EGF-like motifs, a lectin binding region and a CBP-like domain. The central domain is divided in two subdomains, designated GAG-α and GAG-ß, that are encoded by two exons that can be alternatively spliced to give rise to four different versican isoforms (6): V0, containing both GAG-α and GAG-ß; V1, containing GAG-ß; V2, containing GAG-α; and V3, lacking any GAG subdomain. Versican isoforms differ in the size of the G2 subdomain and hence in the number of glycosaminoglycan (GAG) chains.

In melanoma and other tumor cell types, versican has been shown to regulate cellular functions like cell proliferation, adhesion, migration and invasion (3,7-10). Versican is considered to be a pro-proliferative, anti-adhesive and pro-migratory molecule, and hence plays an important role in the development of tumors in many tissues (3,11-13). Some of these actions have been ascribed to specific domains in the molecule. Thus, over-expression of versican G1 domain can enhance cell proliferation and reduce cell adhesion in different cell types (7,14). The G3 domain has also been involved in several processes like cell proliferation and invasion (15-18), and GAG chains have been considered partially responsible for the anti-adhesive properties of versican (19,20).

Versican-HA complexes interact with the cell membrane mainly through the HA receptor, CD44 (21). Like versican, CD44 can be alternatively spliced into a large number of isoforms, of which the one known as the standard isoform (CD44s) has no variable exons (22). Besides HA, CD44 can interact with several molecules like collagen, fibronectin, laminin, osteopontin and chondroitin sulfate chains, and with other cell surface receptors including the ErbB receptor family (22). CD44 can also interact with the cytoskeletal proteins through its cytoplasmic domain. All these interactions allow CD44 to play an important role in tumor progression by enhancing cell proliferation, adhesion, migration and invasion (23-28). Furthermore, it has a central function in HA metabolism, since it has a central role in HA internalization (29,30).

In order to ascertain the role of versican and CD44 in human melanoma cell behavior, the expression of V0 and V1 versican isoforms and the expression of CD44 were silenced

in the SK-mel-131 human melanoma cell line by means of RNAi techniques. The biological effects of versican and CD44 suppression on melanoma cell proliferation, adhesion, migration and HA metabolism, were analyzed in the silenced cell lines.

## Materials and methods

*Cell culture*. The human melanoma SK-mel-131 cell line was obtained from Dr A.N. Houghton (Memorial Sloan-Kettering Cancer Center, NY, USA) (31). Cells were grown at 37ºC in a humidified atmosphere with 5% $CO_2$ in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 IU/ml penicillin, 100 $\mu$g/ml streptomycin and 1 mM glutamine (all from Gibco-BRL/Life Technologies, Rockville, MD, USA). This cell line produces the V0 and V1 isoforms of versican, which are secreted into the extracellular medium (32).

*Versican V0/V1 and CD44 siRNA cloning and cell transfection*. The following small interfering RNA (siRNA) oligonucleotides were used: for versican V0 and V1 silencing: 5'-GATCCCCGGAACTACAGGTACGGCTTCTTT CAAGAGAAGAAGCCGTACCTGTAGTTCCTTTTTA-3' and 5'-AGCTTAAAAAGGAACTACAGGTACGGCTT CTTCTCTTGAAAGAAGCCGTACCTGTAGTTCCGGG-3' primers; for CD44 silencing: 5'-GATCCCCGTATGACA CATATTGCTTCTTCAAGAGAGAAGCAATATGTGTCA TACTTTTTA-3' and 5'-AGCTTAAAAAGTATGACA CATATTGCTTCTCTCTTGAAGAAGCAATATGTGTCA TACGGG-3' primers.

Anti-CD44 primers are based on those described in Tzircotis *et al* (33). Anti-versican primers were designed using the software provided by the Sigma-Aldrich website and directed against the subdomain ß in order to silence this subdomain present in the large isoforms V0 and V1. The oligonucleotides (300 $\mu$g/ml) were annealed in buffer M (Roche Diagnostics, Mannheim, Germany) by heating at 95˚C for 4 min and slowly cooling down to room temperature. The resulting double-strand sequences were cloned into the pSUPERIOR.neo+GFP (anti-CD44) or pSUPERIOR.puro (anti-versican V0 and V1) plasmids (OligoEngine, Seattle, WA, USA) using their *Bgl*II/*Hin*dIII site. siRNA constructs were stably transfected into the SK-mel-131 cell line using Lipofectamine™ reagent (Invitrogen, Paisley, Renfrewshire, UK).

*Protein extraction and Western blotting*. For CD44 analysis, cell extracts from subconfluent cells were prepared in lysis buffer containing 1% NP-40, 150 mM NaCl and 10 mM Tris-HCl, pH 7.4. The amount of protein was normalized after Bradford quantification. For versican analysis, subconfluent cultures grown on 10 cm plates were left in serum-free DMEM for 24 h, conditioned media were collected and a cocktail of protease inhibitors (10 mM EDTA, 5 mM benzamidine and 1 mM PMSF) was added before freezing. For Western blotting, 50 $\mu$g of protein (for CD44) or 50 $\mu$l of conditioned medium (for versican) were resolved on 10% SDS-PAGE gels. Proteins were transferred onto Immobilon™ PVDF membranes (Millipore Corp., Bedford, MA). Membranes were either blotted with a polyclonal antibody directed against versican generated in our laboratory, (1:100) (3), anti-CD44 (1:10) (a kind gift of Dr R. Vilella, Hospital Clinic, Barcelona, Spain) or against ß-actin (1:1000) (Santa Cruz Biotechnology, Heidelberg, Germany). The membranes were developed with the ECL-Plus detection system (GE Healthcare, Amersham, Bucks, UK) and bands were visualized using a LAS-3000 luminescent image analyzer (Fujifilm Corp., Tokyo, Japan).

*Immunocytochemistry*. Cells ($10^5$) were grown in coverslips for 24 h, rinsed with phosphate buffered saline (PBS) and fixed with 3% paraformaldehyde-2% saccharose. Cells were incubated at 4˚C overnight with the primary antibody and subsequently with the secondary antibody labeled with AlexaFluor®488 (Invitrogen, Eugene, OR, USA) or TRITC. Nuclei were visualized with Hoechst 33342. High-power light-microscopic images were digitally captured using a Nikon Eclipse E800 epifluorescence microscope with an integrated camera system.

*Cell proliferation and adhesion assays*. For proliferation assays, $5x10^4$ cells/well were seeded in 6-well plates. After 3 days in culture, cells were detached by trypsinization and counted in a Neubauer chamber at the indicated times.

For adhesion assays, 96-well plates were coated with laminin (10 $\mu$g/ml) or vitronectin (5 $\mu$g/ml) for 2 h at 37˚C; fibronectin (10 $\mu$g/ml), type I collagen (10 $\mu$g/ml) or PBS as a negative control for 16 h at 4˚C, and HA (5 mg/ml) for 16 h under a hood (all substrates were from Sigma). Cells were plated at a density of $4x10^4$ cells/well and allowed to attach to the plate for 2 h (overnight for HA). Non-attaching cells were removed and cells adhering to the plate were fixed with 4% paraformaldehyde in PBS, washed and stained with crystal violet. The extent of adhesion was determined by treating cells with 0.1 M HCl and measuring the absorbance at 620 nm.

*Cell migration assays*. For wound healing assays, cells were grown until confluence was reached. At this point, a scratch was done along the cell monolayer using a pipette tip. Cultures were analyzed with a Nikon Eclipse E800 microscope and pictures were taken with an integrated camera system.

For migration assays, Transwell® chambers (6.5 mm diameter, 0.8 mm pore size membrane, Corning (Corning, NY) were treated with 100 $\mu$g/ml of type I collagen (Sigma) or 1.5 mg/ml of HA (Sigma) for 16 h in a hood. After rinsing, $10^5$ cells were added to the upper chamber and incubated overnight at 37˚C. Cells on the upper side of the membrane were removed with a cotton swab, and cells on the lower side were fixed and stained with crystal violet. Membranes were rinsed, dried and mounted on coverslips to analyze them under the microscope (Nikon Eclipse E800).

*FITC-HA internalization assay*. FITC-conjugated HA was prepared as previously described (34). For the internalization assay, $10^5$ cells were seeded on coverslips and digested with hyaluronidase to remove the surface bound HA. Cells were then incubated with 100 $\mu$g/ml HA-FITC for 1 h at 4˚C to test the binding to the cell surface or for 5 h at 37˚C to test the internalization ability. After incubation, cell membranes were stained with the CellMask® reagent (Invitrogen). Cells were

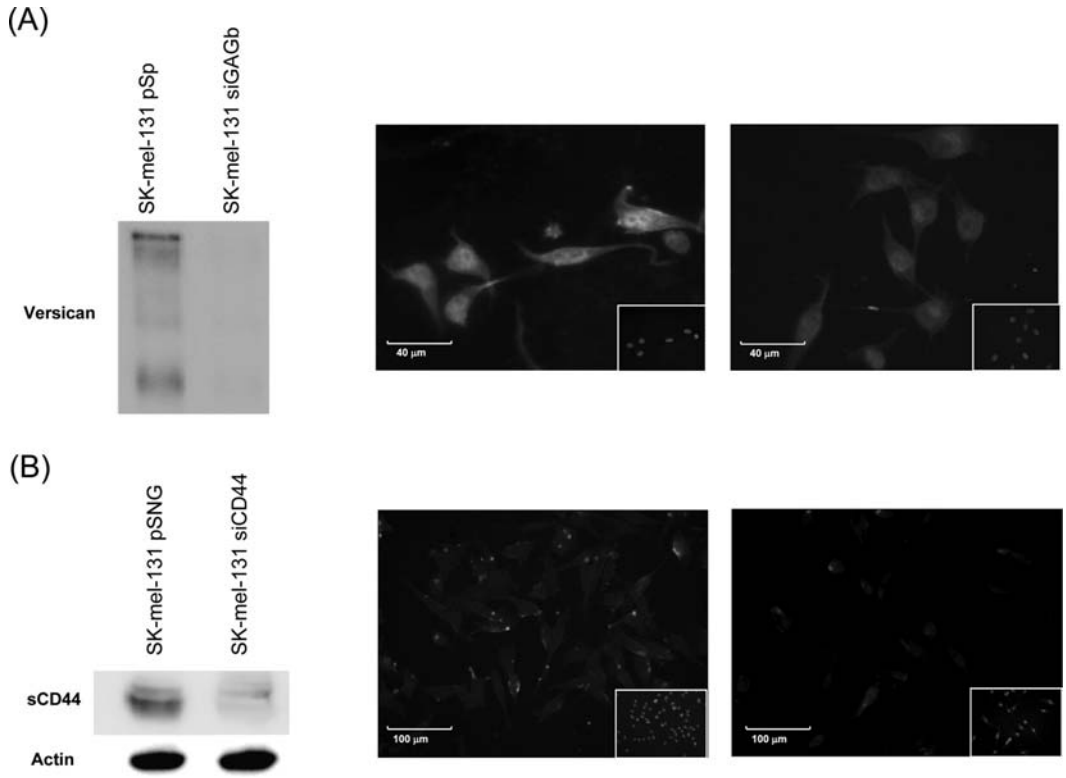


Figure 1. Analysis of versican (A) and CD44 (B) expression after siRNA silencing. For immunoblotting, conditioned media were resolved in 10% SDS-PAGE and blotted with an antibody against versican (1:100) or CD44 (1:1000), respectively. For immunocytochemistry, $10^5$ cells were seeded onto coverslips, fixed and incubated with the corresponding antibody against versican. The signal is given by incubation of the samples with AlexaFluor®488 (for versican) or Alexa®Fluor633 (for CD44).

fixed with a 1% paraformaldehyde solution and visualized using a Leica TCS SP5 AOBS confocal microscope (Servei de Microscopia, UAB, Barcelona). Images were processed with Imaris software.

## Results

*Versican V0/V1 and CD44 silencing in SK-mel-131 human melanoma cells*. The SK-mel-131 human melanoma cell line was stably transfected with the plasmids containing the siRNA sequence against the versican GAGß subdomain or CD44, giving rise to the SK-mel-131 siGAGb and SK-mel-131 siCD44 cell lines, respectively, or with the empty vectors as a control, giving rise to the SK-mel-131 pSp and SK-mel-131 pSNG cell lines, respectively. Efficiency of versican V0/V1 and CD44 inhibition was checked by Western blot analysis and immunocytochemistry (Fig. 1).

*Versican V0 and V1 silencing alters melanoma cell proliferation, migration and adhesion*. Addition of purified versican to the culture medium caused an increase in melanoma cell proliferation, as described previously (3). To check the effect of versican V0 and V1 silencing on melanoma cell growth, a proliferation assay was performed. As shown in Fig. 2A, the proliferation rate was lower in the SK-mel-131 siGAGb cell line compared to the control cell line, SK-mel-131 pSp. Thus, versican V0/V1 silencing exerted a negative effect on melanoma cell growth.

When cell migration was analyzed in wound healing assays, a decrease in migration rate was observed in SK-mel-131 siGAGb cells compared to SK-mel-131 pSp control cells (Fig. 2B). A similar result was observed when cells were allowed to migrate through type I collagen and HA-coated Transwell chambers (Fig. 2C). These observations confirmed the involvement of versican in the migration process in melanoma cells.

The effect of versican V0/V1 on cell adhesion was assessed by plating the cells onto different substrates, namely type I collagen, laminin, fibronectin, vitronectin and HA. As shown in Fig. 2D, SK-mel-131 siGAGb cells presented an increase in cell adhesion to type I collagen, laminin and fibronectin when compared to the control cell line. In contrast, no differences were observed on adhesion to vitronectin or HA. These results confirmed the anti-adhesive role of versican in melanoma cells.

*CD44 silencing alters melanoma cell proliferation, migration and adhesion*. Secondly, the role of the HA-versican complex receptor, CD44, was analyzed by silencing its expression on SK-mel-131 cells, since it has been widely described that CD44 plays a role in cell proliferation, adhesion, migration and invasion (27,28). To check the effect of CD44 silencing on cell growth, a proliferation assay was performed. As shown in Fig. 3A, CD44 silencing caused a delay in cell proliferation rate in the SK-mel-131 cell line, when compared to the control cell line.

When cell migration was checked in wound healing assays, we observed that SK-mel-131 siCD44 cells migrated slower than their control counterpart. CD44 silencing also provoked a decrease in cell migration rate through Transwell chambers

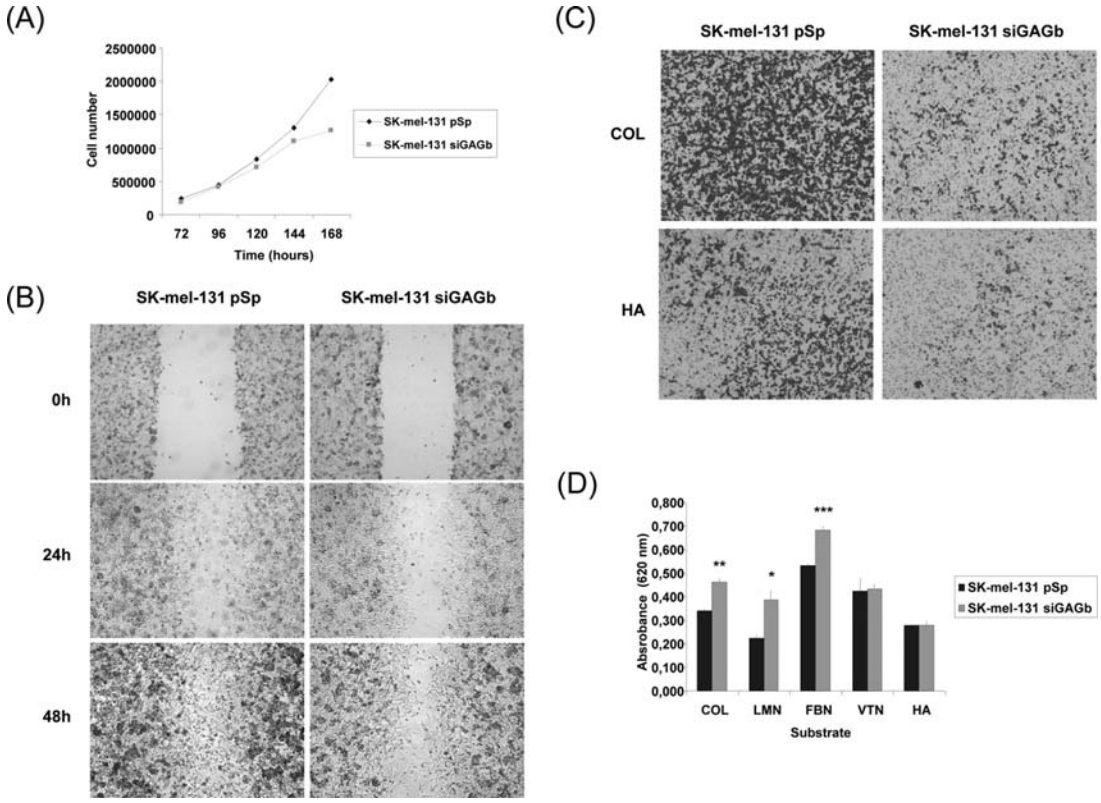


Figure 2. (A) Versican V0/V1 silencing causes a decrease in cell proliferation in SK-mel-131 cells. Six-well plates were seeded with 50000 cells/well and proliferation was quantified as described in Materials and methods. (B) Versican V0/V1 silencing causes a decrease in wound healing ability in SK-mel-131 cells. (C) Versican V0/V1 silencing causes a decrease in cell migration through type I collagen and hyaluronan (HA) in SK-mel-131 cells. Transwell membranes were coated with type I collagen (100 $\mu$g/ml) or HA (5 mg/ml). (D) Effect of versican V0/V1 silencing on cell adhesion. Cells were plated at a density of $4x10^4$ cells/ well onto the specified substrates.

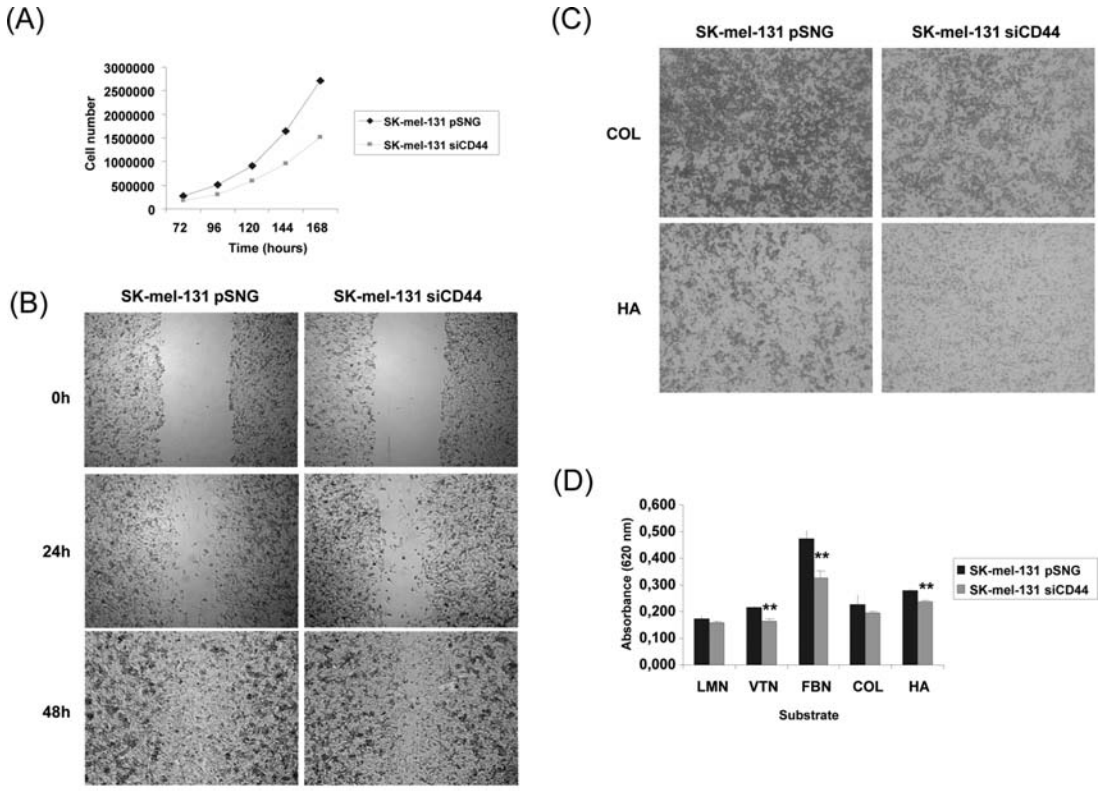


Figure 3. (A) CD44 silencing causes a decrease in cell proliferation in SK-mel-131 cells. Six-well plates were seeded with 50000 cells/well and proliferation was quantified as described in Materials and methods. (B) CD44 silencing causes a decrease in wound healing ability in SK-mel-131 cells. (C) CD44 silencing causes a decrease in cell migration through type I collagen and hyaluronan (HA) in SK-mel-131 cells. Transwell membranes were coated with type I collagen (100 $\mu$g/ml) or HA (5 mg/ml). (D) Effect of CD44 silencing on cell adhesion. Cells were plated at a density of $4x10^4$ cells/well onto the specified substrates.

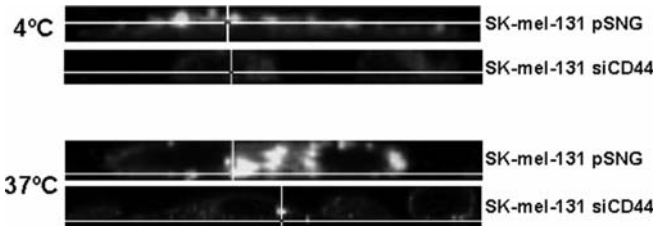


Figure 4. Effect of CD44 silencing on HA internalization. The ability of the cells to bind a FITC-HA complex or to internalize was checked by incubating SK-mel-131 cells with the complex at 4˚C or 37˚C, respectively. The signal is given by incubation with the CellMask reagent.

previously coated with type I collagen or HA (Fig. 3B and 3C), confirming the role of this molecule in cell migration.

In addition to HA, CD44 can bind many other molecules, such as collagen, laminin or fibronectin (35). To check the role of CD44 on the adhesion ability of the SK-mel-131 cell line, cells were seeded on plates previously coated with laminin, vitronectin, fibronectin, type I collagen, or HA, and the extent of adhesion was measured after 2 h. As shown in Fig. 3D, CD44 silencing caused a significant decrease on cell adhesion to vitronectin, fibronectin and HA ($p<0.01$).

*CD44 silencing decreases HA internalization ability in melanoma cells.* Since HA internalization is recognized as a CD44-dependent process, we checked the ability of CD44 silencing to alter the internalization of an exogenous HA-FITC complex. Cells were seeded on coverslips and treated with hyaluronidase to remove endogenous HA. Then FITC-labeled HA was added to the medium and cells were incubated for 1 h at 4˚C to check the binding to the membrane, or for 5 h at 37˚C to allow the cells to internalize it. As shown in a cross-section obtained by scanning confocal microscopy, no FITC-HA appeared to bind to the membrane in SK-mel-131 siCD44 cells when compared to control cells (Fig. 4). When cells were treated for 5 h at 37˚C, FITC-HA was internalized by SK-mel-131 pSNG cells, but when CD44 was silenced, HA internalization appeared to be blocked in this cell line (Fig. 4). Taken together, these results confirm the role of CD44 in HA internalization by regulating HA binding to the cell surface.

## Discussion

In a previous study, we have demonstrated that versican expression in human melanoma cells is correlated with mutations in the BRAF oncogene, early degree of cell differentiation and invasive properties (32,36). Furthermore, addition of purified versican to the culture medium of melanoma cells alters their proliferation, adhesion and migration abilities (3). Thus, versican appears to have an important biological role in the malignant phenotype of melanoma. To confirm this role, the expression of V0 and V1 versican isoforms were silenced in the human melanoma cell line SK-mel-131 by suppressing the common GAG-ß domain present in both large isoforms. This cell line presents an activating mutation in the BRAF gene (V600E) and produces versican V0 and V1 (32). Since versican acts by forming pericellular complexes with HA, which is able to interact with the cell through CD44, this receptor was also silenced in SK-mel-131 cells.

As expected, SK-mel-131 siGAGb cells demonstrated reduced proliferation ability, thus supporting the proposed role for versican in melanoma cells. Silencing experiments have thus led to the same conclusion with positive approaches, during which purified versican was added to the culture media in a melanoma cell line (3). In non-tumor cell lines, a similar approach has also indicated the pro-proliferative role of versican in smooth muscle cells (9), preadipocytes (37) and fibroblasts (38). CD44 silencing also caused a decrease in melanoma cell proliferation, as in other tumor cell lines (39-41), thus supporting the hypothesis that versican can exert its action through its G1 domain, responsible for the interaction with HA and, indirectly, with CD44. The ERK1/2 pathway is constitutively activated in the SK-mel-131 cell line due to the V600E BRAF mutation (32), and activation of the pathway is not affected by suppression of CD44 (not shown). Thus, the antiproliferative effects should be related to other potential interactions of the CD44-transducing pathway. In this sense, disorganization of the cytoskeleton may mediate the decrease of proliferative ability after CD44 silencing (42).

Silencing of V0/V1 versican expression also reduced cell migration in wound healing assays and type I collagen- or HA-coated Transwell chambers. A similar effect has been observed in smooth muscle cells, where versican silencing caused a diminished cell migration in wound healing assays (9), or in prostate cancer cells, in which addition of purified versican to the cells caused an increase in the invasion ability (13). In glioma, treatment of the cells with TGF-ß2 caused an increase in cell migration associated with an increase in versican production (10). CD44 inhibition also caused a reduction in the migration ability of melanoma cells, similarly to other cell types (27,40,41). It has been shown that CD44 participates in cell migration by interaction with the cell cytoskeleton (43). Silencing of CD44 could alter the ability of the cell to rearrange its cytoskeleton and hence would impair cell migration and invasion.

Cell adhesion is closely related to cell migration and invasion, as cells need to detach from the substrate in order to invade other tissues. Silencing of versican V0 and V1 expression in the human melanoma cell line SK-mel-131 caused an increase in cell adhesion to type I collagen, laminin and fibronectin. The anti-adhesive role of versican has been shown in melanoma cells (3), prostate carcinoma cells (20) or neural crest cells (8). This inhibitory effect on cell adhesion may mainly be due to the presence of the GAG chains that might create a more hydrated extracellular matrix less suitable for cell adhesion. In contrast, CD44 silencing caused a decrease in cell adhesion to HA, fibronectin and vitronectin. Since CD44 is the main cell surface receptor for HA, its suppression should lead to a diminished adhesion to this substrate. Similar results have been described in ovarian carcinoma cells (27). No information in the literature exists on the influence of CD44 on cell adhesion to other substrates. CD44 can interact with integrins, altering their binding ability to the ligands (44), and loss of this interaction may thus mediate the decrease in adhesion to fibronectin and

vitronectin. The opposite effects of versican and CD44 on cell adhesion may indicate that this effect may have a different mechanism in each case: in the case of versican it may mainly be related to the GAG chains and in the case of CD44 to specific binding properties.

Finally, other HA-related functions of CD44 are greatly impaired after CD44 silencing, such as the ability to internalize a FITC-HA complex. Melanoma cells lacking CD44 showed a complete loss of the ability to bind and internalize HA, confirming the role of CD44 described in other cell lines (34,45,46).

In conclusion, versican and CD44 play an important role in melanoma cell proliferation, adhesion and migration, contributing to the malignant phenotype of these tumor cells. Both versican and CD44 should be considered as suitable therapeutic targets in malignant melanoma.

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