Podoplanin Associates with CD44 to Promote Directional Cell Migration

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Podoplanin is a transmembrane glycoprotein up-regulated in different human tumors, especially those derived from squamous stratified epithelia (SCCs). Its expression in tumor cells is linked to increased cell migration and invasiveness; however, the mechanisms underlying this process remain poorly understood. Here we report that CD44, the major hyaluronan (HA) receptor, is a novel partner for podoplanin. Expression of the CD44 standard isoform (CD44s) is coordinately up-regulated together with that of podoplanin during progression to highly aggressive SCCs in a mouse skin model of carcinogenesis, and during epithelial-mesenchymal transition (EMT). In carcinoma cells, CD44 and podoplanin colocalize at cell surface protrusions. Moreover, CD44 recruitment promoted by HA-coated beads or cross-linking with a specific CD44 antibody induced corecruitment of podoplanin. Podoplanin–CD44s interaction was demonstrated both by communoprecipitation experiments and, in vivo, by fluorescence resonance energy transfer/fluorescence lifetime imaging microscopy (FRET/FLIM), the later confirming its association on the plasma membrane of cells with a migratory phenotype. Importantly, we also show that podoplanin promotes directional persistence of motility in epithelial cells, a feature that requires CD44, and that both molecules cooperate to promote directional migration in SCC cells. Our results support a role for CD44-podoplanin interaction in driving tumor cell migration during malignancy.

INTRODUCTION

Podoplanin (PA2.26 antigen, Aggrus, or T1a) is a type I transmembrane sialomucin up-regulated in different types of cancer, such as squamous cell carcinomas (SCCs) and testicular germ cell tumors (see Wicki and Christofori, 2007 for a review). Several reports support the involvement of podoplanin in malignant progression. First, it has been shown that podoplanin/Aggrus induces platelet aggregation facilitating tumor-platelet aggregate formation and metastasis (Kunita et al., 2007). Second, we found that podoplanin/PA2.26 antigen promotes either cell scattering or a full epithelial–mesenchymal transition (EMT) associated with cell migration, invasion, and metastasis (Scholl et al., 1999; Scholl et al., 2000; Martín-Villar et al., 2005; Martín-Villar et al., 2006). Third, Wicki and colleagues reported that podoplanin can promote collective tumor cell migration and invasion in a pancreatic cancer mouse model (Wicki et al., 2006). Because podoplanin lacks any obvious enzymatic motif within its structure, all these activities have to be mediated by protein–protein interactions, hence the need to identify its binding partners. Thus, the binding of podoplanin ectodomain to C-type lectin-like receptor 2 (CLEC-2) is involved in podoplanin-induced platelet aggregation (Kato et al., 2008), a process which is attenuated by the interaction of podoplanin with CD9 tetraspanin (Nakazawa et al., 2008). In addition, podoplanin-induced EMT is linked to RhoA activation and requires the association of the podoplanin cytoplasmic tail with ezrin and/or moesin, members of the ERM (ezrin, radixin, moesin) protein family of membrane-cytoskeleton linkers (Martín-Villar et al., 2006).

In this report, we identify the standard isoform of CD44 (CD44s) as a novel partner for podoplanin. CD44 is a widely distributed and highly polymorphic type I transmembrane glycoprotein. Although it is encoded by a single gene, the region comprising the extracellular domain includes 10 vari-

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Abbreviations used: CD44s, CD44 standard isoform; CD44v, CD44 variant isoform; eGFP, enhanced green fluorescent protein; EMT, epithelial–mesenchymal transition; ERM, ezrin, radixin, moesin; FRET/FLIM, fluorescence resonance energy transfer–fluorescence lifetime imaging microscopy; HA, hyaluronan; SCC, squamous cell carcinomas.

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Cells were incubated at 37°C for 6 h before the addition of FBS to 10%. All the reagents were from Invitrogen, Paisley, UK) in Opti-MEM reduced-serum medium (Invitrogen).

Cell Culture

Function depends on the ability of CD44 to bind ERM proteins through its cytoplasmic tail (Ponta et al., 2003). The podoplanin–CD44s interaction described here provides a new mechanism by which both of these glycoproteins cooperate to promote cell migration and tumor progression.

MATERIALS AND METHODS

Cell Culture

Epithelial cell lines (Supplemental Table S1) were grown as described before (Quintanilla et al., 2003). MDCK- and MCA3D podoplanin cell transfectants have been described previously (Scholl et al., 1999; Scholl et al., 2000; Martin-Villar et al., 2006). HEK293T, HT1080, and MDCK-Snail1, -Snail2, and -E47 cells were generous gifts from Dr. Amparo Cano (Instituto de Investigaciones Biomedicas Alberto Sols, Madrid, Spain). HNS cells were kindly provided by Dr. Marcella Flinterman (Oral Pathology Department, King’s College London, London, UK). HaCaT, HEK293T, HT1080, HNS, and MDCK cells were grown in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum, (Bioera, Susses, UK) 1% Pen-Strep (Sigma, Dorset, UK) and L-glutamine (PAA Laboratories GmbH, Germany).

cDNA Constructs and RNA Interference

Full-length wild-type human podoplanin and mutant constructs subcloned into the pcDNA3 and pEGFP vectors have been described elsewhere (Martin-Villar et al., 2006). Human podoplanin and CD44s tagged with Hae and Flag at the C terminus and mRFP-tagged CD44s constructs were obtained by PCR amplification using primers that carry convenient restriction sites to facilitate subcloning into pcDNA3-Hae/Flag and pmRFP-NI vectors. Oligonucleotides used for amplification of all these constructs are described in Table S2 in the Supplemental material.

Short-hairpin RNAs (shRNAs) targeting human podoplanin mRNA were cloned into pLKO.1 vector (Addgene, Cambridge, UK). Lentiviral supernatants were produced in HEK293T cells by cotransfection of pCMV-d8.9 (packaging plasmid), pMD2G (envelope plasmid), and pLKO.1 including targeting shRNA oligonucleotides were transfected with 100 μg/ml for 1 h at 37°C. After three washes with warm PBS to remove unbound oligos, the cells were fixed with 4% paraformaldehyde in PBS for 15 min, washed three times with warm PBS, and processed for immunofluorescence as described below.

Mouse Skin Carcinogenesis

The two-stage mouse skin carcinogenesis (a single DMBA application followed by twice weekly applications of TPA for 16 wk) was performed following standard protocols (Perez-Gomez et al., 2007). Tumors were collected at different time periods after initiation (papillomas at 30 wk and SCCs at 43 wk) and processed for Western blotting. Tumors were histologically typed by H&E staining of paraffin sections and characterized by the expression of the following differentiation/progression markers: keratins K1 and K10, which are lost in SCCs; the extracellular matrix component SPARC, which is induced during progression from papillomas to SCCs; as well as the E-cadherin transcriptional repressor Snail1 and the enzyme lysyl oxidase-like 2, which accumulate in poorly differentiated SCCs, as previously described (Perez-Gomez et al., 2007).

RT-PCR

Reverse transcription was performed as previously described (Martin-Villar et al., 2006). For CD44 analyses specific primers were designed to amplify both human and canine genes: 5′-TATGCTTCAATGCTAGCTTCA-T-3′ and 5′-AGGTGTGTTTGTCCCACTTCAGCA-3′. PCR products were obtained after 35 cycles of amplification with an annealing temperature of 60°C.

Western Blot and Coimmunoprecipitation Experiments

For detection of podoplanin and CD44 in Western blots, cells or tissues were lysed in buffer RIPA (0.1% SDS, 0.5% sodium deoxycholate, 1% Nonidet P-40, 150 mM NaCl, 50 mM Tris-HCl pH 8.0) and a cocktail of protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin and 2 μg/ml leupeptin). Samples containing the same amount of protein (10–30 μg were run on 10% SDS-PAGE and transferred to Immobilon P membranes (Millipore, Bedford, MA). Filters were then immunoblotted with the following Abs: anti mouse podoplanin (PAX-26 kindly provided by Dr. Helen Yarwood; The Institute of Cancer Research, London, UK) and anti human podoplanin (NZ1 mAb purchased from Acris Antibodies, Herford, Germany), rat mAb IM7 against CD44 ectodomain (generously provided by Dr Helen Yarwood; The Institute of Cancer Research, London, UK) and polyclonal Ab raised to the CD44 V3-6 protein (CD44kcyto, Abcam, Cambridge, UK), and mouse mAbs against α-tubulin, β-actin (Sigma) and GAPDH (Roche Diagnostics, Welwyn Garden City, UK).

For coimmunoprecipitation experiments HEK293T cells were transiently cotransfected with expression vectors using Effectene (Qiagen, Crawley, UK), as indicated by the manufacturer. After 24 h, cells were lysed in IP buffer (50 mM Tris-HCl pH 8.0, 100 mM NaCl, EDTA 5 mM, 0.5% Triton X-100, 10% glycerol) containing Complete Protease Inhibitors Cocktail Tablets (Roche Diagnostics). Tagged CD44s-Hae or podoplanin-Flag proteins were immunoprecipitated using anti-Hae (Roche Diagnostics) or anti-Flag (Sigma) mAbs linked to protein A/G resin (Alpha Diagnostic, TX), respectively. After incubation with 750–500 μl of cell lysates for 2 h at 4°C, the resin was then washed five times with lysis buffer and the coimmunoprecipitates were eluted in 1× Laemmli Buffer. Immunoprecipitation of endogenous podoplanin in CarC cells was performed using the PA2.26 mAb for mouse podoplanin and the coimmunoprecipitated products were detected by using the rat mAb IM7 against the CD44 ectodomain.

Coimmunoprecipitation assays of podoplanin–eGFP tagged constructs were performed using an anti-GFP antibody-conjugated resin. The resin was made by cross-linking a GFP polyclonal antibody (MBL International, Woburn, MA) to the protein G beads using a Seize X protein G immunoprecipitation kit purchased from Pierce Biotechnology (Leicester, UK). Coimmunoprecipitation was done following the protocol provided by the manufacturer.

FRET-FLIM Microscopy

FLIM was used to measure FRET between podoplanin eGFP constructs and CD44s-mRFP in MDCK cotransfected cells. Podoplanin-eGFP and CD44s-mRFP constructs were cotransfected at a 1:1 ratio (or higher) with the codon-optimised mammalian expression vector pCD4 in 60 mm dishes of confluent MDCK cells. Cells were fixed and processed for immunofluorescence. Immunofluorescence analysis was performed as described previously (Clark et al., 2005). The podoplanin and CD44 mAbs were purchased from Dako (Glostrup, Denmark). Before CLSM imaging, cells were permeabilized with cold ethanol for 30 min and incubated with 3% bovine serum albumin (BSA) or 1 mg/ml high molecular mass HA (Sigma, H7630). Beads were left uncoated or coated with 0.1 mg/ml for 1 h at 37°C. After three washes with warm PBS, cells were fixed with 4% paraformaldehyde in PBS for 15 min and washed three times with warm PBS. Cells were then washed three times in medium and fixed for immunofluorescence analysis.

Antibody-mediated clustering assays were performed as described previously (Clark et al., 2005). Cells were transfected with the plasmid H2P/9 (generously provided by Dr. Francisco Sánchez-Madrid; Centro Nacional de Investigaciones Cardiovasculares, Madrid, Spain) which was used to transfect cells in serum-starved medium at a concentration of 1 μg/ml for 1 h at 37°C. After three washes with PBS to remove all nontarget Ab, a Cy3-labeled secondary Fc-specific Ab (2 μg/ml) was added for 1 h. Under these conditions the secondary Ab induces the cross-linking of the primary Ab through its Fc-tail. Cells were then fixed and processed as shown in the legend.
Confocal Microscopy and Morphological Analysis

Cells on coverslips were fixed with 3.7% formaldehyde for 30 min and permeabilized with 0.05% Triton-X100/PBS for 10 min. Podoplanin and CD44 staining was carried out using the anti-human podoplanin mAb (NZ-1) and the anti-CD44 H2P/9 mAb. Confocal images were acquired on a Leica TCS-SP2 microscope for Figure 2A or on a Nikon A1 confocal laser-scanning microscope for Figure 2, B and C and Figure 7. Images were assembled using Leica confocal software 2.0 or NIS-Elements AR 3.0.

For morphological analyses cells were allowed to spread for 24 h and then processed for immunofluorescence. The actin cytoskeleton was visualized by staining with Alexa Fluor 568-labeled phalloidin (Invitrogen). Tubulin was detected using a specific mouse anti-α-tubulin Ab (Sigma). Cell area was quantified using the public domain program NIH Image (developed at the US National Institutes of Health).

Cell Migration Assays and Time-Lapse Microscopy

The migratory behavior of the cells was analyzed by using 8-µm pore transwell chambers (Corning, Amsterdam, The Netherlands) and in vitro scratch assays. For transwell assays MDCK cells were cotransfected with podoplanin-eGFP or control siRNA. 2.5 × 10⁴ cells/well were seeded on 24-well chambers (Corning, Amsterdam, NE) and in vitro scratch assays. For wound healing assays podoplanin- and/or CD44-depleted HN5 cells were plated onto collagen I (BD Biosciences, Oxford, UK)-coated 12-well plates and left for 24 h to form a monolayer. Confluent monolayers were scratched with a tip and cells were imaged in an atmosphere of 5% CO₂ at 10× objective of a Leica DMB0008 inverted fluorescence microscope. Migration was analyzed by Imaris 6.4.2 software to obtain cell trajectories and calculate persistence of migration (directionality) was defined as displacement from origin/total cell path. Kinetics of migrating cells along the wound edge was analyzed by Imaris 6.4.2 software to obtain cell trajectories and calculate persistence of migration (directionality) was defined as displacement from origin/total cell path. Kinetics of migrating cells along the wound edge at time 0 h and 18 h. Migration was quantified by calculating the area scored for Figure 2A or on a Nikon A1 confocal laser-scanning microscope for Figure 2, B and C and Figure 7. Images were assembled using Leica confocal software 2.0 or NIS-Elements AR 3.0.

Random migration analysis in MDCK cells was performed by time-lapse fluorescence microscopy. Cells were sparsely plated, grown in the absence of serum, and imaged overnight. Pictures were taken every 5 min with the ×10 objective of a Leica DM/BS008 inverted fluorescence microscope. Migration was analyzed by Imaris 6.4.2 software to obtain cell trajectories and calculate persistence of migration (directionality) was defined as displacement from origin/total cell path.

For wound healing assays podoplanin- and/or CD44-depleted HN5 cells were plated onto collagen I (BD Biosciences, Oxford, UK)-coated 12-well plates and left for 24 h to form a monolayer. Confluent monolayers were scratched with a tip and cells were imaged in an atmosphere of 5% CO₂ at 10× objective. Cell outlines were drawn along the wound edge at time 0 h and 18 h. Migration was quantified by calculating the area scored for Figure 2A or on a Nikon A1 confocal laser-scanning microscope for Figure 2, B and C and Figure 7. Images were assembled using Leica confocal software 2.0 or NIS-Elements AR 3.0.

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Statistics

All experiments were performed at least three times. Data are presented as mean ± SEM. Significance was determined using one-way analysis of variance (ANOVA) followed by Bonferroni’s post test or two-tailed Student’s t test. p < 0.05 was considered statistically significant. All statistical analyses were performed using GraphPad Prism 4.0 software.

RESULTS

The Coordinate Expression of Podoplanin and CD44s Correlates with Malignant Progression and EMT

We have previously shown that ectopic expression of podoplanin in epithelial MDCK cells promoted a dramatic change from an epithelial to a fibroblastic-like morphology linked to down-regulation of epithelial genes, such as E-cadherin, and up-regulation of mesenchymal markers, such as fibronectin (Figure S1 in supplementary material; Martin-Villar et al., 2006). When the levels of CD44 mRNA and protein were analyzed in MDCK cells expressing podoplanin (MDCK-Podo), a switch in the expression from CD44v isoforms to CD44s was associated with the acquisition of a fibroblastic phenotype (Figure 1A). As shown in Figure 1B, this CD44 isoform switch was also seen in EMTs induced by the overexpression of the E-cadherin repressors Snail 1, Snail 2, and E47 in MDCK cells (Peinado et al., 2007).

EMT in mouse skin chemical carcinogenesis is associated with progression from well-differentiated tumors to highly aggressive undifferentiated or spindle cell carcinomas (Akhurst and Balmain, 1999). Because podoplanin was identified as a cell-surface protein induced in keratinocytes during mouse skin carcinogenesis (Gandarillas et al., 1997), we analyzed CD44 and podoplanin expression in cell lines and tumors corresponding to different stages of carcinogenesis (Figure 1, C and D). In both cases, the coordinated up-regulation of podoplanin and CD44s was associated with the undifferentiated phenotype. CD44s was barely detected in the normal epidermis and in benign papillomas, but it was clearly induced in SCCs and further increased in poorly differentiated tumors (Figure 1C). We, therefore, observed an overall increase in CD44 expression that correlated with malignant progression during carcinogenesis in vivo. Furthermore, epithelial premalignant (MCA3D and PB) and weakly metastatic carcinoma (PDV and B9) cell lines expressed low or null amounts of CD44s (Figure 1D); however, its expression was significantly enhanced in highly invasive carcinoma cell lines exhibiting a fibroblastic or spindle phenotype (HaCa4, A5, CarC, and CarB), and this increase occurred concomitantly to down-regulation of CD44v. For a summary of characteristics of the epidermal cell lines used in this study, see Table S1 in supplemental material. Interestingly, podoplanin expression followed a pattern roughly similar to that of CD44s in tumors and cell lines (Figure 1, C and D). The pair of cell lines B9 and A5 representing a homogeneous model for malignant progression is illuminating in this respect. Both cell lines were derived from the same carcinoma, B9 from the squamous component and A5 from the anaplastic spindle region (Burns et al., 1991). While B9 neither expressed CD44s nor podoplanin, both glycoproteins were present in A5 cells (Figure 1D). Furthermore, the ectopic expression of mouse podoplanin in pre-malignant MCA3D keratinocytes promoted an EMT associated with the acquisition of highly invasive and metastatic properties (Scholl et al., 1999; Scholl et al., 2000) and the induction of CD44s expression (Figure 1D). Both podoplanin and CD44s protein levels were also increased in LN11 cells derived from a lymph node metastasis produced by podoplanin-expressing MCA3D cells (Figure 1D and Table S1).

Podoplanin Interacts with CD44s

The subcellular localization of CD44 in podoplanin-expressing cells was analyzed by confocal microscopy. Images revealed that CD44 was distributed along the plasma membrane, concentrated at cell surface protrusions where it colocalized with podoplanin. This was the case for MDCK cells expressing exogenous podoplanin tagged with eGFP (PWT eGFP) and oral carcinoma HN5 cells expressing endogenous podoplanin (Figure 2A). To determine whether the binding of HA to CD44 affected the membrane localization of podoplanin, PWT eGFP cells plated onto coverslips were incubated with 5-µm beads coated with HA. As shown in Figure 2B, podoplanin was recruited by HA-coated beads together with CD44. Binding was specific, as control BSA-coated beads did not trigger recruitment of CD44 or podoplanin. To analyze the possibility of a physical association between podoplanin and CD44, we performed co-clustering experiments in PWT eGFP-expressing HN5 cells. These cells were treated in vivo with an antibody raised to the CD44 ectodomain (HP2/9), and then the antibody was clustered with a secondary antibody. This resulted in the formation of PWT eGFP clusters, whereas no immunofluorescent signal was detected when the control HA-coated beads were used (Figure 2C).
CD44 fluorescent spots on the cell surface and in cell–cell contacts (Figure 2C). Interestingly, CD44 clustering induced corecruitment of PWT eGFP suggesting that both proteins are associated either directly or indirectly within the plasma membrane. Reciprocally, clustering of podoplanin also induced corecruitment of CD44 (Figure S2).

We next studied the interaction of these proteins by coimmunoprecipitation analysis. To this end, HEK293T cells were cotransfected with constructs encoding podoplanin and CD44s tagged with Flag and hemagglutinin (Hae) epitopes at the COOH-terminal end, respectively (see in Figure 5A a schematic representation of these constructs). Expression of both molecules resulted to be heterogeneous in their apparent molecular masses as several bands were detected in the immunoblots (Figure 3 and Supplemental Figure S3). We have previously shown that podoplanin heterogeneity in SDS-PAGE arises from the presence of O-linked carbohydrates in its ectodomain (Scholl et al., 1999; Martin-Villar et al., 2005). Similarly, molecular mass variability of CD44s has been commonly attributed to glycosylation changes, in particular N-linked oligosaccharides (Camp et al., 1991; Skelton et al., 1998). Indeed, removal of N-linked sugars in the CD44s protein confirmed that extracellular modifications were largely responsible for CD44s molecular mass heterogeneity (Supplemental Figure S3, C and D). Immunoprecipitation experiments confirmed that CD44 and podoplanin coprecipitated with each other (Figure 3A). Interestingly, coprecipitated CD44s and podoplanin had a lower molecular mass (45–65 kDa for CD44s, and ~30 kDa for podoplanin; Figure 3A and Supplemental Figure S3) than that of the corresponding mature fully glycosylated forms, indicating that the interaction between podoplanin and CD44s could be dependent on the carbohydrate structure present in the extracellular domain of both molecules. To confirm these results in a different cellular system, we also performed immunoprecipitation assays using the SCC cell line CarC, because it expresses high levels of podoplanin (Supplemental Figure S3, C and D). The occurrence of endogenous partially glycosylated forms of both podoplanin (~30 kDa) and CD44s (~70 kDa) was confirmed in CarC lysates (Figure 3B).
Interestingly, endogenous ~70 kDa CD44s was coprecipitated with a specific Ab recognizing murine podoplanin (PA2.26 mAb). These results demonstrate that podoplanin interacts with CD44s in SCC cells.

**Podoplanin Binds CD44s at Cell-Surface Protrusions**

The interaction of podoplanin with CD44s on the plasma membrane of MDCK cells was also confirmed in vivo by fluorescence resonance energy transfer (FRET) monitored by the acceptor photobleaching method. The mean FRET efficiency value (n = 8) between eYFP-tagged podoplanin (acceptor) and eCFP-tagged CD44s (donor) was 16% (Figure S4). To further analyze the spatial relationship between podoplanin and CD44s in vivo we used fluorescence lifetime imaging microscopy (FLIM) to monitor FRET. This technique enables visualization and quantification of protein–protein interactions by analysis of the donor lifetime decay kinetics (Parsons et al., 2008). Interaction of PWT eGFP (donor) and CD44s-mRFP (acceptor) was observed in single polarized cells, as measured by decreases in eGFP donor fluorescence lifetime relative to its lifetime in control cells expressing PWT eGFP alone (Figure 4B, compare with panel A). FRET was localized at the trailing edge during rear retraction, and on small foci that were distributed throughout the apical surface of the cell across the lamellae. Interestingly, FRET efficiency was significantly decreased in cells in contact. Although specific interaction in the free lamellae at the outer edge of the colonies was sometimes detected, no interaction was observed at cell junctions, despite the presence of high levels of both molecules (Figure 4, B and C). Moreover, stimulation of MDCK cell migration/scattering with EGF or HGF increased the number of cells in which podoplanin–CD44s complexes were present (data not shown).
These results strongly suggest that podoplanin–CD44s interaction may contribute to a motile phenotype.

Podoplanin Binding to CD44s Is not Mediated by ERM Proteins

The fact that podoplanin–CD44s interaction could be monitored by FRET suggested that the molecules may interact directly. However, because both CD44 and podoplanin bind to ERM proteins through their cytoplasmic (CT) tails, we also investigated the possibility that their interaction could be mediated by ERM proteins. To analyze this, podoplanin mutant constructs lacking the CT tail (P<sub>ACCT</sub>) or the ERM binding site (P<sub>NQNN</sub>) were used for FRET/FLIM experiments (Figure 5A). As depicted in Figure 5B-C, preventing the binding of ezrin/moesin to podoplanin (Martin-Villar et al., 2006) did not impair CD44s–podoplanin interaction, indicating that it is not mediated by ERM proteins. To further analyze which regions of the podoplanin molecule are important for podoplanin–CD44s binding we carried out co-immunoprecipitation experiments in HEK293T cells. These experiments were performed coexpressing CD44s–Hae and several podoplanin mutant constructs fused to eGFP, including P<sub>ACCT</sub>, P<sub>NQNN</sub> and a mutant construct lacking the extracellular domain (P<sub>EACF</sub>). All these mutant proteins have been previously characterized (Martin-Villar et al., 2006). The ability of P<sub>EACF</sub> mutant to interact with CD44s in vivo could not be tested in FRET/FLIM experiments due to the fact that the eGFP tag in this construct is located at the NH<sub>2</sub>-terminal end of the fusion protein (Figure 5A). However, coimmunoprecipitation assays showed that only the deletion of the podoplanin extracellular domain prevented its interaction with CD44s (Figure 5D) confirming that ERM proteins are not required for podoplanin–CD44s association. Likewise, these results indicate that the podoplanin ectodomain is a crucial region for its binding to CD44.

Podoplanin-Induced Cell Migration and Directionality Requires CD44

To analyze the contribution of CD44 to podoplanin-induced cell migration, we performed transwell assays using MDCK cells transiently cotransfected with podoplanin cDNA and a small interfering (si)RNA to mediate down-regulation of CD44. Confirming our previous findings (Martin-Villar et al., 2006), P<sub>ACCT</sub> eGFP expression increased MDCK cell migration ~1.7-fold (Figure 6). The knockdown of CD44 (~70% reduction) did not significantly change the migratory behavior of MDCK-eGFP cells. However, CD44 down-regulation prevented podoplanin-enhanced migration, demonstrating that podoplanin requires CD44 to promote cell migration in MDCK cells (Figure 6, A and B).

Our previous observations suggested that podoplanin promotes intrinsic directional motility in MDCK cells (Martin-Villar et al., 2006). To further investigate this, MDCK cells were imaged by fluorescence time-lapse microscopy allowing measurement of the total distance traveled, the displacement from origin, and calculation of mean speed and directional persistence (Figure 6, C and D). The migration pattern of control MDCK cells was erratic, with cells often changing direction, while P<sub>ACCT</sub> eGFP-expressing cells frequently continued migrating in the same direction without turning. Comparison of the migration tracks of a representative number of cells for each condition showed that the directional ratio was significantly increased in podoplanin-expressing cells, and that this was prevented when CD44 was knocked-down. Neither podoplanin expression nor CD44 down-regulation affected the migration speed of MDCK cells (Figure 6, C and D). Altogether, these results demonstrate that CD44 plays a crucial role in podoplanin-mediated migration.

Podoplanin and CD44 Depletion Alters Lamellipodia Extension/Stabilization and Cell Spreading

To further analyze the role of the podoplanin–CD44 complex in the migration of tumor cells, we down-regulated the
expression of both molecules in the SCC cell line HN5, which expresses high levels of endogenous podoplanin and CD44. We used a vector-based small hairpin (sh)RNA approach to deplete podoplanin, in combination with an al-

![Podoplanin–CD44s interaction is not mediated by ERM proteins.](image)

**Figure 5.** Podoplanin–CD44s interaction is not mediated by ERM proteins. (A) Schematic representation of podoplanin and CD44s fusion constructs used for coimmunoprecipitation and FRET/FLIM assays. SP, signal peptide; Ec, ectodomain; TM, transmembrane domain; CT, cytoplasmic tail; QN N, positive charged residues (RK.R) in podoplanin juxtamembrane domain were substituted by uncharged polar amino acids (QN.N) in order to impair podoplanin binding to ERM proteins (Martin-Villar et al., 2006). (B) MDCK cells were cotransfected with CD44s mRFP and podoplanin eGFP mutant constructs, and cells were then imaged by FLIM to detect FRET as depicted in Figure 4. Images show the eGFP multiphoton intensity image and (where appropriate) the corresponding wide-field CCD camera image of the mRFP expression. Control MDCK cells expressing PWT,P/H9004, and P QNN eGFP alone showed a normal GFP lifetime (τ in ns) in the absence of acceptor (CD44s mRFP), while cells coexpressing CD44s mRFP and podoplanin mutant constructs displayed a localized shortening of the eGFP fluorescence lifetime. (C) The bar graph represents average FRET efficiency of 15 cells over three independent experiments. (D) Coimmunoprecipitation assays performed in HEK293T cells coexpressing CD44s Hae and podoplanin eGFP mutant constructs. Total cell lysates were immunoprecipitated with an anti-GFP Ab-conjugated resin as described in Materials and Methods. The coimmunoprecipitated products were detected with an anti-Hae Ab.
ready established siRNA to mediate the knockdown CD44 in human cells (Tzircotis et al., 2005). As depicted in Figure 7A, CD44 expression was efficiently down-regulated (~70%), and similarly, podoplanin protein levels were almost completely suppressed (~90% reduction) by using two different shRNAs (podo-sh1 and -sh2). A significant siRNA-mediated reduction of CD44 levels (~80%) was also achieved in podoplanin-depleted cells. siRNA mediated down-regulation of CD44 seemed not to affect podoplanin expression and vice versa.

Control cells usually displayed a characteristic highly organized leading edge with well-extended lamellipodia extensions, and the microtubule network often aligned parallel to, but localized at a considerable distance from, the leading edge. Interestingly, the single knockdown of CD44 enhanced cell spreading whereas the opposite was found to occur by silencing podoplanin expression alone (Figure 7, B and C). CD44si cells extended wide lamella with clear marginal F-actin ruffles. These cells also showed a well organized microtubule network (Figure 7B). Podoplanin-deficient cells,

Figure 6. Podoplanin-induced migration and directionality in MDCK cells requires CD44. (A) Expression levels of podoplanin (PWT eGFP) and CD44 after PWT eGFP and CD44 siRNA coexpression. β-actin was used as a loading control. Arrowheads indicate fully (black arrowheads) and incompletely glycosylated (open arrowheads) forms of PWT eGFP. (B) Transwell migration assay. Bar graph representing percentage of migrating cells per total number of cells. Results are representative of three independent experiments performed in duplicates. (C) Representative migration tracks of MDCK cells expressing PWT eGFP in the presence or absence of CD44 (n = 30). (D) Average persistence and speed of migration derived from the tracks depicted in C. n = 40–85 cells per bar. Asterisks indicate significant differences in a Student’s t test. **p < 0.005, *p < 0.05.
however, had an extremely disorganized leading edge. These cells were unable to extend typical broad lamellipodia, exhibiting narrower (filopodia-like) protrusions instead, and the microtubules penetrated into the leading edge area (Figure 7B). RNAi-mediated knockdown of endogenous CD44 in podoplanin-deficient cells did not significantly change the cell shape of these cells (Figure 7, B and C). These results point to a role of the podoplanin–CD44 complex in regulating lamellipodia extension/stabilization during cell spreading and migration.

Podoplanin and CD44 Cooperate to Promote Directional Cell Motility during Wound Healing

To assess whether the observed morphological changes in single and double knockdown HN5 cells were coupled with defects in cell motility, we performed an in vitro wound healing assay. Time-lapse video microscopy was used to monitor wound closure. All the control cell lines showed ~95% wound closure 18 h after wounding. Podo-sh1 and sh2 cells covered ~40 and 60% of the control area, respectively, and CD44si cells covered 20–30% in the same period of time. Interestingly, wound closure delay became significantly more evident in double knockdown cells, as only 2–10% of the wound area was covered (Figure 8B and Movie 2 in supplemental material). Moreover, by silencing both molecules the migration of HN5 cells toward the wound was almost suppressed. Altogether, these results strongly indicate that podoplanin and CD44 collaborate to promote directional motility.

DISCUSSION

Podoplanin and CD44 are type I membrane glycoproteins that are anchored to the actin cytoskeleton through association with ERM proteins (Legg and Isacke, 1998; Yonemura et al., 1998; Scholl et al., 1999; Martin-Villar et al., 2006), and both are involved in cell adhesion, motility, and signal transduction despite lacking intrinsic kinase activity (reviewed in Ponta et al., 2003; Wicki and Christofori, 2007). In this study, we report CD44 as a novel binding partner for podoplanin and describe a close relationship between CD44s and podoplanin expression associated with EMT and advanced stages of tumor progression in an experimental model of carcinogenesis. Although studies regarding expression of CD44 isoforms in human cancer are contradictory (Marhaba and Zoller, 2004), it has been proposed that a switch from CD44v to CD44s expression is a feature of poorly differentiated
SCCs (Hudson et al., 1996; Stoll et al., 1999). Accordingly, our conclusion from the analysis of both tumors and cell lines derived from the mouse skin carcinogenesis model is that despite the high heterogeneity of CD44 expression in vivo, a clear CD44s (and podoplanin) up-regulation is observed associated with the loss of differentiation in skin carcinomas. Similarly, increased expression of podoplanin has been associated with poor clinical outcome and metastasis in human SCCs (Yuan et al., 2006; Chuang et al., 2009). Interestingly, a recent study has found that podoplanin and CD44 were coexpressed in cells localized at the periphery of tumor nests in a high proportion of SCCs of the lung, although the distribution of CD44 was broader than that of podoplanin (Shimada et al., 2009). Moreover, CD44 is a common cell-

Figure 8. Knockdown of CD44 and podoplanin in oral carcinoma HN5 cells impairs directional migration during wound healing. (A) Analysis of the migration pattern at the wound edge of podoplanin and CD44 knocked-down HN5 cells. The migration paths of representative cells taken from the wound edge are indicated on an overlay image from the initiation of the imaging (0 h). Images of the wounds at 6 h after the incision was made are shown in the middle panel. Trace of the movement of multiple cells along the wound edge (n = 30) are shown in the graphs of the right panel. (B) Quantification of the wound closure after 18 h. (C) Average persistence of migration (directionality) from the tracks depicted in A. An animate and complete (18h) sequence of these data are shown in Supplemental Movies 1 and 2. P values were obtained using one-way analysis of variance (ANOVA). *p < 0.05; **p < 0.005, ***p < 0.0005.
surface marker for cancer stem cells in solid tumors (Vis-
vader and Lindeman, 2008), and podoplanin has recently
been postulated as a novel candidate marker of SCC stem
cells, where it was coexpressed with CD44 (Atsumi et al.,
2008). These data suggest that there is a subpopulation of
carcinoma cells with stem-like properties that coexpress
podoplanin and CD44. While the biological significance of
the presence of both proteins in cancer stem cells is still
uncertain, it is tempting to speculate that coexpression of
podoplanin and CD44s identifies a cancer stem cell sub-
population with a high potential to disseminate and me-
tastasize because of its association with EMT and the
undifferentiated state of SCCs (Polyak and Weinberg,
2009).

Interestingly, we also found that podoplanin and CD44s
interact to form a dynamic complex at the plasma membrane
of migratory cells. This interaction seems to be direct, as it is
not mediated by their common partners ezrin/moesin, and
might be dependent on extracellular modifications of both
glycoproteins. Supporting this hypothesis is the finding that
the extracellular domain of podoplanin appears to be crucial
for its association with CD44s. Accordingly, podoplanin in-
teractions with other proteins such as CLEC-2 (Kato et al.,
2008) or galectin-8 (Cueni and Detmar, 2009) have been
found to be dependent on the carbohydrate moieties present
in their ectodomains. Moreover, it has been recently re-
ported that recombinant soluble forms of the podoplanin
ectodomain with different degrees of glycosylation can de-
crease cell adhesion and migration of lymphatic endothelial
cells. Interestingly, in this study, the less glycosylated forms
were more effective than the more extensively glycosylated
proteins (Cueni et al., 2010). CD44 is known to undergo
sequential proteolytic cleavages that have an important role
in cell migration (reviewed in Nagano and Saya, 2004). For
this reason, one could argue that the CD44 forms (~45–70
kDa) coprecipitating with podoplanin might be fragments of
the molecule rather than less glycosylated proteins. How-
ever, these forms were recognized either by antibodies
raised to the CD44 NH2-terminal end (IM7: Figure 3B) or to
its CT tail (anti-CD44cyto Ab or anti-Hae Ab; Figure 3A and
Supplemental Figure S3), indicating that these forms are not
processing products of 80-kDa CD44s. Moreover, inhibition
of CD44 proteolysis did not alter the pattern of CD44s co-
coprecipitating forms (Supplemental Figure S3). On the other
hand, removal of N-linked oligosaccharides in the CD44s
molecule gave rise to protein forms of ~45–70 kDa with
similar apparent molecular masses as the ones precipitated
together with podoplanin. Glycosylation has been implicated
in the regulation of CD44-mediated cell binding to
HA, as well as in CD44 association with the actin cytoskel-
eton (Thorne et al., 2004). The fact that podoplanin–CD44s
interaction preferentially occurs when both molecules are
not heavily glycosylated allows us to speculate that this
association could be regulated by the glycosylation status
of both molecules. Further investigations to address the me-
chanisms that regulate podoplanin–CD44s complex formation
and its dependence on differential glycosylation are cur-
rently in progress.

Podoplanin–CD44s interaction appears to occur mainly on
the trailing edge and on discrete foci on the lamella. Because
both proteins can activate RhoA GTPase through binding to
ERM proteins (Hirao et al., 1996; Martin-Villar et al., 2006),
and RhoA-ROCK activity is required for actomyosin-based
cortical contractility leading to detachment of the trailing
edge (Ridley, 2001), podoplanin–CD44s interaction might be
associated with tail retraction during mesenchymal-type of
tumor cell movement (Friedl and Wolf, 2003). However, it is
not clear yet how binding of these molecules to ERM pro-
teins affects podoplanin–CD44s association and whether po-
doplanin–CD44s interaction influences signal transduction
pathways mediated by any of these two molecules. On the
other hand, knockdown of CD44 in HN5 oral SCC cells
resulted in increased spreading and formation of extended
lamella, while the opposite effect was observed upon silenc-
ing podoplanin expression. Moreover, knockdown of CD44
in podoplanin-deficient cells did not change significantly the
shrunken shape characteristic of these cells. These results
also suggest an involvement of podoplanin in lamellipodial
extension/stabilization and spreading that is regulated by
CD44. What strongly emerges from our gain- and loss-of-
function experiments is the notion that podoplanin and
CD44 cooperate to stimulate directional motility. Thus, po-
doplanin enhances the intrinsic directional motility of
MDCK cells migrating either randomly or in a chemotactic
transwell assay, and this effect is abolished by knockdown-
CD44. Likewise, single knockdown of either CD44 or
podoplanin inhibited the migratory potential of HN5 carci-
noma cells in an in vitro wound healing assay, and depletion
of both molecules almost suppressed the migration of HN5
cells toward the wound. Both CD44 and podoplanin appear
to be required for cells to maintain direction during move-
ment, as the knockdown of any of them resulted in less
directional migration. Previous studies have found an im-
portant role for CD44 in mediating directional migration of
different cell types, including cancer cells (Milette-Gonzalez
et al., 2005; Tzircotis et al., 2005; Colone et al., 2008). Inter-
estingly, in neutrophils and fibroblasts, CD44 is characteris-
tically localized at the trailing edge of polarized cells (Jac-
son et al., 1984; Soeava et al., 2001). It has been found that
the binding of CD44 to ezrin is crucial for CD44-dependent
directional motility (Legg et al., 2002). Also, podoplanin
mutant proteins unable to bind ezrin/moesin confer less
motile phenotypes than wild-type podoplanin when ex-
prescribed in MDCK cells (Martin-Villar et al., 2006). How-
ever, it remains to be investigated whether podoplanin associa-
tion with ERM proteins controls podoplanin-dependent di-
rectional motility as occurs with CD44.

Interest in podoplanin has considerably increased over the
years because of its up-regulation in different human tu-
mors, especially those derived from squamous stratified
epithelia. However, many aspects of the biology and func-
tion of this glycoprotein still remain elusive. The lack of any
obvious functional domains in the podoplanin molecule
highlights the need to identify its binding partners. Simi-
larly, the precise mechanisms controlling the structural and
signaling events associated to CD44 have yet to be eluci-
dated. In summary, the data presented here demonstrate
that podoplanin associates with CD44s and that this inter-
action is important for driving directional cell migration in
epithelial and tumor cells. Determining how this interaction
is regulated could help to clarify many open questions in
the biology of both molecules.

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