Podoplanin-mediated cell adhesion through extracellular matrix in oral squamous cell carcinoma

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Podoplanin (PDPN), one of the representative mucin-like type-I transmembrane glycoproteins specific to lymphatic endothelial cells, is expressed in various cancers including squamous cell carcinoma (SCC). On the basis of our previous studies, we have developed the hypothesis that PDPN functions in association with the extracellular matrix (ECM) from the cell surface side. The aim of this study was to elucidate the molecular role of PDPN in terms of cell adhesion, proliferation, and migration in oral SCC cells. Forty-four surgical specimens of oral SCC were used for immunohistochemistry for PDPN, and the expression profiles were correlated with their clinicopathological properties. Using ZK-1, a human oral SCC cell system, and five other cell systems, we examined PDPN expression levels by immunofluorescence, western blotting, and real-time PCR. The effects of transient PDPN knockdown by siRNA in ZK-1 were determined for cellular functions in terms of cell proliferation, adhesion, migration, and invasion in association with CD44 and hyaluronan. Cases without PDPN-positive cells were histopathologically classified as less-differentiated SCC, and SCC cells without PDPN more frequently invaded lymphatics. Adhesive properties of ZK-1 were significantly inhibited by siRNA, and PDPN was shown to collaborate with CD44 in cell adhesion to tether SCC cells with hyaluronan-rich ECM of the narrow intercellular space as well as with the stromal ECM. There was no siRNA effect in migration. We have demonstrated the primary function of PDPN in cell adhesion to ECM, which is to secondarily promote oral SCC cell proliferation.

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Podoplanin (PDPN), a type-I transmembrane sialomucin-like glycoprotein, was originally identified in glomerular podocytes.¹ Because of its characteristic localization in lymphatic endothelial cells,² PDPN has been utilized as an immunohistochemical marker for lymphatics in histopathological studies for neoplastic³ and inflammatory⁴ lesions. Recently, however, PDPN expression has been also confirmed in various types of tumor cells, including squamous cell carcinoma (SCC).^{5–11}

In the field of oral cancer, PDPN has also been regarded as a biomarker in predicting the risk of cancer development and poor clinical outcome.^{10–12} We have also shown the PDPN expression profiles to be a useful immunohistochemical aid in the differential diagnosis of oral carcinoma *in situ* (CIS) from epithelial dysplasia because PDPN positive (+) cells are distributed more widely in CIS than in epithelial

dysplasia, overlapping with Ki-67 + -proliferating cell zones.¹³ In order to provide a background to the cell-proliferating potentials of oral CIS, we have demonstrated the characteristic intercellular deposition and turnover of perlecan, one of the extracellular matrix (ECM) molecules, which results in the widening of intercellular space of the epithelial compartment.^{14–16} In addition, we have reported that PDPN+ cells are characteristically localized within odontogenic tumor-specific architectures, in which ECM signaling seems to be enhanced.⁷

PDPN has been suggested to have a role in cell migration and invasion, through interactions with its binding partners, such as ezrin,¹⁷ CD44,¹⁸ and heat shock protein A9.¹⁹ Those interactions have been shown to induce epithelial–mesenchymal transition (EMT),¹⁷ although the involvement of EMT has been a matter of argument.²⁰ Hence, the actual

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molecular function of PDPN in cancer cells has remained largely unknown. In our previous studies, we have paid attention to the characteristic expression mode of PDPN on the cell border or the basolateral surface of tumor cells,^{7,13} and we have formulated the hypothesis that PDPN has a role in tumor cells' communication with ECM or ECM-mediated cell adhesion. In the present study, we have attempted to examine the function of PDPN by RNA interference (RNAi) with a special reference to cell adhesion and its relationship with the other ECM-signaling molecules using oral SCC cells.

MATERIALS AND METHODS

Clinical Samples

Forty-four cases of oral SCC were collected for the present study from the surgical pathology files of the Division of Oral Pathology, Niigata University Graduate School of Medical and Dental Sciences, during a 4-year period from 2008 to 2011 after histopathological review of those specimens. The surgical samples were fixed in 10% formalin, processed routinely, and embedded in paraffin. Serial sections cut at 4 μ m from paraffin blocks were used for hematoxylin and eosin staining and immunohistochemistry. The experimental protocol for analyzing surgical materials was reviewed and approved by the Ethical Board of the Niigata University Graduate School of Medical and Dental Sciences (Oral Life Science).

Cells and Reagents

SCC cell systems (lines) (ZK-1, ZK-2, and MK-1) were established from SCC arising in the tongue (ZK-1 and ZK-2)²¹ and gingiva (MK-1).²² Salivary gland adenoid cystic carcinoma (ACC) cell systems (lines), ACC2 and ACC3, were established from ACCs of the palatal minor salivary gland and parotid gland, respectively,²³ and ACCM was subcloned from ACC2 cells as a clone with highly metastatic potential.²² SCC cells were cultured in Dulbecco's modified Eagle medium (DMEM; Gibco, Invitrogen, Carlsbad, CA, USA) and ACC cells were cultivated in RPMI 1640 medium (Gibco), both of which contained 10% fetal bovine serum (Gibco), 50 μ g/ml streptomycin, and 50 IU/ml penicillin (Gibco) (DMEM+, RPMI+). They were incubated at 37 °C in a humidified 5% carbon dioxide/95% air atmosphere.

Antibodies

A mouse monoclonal antibody against human PDPN (D2-40, IgG₁), which recognizes the extracellular domain of PDPN, was obtained from Dako (Glostrup, Denmark). Rabbit polyclonal antibodies to human ezrin and ezrin (phosphor T567; p-ezrin (T567)) and a mouse monoclonal antibody to human β -actin (mAbcam 8226, IgG₁) was purchased from Abcam (Cambridge, UK). Rabbit polyclonal antibodies against human ezrin/radixin/moesin (ERM) were purchased from Cell Signaling Technology (Danvers, MA, USA). A rabbit monoclonal antibody against human CD44

(EPR1013Y) was purchased from Epitomics (Burlingame, CA, USA). Mouse and rabbit preimmune IgGs for control experiments were also obtained from Dako.

Immunohistochemistry

Immunohistochemistry was performed using the ChemMate Envision System (Dako), as described elsewhere.²⁴ The sections were treated with 0.3% hydrogen peroxide in methanol for 30 min at room temperature to block endogenous peroxidase activity, and were incubated with 5% milk protein (Morinaga Milk Industry, Tokyo, Japan) in 0.01 M phosphate-buffered saline (PBS, pH 7.4) containing 0.05% Triton X-100 for 1 h at room temperature to block nonspecific protein-binding sites. They were then incubated overnight at 4 °C with the primary antibodies diluted at 1:50 (anti-PDPN) in PBS. After overnight incubation, the sections were incubated with the Envision reagents for 1 h at room temperature and treated with SIGMAFAST DAB With Metal Enhancer Tablet Set (Sigma Chemical, Saint Louis, MO, USA), according to the manufacturer's instructions, to visualize the reaction products. Finally, the sections were counterstained with hematoxylin. For control studies, the primary antibodies were replaced with preimmune IgGs. On the basis of immunostainings in carcinoma cells, SCC cases were evaluated as positive (any positive modes) or not positive (not at all).

Gene Expression Analysis

Total RNA was isolated from the cells using the ISOGEN System (Nippon Gene, Tokyo, Japan). First-strand cDNA was synthesized with the SuperScript III First-Strand Synthesis System (Invitrogen). Following reverse transcription, real-time PCR was carried out using a MiniOpticon Real-Time PCR Detection System CFB-3120 (Bio-Rad Laboratories, Hercules, CA, USA). Amplification of target genes was monitored in real-time and gene expression levels were quantified using CFX Manager (Bio-Rad), according to the manufacturer's instructions. Primer sets used for the real-time PCR experiments are summarized in Table 1. Amplified products of the genes were also checked by conventional agarose gel electrophoresis.

Western Blotting

Cell cultures in 60 mm dishes were lysed with 350 μ l of lysis buffer (50 mM HEPES (pH 7.4), 150 mM NaCl, 1% Triton X-100, a protease inhibitor cocktail (Nacalai Tesque, Kyoto, Japan; 1 mM PMSF, 1 mM Na₃VO₄, and 10 mM NaF), and the supernatant of the cell lysates was recovered. After the total protein was determined, an aliquot of 10 μ g of proteins was subjected to SDS-polyacrylamide gel electrophoresis under reducing condition and the gels were transferred to polyvinylidene difluoride membranes (Bio-Rad). After incubation with 0.5% enhanced chemiluminescence (ECL) blocking agent (GE Healthcare UK, Buckinghamshire, UK) in 50 mM Tris-buffered saline

Table 1 Primer sequences for quantification of gene expression

Gene	Primer sequence
Human podoplanin	F: 5'-CCAAGCGCCACAGCCTCAA-3' R: 5'-GGCACAGAGTCAGAAACGGT-3'
Human CD44	F: 5'-TCCAACACCTCCCAGTATGACA-3' R: 5'-GGCAGGTCTGTGACTGATGTACA-3'
Human ezrin	F: 5'-GCTTTTGTATCAGGTGGTAAAGACT-3' R: 5'-TCCACATAGTGAGGCCAAAGT-3'
Human β -actin	F: 5'-TCACCCACACTGTGCCATCTACGA-3' R: 5'-CAGCGGAACCGCTCATTGCCAATGG-3'

Abbreviations: F, forward; R, reverse.

(pH 7.4) containing 0.1% Tween-20 (TTBS) for 1 h at room temperature, the membranes were further incubated overnight at 4 °C with primary antibodies diluted with TTBS (PDPN, 1:100; CD44, 1:100; ERM, 1:1000; ezrin, 1:1000; p-ezrin (T567), 1:1000; β -actin, 1:5000). Following washing with TTBS, the membranes were reacted with secondary antibodies (ChemMate Envision reagent, Dako, diluted at 1:1000 in TTBS) for 1 h at room temperature. Target protein bands were visualized by ECL Plus western blotting detection reagents (GE), according to the manufacturer's instructions.

Immunofluorescence

Immunofluorescence experiments were performed using Lab-Tek II Chamber Slide System (LabTek, Scotts Valley, CA, USA). Cells were plated at the concentration of 1.2×10^4 cells per well and cultivated for 72 h. The cells were washed with PBS and fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 30 min on ice. To prevent nonspecific protein binding, they were incubated with 2% normal goat serum (Dako) in PBS containing 0.05% Triton X-100 overnight at 4 °C. The cells were then incubated with the primary antibodies (PDPN, diluted at 1:50 in PBS; CD44, 1:250; ezrin, 1:1000) and further with secondary antibodies (Alexa Fluor 568-conjugated goat anti-mouse IgG (H + L) or Alexa Fluor 488-conjugated goat anti-rabbit IgG (H + L; Invitrogen), diluted at 1:200 in PBS for 30 min each at room temperature. Tissue sections were autoclaved in citric acid buffer (pH 6.0) at 120 °C for 10 min. After the pretreatment, sections were blocked with milk protein and then incubated with antibodies against PDPN or CD44 and the secondary antibodies. Finally, cells and tissues were counterstained with Cellstain Hoechst-33258 solution (Dojindo Laboratories, Kumamoto, Japan) diluted at 1:100 in PBS. For control studies, the primary antibodies were replaced with preimmune mouse or rabbit IgGs.

RNA Interference

RNAi experiments were performed using a Stealth RNAi siRNA Duplex Oligoribonucleotides System (Invitrogen). The three human siRNA-targeting PDPN (siPDPN) sequences used in this experiment were as follows: #1, 5'-UGAAG UUGGC AGAUC CUCGA UGCGA-3'; #2, 5'-AAACU UUGCA GUUGA GAACU U-3'; and #3, 5'-UUCAA AUAAU CUUUU AUAGA A-3'. Stealth RNAi-negative control medium GC Duplex #2 (Invitrogen; negative control siRNA (siNC)) and sterile Milli-Q water (mock) were used as negative controls. siRNA transfection was performed with Lipofectamine RNAiMAX reagents (Invitrogen) by reverse transfection method, which was recommended by the manufacturer. Shortly thereafter, ZK-1 cells were incubated with siRNA and Lipofectamine complex for 48 h. Cells were then trypsinized and subcultured in fresh media (time point: 0 h) in order to start siRNA experiments highly effectively from the beginning. The cells were harvested at 72, 120, and 168 h. Efficiencies for RNAi were evaluated by using real-time PCR, western blotting, and immunofluorescence.

DNA Microarray

To determine altered gene expression profiles of ZK-1 cells when their PDPN gene expression was suppressed by RNAi, DNA microarray analysis using the GeneChip Human Genome U133 Plus 2.0 Array (Affymetrix, Santa Clara, CA, USA) was performed for RNA samples from siPDPN and siNC cells at 48 h after transfection. Total RNA was isolated from siPDPN and siNC cells using the ISOGEN system,²² and 100 ng of each aliquot was labeled using the Ambion WT Expression Kit (Ambion, Austin, TX, USA) and the GeneChip WT Terminal Labeling and Control Kit (Affymetrix).²⁵ Scanned data were quantified by the Robust Multichip Average algorithm using the Affymetrix Expression Console Software (Affymetrix). Signal data were then quantile-normalized to calculate Z-scores and ratios.

Cell Proliferation Analysis

To study the role of PDPN in cell proliferation, we determined growth curves of siRNA-transfected ZK-1 cells. siPDPN, siNC, and mock were plated at 1.0×10^5 cells per 60 mm dish in DMEM + . At 24, 48, 72, 96, and 120 h after plating, the cells were collected by trypsinization and the viable cells were counted under a microscope. We also performed an MTS cell-proliferation assay using CellTiter 96 AQueous One Solution Cell Proliferation Assay Kit (Promega, Madison, WI, USA). siPDPN, siNC, and mock cells were plated at 1.0×10^3 cells per well on 96-well microplates ($n=9$ each), and cell viability was evaluated every 24 h according to the distributor's protocol.

Apoptosis Assay

Apoptotic cells were detected by flow cytometry using a MEBCYTO Apoptosis Kit (MBL, Nagoya, Japan).²⁶ siRNA-transfected cells (siPDPN, siNC, and mock) were plated at

the concentration of 1.0×10^5 into 60 mm dishes and incubated for 72 or 120 h at 37 °C. After incubation, cells were treated and stained with Annexin V-FITC and propidium iodide (PI), according to the distributor's protocols. The treated cells were applied on a BD FACS Aria II Cell Sorter (BD Biosciences, Franklin Lakes, NJ, USA) with a single-laser-emitting excitation at 488 nm.

Cell Adhesion Assay

For cell adhesion assay, siPDPN, siNC, and mock cells were plated at 200 cells per well on 96-well microplates in 100 μ l of DMEM + and incubated at 37 °C. At 30, 60, 90, and 120 min after plating, non-adherent cells were removed by gentle washing with PBS. Attached cells were fixed with 100% methanol and then counted under a phase-contrast microscope. To study the effect of the anti-PDPN antibody in cell adhesion, we cultivated ZK-1 cells in DMEM in the presence of 10–100 ng of the antibody and control IgG, from which sodium azide had been excluded by gel filtration. We also examined the effect of calcium in cell adhesion by using DMEM (1 $\times$) liquid (high glucose) without ionized calcium (Invitrogen) for cell suspension. In addition, we studied the participation of hyaluronic acid in cell adhesion. Microplates were coated with 2 mg/ml of swine hyaluronic acid (Seikagaku Biobusiness, Tokyo, Japan) in PBS,²⁷ and siPDPN, siNC, and mock cells were plated onto the plates. For controls, microplates were coated with 1% BSA in PBS. Cell counts at each time point were calculated for mean values from triplicate experiments.

In vitro Scratch-Wounding Assay

An artificial wound was generated on confluent monolayers of siPDPN, siNC, and mock cells in 60 mm culture dishes with a 200- μ l yellow pipette tip (Molecular Bio Products, San Diego, CA, USA). The processes of filling the wound area by migrating cells were observed under a phase-contrast microscope at 0, 6, 12, and 24 h after plating.^{20,28}

Transwell Migration and Matrigel Invasion Assays

Cell migration and invasion were determined by using BD Falcon cell-culture insert-12 well companion tissue-culture plate systems with a pore size of 8 μ m polyethylene terephthalate membrane (BD Biosciences). For the invasion assay, the insert membrane was coated with 40 μ l of BD Matrigel (BD Biosciences), which was diluted at 1:1 with DMEM and air dried. For both assays, cells (siPDPN, 2.0×10^5 ; siNC, 0.87×10^5 ; mock, 0.82×10^5) were seeded in 500 μ l of DMEM in the insert chamber and the lower chamber was filled with 1 ml of DMEM +. After 2 h incubation, floating cells were removed and replaced with fresh DMEM. Following an additional 22-h incubation (total 24 h), cells in the insert chamber were carefully removed with cotton swabs and the bottom membrane was fixed and stained with 100% methanol and 1% toluidine blue. Cells that had traversed the membrane were counted under a

microscope, and the mean values were calculated from triplicate experiments.²⁰

Statistical Analysis

To evaluate the significance of correlation between PDPN expression and clinicopathological characteristics, we used the χ^2 -test or Kruskal–Wallis test (Table 2). Comparative experimental data from cell functional studies were analyzed using the Student's *t*-test. The cut-off for statistical significance was set at $P < 0.05$. All statistical analyses were carried out using the SigmaPlot 2001 for Windows version 7.0 (SPSS, Chicago, IL, USA), KyPlot version 2.0, and Microsoft Excel 2007.

Table 2 Correlation between podoplanin and clinicopathological characteristics in 44 cases of oral squamous cell carcinoma (SCC)

Variable	Podoplanin expression			P-value
	Positive (n = 30)	Not positive (n = 14)	Positive rate (%)	
Gender				0.276 ^a
Male (n = 25)	14	9	56.0	
Female (n = 19)	16	5	64.0	
Age				0.048 ^a
≤ 50 (n = 7)	7	0	100.0	
≥ 50 (n = 37)	23	14	62.2	
Tumor size (mm)				1.765 ^b
≤ 20 (n = 25)	17	8	68.0	
≤ 40 (n = 14)	10	4	71.4	
> 40 (n = 5)	3	2	60.0	
Nodal metastasis				0.837 ^a
+ (n = 21)	14	7	66.7	
– (n = 23)	16	7	69.6	
Local recurrence				0.030 ^a
+ (n = 10)	4	6	40.0	
– (n = 34)	26	8	76.5	
Differentiation				0.002 ^a
Well (n = 35)	27	8	77.1	
Moderately (n = 9)	3	6	33.3	
Lymphatic invasion				0.004 ^a
+ (n = 22)	9	13	40.9	
– (n = 22)	21	1	95.5	
CD44				0.034 ^a
+ (n = 42)	30	12	71.4	
– (n = 2)	0	2	0.0	

^aStatistical analysis: χ^2 -test.

^bStatistical analysis: Kruskal–Wallis test.

RESULTS

Immunohistochemistry and Clinicopathological Analysis

In the present series of SCC cases (Figure 1a-A), PDPN was mainly localized on the cell border of peripheral cells of SCC cell foci (30/44 cases, 68.1%; Figures 1a,B and C), whereas no positive staining for PDPN was obtained in 14 SCC cases (31.8%; Table 2). Even in PDPN+ cases, positive stainings were not observed in SCC cell foci within the lymphatic space (Figures 1a-D and E).

The cases were divided into PDPN+ or PDPN-not positive (–) cases, and their clinicopathological parameters, as listed in Table 2, were examined for their tissue PDPN expression modes. All patients younger than 50 years of age were associated with PDPN+ SCC ($P < 0.05$, Table 2). PDPN+ patients were associated with a lower local recurrence ($P < 0.05$, Table 2). As for the histological features, PDPN+ cases tended to be of the well-differentiated type ($P < 0.01$, Table 2) and free from lymphatic invasion ($P < 0.01$, Table 2). However, there were no significant differences in the other clinical parameters of gender, tumor size, nodal metastasis, and CD44 expression, to which association with PDPN will be described later (Table 2).

Next, we examined PDPN expression at both gene and protein levels in different cell systems from oral SCCs and ACCs. PDPN was specifically expressed in SCC cells but not in ACC cells (Figure 1b). Among the SCC cell systems, the PDPN expression levels were the most pronounced in ZK-1, and thus we chose to use this cell system in the following functional assays. The PDPN protein expression was also confirmed by immunofluorescence; it was confined to the cell border and to the outer boundary of ZK-1 cells (Figure 1c). The cell border expression of PDPN was not obviously seen in ZK-2 cells because they did not form round-shaped colonies, whereas MK-1 cells, which are highly metastatic,²² showed some slight PDPN on the cell border localization (Supplementary Information 1). There was no PDPN expression in ACC cells (not shown).

PDPN Function in Cell Adhesion

Among the three siRNA sequences, all of which were confirmed by RT-PCR to suppress PDPN gene expression, sequence #1 was most effective in suppression and was utilized in the following experiments. When the PDPN gene expression was knocked down by siRNA in ZK-1 cells, it was

Figure 1 Podoplanin (PDPN) expression in oral squamous cell carcinoma (SCC) at tissue (a) and cellular (b, c) levels. (a) Histopathology and immunohistochemistry: (A, D) hematoxylin and eosin (H-E) stain, (B, C, E) immunoperoxidase stain for PDPN, hematoxylin counterstain, (A, B) $\times 50$, (C) $\times 100$; (D, E) $\times 75$; scale bars, 100 μm . (b) Gene and protein: (upper) real-time PCR analysis, relative PDPN gene expression levels for SCC cells (ZK-1, ZK-2, MK-1) and adenoid cystic carcinoma cells (ACC2, ACC3, ACCM) against the ZK-1 level (means $\pm$ s.e. from triplicate experiments), (lower) western blot analysis for PDPN and β -actin in cell lysates of SCC and ACC cells. (c) Immunofluorescence: (left) PDPN, (right) preimmune IgG, Hoechst-33258 (blue) counterstain, $\times 100$, scale bar, 100 μm . PDPN was linearly localized on the cell border of peripheral cells of SCC foci (a-A, B, C), while it tended to be lost in SCC cells invading lymphatics (a-D, E). The PDPN expression was specific to SCC cells, especially to ZK-1, but not to ACC cells (b), and it was also confined to the cell border or the external periphery of ZK-1 cells (c).

significantly suppressed in siPDPN cells for at least 120 h (down to 7% of controls at 72 h, 10% at 120 h; $P < 0.001$) but had slightly recovered at 168 h (36%, $P < 0.01$) after plating (Figure 2a). These gene suppression tendencies were confirmed to be basically the same in the protein level when analyzed by western blotting (Supplementary Information 2a) and immunofluorescence (Supplementary Information 2b). Thus, we carried out the following experiments using 120-h-treated siPDPN cells. Cell numbers were counted every 24 h for 120 h and compared between siPDPN, siNC, and mock. Up to 120 h, cell numbers of siPDPN were obviously

smaller than those of the two controls (72–120 h, $P < 0.001$; 48 h, $P < 0.01$; Figure 2b). This tendency toward a decline in siPDPN cells was also confirmed by the MTS-cell proliferation assay (Supplementary Information 3).

To elucidate the cause for such a cell growth inhibition in siPDPN, we first compared apoptotic cell ratios between siPDPN and controls by Annexin V-FITC and PI double staining. They were 4.6% in siPDPN, 2.3% in siNC, 2.2% in mock at 72 h, and 5.1% in siPDPN, 3.4% in siNC, 2.1% in mock at 120 h (Supplementary Information 4). Although the apoptotic cell ratios in siPDPN at 120 h were approximately

Figure 2 Functions of podoplanin (PDPN) in cell adhesion. (a) Knockdown experimental conditions of PDPN by siRNA in ZK-1. Comparison of relative PDPN gene expression levels determined by real-time PCR between cells transfected by siRNA-targeting PDPN (siPDPN), negative control siRNA (siNC), and mock for a week. (b) Growth curves of ZK-1 cells for 120 h compared between the three conditions are mentioned above. (c) Cell adhesion assays in the three conditions. (d) Cell adhesion assays with the presence and absence of discriminative antibodies to the extracellular domain of PDPN. (e) Cell adhesion assays with the presence or absence of calcium. (f) Cell growth assays started with cell numbers subtracted from control experiments. (b, c, e, f) Closed circle, siPDPN; open circle, siNC; closed triangle, mock. (d) Closed circle, with PDPN monoclonal antibodies; open circle, preimmune IgG; closed triangle, water (mock). (e) Open triangle, siPDPN; closed square, siNC; open square, mock in calcium-free DMEM. All data are means $\pm$ s.e. from triplicate experiments. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. PDPN expression levels were significantly lowered in siPDPN cells ($P < 0.001$) for 120 h (a). Up to 120 h, ZK-1 cells showed an obvious decline in growth (b). Their cell adhesion was gradually suppressed and $\sim 60\%$ inhibited at 120 min (c). Similarly, ZK-1 cell adhesion was suppressed in the presence of PDPN antibodies (d). It was shown to be significantly calcium dependent (e). The numbers of siNC and mock cells were reduced for plating by subtraction to be equal to those of siPDPN cells attached at 2 h after plating in order to start cell growth experiments in optimized conditions. As a result, there was no difference between the three conditions. The delayed cell growth was due to suppressed cell adhesion, and to the fact that PDPN functions directly in cellular attachment and secondly affects cell growth (f).

twofold larger than that in the controls, the final ratio of 5.1% was not considered as explaining the significant suppression of cell growth shown in Figure 2b (down to 34%). Therefore, it was concluded that apoptosis was not the major reason for the suppression in siPDPN cell growth.

Next, we examined the cell adhesion status in siPDPN by using different microplate assays. When ZK-1 cells attached to the plate were counted every 30 min up to 120 min and compared between siPDPN and controls, their cell adhesiveness was more significantly suppressed in siPDPN than in controls (30% of controls at 30 min, $P < 0.05$; 46% at 60 min, $P < 0.01$; 38% at 90 min, $P < 0.001$; 43% at 120 min, $P < 0.001$; Figure 2c). As PDPN was shown to be involved in cell adhesion, we examined the function of the extracellular domain of PDPN in ZK-1 cells by incubation with its discriminative antibody to the extracellular domain (PDPN mAb). After adding PDPN mAb, we counted the number of attached cells every 30 min for 120 min. The addition of PDPN mAb clearly suppressed ZK-1 cell attachment (66% of controls at 30 min; 71% at 60 min, $P < 0.05$; 67% at 90 min, $P < 0.01$; 81% at 120 min, $P < 0.01$; Figure 2d). The results indicated that PDPN-mediated ZK-1 cell adhesion through its extracellular domain.

Furthermore, to examine whether PDPN-mediated ZK-1 cell adhesion was calcium dependent, we performed cell adhesion assays as described above (Figure 2c) by the use of calcium-depleted media. Although control cells were significantly suppressed in their adhesion for 60–120 min after plating in calcium-depleted media, siPDPN cells were not affected at all by calcium depletion for 30–90 min (Figure 2e). To exclude the possibility that gene expression levels for cadherin-family proteins, which are involved in calcium-dependent cell–cell adhesion, were downregulated coordinately with the PDPN gene suppression by RNAi, their gene expression profiles were also analyzed by DNA microarray. However, there was no significant difference in the gene expression levels for cadherin-family members between siPDPN and siNC cells (Table 3 in Supplementary Information 5). The result revealed that PDPN-mediated cell adhesion in ZK-1 cells was calcium dependent.

As the results mentioned above convinced us that the cell growth suppression in siPDPN was due to cell attachment suppression, we again compared cell growth curves between siPDPN and two controls, in which cell numbers for plating were reduced to be equal to the attached cell numbers in siPDPN at 2 h after plating. As a result, there was no difference in the numbers of attached cells between three different conditions at each time point during the 120-h experimental period (Figure 2f). It was hence concluded that the delayed cell growth in siPDPN was due to suppression of ZK-1 cell attachment.

PDPN Function in Cell Migration and Invasion

The effect of PDPN on cell migration and invasion was comparatively examined between siPDPN and controls by

scratch wounding, transwell migration, and Matrigel invasion assays. There was no definite difference between the three different cell conditions in cell migration (Figures 3a and b) or invasion assays (Figure 3c).

PDPN Association with CD44

As a PDPN function in cell adhesion was demonstrated in the experiments mentioned above, we wanted to elucidate the relationship between PDPN and the other cell surface molecules that are involved in cell adhesion. Among them, we first studied siPDPN effects on ezrin and its link proteins radixin and moesin (ERM protein) because the colocalization of PDPN with ezrin or ERM has been demonstrated to function in filopodia formation.¹⁷ However, the ezrin expression at both gene (Figure 4a) and protein (Figures 4b and c) levels, as well as ERM protein expression levels (Figure 4c), were the same in the three cell conditions.

In contrast, the expression of CD44, a hyaluronan receptor of the cell membrane²⁹ and a possible interacting partner for PDPN,¹⁸ at gene (Figure 4a) and protein (Figures 4b and c) levels, was obviously lower in siPDPN than that in the controls. As the change in expression levels for CD44 was in parallel with that of PDPN, we speculated that PDPN was also involved in hyaluronan-mediated cell adhesion. In the next step, we studied how pre-existent hyaluronic acid affects adhesion in siPDPN cells. When plated in the presence and absence of hyaluronic-acid coating, siPDPN did not show significant changes in attachment cell numbers between the two conditions, whereas siNC and mock cells showed significant increases (138%) in cell numbers at 90 min after plating in the presence of hyaluronic acid (siNC, $P < 0.05$; mock, $P < 0.01$; Figure 4d). However, it is necessary to carry out additional functional assays before we can conclude that there is a direct association of CD44 and PDPN in cell–cell adhesion, secondary to cell–ECM adhesion, in oral SCC cells.

To further investigate the coordinated expression profiles between PDPN and CD44, we performed double immunofluorescence for PDPN and CD44 in surgical tissue sections of oral SCC. PDPN and CD44 were exactly colocalized on the cell border at basolateral surfaces of SCC cells as far as they formed cell nests, regardless of their shape and size (Figures 5a–c), although they were restricted to peripheral basaloid SCC cells of larger round-shaped foci (Figure 5a) or to those in slender trabecular foci (Figure 5b). When SCC cells decreased in number in smaller foci, PDPN and CD44 disappeared from their free cell surfaces but remained on the cell border (Figure 5c), and when SCC cells were completely isolated in invasion fronts, PDPN and CD44 were also lost at the same time from the cells (Figure 5d). The results indicate that PDPN and CD44 did not function in invasion of SCC cells in their singular form but that they were needed for maintaining SCC cell adhesion in order to form cancer cell nests. However, when evaluated on a case-by-case basis, most

Figure 3 Functions of podoplanin (PDPN) in cell migration and invasion. **(a)** Scratch-wounding assays on confluent monolayers of siRNA-targeting PDPN (siPDPN), siNC, and mock ZK-1 cells at 0, 6, 12, and 24 h after scratching. **(b)** Transwell migration assays of ZK-1 cells in the three conditions for 24 h. Migrating cell numbers were counted in 1 mm² of opposite surfaces of filter membranes at 24 h after seeding. **(c)** Matrigel invasion assays of ZK-1 cells in the same conditions are mentioned above. Filter membranes were coated with Matrigels and were air-dried before seeding. Migrating cell numbers were counted in the same way. All experiments were performed in triplicate. There was no definite difference in ZK-1 cell migration between siPDPN, siNC, and mock conditions either by scratch wounding **(a)** or transwell migration **(b)** assays. The knockdown treatments did not affect ZK-1 cell invasion, either **(c)**.

cases were found to be CD44 positive because their large and differentiated SCC foci, which were present in every case, were positive for CD44 ($P < 0.05$, Table 2).

DISCUSSION

In the present study, we have demonstrated for the first time that the most fundamental function of PDPN in oral SCC cells is mediating cell adhesion through the crosstalk between CD44 and hyaluronic acid, although its functions had been mainly correlated to cell proliferation^{30,31} and migration^{32,33} in the literature. The PDPN contribution to cell proliferation has now been shown to be a secondary event to cell adhesion, and the present PDPN inhibition by siRNA did not affect cell migration, as shown in either scratch-wound or transwell migration assays. These *in vitro* results also reflected in the tissue-level observation that PDPN⁺ cells tended to locate in the basal or peripheral zone of SCC cell nests, in which Ki-67⁺ proliferating cells were not always simultaneously overlapped.¹³ In addition, there was no involvement of PDPN in the apoptotic process of oral SCC cells. Although PDPN⁺ SCC cases were clinicopathologically related to their degrees of differentiation and lymphatic invasion, we did not extend our experiments to include cellular differentiation and lymphatic invasion in this study.

PDPN has long been known as one of the lymphatic endothelial markers² and has been widely utilized in the field of lymphatic research.^{3,4} However, it has recently attracted attention in areas of cancer research because various kinds of tumor cells have been shown to express PDPN.^{5–9,34} In oral cancer, PDPN expression has been related to poor clinical outcomes, including lymph node metastasis or to malignant transformation of precancerous lesions, although its molecular function has not been well understood.^{10–13,35} To elucidate its function, we carried out comparative immunohistochemical studies between PDPN and the other molecules, which are related to cell proliferation or differentiation in oral mucosal malignancies¹³ or odontogenic tumors.⁷ Its expression profile in oral CIS was almost identical to that of SCC, and the immunohistochemistry for PDPN was helpful in the differential diagnosis of CIS from epithelial dysplasia.¹³ In odontogenic tumors, its immunolocalization pattern was related to intraepithelial signaling pathways of ECM molecules.⁷ In both instances, the area of PDPN distribution overlapped with Ki-67 or PCNA⁺ areas but was not always identical to them. Making use of these previous morphological data,^{7,13} we were able to narrow down the function of PDPN to ECM-related signaling from

Figure 4 Podoplanin (PDPN)-dependent CD44 expression and hyaluronan recognition. **(a)** Gene expression levels for CD44 and ezrin in siRNA-targeting PDPN (siPDPN), siNC, and mock ZK-1 cells determined at 72 h by real-time PCR. **(b)** Immunofluorescence for CD44 (upper) and ezrin (lower) in ZK-1 cells in the three conditions mentioned above, Hoechst-33258 (blue) counterstain, $\times 100$, scale bar, 100 μ m. **(c)** Western blot analysis for CD44, ezrin/radixin/moesin (ERM), ezrin, ezrin T567 phosphorylation site (p-ezrin (T567)), and β -actin in ZK-1 cell lysates from the three conditions. **(d)** Cell adhesion assays in the presence and absence of 2 mg/ml of hyaluronic-acid coating for 120 min. Open triangle, siPDPN; closed square, siNC; open square, mock on hyaluronic-acid-coated wells. All data are means $\pm$ s.e. from triplicate experiments. * $P < 0.05$, ** $P < 0.01$. CD44 mRNA expression levels significantly decreased in siPDPN cells, whereas ezrin levels remained unchanged **(a)**. CD44 protein levels were most completely suppressed in siPDPN ZK-1 cells, whereas there were no changes in ezrin expression levels **(b)**. The same tendencies were obtained in western blotting. ERM, ezrin, and p-ezrin levels were unchanged **(c)**. The suppressed adhesion in siPDPN ZK-1 cells was recovered slightly on hyaluronic-acid-coated wells at and after 90 min, although the difference was not statistically significant. In contrast, in the controls hyaluronic-acid coating enhanced significantly cell adhesion at 90 min. The results indicated that PDPN was involved in hyaluronan-mediated cell adhesion and that the CD44 expression was also regulated by PDPN **(d)**.

the cell surface side. In fact, the present study using the siRNA method has shown that PDPN is obviously involved in cell-ECM adhesion.

PDPN was also shown to mediate ZK-1 cell adhesion by its extracellular domain because the neutralizing monoclonal

antibody (D2-40) we used in the present study had already been documented to recognize the extracellular platelet aggregation-stimulating domain-1 and -2 of PDPN.³⁶ In addition, we confirmed that the PDPN-mediated adhesion was calcium dependent without any expressional switching

Figure 5 Immunohistochemical colocalization of podoplanin (PDPN) and CD44 in different shapes of oral squamous cell carcinoma (SCC) foci. (a) Large round-shaped foci; (b) trabecular foci; (c) small foci; (d) isolated cells. (upper row) hematoxylin and eosin (H-E) stain, immunofluorescence for PDPN (red; second), CD44 (green; third), Hoechst-33258 (blue; second from bottom), and merge (bottom). (a, b) $\times 150$, (c, d) $\times 300$, scale bars, $50\mu\text{m}$. PDPN and CD44 were mostly colocalized on the cell border at the basolateral surface of oral SCC cells as far as they formed carcinoma cell nests (a–c), although they were restricted to peripheral cells in larger foci (a). However, both PDPN and CD44 disappeared from SCC cells when they were isolated but did not form nests (d). The results indicated that PDPN and CD44 did not function in the invasion of SCC cells in singular form but that they were needed for formation of SCC cell nests.

among cadherin-family members, which has never been reported. On the basis of these lines of evidence, we wanted as our next step to investigate the behavior of CD44, one of the representative hyaluronan receptors, in cell–matrix adhesion^{37–39} because its interaction with PDPN had been reported.¹⁸ As the expression level of CD44 in siPDPN cells was repressed in parallel with that of PDPN, both PDPN and CD44 were considered to be coordinately involved in hyaluronan-mediated cell adhesion. However, at the present time it is difficult for us to explain how this phenomenon occurs because CD44 mRNA levels were not completely suppressed. We simply speculate that unpaired CD44 molecules, which were unable to be paired with PDPN, were degraded. In contrast to the results from MDCK cells or oral SCC-derived HN5 cells by Martin-Villar *et al*,¹⁸ no

apparent physical interaction between PDPN and CD44 was demonstrated in our co-immunoprecipitation/pull-down assay using ZK-1 cells (data not shown). Further investigations are needed for confirming the actual interaction between PDPN and CD44 in oral SCC cells; however, it is at least concluded that PDPN is involved in cell–ECM adhesion in the ECM-rich intercellular space of the epithelial compartment of oral SCC and CIS, which we have called ‘intraepithelial stroma’.^{14,15}

Regarding the involvement of PDPN in cell movement, migration, and invasion, Martin-Villar *et al*¹⁷ showed the PDPN interaction with ezrin in its cytoplasmic tail and with CD44 in its extracellular domain,¹⁸ which promoted EMT and directional cell migration. In contrast, Wicki *et al*²⁰ reported the PDPN-induced collective cell migration and

invasion by filopodia formation via the downregulation of the activities of RhoA GTPase activities, even in the absence of EMT. On the basis of our results on PDPN involvement in cell adhesion, we intended to correlate this particular function with invasive or migratory properties of oral SCC cells. However, we could not demonstrate any effects of PDPN in the ZK-1 cell migration and invasion or in the expression levels of ERM-related proteins.^{40,41} A similar tendency had already been reported in lung SCC cells.⁴²

Recently, we have reported that the immunohistochemical expression of PDPN was closely related to the degree of malignancy in the oral mucosa,¹³ but that the PDPN expression mode in odontogenic tumors was related to ECM-signaling pathways.⁷ In both of those studies, PDPN+ cells were shown to be distributed in areas narrower than Ki-67+/PCNA+/-proliferating cell areas, which indicated that PDPN+ cells were located in a close relationship with cell-proliferating events, but that they did not completely overlap. From the present *in vitro* study, it is now possible to interpret reasonably those immunohistochemical profiles in relation to cell adhesion with ECM molecules, especially the cell border immunolocalization of PDPN, which indicates that PDPN-mediated cell adhesion requires the presence of ECM in the intercellular space. With the presence of PDPN, SCC cells are tethered to each other with ECM. The clinical significance of PDPN expression in oral SCC has been correlated to lymphatic invasion. In the present study, SCC cases without PDPN+ cells were histologically classified into less-differentiated types, in which lymphatic invasion was more frequently observed. The result contradicted that of previous reports in which the PDPN expression itself was emphasized in malignancies, yet no detailed attention has paid to its relation to cell adhesion.^{10–12,35} We would like to suggest the possibility that the loss of PDPN expression is because of the decreased differentiation of oral SCC cells, which also results in their invasive or scattering tendencies.

In conclusion, PDPN, in collaboration with CD44, tethers SCC cells to hyaluronan-rich extracellular matrices to inhibit their scattering. PDPN contributes to attachment of SCC cells to ECM but not directly to their proliferation, which must be an effect that is secondary to established cell adhesion. Thus, it may not directly function in either cell migration or invasion.

In the next step, it is necessary to elucidate the mechanism by which CD44 interacts with PDPN in the cell surface, and to explain how their expression levels are coordinated with each other.

Supplementary Information accompanies the paper on the *Laboratory Investigation* website (<http://www.laboratoryinvestigation.org>)

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DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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