Periostin Secreted by Epithelial Ovarian Carcinoma Is a Ligand for $\alpha_V\beta_3$ and $\alpha_V\beta_5$ Integrins and Promotes Cell Motility

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Periostin Secreted by Epithelial Ovarian Carcinoma Is a Ligand for $\alpha_V\beta_3$ and $\alpha_V\beta_5$ Integrins and Promotes Cell Motility[1]

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ABSTRACT

Periostin (PN) is a secreted protein that shares a structural homology to the axon guidance protein fasciclin I in insects. Previously, we reported that PN expression is up-regulated in epithelial ovarian tumors. We further examined the role of PN in ovarian cancer. PN is expressed in several normal tissues but not in normal ovaries and has a tendency for higher expression in fetal tissues. Ovarian cancer cells secrete PN, which can accumulate in malignant ascites of ovarian cancer patients. Purified recombinant PN supports adhesion of ovarian epithelial cells that can be inhibited by monoclonal antibodies against $\alpha_V\beta_3$ or $\alpha_V\beta_5$ integrin, but not by anti-β_1 integrin antibody. Furthermore, $\alpha_V\beta_3$ integrin, but not β_1 integrins, colocalizes to the focal adhesion plaques formed on PN. Cells plated on PN form fewer stress fibers and are more motile compared with those plated on fibronectin. We propose PN functions as a ligand for $\alpha_V\beta_3$ and $\alpha_V\beta_5$ integrins to support adhesion and migration of ovarian epithelial cells.

INTRODUCTION

EOC[3] arises from the epithelial cells covering the surface of the ovaries (1, 2). EOC is a highly malignant disease, usually presenting with widespread i.p. metastasis (3). The i.p. dissemination is believed to be initiated by exfoliation of cancer cells, followed by their adhesion to the mesothelium that covers the peritoneal cavity. Although the precise mode of i.p. spread of EOC is not known, there are several unique features of ovarian epithelium that may facilitate this process. First, the ovarian epithelial cells can adopt a mesenchymal phenotype and lack the tight cell junctions (4), making them prone to exfoliation. Second, the ovarian epithelium is in direct contact with the peritoneal cavity. Once shed from the tumor, cancer cells are free to disseminate throughout the peritoneal cavity, carried by the flow of the peritoneal fluid.

The adhesion of ovarian epithelial cells to the ECM involves both integrin-dependent and independent mechanisms (5–8). Integrins are transmembrane heterodimeric receptors involved in both cell-cell and cell-ECM interactions (9). The functions of integrins are not limited to cell adhesion, but also involve activation of cytosolic signaling cascades to mediate cell proliferation, cell survival, and cell migration (10, 11). Integrin expression is frequently altered in cancer cells (12, 13), which together with the changes in the ECM composition alters the adhesion and motility of cancer calls. Malignant ovarian epithelial cells also secrete their own ECM proteins including fibulin-1, tenascin-c, and VN (7, 14, 15).

PN (formerly called osteoblast-specific factor-2) was originally identified as a 811-amino acid protein secreted by osteoblasts (16). It shares a structural homology to insect fasciclin I and can bind heparin (17) and support adhesion of osteoblasts (18), leading to a hypothesis that it functions to recruit and attach osteoblasts to the periosteum. Previously, we reported that PN mRNA expression is up-regulated in ovarian tumors (19). In addition, two recent reports showed that serum levels of PN are elevated in patients with thymoma (20) and non-small cell lung cancer (21).

We further characterized the expression and function of PN. PN transcripts were expressed in a number of normal organs, with a tendency for higher expression in fetal tissues. IHC analysis using polyclonal anti-PN antisera showed specific staining within the cancer cells in epithelial ovarian tumors. Purified recombinant PN promoted $\alpha_V\beta_3$- and $\alpha_V\beta_5$-dependent cell adhesion and spreading. Interestingly, ovarian epithelial cells spread on PN formed less stress fibers and were more motile, suggesting that PN secreted by the cancer cells may enhance their motility and invasiveness.

MATERIALS AND METHODS

PN cDNA. The full-length PN cDNA (periostin-bm) was derived from the expressed sequence tag clone ID:1091416 (GenBank accession no. AA599197). The coding sequence of this clone consists of 782 amino acids and differs from the published sequence (GenBank accession no. D13666) at the amino acid position 290 (Ile rather than Phe) and 421 (Asp rather than Val).

Anti-PN Antibodies. Rabbit anti-PN antibodies were generated using bacterially expressed NH_2-terminal hexa-histidine-tagged PN as the immunogen. The coding region of PN was cloned into the pQE60 (Qiagen Inc., Valencia, CA) vectors and transformed into bacterial cells (BL21). The bacteria were cultured in LB-amp at 37°C to an early exponential phase of growth ($OD_{600} \approx 0.5$) before the addition of 1 mM isopropyl-1-thio-β-D-galactopyranoside. After and additional 3 h, bacteria were harvested and PN-his proteins were purified under denaturing conditions using Ni-NTA beads (Qiagen Inc.).

Ovarian Epithelial Cells. HOSE and CSOC correspond to epithelial cells derived from the normal ovary and EOC. These primary cultures were prepared as described previously (19, 22) and maintained in MCDB 105:199 (1:1) medium supplemented with 10% FBS, penicillin (100 units/ml), and streptomycin (100 μg/ml). H281-hTERT was derived from HOSE cultures by transducing the catalytic subunit of hTERT (23). CSOC848-hTERT and CSOC272-hTERT/E7 were derived from CSOC culture by transducing hTERT alone or hTERT and the human papilloma virus E7 subunit. These cells have been passaged >70 times and are considered continuous cell lines. Sk-ov-3 human ovarian carcinoma cells were obtained from the American Type Culture Collection (Manassas, VA) and maintained in McCoy's 5A medium supplemented with 10% FBS.

Immunoblot Analysis of PN. When cell growth reached confluency, the culture media was replaced to complete medium without serum and incubated for an additional 4–5 days to obtain conditioned media. Ovarian ascites samples were from women with ovarian cancer (FIGO stage III or IV) undergoing debulking procedures. Nonovarian ascites were from patients undergoing therapeutic paracentesis for clinically indicated reasons. The conditioned media and ascitic fluids were centrifuged at 10,000 × *g* for 15 min and fractionated on a 6% SDS-polyacrylamide gel under reducing condition. The proteins were transferred to a nitrocellulose membrane and blotted with

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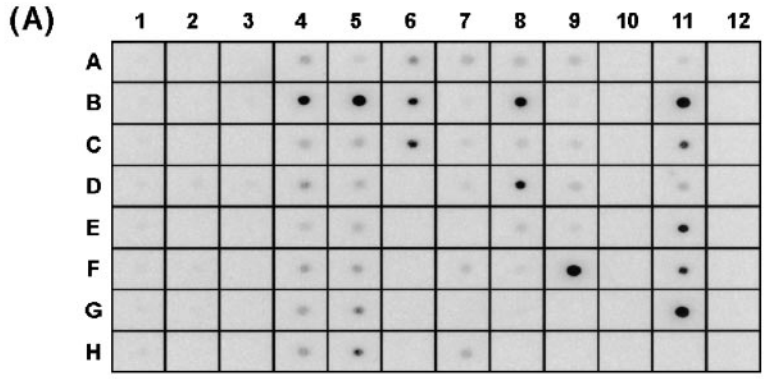
[3] The abbreviations used are: EOC, epithelial ovarian cancer; ECM, extracellular matrix; PN, periostin; FN, fibronectin; VN, vitronectin; IHC, immunuhistochemistry; HOSE, human ovarian surface epithelia; CSOC, Cedars Sinai ovarian carcinoma; hTERT, human telomerase; FBS, fetal bovine serum; mAb, monoclonal antibody.



	1	2	3	4	5	6	7	8	9	10	11	12
A	Whole Brain	Left Cerebell.	Substan. Nigra	Heart	Esophagus	Colon	Kidney	Lung	Liver	HL-60	Fetal Brain	C
B	Cerebral Cortex	Right Cerebell.	Accum. Nucleus	Aorta	Stomach	Colon	Skeletal Muscle	Placenta	Pancreas	HeLa	Fetal Heart	C
C	Frontal Lobe	Corpus Callosum	Thalamus	Left Atrium	Duodenum	Rectum	Spleen	Bladder	Adrenals	K-562	Fetal Kidney	C
D	Parietal Lobe	Amygdala	Pituitary	Right Atrium	Jejunum		Thymus	Uterus	Thyroid	MOLG-4	Fetal Liver	C
E	Occipital Lobe	Caudate nucleus	Spinal Cord	Left Ventricle	Ileum		PBL	Prostate	Salivary Gland	Raji	Fetal Spleen	C
F	Temporal Lobe	Hippo-campus		Right Ventricle	Ileocecum		Lymph Node	Testis	Mammary Gland	Daudi	Fetal Thymus	C
G	Paracen. Gyrus	Medulla		IV septum	Appendix		Bone Marrow	Ovary		SW480	Fetal Lung	C
H	Pons	Putanum		Heart Apex	Colon		Trachea			A549		C

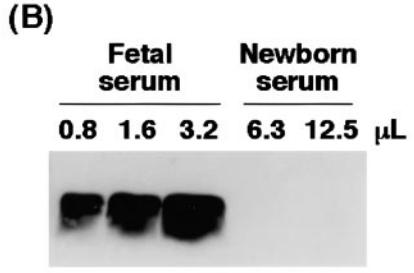


Fig. 1. Oncofetal pattern of PN expression. *A*, a multiple tissue expression array (BD Biosciences Clontech, Palo Alto, CA) that contains mRNA from 76 human tissues and cancer cell lines (*bottom*) arrayed on a nylon membrane was hybridized with ^{32}P-labeled PN cDNA probe (*top*) to determine the tissue distribution of periostin expression. *B*, indicated amounts of FBS (Gemini Bio-Products, Woodland Hills, CA) and newborn calf serum (Gemini Bio-Products) were immunoblotted with anti-PN antibodies.

anti-PN antibodies (1:5,000). After incubation with a horseradish peroxidase-conjugated antirabbit antibody (1:10,000; Transduction Laboratories, Lexington, KY), protein bands were visualized by chemiluminescence (Pierce Chemical Co., Rockford, IL).

IHC. Paraffin-embedded ovarian tissue slides were processed for antigen retrieval by heating in 10 mM sodium citrate (pH 6.0) at 95°C for 25 min. The slides were blocked with 3% goat serum in 25 mM Tris-HCl, 150 mM NaCl (pH 7.5) for 30 min and then incubated with anti-PN antibodies (1:1200) for 30 min. Subsequent substrate-chromogen development was carried out using a DAKO EnVision+ System, Peroxidase (DAB) kit (DAKO, Carpinteria, CA).

Preparation of Recombinant PN from Sf-9 Cells. A COOH-terminal hexa-histidine-tagged PN (PN-his) was expressed in the insect Sf-9 cells using the Bac-to-Bac Baculovirus Expression System (Invitrogen Corp., Carlsbad, CA). Briefly, a hexa-histidine tag was added to the COOH terminus of the PN cDNA using PCR, and the resulting fusion construct was cloned into the pFastBac1 plasmid. The isolated recombinant plasmid was transformed into *Escherichia coli* DH10BAC cells harboring a baculovirus shuttle vector, and white colonies representing the clones that have undergone transposition were isolated. High molecular weight DNA was prepared from the isolated clones and used to transfect Sf-9 cells. The recombinant baculovirus was prepared as conditioned culture media and used to infect 2×10^9 Sf-9 cells at a multiplicity of infection of 5. PN-his was purified from 1000 ml of conditioned medium obtained 72 h after the infection. Briefly, the conditioned medium was adjusted to pH 8 with 1 M Tris and loaded onto a 10-ml heparin-Sepharose column (Amersham Biosciences, Piscataway, NJ) equilibrated with wash buffer [0.1 M NaCl in 20 mM Tris-HCl (pH 8)]. The column was washed with 10× bed volumes of wash buffer, and the bound proteins were eluted with 1 M NaCl in 20 mM Tris-HCl (pH 8). PN-his protein in the pooled fractions was bound to Ni-NTA beads (Qiagen Inc.). After washing the Ni-NTA beads extensively in a buffer containing 20 mM Tris-HCl (pH 8), 500 mM KCl, 20 mM imidazole, and 10% (v/v) glycerol, PN-his protein was eluted in a buffer containing 20 mM Tris-HCl (pH 8), 100 mM KCl, 100 mM imidazole, and 10% (v/v) glycerol.

Solid Phase Binding Assay. Exponentially growing ovarian epithelial cells were harvested by treatment with 0.05% trypsin/0.02% EDTA and suspended in serum-free media supplemented with soybean trypsin inhibitor (0.5 mg/ml). Fifty thousand cells were added to the 96-well plates that have been coated with PN (0.5–10 μg/ml), FN (0.5–10 μg/ml; Roche Diagnostic Corp., Indianapolis, IN), or BSA (1% w/v). After a 1-h incubation at 37°C, the wells were washed three times with Tris-buffered saline, fixed with 3.7% (v/v) formaldehyde in Tris-buffered saline for 30 min, and stained with 1% (w/v) toluidine blue overnight. After washing with distilled water until no trace of free dye was visible, the cells were lysed in 2% (w/v) SDS for 10 min. The absorbance (600 nm) of toluidine blue was measured and converted into cell number, using a standard curve generated from cells bound to poly-L-lysine (1 mg/ml)-coated wells. To demonstrate the specificity of cell binding to PN, the coated wells were preincubated with anti-PN antibodies (1:25 and 1:100) for 30 min, before carrying out the adhesion assay. Inhibitory anti-integrin mAbs LM609 (anti-$\alpha_V\beta_3$; Chemicon Inc., Temecula, CA) and PIF6 (anti-$\alpha_V\beta_5$; Chemicon Inc.) were used at 10 μg/ml. The mAb P4C10 (anti-β_1; Invitrogen Corp.) was used at 1:100 dilution.

***In Situ* Immunofluorescence.** Cells in serum-free growth media [MCDB 105:M199 (1:1) supplemented with 1% BSA] were plated on FN-coated (10 μg/ml), PN-coated (10 μg/ml), or VN-coated (5 μg/ml; Promega Corp., Madison, WI) glass coverslips. After 5 h, cells were fixed with 3.7% formaldehyde in PBS for 10 min and permeabilized with 0.5% Triton X-100 for 5 min. Focal adhesions were visualized by staining paxillin with anti-paxillin antibody (BD Transduction Laboratories, San Diego, CA). Integrin staining was carried out the mAb TS2/16 (anti-β_1; American Type Culture Collection), LM609 (Chemicon Inc.), or PIF6 (Chemicon Inc.) for 1 h before fixation. Cy-3-conjugated goat antimouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) was used as the secondary antibody. Actin filaments were stained with rhodamine-conjugated phalloidin (Molecular Probes Inc., Eugene, OR).

Time-Lapse Migration Microscopy. CSOC cells were plated on Delta-T glass dishes (0.5 mm; Bioptechs Inc., Butler, PA) that have been coated with PN or FN at 10 μg/ml or VN at 5 μg/ml. One and a half hours after plating the cells, the medium was refreshed and cell migration was monitored from images captured at 20-min interval from a Nikon Diaphot microscope equipped with

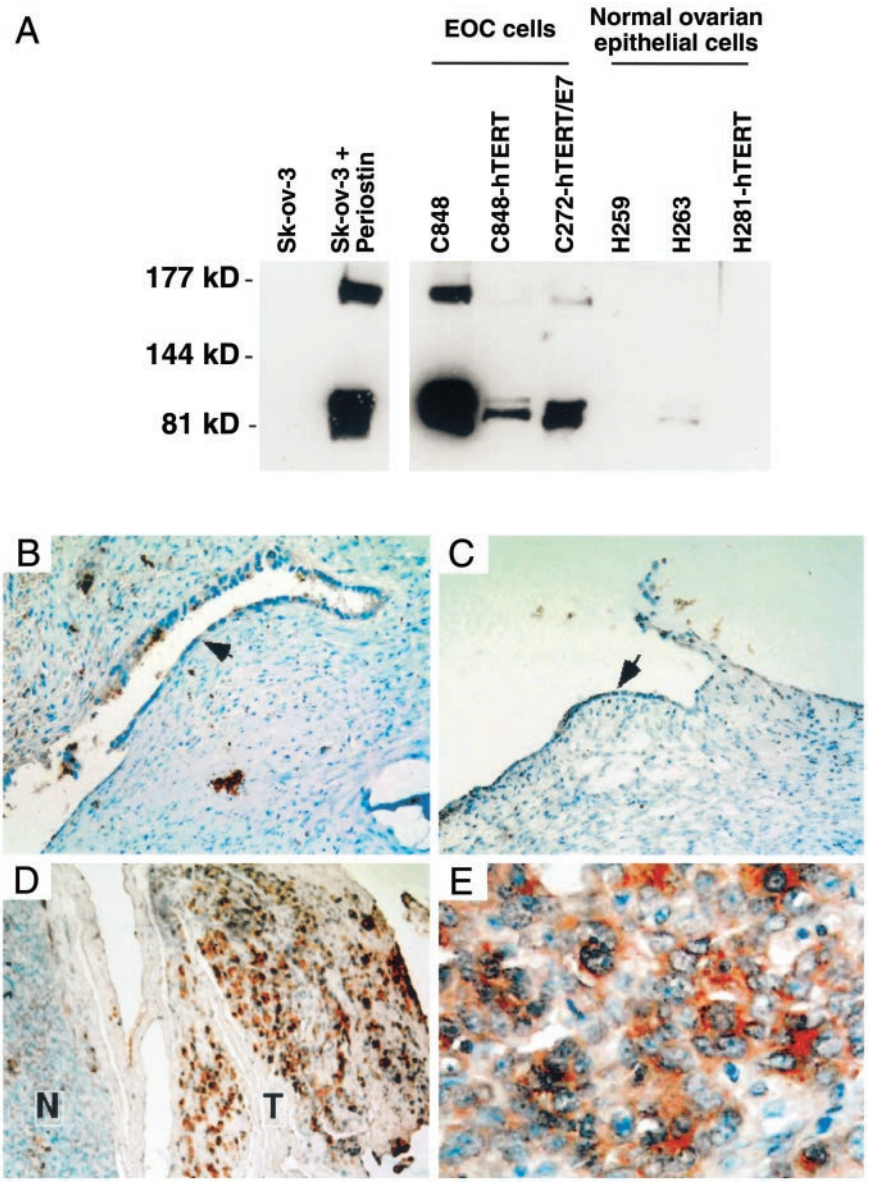


Fig. 2. PN is produced by EOC cells. *A*, cells were grown to confluency in complete growth media, rinsed to remove any traces of serum, and cultured for 96–120 h in growth media in the absence of serum. The conditioned media (10 μl) were immunoblotted with anti-PN antibodies. Sk-ov-3 cells transfected with the PN cDNA is shown for comparison. *C848*, EOC-derived primary epithelial culture; *C848-hTERT*, EOC-derived epithelial cell line expressing hTERT; *C272-hTERT/E7*, EOC-derived epithelial cell line expressing hTERT and the HPV16 E7 protein; *H259* and *H263*, primary epithelial cultures derived from normal ovaries; *H282-hTERT*, normal ovarian epithelial cell line expressing hTERT. *B–E*, IHC staining for PN in paraffin-embedded slides of normal ovaries (*B* and *C*) and EOC (*D* and *E*) are shown. The tissue shown in *D* contains both tumor (*T*) and adjacent normal ovarian tissue (*N*). The *arrows* in *B* and *C* indicate the surface epithelial cell layer.

a digital camera. The positions of the nuclei ($n = 39$ for FN; $n = 31$ for PN; $n = 42$ for VN) were tracked to measure cell movement. Cell velocity was calculated in micrometers per 8 h using the Image-Pro software (Media Cybernetics, Silver Springs, MD). Cell migration was carried out under serum-free conditions.

RESULTS

Tissue Distribution of PN Expression. PN was expressed in a wide range of normal adult tissues, notably in the aorta, stomach, lower gastrointestinal tract, placenta, uterus, and breast (Fig. 1*A*). Expression of PN in the normal ovaries was negligible. Interestingly, the expression of PN was high in the majority of fetal tissue. This fetal pattern of expression was also suggested when the circulating level of PN in bovine serum was estimated. In immunoblot analysis, a strong ~90 kDa band corresponding to PN can be readily detected from 0.8 μl of FCS (Fig. 1*B*). We were unable to detect a specific signal for PN in up to 12.5 μl of newborn calf (Fig. 1*B*).

Ovarian Cancer Cells Secrete PN. Anti-PN antisera detect a family of closely migrating proteins of ~90 kDa in the conditioned media of cultured epithelial cells derived from EOC (Fig. 2*A*). PN was either absent or low in the conditioned media of the normal ovarian epithelial cells. PN undergoes alternative splicing (18), which accounts for the appearance of multiple bands of ~90 kDa size. The immunoblot analysis also revealed an additional higher molecular weight band migrating ~170 kDa. This form is also seen in the conditioned media of Sk-ov-3 cells that have been transfected with the PN cDNA and is likely to represent a covalently linked multimer rather than an alternatively spliced isoform.

We next examined the expression of PN in ovarian tumor by IHC. In the normal ovary, PN expression was negligible in the stroma or the surface epithelia (Fig. 2, *B* and *C*). Anti-PN antisera (1:1200 dilution) stained tumor nodule, but not the adjacent normal ovarian tissue (Fig. 2*D*). In another EOC sample, intense staining could be seen in the carcinoma cells (Fig. 2*E*). Staining with preimmune sera in the same tumor samples was negligible, even at a 3-fold higher concentration (1:400 dilution; data not shown).

PN Accumulates in the Ovarian Ascites. EOC often disseminates into the peritoneal cavity as tumor implants and creates large volumes of ascites. Immunoblot analysis revealed the presence of PN in 20 of the 21 ascites from ovarian cancer patients (Fig. 3*A*). The concentra-

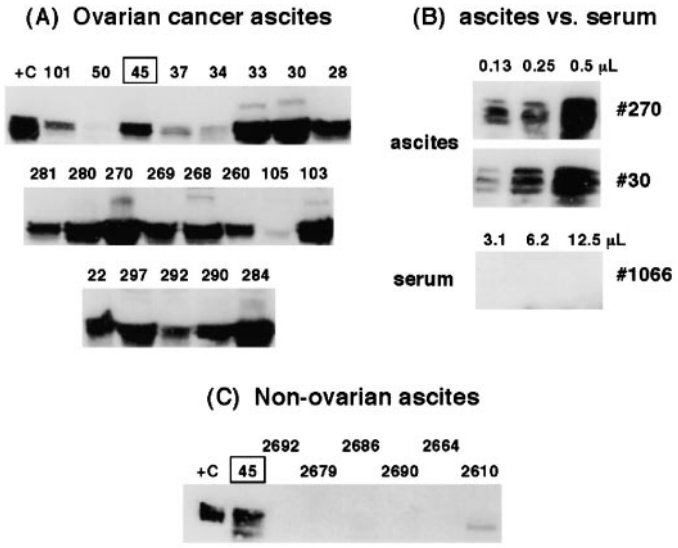


Fig. 3. PN accumulates in ascites of women with ovarian cancer. *A*, equal volumes of malignant ascites from patients with ovarian cancer were immunoblotted with anti-PN antibodies. The conditioned media of the C272-hTERT/E7 cell line was used as the positive control. *B*, indicated volumes of ascites (270 and 30) and serum from one patient (1066) were immunoblotted with anti-PN antisera. *C*, equal volumes of ascites from patients with pancreatic cancer (2692), leiomyoma (2679), uterine sarcoma (2686), multiple myeloma (2690), cirrhosis (2664), and breast cancer (2610) were immunoblotted with anti-PN antisera. The conditioned media of the C272-hTERT/E7 cell line was used as the positive control. For comparison, ascites from an ovarian cancer patient (45) is included.

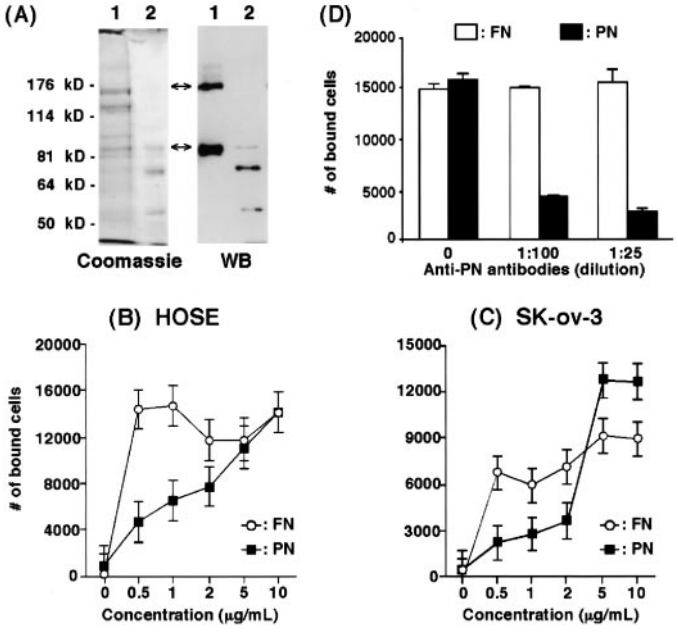


Fig. 4. Recombinant PN supports cell adhesion. *A*, Ni-NTA column-purified recombinant PN-his protein produced in insect cells (*Lane 1*) and bacterial cells (*Lane 2*) were fractionated by SDS-PAGE and stained with Coomassie (*left*) or immunoblotted with anti-PN antibodies (*right*). *B* and *C*, primary human ovarian epithelial cells (HOSE) and Sk-ov-3 were added to a 96-well plate (15,000/well) that had been coated with 0.5–10 μg/ml of PN (■) or FN (○) and incubated at 37°C for 1 h. *D*, Sk-ov-3 cells were adhered to a 96-well plate coated with 10 μg/ml of PN or FN in the presence of anti-PN antibodies. The dilutions of anti-PN antibodies are indicated. Cell adhesion was carried out under a serum-free condition in McCoy's 5A. Bound cells were enumerated from dye uptake (see "Materials and Methods").

tion of PN in ascites was variable but estimated at ~1 μg/ml (our unpublished results). In a semiquantitative immunoblot analysis, the concentrations of PN in the ascites of patients 270 and 30 were >100-fold higher than that in serum (Fig. 3*B*). PN was also in the ascites of a breast cancer patient (2610), but was absent or low in ascites from nonovarian cancer patients (Fig. 3*C*).

Recombinant PN Supports the Adhesion of Ovarian Epithelial Cells. Baculovirus-produced recombinant PN migrated as a ~90 kDa protein, similar to the endogenous protein (Fig. 4*A*). The Ni-NTA column purified PN fraction contained additional protein bands, but was free of FN or VN (our unpublished results). PN-coated surfaces supported the attachment of HOSE and Sk-ov-3 in a concentration-dependent manner (Fig. 4, *B* and *C*). At a coating concentration of 5 μg/ml, PN supported cell adhesion equivalent to FN. Anti-PN antibodies specifically inhibited the adhesion of Sk-ov-3 cells to PN, reducing cell adhesion by >82% at 1:25 dilution (Fig. 4*D*). Anti-PN antibodies did not affect cell adhesion to FN.

The adhesion of Sk-ov-3 cells to PN-coated surface required divalent cation (our unpublished results). The addition of manganese, which can increase the ligand-binding affinity of some integrins, including $\alpha_V\beta_3$ (24), further enhanced adhesion of Sk-ov-3 cells to PN nearly 2-fold (Fig. 5*A*). The effect on manganese on the adhesion of Sk-ov3 cells to FN was less dramatic. Sk-ov-3 cells express the β_1, $\alpha_V\beta_3$, and $\alpha_V\beta_5$ integrins (Fig. 5*B*). The attachment of Sk-ov-3 cells to a PN-coated plate was inhibited by anti-$\alpha_V\beta_3$ (LM 609; $P < 0.001$) or anti-$\alpha_V\beta_5$ mAb (PIF6; $P < 0.001$), and further inhibited by the addition of both antibodies (Fig. 5*C*). Conversely, while the function blocking mAb to β_1 integrins (P4C10) inhibited the attachment of Sk-ov-3 cells to FN ($P < 0.001$; Fig. 5*D*), it did not affect the attachment of Sk-ov-3 cells to PN. The adhesion of Sk-ov-3 cells to FN was partially inhibited by mAb PIF6 ($P = 0.253$).

Ovarian Epithelial Cells Display Motile Phenotype When Spread on PN. On FN, ovarian epithelial cells form well-defined focal adhesion plaques throughout the cell body (Fig. 6*A*). Dense stress fibers emanating from the focal adhesion plaques can be visu-

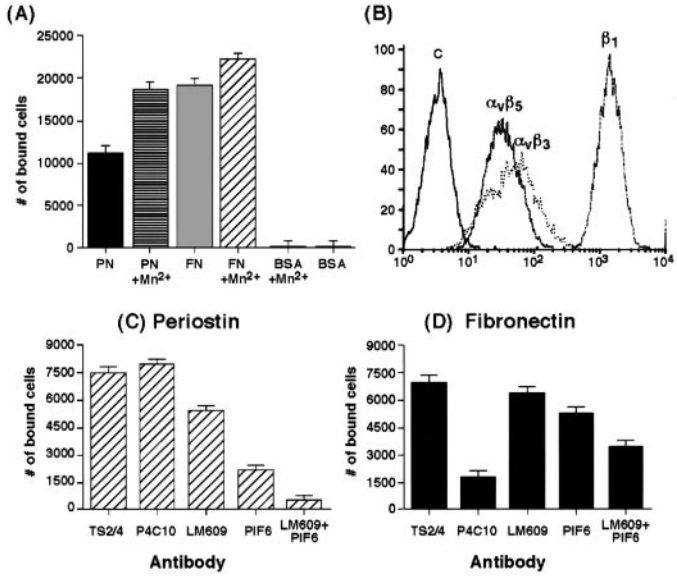


Fig. 5. PN adhesion is α_V integrin dependent. *A*, Sk-ov-3 cells (25,000) were added to a 96-well plate that had been coated with PN (10 μg/ml), FN (10 μg/ml), or BSA (1% v/v). Cell adhesion was carried out under a serum-free condition in McCoy's 5A (contains 0.8 mM Mg^{2+}). When indicated, $MnCl_2$ was added to 1 mM. After 1 h at 37°C, bound cells were enumerated from dye uptake. *B*, surface expression of β_1 and α_V integrins were analyzed by fluorescence-activated cell sorting. Sk-ov-3 cells were incubated with mAb against β_1-integrin (P4C10), $\alpha_V\beta_3$ (LM609), $\alpha_V\beta_5$ (PIF6), or $\alpha_L\beta_2$ (TS 2/4) for 30 min at 4°C, followed by FTIC-labeled goat antimouse IgG. Leukocyte integrin $\alpha_L\beta_2$ is not expressed in nonhematopoeitic cells, and the staining with TS2/4 mAb served as the control. *C* and *D*, Sk-ov-3 cells were incubated with P4C10, LM609, PIF6, or TS2/4 mAb before adding to a 96-well plate (10,000/well) that had been coated with PN or FN at 10 μg/ml. Cell adhesion was carried out in the absence of serum. After 1 h at 37°C, bound cells were enumerated from dye uptake.

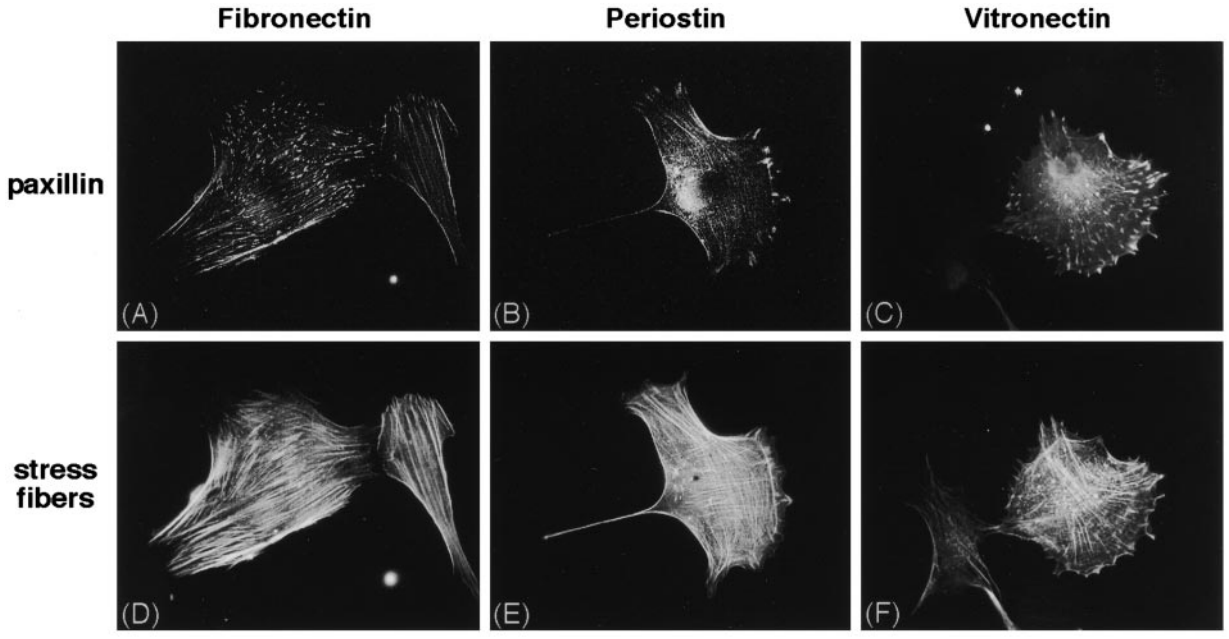


Fig. 6. Cells plated on PN have a motile phenotype. C848-hTERT cells were cultured without serum on glass coverslips that have been coated with FN (*A* and *D*), PN (*B* and *E*), or VN (*C* and *F*). After 5 h at 37°C, the cells were fixed and stained with anti-paxillin (*A–C*) or rhodamine-conjugated phalloidin (*D–F*).

alized in these cells with rhodamine-conjugated phalloidin (Fig. 6*D*). On PN, ovarian epithelial cells formed less stress fibers and focal adhesion plaques. In addition, the focal adhesion plaques were distributed mainly at the forward edge of the cell (Fig. 6*B*). This distribution of the focal adhesion plaques to the forward edge was unique to the cells spread on PN and was not seen in the cells spread on VN which, similar to PN, mediates $\alpha_V\beta_3$- or $\alpha_V\beta_5$-dependent cell spreading (Fig. 6*C*).

The type of integrins localizing to the focal adhesion plaques was also influenced by the composition of ECM. When cells were seeded onto substrates coated with FN in the absence of serum, β_1 integrins were localized to punctated regions throughout the cell body, similar to the localization of the focal adhesion protein paxillin (Fig. 7*A*). Under these conditions, $\alpha_V\beta_3$ integrins were distributed diffusely and could not be localized to the focal adhesion plaques (Fig. 7*D*). On a PN-coated surface, an entirely different pattern emerged, where $\alpha_V\beta_3$ integrins were found in a punctated pattern at the periphery of plasma membrane, similar to the distribution of focal adhesion protein paxillin, whereas the distribution of β_1 integrins were diffuse (Fig. 7, *B* and *E*). On VN, both β_1 and $\alpha_V\beta_3$ integrins localized to the focal adhesion plaques (Fig. 7, *C* and *F*).

PN Enhances Cell Motility. When ovarian epithelial cells were plated on a PN-coated surface, they frequently adopted a fan shape with a definable forward and trailing edge (see Figs. 6 and 7). This

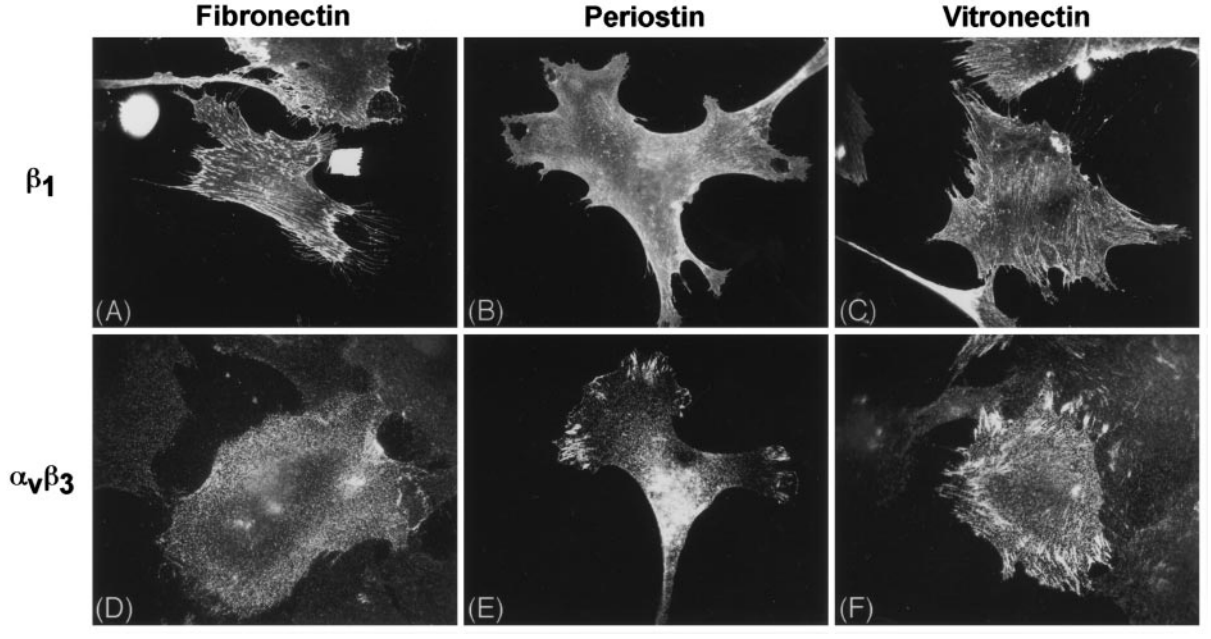


Fig. 7. PN directs $\alpha_V\beta_3$ integrins to focal adhesion plaques. C848-hTERT cells were cultured without serum on glass coverslips coated with FN (*A* and *D*), PN (*B* and *E*), or VN (*C* and *F*). After 4 h, cells were stained with anti-β_1-integrin (*A–C*) or $\alpha_V\beta_3$ integrin (*D–F*) antibodies for 1 h and then were fixed and incubated with goat Cy-3 conjugated antimouse IgG.

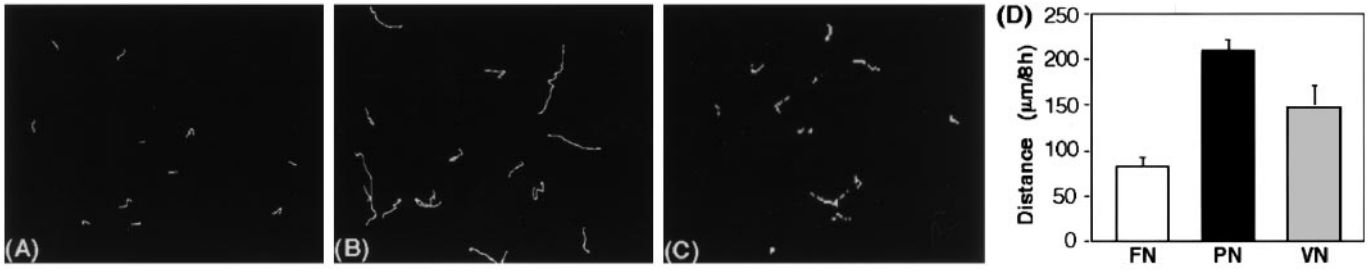


Fig. 8. PN enhances cell motility of ovarian epithelial cells. C848-hTERT cells were plated on (*A*) FN-coated (10 μg/ml), (*B*) PN-coated (10 μg/ml), or (*C*) VN-coated (5 μg/ml) Delta-T dishes, and cell migration was monitored with a time-lapse photography over an 8-h period. Cell movements were traced from digital images that have been captured at a 20-min interval (*A–C*) to obtain cell velocity (*D*; see "Materials and Methods").

finding and the reduced number of stress fibers in these cells suggested that cells might be more motile on PN. We monitored the movement of cells with time-lapse microscopy (Fig. 8). On a PN-coated surface, cell motility was significantly higher (210 $\pm$ 13 μm/8 h) compared with a FN-coated surface (80 $\pm$ 10 μm/8 h; $P < 0.001$). The motility of ovarian cells on a VN-coated surface was 148 $\pm$ 22 μm/8 h ($P < 0.05$), better than on FN, but not as efficient as on PN.

DISCUSSION

PN is structurally similar to fasciclin I, an insect neuronal adhesion protein (25, 26), and is composed of four internal repeat domains of about 120–160 amino acids. The only other fasciclin domain protein in mammals is βig-h3, which was identified as a transforming growth factor-β-induced gene in human adenocarcinoma cells (27). The fasciclin domain-containing proteins function as adhesion molecules. Fasciclin I can mediate homotypic cell adhesion (28), whereas purified recombinant βig-h3 supports $\alpha_1\beta_1$ integrin-dependent adhesion of chondrocytes and fibroblasts (29). βig-h3 contains an RGD motif near the COOH terminus, but this integrin recognition site can be deleted without affecting cell adhesion (29). PN does not contain an RGD motif.

The COOH-terminal region of PN, outside the four fasciclin domains, undergoes alternative splicing to generate multiple PN isoforms. The immunoblot also revealed a larger form of PN migrating at ~170 kDa that could not be accounted for by alternative splicing. ECM proteins, notably FN and VN, form disulfide-bound dimer or multimer (30, 31). The ~170-kDa form likely is a multimer (probably a dimer) of PN. This form is stable under reducing condition, indicating that it is not disulfide bound. The functions of different isoforms of PN need further investigation.

PN is overexpressed in a number of human tumors. To date, our group has examined PN expression in over 40 ovarian tissues and found that up to 30% of tumors are strongly positive for PN staining, with another 46% showing weak to moderate staining (our unpublished results). Besides EOC, PN expression is up-regulated in glioblastoma (32), non-small cell lung cancers (33), and melanoma (our unpublished results), and the serum levels of PN are elevated in patients with thymoma (21) and lung cancer (20). PN is expressed in most normal tissues, except in the brain, ovary, and hematopoeitic organs. The broad tissue distribution of PN expression suggests that it has a more generalized function that is not limited to bone formation. PN expression tends to be higher in fetal tissues, and the serum level of PN in fetal calf is significantly higher than that in newborn calf. This preferential expression in fetal tissue, together with its up-regulation in tumors, suggests PN has an "oncofetal" pattern of expression, similar to VN and the ED-B isoform of FN (34–36).

We have not been able to detect any significant change in the serum levels of PN in women with ovarian cancer compared with the normal controls (our unpublished results). However, the majority of ascites from ovarian cancer patients contains high levels of PN. We have shown that ovarian epithelial cancer cells secrete PN, which most likely accounts for the accumulation of PN in the ascites. Alternatively, carcinomatosis and the generalized inflammatory process associated with it may up-regulate PN expression from the mesothelial cells lining the peritoneal cavity. One contributing factor for the development of ascites is the increased permeability of vessels (37). A passive transfer of PN in circulation to the peritoneal cavity, however, is viewed unlikely because the level of PN in ascites can exceed 100 times that in serum or plasma.

Purified PN supports integrin-dependent adhesion and spreading of ovarian epithelial cells. First, the adhesion requires divalent cation and can be stimulated by Mn^{2+}, which increases the ligand-binding activity of several integrins (38). Second, the mAbs to $\alpha_V\beta_3$ or $\alpha_V\beta_5$ integrin suppressed the adhesion individually, and completely abolished the adhesion when combined. Third, when cells were allowed to spread on PN, $\alpha_V\beta_3$ integrin localized to the focal adhesion plaques, whereas the β_1 integrins were distributed diffusely throughout the cell. On FN, an opposite staining pattern emerged with the β_1 integrins localizing to the focal adhesion plaques and $\alpha_V\beta_3$ integrin being distributed diffusely. The dependence of cell surface distribution of integrins on ECM composition has been reported previously (39, 40). Altogether, these findings indicate that $\alpha_V\beta_3$ and $\alpha_V\beta_5$ integrins play a pivotal role in the PN-induced cell adhesion and spreading.

PN also confers more motile features to the adherent cells. Compared with cells spread on FN or VN, cells spread on PN display a recognizable front and trailing edge. In these cells the focal adhesion plaques are localized to the front of the cell, rather than being distributed throughout the ventral surface, as seen in cells spread on FN or VN. In addition, cells form fewer stress fibers on PN. The formation of focal adhesion plaques and organized actin stress fibers requires tension (41), which in part depends on the rigidity of the substrate (42). One possible explanation for our finding is that PN, compared with FN or VN, is a more pliable substrate. Alternatively, PN, through clustering α_V integrins, may trigger a different set of signals that favors motile features. The different morphological features of cells spread on FN *versus* PN correlate with cell motility. When cell motility was followed by time-lapse microscopy, cells were more motile on PN than FN.

Our finding that ovarian epithelial carcinoma cells secrete PN, which then accumulates in ascites, suggests that this oncofetal protein may play a role in the pathogenesis of EOC. PN, through promoting $\alpha_V\beta_3$ or $\alpha_V\beta_5$ integrin-dependent adhesion and migration of ovarian epithelial cells could promote i.p. dissemination. Another common feature of EOC is large volume ascites, stemming from neovascularization and vascular endothelial growth factor-induced increase in vascular permeability. One factor believed to be important in the

recruitment and proliferation of endothelial cells is the production of VN at the site of tumor. Like VN, PN could potentially stimulate neovascularization by serving as an "onco-matrix" protein to support $\alpha_V\beta_3$ or $\alpha_V\beta_3$ integrin dependent migration of endothelial cells.

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REFERENCES

1. Auersperg, N., Edelson, M. I., Mok, S. C., Johnson, S. W., and Hamilton, T. C. The biology of ovarian cancer. Semin. Oncol., *25:* 281–304, 1998.
2. Young, R. C., Perez, C. A., and Hoskins, W. J. Cancer of the ovary. *In:* V. T. DeVita, S. Hellman, and S. A. Rosenberg (eds.), Cancer: Principles and Practice of Oncology, pp. 1226–1263. Philadelphia: Lippincott Williams & Wilkins, 2001.
3. Perez, R. P., Godwin, A. K., Hamilton, T. C., and Ozols, R. F. Ovarian cancer biology. Semin. Oncol., *18:* 186–204, 1991.
4. Auersperg, N., Pan, J., Grove, B. D., Peterson, T., Fisher, J., Maines-Bandiera, S., Somasiri, A., and Roskelley, C. D. E-cadherin induces mesenchymal-to-epithelial transition in human ovarian surface epithelium. Proc. Natl. Acad. Sci. USA, *96:* 6249–6254, 1999.
5. Cannistra, S. A., Kansas, G. S., Niloff, J., DeFranzo, B., Kim, Y., and Ottensmeier, C. Binding of ovarian cancer cells to peritoneal mesothelium in vitro is partly mediated by CD44H. Cancer Res., *53:* 3830–3838, 1993.
6. Strobel, T., and Cannistra, S. A. β1-Integrins partly mediate binding of ovarian cancer cells to peritoneal mesothelium in vitro. Gynecol. Oncol., *73:* 362–367, 1999.
7. Carreiras, F., Cruet, S., Staedel, C., Sichel, F., and Gauduchon, P. Human ovarian adenocarcinoma cells synthesize vitronectin and use it to organize their adhesion. Gynecol. Oncol., *72:* 312–322, 1999.
8. Cruet, S., Salamanca, C., Mitchell, G. W., and Auersperg, N. $\alpha v\beta 3$ and vitronectin expression by normal ovarian surface epithelial cells: role in cell adhesion and cell proliferation. Gynecol. Oncol., *75:* 254–260, 1999.
9. Hynes, R. O. Integrins: versatility, modulation, and signaling in cell adhesion. Cell, *69:* 11–25, 1992.
10. Schwartz, M. A., Schaller, M. D., and Ginsberg, M. H. Integrins: emerging paradigms of signal transduction. Annu. Rev. Cell Dev. Biol., *11:* 549–599, 1995.
11. Lafrenie, R. M., and Yamada, K. M. Integrin-dependent signal transduction. J. Cell. Biochem., *61:* 543–553, 1996.
12. Varner, J. A., and Cheresh, D. A. Integrins and cancer. Curr. Opin. Cell Biol., *8:* 724–730, 1996.
13. Mizejewski, G. J. Role of integrins in cancer: survey of expression patterns. Proc. Soc. Exp. Biol. Med., *222:* 124–138, 1999.
14. Clinton, G. M., Rougeot, C., Derancourt, J., Roger, P., Defrenne, A., Godyna, S., Argraves, W. S., and Rochefort, H. Estrogens increase the expression of fibulin-1, an extracellular matrix protein secreted by human ovarian cancer cells. Proc. Natl. Acad. Sci. USA, *93:* 316–320, 1996.
15. Wilson, K. E., Bartlett, J. M., Miller, E. P., Smyth, J. F., Mullen, P., Miller, W. R., and Langdon, S. P. Regulation and function of the extracellular matrix protein tenascin-C in ovarian cancer cell lines. Br. J. Cancer, *80:* 685–692, 1999.
16. Takeshita, S., Kikuno, R., Tezuka, K., and Amann, E. Osteoblast-specific factor 2: cloning of a putative bone adhesion protein with homology with the insect protein fasciclin I. Biochem. J., *294:* 271–278, 1993.
17. Sugiura, T., Takamatsu, H., Kudo, A., and Amann, E. Expression and characterization of murine osteoblast-specific factor 2 (OSF-2) in a baculovirus expression system. Protein Expr. Purif., *6:* 305–311, 1995.
18. Horiuchi, K., Amizuka, N., Takeshita, S., Takamatsu, H., Katsuura, M., Ozawa, H., Toyama, Y., Bonewald, L. F., and Kudo, A. Identification and characterization of a novel protein, periostin, with restricted expression to periosteum and periodontal ligament and increased expression by transforming growth factor β. J. Bone Miner. Res., *14:* 1239–1249, 1999.
19. Ismail, R. S., Baldwin, R. L., Fang, J., Browning, D., Karlan, B. Y., Gasson, J. C., and Chang, D. D. Differential gene expression between normal and tumor-derived ovarian epithelial cells. Cancer Res., *60:* 6744–6749, 2000.
20. Sasaki, H., Auclair, D., Kaji, M., Fukai, I., Kiriyama, M., Yamakawa, Y., Fujii, Y., and Chen, L. B. Serum level of the periostin, a homologue of an insect cell adhesion molecule, in thymoma patients. Cancer Lett., *172:* 37–42, 2001.
21. Sasaki, H., Auclair, D., Fukai, I., Kiriyama, M., Yamakawa, Y., Fujii, Y., and Chen, L. B. Serum level of the periostin, a homologue of an insect cell adhesion molecule, as a prognostic marker in non-small cell lung carcinomas. Cancer (Phila.), *92:* 843–848, 2001.
22. Karlan, B. Y., Baldwin, R. L., Cirisano, F. D., Mamula, P. W., Jones, J., and Lagasse, L. D. Secreted ovarian stromal substance inhibits ovarian epithelial cell proliferation. Gynecol. Oncol., *59:* 67–74, 1995.
23. Meyerson, M., Counter, C. M., Eaton, E. N., Ellisen, L. W., Steiner, P., Caddle, S. D., Ziaugra, L., Beijersbergen, R. L., Davidoff, M. J., Liu, Q., Bacchetti, S., Haber, D. A., and Weinberg, R. A. hEST2, the putative human telomerase catalytic subunit gene, is up-regulated in tumor cells and during immortalization. Cell, *90:* 785–795, 1997.
24. Felding-Habermann, B., O'Toole, T. E., Smith, J. W., Fransvea, E., Ruggeri, Z. M., Ginsberg, M. H., Hughes, P. E., Pampori, N., Shattil, S. J., Saven, A., and Mueller, B. M. Integrin activation controls metastasis in human breast cancer. Proc. Natl. Acad. Sci. USA, *98:* 1853–1858, 2001.
25. Zinn, K., McAllister, L., and Goodman, C. S. Sequence analysis and neuronal expression of fasciclin I in grasshopper and *Drosophila*. Cell, *53:* 577–587, 1988.
26. Wang, W. C., Zinn, K., and Bjorkman, P. J. Expression and structural studies of fasciclin I, an insect cell adhesion molecule. J. Biol. Chem., *268:* 1448–1455, 1993.
27. Skonier, J., Neubauer, M., Madisen, L., Bennett, K., Plowman, G. D., and Purchio, A. F. cDNA cloning and sequence analysis of β ig-h3, a novel gene induced in a human adenocarcinoma cell line after treatment with transforming growth factor-β. DNA Cell Biol., *11:* 511–522, 1992.
28. Elkins, T., Hortsch, M., Bieber, A. J., Snow, P. M., and Goodman, C. S. *Drosophila* fasciclin I is a novel homophilic adhesion molecule that along with fasciclin III can mediate cell sorting. J. Cell Biol., *110:* 1825–1832, 1990.
29. Ohno, S., Noshiro, M., Makihira, S., Kawamoto, T., Shen, M., Yan, W., Kawashima-Ohya, Y., Fujimoto, K., Tanne, K., and Kato, Y. RGD-CAP ((β)ig-h3) enhances the spreading of chondrocytes and fibroblasts via integrin $\alpha(1)\beta(1)$. Biochim. Biophys. Acta, *1451:* 196–205, 1999.
30. Mosher, D. F., and Johnson, R. B. In vitro formation of disulfide-bonded fibronectin multimers. J. Biol. Chem., *258:* 6595–6601, 1983.
31. Declerck, P. J., De Mol, M., Alessi, M. C., Baudner, S., Paques, E. P., Preissner, K. T., Muller-Berghaus, G., and Collen, D. Purification and characterization of a plasminogen activator inhibitor 1 binding protein from human plasma. Identification as a multimeric form of S protein (vitronectin). J. Biol. Chem., *263:* 15454–15461, 1988.
32. Lal, A., Lash, A. E., Altschul, S. F., Velculescu, V., Zhang, L., McLendon, R. E., Marra, M. A., Prange, C., Morin, P. J., Polyak, K., Papadopoulos, N., Vogelstein, B., Kinzler, K. W., Strausberg, R. L., and Riggins, G. J. A public database for gene expression in human cancers. Cancer Res., *59:* 5403–5407, 1999.
33. Sasaki, H., Lo, K. M., Chen, L. B., Auclair, D., Nakashima, Y., Moriyama, S., Fukai, I., Tam, C., Loda, M., and Fujii, Y. Expression of periostin, homologous with an insect cell adhesion molecule, as a prognostic marker in non-small cell lung cancers. Jpn. J. Cancer Res., *92:* 869–873, 2001.
34. Felding-Habermann, B., and Cheresh, D. A. Vitronectin and its receptors. Curr. Opin. Cell Biol., *5:* 864–868, 1993.
35. Castellani, P., Dorcaratto, A., Pau, A., Nicola, M., Siri, A., Gasparetto, B., Zardi, L., and Viale, G. The angiogenesis marker ED-B+ fibronectin isoform in intracranial meningiomas. Acta Neurochir., *142:* 277–282, 2000.
36. Midulla, M., Verma, R., Pignatelli, M., Ritter, M. A., Courtenay-Luck, N. S., and George, A. J. Source of oncofetal ED-B-containing fibronectin: implications of production by both tumor and endothelial cells. Cancer Res., *60:* 164–169, 2000.
37. Nagy, J. A., Masse, E. M., Herzberg, K. T., Meyers, M. S., Yeo, K. T., Yeo, T. K., Sioussat, T. M., and Dvorak, H. F. Pathogenesis of ascites tumor growth: vascular permeability factor, vascular hyperpermeability, and ascites fluid accumulation. Cancer Res., *55:* 360–368, 1995.
38. Plow, E. F., Haas, T. A., Zhang, L., Loftus, J., and Smith, J. W. Ligand binding to integrins. J. Biol. Chem., *275:* 21785–21788, 2000.
39. Singer, I. I., Scott, S., Kawka, D. W., Kazazis, D. M., Gailit, J., and Ruoslahti, E. Cell surface distribution of fibronectin and vitronectin receptors depends on substrate composition and extracellular matrix accumulation. J. Cell Biol., *106:* 2171–2182, 1988.
40. Dejana, E., Colella, S., Conforti, G., Abbadini, M., Gaboli, M., and Marchisio, P. C. Fibronectin and vitronectin regulate the organization of their respective Arg-Gly-Asp adhesion receptors in cultured human endothelial cells. J. Cell Biol., *107:* 1215–1223, 1988.
41. Burridge, K., and Chrzanowska-Wodnicka, M. Focal adhesions, contractility, and signaling. Annu. Rev. Cell Dev. Biol., *12:* 463–518, 1996.
42. Felsenfeld, D. P., Schwartzberg, P. L., Venegas, A., Tse, R., and Sheetz, M. P. Selective regulation of integrin–cytoskeleton interactions by the tyrosine kinase Src. Nat. Cell Biol., *1:* 200–206, 1999.