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Osteopontin-dependent CD44v6 expression and cell adhesion in HepG2 cells

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The interaction of osteopontin (OPN) with CD44 and $\alpha_v\beta_3$-integrin has been implicated in numerous signal transduction pathways that may promote cancer metastasis. CD44v6 is a splice variant of CD44 which has been identified as a marker of cancer progression. In this study, immortalized liver carcinoma cells (HepG2) were used to examine the effect of OPN on two isoforms of CD44: CD44 standard (CD44s) and CD44v6. Western blots demonstrated that OPN up-regulated plasma membrane CD44v6 protein expression in a concentration- and time-dependent fashion. CD44v6 levels returned to control levels when OPN–$\alpha_v\beta_3$-integrin binding was blocked by an RGD peptide or tyrosine kinase activity was inhibited. OPN significantly increased CD44v6 protein synthesis, while simultaneously decreasing protein degradation. Steady-state mRNA levels of both CD44s and CD44v6 were unaltered in the presence of OPN stimulation. OPN increased HepG2 *in vitro* adhesion to hyaluronate (HA); excess soluble HA extinguished OPN-mediated HepG2 adhesion, indicating CD44 dependence. In conclusion, OPN binds to the $\alpha_v\beta_3$-integrin to increase plasma membrane CD44v6 expression and augment *in vitro* adhesion to HA. This may contribute to the mechanism by which OPN enhances metastatic behavior in hepatocellular cancer cells.

Introduction

Cancer progression depends on an accumulation of metastasis-supporting genetic modifications and physiological alterations. Often, these physiological changes are regulated by cell signaling molecules, which target signal transduction pathways and, ultimately, gene expression. One such molecule, osteopontin (OPN), functions as both a cell attachment protein and a cytokine that signals through two cell adhesion molecules: $\alpha_v\beta_3$-integrin and CD44 (1). Initially discovered as an inducible, tumor promoter gene, OPN is often overexpressed in human tumors, is the major phosphoprotein secreted by malignant cells in patients with advanced metastatic cancer and has been implicated in tumor cell migration and metastasis (2–4). Data suggest that OPN plays a key role in cell adhesion and the potential migration/aggregation of neoplastic cells and macrophages (5). OPN is significantly overexpressed in hepatocellular cancer (HCC) and correlates with capsular infiltration (6,7). Most recently, using expression profiles of HCC samples with and without intrahepatic metastases, OPN was found to be profoundly overexpressed in metastatic lesions (8). An OPN antibody effectively blocked *in vitro* cell invasion and inhibited pulmonary metastases in nude mice. The underlying mechanism by which OPN may induce tumor metastases has not been well characterized. However, it is well known that binding of OPN with CD44 or $\alpha_v\beta_3$-integrin initiates a number of signal transduction pathways that control cytoskeletal reorganization, cell survival, cell proliferation and cell migration (1).

CD44 is a member of the immunoglobulin superfamily that is expressed on most epithelial and non-epithelial cells. Functionally, CD44 binds hyaluronate (HA) in the extracellular matrix (ECM) to maintain tissue/organ structure, promote cell aggregation and mediate cell movement. There are multiple isoforms of CD44 resulting from splice variation. The standard CD44 (CD44s) isoform aids in maintenance of the 3-dimensional tissue/organ stucture. While CD44s expression does not necessarily convey metastatic potential, CD44 variant isoforms, especially CD44v6, have been identified as protein markers for metastatic behavior in hepatocellular, breast, colorectal and gastric cancers (9–12). In many biological systems, receptor expression is regulated by the local concentration of its ligand. In this regard, we examined the role of OPN in CD44s and CD44v6 regulation and function in the HepG2 hepatocellular cancer cell line. This analysis has not been previously performed. Our findings suggest that OPN may enhance metastatic potential in hepatocellular cancer cells by increasing plasma membrane CD44v6 expression and augmenting cell adhesion to HA.

Materials and methods

Cell culture

HepG2 cells (ATCC HB 8065) were obtained from the American Type Culture Collection (Manassas, VA). The cells were grown as monolayer cultures in Dulbecco's modified Eagle's medium (DMEM) with the addition of 10% fetal bovine serum (FBS). The medium was changed every 3–4 days and the cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2. In selected instances, the cells were treated for various incubation periods (0, 1, 3, 9 and 12 h) with human OPN (0, 1, 2, 5 and 10 nM). Protein half-life determinations were performed by addition of anisomycin (10 μg/ml) following 12 h of OPN (5 nM) incubation. Human hepatocytes isolated from surgical pathology specimens were a gift from Dr Donald C.Rockey (Duke University, Durham, NC). In selected instances, cytoplasmic and plasma membrane protein fractions were isolated using a ProteoPrep Universal Extraction System (Sigma-Aldrich, St Louis, MO).

Immunoblot analysis

Cells were lysed in buffer (0.8% NaCl, 0.02% KCl, 1% SDS, 10% Triton X-100, 0.5% sodium deoxycholic acid, 0.144% Na_2HPO_4 and 0.024% KH_2PO_4, pH 7.4) and centrifuged at 12 000 *g* for 10 min at 4°C. Protein concentration was determined by absorbance at 650 nm using protein assay

Abbreviations: DMEM, Dulbecco's modified Eagle's medium; ECM, extracellular matrix; FBS, fetal bovine serum; HA, hyaluronate; HCC, hepatocellular cancer; HGF, hepatocyte growth factor; OPN, osteopontin; PBS, phosphate-buffered saline.

reagent (Bio-Rad, Hercules, CA). Cell lysates (50 μg/lane) were separated by 12% SDS–PAGE and the products were electrotransferred to PVDF membrane (Amersham Pharmacia, Piscataway, NJ). The membrane was blocked with 5% skimmed milk in phosphate-buffered saline (PBS) containing 0.05% Tween for 1 h at room temperature. After being washed three times, blocked membranes were incubated with antibody directed against human CD44v6 (mouse monoclonal Ab; R & D Systems, Minneapolis, MN), CD44s (mouse monoclonal Ab; Santa Cruz Biotechnology, Santa Cruz, CA) and β-actin (goat polyclonal Ab; Santa Cruz Biotechnology) for 1 h at room temperature, washed three times in PBS containing 0.05% Tween and incubated with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. After an additional three washes, bound peroxidase activity was detected with the ECL detection system (Amersham Pharmacia).

RNA preparation and northern blot analysis

Total RNA was isolated using Trizol reagent (Gibco BRL, Rockville, MD). RNA samples (10 μg/lane) were fractionated by electrophoresis on a 1% agarose formaldehyde gel and transferred to Hybond-C nylon membrane (Amersham Pharmacia). [^{32}P]dATP-labeled probes for CD44s (exons 1–5, nt 179–849) and CD44v6 (exon 6, nt 846–974) were constructed based upon GenBank accession no. NM000610. cDNA probes were constructed to bridge two contiguous exons and were prepared by random primer labeling, followed by purification using a Sephadex G-50 mini-column (BioMax Inc., Odenton, MD). Hybridization was performed at 42°C for 18 h in ULTRAhyb hybridization buffer (Ambion, Austin, TX). Following hybridization, filters were washed twice and subjected to autoradiography using Fuji film for a period of 14 h. β-Actin was utilized as the housekeeping gene. Quantification was performed using a Phosphorimager (Molecular Dynamics Storm 840).

Pulse–chase study of OPN-mediated CD44v6 protein synthesis

Pulse–chase studies were performed to determine rate of synthesis of CD44v6 in the setting of OPN (5 nM) stimulation. HepG2 cells were subcultured at a density of 1.0×10^5 cells/well. After 24 h, cells were washed with PBS and then were incubated with 1 ml of FBS-, methionine- and cysteine-free DMEM supplemented with 100 μCi/ml of L-[^{35}S]methionine and L-[^{35}S]cysteine [Promix L-(^{35}S); Amersham Pharmacia Biotech] *in vitro* cell labeling mixture and incubated for 30 min. The cells were chased with fresh FBS medium containing cold methionine and cysteine. At each time point, the medium was collected and the cells were washed with PBS and suspended in 300 μl of disruption buffer (10 mmol/l Tris–HCl, pH 7.5, 150 mmol/l NaCl, 5 mmol/l EDTA, 10 mmol/l benzamidine, 1 mmol/l phenylmethylsulfonyl fluoride and 1% Nonidet P-40) and lysed with three repetitive freeze–thaw cycles. The cell lysates were adjusted to equal protein concentration with disruption buffer and subjected to immunoprecipitation. A total of 500 μl of cell lysate (100 μg total protein) was mixed with an equal volume of disruption buffer lacking EDTA and Nonidet P-40 and immunoprecipitated with antibody directed against human CD44v6 (mouse monoclonal Ab; R & D Systems) overnight at 4°C, followed by incubation with 40 μl of protein A beads (Amersham Biosciences) for 2 h at 4°C. The immunoprecipitates were washed, solubilized, subjected to SDS–PAGE and ^{35}S incorporation determined.

Cellular adhesion to immobilized hyaluronan

Flat-bottomed 96-well tissue culture plates (Costar, Corning, NY) were coated with 5 mg/ml HA and HepG2 cell adhesion was determined in the presence and absence of 5 nM OPN. For experiments where potassium hyaluronan was used to compete for adhesion, the cells were preincubated with potassium hyaluronan at the concentrations indicated for 30 min at room temperature. Samples of 2×10^5 cells were added to each well and the 96-well plate incubated at 37°C for 60 min in serum-free medium. Non-adhered cells were removed by washing. Adhered cells were fixed with 1.0% glutaraldehyde and stained with 0.1% (w/v) crystal violet solution. Cells were washed, solubilized overnight in 1.0% Triton X-100 and absorbance readings recorded at 570 nm. The results obtained were corrected by the subtraction of background staining of the underlying matrix (13). In selected instances, Lipofectin (Invitrogen) was used to transfect antisense and sense oligonucleotides for CD44v6 into HepG2 cells for 12 h. The sequence for the v6 exon antisense oligonucleotide was 5′-AATACCATTAACCAGGTAGT-3′.

In vitro cell proliferation assay

Cells were seeded into 96-well tissue culture plates in triplicate at a density of 2×10^3 cells/well and proliferation monitored daily over a period of 3 days using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (13).

Matrigel invasion assay

Matrigel diluted in 4H medium was solidified onto a Transwell polycarbonate membrane. HepG2 cells (2×10^5 cells/well) suspended in DMEM medium in the presence or absence of 5 nM OPN were added to the top of the well. Transwell migration studies were performed with 5 nM hepatocyte growth factor (HGF) as an attractant. Wells were incubated at 37°C in humidified air containing 5% CO_2 for 72 h. The cells on the upper side of the polycarbonate membrane were wiped off and the remaining cells that traversed the Matrigel and spread on the lower surface of the filter were collected separately. An MTT assay was then performed on harvested cells. The relative invasive rate was calculated as the percent OD of the cells from the top of the membrane to the overall OD from the total cells.

Statistical analysis

Data are expressed as means ± SEM of three or four experiments. Statistical analyses were performed using Student's *t*-test. P values <0.05 were considered significant.

Results

Steady-state levels of CD44s and CD44v6 mRNA are not altered by OPN

Northern blots were performed to determine the effect of OPN on CD44s and CD44v6 mRNA expression (Figure 1). HepG2 cells were exposed to 5 nM OPN for incubation periods of 0, 4, 8 and 12 h. There was no discernible change in CD44s and CD44v6 mRNA levels induced by OPN.

OPN increases CD44v6 protein expression

OPN was initially found to be constitutively expressed in HepG2 cells, but not in normal human hepatocytes (Figure 2A). HepG2 cells were then stimulated with OPN (0, 1, 2, 5 and 10 nM) for a period of 12 h. Previous work has demonstrated elevated OPN serum levels of 8–15 nM in patients with metastatic liver, breast and prostate cancer (14). CD44s and CD44v6 protein expression was then determined by immunoblot analysis (Figure 2B and C). When compared with that noted in the absence of OPN stimulation, CD44v6 levels increased approximately 8- and 6-fold in the presence of

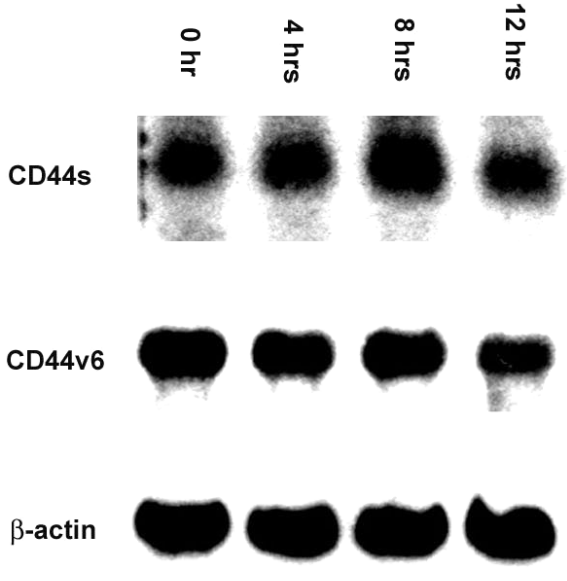


Fig. 1. Osteopontin and CD44s and CD44v6 mRNA expression. HepG2 hepatocytes were incubated in the presence of OPN (5 nM) for 0 (Control), 4, 8 and 12 h. Total RNA was isolated using Trizol reagent (Gibco BRL). RNA samples (10 μg/lane) were fractionated by electrophoresis on a 1% agarose formaldehyde gel and transferred to Hybond-C nylon membrane (Amersham Pharmacia Inc.). [^{32}P]dATP-labeled probes for CD44s (exons 1–5, nt 179–849) and CD44v6 (exon 6, nt 846–974) were constructed based upon GenBank accession no. NM000610. cDNA probes were prepared by random primer labeling, followed by purification using a Sephadex G-50 mini-column (BioMax Inc.). Hybridization was performed at 42°C for 18 h. The blot is representative of four separate experiments.

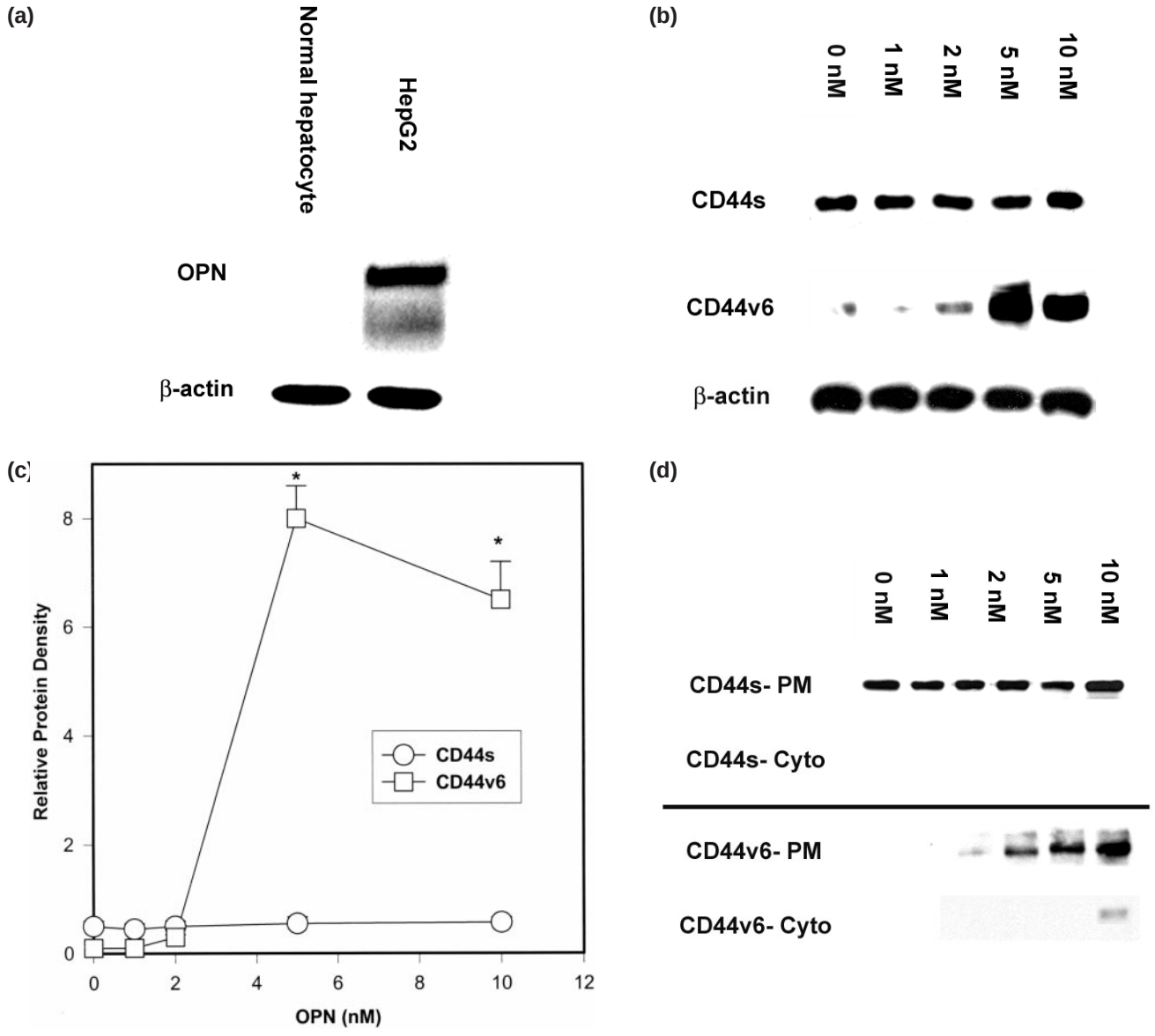


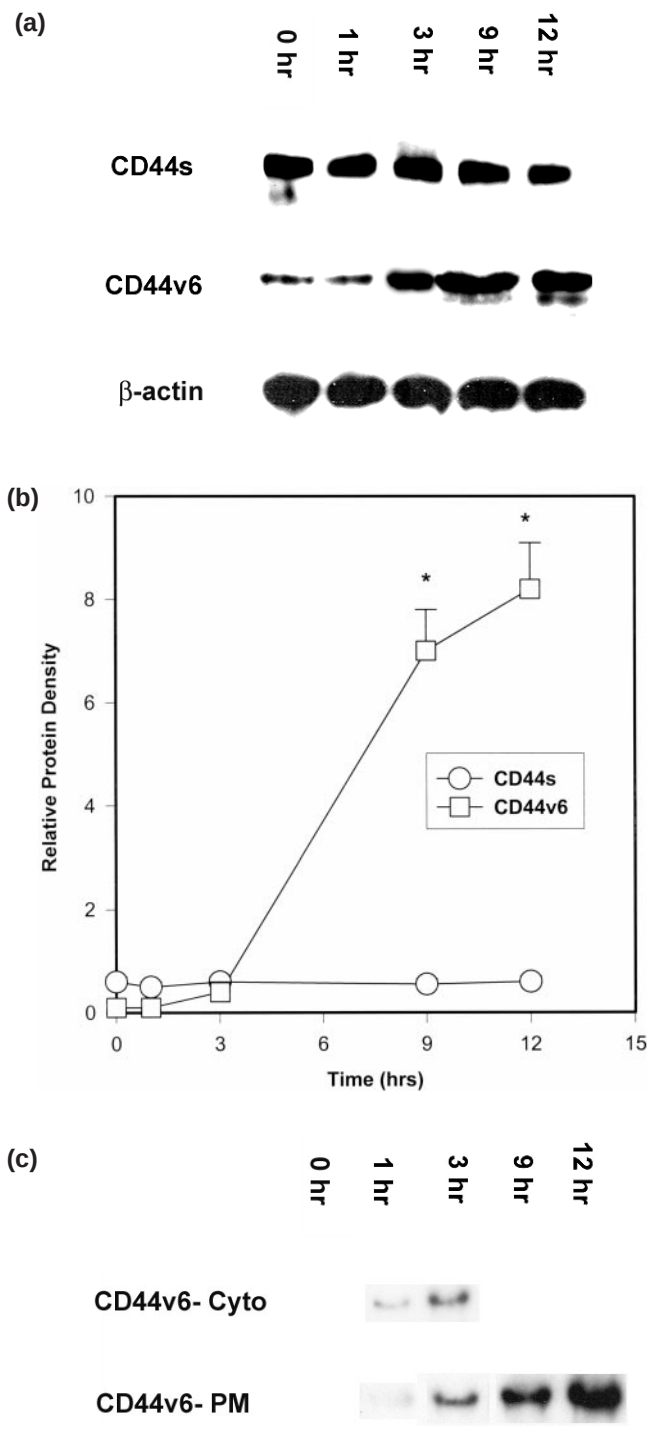
Fig. 2. Osteopontin and HepG2 expression of CD44s and CD44v6 protein. (**A**) OPN expression in HepG2 and normal human hepatocytes. Cells were lysed and centrifuged at 12 000 *g* for 10 min at 4°C. Cell lysates (50 μg/lane) were separated by 12% SDS–PAGE and the products were electrotransferred to PVDF membrane (Amersham Pharmacia). Blocked membranes were then incubated with antibody directed against human OPN (Santa Cruz Biotechnology) for 1 h at room temperature, washed and then exposed to horseradish peroxidase-conjugated secondary antibody. Bound peroxidase activity were detected by the ECL detection system (Amersham Pharmacia). The blot is representative of three experiments. (**B**) OPN-dependent HepG2 expression of CD44s and CD44v6 proteins. HepG2 cells were incubated in the presence of OPN (0–10 nM) for a period of 12 h. Immunoblot analysis was performed as previously described. Primary antibodies were directed against human CD44v6 (mouse monoclonal Ab; R & D Systems), CD44s (mouse monoclonal Ab; Santa Cruz Biotechnology) and β-actin (Santa Cruz Biotechnology). The blot is representative of three experiments. (**C**) Dose–response relationship between OPN and CD44s and CD44v6 expression. A graphical summary of OPN-dependent CD44s and CD44v6 expression. Laser densitometric analysis was performed relative to β-actin protein. Data represent a total of three experiments. $^{*}P < 0.01$, CD44v6 versus CD44 s. (**D**) Plasma membrane and cytoplasm expression of CD44s and CD44v6 protein. HepG2 cells were incubated in the presence of OPN (0–10 nM) for a period of 12 h. Cytoplasmic and plasma membrane protein fractions were isolated using the ProteoPrep Universal Extraction System (Sigma-Aldrich). Immunoblot analysis was performed as previously described. Data are expressed as means ± SEM. The blot is representative of three experiments.

5 and 10 nM OPN, respectively ($P < 0.001$ for 0 nM OPN versus 5 and 10 nM OPN). CD44s expression was unaltered by OPN. Following isolation of cytoplasmic and plasma membrane protein fractions, CD44s and CD44v6 expression in all treament groups was largely confined to the plasma membrane fraction (Figure 2D). These data indicate that OPN increases plasma membrane expression of CD44v6.

The time-dependence of OPN-mediated increase in CD44s and CD44v6 expression was then determined in the presence of 5 nM OPN (Figure 3A and B). Augmented CD44v6 expression was detected within 3 h, with maximal expression at 12 h. Again, CD44s expression was unaltered. Following isolation of cytoplasmic and plasma membrane fractions, CD44v6 protein levels were examined at 0, 1, 3, 9 and 12 h following 5 nM OPN stimulation (Figure 3C). CD44v6 was simultaneously expressed in both cytoplasm and plasma membrane at 3 h. Thereafter, the vast majority of CD44v6 was present exclusively in the plasma membrane fraction.

OPN-mediated increase in CD44v6 expression is RGD and tyrosine kinase dependent

The hexapeptide GRGDSP blocks binding of RGD-containing proteins, such as OPN, to cell surface integrins. OPN–integrin binding is thought to be an RGD-dependent process, while OPN–CD44 binding is an RGD-independent process (1,4). To determine the potential contributory role of OPN binding to $\alpha_v\beta_3$-integrin, immunoblots were performed to determine the effect of 10-fold excess GRGDSP (50 nM) on CD44s and CD44v6 expression in the presence of 5 nM OPN (Figure 4). In the presence of RGD blockade, CD44s expression was unaltered by OPN treatment. In contrast, in the presence of 5 nM OPN, GRGDSP restored the OPN-mediated increase in CD44v6 to levels equivalent to that noted in the absence of OPN. HA (500 nM) was then added in excess as a specific competitor of OPN binding to CD44. In this setting, OPN + HA treatment did not alter CD44v6 or CD44s protein expression compared to that of OPN treatment alone. These results indicate that OPN acts via the $\alpha_v\beta_3$-integrin rather than CD44 to increase CD44v6 expression. Immunoblot studies were repeated in the presence of the tyrosine kinase inhibitor tyrphostin B46 (40 μM) and the serine/threonine kinase inhibitor staurosporine (1 μM). In this instance, inhibition of tyrosine kinase activity ablated the OPN-induced increase in CD44v6 protein expression. CD44s expression was unaltered.



OPN alters CD44v6 protein synthesis and degradation

OPN does not alter steady-state CD44v6 mRNA levels. We therefore examined the effect of 5 nM OPN on CD44v6 protein synthesis and degradation (Figure 5). Pulse–chase studies were performed with L-[^{35}S]methionine and L-[^{35}S]cysteine in HepG2 cells in the presence and absence of OPN. CD44v6 was immunoprecipitated from cell lysates at 0, 3, 6 and 12 h (Figure 5A). The results indicate that OPN induces a significant increase in CD44v6 synthesis, with total synthesis increased by approximately 5- and 7-fold following OPN stimulation for 6 and 12 h ($P = 0.0001$ by ANOVA for 5 nM OPN).

In the presence of anisomycin, a protein synthesis inhibitor, immunoblots of CD44v6 were performed to determine CD44v6 half-life in the presence of 5 nM OPN (15) (Figure 5B and C). Anisomycin was added 12 h following OPN stimulation. Unstimulated cells (0 nM OPN) served as controls. β-Actin was chosen as a reference protein as its half-life is ~5–6 h (16). CD44v6 protein is expressed at low levels in the absence of OPN, therefore, a 5-fold greater number of cells was required in the untreated (0 nM OPN) group to obtain an adequate signal. This is reflected in the increased amounts of β-actin in this group. Normalized CD44v6 expression at time 0 was then set to 100% in both groups, OPN-treated and OPN-untreated. Nevertheless, in the presence of 5 nM OPN, CD44v6 protein was degraded at a significantly slower rate. Semi-logarithmic plots further demonstrate that the protein half-lives of CD44v6 in the absence and presence of OPN were 80 and 135 min, respectively ($P = 0.0001$ by ANOVA

Fig. 3. Time dependence of osteopontin and HepG2 expression of CD44s and CD44v6 protein. (**A**) HepG2 cells were incubated in the presence of OPN (5 nM) for periods of 0 (control) to 12 h. Cells were lysed and centrifuged at 12 000 *g* for 10 min at 4°C. Cell lysates (50 μg/lane) were separated by 12% SDS–PAGE and the products were electrotransferred to PVDF membrane (Amersham Pharmacia). Blocked membranes were then incubated with antibody directed against human CD44v6 (mouse monoclonal Ab; R & D Systems) and CD44s (mouse monoclonal Ab; Santa Cruz Biotechnology) for 1 h at room temperature, washed and then exposed to horseradish peroxidase-conjugated secondary antibody. Bound peroxidase activity was detected with the ECL detection system (Amersham Pharmacia). The blot is representative of three experiments. (**B**) Time–response relationship between OPN and CD44s and CD44v6 expression. A graphical summary of time-dependent OPN-mediated CD44s and CD44v6 expression. Laser densitometric analysis was performed relative to β-actin protein. Data are expressed as means ± SEM. Data represent a total of three experiments. $^*P < 0.01$, CD44v6 versus CD44s. (**C**) OPN and CD44v6 expression in cytoplasm and plasma membrane. HepG2 cells were incubated in the presence of OPN (5 nM) for periods of 0–12 h. Cytoplasmic and plasma membrane protein fractions were isolated using the ProteoPrep Universal Extraction System (Sigma-Aldrich). Immunoblot analysis was performed, as previously described. The blot is representative of three experiments.

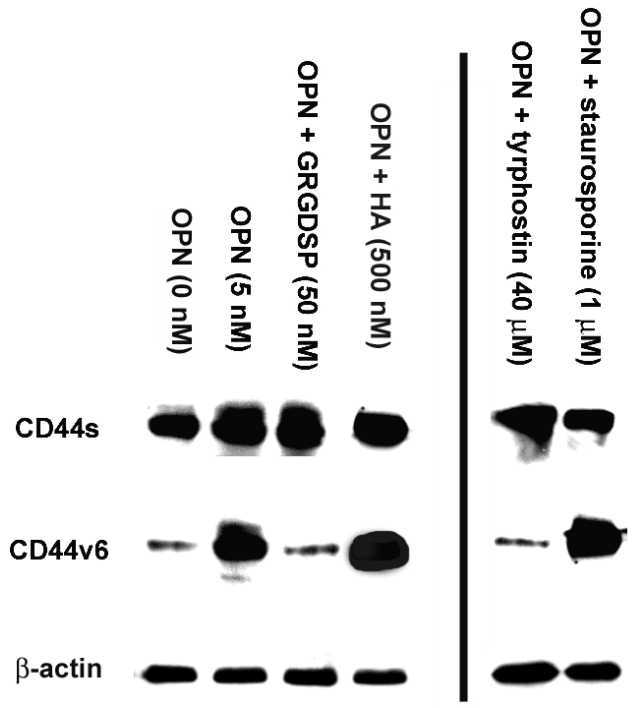


Fig. 4. Signal transduction of OPN-mediated CD44s and CD44v6 protein expression. HepG2 cells were incubated in the presence of OPN (5 nM) for a period of 12 h. In selected instances, GRGDSP (50 nM), a competitive ligand inhibitor of integrin binding, HA (500 nM), a competitive ligand for CD44 binding, tyrphostin (40 μM), a tyrosine kinase inhibitor, or staurosporine (1 μM), a serine/threonine kinase inhibitor, was added with OPN. Immunoblots were performed to detect CD44s or CD44v6 protein. Cells were lysed and centrifuged at 12 000 *g* for 10 min at 4°C. Cell lysates (50 μg/lane) were separated by 12% SDS–PAGE and the products were electrotransferred to PVDF membrane (Amersham Pharmacia). Blocked membranes were then incubated with antibody directed against human CD44v6 (mouse monoclonal Ab; R & D Systems) and CD44s (mouse monoclonal Ab; Santa Cruz Biotechnology) for 1 h at room temperature, washed and then exposed to horseradish peroxidase-conjugated secondary antibody. Bound peroxidase activity was detected by the ECL detection system (Amersham Pharmacia). The blot is representative of three experiments.

for both 5 and 0 nM OPN). In HepG2 cells, we conclude that CD44v6 protein synthesis and degradation are significantly altered in the presence of OPN to increase total CD44v6 protein content.

OPN and HepG2 proliferation and migration

To investigate whether OPN (0 and 5 nM) treatment of HepG2 cells may enhance growth factor binding and, hence, proliferation *in vitro*, cell growth was monitored over a period of 3 days in the absence and presence of added heparin-binding epidermal growth factor (10 pM to 10 nM), basic fibroblast growth factor (10 pM to 10 nM) and hepatocyte growth factor (HGF) (10 pM to 10 nM). No differences were found between the proliferation rates of 0 and 5 nM OPN-treated HepG2 cells in the absence or presence of added growth factors (data not shown). Increased cell invasion is a key phenotypic advantage of malignant cells favoring metastasis. Cell invasion was studied in Matrigel-coated transwells. Transwell migration studies were performed with 5 nM HGF as an attractant. In this setting, HepG2 cells treated with 5 nM OPN did not exhibit a level of Matrigel invasion that was statistically different from that noted in the absence of OPN (data not shown).

OPN and HepG2 adhesion to hyaluronan

The effect of OPN on HepG2 hyaluronan binding was examined by adhesion assays on immobilized hyaluronan (Figure 6). In the absence of OPN, HepG2 cells displayed modest levels of binding to immobilized hyaluronan, which was decreased by preincubation of the cells with excess soluble hyaluronan (250 and 500 μg/ml), suggesting that adhesion may be dependent upon a CD44 isoform. In contrast, OPN (5 nM)-treated cells showed an ~2-fold increased affinity for hyaluronan. Again, soluble HA extinguished OPN-dependent adhesion, suggesting that this was CD44 dependent. Antisense and sense CD44v6 oligonucleotides were then added with OPN. Addition of antisense CD44v6 oligos completely eliminated CD44v6 protein expression, as determined by western blot assays (data not shown). In the presence of 5 nM OPN and antisense CD44v6, cell adhesion to HA was not statistically different from that noted in the absence of OPN. Addition of sense CD44v6 did not alter OPN-dependent adhesion to HA. Trypan blue exclusion demonstrated that free soluble HA at concentrations of 250 and 500 μg/ml did not alter HepG2 cell viability. Cell viability was routinely >90% in the presence of HA, OPN, sense CD44v6 and/or antisense CD44v6. These data suggest that the OPN-mediated increase in HepG2 adhesion to HA is CD44v6-dependent.

Discussion

In this study, HepG2 cells stimulated with OPN were found to specifically up-regulate CD44v6 expression on the plasma membrane as the result of both increased protein synthesis amd decreased protein turnover. This OPN effect was dependent upon tyrosine kinase activity and $\alpha_v\beta_3$-integrin binding. Although enhanced CD44v6 expression did not alter HepG2 proliferation or migration, increased *in vitro* adhesion to HA was noted. This could be extinguished by antisense-mediated ablation of CD44v6 protein expression. These data indicate that OPN may enhance metastatic potential in HCC by increasing CD44v6 expression, resulting in increased adhesion to ECM proteins. The effect of OPN on CD44 expression has not been previously examined in the setting of HCC. These findings represent a novel mechanism by which a ligand, OPN, interacts with one of its receptor types, $\alpha_v\beta_3$-integrin, to functionally up-regulate expression of an isoform of its second receptor, CD44v6.

OPN is a highly hydrophilic and negatively charged sialoprotein of ~298 amino acids which contains a Gly-Arg-Gly-Asp-Ser sequence. It is a secreted protein which binds to both cell surface $\alpha_v\beta_3$-integrins bearing an RGD binding motif and/or CD44. Its known regulatory functions include cell adhesion and migration, tumor growth and metastasis, atherosclerosis, aortic valve calcification and repair of myocardial injury. Recently, OPN has received a great deal of attention as a primary marker and, perhaps, mediator of metastatic spread in breast, prostate, colorectal and melanoma cancers, among others (4,5,17,18). In a pathology study of HCC, Gotoh and colleagues demonstrated that both OPN transcript and protein were significantly overexpressed in HCC with capsular infiltration, compared with HCC without capsular infiltration. Moreover, OPN-positive cancer cells were often dispersed in the periphery of cancer nodules and adjacent to stromal cells (6). Using a supervised machine learning algorithm, Ye *et al*. examined human HCC samples for the gene expression

signature of primary HCCs with accompanying metastasis. OPN was identified as the lead gene which was overexpressed in metastatic HCC. An OPN-specific antibody effectively blocked HCC cell invasion *in vitro* and inhibited pulmonary metastasis of HCC cells in nude mice. The authors conclude that OPN expression in HCC confers a metastatic phenotype (8). Another study indicates that OPN is a major transcription target induced by HGF and may contribute to HGF induced cell–cell association and cell growth and invasiveness (3). However, the underlying mechanism by which OPN imparts a metastatic phenotype is unknown.

CD44 is a transmembrane cell adhesion molecule that is the major cell surface receptor for HA. It is implicated in a number of cellular functions, including lymphocyte homing, leukocyte activation, extracellular matrix adhesion, embryogenesis and wound healing. Multiple high molecular weight isoforms (CD44v) of the core molecule (CD44s) may be generated through alternative splicing of 10 variable exons which encode part of the extracellular domain. Up-regulation of Cd44s and CD44v expression characterize multiple human malignancies, including colorectal, breast, pancreatic, gastric and melanoma cancers (9,10,12). With respect to HCC, multiple studies have demonstrated a correlation between CD44s and CD44v expression and a HCC metastatic phenotype. Mathew and colleagues found that CD44s and CD44v6 expression in HCC correlated significantly with vascular invasion, but not with tumor grade or proliferation indices. These findings imply a relationship between CD44 and metastatic behavior (19). In another series of pathology studies, Endo and Terada found that CD44v6 expression correlated signficantly with vascular invasion and p53 overexpression (11). Most recently, in a human HCC cell line that does not express CD44v, Barbour and co-workers expressed the full-length CD44v2-10 isoform. They found that CD44v2-10 resulted in pulmonary metastases in SCID mice, but had no effect on primary tumor development. In findings analogous to ours, Barbour *et al*. found that CD44v did not alter *in vitro* HCC proliferation or migration, although cell adhesion was significantly altered (13). The relationship between OPN and CD44 as potent enhancers of cancer metastatic behavior has already been examined in the context of OPN as a ligand which binds to the CD44 receptor. Weber *et al*. demonstrated that OPN binds specifically to CD44s in a dose-dependent fashion and induces chemotaxis. OPN binding to CD44 is RGD independent (20). With respect

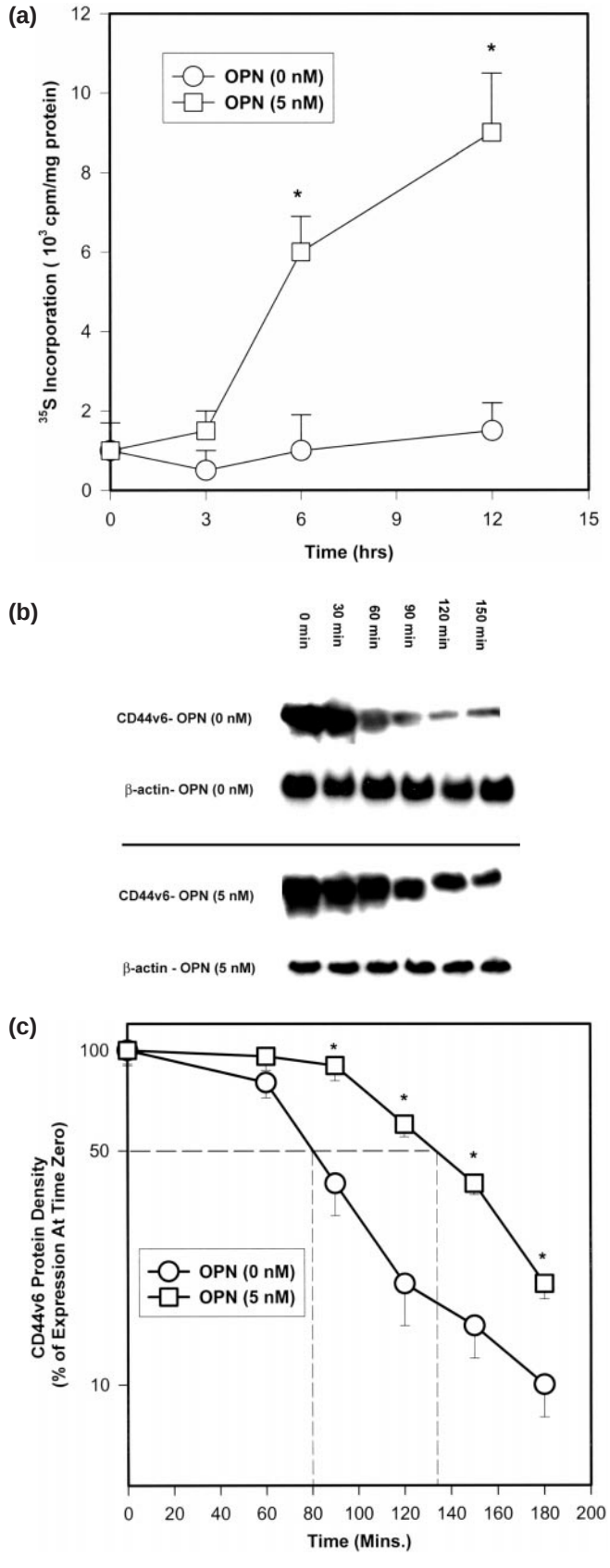


Fig. 5. OPN and CD44v6 protein metabolism. (**A**) Pulse–chase experiments were performed utilizing L-[^{35}S]methionine and L-[^{35}S]cysteine. HepG2 cells were stimulated with 0 or 5 nM OPN. At 0, 3, 6 and 12 h, CD44v6 protein was immunoprecipitated and ^{35}S incorporation determined. Data are expressed as means ± SEM. The plot is representative of three experiments. $^{*}P < 0.01$, 5 nM versus 0 nM OPN. (**B**) HepG2 cells were incubated in the presence or absence of OPN (5 nM) for a period of 12 h. Anisomycin (10 μg/ml) was then added as an inhibitor of protein synthesis. Because of low levels of CD44v6 protein expression in the absence of OPN, a 5-fold greater number of cells was required in the unstimulated group; this is reflected in the increased β-actin signal. Normalized CD44v6 expression at time 0 was then set to 100% in both groups, OPN-treated and OPN-untreated. Thereafter, immunoblots were performed on cell lysates to determine CD44v6 protein levels. Cells were lysed and centrifuged at 12 000 *g* for 10 min at 4°C. Cell lysates (50 μg/lane) were separated by 12% SDS–PAGE and the products were electrotransferred to PVDF membrane (Amersham Pharmacia). Blocked membranes were then incubated with antibody directed against human CD44v6 (mouse monoclonal Ab; R & D Systems) for 1 h at room temperature, washed and then exposed to horseradish peroxidase-conjugated secondary antibody. Bound peroxidase activity was detected by the ECL detection system (Amersham Pharmacia). The blot is representative of three experiments. (**C**) Semi-logarithmic plot of CD44v6 protein degradation. Laser densitometric analysis was performed relative to β-actin protein. Data are expressed as means ± SEM. Data represent a total of three experiments. $^{*}P < 0.01$, 5 nM versus 0 nM OPN.

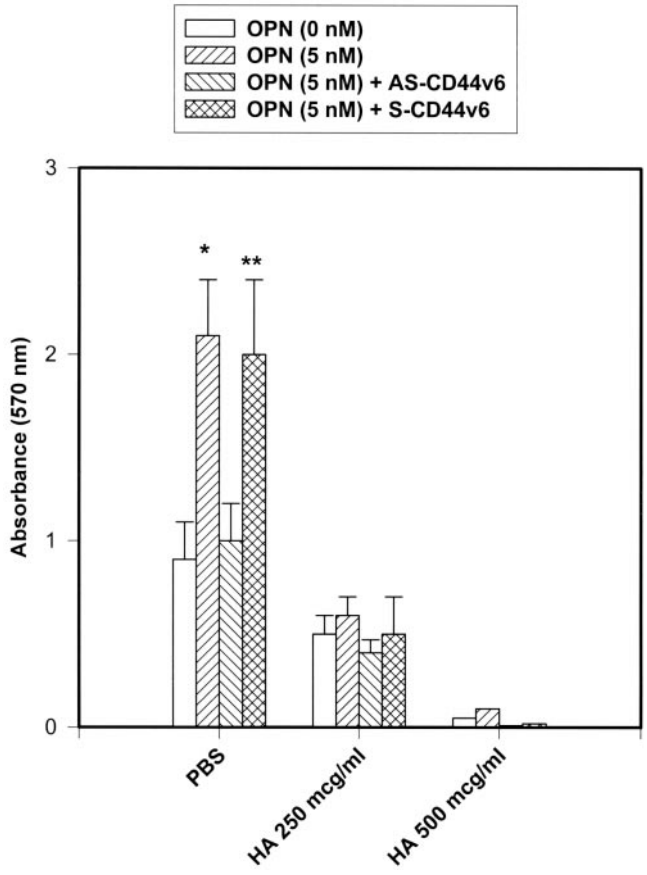


Fig. 6. HepG2 cell adhesion to HA. Flat-bottomed 96-well tissue culture plates (Costar) were coated with 5 mg/ml HA and control and OPN-treated HepG2 cell adhesion was determined. For experiments in which potassium hyaluronan was used to compete for adhesion, the cells were preincubated with potassium hyaluronan at the concentrations indicated for 30 min at room temperature. Samples of 2×10^5 cells were added to each well and the 96-well plate incubated at 37°C for 60 min. The bound cells were stained with crystal violet, lysed and the absorbance at 570 nm measured. In selected instances, Lipofectin (Invitrogen) was used to transfect antisense (AS) and sense (S) oligonucleotides for CD44v6 into HepG2 cells for 12 h before determination of cell adhesion. Data are expressed as means ± SEM of three experiments. $^{*}P < 0.01$, 5 nM versus 0 nM OPN or 5 nM OPN + AS CD44v6; $^{**}P < 0.01$, 5 nM OPN + S CD44v6 versus 0 nM OPN or 5 nM OPN + AS CD44v6.

to the CD44v isoforms, Uede and colleagues showed that only v6 or v7 had to be present in CD44v to bind OPN. It is clear that the bulk of experimental data indicates that OPN interacts with CD44s and CD44v isoforms to convey a metastatic phenotype (21).

Recently, in osteoclasts from $OPN^{-/-}$ mice, Chellaiah and colleagues examined OPN–CD44 interactions (22–24). Osteoclasts from $OPN^{-/-}$ mice are hypomotile and partially disabled in bone resorption. Treatment of $OPN^{-/-}$ osteoclasts with exogenous OPN partially restored the phenotype due to activation of $\alpha_v\beta_3$-integrin, stimulating motility and increasing expression of CD44 on the basolateral plasma membrane surface. Antibody blockade of CD44 and β_3 ablated the stimulatory actions of OPN on motility and bone resorption. These authors concluded that surface expression of CD44 was a key function of OPN. Subsequently, they showed that an OPN/$\alpha_v\beta_3$-generated outside-in Rho signaling pathway was required for the surface expression of CD44 and motility of osteoclasts. One downstream effect of the Rho signaling pathway was targeting of the CD44 complex to the plasma membrane. OPN-deficient osteoclasts fail to efficiently move CD44 to the surface of the plasma membrane and exogenous addition of OPN or Rho is sufficient to partially rescue the phenotype. Restoration of the phenotype by exogenous OPN was blocked by antibodies to the β_3-integrin. Subsequently, Chellaiah *et al*. demonstrated that OPN stimulated ROK-α phosphorylation and its interaction with CD44. To further assess the role of ROK-α on the phosphorylation of CD44 and ERM proteins, they used the recently identified ROK inhibitor Y27632 and found that it significantly inhibited OPN- and Rho_{Val14}-induced phosphorylation of CD44 and the associated complex. Their analyses demonstrated that ROK-α activation by Rho resulted in the phosphorylation of CD44. Inhibition of CD44 phosphorylation and surface expression of CD44 by Y27632 identified ROK-α as the primary candidate required for CD44 surface expression. Although their studies utilized a different cell type, their results mirror ours in that OPN-dependent membrane expression of CD44 was mediated by phosphorylation and OPN–$\alpha_v\beta_3$-interactions. Unfortunately, these authors did not examine VD44 splice variants or the translational and transcriptional basis for increased OPN-mediated CD44 expression in their system.

With the exception of the studies above, the potential interplay between OPN and CD44 as ligand and receptor has otherwise not been previously examined. Certainly, feedback regulation of receptor function and expression by ligand binding is a well-characterized cellular phenomenon. However, in this instance our data suggest that OPN binds to one class of receptor, $\alpha_v\beta_3$-integrin, to up-regulate expression of a second class of receptor, the CD44v6 isoform. Increased CD44v6 is tyrosine kinase-dependent and insensitive to competition by HA. However, HA can inhibit the increased cell adhesion noted in the presence of OPN-mediated up-regulation of HepG2 cell surface CD44v6. In conclusion, our observations suggest a unique mechanism by which OPN enhances *in vitro* cell adhesion by HepG2 cells. These findings may have relevance for OPN and metastatic behavior of HCC.

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