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ORIGINAL ARTICLE

Osteopontin (OPN) Isoforms, Diabetes, Obesity, and Cancer; What Is One Got to Do with the Other? A New Role for OPN

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Abstract Alternative splicing of osteopontin (OPN) produces three isoforms: OPNa, OPNb, and OPNc. The aims of this study were to examine the expression profile of OPN isoforms in sera from patients with pancreatic lesions and to determine their correlation with the presence of comorbid systemic inflammatory conditions, such as diabetes and/or obesity. Sera from 90 patients undergoing pancreatic surgery and 29 healthy volunteers were analyzed. Seventeen patients were diabetics, 17 were obese, and 6 had both diabetes and obesity. In patients with pancreatic lesions, OPNb was expressed in 48 % of the patients' sera, OPNc in 34 %, and both in 5 %. The presence of diabetes and/or obesity was associated with complete disappearance of OPNb and expression of only OPNc. OPNc presence was significantly associated with diabetes and obesity (OR=7.06 [95 % CI 1.97–23.3]; $p=0.003$). No OPNb or OPNc was detected in the normal sera. Overexpression of OPNb and OPNc isoforms in PDA cells significantly ($p<0.05$) increased their activity in soft-agar colony formation and wound healing assays, induced the transcription of interleukin (IL)-6, and reduced tumor necrosis factor alpha (TNF-α), interferon gamma (IFN-γ), and IL-10. Our data show for the first time the significant association between serum OPNc and diabetes and/or obesity. Unraveling the functional role of OPN isoforms in systemic inflammation is essential to understanding their significance as therapeutic targets in diabetes and obesity, and during metastasis development in PDA.

Keywords Osteopontin · OPN · Diabetes · Obesity · Pancreatic ductal adenocarcinoma

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Introduction

Pancreatic ductal adenocarcinoma (PDA) is the fourth leading cause of adult cancer death in the USA with an overall 5-year survival rate of less than 5–6 %.[1,2] American Cancer Society estimates that 45,220 people will be diagnosed with PDA in 2013 while 38,460 will succumb to its disease process. The high mortality rate stems from that at the time of presentation, over 85 % of the tumors have already infiltrated adjacent tissues or metastasized.

Several factors have been shown to increase the risk of developing PDA including diabetes and obesity. Patients with new onset diabetes or who had diabetes for less than 5 years have a higher risk of developing PDA compared to those without diabetes (odds ratio 2.1, 95 % confidence interval (CI)=1.9–2.3).[3] Epidemiological studies have shown that cancers of the colon, breast, endometrium, kidney, esophagus, gastric, pancreas, gallbladder, and liver have a strong association with obesity.[4] An increased risk of PDA is seen in obese individuals (BMI >30) versus those with normal weight (BMI <25) (relative risk 2.08, CI 1.48–2.93), confirming the association with the metabolic syndrome.[5]

The link between inflammation and cancer was introduced over 150 years ago by Virchow in 1858 when he observed that cancers frequently occurred at sites of chronic irritation.[6] Diabetes and obesity are also associated with a state of chronic inflammation.[6] Interleukin (IL)-6 and tumor necrosis factor alpha (TNF-α) are increased in overweight individuals and in diabetics, leading to increased hepatic production of C-reactive protein, an acute-phase protein which activates the complement system.[7,8]

Osteopontin (OPN), a secreted phosphoprotein, plays a large role in normal physiology as well as in various disease states. The protein is widely expressed in tissues of the kidney and brain, and in cells like macrophages, vascular smooth muscle cells, and epithelial cells.[9] OPN, utilizing intracellular and extracellular signaling mechanisms, affects cell migration, adhesion, and survival.[10] Through its actions on cells of the immune system, OPN plays a large role in chronic inflammation and autoimmune diseases.[10] Elevated levels of the secreted protein have been implicated in disease states of chronic inflammation like Crohn's disease[11] and rheumatoid arthritis, as well as in cancer.[12]

OPN undergoes alternative splicing to produce three splice variants, OPNa, OPNb, and OPNc[13] each of which are thought to have specific effects that are not completely understood. Previously, we demonstrated the presence of high levels of pancreatic OPNc in PDA patients who are smokers[14], and in fine needle pancreatic sample aspirates from patients with metastatic lesions.[15] Our research has also shown that OPNc plays a role in PDA cell inflammation.[16,17]

In this study, we explored the presence and pattern of expression of OPN isoforms in sera from patients with pancreatic lesions, PDA and intraductal papillary mucinous neoplasm (IPMN), and tested the hypothesis that their presence is associated with chronic inflammatory comorbidities, such as diabetes and obesity. In vitro, we analyzed the effect of over-expressing OPN isoforms on the inflammatory phenotype of PDA cells.

Methods

Serum Acquisition Serum samples were retrospectively obtained from 90 patients at Thomas Jefferson University between 2005 and 2012 prior to surgical resection of a pancreatic mass. Serum samples were prepared and stored at −80 °C until analyzed. Fifty-eight patients had pathologically confirmed PDA, and 32 had IPMNs. All patients signed an appropriate consent form, and clinical data were obtained from the patients' chart. The study was approved by the Institutional Review Board at Thomas Jefferson University, and all patients consented to having their serum drawn and analyzed. Clinical data, tumor characteristics, and AJCC staging of these 90 patients are shown in Table 1.

Table 1 Clinicopathological characteristics of PDA and IPMN patients

	PDA, n=58	%
Average age (years)	65.82	
<60 (mean 54.2)	18	31
>60 (mean 71.0)	40	69
M	26	45
F	32	55
BMI		
Normal weight (<25)	24	41
Overweight (25–29.9)	22	37
Obese (>30)	12	21
DM	19	33
AJCC stage		
I	2	3
II	45	78
III–IV	11	19
Grade		
G1	5	9
G2	35	60
G3–G4	18	31
Specimen lymph nodes		
N0	3	5
N1–N2	16	26
N3–N5	17	27
>N6	22	36
	IPMN, n=32	%
Average age (years)	66.34	
<60 (mean 54)	12	38
>60 (mean 74)	20	63
M	14	43
F	21	65
BMI		
Normal weight (<25)	10	31
Overweight (25–29.9)	11	34.5
Obese (>30)	11	34.5
DM	5	16

BMI body mass index, *DM* diabetes mellitus, *PDA* pancreatic ductal adenocarcinoma, *IPMN* intraductal papillary mucinous neoplasm, *AJCC* American Joint Committee on Cancer staging

Study Design Based on the presence of obesity and diabetes, patients were arranged into four groups (Table 2) and a normal control group: group 1 (n=50) included patients with a pancreatic lesion (PDA or IPMN) and no obesity or diabetes; group 2 (n=17) included patients with a pancreatic lesion and obesity but without diabetes; group 3 (n=6) included patients with a pancreatic lesion and both, obesity and diabetes; and group 4 (n=17) included patients with a pancreatic lesion and diabetes but without obesity. Normal controls (n=29) were obtained from healthy age-matched volunteers. Obesity was defined as a BMI >30, and diabetes was assigned if the patient

Table 2 Groups assignments with correlating expression of OPN isoforms

	Group 1 +Pancreatic lesion −Obesity −Diabetes *n* (%)	Group 2 +Pancreatic lesion +Obesity −Diabetes *n* (%)	Group 3 +Pancreatic lesion +Obesity +Diabetes *n* (%)	Group 4 +Pancreatic lesion −Obesity +Diabetes *n* (%)	Normal control −Pancreatic lesion −Obesity −Diabetes *n* (%)
Patient (*n*)	50	17	6	17	29
OPNa	0(0)	0(0)	0(0)	0(0)	0(0)
OPNb	24 (48)	0 (0)	0 (0)	0 (0)	0 (0)
OPNc	17 (34)	17 (100)	6 (100)	17 (100)	0 (0)

Pancreatic lesion (PDA or IPMN), obesity (BMI >30)

listed diabetes on the intake questionnaire and/or was on glucose lowering medications such as oral hypoglycemic or parenteral insulin.

RNA Extraction and Semi-Quantitative PCR Serum RNAs were isolated using TRIzol reagent (Life Technologies, Gaitherburg, MD), DNase-digested, and cDNAs were prepared using ImProm-II™ Reverse Transcription System (Promega), then subjected to semi-quantitative PCR using master mix (Promega). The primers used were OPNa forward, 5′-ATCTCCTAGCCCCACAGAAT-3′, reverse, 5′-CATCAG ACTGGTGAGAATCATC-3′; OPNb forward, 5′-AAAATC AGTGACCAGTTCATCAG-3′, reverse, 5′-ATCTCCTAGC CCCAGAGAC-3′; and OPNc forward, 5′-TCAGGAAAAG CAGAATGCTG-3′, reverse, 5′-GTCAATGGAGTCCTGG CTGT-3′. Upstream and downstream primers that could anneal with the 3′-untranslated region of human GAPDH were included in the PCR reaction as an internal standard, forward, 5′-TGAAGGTCGGAGTCAACGGATTTGGT-3′, reverse, 5′-CATGTGGGCCATGAGGTCCACCAC-3′. The linear range of amplification for each set of primers was determined to ensure that we used a number of cycles in the linear range. PCR products were electrophoresed on 2 % agarose gels and band intensities were quantified using Kodak Electrophoresis Documentation and Analysis System 290 (EDAS 290).

Real-Time Reverse Transcription Polymerase Chain Reaction Total RNA was isolated from PDA cells or sera using TRIzol reagent (Life Technologies, Gaitherburg, MD). RNAs were quantified, and input amounts were optimized for each amplicon. OPN, a panel of inflammatory cytokines including interferon gamma (IFN-γ), tumor necrosis factor alpha (TNF-α), IL-10, and IL-6), and GAPDH (internal control) were used. Primers and probes were designed with the help of Primer Express Software (Applied Biosystems; Foster City, CA). cDNA was prepared, diluted, and subjected to real-time PCR using the TaqMan technology (7500 Sequence Detector; Applied Biosystems). The relative messenger RNA (mRNA) levels were presented as unit values of $2[C_{T\ (GAPDH)} - C_{T\ (gene\ of\ interest)}]$, where C_T is the threshold cycle value defined as the fractional cycle number at which the target fluorescent signal passes a fixed threshold above baseline.

Cell Culture MiaPaca-2 and AsPC-1 PDA cells (American Type Culture Collection; Manassas, VA) were used for the following studies. Cells were cultured in six-well plates and maintained in DMEM with 10 % fetal bovine serum (FBS) in an incubator with set conditions of a humid atmosphere of 5 % CO_2/5 % air at 37 °C.

Transient Transfection of OPN Isoforms pCDNA3vector containing a truncated splice variant OPNa (NM_001040058), OPNb (NM_00058), OPNc (NM_001040060), and empty vector for control were a generous gift from Dr. X Wang, Center for Cancer Research, NCI, Bethesda, MD (17). MiaPaca-2 and AsPC-1 cells were transfected with 0.5 μg OPN isoforms plasmid DNA using TransFast (Promega, Madison, WI), and lysates were harvested after 24 h for initial RT-PCR testing of expression of the OPN isoforms. Proof of transfection was evaluated by RT-PCR from a pooled population of cells. Pooled populations of transfected cells were used in scratch closure, cell proliferation, soft-agar and colony formation assays. Comparisons were made between cells transfected with each isoform and empty vector. Assays were performed in triplicate, and the data were collected independently and expressed as mean±SE.

shRNA Sequences and Constructs MISSION® shRNA lentiviral particles against total OPN and GFP as a control were purchased from Sigma-Aldrich Corporation. MiaPaca-2 and AsPC-1 cells were infected as directed by the manufacturer. Cells were examined for OPN expression by real time PCR and were subjected to scratch closure, cell proliferation and soft-agar colony formation assays. Comparisons were made between cells infected with shOPN and shGFP as a control. Assays were performed in triplicate, and the data were collected independently and expressed as mean±SE.

MTT Assay To examine the effect of OPN isoform overexpression or OPN downregulation on in vitro cell viability, MiaPaca-2 and AsPC-1 cells were plated in quintuplicate in 96-well plates and incubated in full-growth media at 37 °C and 5 % CO_2. Cells were grown for 72 h, and their viability was examined using 3-[4,5-cimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) conversion assay (Sigma), and optical density was read at 560 nm. Optical density is directly correlated with cell quantity.

Soft-Agar Colony Formation Assay Base agars were made by combining equal volumes of 1 % agarose and 2× DMEM/10 % FBS, both at 40°. Warm base agar (1.5 ml) was plated into six-well plates and allowed to polymerize. The top agar was produced by combining equal volumes 5×10^3 transfected cells/ml in 1× DMEM/10 % FBS with warm 0.7 % agarose. Two milliliters of the agarose cell mixture was placed on top of each base coat. Plates were incubated at 37°, 5 % CO_2. At 21 days, colonies were stained with 0.5 ml of crystal violet and analyzed using a dissecting microscope and ImageJ software.

Cell Migration Assay MiaPaca-2 and AsPC-1 cells overexpressing OPNa, OPNb, or OPNc or downregulating OPN were plated and grown to 90 % confluency in a six-well plate (Falcon Becton Dickinson). Cells were scratched with a sterile 1000 μl pipette tip in each well and washed with PBS to remove cell debris, and the medium was then added. After 48 h, the wounds were observed and images were taken in ×200 magnification. The extent to which the wound had closed over 48 and 72 h was calculated and expressed as a percentage of the control at 0 h. The data are representative of three independent experiments.

Statistical Analysis Frequency of obesity and diabetes and mean BMI by presence or absence of the OPN isoforms was calculated. Logistic regression models with obesity and diabetes as outcomes and OPN subgroup were calculated, both unadjusted and adjusted for the presence of malignancy or not. The same predictors were used in a linear regression model exploring the association with BMI. Additionally, OPN subgroups and inflammation markers were cross-tabulated with cancer stage. Cross-tabulations between disease (limited to PDA and IPMN) and OPN subgroups and inflammation markers were also calculated. Differences were considered significant with a p value ≤0.05. All in vitro experiments were performed three to four times. Data were analyzed for statistical significance by ANOVA with post hoc Student's t test analysis. Data are presented as mean±SEM. Continuous normally distributed variables were analyzed by Student's t test. Analyses were performed with the assistance of a computer program (JMP 5 Software SAS Campus Drive, Cary, NC). Differences were considered significant at p value ≤0.05.

Results

Serum OPN Isoform Expression Profile Table 2 and Fig. 1a show that in group 1 (+pancreatic lesion/−obesity/−diabetes), patients sera predominantly expressed OPNb (48 %) and OPNc (34 %), with 10 % of the samples expressing both isoforms. In the presence of diabetes and/or obesity (groups 2–4), there was complete disappearance of the OPNb and presence of OPNc in 100 % of the samples (Fig. 1b–d). OPNa was not present in any of the four groups.

Descriptive statistics and measures of association between lesion type (PDA or IPMN) and OPN isoform expression (Table 3) did not show any significant differences. Similarly, there were no significant associations between the type of pancreatic lesion and diabetes and/or obesity (Table 3).

However, when looking at OPN isoforms OPNb and OPNc in the context of systemic inflammation comorbidities, i.e., diabetes and/or obesity, it was clear that the presence of OPNc and the absence of OPNb have a significant association with diabetes and/or obesity (Table 4). Logistic regression analysis adjusted for disease status (PDA or IPMN) show that the odds of having diabetes and/or obesity when OPNc is present are seven times more common than without OPNc (Table 5). Interestingly, OPNb was consistently absent in patients with diabetes and/or obesity with an OR of 0.1.

Effect of Systemic Inflammation on Total Serum OPN mRNA Based upon the above results and since there was no difference in OPN isoform expression between groups 2, 3, and 4, we combined them into a new group 2 (+pancreatic lesion/+obesity and/or diabetes) while group 1 remained the same (+pancreatic lesion/−obesity/−diabetes). Real-time PCR analysis of total OPN mRNA in sera from groups 1 and 2 showed significantly higher levels in both groups when compared to normal controls ($p<0.05$) (Fig. 2). These data indicate that while OPN isoforms differentiate between the presence or absence of obesity and/or diabetes, total OPN does not.

OPN Overexpression in PDA Cells Next, we wanted to determine the impact of each OPN isoform on the biological behavior of PDA cells. We transiently transfected MiaPaca-2 and AsPC-1 cells with plasmids containing OPNa, OPNb, OPNc isoforms, or empty vector (pCDNA3). In MiaPaca-2 cells, RT-PCR showed that the transiently transfected cells had significantly higher levels of the individual OPN isoforms (Fig. 3a) and total OPN (Fig. 3b). OPNc-transfected cells expressed the highest levels of OPN mRNA (>400-fold increase). Similar data were obtained from AsPC-1 cells (data not shown).

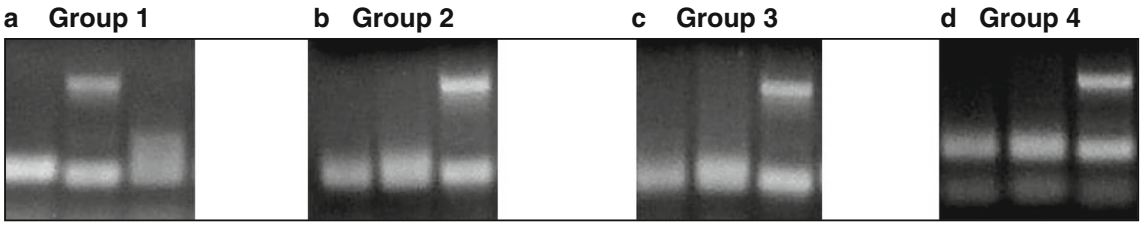

Fig. 1 Representative ethidium-bromide gel showing OPN isoforms expressed in sera of patients with a pancreatic lesion. No patients expressed the OPNa isoform. **a** Group 1 (+pancreatic lesion/−obesity/−diabetes) expressed OPNc in 34 %, OPNb in 48 %, and both isoforms in 5 % of the serum samples. **b** Group 2 (+pancreatic lesion/+obesity/−diabetes) expressed OPNc in 100 % of the samples. **c** Group 3 (+pancreatic lesion/+obesity/+diabetes) and **d** group 4 (+pancreatic lesion/−obesity/+diabetes) expressed OPNc in 100 % of the serum and no OPNb

OPN Downregulation in PDA Cells We also wanted to determine the impact of total OPN downregulation on the biological behavior of PDA cells. We infected MiaPaca-2 and AsPC-1 cells with MISSION® shRNA lentiviral particles against total OPN or GFP as a control. In MiaPaca-2 and AsPC-1 cells, PCR showed that infected cells had significantly lower levels of total OPN (Fig. 3c).

Effect of OPN on PDA Cell Biological Behavior OPN isoform overexpression or downregulation had no effect on PDA cell proliferation as demonstrated by MTT assay (data not shown).

In MiaPaca cells, both OPNb- and OPNc-transfected cell lines showed significantly ($p<0.001$) increased numbers and size of colonies compared to control. This suggests a correlation between isoform expression and the pro-metastatic characteristic of anchorage independence. The effect of OPNc was most pronounced (Fig. 3d). Similar data were obtained from AsPC-1 cells ($p<001$).

In MiaPaca cells, OPNb- and OPNc-transfected cells showed significant ($p<0.001$) increase in migration across the wound as compared to both control (Fig. 3e). Similar data were obtained from AsPC-1 cells ($p<0.001$).

OPN-depleted MiaPaca-2 and AsPC-1 cells showed ($p<0.05$) significant reduction of the number and size of colonies in soft agar when compared to control (Fig. 3f). There was also a significant reduction ($p<0.02$) in migration across the wound space when compared to control (Fig. 3g).

Table 3 The association of a pancreatic lesion with respect to OPN isoform expression and diabetes and/or obesity

	IPMN (n=32) n (%)	PDA (n=58) n (%)	p value n (%)
OPNa	0 (0)	0 (0)	n/a
OPNb	7 (23.3)	19 (32.8)	0.276
OPNc	25 (83.3)	43 (74.1)	0.674
DM	5 (16.7)	18 (31.0)	0.109
Obesity	11 (36.7)	12 (20.7)	0.154
DM or obesity	15 (46.9)	25 (43.1)	0.730

n/a not applicable

These data confirm the role of OPN in enhancing PDA cell metastatic behavior [18].

The Effect of Overexpression of OPN Isoforms on Markers of Inflammation Next, we evaluated cells that overexpress OPN isoforms for the presence of a panel of inflammation-related markers. IL-6 expression was significantly ($p<0.05$) increased with overexpression of OPNb and OPNc (Fig. 4a). IL-10 and TNF-α were significantly suppressed by all OPN isoforms (Fig. 4b, c). Interestingly, IFN-γ was significantly suppressed by only OPNa (Fig. 4d). These data indicate that while only OPNb and OPNc impacted PDA cell migration and colony formation, all OPN isoforms modified the PDA inflammatory phenotype.

Discussion

OPN is a 44-kDa acidic glycoprotein that is subjected to posttranslational modifications that alter its function.[13,19] OPN mRNA undergoes alternative splicing, resulting in isoforms that are smaller than full-length OPN. Alternative splicing leads to deletions in the N-terminal portion of OPN that lies upstream from the central integrin and C-terminal CD44 binding domains. OPNa is the full-length isoform, whereas OPNb lacks exon 5 (a site for serine and threonine phosphorylation) and OPNc lacks exon 4 (site for transglutaminase cross linking).[20] Previous studies have shown a role for

Table 4 Association between OPN isoform OPNb and OPNc and the presence of diabetes and/or obesity

	OPNb			OPNc		
	Present (n=26) n (%)	Absent (n=64) n (%)	p value	Present (n=68) n (%)	Absent (n=22) n (%)	p value
Diabetes	3 (12)	20 (31)	0.052	20 (29)	3 (14)	0.140
Obesity	0 (0)	23 (36)	0.000*	23 (33)	0 (0)	0.002*
DM or obesity	5 (19)	37 (58)	<0.001*	37 (54)	3 (14)	0.001*

*$p<0.05$, significant

Table 5 Logistic regression analysis adjusted for pancreatic lesion showing the association between OPNb and OPNC to diabetes and/or obesity

Predictors	OPNb			OPNc		
Outcomes	OR	95 % CI	*p* value	OR	95 % CI	*p* value
Diabetes	0.26	(0.7, 0.96)	0.435	2.73	(0.73, 10.26)	0.676
Obesity	0.01	(<0.001, 1.20)	0.603	56.85	(0.67, inf)	0.074
DM or obesity	0.10**	(0.03, 0.36)	0.000*	7.06***	(1.97, 25.30)	0.003*

*$p<0.05$, significant

**$p<0.0002$

***$p<0.003$

OPN isoforms in promoting metastasis in different cancers.[13] In this study, we describe for the first time (a) the presence of OPN isoforms in the sera from patients with benign and malignant pancreatic lesions, (b) a significant association between the presence of serum OPNc with diabetes and/or obesity, and (c) the potential common role played by OPNb and OPNc in PDA, diabetes, and obesity through promoting metastatic cell behavior and mediating a chronic inflammatory phenotype. Through their proinflammatory and prometastatic properties, OPNc and OPNb may prove to be effective common novel therapeutic targets for PDA, diabetes, and obesity.

Inflammation and cancer are closely related and frequently coexist. While acute inflammation inhibits cancer, chronic inflammation leads to cancer promotion.[6] OPN regulates and promotes inflammation in macrophages, T cells, and dendritic cells.[10] The presence of OPN isoforms in tumor tissues has been described in several cancers, but their exact role in relation to the clinical/pathological features is not clear.[13] Our research shows for the first time that OPN isoform mRNA can be isolated not only from the sera of patients with PDA but also from patients without cancer. Circulating mRNA in serum is a relatively newly developed area for novel diagnostic serum studies. Cellular mRNA is released into the serum and stored in exosomes, which provide communication between cells and can act as transporters of translatable mRNA.[21] OPN mRNA and its isoforms were compared in patients with PDA, IPMN, and healthy, normal controls. Our results show that in the sera of patients with pancreatic lesions, OPNa was absent, OPNb was the predominant isoform (48 %), while OPNc was present in only 34 % of the samples. In states of comorbid systemic chronic inflammation (diabetes and obesity), the expression pattern of OPN isoforms changed; OPNb disappeared completely, and 100 % of patients' sera expressed only OPNc (Table 4). Our logistic regression model adjusted for pancreatic lesion showed that the odds of having diabetes and/or obesity in a patient with OPNc are seven times the odds when compared to patients without OPNc (Table 5). While prior studies have shown the association of total OPN with inflammation, this is the first report to describe that the presence of chronic inflammatory comorbidities such as diabetes and obesity affect the serum pattern for OPN isoforms expression.

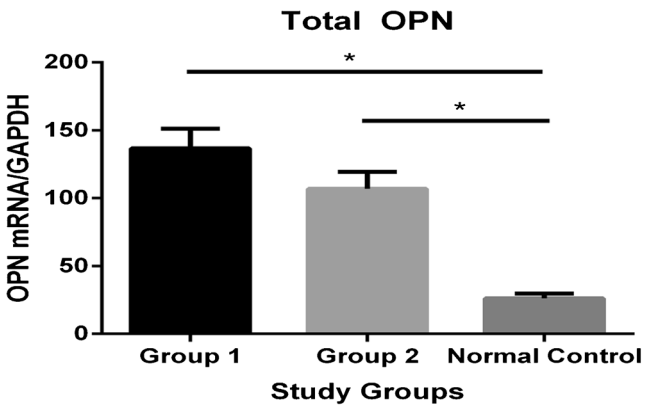

Fig. 2 Real-time PCR of sera from patients in group 1 (+pancreatic lesion/−obesity/−diabetes) and group 2 (+pancreatic lesion/with obesity and/or diabetes) and normal control. Statistically significant differences were observed between group 1 and normal control, and group 2 and normal control. Data presented as mean±SEM; *$p<0.05$, statistically significant result

When we realigned our patients into groups 1 and the new group 2 and quantified total serum OPN mRNA, it was clear that total OPN did not significantly change when comorbidities of diabetes and obesity were factored in (Fig. 2). Looking at the same two groups with respect to OPN isoform expression, it was clear that patients with a pancreatic lesion without diabetes and obesity predominantly express OPNb, and patients with pancreatic lesion and obesity and/or diabetes only express OPNc. Thus, we introduce here the presence of OPNc in patients' sera and absence of OPNb as discriminatory between the presence and absence of diabetes and/or obesity.

Our in vitro studies in PDA cells after overexpressing OPN isoforms showed that OPNc and to a lesser extent OPNb induce colony formation and cell migration (Fig. 3c, d). These results are consistent with previous studies describing the same roles for the two isoforms in prostate[22] and ovarian[23] cancers. Although our studies show that OPNb and OPNc isoforms did not affect PDA cell proliferation, this might be related to their presence in 2D cultures. Studies to explore the effect of OPN isoforms in 3D cultures, which provide an extracellular environment similar to where cells routinely operate in vivo[24] are currently ongoing in our laboratory.

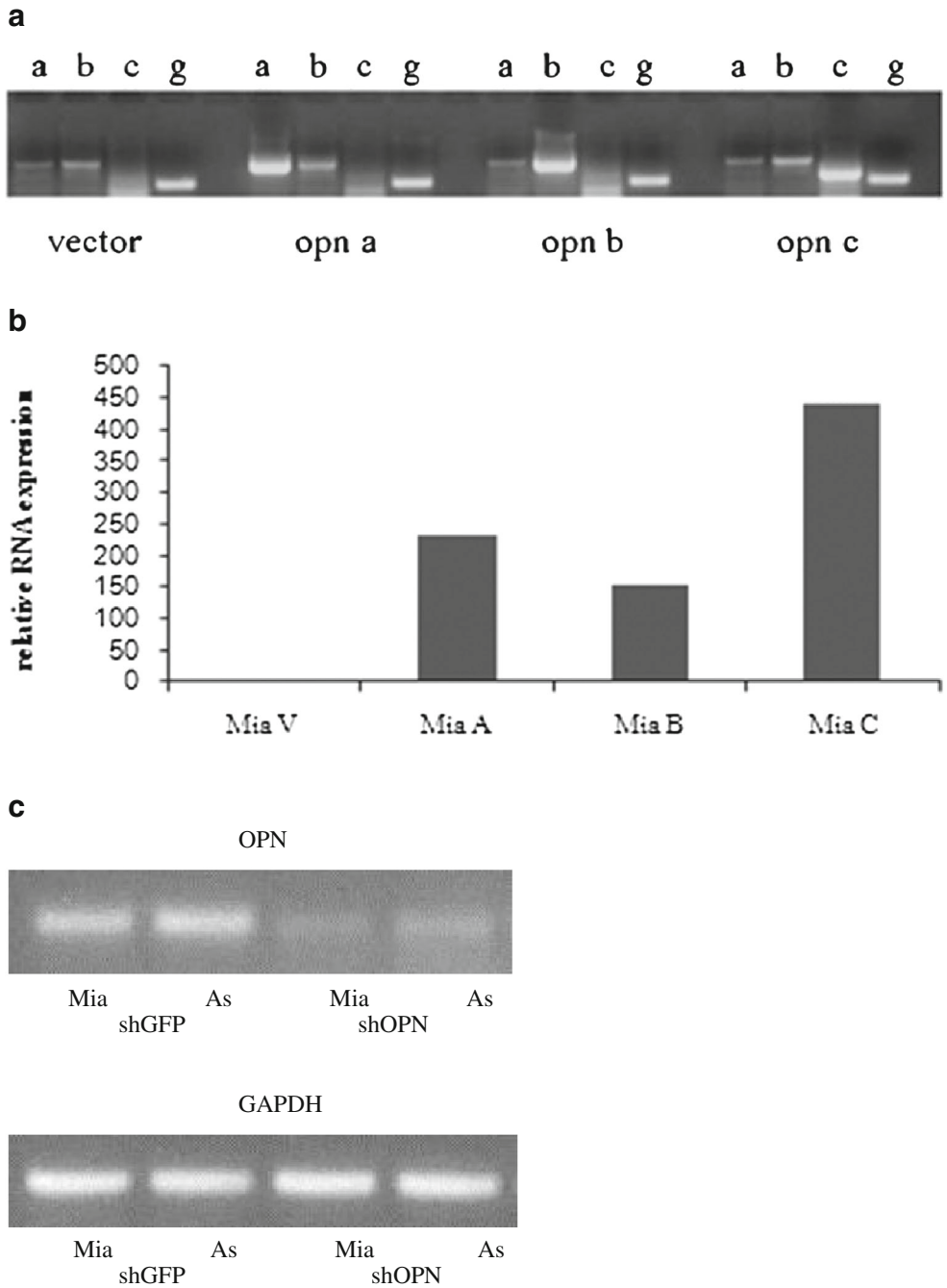

Fig. 3 Effect of overexpression and downregulation of OPN on PDA cell biological behavior. **a** Representative ethidium bromide gel and RT-PCR demonstrating successful transfection of MiaPaca-2 cells with OPNa, OPNb, OPNc, nd empty vector. **b** Real-Time PCR showing high levels of total OPN mRNA after overexpression of the different isoforms. **c** Representative ethidium bromide gel showing RT-PCR demonstrating downregulation of total OPN after successful infection of MiaPaca-2 (Mia) and AsPC-1 (As) cells with shOPN and shGFP as a control. **d** *Upper panel*: representative image showing colony formation assay in MiaPaca cells transfected with OPNa, OPNb, and OPNc. Increased colony formation is evident after 1 week of growth with the greatest effect of produced by OPNc, ×200 original magnification. *Lower panel*: quantification of colonies showing significant colony formation in cells with overexpression of OPNb and OPNc. **e** *Upper panel*: representative image of cell migration assay showing cells overexpressing OPNa, OPNb, OPNc, and empty vector, ×200 original magnification. *Lower panel*: significant migration can be seen in cells with overexpression of OPNb and OPNc at the 48- and 72-h time points compared to empty vector. **f** *Upper panel*: representative image showing colony formation assay in MiaPaca-2 and AsPC-1 cells infected with shOPN and shGFP. Decreased colony formation is evident after 1 week of growth, ×200 original magnification. *Lower panel*: quantification of colonies showing significant colony formation reduction in cells with low levels of OPN. **g** *Upper panel*: representative image of cell migration assay showing cells with OPN depletion and shGFP as a control, ×200 original magnification. *Lower panel*: reduction in the rate of migration can be seen in cells with low levels of OPN at the 48- and 72-h time points compared to shGFP as a control. Values are expressed as mean±SEM of three experiments. *$p<0.005$, #$p<0.05$ versus control levels, using one-way repeated ANOVA with subsequent all pairwise comparison procedure by Student's *t* test

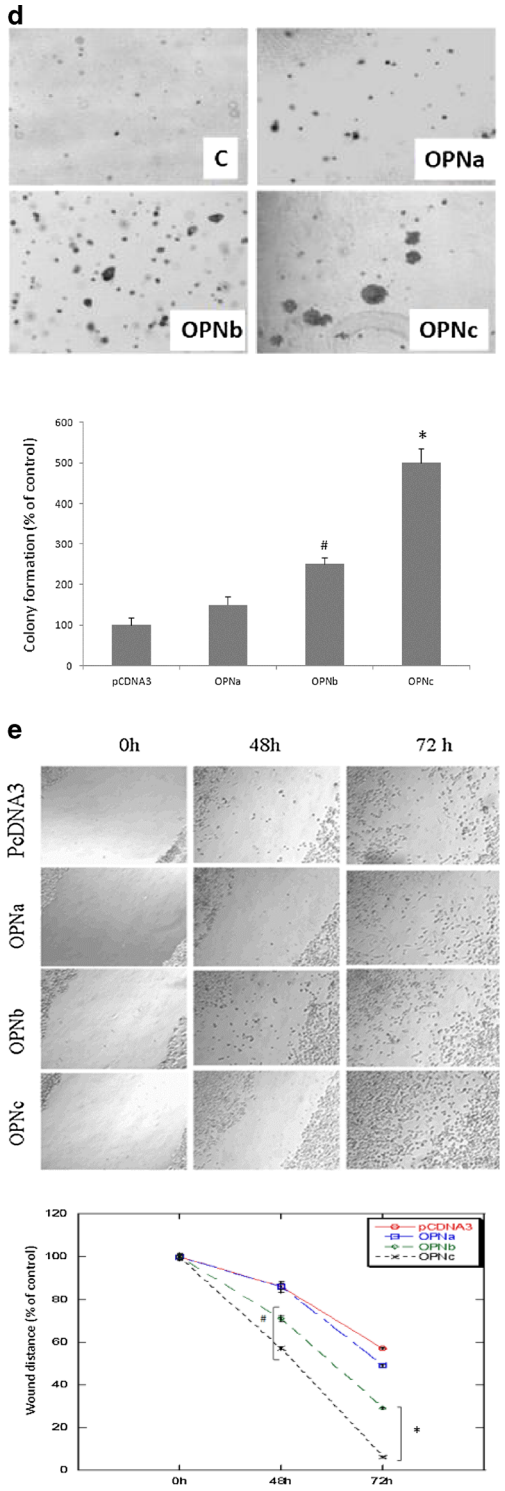

Fig. 3 (continued)

Analyzing the effect of the different OPN isoforms on the inflammatory phenotype of PDA cells, we show here for the first time that OPNb and OPNc induce a chronic inflammatory phenotype through increasing the levels of IL-6. OPNb induced a 40-fold increase in IL-6 mRNA, while OPNc induced it by ~100-fold (Fig. 4a). IL-6 in combination with its soluble receptor play a key role in the transition from acute to chronic inflammation by changing the nature of leukocyte infiltrate from neutrophils to macrophages. In addition, IL-6 exerts stimulatory effects on T and B cells, thus favoring chronic inflammatory responses.[25] High levels of circulating IL-6 and its receptor have been demonstrated in PDA,[26] and their signaling leads to activation of Stat3/Socs3 and promotion of PanIN progression into PDA.[27] A similar IL-6-Stat3/Socs3 signaling pathway was described in obesity and type 2 diabetes leading to a state of low-grade chronic inflammation.[28–31] Although OPN has been shown to colocalize with STAT-3 in human melanomas,[32] its relationship with IL-6 and their role in promoting chronic inflammation have not been described previously. Our results potentially implicate OPNb and OPNc as upstream effectors for IL-6 to promote chronic inflammation. Studies to explore this relationship and its role in the pathogenesis of diabetes, obesity, and pancreatic malignancy are currently being planned.

Our data show that all OPN isoforms significantly reduce the expression levels of IL-10 (Fig. 4b). IL-10 is a broadly acting immune inhibitory cytokine that is generally thought to support tumor growth. However, studies have shown that IL-10 stimulates natural killer (NK) cells both in vitro and in vivo.[32] In vivo treatment of tumor with IL-10 or tumor cells secreting IL-10 inhibit tumor metastasis by NK-dependent mechanism.[33,34] Furthermore, ablation of IL-10 was shown to promote tumor growth and metastasis through inhibition of inflammatory cytokine production and hampering the development of regulatory T cells and myeloid-derived suppressor cells.[35] Thus, unlike the majority of host immune system that is stimulated by IL-10 blockade during cancer therapy, OPN-mediated inhibition of IL-10 may be one of the mechanisms that enhance early metastasis in PDA.

IFN-γ has long been associated with cytostatic/cytotoxic and antitumor functions.[36] It was suggested that IFN-γ takes part in tumor surveillance functions by enhancing tumor cell immunogenicity, as mice that were insensitive to IFN-γ exhibited enhanced methylcolanthrene-induced tumor growth.[37] This observation was supported by the finding that approximately one third of melanoma and lung adenocarcinoma cell lines had inactivating mutations in the IFN-γ. Our data show that OPNa significantly reduced IFN-γ, suggesting its potential involvement in active evasion of tumor cells from tumor surveillance. Since we could not recognize OPNa in our serum samples, it would be important to determine whether OPNa plays an earlier role in tumor development through its effect on IFN-γ and possible other targets.

TNF-α has potent tumor suppressor activities and its significant inhibition by the three OPN isoforms my serve to enhance PDA cell survival. We have shown recently that overexpression of TNF-α in breast cancer cells enhances their autophagy, promotes mitochondrial dysfunction, and was enough to prevent their growth in vivo.[38] Other studies have shown that dendritic cell exosomes, which contain TNF-α, can kill melanoma, colon cancer, and squamous cell

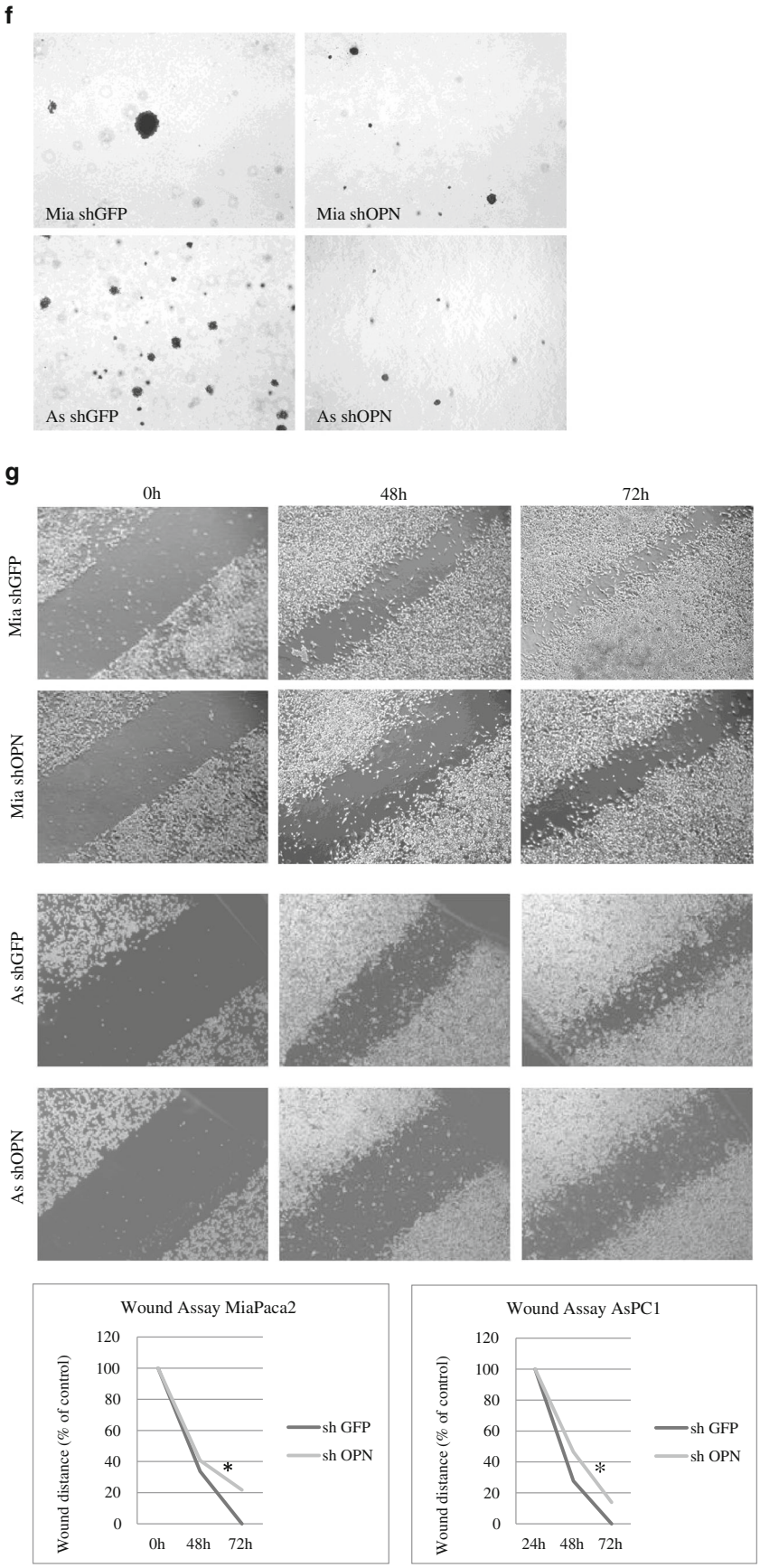

Fig. 3 (continued)

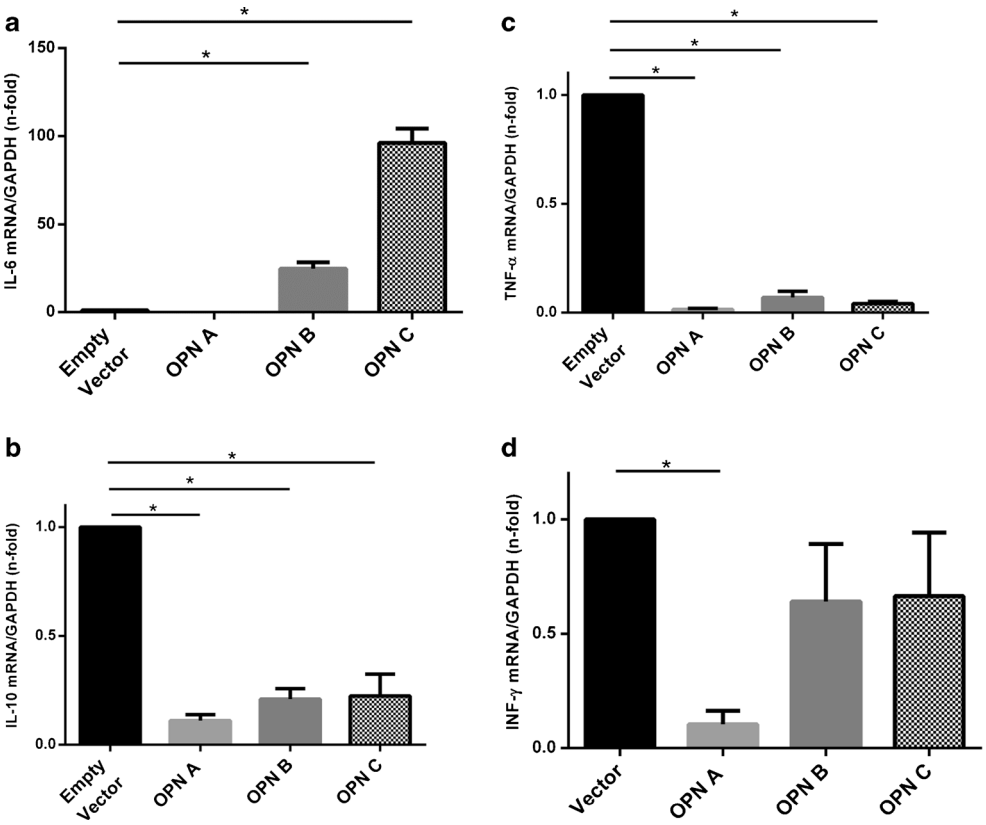

Fig. 4 Q-PCR of MiaPaca Cells transfected with OPN isoforms OPNa, OPNb, and OPNc. **a** OPNb and OPNc induce expression of IL-6 mRNA ($p<0.05$) compared to empty vector. **b** OPNb and OPNc suppress expression of IL-10 mRNA ($p<0.05$) compared to empty vector. **c** OPNb and OPNc suppress expression of TNF-α mRNA ($p<0.05$) compared to empty vector. **d** OPNa suppresses expression of IFN-γ mRNA production ($p<0.05$) compared to empty vector. Data presented as mean±SEM; *$p<0.05$, statistically significant result

carcinoma cells.[39] Studies from our lab have shown that overexpressing TNF-α in PDA cells significantly inhibit the transcription of OPN (unpublished observation). It is plausible to primarily postulate that OPN and TNF-α cross talk and their mutual regulation contribute to progression of PDA. It is also probable that TNF-α regulation by OPN in PDA cells is not related to its function as a promoter for acute and chronic inflammation.[40,41] It remains to be determined, however, whether OPN isoforms are produced by other inflammatory cells and whether they cross talk with other cytokine and chemokines to dictate the final inflammatory phenotype of the tumor microenvironment.

Study Limitations

Our in vitro mRNA studies were performed in two PDA cell lines, the addition of other PDA cells and evaluation of their interaction in the tumor microenvironment would strengthen and elaborate on our findings. While we pursued a limited number of inflammatory cytokines consisting of IFN-γ, TNF-α, IL-10, and IL-6, expanding this set to include additional cytokines and chemokines would help construct a more comprehensive inflammatory phenotype. Since this was a pilot study, increasing our patient cohort to include patients without pancreatic disease who are diabetic and/or obese are required to determine the specificity of OPNc to states of systemic inflammation. Those experiments are currently in preparation.

Conclusion

In this study, we show that OPN mRNA can be isolated from the serum of patients with and without pancreatic cancer. The specific OPN isoforms do not vary between patients with cancer and premalignant lesions although the presence of comorbidities that manifest with chronic systemic inflammation is associated with a change in isoform expression. Patients who have comorbidities of diabetes and/or obesity undergo an isoform expression modification from predominantly OPNb to completely OPNc. Although our in vitro studies show that OPNb and OPNc promote colony formation, cell migration, and enhance an inflammatory gene profile in PDA cells that is permissible of chronic inflammation and tumor evasion, it remains to be determined whether the serum isoform shift is the result or the cause of systemic inflammation. Elucidation of these mechanisms may establish OPN as a major link between obesity, diabetes, and PDA.

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