Instituto Nacional de Câncer
Coordenação de Pesquisa
Programa de Carcinogênese Molecular

Caracterização funcional das isoformas variantes de '*splicing*' da osteopontina nos carcinomas de ovário e próstata.

Tatiana Martins Tilli

Orientação: Dra Etel Rodrigues Pereira Gimba

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Tese apresentada ao Instituto
Nacional de Câncer para obtenção do título de doutor

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Tese de doutorado – Instituto Nacional de Câncer, Pós-Graduação em Oncologia, 2012.

Referências Bibliográficas: f161-169

1- Câncer de Ovário e Próstata. 2- Isoformas variantes de '*splicing*' da Osteopontina. 3- Caracterização funcional.

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III. Caracterização funcional das isoformas variantes de '*splicing*' da osteopontina nos carcinomas de ovário e próstata

“O degrau de uma escada não serve simplesmente para que alguém permaneça em cima dele, destina-se a sustentar o pé de um homem pelo tempo suficiente para que ele coloque o outro um pouco mais alto”.

Thomas Huxley

AGRADEÇO....

A Deus. Nesta área acadêmica aprendi a acreditar somente naquilo passível de comprovação. Mas, o que realmente senti e constatei é a presença Dele em todos os momentos de minha vida. Esta presença nunca me faltará, pois Ele é fiel. Obrigada por ter me concedido à vida e com ela as oportunidades.

Aos meus queridos pais, irmã, noivo e cunhado, verdadeiros amigos, que viveram comigo esta caminhada em busca de meus ideais e sonhos, incentivando-me a prosseguir, quais fossem a natureza dos obstáculos. Pai e mãe, vocês abriram as portas para o meu futuro, me apoiaram e me encheram de amor e carinho. Vocês são a minha vida. Família: Obrigada, por mais essa etapa que vencemos juntos! Amo vocês.

À minha querida orientadora e amiga Etel. Nossa história começou na minha iniciação científica, desde então passamos por muitos momentos juntas. Durante esses anos nos tornamos amigas, compartilhamos carinho, respeito, confiança e muito trabalho! MUITO OBRIGADA por me ajudar, apoiar e a transformar meus sonhos em realizações.

Ao grupo de marcadores moleculares em neoplasias, a Kivvi Mello, Aline Matos, Luciana Bueno, Ana Emília, Vanessa Franco, Fabrício Ribeiro; vocês fazem parte da minha história. Muito obrigada a vocês que me ajudaram a resolver os problemas com discussões construtivas ou com o apoio nos experimentos.

Aos meus orientadores durante o estágio de doutorado sanduíche, Dra Akeila Bellahcène e Dr Vincent Castronovo da Universidade de Liège, Bélgica. Agradeço ao apoio, respeito, confiança e aprendizado.

Aos amigos que conheci no *Metastasis Research Lab,* Clara Novo, Lola Vanoorschot, Naima Maloujahmoum, Veronique Goffin, Mathieu Fontaine, Paul Peixoto, Vincent Hennequière, que me ajudaram muito durante o período de doutorado sanduíche.

Ao Dr Marcelo Barcinski. A convivência com o senhor foi um aprendizado. Obrigada pelo carinho e respeito.

Aos pesquisadores Adriana Bonomo, Martin Bonamino e Ana Carolina Monteiro, pesquisadores que respeito e admiro. Gostaria de agradecer as discussões para a execução desta tese.

Ao Dr João Luiz Wanderley, que sempre fará parte da minha história acadêmica. Agradeço pelo apoio, disponibilidade e pela preciosa calma. Admiro sua competência e o respeito. Obrigada.

Aos pesquisadores do Programa de Biologia Celular, especialmente ao Dr João Viola e Dr Bruno Robbs, o meu sincero agradecimento, pois contribuíram ativamente para a minha formação acadêmica.

Agradeço ao coordenador da Pós Graduação do INCa, ao Dr Luiz Felipe Ribeiro Pinto pelo apoio e incentivo durante minha formação acadêmica.

Ao Dr Eloisio Alexsandro Silva e seus alunos do Serviço de Urologia da UERJ, pela coleta das amostras de pacientes com câncer de próstata. Agradeço pelo carinho e respeito.

Ao Serviço de Ginecologia do INCa, pela coleta de amostras de ovário.

À equipe técnica do Programa de Carcinogênese Molecular, por todo o respeito, carinho e competência. Fernanda Jorge, Ana Paula, Andréia e Rosimar quero agradecer ao apoio que recebi de vocês: muito obrigada. Ah, obrigada pela 'Terapia do Café'!

À Sueli e Thaís, agradeço pelo apoio, carinho, milhares de compras e paciência.

Ao suporte financeiro da Fundação Swiss Bridge, Fundação do Câncer, Ministério da Saúde, CAPES, CNPq e FAPERJ que tornaram possível a execução desse projeto.

ÍNDICE

RESUMO

A osteopontina (OPN) é um dos transcritos que apresenta maiores aumentos no nível de expressão no câncer de ovário (CO) e próstata (CaP), e está envolvida na tumorigênese e metástase. O gene que codifica a OPN está sujeito à '*splicing'* alternativo, gerando 3 mensagens, denominadas de OPNa, OPNb e OPNc. O objetivo deste projeto é a caracterização do perfil de expressão e do papel funcional das isoformas variantes de '*splicing'* da OPN na tumorigênese e progressão tumoral no CO e CaP. A OPNc encontra-se especificamente expressa nos tumores malignos de ovário. No CaP, observamos que as isoformas apresentam maior nível de expressão na amostras de CaP quando comparado com o tumor benigno da próstata. A expressão diferencial das isoformas da OPN nas amostras tumorais e não tumorais de ovário e próstata identificam as isoformas como potenciais novos biomarcadores para estas neoplasias. Observamos que a OPNc, no modelo de ovário e próstata, e a OPNb, no modelo de próstata, apresentam efeitos estimulatórios sobre a proliferação, migração, invasão, formação de colônia e crescimento de tumores *in vivo*. A OPNc nos dois modelos tumorais estimula a proliferação celular da IOSE e RWPE-1, indicando que a OPNc apresenta características pró-tumorigênicas. Descrevemos também que os efeitos da OPNb e OPNc são mediados pela via de sinalização PI3K/Akt. Com base na observação de que tumores que superexpressam a OPNc apresentam altos níveis de expressão de marcadores típicos de angiogênese nos tumores ovarianos, tais como VEGF-A, Flk-1 e CD34, investigamos o papel funcional e o mecanismo molecular pelo qual a OPNc estimula a angiogênese no CO. Nossos dados demonstram que a OPNc secretada interage com receptores do tipo integrinas de forma RGD-dependente, possivelmente ativando a via de sinalização PI3K/Akt. Observamos também que a OPNc secretada e a via de sinalização PI3k/Akt modulam a expressão de VEGF-A, c-Fos e c-Jun e a fosforilação de c-Jun. Adicionalmente, observamos que o meio condicionado da OPNc induz a proliferação, migração e adesão das células endoteliais HUVEC, de forma a contribuir para a neovascularização. Através de PCR em tempo real, caracterizamos que a OPNc altera a expressão de 34 genes, no modelo de CO, e 16 genes, no CaP, essenciais para a transformação e progressão tumoral destas neoplasias. Os resultados gerados por este estudo contribuem para o melhor entendimento da biologia e dos mecanismos moleculares do CO e CaP. O papel crucial destas isoformas em distintas etapas da progressão destes tumores indicam a OPNb e OPNc como potenciais alvos terapêuticos para o CO e CaP.

ABSTRACT

Osteopontin (OPN) is one of the proteins overexpressed in ovarian carcinoma (OC) and prostate cancer (PCa), and is involved in tumorigenesis and metastasis. Alternative splicing of OPN (OPN-SI) leads to 3 isoforms, OPNa, OPNb, and OPNc. This study aims to characterize the expression profile and functional roles of OPN isoforms in OC and PCa tumor progression. In OC, OPNc is specifically expressed in ovarian tumor samples. PCa tissue samples presented significantly higher levels of OPN-SI transcripts than in BPH specimens. OPN-SI mRNA expression were positively correlated with Gleason Score. The OPNc isoform was the most upregulated variant and the best marker to distinguish patients groups, presenting sensitivity and specificity of 90% and 100%, respectively. OPNc, in OC and PCa, and OPNb, in PCa, significantly activated OvCar-3 and PC-3 cell proliferation, migration, invasion, anchorage-independent growth and tumor formation *in vivo*. Additionally, we have also shown that some of the OPNc-dependent protumorigenic roles are mediated by PI3K/Akt signaling pathway. OPNc stimulated immortalized ovarian and prostate epithelial cell proliferation, indicating a role for this isoform in OC and PCa tumorigenesis. Functional assays using OPNc conditioned medium and an anti-OPNc antibody have shown that most cellular effects observed here were promoted by the secreted OPNc. We identified that OPNc can affect the expression of genes involved in cancer pathways in these tumor models. Tumors formed by OvCar-3 OPNc-overexpressing cells present high expression levels of typical angiogenesis markers, as VEGF-A, VEGFR-2 and CD34. Based on this observation, we also investigated the molecular mechanisms by which OPNc stimulates angiogenic processes. Our data showed that OPNc overexpression activates VEGF-A expression and secretion, also through the PI3K/Akt pathway. This splicing isoform is also able to activate the expression of c-Fos, c-Jun and phospho-c-Jun. OPNc role on activating the phosphorylation of c-Jun is mediated by integrin receptor, in an RGD-dependent manner. OPNc-conditioned medium is able to induce HUVEC endothelial cells proliferation, migration and adhesion. According to our data, contributes to the physiopathology of ovarian and prostate cancer progression and tumorigenesis. Altogether, the data open possibilities of new therapeutic approaches that selectively down regulate OPNc, altering its properties favoring OC and PCa tumor progression.

Lista de abreviaturas, unidade de medida e fórmulas químicas

℃	graus Celsius
μg	micrograma
μL	microlitro
μM	micromolar
AP1	ativador de proteína 1
BSP	sialoproteína óssea
CaP	câncer de próstata
cDNA	ácido desoxirribonucléico complementar
CO	câncer de ovário
CO_2	gás carbônico
DAB	diaminobenzidina
DMP1	dentina proteína 1 de matriz
DNA	ácido desoxirribonucléico
DNase	desoxirribonuclease
DNTPs	desoxirribonucleotídeos trifosfato
D.O.	densidade ótica
DSPP	dentina sialofosfoproteína
EDTA	ácido etilenodiaminotetracético
EG	escore de Gleason
EGFR	receptor de fator epidérmico de crescimento
ERK	sinal extracelular-relacionado com cinase
GFP	“green fluorescent protein”
HA	hialuronato
HPB	hiperplasia prostática benigna
IPTG	isopropil-beta-D-tiogalactopiranosídeo
Kb	kilobases
KDa	kiloDaltons
M	molar
MEPE	fosfoglicoproteína de matriz extracelular
mg	miligrama

ml	mililitro
mM	milimolar
MMPs	metaloproteinases
ng	nanograma
NIK	fator nuclear induzido por cinase
nm	nanômetros
OPN	osteopontina
OPNa	osteopontina a
OPNb	osteopontina b
OPNc	osteopontina c
Pb	pares de base
PBS	salina tamponada com fosfato
PBS-T	salina tamponada com fosfato contendo Tween 20 a 0,05%
PCR	reação em cadeia da polimerase
PI3-K	fosfatidilinositol 3-cinase
PIA	atrofia inflamatória proliferativa
PIN	neoplasia intraepitelial prostática
PLC- γ	fosfolipoase-C-γ
PSA	antígeno específico da próstata
PTMs	modificações pós-tradução
qRT-PCR	PCR quantitativo em tempo real
RGD	trinca de aminoácidos: glicina-arginina-aspartato
RNA	ácido ribonucléico
RPM	rotações por minuto
RT-PCR	reação em cadeia da polimerase - transcriptase reversa
SDS	sulfato dodecil de sódio
SFB	soro fetal bovino
SIBLING	“small integrin-binding ligand N-linked glycoproteins”
TNM	tumor, linfonodos e metástases
U	unidade (s) de enzima
UPA	ativador de plasminogênio do tipo uroquinase
UPAR	receptor do ativador de plasminogênio do tipo uroquinase
VEGF	fator de crescimento endotelial

Prefácio

A presente tese de doutorado tem por objetivos principais caracterizar o papel funcional de variantes de '*splicing*' da proteína osteopontina (OPN) nos modelos de carcinoma de ovário (CO) e de câncer de próstata (CaP), destacando seus perfis de expressão, funções tumor-específicas, papéis em distintas etapas da progressão tumoral e suas possíveis aplicações como biomarcadores para estas neoplasias. Utilizamos na presente tese, um formato em que temos uma introdução geral, seguida de 05 capítulos distintos, quais sejam:

1) Capítulo I – Caracterização do perfil de expressão e o papel funcional das isoformas da OPN na tumorigênese e progressão tumoral nos tumores ovarianos, utilizando modelos *in vitro* e *in vivo.*

- Osteopontin-c Splicing Isoform Contributes to Ovarian Cancer Progression; Tilli *et al. Molecular Cancer Research*, 2011; 9(3):280-93.

2) Capítulo II - **A osteopontina-c secretada induz a expressão de VEGF em células tumorais de ovário.**

- Osteopontin-c is more effective than OPNa and OPNb isoforms to promote VEGF expression and tumor-associated angiogenesis in ovarian cancer.

3) Capítulo III – Caracterização do perfil de expressão das isoformas da OPN nas amostras de câncer de próstata em comparação com os tumores benignos da próstata.

- Expression analysis of osteopontin mRNA splice variants in prostate cancer and benign prostatic hyperplasia; Tilli *et al. Experimental and Molecular Pathology* 92 (2012) 13–19.

4) Capítulo IV – Identificação do papel funcional das isoformas da OPN na tumorigênese e progressão tumoral em câncer de próstata, utilizando modelos *in vitro* e *in vivo.*

- Both Osteopontin-c and Osteopontin-b Splicing Isoforms Exert Pro-Tumorigenic Roles in Prostate Cancer Cells. *Prostate* 2012; 72(15):1688-99.

5) Capítulo V – Identificação das vias de sinalização alteradas pela superexpressão da OPNc no CO e CaP.

- Osteopontin-c splicing isoform activates ovarian and prostate tumor progression features by modulating the expression of key cancer-related genes.

Estes distintos capítulos estão apresentados no formato de artigos já publicados (Capítulos I, III e IV), em fase final de preparação para publicação (Capítulo V) ou como texto em português, ainda fora do formato final de um artigo (Capítulo II). Cada capítulo apresenta o resumo do conjunto de dados, introdução, materiais e métodos, resultados, discussão e referências bibliográficas. No caso dos artigos já publicados, seguimos o formato de cada revista.

Após a apresentação de todos os capítulos, apresentamos uma discussão geral, referências bibliográficas, conclusões e perspectivas, com base nos dados apresentados. Uma seção final contém 02 anexos, nos quais apresento:

Anexo I - **Atividades extras, as quais também foram realizadas no período de meu doutoramento sanduíche.**

- **Investigação do perfil de expressão e papel funcional das isoformas da OPN em células tronco tumorais de glioma.**

Anexo II - Revisão submetida para a Revista *Cancer Letters*. Número da submissão: CAN-D-12-01811-sob revisão.

- Osteopontin splicing isoforms: known roles, potential clinical applications and activated signaling pathways.

1. Introdução

O câncer é uma doença genética, resultante do acúmulo de mutações no genoma. Embora, os fatores ambientais e outros fatores não-genéticos apresentem um papel importante em diversas etapas da tumorigênese (Michor F.*, et al.,* 2004), estas mutações estão associadas ao descontrole de programas essenciais como proliferação, morte e diferenciação celular. Acredita-se que o genoma das células transformadas seja instável e desta instabilidade resulte a aquisição cumulativa de mutações que podem converter uma célula normal em uma célula tumoral. Hanahan e Weinberg (2011) agruparam as capacidades adquiridas por uma célula tumoral em classes de alterações que interferem com a fisiologia normal de células e tecidos: (1) auto-suficiência quanto a fatores de crescimento; (2) insensibilidade a fatores inibitórios de proliferação; (3) evasão da apoptose ou morte celular programada; (4) potencial replicativo ilimitado; (5) angiogênese; (6) invasão tecidual e metástase; (7) inflamação; (8) reprogramação do metabolismo energético; (9) evasão do sistema imune; e (10) instabilidade genômica e mutação. Neste contexto de alterações celulares, destaca-se a alteração das vias de sinalização que medeiam esse fenótipo tumoral.

As atuais abordagens em grande escala, como os microarranjos de DNA, RNA e de tecidos, proteoma, metaboloma e epigenoma, constituem importantes ferramentas para estudos em grande escala de mudanças na expressão gênica em neoplasias. Através destas ferramentas, já foram descritas uma série de mutações somáticas em tumores, que indicam a ativação constitutiva de circuitos de sinalização, sendo muitas destas alterações tumor e tecido-específicas (Hanahan e Weinberg, 2011; Nelson *et al.*, 2004; Datta *et al.,* 2007; Kosari *et al.,* 2008; McDonnell *et al.*, 2008). Neste sentido, uma melhor caracterização de genes diferencialmente expressos em células tumorais em relação ao tecido normal, de informações a respeito do papel funcional destes produtos gênicos de expressão aberrante relacionados

a tumorigênese (Joos et al., 2007; Reynolds 2008); apresentam potencial de identificação de novos biomarcadores e alvos terapêuticos em neoplasias.

Os modelos de tumores de carcinomas de ovário (CO) e próstata (CaP) são os focos de estudo do presente trabalho. Nestas neoplasias, o estudo do perfil de expressão e papel funcional de produtos gênicos envolvidos na progressão destes tumores são especialmente importantes a caracterização e descrição de novos biomarcadores, alvos terapêuticos e compreensão dos complexos mecanismos que levam ao desenvolvimento destes tumores. Nesse contexto, e dentre o grande número de alterações genéticas e epigenéticas associadas à carcinogênese destas neoplasias, selecionamos a proteína osteopontina (OPN) como alvo comum de nossos estudos. A OPN encontra-se superexpressa nestas neoplasias e apresenta uma série de importantes características que serão apresentadas a seguir, e que reforçam sua escolha deste produto gênico como alvo de nossos estudos.

1.1. A biologia do câncer de ovário

No Brasil, o câncer de ovário (CO) corresponde à cerca de 2 a 3% dos tumores femininos e a quarta causa de morte por câncer em mulheres sendo o mais letal dos tumores ginecológicos. No Brasil, existe um risco estimado de 6 casos a cada 100 mil mulheres (estimativa INCa 2012). Dois terços das pacientes são diagnosticadas com doença metastática e apresentam uma sobrevida de 30% (Kim *et al*., 2011; Davidson *et al*., 2010).

O câncer do ovário pode ser originado de três tipos celulares: células epiteliais, germinativas e estromais. Oitenta por cento dos tumores ovarianos são originados das células epiteliais (Figura 01). Os tumores derivados das células germinativas ocorrem mais frequentemente na juventude, em geral até os 20 anos de idade.

O CO é uma doença de causas multifatoriais, de forma que o acúmulo de mutações genéticas e fatores como idade avançada, nuliparidade (nunca ter tido filhos), histórico familiar para câncer de mama e ovário, fatores

ambientais, raça, terapia de reposição hormonal e dieta alimentar estão associados a um risco aumentado de desenvolvimento do CO (Hinds *et al.*, 2010; Vlahos *et al*., 2010; D'Angelo *et al.*, 2010; Rubin *et al.*, 2006).

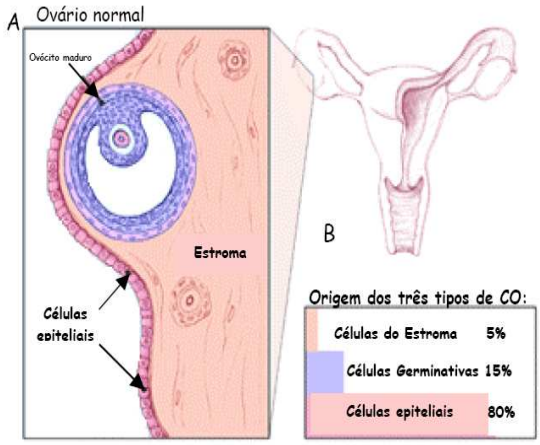

Figura 01 – A - Representação dos tipos celulares do tecido ovariano normal: células epiteliais, germinativas e estromais. B - Incidência da origem de tumor dos três tipos celulares componentes do ovário.

O carcinoma de ovário apresenta biologia e comportamento distintos em níveis clínicos, celulares e moleculares. Clinicamente, os carcinomas de ovário em geral apresentam-se como uma massa sólida na região pélvica. Embora o câncer de ovário tenha sido caracterizado como um câncer "silencioso", mais de 80% dos pacientes apresentam sintomas, mesmo quando a doença encontra-se limitada aos ovários. Contudo, os sintomas são compartilhados com muitas outras condições gastrointestinais, genitourinárias e ginecológicas e não têm demonstrado utilidade para o diagnóstico precoce. Nas mulheres em idade reprodutiva, anormalidades menstruais são observadas em aproximadamente 15% dos casos (Hinds *et al.*, 2010; Vlahos *et al*., 2010).

O CO é caracterizado por um crescimento heterogêneo, rápido e altamente metastático (Figura 02). Diversos subtipos histológicos de CO epitelial são reconhecidos, incluindo o tipo seroso papilífero (50 – 60 % dos

tumores ovarianos), mucinoso (4%), endometrióide (25%) e células claras (4%) (Köbel *et al.*, 2008; Farley *et al.*, 2008). Cada um destes subtipos está associado com diferentes fatores de risco, mecanismos de patogênese, eventos moleculares durante a tumorigênese e comportamento natural específicos, sendo caracterizados por distintos perfis de expressão gênica. É importante classificar corretamente os carcinomas ovarianos, uma vez que alguns tumores, tais como os mucinosos e os carcinomas de células claras parecem exibir uma resposta não satisfatória à quimioterapia baseada em drogas do tipo platina (Narod *et al.*, 2002; Shih *et al*., 2004; Zorn *et al*., 2005; Köbel *et al.,* 2008).

Histologicamente, os tumores ovarianos dividem-se em duas categorias: benígnos e malígnos. Contudo, os tumores malígnos dividem-se em '*borderline*' e invasores. Os tumores do tipo '*borderline*' são responsáveis por 5% a 20% dos tumores epiteliais malígnos do ovário e apresentam baixo potencial malígno, desta forma apresentam um prognóstico muito melhor que os carcinomas invasores. Os tumores benígnos não apresentam estratificação e não apresentam células atípicas (Kobel *et al*., 2008; Ouellet *et al.*, 2005). Os tumores denominados como '*borderline*' apresentam proliferação epitelial com estratificação e citologia atípica sem invasão estromal, embora a microinvasão possa ser observada (Tavassoli 1988; Cvetkovic 2003). Ao contrário, os tumores invasivos apresentam um alto índice mitótico, citologia atípica e invasão estromal (Burger *et al.,* 2000; Cvetkovic 2003).

O tumor de ovário progride por adesão das células malígnas na cavidade abdominal, na região do peritôneo (Kim *et al*., 2011) (Figura 02). Estas células podem proliferar na superfície do fígado, intestinos, bexiga, diafragma e no omento. Nestes sítios, impedem a drenagem de líquidos da cavidade abdominal, causando um acúmulo desse líquido, conhecido como ascite. O CO também pode disseminar-se para linfonodos pélvicos e peri-aórticos.

O diagnóstico de doença benígna deve ser preciso, uma vez que o procedimento cirúrgico em geral é suficiente para controlar a doença sem a

necessidade de quimioterapia (Trope *et al.*, 1998). Apesar de grandes esforços para desenvolver uma estratégia efetiva de rastreamento, somente 20% dos carcinomas de ovário são diagnosticados enquanto estão ainda limitados aos ovários (estadio I). Neste estágio, até 90% dos pacientes podem ser curados ao utilizar as terapias atualmente disponíveis. Após a doença ter se disseminado para os órgãos pélvicos (estadio II), para o abdômen (estadio III) ou além da cavidade peritoneal (estadio IV), os índices de cura diminuem substancialmente. Contudo, os critérios histológicos que permitem a distinção entre doença benígna, '*borderline*' e invasiva nem sempre são bem definidos. Uma classificação errônea pode resultar em abordagens terapêuticas sub-ótimas ou às vezes mais agressivas para a paciente. Estas características ressaltam a necessidade da caracterização de novos biomarcadores para o diagnóstico, prognóstico e de determinação da conduta terapêutica destas doenças ovarianas.

As correlações obtidas entre os resultados de recentes estudos genéticos, moleculares e histopatológicos, gerou uma recente proposta para um novo modelo de carcinogênese do ovário, o chamado modelo dualístico (Schlumbrecht *et al*., 2010; Kurman & Shi, 2008; Shi & Hirman, 2005; Shi & Kurman, 2004). Neste modelo, todos os tumores ovarianos epiteliais são divididos em dois grupos, designados Tipo I e Tipo II (Figura 02). As categorias são definidas assim: (1) Baixo Grau ou Tipo I, um carcinoma composto de células uniformes com atipia nuclear leve à moderada e em geral com baixo nível mitótico, tumores que surgem de maneira gradual a partir dos tumores ('*borderline*') de baixo potencial de malignidade e; (2) Alto Grau ou Tipo II, uma carcinoma composto de células pleomórficas com importante atipia nuclear e alto índice mitótico, tumores de desenvolvimento *de novo* (nenhuma lesão precursora identificada) (Shi *et al.,* 2008). Este modelo tem se mostrado um bom método para distinguir os carcinomas que apresentam diferentes características histológicas, patogenéticas, moleculares, imunohistoquímicas e clínicas.

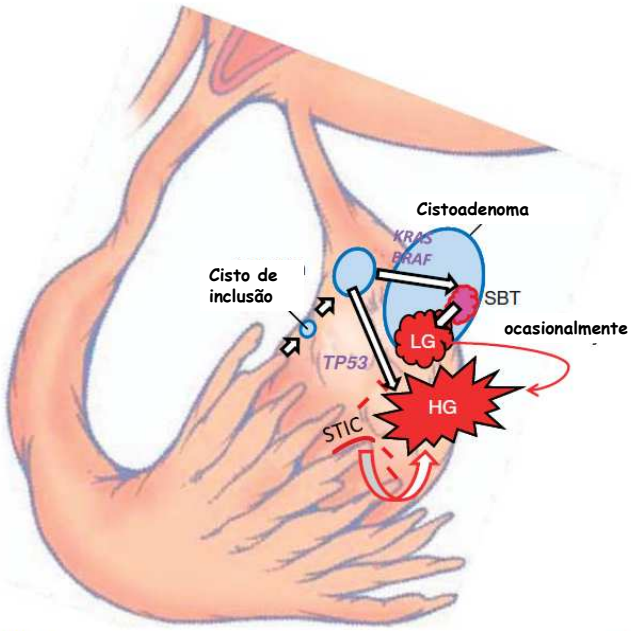

Figura 02 – Hipótese para a carcinogênese do ovário – o modelo dualístico. Desenvolvimento dos tumores de baixo grau (LG) e alto grau (HG) do carcinoma seroso de ovário. O mecanismo que envolve o desenvolvimento do tumor seroso é o implante de células das fimbrias da tuba uterina no ovário, gerando cisto de inclusão. Dependendo do tipo de mutação molecular KRAS/BRAF ou TP53, tem se a origem dos tumores de baixo ou alto grau, respectivamente. Os tumores de baixo grau apresentam a seguinte progressão: cisto de inclusão – cistoadenoma – tumo borderline (SBT) – tumor de ovário de baixo grau. Por outro lado, os tumores de alto grau apresentam a seguinte origem: podem ser originados dos cistos de inclusão ou a partir de células transformadas da tuba iterina (STIC). Modificado de Kurman *et al.*, 2011.

1.1.1. Tipos histológicos do câncer de ovário

- **Carcinoma seroso do ovário (CSO)**

Estudos recentes têm mostrado dois tipos distintos de CSOs (McCluggage, 2009). Embora denominados de CSOs de alto e baixo grau, não são duas graduações da mesma neoplasia, mas sim dois tipos de tumores distintos com diferentes mecanismos de patogênese, eventos moleculares, comportamentos e prognósticos. Acredita-se que o CSO de baixo grau surge a partir de um cistoadenoma benigno, o qual por sua vez evolui para um cistoadenoma *'borderline'*. Depois, segue para um CSO invasivo de baixo grau. Assim, há uma sequência bem definida de conversão de um adenoma para carcinoma. No entanto, não está provado que todos os CSOs de baixo grau surgem de tumores *'borderline'* pré-existentes. Por outro lado, o CSO de alto grau parece surgir diretamente da superfície do epitélio ovariano ou de cistos de inclusão corticais sem nenhuma lesão precursora definida. Há evidências de que muitos CSOs de alto grau podem ser na verdade derivados a partir do epitélio das porções distais das fímbrias da trompa de falópio (Kurman *et al.*, 2011).

Importantes eventos moleculares diferem o CSOs de alto e baixo grau. O CSO de baixo grau está associado à mutações no gene *KRAS* ou *BRAF* em aproximadamente dois terços dos casos. Estas mutações acontecem precocemente na evolução dos CSOs de baixo grau, uma vez que são encontrados em áreas *'borderline'* e benignas no mesmo tumor. As mutações nos genes *KRAS* e *BRAF* são mutuamente exclusivas. Por outro lado, o CSOs de alto grau contém, na maioria dos casos, uma mutação no gene p53 que ocorre precocemente no desenvolvimento do tumor (Kurman *et al.*, 2011).

- **Carcinomas Mucinosos**

Há também dois tipos distintos de adenocarcinoma mucinoso primário do ovário e tumores *'borderline'* mucinosos. Estes tumores podem ser do tipo

intestinal (o mais comum) e o raro carcinoma endocervical (também conhecido como, tipo Mülleriano). Os tumores ovarianos mucinosos do tipo intestinais expressam tipicamente, marcadores entéricos tais como CH20, CDX2, CEA, vilina e CA19.9 (Kurman *et al.*, 2011). As neoplasias mucinosas do tipo endocervicais são geralmente negativas para estes marcadores. Por outro lado, os tumores do tipo endocervicais geralmente expressam marcadores Müllerianos, tais como o CA125, o receptor de estrogênio (ER) e o receptor de progesterona (PR), que são negativos nas neoplasias do tipo intestinais.

Há poucos estudos dos eventos moleculares nos tumores mucinosos ovarianos do tipo endocervical. Os carcinomas mucinosos, assim como os CSOs de baixo grau, provavelmente surgem através de uma sequência adenoma-carcinoma a partir de um cistoadenoma benigno, passando por um cistoadenoma *'borderline'*, chegando depois a um carcinoma intraepitelial e um carcinoma mucinoso. Os tumores mucinosos ovarianos do tipo intestinal comumente exibem mutações no gene KRAS e mutações idênticas foram demonstradas em áreas benignas, *'borderline'* e malignas dentro do mesmo tumor, sugerindo que as mutações no gene KRAS sejam um evento precoce na evolução destes tumores. Mutações no gene BRAF não foram encontradas nos tumores mucinosos ovarianos do tipo intestinal.

- **Carcinoma endometrióide**

A maioria dos carcinomas endometrióides são de baixo grau e de estadiamento inicial. Em geral, mas não sempre, surgem de endometriose e/ou de adenofibromas benignos oude tumores *'borderline'* pré existentes. A tipagem do adenocarcinoma endometrióide é geralmente direta, embora surjam problemas na distinção entre um adenocarcinoma endometrióide de grau (1) e um adenofibroma endometrióide *'borderline'* (McCluggage, 2009).

- **Carcinoma de célula claras**

A maioria dos carcinomas ovarianos de células claras surgem a partir da endometriose. Estes carcinomas ovarianos são caracteristicamente compostos de células com citoplasma claro abundante, em geral com proeminentes membranas celulares. A presença de áreas mais típicas de em associação com adenocarcinoma seroso endometrióide ou seroso são indícios úteis para o diagnóstico (algumas vezes ocorre uma combinação de adenocarcinoma de células claras e endometrióide).

Lesões precursoras para o carcinoma ovariano de células claras não foram bem descritas. WT1 é geralmente negativo no carcinoma de células claras, assim como o p53. Contudo, os eventos moleculares relacionados ao desenvolvimento do carcinoma de células claras não foram extensivamente estudados.

Considerando a biologia heterogênea do carcinoma de ovário, em especial destaque aos diversos tipos histológicos e as vias de sinalização envolvidas na tumorigênese; o reflexo nas limitações de diagnóstico sensível e específico, e ainda, pouco conhecimento na biologia desta neoplasia; existe a necessidade da melhor caracterização de produtos gênicos e seu papel na progressão dos tumores ovarianos.

1.1.2. Diagnóstico do câncer de ovário

Atualmente, os métodos de diagnóstico do CO mais utilizados são: a ultra-sonografia abdominal e pélvica, incluindo a ultra-sonografia transvaginal e a tomografia computadorizada. Em contraste com a mama, próstata e cólon, os ovários são anatomicamente mais difíceis de visualizar durante os exames clínicos de imagem, dificultando o diagnóstico por exames de imagem.

Com isso, a dosagem dos marcadores tumorais constitui um método de alta importância tanto no diagnóstico como no seguimento das intervenções terapêuticas das pacientes com CO. Os principais marcadores atualmente

utilizados são o CA125 e o antígeno carcinoebrionário (CEA) para tumores epiteliais e o ßHCG e alfa-fetoproteína (AFP) para o câncer de células germinativas (Samulak *et al.,* 2010; Mrochem *et al.,* 2008).

O CA125, glicoproteína pertencente à família das mucinas, é o marcador sorológico mais comumente associado ao câncer de ovário. O nível de CA125 encontra-se elevado (acima de 30U\mL) em 50% das pacientes com CO estágio I e em 90% do estágio II. Entretanto, pode encontrar-se elevado em diversas neoplasias, tais como: tumores malígnos e benígnos de ovário, endométrio, mama, pulmão, bexiga, fígado, cólon e linfoma não-Hodgkin (Davidson *et al*., 2010; Farley *et al.*, 2008). Outras condições clínicas ginecológicas, tais como endometriose, cistos hemorrágicos ovarianos, menstruação, doença inflamatória pélvica aguda e a gestação podem estar acompanhadas de elevação dos níveis de CA125. Outras patologias também estão associadas à elevação do CA125, como por exemplo, a pancreatite aguda, doenças inflamatórias intestinais, cirrose hepática, hepatite crônica ativa, diverticulite, pericardite e lupus eritematoso sistêmico (Davidson *et al*., 2010; Moore *et al.*, 2008). A principal aplicação do CA125 é permitir o monitoramento da quimioterapia e predizer a recaída em casos de tumor epitelial de ovário (Chambers *et al.*, 2006; Moore *et al.*, 2008).

1.2. A biologia do câncer de próstata

No Brasil, o câncer de próstata (CaP) é a segunda causa de morte por neoplasias entre os homens brasileiros. O câncer de próstata é uma doença multifatorial, com isso o acúmulo de mutações genéticas e fatores como idade avançada, histórico familiar, fatores ambientais, raça e dieta alimentar estão associados a um maior risco de desenvolvimento do CaP (Reynolds 2008; McDonnell *et al.,* 2008). O CaP é caracterizado por um crescimento heterogêneo, variando de tumores de crescimento lento a lesões altamente metastáticas de crescimento muito rápido (Koeneman *et al.,* 2008; Reynolds 2008).

O CaP, assim como em outras neoplasias, resulta do acúmulo de alterações genéticas e epigenéticas que transformam o epitélio glandular normal em lesões pré-neoplásicas e em seguida para o carcinoma com propriedades invasivas (Kopp *et al.,* 2011; Fenic *et al.,* 2008). O CaP se desenvolve a partir de uma atrofia inflamatória proliferativa (PIA), a qual é caracterizada pelo aumento da proliferação das células epiteliais com a presença de grandes infiltrados inflamatórios (Fenic *et al.,* 2008). Análises histológicas demonstram a associação entre PIA e o processo inflamatório, sugerindo que a inflamação crônica pode resultar em dano às células epiteliais, desta forma causando lesões proliferativas (Gonzalgo *et al.*, 2003; Fenic *et al.*, 2008). Neste contexto, as células epiteliais altamente proliferativas da PIA perdem a estrutura colunar e passam a exibir células em formato cuboidal. Além disso, os ácinos começam a perder as suas invaginações características.

Diversas evidências sugerem que lesões da PIA podem ser precursoras da neoplasia intraepitelial da próstata (PIN) (Woenckhaus *et al.,* 2008) (Figura 03). A PIN é caracterizada por apresentar células de formas variadas e aumento no tamanho do núcleo das células dos ductos e ácinos. No contexto de PIN, a membrana basal apresenta-se intacta, sem invasão de estroma e com baixa secreção de PSA. A progressão da PIN dá origem então ao CaP localizado, caracterizado principalmente pela perda da camada de células basais e da arquitetura normal da glândula. O CaP avança para a doença metastática, caracterizada por ausência de membrana basal, grande invasão do estroma e formação glandular inexistente. Todas essas alterações histopatológicas são acompanhadas por mudanças na expressão gênica e também de deleções de regiões cromossômicas, que contribuem para a progressão do tumor de próstata (De Marzo *et al.*, 2008; Koeneman *et al.,* 2008; Gonzalo *et al.*, 2003).

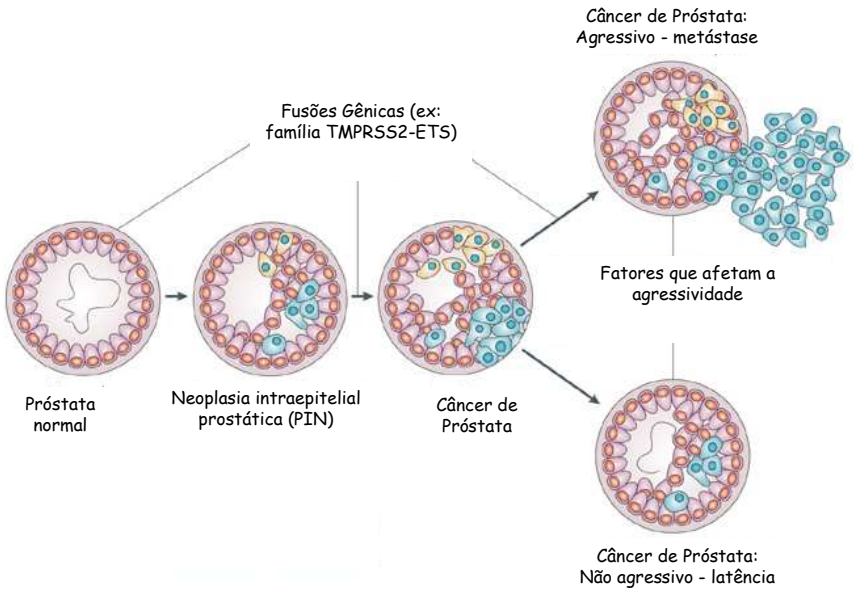

Figura 03 – Modelo de progressão do CaP. O CaP se desenvolve a partir de uma atrofia inflamatória proliferativa (PIA), que é precursora da neoplasia intraepitelial da próstata (PIN). A progressão de PIN origina o CaP localizado, dependendo das alterações genéticas, esse tumor apresenta um potencial agressivo ou não agressivo. (Modificado de Witte *et al*., 2009).

1.2.1. Tumor Benigno da Próstata (HPB)

Há também uma outra alteração do tecido prostático, a hiperplasia prostática benigna (HPB), que consiste no aumento do volume da próstata. A HPB possui uma elevada incidência em homens com mais de 50 anos e é uma das principais patologias urológicas, causando transtornos urinários aos pacientes (Cheng *et al.,* 2011; Schauer *et al.,* 2009). Entretanto, a HPB não está envolvida na tumorigênese da próstata, por não ser considerada uma lesão pré-maligna. A HPB origina-se na zona de transição da próstata e é caracterizada por progressivas alterações histológicas que se iniciam no estroma, causando um aumento no número de células do epitélio e do estroma (Cheng *et al.,* 2011; Bullock *et al.,* 2006). Na patogênese da HPB, as alterações celulares incluem uma maior proliferação celular, diferenciação,

apoptose e senescência nas células componentes do estroma e epitélio (Lee *et al.*, 2004).

A HPB é também uma doença multifatorial, com isso os fatores ambientais em conjunto com os fatores genéticos e endócrinos estão associados a um maior risco de desenvolvimento de HPB. Nenhuma alteração genética específica parece estar associada com o surgimento desta patologia (Cheng *et al.,* 2011; Sampson *et al.,* 2007). Por outro lado, encontra-se descrito na literatura uma expressão gênica diferencial entre o tumor benígno e o tecido prostático normal, principalmente de citocinas e fatores de crescimento, sugerindo um papel importante da inflamação crônica na patogênese da HPB (Cannon *et al.*, 2008; Schauer *et al.,* 2008; Sampson *et al.,* 2007).

1.2.2. Diagnóstico do câncer de próstata

Os métodos de diagnóstico do CaP mais utilizados são: a dosagem sérica do antígeno específico da próstata (PSA), o exame do toque retal, a ultra-sonografia transretal e a biópsia da próstata (Mottet *et al.,* 2011).

Por ser um método não invasivo, a detecção sérica do PSA surgiu em 1980 como teste promissor, e até hoje é amplamente utilizada para diagnóstico e monitoramento de resposta ao tratamento e de recidivas de pacientes com CaP (Cao *et al.,* 2011; Vickers *et al.*, 2008). O PSA é uma serina protease de 34 KDa pertencente à família das kalicreínas. Essa protease é secretada no fluido prostático, onde sua função é a degradação de proteínas de alto peso molecular, inibindo a coagulação do sêmen (Coffey *et al.,* 1993). O tumor da próstata provoca um aumento da concentração sérica do PSA em aproximadamente 10^5 vezes (Vickers *et al.*, 2008). No entanto, esse marcador possui baixo valor de sensibilidade e especificidade, uma vez que a elevação do nível sérico desta glicoproteína também é observada em outras alterações e manipulações da próstata como a HPB, prostatites e a ejaculação (Cao *et al.,* 2011; Vickers *et al.*, 2008). O PSA apresenta alto valor

prognóstico, visto que seus níveis estão diretamente relacionados à progressão ou regressão do tumor pós-tratamento (Cao *et al.,* 2011; Koeneman *et al.,* 2008).

O exame do toque retal constitui uma importante ferramenta no diagnóstico do CaP, entretanto apresenta baixo valor preditivo em identificar tumores que estão confinados ao órgão ainda em estágio inicial (Koeneman *et al.,* 2008). Somente as porções posterior e lateral da próstata conseguem ser palpadas durante o exame, deixando cerca de 40% dos tumores fora de seu alcance.

Além do PSA e do toque retal, o diagnóstico de CaP também é dado pela análise da ultra-sonografia transretal e da biópsia de tecidos prostáticos, as quais são indicados sempre que houver anormalidades no toque retal e/ou alteração no nível de PSA. O diagnóstico final de CaP é dado pelo estudo histopatológico do tecido obtido pela biópsia. A origem do CaP é multifocal, com isso podem ocorrer distintos focos de PIN e de CaP em uma mesma glândula prostática. Cada um destes focos pode apresentar diferentes graus de diferenciação celular. O CaP é comumente graduado pelo método descrito por Gleason (Gleason 1996), que reflete a arquitetura glandular. As lesões de baixo grau representam lesões mais diferenciadas e as de alto grau as menos diferenciadas (Figura 04). Como a origem do CaP é multifocal, o diagnóstico final é dado pelo Escore de Gleason (EG), representando distintos padrões do mesmo tumor. O EG é a soma dos graus das duas subpopulações predominantes de células no tecido (Turker *et al*., 2011; Lapointe *et al*., 2004). Mesmo utilizando parâmetros clínicos como o EG, níveis séricos de PSA e o estadiamento do tumor, ainda permanece difícil de predizer o prognóstico dos pacientes (Singh *et al.*, 2002).

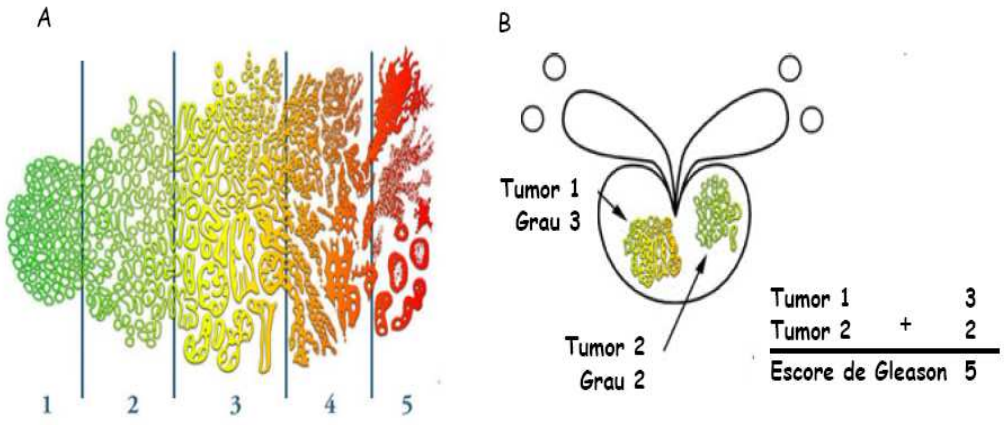

Figura 04 – A - Representação esquemática dos graus de diferenciação das células tumorais prostáticas. B – Escore de gleason é a soma dos graus das duas subpopulações predominantes de células no tecido.

1.3. Osteopontina (OPN): um importante biomarcador tumoral e seu papel na tumorigênese

1.3.1. Família SIBLING

A família SIBLING (do inglês, *small integrin-binding ligand N-linked glycoproteins*) agrupa cinco glicofosfoproteínas secretadas para a matriz extracelular, incluindo a osteopontina (OPN), a sialoproteina óssea (BSP), a dentina proteína 1 de matriz (DMP1), a dentina sialofosfoproteína (DSPP) e a fosfoglicoproteína de matriz extracelular (MEPE). Os genes que codificam esta família de proteínas localizam-se no cromossomo 4 (Figura 05). O nome SIBLING refere-se a esta família pelas características gênicas e bioquímicas, como a presença da sequência de aminoácidos RGD (glicina-arginina-aspartato) e modificações pós-tradução semelhantes, como as glicosilações e

fosforilações. Estas proteínas são solúveis e exercem suas principais funções na matriz extracelular, atuando como moduladores da adesão celular e como fatores autócrinos e parácrinos em relação à interação com os receptores de membrana (Fedarko *et al.,* 2008).

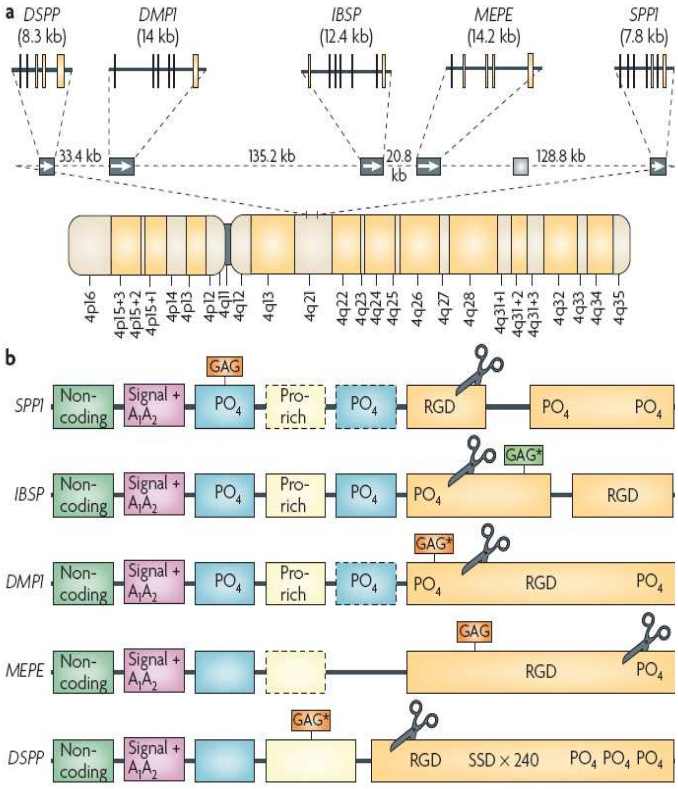

Figura 05 - Localização cromossômica e as similaridades no arranjo de éxons e íntrons dos genes codificadores de proteínas da família SIBLING. A - Os genes que codificam esta família estão agrupados no cromossomo 4. Entre os genes MEPE e o gene da OPN (SPP1) está localizado o pseudogene HSP90AB, que encontra-se representado na figura por um retângulo em cinza claro. As linhas verticais representam os éxons. B – A estrutura das proteínas da família SIBLING. As proteínas SIBLING são compostas por aminoácidos hidrofílicos. O primeiro éxon não é traduzido e o segundo contêm o códon de iniciação e o peptídeo sinal, indicando que a proteína é secretada. Os éxons 3 e 5 contêm seqüências consenso para fosforilação de serina (PO4). O éxon 4 apresenta sequência rica em prolina e pode sofrer processamento alternativo de RNA (retângulo desenhado em pontilhado). A trinca de aminoácidos RGD é encontrada em um dos dois últimos éxons. Todas estas proteínas contêm N ou O-oligossacarídeos em sua estrutura. O sítio de clivagem por proteases

específicas, como a trombina e as metaloproteinases de matriz, está representado por uma tesoura, sendo uma sequência conservada em muitas espécies (Modificado de Fedarko *et al.*, 2008).

1.3.2. Funções e perfil de expressão da OPN

Prince *et al.* (1989), sequenciaram e descreveram as características estruturais da OPN, que incluem a sequência de aminoácidos de interação com as integrinas, a sequência RGD (arginina-glicina-aspartato), nove resíduos consecutivos de ácido aspártico e os sítios de interação com cálcio e heparina (Figura 05).

A OPN apresenta múltiplas funções biológicas bem descritas e caracterizadas, especialmente relacionadas ao desenvolvimento da resposta imune, ativando funcionalmente células dendríticas e polarizando o fenótipo para a resposta Th1 (Qu Z *et al*., 2010; Anborgh *et al.,* 2010**;** Schack *et al.,* 2009; Weiss *et al.,* 2005). Apresenta um importante papel como citocina inflamatória, na calcificação e remodelamento de tecidos mineralizados, na adesão celular, na angiogênese, na carcinogênese (Beausoleil *et al*., 2011; Weber *et al.*, 2010; Stromnes *et al*., 2007). A OPN apresenta diversas modificações pós-tradução (PTMs), como a fosforilação, a glicosilação e o processamento proteolítico, os quais conferem efeitos significativos na estrutura e nas propriedades biológicas desta proteína (Qin *et al.*, 2004; Sodek *et al.*, 2000) (Figura 06). As PTMs da OPN são tecido e célula-específicas, refletindo as diversas funções desta proteínas em diferentes sistemas fisiológicos (Anborgh *et al.,* 2010; Christensen *et al.,* 2010; Sorensen *et al.*, 2005).

A OPN encontra-se constitutivamente expressa em tecidos normais. Contudo, sua expressão está elevada em determinadas condições, como por exemplo, no câncer, no remodelamento vascular e na resposta inflamatória e imune (Kuo *et al.*, 2008). O aumento nos níveis de expressão da OPN é observado em diversas neoplasias, como nos carcinomas de cólon, mama,

pulmão, estômago, endométrio, tireóide, ovário, próstata, esôfago e gliomas (Weber *et al.*, 2011; Weber *et al.*, 2010; Brown *et al.,* 1994; Chambers *et al.,* 1996; Tuck *et al.,* 1998; Casson *et al.,* 1997; Thalman *et al.,* 1999; Saitoh *et al.,* 1995). No CaP, a expressão desregulada da OPN é observada em eventos iniciais da tumorigênese, como na atrofia proliferativa (PIA) e a intraepitelial (PIN) (Khodavirdi *et al*., 2006). O nível de expressão da OPN correlaciona-se com o potencial metastático de alguns tumores, em especial no CO e CaP (Carlinfante *et al.,* 2003).

1.3.3. Associação da OPN com a tumorigênese

Chambers *et al.* (1992) demonstraram a relação entre a OPN e o câncer quando transformou a linhagem NIH-3T3 com uma construção contendo o gene Ras fusionado ao gene da OPN, observando que esta linhagem não-tumorigênica adquiriu um fenótipo malígno.

Uma das primeiras demonstrações do papel funcional da OPN na tumorigênese foi comprovada através de ensaios com cDNA antisenso. Estes resultados mostraram que as propriedades tumorigênicas e metastáticas da OPN foram inibidas em diversos tipos celulares (Behrend *et al.*, 1994; Gardner *et al.*, 1994; Su *et al.*, 1995; Feng *et al.*, 1995). Posteriormente, a transfecção do cDNA da OPN em células epiteliais mamárias de rato induziu um fenótipo metastático (Oates *et al.*, 1996). Estes estudos sustentam a idéia de que a OPN não somente está associada com a tumorigênese, mas também contribui funcionalmente para o comportamento malígno das células de tumorais.

1.3.4. Interação da OPN com os receptores de superfície celular

As diversas funções fisiológicas da OPN refletem a habilidade da OPN secretada de interagir com as moléculas de transdução de sinal. Encontra-se descrito que a OPN inibe a apoptose e contribui para a sobrevivência celular através de sua fosforilação, interação com CD44, ativação de NF-kappa B,

alteração da expressão de proteínas pro-apoptóticas e ativação da via de sinalização de PI3K/Akt (Song *et al.,* 2008). A OPN é capaz de sinalizar através de distintos heterodímeros de integrinas e de CD44 e seus variantes de *'splicing'* para aumentar a adesão celular, o comportamento migratório e invasivo, ativar o processo metastático, promover a formação de colônias e a induzir células inflamatórias associadas a tumores e também a expressão de fatores angiogênicos (Iczkowski *et al.,* 2010; Bellahcène *et al*., 2008).

Devido às múltiplas funções da OPN, múltiplos domínios funcionais foram identificados. As regiões N-terminal e C-terminal, o sítio de clivagem de trombina e a sequência RGD da OPN representam importantes domínios desta proteína (Figura 06 e 07). O principal domínio funcional da OPN é o domínio de ligação a integrina arginina-glicina-aspartato (RGD), o qual é responsável pela migração e sobrevivência celulares. A sequência RGD está localizada no éxon 6 da OPN humana e contém um domínio funcional que se liga às integrinas. O sítio de clivagem com trombina se localiza há 6 aminoácidos de distância do motivo RGD. A trombina cliva a OPN e produz dois fragmentos, os quais apresentam os domínios de ligação à integrina e CD44. Um outro domínio funcional, denominado de WLNPDP é capaz de promover migração e sobrevivência de leucócitos e neutrófilos através destes sítios alternativos de uma maneira independente de RGD (Dai *et al*., 2009). A clivagem da OPN humana pela trombina também expõe a sequência de adesão SVVYGLR, que é um ligante de integrinas. A ligação da OPN à diferentes integrinas via RGD resulta em distintas consequências funcionais, que podem depender tanto do tipo de integrina específica quanto do tipo celular. A ligação à integrina depende não somente do motivo RGD, mas também de sítios da OPN independentes de RGD, como descrito anteriormente. A extremidade C-terminal da OPN se liga ao CD44 e regula vários eventos celulares independentes de modificações pós-tradução.

As células tumorais expressam uma variedade de integrinas e, dependendo do grau de diferenciação do tumor, diversas integrinas podem estar hipo ou hiper-expressas (Schneider *et al.,* 2011; Cox *et al.,* 2010; Karlou

et al., 2010). A OPN interage com as diferentes integrinas, via sequência RGD-dependende ou independente, incluindo as formas αvβ3, αvβ1, αvβ5, α4β1, α9β1 e α8β1 (Fedarko *et al.,* 2008). Dentre estas integrinas, especialmente a αvβ3 está correlacionada com a malignidade e formação de metástases (Varner *et al.*, 1996; Seftor *et al.*, 1999). A interação da OPN com as integrinas depende não somente da sequência RGD, mas também de sítios específicos de fosforilação da OPN (Weber *et al.,* 2002).

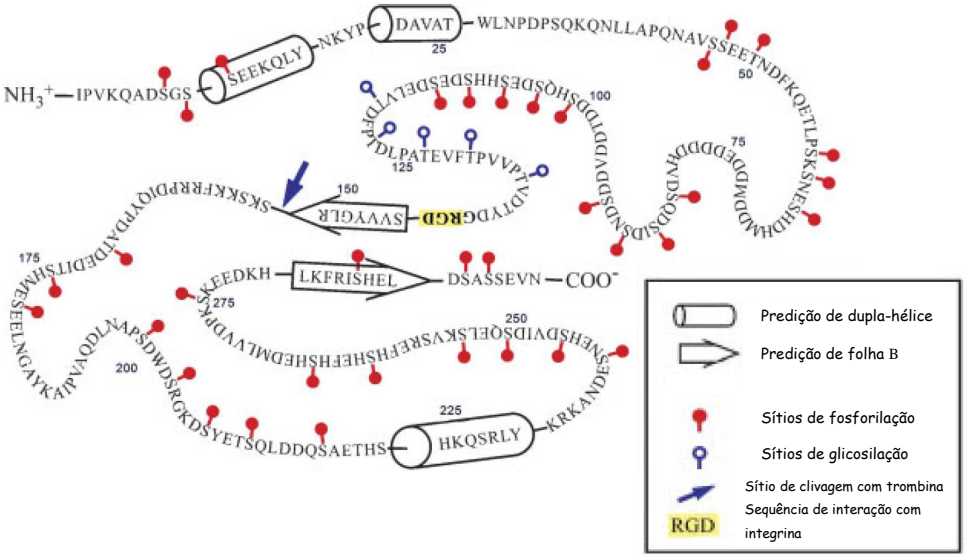

Figura 06 - Estrutura molecular da OPN. O modelo de predição da estrutura secundária da OPN foi realizado através de análise de bioinformática (APSSP2, Raghava *et al.*, 2002). A OPN apresenta uma estrutura desordenada, com isso sua estrutura final depende da interação com o parceiro protéico e do microambiente (Modificado de Kazanecki *et al*., 2007).

A família de receptores CD44 inclui múltiplas isoformas da proteína codificadas pelo mesmo gene e geradas por processamento alternativo do RNA (Desai *et al.*, 2007; Weber *et al.,* 1996; Zohar *et al.,* 2000). Diversas isoformas deste receptor encontram-se superexpressas em neoplasias (Ponta *et al.,* 1998; Goodison *et al.,* 1999). O CD44 é o principal receptor de interação com hialuronato (HA) e suas diversas isoformas podem interagir com a OPN.

Weber *et al.* (1996) demonstraram as interações do receptor CD44 com OPN e HA em células de fibroblasto murino que superexpressavam este receptor. Foi observado que a OPN, mas não o HA, pode induzir quimiotaxia dependente de CD44. Por outro lado, o HA, mas não a OPN, pode induzir agregação dependente de CD44. Esta é a primeira evidência de que a OPN e o HA podem interagir com estes receptores e induzir diferentes fenômenos.

A OPN ativa a via de sinalização de NIK (fator nuclear induzido por cinase)-ERK (sinal extracelular-relacionado com cinase) e MEKK1-JNK1, promovendo a migração celular por ativação do fator de transcrição AP-1 (Robertson *et al.*, 2010). Adicionalmente, induz a sobrevivência celular, através da ativação da via fosfolipoase-C-γ(PLC- γ)-proteína cinase C (PKC)-fosfatidilinositol 3-cinase (PI3K)- via Akt. A via de Akt, por sua vez, regula progressão no ciclo celular, a sobrevivência celular mediada por fatores de crescimento, a migração celular e o crescimento independente de ancoragem (Kundu *et al.,* 2006).

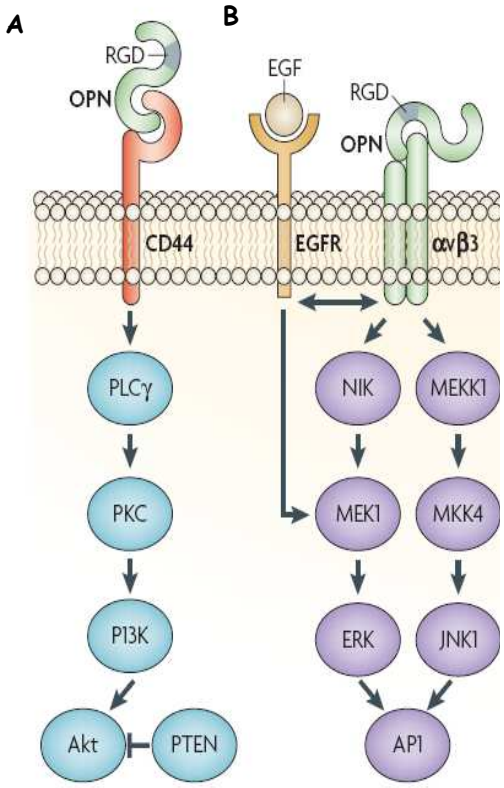

Figura 07 - Interações da osteopontina com receptores de membrana e sinalização intracelular. A OPN pode interagir com as integrinas e com CD44. A – A interação com CD44 ativa a via da fosfolipase-C-γ(PLC- γ)-proteína cinase C (PKC)-fosfatidilinositol 3-cinase (PI3K)- via Akt, culminando com a liberação de sinais anti-apoptóticos para as células tumorais. B – A OPN interagindo com as integrinas e ativando as vias de sinalização de NIK (fator nuclear induzido por cinase)-ERK (sinal extracelular-relacionado com cinase) e MEKK1-JNK1, ativando AP1. A OPN estimula a transativação do receptor de fator epidérmico de crescimento (EGFR) com fosforilação de ERK e ativação de AP1 (Modificado de Fedarko *et al.*, 2008).

1.3.5. Papel funcional da OPN na progressão tumoral

- **Indução de proteases mediada pela OPN**

As integrinas são capazes de interagir com diversas proteínas constituintes da matriz extracelular, aumentando a complexidade de sinalização na membrana celular. Por exemplo, as integrinas podem interagir com o ativador de plasminogênio do tipo uroquinase (uPA) e seu respectivo receptor (uPAR), os quais apresentam importantes papéis na metástase (Wang *et al.,* 2010; Kundu *et al.,* 2005; Chambers *et al.*, 2001). A uPA é uma das proteases superexpressas em tumor. Esta enzima proteolítica apresenta um importante papel na metástase e na progressão tumoral, por induzir a degradação de componentes da matriz extracelular e facilitar a migração e invasão (Wolff *et al.,* 2011). A expressão de uPA pode também promover migração celular de forma indireta, por ativação da cascata de sinalização de MAP cinases, incluindo ERK1 e 2. Em carcinoma hepatocelular e de mama, a OPN apresenta um importante papel funcional na invasividade e na indução de expressão de uPA (Chen *et al.*, 2011; Tuck *et al.,* 1999).

A OPN induz a expressão e atividade da família de metaloproteinases (MMPs), que podem contribuir para a formação de metástases (Georges *et al*., 2010; Belotti *et al*., 2003) (Figura 08). Castellano *et al.* (2008) demonstraram uma correlação positiva entre a concentração plasmática da OPN, altos níveis e alta atividade de MMP-9 com a progressão do CaP. Para a progressão do carcinoma de ovário a MMP-2 e MMP-9 apresentam um importante papel na degradação da matriz extracelular promovendo a invasão tecidual (Belotti *et al.*, 2003).

Figura 08 – Papel da OPN na progressão tumoral. Este modelo representa o papel da OPN na regulação da transformação da célula normal em célula tumoral. A OPN é superexpressa pelas células tumorais, interagindo com as integrinas e CD44, ativando uma sinalização específica intracelular. A fosforilação e ativação de cinases (PI3K, NIK, PLC, MAPK) induz a transativação de fatores de transcrição, incluindo NF-κB e AP-1. A OPN contribui com a tumorigênese inibindo a apoptose e ativando proteases que degradam a matriz extracelular, promovendo a migração celular, crescimento tumoral e formação de metástases (Modificado de Kundu *et al.*, 2006).

A formação de metástases é um processo complexo, caracterizado por múltiplos estágios: proliferação das células tumorais, inibição da apoptose, migração, invasão tecidual e de capilares e vasos linfáticos, resistência aos ataques do sistema imune e capacidade de invasão e proliferação celular no sítio tumoral secundário (Weber *et al.*, 2011; Weber *et al.*, 2010; Kundu *et al.*, 2006). A correlação observada entre altos níveis de expressão da OPN em células tumorais e a subsequente disseminação metastática é sustentada por ensaios de aumento ou diminuição dos níveis de expressão da OPN (Wai *et al.,* 2004). A superexpressão da OPN em situação metastática pode ser observada em diversas neoplasias, como pulmão, próstata, mama, mieloma e ovário (Weber *et al*., 2011; Zhang *et al.,* 2011; Weber *et al.*, 2010; Bellahcene *et al.,* 2008). Estudos funcionais utilizando superexpressão da OPN em duas linhagens de câncer de próstata (LNCaP e PC-3) revelaram que a OPN aumenta a invasividade e a proliferação de células de CaP em modelos de ensaios funcionais *in vitro* e *in vivo* (Khodavirdi *et al.,* 2006; Angelucci *et al.*,

2004). A OPN aumenta a invasividade das células tumorais do pâncreas e das células tumorais de pulmão (Kolb *et al.,* 2005; Hu *et al.,* 2005).

- **Angiogênese e OPN**

A metástase é um processo complexo, constituído de variadas etapas, e que resulta das interações entre as células tumorais e o microambiente tecidual onde estas células se encontram. Durante a disseminação do tumor, as células tumorais devem ser capazes de se soltar do tumor primário (perda de interação célula-célula) e escapar do tecido de origem. Precisam também invadir a matriz extracelular, migrar ativamente pelo estroma intersticial, induzir a formação de novos vasos sanguíneos e/ou linfáticos (angiogênese), essenciais para a expansão da massa tumoral. Por estes mesmos vasos, as células podem alcançar a corrente sanguínea ou linfática, após atravessar a membrana basal e o endotélio dos vasos, sobreviver na circulação, interagir com o endotélio vascular, extravassar e ainda, proliferar no parênquima do órgão-alvo (Anisimov *et al*., 2009; Laconi *et al.,* 2008; Fedarko *et al*., 2008). O processo de angiogênese é também crítico para a expansão das células do tumor primário e dos focos metastáticos. Ao longo deste processo, as células tumorais interagem com diversos elementos do hospedeiro, que atuam facilitando o processo de metastatização (Nguyen *et al.,* 2009; Horak *et al.*, 2008). Este processo resulta do balanço entre fatores estimulatórios (VEGF, PDGF, TGF-α e β, FGF-2 e citocinas pró-inflamatórias) e inibitórios da angiogênese (interferons, angiostatina, endostatina, trombospondina e inibidores de MMPs).

É bem conhecido que a OPN (isoforma completa da OPN) induz a formação de novos vasos sanguíneos, sustentando a progressão tumoral e a metástase. A relação da OPN com este processo está relacionada predominantemente com a interação desta proteína com a integrina do tipo αvβ3, um importante marcador de angiogênese. Por outro lado, a OPN estimula a angiogênese por induzir a expressão do fator de crescimento endotelial (VEGF) (Wang *et al.,* 2011; Belotti *et al*., 2003; Chambers *et al.,*

2001). Brooks *et al.* (1994) demonstraram através de experimentos de angiogênese *in vivo* utilizando membrana corio-alantóica de embrião de galinha, que a expressão da integrina αvβ3 aumenta durante a angiogênese. Na situação em que a expressão desta integrina é bloqueada, a angiogênese é inibida. A integrina αvβ3 e a OPN encontram-se superexpressas durante o processo tumorigênico (Robertson *et al*., 2010). A superexpressão de VEGF em pacientes com carcinoma de ovário está associada com a transformação do epitélio normal em tumoral, estimula a formação de ascite e suprime a resposta imune anti-tumoral (Zhang *et al*., 2003; Schumacher *et al*., 2007; Belotti *et al*., 2003).

1.3.6. Aspectos clínicos da OPN como marcador em neoplasias

A correlação entre os níveis elevados de expressão da OPN com a invasividade tumoral foi caracterizado por diversos estudos clínicos que demonstraram um aumento da concentração plasmática da OPN em pacientes com doença metastática (Zhang *et al.,* 2011; Weber *et al*., 2011; Jeong *et al.*, 2006; Qin *et al.,* 2006). A concentração plasmática da OPN em pacientes com doença avançada em comparação aos indivíduos saudáveis ou com doença localizada indica que o nível sérico de OPN pode ser sensível e específico na predição da evolução dos carcinomas de cólon, próstata, pâncreas, mama, ovário, dentre outros (Zhang *et al.,* 2011; Weber *et al*., 2011; Kuo *et al.*, 2008). A expressão da OPN circulante está positivamente associada com a presença de metástases no carcinoma de próstata e ovário, e negativamente relacionada com a sobrevida dos pacientes com estas neoplasias (Zhang *et al.,* 2011; Fedarko *et al*., 2001; Mok *et al.,* 2002). A alta concentração sérica da OPN, em pacientes com CO pós-cirúrgico, é indicativo de recorrência do tumor ovariano (Brakora *et al.,* 2004). A OPN sérica, em conjunto com outros marcadores ósseos podem constituir um painel de marcadores para diagnóstico de metátases ósseas, além de auxiliar na predição da sobrevida de pacientes com CaP (Lein *et al.*, 2007). Tem sido

demonstrado que a OPN pode ser clinicamente útil ao ser avaliada em conjunto com o CA125 na detecção do CO recorrente (Schorge *et al.,* 2004).

1.3.7. Processamento alternativo de transcritos da OPN

O '*splicing*' alternativo é um dos mecanismos utilizados pelas células tumorais para gerar variabilidade na estrutura e função da proteína osteopontina. A OPN secretada de vários tipos celulares apresentam características estruturais diversas, sendo que as formas derivadas de tumores apresentam tamanhos moleculares menores que a OPN secretada a partir de células não transformadas (Mirza *et al.*, 2008). A variabilidade de estrutura e função da OPN é conferida por processamento alternativo do RNA mensageiro da OPN e por modificações pós-tradução desta proteína (Christensen *et al.*, 2007; Seo *et al.,* 2003).

O gene da OPN está sujeito a processamento alternativo, gerando 3 mensagens, denominadas de osteopontina-a (OPNa, a isoforma completa), osteopontina-b (OPNb, com deleção do éxon 5) e osteopontina-c (OPNc, com deleção do éxon 4) (Figura 09). O domínio N-terminal da OPN, o qual inclui a região codificada pelos éxons 4 e 5, não apresenta papel fisio-patológico caracterizado.

O '*splicing*' alternativo da OPN foi primeiramente descrito em células de glioma (Saitoh *et al.,* 1995), posteriormente no carcinoma hepatocelular, mama, pulmão não-pequenas células, cabeça e pescoço e mesotelioma (Takafuji *et al.,* 2007; He *et al.*, 2006; Ivanov *et al*., 2009; Blasberg *et al*., 2009; Courter *et al.*, 2010). Esses dados indicam que o perfil de expressão e o papel funcional das isoformas da OPN é tecido e tumor específico. Por exemplo, no câncer de mama, a OPNc não altera o perfil proliferativo, mas estimula o crescimento independente de ancoragem (He *et al.*, 2006). Por outro lado, no mesotelioma, câncer de pulmão e cabeça e pescoço, a OPNa e OPNb, mas não a OPNc, são capazes de estimular características pró-tumorigênicas (Ivanov *et al*., 2009; Blasberg *et al*., 2009; Courter *et al.*, 2010). No câncer de

pulmão, a superexpressão da OPNc está associada com a diminuição das propriedades anti-angiogênicas, enquanto a OPNa e OPNb estimulam a angiogênese (Blasberg *et al*., 2009). Até o presente momento, no CO e CaP, ainda não é conhecido o papel funcional de cada uma das isoformas nestes tumores.

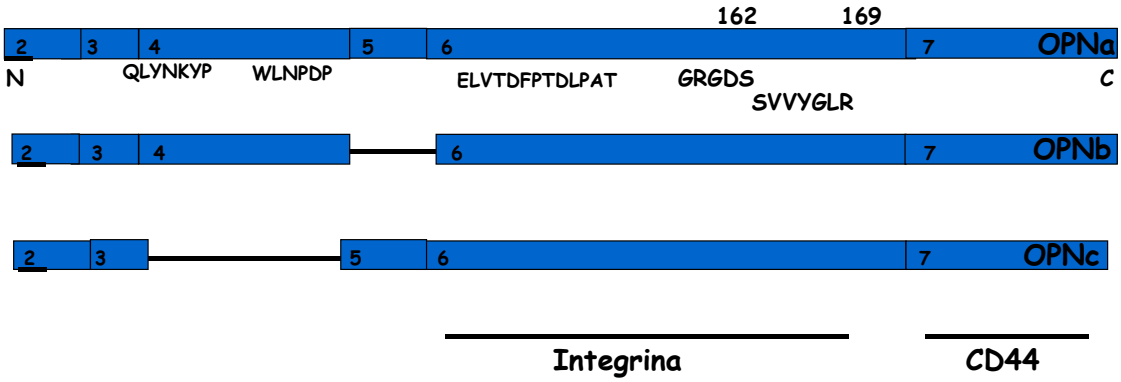

Figura 09 – Estrutura das isoformas variantes de '*splicing*' da OPN. Características estruturais das isoformas da OPN. O gene da OPN apresenta seis éxons traduzidos. A OPNc perde o éxon 4 e a OPNb perde o éxon 5. A sequência RGD de interação com as integrinas é mantida nas três isoformas, assim com a sequência de interação com CD44. As sequências QLYNKYP, WLNPDP, ELVTDFPTDLPAT e SVVYGLR também interagem diretamente com as integrinas.

1.4. Variantes de '*splicing*' e câncer

Análises genômicas em grande escala têm indicado que até 95% do genoma humano pode apresentar formas de processamento alternativo de RNA (do inglês – '*splicing*' alternativo, também denominado como AS), sugerindo que o AS, juntamente com várias modificações pós-tradução, seriam as chaves moleculares para a geração da complexidade proteômica (Norris *et al*., 2012; Luco *et al*., 2011; Xing 2007). Mutações na sequência de

nucleotídeos dos elementos regulatórios de '*splicing*' e alterações nos componentes da maquinaria deste processo resultam em mudanças no sítio de '*splicing*' de diversos genes relacionados com a progressão tumoral (por exemplo, CD44, MDM2 e FHIT) e na susceptibilidade ao câncer (por exemplo, BRCA1 e APC) (David *et al.,* 2010; Srebrow *et al.*, 2006; Pajares *et al.,* 2007). Pelo menos em alguns casos, mudanças no '*splicing*' desempenham um papel significativo na tumorigênese, tanto inativando genes supressores tumorais quanto promovendo ganho de função de proteínas que promovem o desenvolvimento tumoral. O '*splicing*' alternativo de produtos gênicos pode resultar em alterações de localização celular e de estrutura das proteínas, eventualmente removendo sítios de modificações pós-tradução e de susceptibilidade à degradação pelo proteasoma (David *et al.,* 2010; Hu *et al.*, 2005; Song *et al.*, 2005). A identificação de isoformas variantes de '*splicing*' câncer-específicas fornece uma nova fonte promissora de descoberta de biomarcadores para fins diagnósticos e prognósticos e também no adequado direcionamento para intervenção terapêutica (Tilli *et al.*, 2011; Skotheim *et al.*, 2007). Recentemente, uma série de publicações tem demonstrado a importância da expressão diferencial de isoformas de '*splicing*' na evolução clínica de pacientes com câncer (Li *et al*., 2011; Hayes *et al*., 2010; Venables *et al.*, 2006).

Mills *et al.* (2005) demonstraram que o gene da p53, um importante regulador da apoptose, apresenta isoformas variantes de '*splicing*' com alterações funcionais em diversas neoplasias. O fator de transcrição KLF6, um importante supressor tumoral que interage com p53, produz isoformas oncogênicas variantes e encontra-se superexpresso especialmente em tumor de próstata (Narla *et al.*, 2005). O processamento alternativo do mRNA também é observado em algumas caspases, exercendo um papel protetor das células tumorais à morte por apoptose (Solier *et al.*, 2004).

O CO e o CaP são causados por alterações genéticas que alteram a regulação da proliferação celular, morte celular programada e senescência. Entretanto, as alterações genéticas e as potenciais vias de sinalização relacionadas com estes processos não se encontram bem caracterizadas (Gross *et al*., 2010; Kurman *et al*., 2010; Reynolds, 2008). O '*splicing*' alternativo do mRNA é um importante mecanismo gerador de diversidade genética associado com a tumorigênese e com a progressão tumoral (Pajares *et al*., 2007) e também é um dos mecanismos pelo qual as células tumorais podem alterar a estrutura e a função da OPN, conforme observado em outros modelos tumorais (Mirza *et al.,* 2008; He *et al*., 2006; Ivanov *et al*., 2009; Blasberg *et al*., 2009; Courter *et al.,* 2010). No entanto, o perfil de expressão e o papel funcional das isoformas da OPN não se encontram ainda descritos para o CO e CaP, cujas neoplasias são foco deste trabalho.

2. OBJETIVOS

Com base no contexto apresentado acima e na necessidade de caracterização das vias moleculares pelas quais a OPN e suas isoformas variantes de '*splicing'* atuam na progressão dos tumores de ovário e próstata e na necessidade do desenvolvimento de nossos marcadores e potenciais novos alvos para a terapia destes tumores, o presente projeto apresenta os seguintes objetivos:

2.1. Objetivo geral

Investigar o perfil de expressão e o papel funcional das 3 diferentes isoformas variantes de '*splicing*' da OPN na tumorigênese e progressão tumoral do CO e CaP.

2.2. Objetivos específicos

1. Identificar o perfil de expressão das isoformas da OPN nas amostras de pacientes e linhagens de câncer de ovário e próstata.

2. Caracterizar *in vitro* e *in vivo* o papel funcional de cada uma das isoformas da OPN em diferentes aspectos da progressão tumoral.

3. Caracterização das vias de sinalização e mecanismos moleculares pelos quais as isoformas são capazes de mediar seus efeitos celulares.

Capítulo I

A primeira parte dos resultados dessa tese foi publicada no periódico *Molecular Cancer Research* em 2011; 9(3):280-93.

O objetivo deste estudo foi avaliar o perfil de expressão e o papel funcional das isoformas da OPN na tumorigênese e em aspectos relacionados à progressão dos tumores ovarianos, utilizando modelos *in vitro* e *in vivo*. Este foi o primeiro estudo a mostrar que a OPNc encontra-se expressa somente nas amostras de pacientes de carcinoma de ovário e tumor '*borderline*'. Esse mesmo perfil de expressão foi observado nas linhagens tumorais de ovário. Estes resultados indicam que a OPNc é especificamente expressa nas amostras tumorais de ovário, e essa expressão diferencial identifica essa isoforma como potencial novo biomarcador para esta neoplasia. Observamos que a OPNc apresenta efeitos estimulatórios sobre a proliferação, migração, invasão, formação de colônia e o crescimento de tumores xenotransplantados a partir de células OvCar-3 que superexpressam esta isoforma. A partir dos tumores formados *in vivo* pela superexpressão da OPNc, observamos um significativo aumento de expressão do mRNA de *Vegf*, *Mmp-2* e *Mmp-9,* quando comparado com os tumores formados pela superexpressão da OPNa, OPNb e vetor vazio. Adicionalmente, demonstramos que esta isoforma estimula a proliferação celular de uma linhagem não tumoral de ovário (IOSE), indicando que a OPNc também apresenta características pró-tumorigênicas. Os ensaios funcionais utilizando o meio condicionado e o anticorpo anti-OPNc demonstraram que os efeitos pró-tumorigênicos são estimulados pela OPNc secretada. Descrevemos também que os efeitos da OPNc são mediados pela via de sinalização PI3K/Akt. Os resultados gerados por este estudo contribuem para o melhor entendimento da biologia e dos mecanismos moleculares dos tumores de ovário. Em associação aos dados da literatura, os resultados gerados no presente estudo reforçam que o papel funcional das isoformas da OPN é tumor e tecido específico. O papel crucial desta isoforma em distintas etapas da progressão deste tumor indica a OPNc como potencial alvo terapêuticos para o CO.

Cancer Genes and Genomics

Molecular Cancer Research

Osteopontin-c Splicing Isoform Contributes to Ovarian Cancer Progression

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Abstract

Ovarian carcinoma is one of the most aggressive gynecological diseases and generally diagnosed at advanced stages. Osteopontin (OPN) is one of the proteins overexpressed in ovarian cancer and is involved in tumorigenesis and metastasis. Alternative splicing of OPN leads to 3 isoforms, OPNa, OPNb, and OPNc. However, the expression pattern and the roles of each of these isoforms have not been previously characterized in ovarian cancer. Herein, we have evaluated the expression profiling of OPN isoforms in ovarian tumor and nontumor samples and their putative roles in ovarian cancer biology using *in vitro* and *in vivo* functional assays. OPNa and OPNb were expressed both in tumor and nontumor ovarian samples, whereas OPNc was specifically expressed in ovarian tumor samples. The isoform OPNc significantly activated OvCar-3 cell proliferation, migration, invasion, anchorage-independent growth and tumor formation *in vivo*. Additionally, we have also shown that some of the OPNc-dependent protumorigenic roles are mediated by PI3K/Akt signaling pathway. OPNc stimulated immortalized ovarian epithelial IOSE cell proliferation, indicating a role for this isoform in ovarian cancer tumorigenesis. Functional assays using OPNc conditioned medium and an anti-OPNc antibody have shown that most cellular effects observed herein were promoted by the secreted OPNc. According to our data, OPNc-specific expression in ovarian tumor samples and its role on favoring different aspects of ovarian cancer progression suggest that secreted OPNc contributes to the physiopathology of ovarian cancer progression and tumorigenesis. Altogether, the data open possibilities of new therapeutic approaches for ovarian cancer that selectively down regulate OPNc, altering its properties favoring ovarian tumor progression. *Mol Cancer Res; 9(3); 280–93. ©2011 AACR.*

Introduction

Ovarian carcinoma is composed of a heterogeneous group of tumors derived from surface epithelia or inclusions (1). A vast majority of ovarian tumors arise due to accumulation of genetic damage, but the specific genetic pathways involved on the development of epithelial ovarian tumors are largely unknown. An improved understanding of the molecular basis of ovarian carcinogenesis and tumor progression could allow the establishment of new ovarian cancer markers and also the identification of new targets for better treatment options.

Many gene products are involved in ovarian cancer progression, as recently reviewed (2, 3). Among those genes, osteopontin (OPN, 2ar, Spp1) is recognized as a key prognostic marker during ovarian cancer progression (4, 5), which is overexpressed in ovarian cancer in relation to normal ovarian tissues (6). An increase in OPN levels has been correlated to tumor staging, invasiveness, and grade (7–9), as well as to the presence of ovarian cancer peritoneal metastasis (10). Furthermore, plasma OPN has been shown to have potential clinical utility in detecting recurrent ovarian cancer (11).

OPN mRNA is subject to alternative splicing, resulting in isoforms that are smaller than full-length OPN (12, 13). Alternative splicing leads to deletions in the N-terminal portion of OPN, located upstream from the central integrin and C-terminal CD44 binding domains (14). OPNa is the full-length isoform, whereas OPNb lacks exon 5 and OPNc lacks exon 4 (15). OPN splicing isoforms present tissue and tumor-specific roles (16–19). All current available data about OPN in ovarian cancer relates to OPN in general,

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doi: 10.1158/1541-7786.MCR-10-0463

without distinguishing among the various isoforms, and OPN splicing in this tumor has not been characterized previously. Here we examine the putative roles of each splice variant in ovarian cancer biology by using *in vitro* and *in vivo* tumor models. Our data show that the overexpression of OPNc increases OvCar-3 cell growth, migration, invasion, anchorage-independence and tumor formation *in vivo*, suggesting a possible functional role for OPNc in ovarian cancer progression. Our observations indicate that OPNc promotes ovarian cancer progression through activating the Phosphatidylinositol-3 Kinase (PI3K)/Akt signaling pathway.

Material and Methods

Tumor specimens

We evaluated nontumoral ovarian tissues (11 samples), benign tumors (8 samples), borderline tumors (6 samples), and ovarian carcinoma (15 samples). Tumors were classified according to the standard criteria from FIGO (20). Ovarian tissues samples were collected at Instituto Nacional de Cancer, Brazil from May, 2006 to January, 2008. This study was approved by the local Ethics Committee and informed consent was obtained from all patients. Ovarian tumor samples were obtained from patients who underwent resection of a tumor lesion of the ovary. Ovarian nontumoral tissue samples were histopathologically evaluated and were negative for tumor cells. These were obtained from patients who underwent hystectomy or anexectomy due to other gynecologic malignancies, such as endometrial carcinoma tumors, cervical uterus tumors, or an ovarian follicular cyst. Histology of patient tissue specimens and some features of those samples are presented on Supplementary Table S1. Ovarian tissues samples were stored in RNA Later (Ambion) at −20°C until processing. Patients presenting ovarian borderline tumors were followed up from 10 to 37 months and presented no evidence of ovarian disease.

Cell culture and the generation of OPN isoform overexpressing ovarian cancer cells

As a model to examine the putative roles of OPN isoforms in ovarian carcinoma, we used herein ovarian cancer and normal ovarian cell lines. We used 3 ovarian cancer cell lines, OvCar-3 (ovarian papillary adenocarcinoma), ES2 (clear cell carcinoma), and MDAH 2774 (endometrioid ovarian cancer), and an immortalized ovarian surface epithelium (IOSE) normal cell line. Cell lines were cultured in standard conditions. OvCar-3, ES2, and MDAH 2774 cell lines were supplied from Clinical Research Service, Instituto Nacional de Cancer, Brazil. IOSE cell line was a kind gift from Dr. Nelly Auersperg from University of British Columbia, Canada. Cell lines were cultured in a humidified environment containing 5% CO_2 at 37°C. All cell lines were cultured in RPMI medium supplemented with either 20% (OvCar-3) or 10% (ES2, MDAH 2774, and IOSE) fetal bovine serum (FBS), 100 IU/mL penicillin, and 100 mg/mL streptomycin. For all functional assays, OvCar-3 was cultured in RPMI with 2% FBS for 48 hours.

The open reading frame of OPN splice variants, OPNa, OPNb, and OPNc were cloned into pCR3.1 mammalian expression vector as previously described (17) and these DNA constructs were used for transfection into OvCar-3 cells. Transfections were carried out using Lipofectamine 2000 (Invitrogen). The OPN isoforms/pCR3.1 plasmids or the vector alone were transfected into OvCar-3 cells and the stably expressing cell clones were selected with 600 μg/mL of G418 in the culture medium. Six OPN isoform overexpressing clones (OPNa1, OPNa4, OPNb1, OPNb3, OPNc1, and OPNc5) and one empty vector control clone (empty vector) were selected. Each clone was tested individually on functional assays, as indicated.

Quantitative real-time RT-PCR

RNA was extracted from the cell lines and tumor tissues using the RNeasy kit (Qiagen). cDNA synthesis was carried out using SuperScript II (Invitrogen). OPN splice variants were amplified with specific primer pairs (Supplementary Table S2). All PCR reactions were conducted using the SYBR Green detection reagent (Applied Biosystems). Conditions for OPN isoforms PCR amplification were 50°C for 2 minutes, 94°C for 5 minutes followed by 10 cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 90 seconds; at the end of each cycle the temperature decreased 0.5°C, followed by 30 cycles of 94°C for 30 seconds, 50°C for 30 seconds, and 72°C for 90 sec, 72°C for 15 minutes, and finally a melting curve analysis (60–90°C with a heating rate of 0.2°C/sec and continuous fluorescence measurement). Product purity, size, and absence of primer dimers were confirmed by the DNA melting curve analysis and agarose gel electrophoresis. Relative gene expression of the target gene was calculated by using the ΔCT method. GAPDH and actin amplification were used as normalization controls for OPN isoforms, MMPs, or VEGF transcription level evaluation. Conditions for amplification of these genes are detailed in Supplementary Information.

Cell proliferation, sub-G_0, cell migration, invasion, and soft agar growth assays

Details about all these assays are presented in the Supplementary Information. Cell proliferation was analyzed by crystal violet, exclusion of trypan blue, and incorporation of (^{3}H) thymidine assays. Sub-G_0-G_1 analyses were conducted by using propidium iodide staining and fragmented DNA content using flow cytometry. Cell proliferation assays for OPN depletion using anti-OPNc antibody were evaluated in the absence or with the addition every other day of anti-OPNc antibody (Gallus Immunotech) at 4 μg/mL. Proliferation assays were also carried out using anti-rabbit IgG goat antibody (Pierce) as immunoglobulin control (4 μg/mL). Cell migration assays were evaluated by *in vitro* wound closure assays, as described by others (21). Transwell Invasion Assays were carried out as reported (22) and anchorage independent growth was analyzed in soft agar medium.

Preparation of cell lysates

Cells were harvested and rinsed twice with PBS. Total cell extracts were prepared with Cell Lysis Buffer (Cell Signaling Technology), sonicated and cleared by centrifugation at 15.000 g, 4°C. Total protein concentration was measured using the BCA assay kit (BioRad) with bovine serum albumin as a standard, according to the manufacturer's instructions.

Treatment of cells with LY294002 inhibitor and immunoblotting

LY294002, a PI3K inhibitor, was obtained from Cell Signaling Technology. Nontransfected OvCar3 cells and those overexpressing each OPN splice variant or empty vector control were cultured as described in Supplementary Information and above for cell proliferation, migration, and soft agar colony assays and then treated with 50 μmol/L of LY294002, according to the following protocols. For proliferation assays, 4 hours after cells were seeded, the medium was removed and replaced with culture medium in the presence of either LY294002 dissolved in DMSO and diluted in media was maintained for all proliferation kinectics. On cell migration assays, culture media containing or not LY294002 inhibitor was added just after conducting streaks on cell culture. Finally, on soft agar colony assays, culture media containing or not LY294002 was changed every 3 days.

Total cell lysates containing 50 μg total protein was subjected to 10% SDS/PAGE and the resolved proteins transferred electrophoretically to nitrocellulose membranes (Millipore). To examine PI 3-Kinase activation, we analyzed the levels of Ser^{473} phosphorylation of Akt, a downstream effector of PI 3-Kinase. After blocking with PBST (phosphate buffered saline containing 0.05% Tween 20) containing 5% bovine serum albumin for 1 h at room temperature, membranes were incubated with Akt and Phospho-Akt antibodies (Cell Signaling Technology) according to the manufacturer's instructions. The horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (Pierce, Rockford) were diluted 1:1000 in PBST containing 5% bovine serum albumin and incubated for 1 h at room temperature. Chemoluminescensce detection (Amersham Biosciences) was conducted in accordance with the manufacturer's instructions. The densitometric signal was quantified using ImagePro Plus software version 4.0 (Media Cybernetics, Silver Spring, MD) and normalized to that of loading control as appropriate. Blots were conducted in triplicate in at least 3 independent experiments. Mean densitometric values were calculated.

In vivo studies

For the analysis of tumor progression *in vivo* we used athymic BALB/$c^{nude/nude}$ mice. Transfected cell clones were trypsinized, washed, and resuspended in PBS. Mice were injected subcutaneously (s.c.) into the left flank with 5×10^6 cells. Tumor volumes (V) were analyzed every 5 days using the following formula: $V\ (mm^3) = \text{width} \times \text{length}^2 \times 0.52$. A total of 25 mice were randomly assigned to five groups (5 mice/group) corresponding to five transfectant clones (wild type OvCar-3, OvCar-3 cells transfected with either OPNa, OPNb, OPNc, or empty vector). Pictures were taken 15 days after injection.

Data and statistical analysis

All results are presented as mean ± standard error of at least 3 independent experiments. For *in vitro* and *in vivo* data assays, statistical comparisons among the groups were conducted by the Student's t-test or ANOVA. $P < 0.05$ was considered significant. P values are indicated in the Figure legends.

Results

OPNc is specifically expressed in ovarian tumor samples

As a first step to investigate the expression profiling of distinct OPN splice variants in ovarian carcinoma, we analyzed OPNa, OPNb and OPNc RNA levels in ovarian cell lines and human ovarian tumor and nontumor tissues. OPNa and OPNb were expressed in tumor and nontumor cell lines and tissues, whereas OPNc was only expressed in tumor samples (Fig. 1A and B). In ovarian tumor cell lines, OPNa and OPNb present higher expression level as compared with an immortalized ovarian surface epithelium (IOSE) cell line. In ovarian malignant and borderline tumor samples, these isoforms presented variability in expression levels. Notably, OPNc expression was not detected in benign as well as in normal ovarian tissues (Fig. 1B). These findings suggest that OPN isoforms present differential expression patterns in tumor and nontumor ovarian samples, with OPNc only expressed in tumor samples. These data, together with previous reports showing that OPN isoforms have tumor-specific expression and functional properties (16–19), prompted us to investigate their functional roles in ovarian cancer biology.

Ectopic expression of OPNc is associated with increased cell proliferation

To examine the contributions of each OPN isoform in ovarian cancer progression, we used *in vitro* and *in vivo* gain-of-function experiments. We chose the approach to ectopically overexpress each of these splicing isoforms in OvCar-3 cells, once these cells are a well-established model for ovarian cancer functional studies (23, 24).

A number of cellular events are associated with tumor progression and an increase of cell proliferation is one of them. We first asked whether the proliferation rates of OvCar-3 cells overexpressing OPNa, OPNb and OPNc were altered as compared with an empty vector control clone. As shown in Fig. 2A, OPNc overexpressing cells present higher proliferation rates as compared with OPNa, OPNb, empty vector, and OvCar-3 nontransfected cell clones in the range of 4 to 96 hours of cell culture. The same proliferation behavior was observed when testing stably expressing clones presenting different OPN isoform

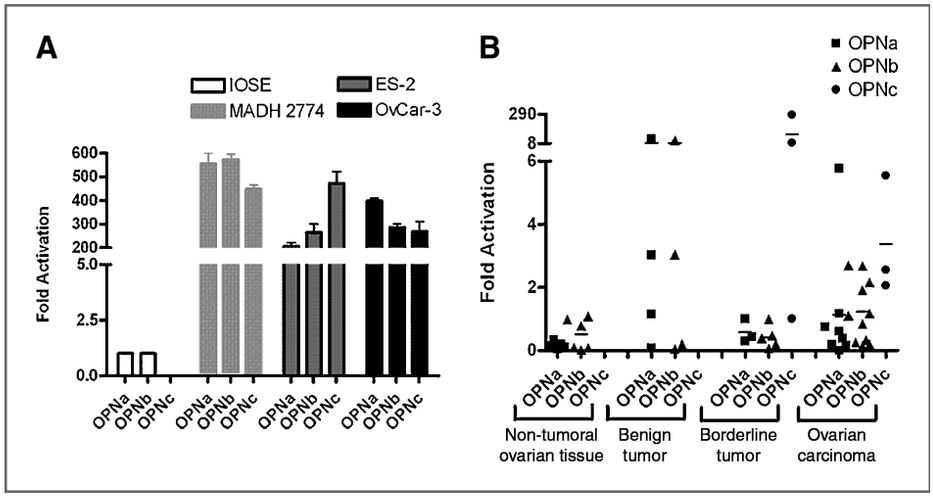

Figure 1. OPNc is specifically expressed in ovarian tumor samples. OPN isoform expression levels were analyzed by real-time PCR using isoform specific primers and were represented by fold activation and GAPDH amplification as a control for template amount. Fold activation represents the increase of expression level of each OPN isoform in relation to IOSE cell line and an ovarian nontumor tissue. A, OPN splice variants present variable expression levels in different ovarian cell lines. Ovarian tumor cell lines (MADH 2774, ES-2, and OvCar-3) and an immortalized ovarian surface epithelium (IOSE) normal cell line (IOSE) were tested. OPNc was only expressed in the ovarian tumor cell lines, but not in the IOSE cell line, whereas OPNa and OPNb were expressed in all cell lines analyzed. Results are representative of 3 independent experiments and horizontal bars represents standard deviations (SD) B, OPNa, OPNb, and OPNc expression levels in different ovarian tissue samples, as represented by the legends on the right and on abscissa axis. Each OPN isoform is represented by a different symbol as indicated. Patient data are shown on Supplementary Information. Horizontal bars represent the median splice variant expression level. OPNc was only expressed in ovarian carcinoma and ovarian borderline tumor samples, and not in ovarian benign tumors or normal tissues, while OPNa and OPNb were expressed in all ovarian tissues analyzed.

expression levels, as shown on Figure 2A and Supplementary Figure 1. Similar results were obtained when analyzing proliferation rates by tritiated thymidine incorporation (Fig. 2B) and trypan blue exclusion (data not shown) assays. We further tested whether OPNc could affect cell proliferation even under serum-starving conditions (Fig. 2C), which are known to induce cell cycle arrest and apoptosis in ovarian cancer (25, 26). In this experimental condition, OvCar-3 cells overexpressing OPNa, OPNb, empty vector, and OvCar-3 nontransfected cells exhibited reduced proliferation rates as compared with OPNc overexpressing clones. These results suggest that OPNc increases the ability of OvCar-3 cells to grow independently of growth factors, a typical feature of a protein involved in tumor progression (27). Consistently, these results support the hypothesis that expression of OPNc modulates the growth of ovarian cancer cells, even in serum-starved conditions. We then hypothesized that the higher proliferation rates observed for OPNc overexpressing clones could be explained by induction of cell death promoted by either OPNa or OPNb overexpression. Hence, we assessed the susceptibility to cell death of OvCar-3 cells transfected with either OPNa, OPNb, OPNc, or with empty vector. All these cell clones presented a low proportion of cells presenting fragmented DNA content, as shown by flow cytometry, which is indicative of a low proportion of cells undergoing apoptosis (Supplementary Fig. S2A and B). These results further confirm the potential role of OPNc on favoring ovarian cancer cell proliferation, which is not associated with stimulated cell death promoted by OPNa or OPNb.

OvCar-3 cells overexpressing OPNc present faster migration, invasion, and increased MMP2, MMP9, and VEGF expression

Next we examined whether OPN splicing isoforms are able to induce additional altered phenotypes associated with tumor progression in OvCar-3 cells. Cell migration, a component of cellular invasion, contributes to several important steps in tumor progression. We asked whether full-length OPNa or the splice variants, OPNb and OPNc, are able to activate OvCar-3 cell migration. OvCar-3 cells overexpressing OPN splicing isoforms or empty vector were subjected to an *in vitro* wound closure assay (Fig. 3A). The assays were conducted over 72 hours and in the presence of mitomycin C, which suppresses the proliferation of rapidly growing cells. Migration of OPNc expressing cells was more extensive as compared with OPNa, OPNb, empty vector-expressing cells and OvCar-3 nontransfected cells. Eighteen hours after scratching, OPNc activated OvCar-3 cell motility into the wound. After 24 hours, OPNc expressing cells completely closed the wound edges. In contrast, OPNa, OPNb, vector controls and OvCar-3 cells did not completely close the wound until 72 hour time point (data not shown).

Higher cancer cell mobility combined with increased expression of proteases that degrade the extracellular matrix is generally predictive of invasive capability (2). We next tested the effects of OPN isoforms overexpression on OvCar-3 invasive capacity by using transwells coated with Matrigel. OPNc overexpression stimulated OvCar-3 invasion capacity by at least 5.0-fold ($P < 0.001$), as compared with cells overexpressing OPNa, OPNb and empty vector control (Fig. 3B and C).

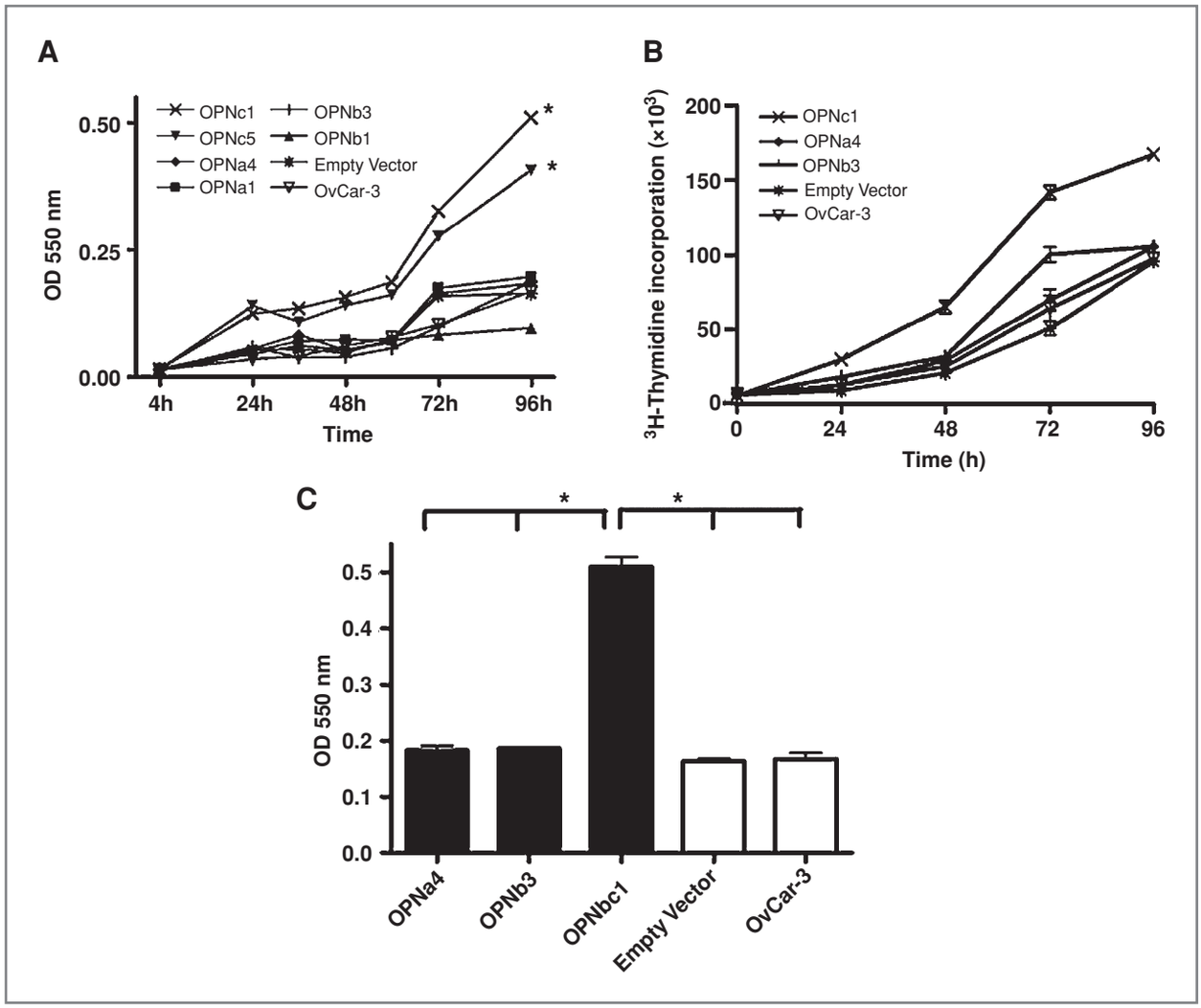

Figure 2. OPNc alters the proliferative profile of OvCar-3 cells. (A) Stably transfected cells with either empty vector, OPNa, OPNb, OPNc, or OvCar-3 nontransfected cells were plated as indicated in Material and Methods and Supplementary Information. On every consecutive day, 3 wells per cell line were harvested and the total number of cells was measured. Proliferation kinetics analysis was evaluated by crystal violet staining. OPNc had a higher proliferation rate as compared with OPNa, OPNb, and empty vector. Clones OPNa4, OPNb3 and OPNc1 had similar expression levels of each OPN splicing isoform, whereas clones OPNa1, OPNb1, and OPNc5 present differential expression levels (Supplementary Figure 1). *, $P < 0.05$ vs. empty vector control clone B, cell proliferation analyzed by (^{3}H) thymidine incorporation. ^{3}H-thymidine was added and the cells were washed, fixed and counted. C, OPNc accelerates cell proliferation under reduced-serum conditions. Cells were grown in 0.2% FBS and cell numbers were counted by crystal violet staining at 96 hours after plating. O.D., optical density measured at 550 nm. *, $P < 0.0001$ versus empty vector control clone.

Matrix metalloproteinase (MMP)-2 (gelatinase A) and MMP-9 (gelatinase B) are key players in the process of degrading extracellular matrix during tumor cell invasion. Ovarian cancer cells express MMP2, MMP9 and vascular endothelial growth factor (VEGF) and their increased expression is associated with their invasive and metastatic potential. Besides, a functional interplay between VEGF and MMP2/MMP9 expression has been described in ovarian carcinomas (28). There was a significant increase in MMP-2, MMP-9 and VEGF mRNA levels ($p < 0{,}002$) in OvCar-3 cells overexpressing OPNc as compared with OPNa, OPNb and empty overexpressing clones (Fig. 3D–F).

Secreted OPNc is involved in tumor progression features

Because OPN is predominantly a secreted protein, we sought to investigate whether the effects observed on ovarian cell proliferation, migration and invasion were mainly associated with the secreted OPN splicing isoforms.

We evaluated proliferation-kinetics of nontransfected OvCar-3 and IOSE cells in the presence of conditioned culture medium produced by OPNa, OPNb, OPNc, and empty vector overexpressing cell lines (Figs. 4A and B). IOSE cells were also tested in order to investigate the putative roles of secreted OPN isoforms on activating proliferation in nontumoral ovarian cells, as indicative of

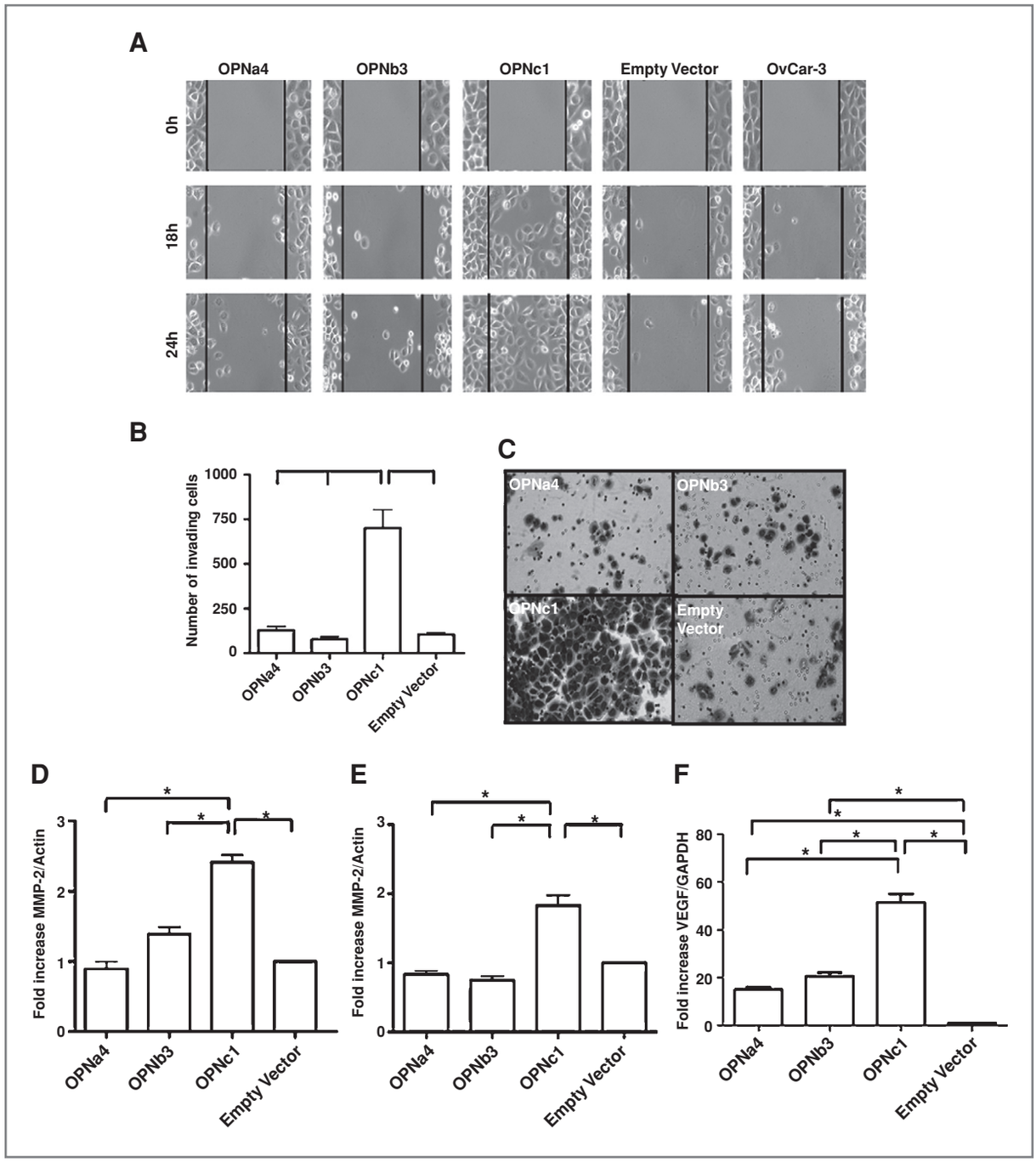

Figure 3. Osteopontin-c supports cell migration and invasion. Cell clones of OPNa1, OPNb3, OPNc1, empty vector, and OvCar-3 s nontransfected cells were plated as indicated in Supplementary Information and analyzed for cell migration and invasion by wound closure and transwell invasion assays, respectively. A, OPNc activates OvCar-3 cell migration. Phase-contrast micrographs photographs were taken at 0, 18, and at 24 hour after subjected to migration are shown. For each cell line, 3 plates were used per experiment. B, OPNc activates OvCar-3 cell invasion. The effects of OPN splice variants overexpression in OvCar-3 cell invasion capacity was determined by transwell invasion assays. Invading cells were stained with crystal violet and the number of cells manually counted. The data are reported as average of number of invading cells $\pm$ S.D. of 3 independent experiments and six microscopic fields were counted per insert. *, $P < 0{,}001$ C, representative image of invading cells stained with crystal violet described in B; D–E, induction of MMP2 (D), MMP9 (E), and VEGF (F) mRNA expression in cells overexpressing OPNc. Total RNA from cells overexpressing OPNa, OPNb, OPNc, and empty vector control was prepared to conducted quantitative real-time PCR (qRT-PCR) using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) or actin as internal controls. The amount of targets was analyzed using the comparative CT method, where the threshold cycle (CT) values of each target sequence are given by the $2^{-\Delta\Delta CT}$ formula. We present the data as log *n*-fold change in gene expression normalized to the endogenous reference genes (GAPDH or actin) relative to the expression of cells overexpressing empty vector control.*, $P < 0.002$.

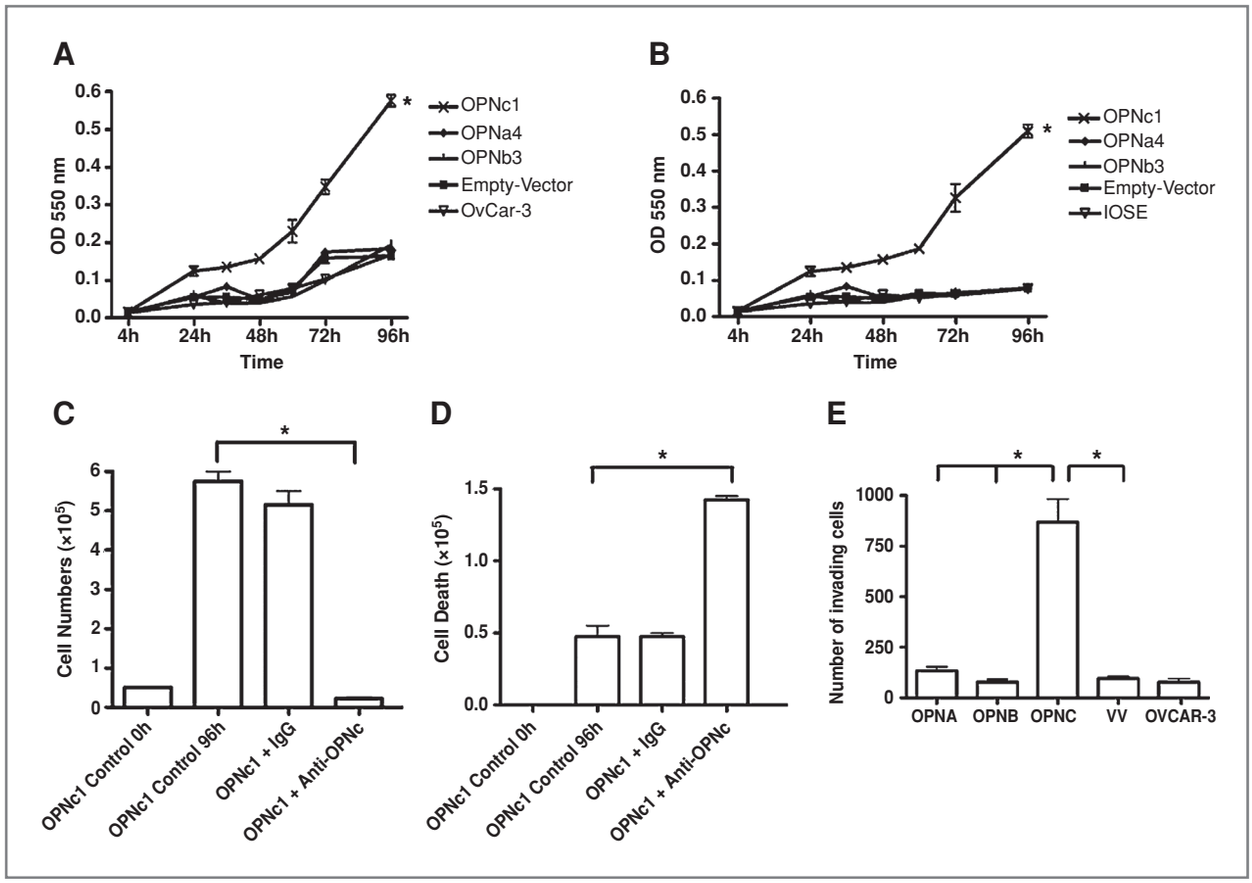

Figure 4. Secreted OPNc is mostly involved in OPNc pro-proliferative features. OvCar-3 A, and IOSE non-transfected cells B, were assayed for cell proliferation rates by crystal violet staining after incubation with conditioned medium from OPNa, OPNb, OPNc, and empty vector overexpressing cells. Legend on the right indicate cell clones tested. All results are representative of at least 3 independent experiments. O.D., optical density measured at 550 nm. The standard deviations (error bars) indicate the variability within each experiment. *, $P < 0.05$ vs. IOSE nontransfected cells C, D, 5×10^4 of OvCar-3 cells overexpressing OPNc (clone OPNc1) were plated and the total number of cells as indicative of cell proliferation (C) and cell death (D) was measured by trypan blue exclusion staining. Cell number was measured at 0 hour time point (bar OPNc1 control 0 h) and after 96 of cell culture (bar OPNc1 control 96 hours). Cells were treated with anti-OPNc antibody (bar OPNc1 + anti-OPNc) or with rabbit anti-human IgG (bar OPNc1 + IgG) at the concentration of 4 μg/mL for 96 hours. Significant differences in cell number are represented by asterisk. *, $P < 0.0001$ vs. OPNc1 control 96 hour. E, OPNc conditioned media is a chemoattractant for OvCar-3 cells invasion capacity. Matrigel invasion assays were conducted using conditioned media form cells overexpressing OPNa, OPNb, OPNc, and empty vector control as chemoattractant for OvCar-3 cell invasion thorugh transwell invasion assays. Invading cells were stained with crystal violet and cell counting was carry out manually. The data are reported as average of number of invading cells ± S.D. of 3 independent experiments and six microscopic fields were counted per insert. *, $P < 0.05$.

early steps of tumorigenesis. These assays were conducted in serum-free conditions, in order to eliminate skewing of the results by cell activation promoted by growth factors. OvCar-3 and IOSE cells cultured in OPNc conditioned medium displayed higher proliferation rates as compared with cells cultured with OPNa, OPNb, or empty vector conditioned medium (Figs. 4A and B). A serial dilution analysis of OPNc conditioned medium promoted a proportional decrease on OvCar-3 and IOSE cell proliferation rates (Supplementary Figure 3).

We then show that these changes in cell proliferation were directly dependent on the secreted OPNc protein, as the addition of a neutralizing polyclonal anti-OPNc antibody, but not control immunoglobulin, to the cultures every other day with the exchange of medium, significantly suppressed proliferation rates promoted by OPNc OvCar-3 overexpressing cells (Fig. 4C). Accordingly, OPNc OvCar-3 overexpressing cells treated with the anti-OPNc antibody, also presented an increase in cell death, further evidencing a survival role for OPNc in this ovarian tumor cell line (Fig. 4D). In contrast, the antibody incubation had no significant effects on cell proliferation and cell death of control clones formed by nontransfected or OPNa OvCar-3 overexpressing cells (Supplementary Figure 4A–D), indicating that cell death induced by the depletion of OPNc using the anti-OPNc neutralizing antibody was specific for cells overexpressing this isoform.

Altogether, these data indicate that secreted OPNc not only activates the proliferation of an ovarian tumoral cell, such as OvCar-3, but also stimulates proliferation of ovarian normal cells, indicating a role of secreted OPNc not only on

ovarian tumor progression, but also in ovarian tumorigenesis and cell survival.

To obtain further functional insights related to migration and invasion profiles modulated by OPN isoforms, we next evaluated whether the effect on cell migration and invasion was mediated by the secreted OPN isoforms. Wound closure assays were carried out with nontransfected OvCar-3 cells cultured in the presence of conditioned culture medium from clones transfected with each OPN splicing isoform. Similarly to OPNc overexpressing clones, as observed on Figure 3A, OvCar-3 cells cultured in the presence of OPNc conditioned medium completely closed the wound edge 18 h after scratching (Supplementary Figure 5). On the other hand, cells cultured with conditioned medium from with either OPNa, OPNb or empty vector-overexpressing cells showed limited migration (Supplementary Figure 5) and did not reach this stage until 72 hours after scratching (data not shown). Similarly, when testing conditioned media from cells overexpressing each isoform as chemoattractant for nontransfected OvCar-3 cells on transwell invasion assays, conditioned media from cells overexpressing OPNc significantly stimulated OvCar-3 cell invasion (Fig. 4E).

Taken together, these results indicate that secreted OPNc acts as an activating factor for ovarian cell proliferation, migration and invasion, typical features of tumor progression.

OPNc enhances soft agar colony formation

Soft agar colony formation reflects a combination of growth rate (cell cycle progression) and anchorage independence (antianoikis). The ability to form colonies in semisolid medium correlates well with tumor malignancy and metastasis formation. We further analyzed the effect of OPN splicing isoform overexpression on the potential of OvCar-3 to form colonies in soft agar. OPNc significantly increased the number of colonies formed, whereas OPNb inhibited this process. OPNa isoform promoted no significant effect on the number of colonies formed (Fig. 5A). OPNa, OPNb, and OPNc promoted an increase in the size of colonies formed (Fig. 5B) as compared with control cells, but for OPNc overexpressing cells these effects were even stronger. These data indicate that OPNc can act as a strong activating factor on soft agar colony formation, whereas OPNb can inhibit the number of colonies formed.

OPNc enhances tumor growth *in vivo*

To assess the roles of OPN splicing isoforms on ovarian progression *in vivo*, equal numbers of OvCar-3 cells stably transfected with OPNa, OPNb, OPNc, empty vector, or nontransfected OvCar-3 cells were implanted subcutaneously into athymic nude mice and the growth of the implanted tumors was measured. The animals were monitored for tumor growth and xenograft tumors volumes were measured. Five days after the injections, small tumors were observed in all the groups of mice. Notably, cells overexpressing OPNc resulted in extremely rapid tumor growth and formation of larger ($\geq$200 mm^3) tumors between 10 and 15 days after s.c. injection (Fig. 5C and 5D). In these tumors, 3 known markers of tumor progression, MMP2, MMP9, and VEGF were consistently upregulated ($P < 0.001$; Fig. 5E). In contrast, tumors resulting from OPNa, OPNb or empty vector expressing clones were of comparable size to those generated with OvCar-3 nontransfected cells. Taken together, these results indicate that overexpression of OPNc is able to enhance tumor growth rates and progression of xenograft tumors formed by OvCar-3 cells.

OPNc mediates ovarian carcinoma progression features through the PI3K/Akt pathway

OPN bind to a variety of cell surface integrins, CD44 and epidermal growth factor receptor (EGFR) and function as signal transducers to promote cell proliferation, adhesion, motility and survival through activating kinase cascades, and transcription factors (2). Among signaling pathways typically activated on ovarian tumor progression, phosphatidylinositol 3-kinase (PI3K) present an important prosurvival role (29–33). Previous reports indicate that the gene encoding the p110α catalytic subunit of (PI3K) is increased in copy number in ovarian cancer cells (32, 33). PI3K binds to and is activated by several receptor and non receptor tyrosin kinases and its oncogenic form can induce cellular transformation. The best known downstream target of PI3K is the serine-threonine Akt, which transmits the angiogenic and oncogenic signals from growth factors. In ovarian tumorigenesis, this pathway plays critical roles in invasion and metastasis (32). Given these observations, we sought to determine if the overexpression of each OPN isoform would modulate PI3K activity and Akt phosphorylation (Ser^{473}) and whether this signaling pathway is important to mediate specific OPN splicing variant effects on ovarian cancer progression.

OPNc overexpression, but not the remaining isoforms, resulted in a 2-fold increase in Akt (Ser^{473}) phosphorylation (Fig. 6A). ERK 1/2 phosphorylation was not observed on cell extracts overexpressing OPNa, OPNb and OPNc splice variants (data not shown). Having shown that OPNc overexpression induces activation of PI3K/Akt pathway, we next examined the effects of inhibiting this pathway on cells overexpressing each OPN isoform. We used a specific small molecule inhibitor of PI3K, LY294002, to assess the effects of inhibiting PI3K on OvCar-3 cells proliferation, migration, and soft agar colony formation (Fig. 6 B–E).

To elucidate whether PI3K affects the proliferation of OvCar-3 cells overexpressing each OPN isoform we evaluated proliferation kinectics by crystal violet assay in the absence and presence of LY294002. Optical density at 550 nm, reflecting total cell number, was counted 24, 48, 72, and 96 hours after the incubation. As shown on Figure 6B, proliferation rates of OvCar-3 cells overexpressing OPNc was greatly increased over the period of 96 h in culture in the absence of LY294002. The proliferation of OPNc overexpressing cells was slightly decreased by the addition of 50 μmol/L LY294002 24 and 48 hours after the treatment. However, after 72 and 96 hours of the treatment,

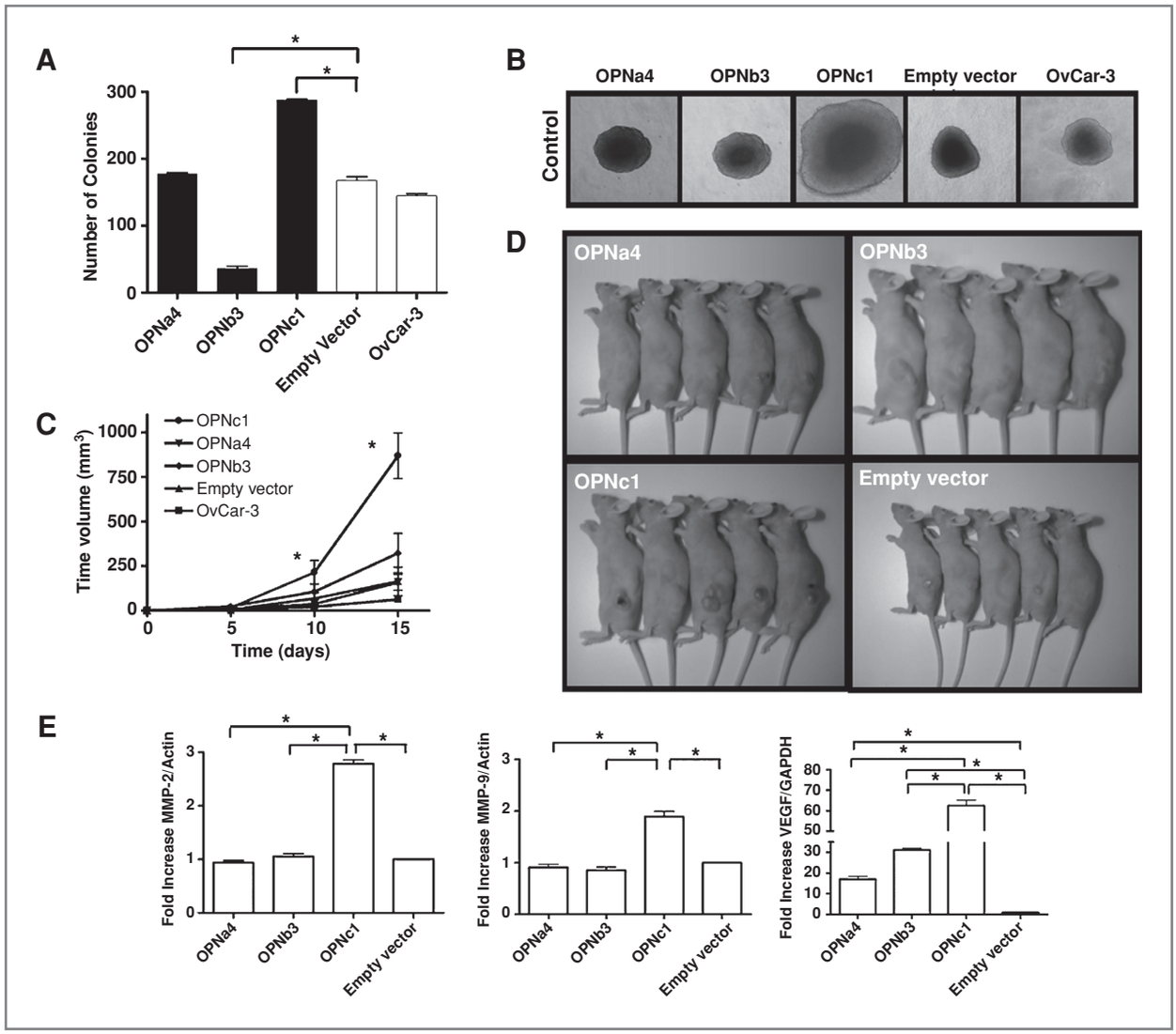

Figure 5. Expression of the OPNc enhances the colony formation in soft agar and potentiates tumor *in vivo* formation. Soft agar assay was carried out to assess the ability of stably transfected cell lines to grow in an anchorage independent environment as described in Supplementary Information. Plates were examined microscopically for growth after 30 days. A, quantification of the number of OvCar-3 cell colonies grown in semisolid agarose medium transfected either with empty vector, OPNa, OPNb, or OPNc expression vectors. *, $P < 0.01$ vs. empty vector cells. B, phase-contrast microscopy of 2 representative OvCar-3 colonies. Cell overexpressing OPNc form colonies with larger size than those overexpressing OPNa, OPNb, empty vector, or OvCar-3 nontransfected cells. Pictures were obtained in 5× magnification. All results are representative of at least 3 independent experiments. C, OvCar-3 transfected and nontransfected cells were implanted s.c. in the right flanks of BALB/c nude mice and the animals were monitored for tumor formation. Tumor growth rates were measured every 5 days. Cells overexpressing OPNc present higher tumor volumes. *, $P < 0.01$ vs. empty vector cells. D, enhanced tumor growth in mice injected with OvCar-3 cells overexpressing OPNc. Representative pictures of tumors grown in nude mice. Tumor development was monitored over 15 days. E, induction of MMP2 (left panel), MMP9 (middle panel) and VEGF (right panel) mRNA expression in xenograft tumors overexpressing OPNc. Total RNA from xenograft tumors grown from cells overexpressing OPNa, OPNb, OPNc, and empty vector control was prepared to conduct quantitative real-time PCR (qRT-PCR) using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) or actin as internal controls. The amount of targets was analyzed using the comparative CT method, where the threshold cycle (CT) values of each target sequence are given by the $2^{-\Delta\Delta CT}$ formula. We present the data as log *n*-fold change in gene expression normalized to the endogenous reference genes (GAPDH or actin) relative to the expression of cells overexpressing empty vector control. *, $P < 0{,}001$.

the proliferation of the OPNc overexpressing cells was significantly inihibited by LY294002. These results indicate that PI3K may play a role in the induction of proliferation promoted by OPNc splicing isoform. Cells overexpressing OPNa, OPNb, and empty vector control as well as nontransfected OvCar-3 cells present no modification on proliferation rates after LY294002 treatment (Fig. 6B).

To determine whether the inhibition of PI3K activity by LY294002 also affects OvCar-3 cell migration, cells overexpressing each OPN isoform were treated with LY294002

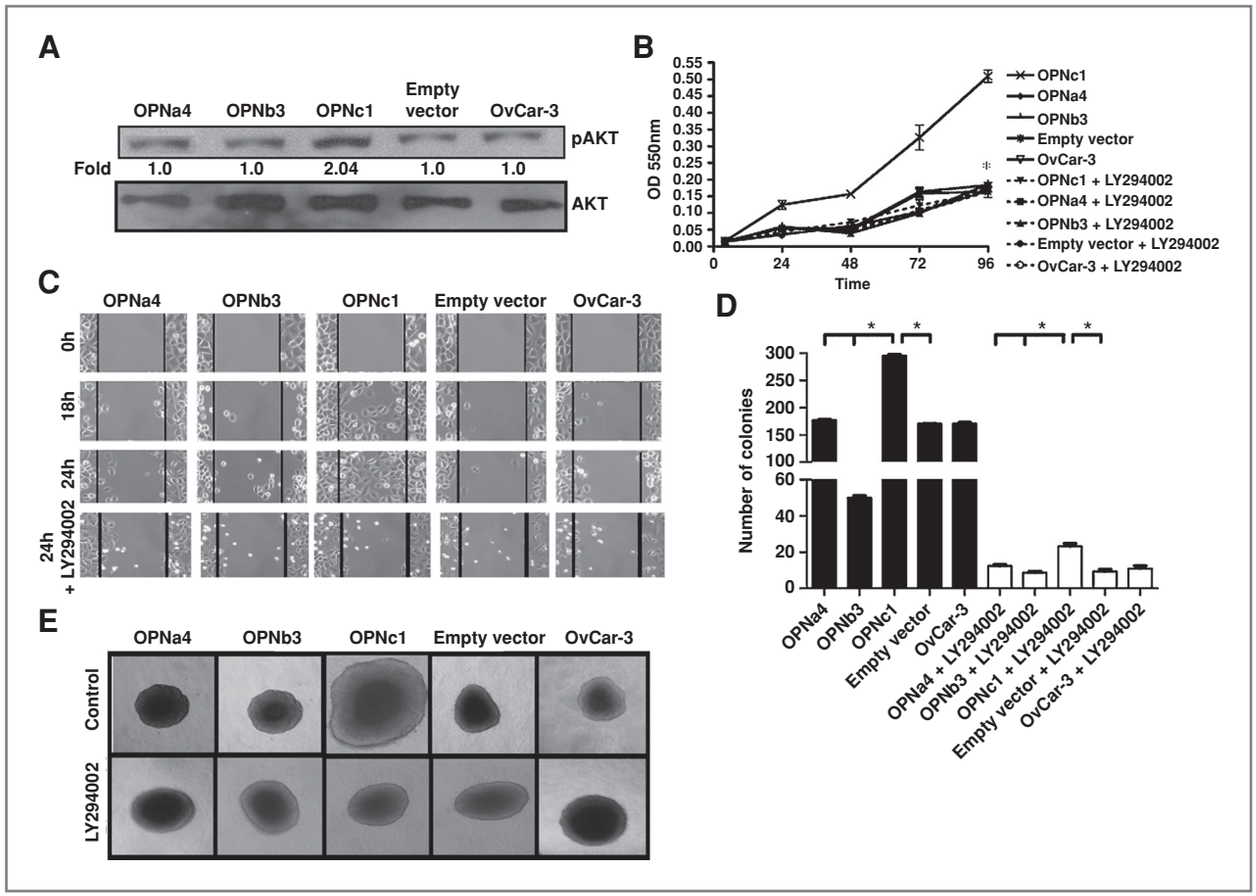

Figure 6. PI3K/Akt signaling pathway mediate ONPc roles on activating tumor progression features. A, PI3K/Akt signaling is activated in OvCar-3 cells overexpressing OPNc. Total Akt and p-Akt levels in OvCar-3 cells overexpressing OPNa, OPNb, OPNc, and empty vector control were measured using Western blotting using their specific antibodies. Non phospho Akt were used as a loading control. Fold changes were calculated based on densitometric quantification; B, OPNc-induced OvCar-3 cell proliferation is specifically blocked by the PI3K inhibitor. Growth curves of OvCar-3 overexressing clones and nontransfected OvCar-3 cells cultured with or without PI3K LY294002 inhibitor are plotted. LY294002 was added 4 hours after cell plating and culture media maintained for all proliferation kinectics as measured by crystal violet assay.). *, $P < 0.0001$ vs. empty vector control clone; C, OPNc-induced OvCar-3 cell migration is blocked by the PI3K inhibitor. OvCar-3 cells were seeded, and migration capacity was evaluated after carrying out streaks in wound closure assay. LY294002 inhibitor was added just after carrying out the streaks and cell migration was analyzed at the 24 hour time point; D, OPNc overcomes LY294002 effects on inhibiting the number of OvCar-3 cells colonies formed in soft agar. Colonies were cultured in soft agar in the presence or absence of LY294002 inhibitor added to culture medium, which was changed every 3 days until reaching 30 days of culture. *, $P < 0{,}002$; E, the increase of size of colonies of OvCar-3 formed in soft agar promoted by OPNc overexpression was reversed by the PI3K inhibitor. OvCar-3 cells overexpressing OPNa, OPNb, OPNc, and empty vector control were cultured in the presence and absence of LY294002 and the the size of colonies was evaluated until reaching 30 days of cell culture in soft agar. Original magnification 5×.

as described above and cell migration was evaluated by wound closure assay. The treatment of OvCar-3 cells with 50 μmol/L of LY294002 for 24 hours specifically inhibited the migration of cells overexpressing OPNc (Fig. 6C).

Similarly, the inhibition of PI3K activity significantly reduced the size of colonies of anchorage independent growth formed by OvCar-3 cells overexpressing OPNc. The number of colonies formed decreased 5- to 12-fold after LY294002 treatment on cells OvCar-3 overexpresing either OPNa, OPNb, OPNc, empty vector control, and wild-type OvCar-3 cells. However, cells overexpressing OPNc treated with LY294002 could better overcome the effects of this inhibitor, once the number of colonies formed was ∼50% higher as compared with clones overexpressing OPNa, OPNb, emtpty vector, and OvCar-3 cells nontransfected cells. These results indicate that OPNc probably exert an antagonist effect over LY294002 treatment on soft agar colony formation, probably through OPNc roles on activating ovarian cancer cell survival. Taken together, these results indicate that the PI3K signaling pathway is required for some OPNc oncogenic features related to ovarian cancer progression.

Discussion

We have shown here the first description of OPN alternative splicing expression in ovarian carcinoma cell lines and human tissue samples. We further show that the OPNc

isoform, when overexpressed in OvCar-3 cells, is able to promote several aspects of ovarian cancer tumor progression, both *in vitro* and *in vivo*. Additionally, this paper is the first description of the involvement of PI3K signaling pathway mediating OPN isoform-specific roles on promoting tumor progression features. Ovarian malignant transformation is caused by genetic modifications that disrupt the regulation of proliferation, programmed cell death and senescence, but the specific genetic pathways involved in these processes are poorly understood (34, 35). Alternative pre-mRNA splicing is an important molecular mechanism associated with tumorigenesis and cancer progression (36) and has been shown to be one of the mechanisms by which cancer cells alter the structure and function of OPN (16–19, 37). Based on these assumptions, we believe that describing the functional significance of OPN splicing isoforms in ovarian cancer would improve the understanding of signaling pathways involved in the progression of this complex neoplasic disease. Alternative splicing of OPN has been described in mesothelioma, breast cancer, hepatocellular carcinoma, glioma, lung cancer and head and neck squamous cell carcinoma (HNSCC) and the roles of different splicing isoforms on cancer growth and progression has been reported (16–19, 37). However, OPN splicing and its resulting products has not been examined previously in ovarian cancer.

To address these issues, we firstly explored the occurrence of OPN isoforms in ovarian tumor and nontumor cell lines and tissues. Our results have shown that OPNa and OPNb are expressed in all ovarian cell lines analyzed, both normal and tumor, but OPNc is only expressed in tumor samples, including tumor cell lines and malignant and borderline tumor tissues. Therefore, our data indicate that OPNc isoform is specifically expressed in ovarian tumor samples, as previously described for breast cancer cells (16, 17). In contrast, OPNc is barely detected in mesothelioma tumors and in lung cancer samples, whereas OPNa and OPNb showed upregulation in these samples (18, 19). Our data further support previous findings showing that OPN isoforms present tumor-specific expression patterns.

The differential expression profiling of OPNa, OPNb and OPNc expression in ovarian samples and the important cellular effects due to OPNc overexpression observed herein, could be the basis for future studies to better investigate their potential use of OPNc as an ovarian cancer biomarker. Examining the expression of these isoforms in an appropriate representative sample of ovarian tissues containing different tumor subtypes and clinical behavior could provide additional information about the involvement of these isoforms in ovarian cancer tumorigenesis and progression.

We then examined the functional roles of each OPN splicing isoform in ovarian cancer progression by using *in vitro* and *in vivo* tumor models. We show here that OvCar-3 cells that overexpress OPNc have increased proliferation, migration and invasion, whereas OPNa and OPNb did not have these effects. Additionally, OPNc significantly increased the number and size of colonies formed in soft agar assay. Interestingly, OPNb inhibited the number of colonies formed, suggesting a potential negative regulatory role of this isoform in the formation of tumor metastasis. Opposing roles for OPN isoforms have also been described previously in breast cancer cells when comparing OPNc versus OPNa (17). In general, tumor cells express splice variants involved in cancer progression with different potential to support metastasis. Among these examples, only certain splice variants of HIF1a (38), Syk (39), CD44 (40) and S100A4 (41) gene products favor metastasis formation, whereas others act as inhibitors. Although we do not provide here direct evidence about the roles of individual OPN isoforms on modulating tumor metastasis, the roles of OPNc on activating MMPs, VEGF, tumor migration, and invasion give strong support to the putative roles of OPNc on stimulating ovarian cancer metastasis, once all this events act cooperatively to facilitate metastasis of cancer. Future studies with appropriate animal tumor models should be conducted in order to elucidate the roles of OPN isoforms on ovarian carcinoma metastasis formation. Altogether our results clearly show that OPNc contributes to ovarian cancer progression and malignancy.

We also observed herein that OPNc stimulate OvCar-3 cell proliferation rates independently of growth factors, a typical feature of a protein involved in tumor progression (27). OPNc could stimulate growth signal autonomy by different strategies, such as alteration of extracellular growth signals, of transcellular transducers of those signals or of intracellular circuits that translates those signals into action (26). Literature has provided evidence that total OPN affect the expression of genes involved in multiple aspects of tumor progression and malignant growth, including those involved on self-sufficiency in growth signals (42, 43). In ovarian carcinoma, among all isoforms, OPNc could better stimulate these signals as compared with the remaining isoforms. As shown herein, OPNc overexpression stimulate PI3K signaling pathway, which could be one of the molecular mechanisms of activating transducer growth signals and signaling pathways activating cell proliferation and/or rescuing cells from apoptotic stimuli such as serum starvation. Our data also give support to the hypothesis that the increase in cell proliferation observed for cells overexpressing OPNc, as compared with OPNa, OPNb and vector control, is not due to differential levels of cell death promoted by these OPN isoforms, but rather to an enhancement of cell survival promoted by OPNc. This hypothesis was further reinforced by the observation that when treating OPNc overexpressing cells with an anti-OPN antibody, cells were induced to die, again supporting a role of OPNc in ovarian cancer cell survival. Previous reports have been showing that OPN is able to prevent cell death in response to diverse stress stimuli, including serum starvation (17, 37) and that OPN has been shown to inhibit apoptosis in several systems. According to our data, we propose that OPNc may enhance OvCar-3 survival due to an enhancement of proliferation. Further studies should be carried out to determine whether apoptosis evasion is also involved in OPNc survival roles in ovarian cancer. Besides promoting higher proliferating rates in OvCar-3 tumor cells, we have

also shown that secreted OPNc stimulates IOSE nontumoral cell line proliferation. These data indicate that OPNc is not only involved in ovarian cancer progression and survival, but may also contribute to early steps of ovarian cancer tumorigenesis process.

Current available data about OPNc roles in other tumor models show that the effects of OPNc may vary among different tumor types. For example, in breast cancer cells, OPNc does not affect cell growth, but can stimulate soft agar colony formation (16). By contrast, in mesothelioma and non-small–cell lung cancer (NSCLC) and head and neck squamous cell carcinoma (HNSCC) tumors, isoforms OPNa and OPNb, but not OPNc, are able to stimulate pro-tumorigenic behaviors (18, 19, 37). In lung cancer, OPNc overexpression was associated with decreased angiogenic properties, whereas OPNa and OPNb present opposite roles (19). Therefore, OPN splicing isoforms seem to have cell, tissue and tumor specific and even opposing roles in different tumor progression models. One such example of this opposite roles has been observed when evaluating OPNa functional roles in another ovarian cancer cell line (31). In this model, it was observed that OPNa promoted ovarian cancer progression and survival, in contrast to what has been observed in our study. However, in this study they did not compared their results with the remaining OPN isoforms.

OPNc lacks exon 4, and it is thus likely that the roles of OPNc in tumor progression are related to the absence of the protein sequence contained in exon 4. However, the physiological and pathophysiological roles of the N-terminal domain of OPN, including exon 4, are still poorly understood. Some evidence about functional properties of exon 4 suggests that this region, as well as exon 5, may play a critical role in OPN solubility, making soluble OPN available for receptor ligation (17). Another layer of added complexity is that, besides alternative splicing, post translational modifications (PTMs) of OPN, especially phosphorylation, are functionally important (17, 44–46). Recent reports have also shown that the degree of phosphorylation of OPN isoforms produced by different tumor cell types can regulate its roles and receptor interactions (37). Based on these data, we propose here that the deletion of exons 4 and 5 could alter the pattern of PTMs, promoting functional modifications. According to previous reports evaluating OPN PTMs in tumor cells, OPN splicing isoforms produced by different cell types exhibited different functional properties (46). Similar functional modifications could affect OPNc and OPNb isoforms in ovarian cancer cell lines, due to alterations on their PTMs patterns as a consequence of exon deletion. Further work is required to elucidate PTMs patterns of OPN isoforms and their impact on their roles in ovarian cancer cells.

The molecular mechanisms by which OPN splice variants modulate tumorigenic roles are still unclear and very few data are currently available. Previous reports on hepatocellular carcinoma cell lines showed that OPNa and OPNb activated migration-associated signaling pathways by increasing the expression of urokinase-type plasminogen activator and the phosphorylation of p42/p44 MAP Kinase, but these pathways were not activated by OPNc (47). Another report showed that OPNa and OPNb overexpression promoted tumor growth in pancreatic cancer and fibrosarcoma cells due to less apoptosis and that these effects were signaled mainly through the activation of FAK and NF-kB (37). Most data about signaling pathways activated by human OPN relates to total OPN. Besides post translational modifications, OPN functional diversity results from differential binding to seven types of integrins and specific splice variants of CD44 (26, 47). The engagement of the integrin receptor $\alpha v\beta 3$ by OPN increases the c-Src-mediated phosphorylation of EGFR, activating PI3K-dependent Akt and mitogen-activated protein kinase 1 (MEK1)-dependent ERK1/2 pathways. In contrast, the binding of OPN to CD44 receptors activates the PLC-γ-dependent Akt pathways (47). Besides, crosstalks of these signaling pathways also exists (2). Our data showed that OvCar-3 cells overexpressing OPNc specifically activate PI3K/Akt signaling pathway and that OPNc roles on activating OvCar-3 cell proliferation, migration and soft agar colony formation are mediated by this pro-survival pathway. OPNc roles on overcoming the effects induced by the PI3K inhibitor LY294002 on the number of colonies formed in soft agar are probably related to OPNc signals on counteracting the effects of this molecule as a typical inhibitor of ovarian tumor growth by inducing apoptosis (32). In summary, these results suggest that OPN splice variants differentially couple to signaling pathways to modulate ovarian cancer progression and that previous description of PI3K/Akt pathway involvement on survival-promoting and stimulating cell cycle progression functions of OPN in ovarian carcinoma (31) are probably mainly mediated by OPNc splicing isoform. Our data also reinforce the notion that OPN splicing isoform-specific roles on tumor progression are a result of differential activation of cell surface receptors and signaling pathways.

OPN is predominantly known as a secreted protein (sOPN) and there is increasing knowledge that OPN binding to its receptors and the signaling pathways so activated, mediate different OPN functions (2). Our results suggest that the functional effects we described herein are mediated primarily by secreted OPNc. Nevertheless, based on our data, we cannot exclude the possibility that a putative intracellular OPN (iOPN), recently described (48–51), could act in addition to secreted OPNc and contribute to the effects we describe. Considering the different roles OPNc has in different tumor types, we hypothesize that besides posttranslational modification and proteolytic cleavage (46, 52), an unbalanced proportion of putative iOPN and sOPN isoforms could also modulate the tumor- and tissue-specific functional roles of this splicing isoform, as has been described by others (49, 50). Further studies will be necessary to determine the putative occurrence, regulation, and functional roles of both iOPN and sOPN in tumor cells, specially the occurrence of secreted and intracellular OPNc isoforms in ovarian cancer and their

corresponding roles in this cancer.In conclusion, based on the observation of differential expression of OPN splicing isoforms in tumor and nontumoral ovarian samples, with OPNc identified only in tumor cell lines and tissues, whereas OPNa and OPNb expressed in tumor and non-tumor tissue and cell lines, we examined the functional roles of each isoform in ovarian cancer biology. We found that OPNc overexpression stimulating cell proliferation, migration, invasion, colony formation and tumor growth denotes its important role on ovarian cancer progression. Our data also show that at least some of these OPNc tumorigenic roles are mediated by PI3K/Akt signaling pathways. The observed involvement of OPNc in ovarian cancer progression contributes to a better knowledge about the molecular mechanisms related to this important and complex gynecological malignancy. Together with previous published results on other tumor models, our data reinforce the notion that OPN splicing isoforms may have tumor and tissue-specific roles. An in-depth knowledge of specific behavior of the OPNc isoform and its corresponding gene expression control may open the possibilities of new therapeutic approaches that selectively down regulate OPNc altering its properties which favor tumor progression.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank the Swiss Bridge Foundation, CNPq, FAPERJ, CAPES, and MS-FAF for financial support and Dr. Ann Chambers for critical reading of this manuscript.

Grant Support

Swiss Bridge Foundation, FAPERJ, CAPES, CNPq, Ministério da Sáude and Fundação do Câncer.

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Received October 13, 2010; revised January 11, 2011; accepted January 13, 2011; published OnlineFirst January 24, 2011.

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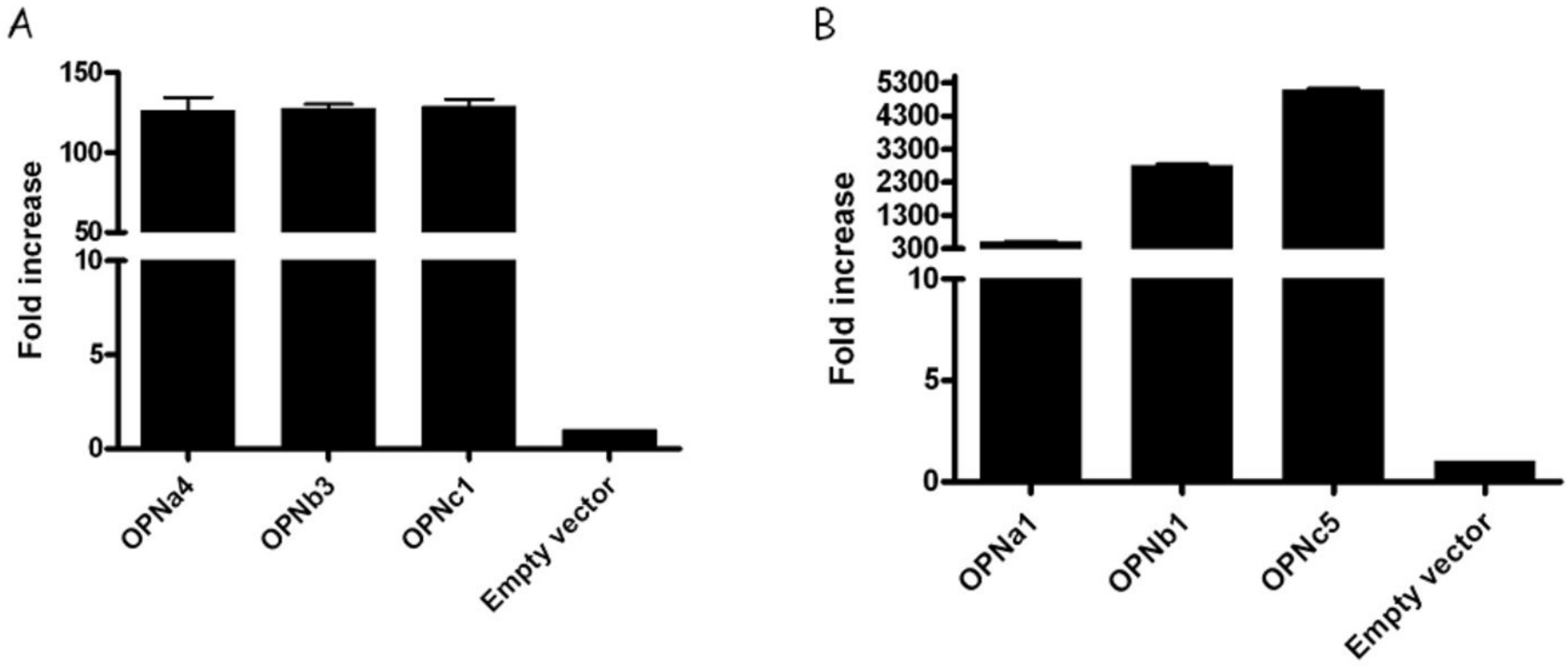
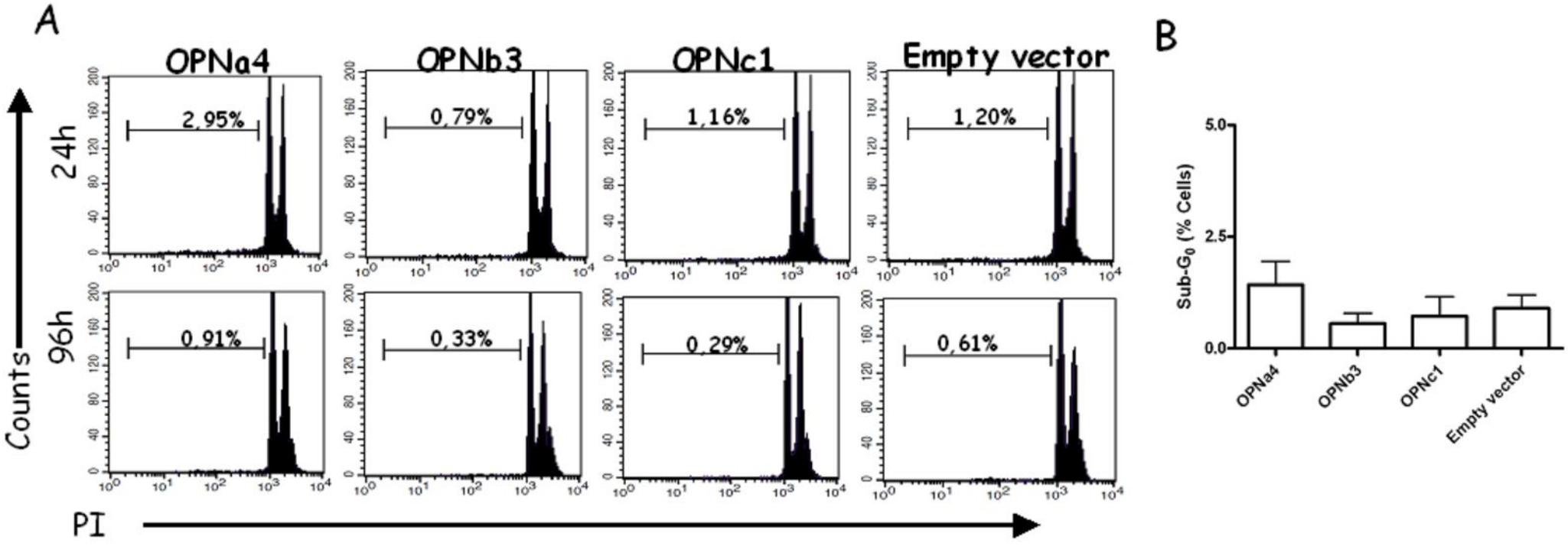
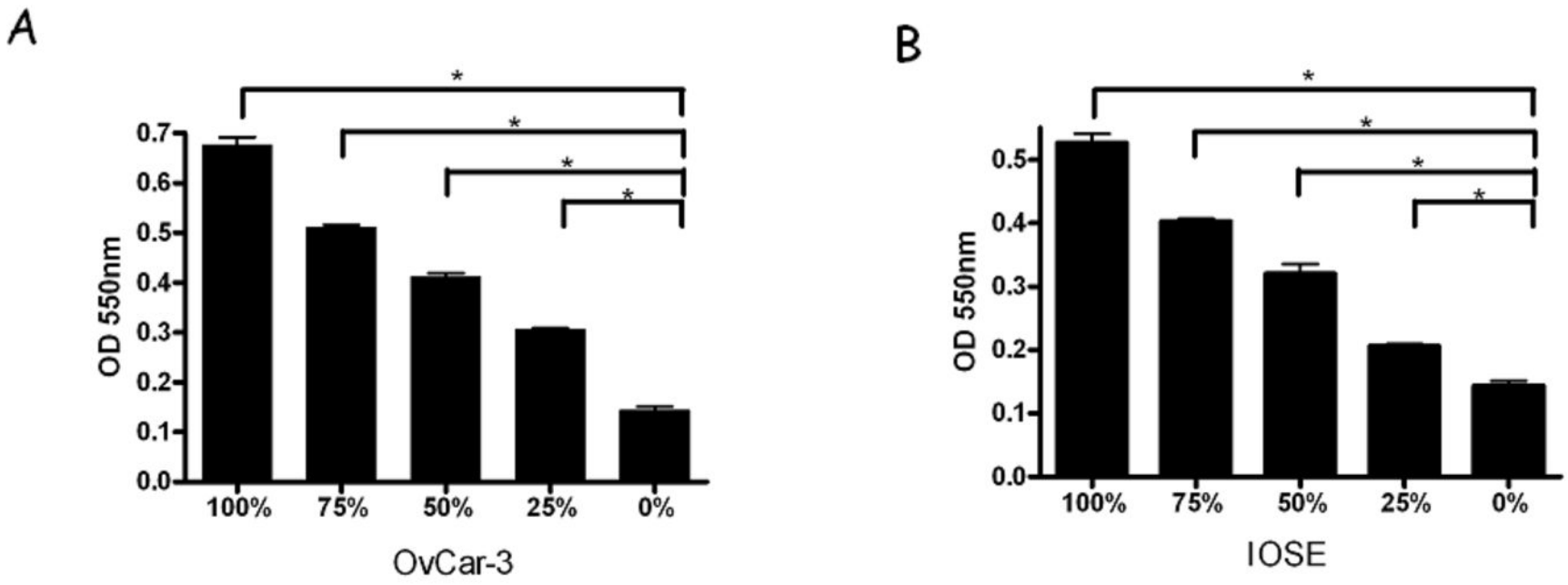


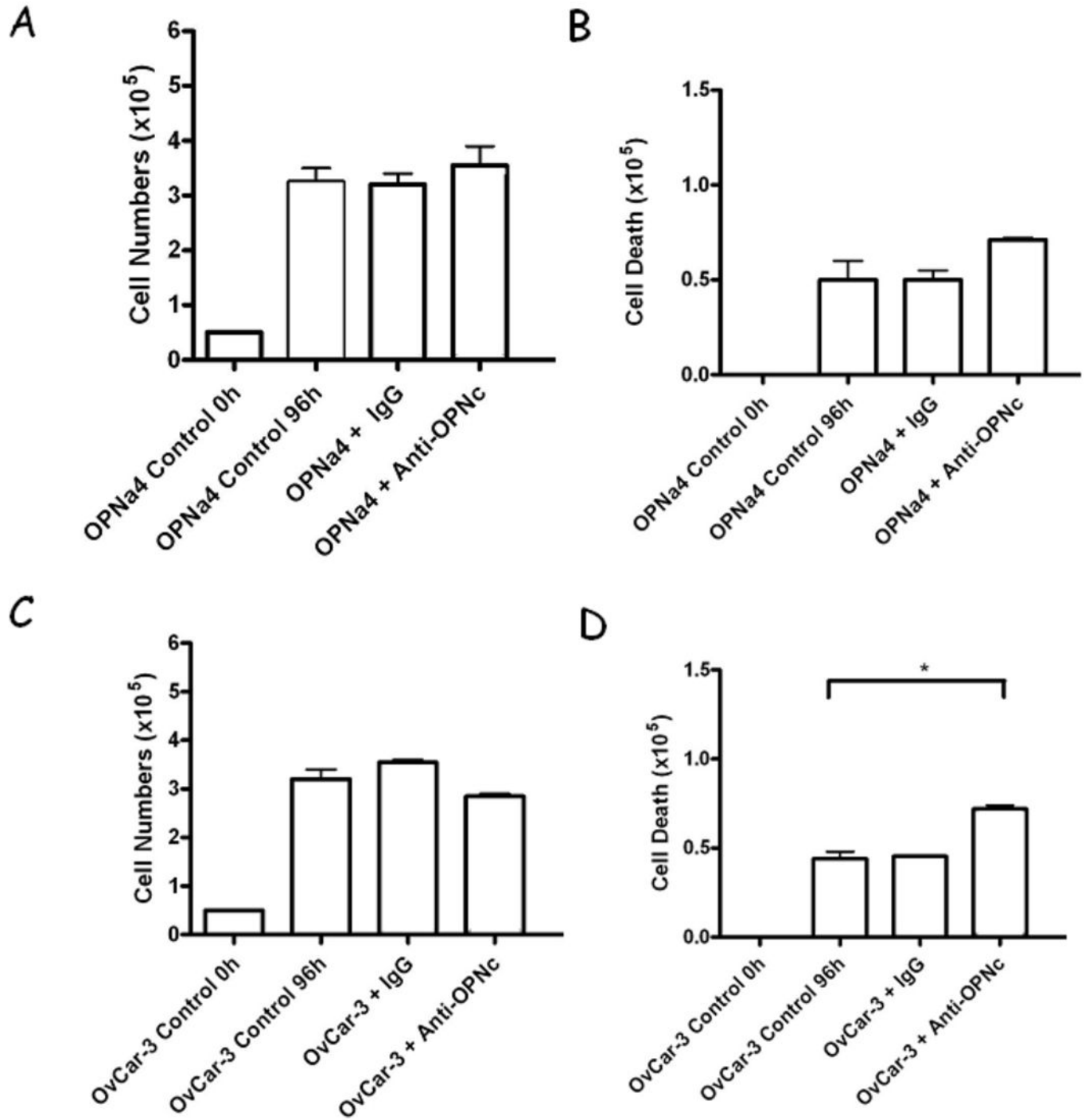
Supplementary Figure 1



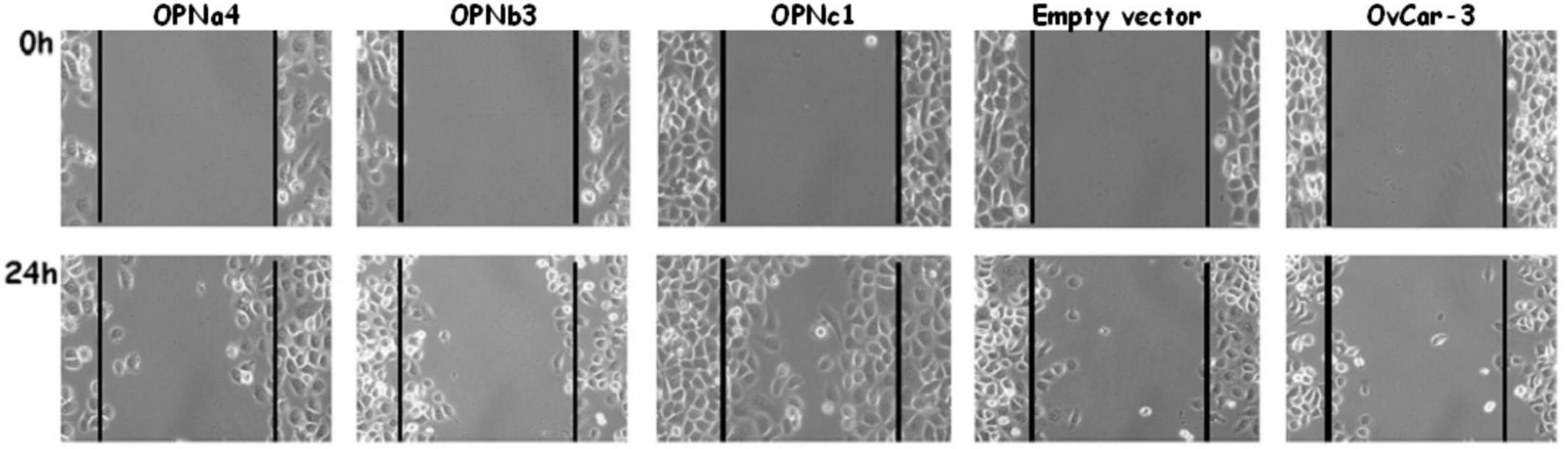
Supplementary Figure 2



Supplementary Figure 3



Supplementary Figure 4



Supplementary Figure 5

Supplementary Figure legends

Supplementary Figure 1. Overexpression of OPN isoforms in OvCar-3 cell lines. (A) OPN isoforms expression levels were analysed by qRT-PCR using isoform specific primers as presented on material and method section. Fold increase represents the augment of expression level of each OPN isoform in relation to empty vector control clone. Transfectant clones OPNa4, OPNb3 and OPNc1 expressed similar levels of OPNa, OPNb and OPNc isoforms, respectively, as compared to the empty vector control clone; (B) Transfectant clones OPNa1, OPNb1 and OPNc5 transfectant clones exhibit differential expression levels of OPNa, OPNb and OPNc isoforms, respectively, as compared to the empty vector control clone. Different cell clones for each OPN isoform was tested isolatedly and presented similar proliferation behavior. The standard deviations (error bars) indicate the variability within each experiment.

Supplementary Figure 2. OPNa, OPNb and OPNc overexpressing cells present similar cell death rates. Cell clones of OPNa4, OPNb3 e OPNc1 and empty vector were plated and cellular DNA was stained with propidium iodide (PI) for staining fragmented DNA (sub-G0/G1 population) as an indicative of cells undergoing apoptosis. (A) The cellular DNA was stained with PI and analysed by flow cytometry 24, 48, 72 and 96 h after being plated. Representative plots were shown at 24 and 96 h time points. The percentages of cell with hypodiploid DNA presenting cell death (sub-G0/G1 fraction) after PI staining are indicated in each panel and were analysed from FL2-A versus cell counts. (B) Histograms reflect the proportion of cells at sub-G0/G1 fraction (%) at 96 h time point incubation. Data are presented as mean ± S.D. (represented by horizontal bars). The cell counts are shown in %, were analysed by

CellQuest software and were plotted. Results are representative of one of at least three independent experiments.

Supplementary Figure 3. Secreted OPNc induced cell proliferation in a dose-dependent manner. OvCar-3 (A) and IOSE (B) non-transfected cells were assayed for cell proliferation rates by crystal violet staining after 96h incubation with different dilutions of conditioned medium from OPNc overexpressing cells. Abscissa axis represents the percentage of OPNc conditioned media used to culture OvCar-3 and IOSE non-transfected cells. OPNc complete conditioned media (100%) was serially diluted in RPMI medium, generating 75%, 50%, 25% and 0% of conditioned culture medium. All results are representative of at least three independent experiments. O.D., optical density measured at 550 nm. The standard deviations (error bars) indicate the variability within each experiment.. * $p < 0.0001$ vs. cells incubated with 0% of OPNc conditioned medium.

Supplementary Figure 4. Anti-OPNc antibody specifically alter the proliferation and cell deathof OPNc overexpressing cells. OvCar-3 overexpressing OPNa (A and B) or OvCar-3 non-transfected cells (C and D) were treated with anti-OPNc antibody at the concentration of 4 μg/mL for 96 h. Total number of cells (A and C) and cell death (B and D) was measured by Trypan blue exclusion staining. Significant differences are represented by asterisk. * $p < 0.0001$ vs. cell number of clones of OvCar-3 non-transfected cells or cells overexpressing OPNa after 96 h of cell culture without anti-OPNc antibody incubation.

Supplementary Figure 5. Secreted OPN-c enhanced cell migration. OvCar-3 non-transfected cells were plated as indicated in Material and Methods and incubated with conditioned medium from OPNa,OPNb, OPNc, empty vector and OvCar-3 non-transfected cells and then and analyzed for cell migration by wound closure assay. Phase-contrast micrographs photographs taken at 0 and at 24 h after subjected to migration are shown. For each cell line, three plates were used per experiment.

Supplementary Information

Material and Methods

Cell proliferation studies. Cell proliferation was analyzed at the indicated time points by crystal violet, exclusion of Trypan blue and incorporation of [^{3}H] thymidine. For the analyses by crystal violet and Trypan blue, wild-type OvCar-3, OvCar-3 cells transfected either with OPNa, OPNb or OPNc plasmid constructs or pCR3.1 empty vector were plated in triplicate in 24-well microtiter plates. For these assays, 5 X 10^{4} cells were plated per well. For crystal violet incorporation assays, cells were washed twice with PBS and fixed in glutharaldeyde for 10 min, followed by staining with 0.1% crystal violet and solubilization with 0,2 % Triton X-100. Microtiter plates were read on a spectrophotometer at 550 nm. Viable and non-viable cells were counted using a Neubauer's chamber. Total number of cells/well for each cell clone group was calculated and plotted against each time point. In order to test whether secreted OPN isoforms were performing the observed cell behavior, wild type OvCar-3 and IOSE cells were incubated with conditioned media obtained from OvCar-3 cells transfected with OPNa, OPNb, OPNc or empty vector, and cell proliferation was measured by crystal violet incorporation. For the [^{3}H] thymidine incorporation assays, cells were plated at a density of 2.5 X 10^{3} per well in 96-well dishes. At appropriated time points after plating, 5 μCi/mL [^{3}H] thymidine was added for 8 hs. Cells were then harvested, and [^{3}H]thymidine incorporation was analyzed by using a scintillation counter.

Sub-G0/G1 analyses. Six-well microtiter plates were inoculated with 5 X 10^5 cells for each assay. On the indicated days, the cells were trypsinized and washed once with phosphate-buffered saline (PBS) before staining with propidium iodide (75 μM). Analysis of the DNA content was done by collecting 20,000 events for sub-G_0 analysis using a FACScalibur flow cytometer and CellQuest software (BD Biosciences).

Analysis of cell migration by wound closure assay. A wound closure assay was performed as described previously [21]. Wild-type OvCar-3 or OvCar-3 cells transfected with either OPNa, OPNb, OPNc or empty vector were grown in six-well microtiter plates to near-confluent level in RPMI medium containing 20% FBS. Mitomycin C (5 μg/ml; Sigma) was added to inhibit cell proliferation, and the cells were incubated for additional 3 hs. Multiple uniform and constant streaks were made on the monolayer culture with 10 μl pipette tips. Plate dishes were immediately washed with PBS to remove detached cells. Cell migration was monitored for 72 hs, and pictures were taken at 0, 18, 24, 48, and 72 hs time points with a digital camera attached to an inverted Nikon phase contrast microscope. In order to evaluate cell effects and behaviour promoted by secreted OPN splicing isoforms, wild-type OvCar-3 cells were incubated with conditioned media obtained from OvCar-3 cells transfected with either OPNa, OPNb, OPNc or empty vector, and wound assay was conducted.

Transwell Invasion Assays. To determine the effect of OPN isoforms on the invasion potential of OvCar-3 cells, invasion *in vitro* assays were performed as reported (Christian Klausen 2009). Cell culture inserts (24-well, pore size 8 μm; BD

Biosciences) were seeded with 25,000 wild-type OvCar-3 cells, OvCar-3 cells transfected either with OPNa, OPNb or OPNc plasmid constructs or pCR3.1 empty vector in 500 μL of medium with 0.1% FBS. Pre-coated inserts with growth factor reduced Matrigel (40 μL, 1 mg/mL; BD Biosciences) were used. As chemotactic agents added at the lower chamber, 750 μL of culture medium containing 20% FBS or conditioned medium obtained from OvCar-3 cells transfected with OPNa, OPNb, OPNc or empty vector have been used. Noninvading cells were wiped from the upper side of the membrane and cells on the lower side were fixed in glutharaldeyde followed by staining with 0.1% crystal violet. The lower side of the filter was photographed by using a phase-contrast microscope at 10 fold magnification and the number of cells was manually counted. Each individual experiment had triplicate inserts and six microscopic fields were counted per insert.

Soft agar growth. Six-well plates were coated with 1.2% agarose-supplemented growth medium to resist cell adhesion. OvCar-3 wild-type or OvCar-3 cells transfected with either OPNa, OPNb, OPNc or empty vector were then trypsinized, and 5 X 10^2 cells were resuspended in 3 ml of growth medium containing 0.6% agarose. After being plated, colonies were allowed to grow for 4 weeks. Representative colonies were visualized by both phase contrast microscopy and the total number of colonies was determined.

Real time PCR for GAPDH, actin, MMPs and VEGF. Conditions for MMP-2 and MMP-9 PCR amplification by realt time PCR were 50°C for 2 min, 94°C for 5 min followed by 40 cycles of 94°C for 30 sec, 58°C for 30 sec and 72°C for 90 sec; 72°C for

15 min and finally a melting curve analysis (60–90°C with a heating rate of 0.2°C and continuous fluorescence measurement). Conditions for VEGF PCR amplification were 50°C for 2 min, 94°C for 5 min followed by 40 cycles of 94°C for 30 sec, 60°C for 30 sec and 72°C for 90 sec; 72°C for 15 min and finally a melting curve analysis (60–90°C with a heating rate of 0.2°C and continuous fluorescence measurement).

Table I. Specimen and clinical data of ovarian samples used in this study

Patient Number	Histologic Type	Grade	FIGO	CA-125 (U/mL)
Ovarian Carcinoma (n=15)				
Age (mean, years) - 60				
66	Mixed	3	Ic	264,81
52	Serous	3	Ic	81.4
73	Serous	3	IIIc	875
45	Serous	3	IIIc	1730
58	Serous	3	IIIc	135
70	Serous	3	IIIc	43.2
59	Serous	ND	IIIc	3189
69	Serous	2	IV	2411
53	Serous	3	Ia	38.6
43	Serous	3	IIIc	1853
78	Serous	3	IIa	1130.94
61	Serous	2	Ic	125
67	Serous	3	IIIc	3243.79
52	ND	3	IIIc	375.7
68	Endometrioid	2	Ia	46.2
Borderline Tumors (n=06) (¥)				
Age (mean, years) - 46				
31	Serous	NA	Ia	9.3
30	Serous	NA	IIIa	35.5
48	Serous	NA	Ia	37.6
80	Mucinous	NA	Ia	15.9
28	Mucinous	NA	IIIc	128
61	Mucinous	NA	IIIc	222
Benign Tumors (n=08)				
Age (mean, years) - 48				
44	Mucinous Cystadenoma	NA	NA	16.5
63	Serous Cystadenoma	NA	NA	5.28
60	Serous Cystadenoma	NA	NA	22.4
51	Serous Cystadenofibroma	NA	NA	33.9
38	Stromal tumors of tecoma type	NA	NA	211
58	Germline tumor of teratoma type	NA	NA	8.02
26	Germline tumor of teratoma type	NA	NA	19.2
45	Germline tumor of teratoma type	NA	NA	11.4
Non-Tumoral Tissues (n=11) (*)				
Age (mean, years) - 62				
58	Primary endometrious tumors	2	Ib	ND
74	Primary endometrious tumors	3	IIIc	6.25
67	Primary endometrious tumors	2	Ib	ND
63	Primary endometrious tumors	2	Ib	10.81
64	Primary endometrious tumors	1	Ic	ND
63	Primary endometrious tumors	3	Ib	8.23
81	Primary endometrious tumors	3	IIb	25.65
69	Primary endometrious tumors	3	IIIc	ND
63	Primary endometrious tumors	2	Ic	18.60
44	Cervical cancer	2	Ib1	ND
41	Folicular cyst	NA	NA	ND

(*) Morphological and histological normal ovary derived from patients that performed hysterectomy or anexectomy due to other gynecological malignancies; (¥) Follow-Up of 10 – 37 months; FIGO, FIGO stage; NA, not applicable; ND, not defined.

Supplementary Table 2. Forward and reverse oligonucleotide sequences used for osteopontin splicing isoforms, MMP-2, MMP-9, VEGF, Actina and GAPDH specific amplification.

Gene	*Oligonucleotide Name*	*Sequence 5' – 3'*
OPNa	OPNaF	ATC TCC TAG CCC CAC AGA AT
	OPNaR	CAT CAG ACT GGT GAG AAT CAT C
OPNb	OPNbF	CTC CTA GCC CCA CAG ACC CT
	OPNbR	TAT CAC CTC GGC CAT CAT ATG
OPNc	OPNcF	CTG AGG AAA AGC AGA ATG
	OPNcR	AAT GGA GTC CTG GCT GT
MMP-2	MMP2F	AAA ATG GAT CCT GGC TTC CC
	MMP2R	TAG CCA GTC GGA TTT GAT GC
MMP-9	MMP9F	TGA CAG CGA CAA GAA GTG
	MMP9R	CAG TGA AGC GGT ACA TAG G
VEGF	VEGF-F	CTT GCC TTG CTG CTC TAC C
	VEGF-R	CAC ACA GGA TGG CTT GAA G
Actina	Actina-F	TGA CCC AGA TCA TGT TTG AGA
	Actina-R	ACT CCA TGC CCA GGA AGG A
GAPDH	GAPDH-F	TGA CCC CTT CAT TGA CCT CA
	GAPDH-R	AGT CCT TCC ACG ATA CCA AA

1. Introdução

A angiogênese é definida como a formação de novos vasos sanguíneos a partir de vasos pré-existentes, em um processo envolvendo migração e proliferação de células endoteliais já existentes. A hipótese de que o crescimento dos tumores e metástases é dependente de angiogênese foi primeiramente proposta por Judah Folkman em 1971 (Folkman 1971; Folkman 1995). Em um estágio pré-vascular, as células tumorais podem obter nutrientes e oxigênio por difusão passiva na distância aproximada de 0,2 a 0,5 mm, levando a um crescimento tumoral de aproximadamente 2 a 3 mm^3. A taxa de crescimento de células tumorais sem neovascularização fica em equilíbrio com a taxa de mortalidade. Os novos vasos sanguíneos suprem as células tumorais de oxigênio e nutrientes, removendo também os produtos de seu metabolismo. Como resultado da angiogênese, ocorre o crescimento dos tumores, o que pode inclusive facilitar a formação de metástases. Ao contrário da angiogênese observada em tecidos normais, a angiogênese tumoral apresenta vasos com diâmetros irregulares, paredes finas, fluxo sanguíneo aberrante e áreas de necrose. O balanço entre fatores pró e anti-angiogênicos controla a geração de novos vasos e, portanto, o suplemento sanguíneo do tecido. Exemplo destas moléculas são os Fatores de Crescimento de Fibroblastos (FGF), Fator de Crescimento Vascular Endotelial (VEGF), Angiopoitina (Ang1), Fator de Crescimento Tumoral (TGF) e Fator de Crescimento Derivado de Plaquetas (PDGF).

Dentre todos estes fatores pró-angiogênicos, destacamos o VEGF. O VEGF é uma proteína homodimérica de 45 KDa, que atua como um fator de crescimento específico de células endoteliais e um dos principais reguladores da angiogênese sob condições normais e patológicas. Foi inicialmente identificado com base em sua habilidade de estimular a permeabilidade vascular. Posteriormente, demonstrou tratar-se de um fator angiogênico e um mitógeno específico de células endoteliais. A importância crítica do VEGF na angiogênese foi demonstrada em camundongos, nos quais a deleção de ambos ou um único alelo do VEGF mostrou-se letal durante o desenvolvimento fetal, devido à interrupção da angiogênese embrionária. Sua expressão

aumentada de maneira anormal também induz a morte fetal, devido à hiper-vascularização e consequentemente, aumento da quantidade e do volume celular, levando ao aumento do tamanho dos tecidos (Hanahan, 1997; Stouffer e col., 2001). O VEGF pertence à família de proteínas VEGF/PDGF, sendo composta pelos seguintes membros: VEGF-A, VEGF-B, VEGF-C, VEGF-D e VEGF-E (Ferrara *et al,* 1997; Takahashi *et al.*, 2005). O subtipo VEGF-A é o mais conhecido e estudado por sua relação com a angiogênese. Dados da literatura demonstraram que no promotor do VEGF humano existem três domínios de interação com o fator de transcrição AP-1 (Pagès et al., 2005). AP-1 é um fator de transcrição dimérico composto por várias famílias de proteínas que apresentam em comum, domínios essenciais para dimerização e ligação ao DNA. As subfamílias Jun (c-Jun, JunB e JunD) e Fos (c-Fos, FosB, Fra-1 e Fra-2) correspondem a maior parte das proteínas que compõem AP-1. A combinação entre as diferentes proteínas determina a especificidade e afinidade de ligação e os genes que serão regulados. Os efeitos da família Jun na transformação neoplásica é fundamentalmente contexto-dependente, de maneira que clinicamente, JunB pode ser um oncogene e um supressor de tumor. Por outro lado, a relevância de JunD como regulador de crescimento é ainda pouco clara. O fator c-Jun, por outro lado, parece induzir a proliferação e proteger a células da apoptose. AP-1 exerce seu efeito oncogênico ou anti-oncogênico regulando genes envolvidos na proliferação, diferenciação, apoptose, angiogênese e metástase. A hipóxia, o estresse oxidativo e as citocinas podem aumentar a expressão do VEGF através da síntese das proteínas JUN e FOS e aumento da atividade de ligação do AP-1.

O CO é caracterizado por um crescimento heterogêneo, rápido e altamente metastático. A superexpressão de VEGF em pacientes com carcinoma de ovário está associada com a transformação do epitélio normal em tumoral, estimulando a formação de ascite e suprimindo a resposta imune anti-tumoral (Zhang *et al*., 2003; Schumacher *et al*., 2007; Belotti *et al*., 2003). Hu *et al.* (2005) mostraram a importância da via de sinalização PI3k/Akt na formação de ascite e indução da angiogênese em tumores ovarianos, por meio da indução da expressão de VEGF. Além disto, células OvCar-3 tratadas com ácido

lisofosfatídico (LPA) apresentam indução da expressão de VEGF-A mediada pelo fator de transcrição AP-1 (Hu *et al*., 2001).

É bem conhecido que a isoforma completa OPN é capaz de induzir a formação de novos vasos sanguíneos, sustentando a progressão tumoral e a metástase do CO. A relação da OPN com este processo está relacionada predominantemente com a interação desta proteína com a integrina do tipo αvβ3, um importante marcador de angiogênese. Por outro lado, a OPN estimula a angiogênese por induzir a expressão do VEGF (Wang *et al.,* 2011; Belotti *et al*., 2003; Chambers *et al.,* 2001). Brooks *et al.* (1994) demonstraram através de experimentos de angiogênese *in vivo,* utilizando membrana corio-alantóica de embrião de galinha, que a expressão da integrina αvβ3 aumenta durante a angiogênese. Na situação em que a expressão desta integrina é bloqueada, a angiogênese é inibida. A integrina αvβ3 e a OPN encontram-se superexpressas durante o processo tumorigênico (Robertson *et al*., 2010). Tendo como enfoque a angiogênese e as isoformas de '*splicing*' da OPN, Blasberg *et al*. (2010) mostraram em células de tumores de pulmão não pequenas células (NSCLC), que a superexpressão da OPNc está associada com a diminuição da angiogênese, enquanto que a OPNa e OPNb apresentam papéis opostos. Entretanto, as vias de sinalização relacionadas às funções destas isoformas neste tipo tumoral ainda não foram caracterizadas. Especificamente no caso de tumores ovarianos, ainda não é conhecido o papel funcional e o mecanismo molecular pelos quais cada isoforma de '*splicing*' da OPN atua nas vias relacionadas à angiogênese no CO.

Dados anteriormente gerados por nosso grupo de pesquisa demonstraram que a isoforma OPNc é um importante produto gênico envolvido na tumorigênese e progressão dos tumores de ovário e próstata (Capítulos I, III e IV desta tese de doutorado). Observamos que as células OvCar-3 que superexpressam a OPNc induzem a expressão do mRNA do *Vegf in vitro* e *in vivo*, indicando o potencial papel funcional desta isoforma no processo de angiogênese. Demonstramos também que células OvCar-3 que superexpressam esta isoforma ativam de forma significativa a adesão, proliferação e migração de células endoteliais HUVEC, além de ativar uma

série de outros transcritos pró-angiogênicos (Capítulo V desta tese de doutorado). Estes dados em conjunto reforçam o possível envolvimento da OPNc na ativação da angiogênese em tumores de ovário. O presente trabalho tem por objetivo investigar os mecanismos moleculares capazes de mediar o efeito da OPNc na ativação da expressão de VEGF-A em células de tumores ovarianos. Demonstramos que tumores formados pela superexpressão da OPNc apresentam maior expressão de VEGF-A, VEGFR-2 e CD34. Mostramos que a OPNc interage com as integrinas de forma RGD-dependente, ativando a via de sinalização PI3K/Akt, a qual medeia a ativação de expressão de c-Fos, c-Jun e fosforilação de c-Jun. Estes dados indicam o envolvimento destes fatores transcricionais na indução da expressão de VEGF-A.

2. Objetivos

2.1. Objetivo Geral

1. Investigar os mecanismos moleculares pelos quais a OPNc ativa a expressão de VEGF-A nas células tumorais de ovário.

2.2. Objetivos Específicos

1. Identificar e caracterizar o efeito da superexpressão da OPNc em eventos relacionados ao processo angiogênico.
2. Investigar as vias de sinalização envolvidas na ação da OPNc na ativação da expressão de VEGF-A.

3. MATERIAIS E MÉTODOS

3.1. Cultura de células

Foi utilizada neste trabalho a linhagem celular humana tumoral de ovário, OvCar-3. Esta linhagem foi cultivada em meio RPMI 1640 (Sigma Aldrich), suplementado com 20% de soro fetal bovino (SFB - Invitrogen) inativado, 100U/mL de penicilina e 100µg/mL de estreptomicina. As células HUVEC foram isoladas como descrito por Jaffe *et al.* (1973). Esta linhagem foi cultivada em meio MCDB131 (GIBCO) suplementado com 20% de SFB (Invitrogen), 2mM L-glutamina, 50 µg/mL de heparina, e 50 µg/mL de fator de crescimento endotelial (ECGS). As culturas foram mantidas em estufa a 37℃ e 5% de CO_2.

3.2. Superexpressão das isoformas da OPN em linhagem de CO

Para superexpressão das três diferentes isoformas de *splicing* da OPN na linhagem OvCar-3, transfectamos as células com os plasmídeos pCR3.1, contendo clonados os cDNAs de cada uma das isoformas da OPN, sendo em seguida selecionados cinco clones isolados de expressão estável destas três diferentes isoformas (Tilli *et al*., 2011).

Dentre os clones isolados selecionados, foram utilizados para os ensaios do presente estudo duas distintas séries de clones; Série 1: OPNa5, OPNb2, OPNc3 e vetor vazio2 (VV2); e Série 2: OPNa6, OPNb3, OPNc1 e VV3 (Figura 01). Os extratos celulares totais de cada clone individual foram preparados para extração de RNA e proteína total.

3.3. RT-PCR e PCR em tempo real

Para avaliar o nível de expressão dos transcritos das três diferentes isoformas da OPN na linhagem celular Ovcar-3, utilizamos o RNA total extraído dos clones celulares utilizando-se o Kit RNeasy (Qiagen), seguindo o protocolo descrito pelo fabricante. Três microgramas de cada amostra de RNA total foram reversamente transcritas para cDNA, segundo o protocolo do kit “SuperScript II First-Strand Synthesis System for RT-PCR” (Invitrogen).

Os ensaios de PCR quantitativo em tempo real (qRT-PCR) foram realizados utilizando o sistema de detecção de SYBR Green (SYBR™ Green PCR Master Mix, Applied Biosystems) e oligonucleotídeos específicos para cada umas das isoformas (Tilli *et al.*, 2011). Os tamanhos para os produtos da PCR são de 216 pb para OPNa, 282 pb para OPNb, 149 pb para OPNc e 419 pb para GAPDH, o qual foi utilizado como gene de expressão constitutiva. O volume final da reação da PCR foi de 20 μL, a qual contém 10 μL de SYBR Green, 5 μL da solução 800 nM de mistura dos oligonucleotídeos senso e antisenso e 5 μL de cDNA (sintetizado a partir de 0,1 e 0,2 μg de RNA total). O programa de ciclagem consistiu de uma incubação inicial a 50℃ por 2 min e 95℃ por 10 minutos, seguido de 10 ciclos de 95℃ p or 15 segundos (desnaturação), 55℃ por 1 minuto (anelamento) e 72 ℃ por 30 segundos (extensão), sendo que a cada ciclo a temperatura de anelamento diminuiu em 0,5℃; Esta etapa foi seguida por 30 ciclos de 95℃ por 15 segundos (desnaturação), 50℃ por 1 minuto (anelamento) e 72 ℃ por 30 segundos (extensão). Ao término da ciclagem, era realizada uma curva de dissociação com o objetivo de confirmar a especificidade de amplificação. A curva de dissociação foi realizada com um aumento de temperatura que se inicia em 60℃ e estende-se até 90℃, com leituras de fluores cência efetuadas a cada 0,2℃.

Todas as amostras foram amplificadas em duplicatas. As reações de qRT-PCR foram realizadas e analisadas no aparelho *CFX 96 Real Time System* (Bio Rad). O nível de expressão relativa das isoformas de OPN foi calculado pelo método de quantificação relativa, utilizando a fórmula $2^{-\Delta\Delta CT}$.

3.4. Preparação do Meio Condicionado (MC)

Ao atingir cerca de 80% de confluência, as células transfectadas com as isoformas da OPN e com o VV foram lavadas com PBS 1x (salina tamponada com fosfato) e as células foram cultivadas em meio RPMI sem adição de SFB por 48hs. Após esse tempo, o meio de cultura foi centrifugado e utilizado nos ensaios funcionais. Para todos os ensaios foram utilizados meio condicionado fresco.

3.5. Ensaio de proliferação celular

As células endoteliais foram plaqueadas em placas de 24 poços com $2x10^4$ células por poço e cultivadas em 5% de CO_2 à 37ºC por 24 e 48 hs. O ensaio foi realizado em triplicata, em três experimentos independentes. A proliferação de células HUVEC foi analisada mediante o cultivo destas células na presença de MC secretado pelos clones celulares de superexpressão das três isoformas de splicing da OPN (OPN-IS) (OPNa, OPNb e OPNc) e do VV. O meio condicionado secretado por cada um destes clones foi denominado de OPNa-MC, OPNb-MC, OPNc-MC e VV-MC. Em cada tempo experimental avaliado, as células foram lavadas duas vezes com PBS 1x e fixadas por 10 minutos com glutaraldeído 1% em PBS por 15 minutos. As células foram lavadas com H_2O destilada e coradas com cristal violeta 0,1%, seguindo-se duas lavagens de 10 minutos com H_2O destilada para retirada do excesso de corante. As células coradas foram solubilizadas em 0,2% de Triton X-100 e 100µl da suspensão de células foram lidos em 550nm no espectrofotômetro SpectraMax 90 (Molecular Devices, Sunnyvale, CA). A densidade ótica (D.O.) obtida neste comprimento de onda é proporcional ao número de células aderidas. A proporção de células em proliferação era estimada com base na D.O. obtida. Os resultados são representados na forma de absorbância à 550nm, em função do tempo de cultivo após o plaquamento das células e incubação com o MC.

Ensaios de proliferação de células HUVEC também foram realizados na presença de anticorpo policlonal anti-OPNc (4µg/mL - *Gallus* Imunotech), anticorpo anti-IgY (4µg/mL - Promega), anticorpo monoclonal anti-VEGF (4µg/mL - R&D Systems) ou de VEGF recombinante (1.2ng/mL - GIBCO).

3.6. Preparação dos extratos protéicos celulares

As células foram lavadas com PBS 1x e tripsinisadas. O precipitado de células foi ressuspendido em SDS 1% com adição do inibidor de protease e fosfatase 1X. A dosagem total de proteínas foi realizada pelo kit *BCA assay* (BioRad), de acordo com as instruções do fabricante.

3.7. Tratamento das células com LY294002 e imunoblot

Os clones de superexpressão das isoformas da OPN e VV foram cultivados na presença do inibidor específico da via de PI3K, o LY294002 na concentração final de 50µM (Cell Signaling Technology). Os extratos celulares totais de células cultivadas nestas condições foram avaliados por imunoblot com relação ao perfil de expressão de VEGF-A, c-Fos, c-Jun e fosforilação de c-JunNos ensaios de imunoblot, 25 µg de proteína total foram resolvidos em gel de SDS-poliacrilamida 10% ou 8% e transferidos para membrana de PVDF (Milipore). A membrana era bloqueada em solução de bloqueio (5% BSA, 0.05% de Tween 20 em TBS (Tris-HCl salino) 1X (TBST) por 1 hora. A membrana era então incubada com os anticorpos primários em solução de bloqueio por 18 horas. Os ensaios foram realizados com os anticorpos anti-OPN (R&D systems), anti-c-Fos (Cell Signaling), anti-c-Jun (Santa Cruz), anti-P-c-Jun (Cell Signaling), anti-Akt (Cell Signaling), anti-pAkt (Cell Signaling) e anti-VEGF (R&D systems). Com o término da incubação, a membrana foi lavada 3 vezes por 5 minutos em agitação com TBST. A incubação com anticorpo secundário anti-IgG coelho ou cabra (1: 1.000) conjugado a peroxidase (Pierce, Rockford, IL, USA) era realizada por 1 hora. As bandas imunoreativas foram visualizadas com a adição do substrato da peroxidase *ECL plus Western Blotting Detection Reagents* (Amersham Biosciences, Piscataway, NJ, USA), sendo as membranas em seguidas expostas a filmes de raios-X (Kodak BioMax Light Film).

A análise de densitometria das bandas foi quantificada usando o programa *ImageJ* (http://rsb.info.nih.gov/ij/). A normalização foi realizada com o extrato de clones celulares VV. Estes ensaios foram realizados em triplicata e em três experimentos independentes.

3.8. Imunohistoquímica

Para a técnica de imunohistoquímica, foram utilizados os seguintes anticorpos primários; anticorpo monoclonal anti-VEGF (1:100 / SC-7269; Santa Cruz Biotechnology, Santa Cruz, CA), anticorpo policlonal anti-VEGFR2 (Flk-1, 1:200 / SC-6251; Santa Cruz Biotechnology, Santa Cruz, CA) e anticorpo anti-

CD34 (1:200 / SC-74499; Santa Cruz Biotechnology, Santa Cruz, CA). Para a inibição da atividade da peroxidase endógena, os cortes histológicos foram tratados com solução de H2O2 3% em 0,01mol/L de PBS, pH7.5. A recuperação antigênica foi realizada incubando as preparações histológicas em tampão citrato (pH 6) e aquecimento em microondas por 5 minutos. Com o objetivo de inibir as reações inespecíficas, as lâminas foram incubadas em solução de PBS contendo 10% de soro normal de cabra e 5% de BSA durante 30 minutos. Os anticorpos primários foram incubados por 12 horas, a 4℃, seguido da aplicação do complexo estreptavidina-biotina-peroxidase (LSAB, Dako) e revelação com diaminobenzidina tetraidroclorido (Sigma, St. Louis, MO). Posteriormente, os cortes foram contra-corados com hematoxilina de Harris por 30 segundos e lavados em cinco banhos de água destilada, sendo que o terceiro banho continha hidróxido de amônia para conferir a coloração azulada da Hematoxilina. Finalmente procedeu-se à desidratação dos cortes em dois banhos de álcool (um minuto cada) seguido por quatro banhos de xilol (um minuto cada). As lâminas foram montadas com Enthelam (Merck) para fixação das lamínulas em resina e avaliadas no microscópio óptico (Olympus BH-2, Japão). As imagens das preparações histológicas foram capturadas utilizando um sistema de vídeo-câmera acoplado a um microscópio óptico (Olympus BH-2) e Câmera digital Coolpix 990 (Nikon).

Um total de 10 campos, escolhidos de forma randômica, foi fotografado. A quantificação da marcação foi realizada através do software Image Pro Plus 4.5.1 (Media Cybernetics, Silver Spring, MD). A quantificação da marcação foi realizada conforme descrita por Donnez *et al.* (1998). Este método envolve a análise da distribuição e intensidade de marcação no epitélio e estroma.

3.9. Análises Estatísticas

Para a análise de significância estatística foi utilizado o programa: GraphPrism. Para a análise dos ensaios funcionais de proliferação e análises de imunohistoquímica foi utilizado o teste de ANOVA e o teste de comparação de Dunnett, o valor de p está indicado nas legendas.

4. Resultados

4.1. A OPNc secretada induz a expressão de VEGF em células OvCar-3

Numerosos eventos celulares estão associados com a progressão tumoral, dentre eles a angiogênese. Demonstramos previamente que a superexpressão da OPNc induz a superexpressão do transcrito de *Vegf* em células OvCar-3. Adicionalmente, observamos que as células OvCar-3 que superexpressam a OPNc formam, em camundongos atímicos, tumores que também apresentam maiores níveis de expressão de *Vegf*, em comparação aos demais clones celulares que superexpressam a OPNa, OPNb e VV (Tilli *et al*., 2011). Com base nestes dados, investigamos nos tumores xenotransplantados resultantes destes clones de superexpressão, a expressão de VEGF-A, de seu receptor (VEGFR-2) e de um marcador típico de células endoteliais, CD34. Observamos que a superexpressão da OPNc, mas não das demais isoformas e o VV, estimula de forma significativa a expressão de VEGF-A, VEGFR-2 e CD34 ($p < 0.0001$) (Figuras 02,03,04A-B). Também observamos que as células OvCar-3 que superexpressam a OPNc apresentam maior indução da secreção de VEGFA, em comparação com os demais clones (aumento de 3 vezes, $p < 0.0001$ – Figura 05).

A OPN é majoritariamente secretada e exerce suas principais funções na matriz extracelular. Por meio de cultivo das células OvCar-3 na presença de MC obtido de células que superexpressam as distintas isoformas da OPN e o VV, observamos que vários aspectos ligados à características que favorecem a progressão de células de CO estão relacionadas à isoforma secretada da OPNc (Tilli *et al*., 2011). Neste contexto, nos perguntamos se a indução da expressão de VEGF-A como resultado da superexpressão da OPNc também é majoritariamente modulada pela forma secretada. Células OvCar-3 não transfectadas foram cultivadas na presença de MC oriundos das duas séries de clones de OvCar-3 que superexpressam as 3 isoformas da OPN e VV. Células OvCar-3 cultivadas nestas condições tiveram seus extratos protéicos totais extraídos e a expressão de VEGF-A foi analisada por imunoblot. As células OvCar-3 cultivadas com o OPNc-MC apresentaram aumento de 4 vezes na

expressão protéica de VEGF-A, quando comparadas com as células OvCar-3 cultivas com o OPNa-MC, OPNb-MC ou VV-MC. Resultados semelhantes foram observados com o MC proveniente das duas séries de clones isolados de superexpressão (Figura 06).

Uma vez que os tumores formados pela superexpressão da OPNc são mais vascularizados, conforme indicado pela marcação com CD34, avaliamos a contribuição do OPNc-MC na proliferação das células endoteliais HUVEC. O meio OPNc (OPNc-MC) contém altos níveis de expressão desta isoforma (Figura 01) e de VEGF-A secretado (Figura 05). Realizamos ensaios de proliferação de células endoteliais HUVEC, sendo cultivadas por até 48 hs na presença de OPNc-CM, VEGF recombinante ou apenas com o meio de cultivo usual destas células. Na Figura 05 mostramos que a concentração de VEGF-A secretado presente no meio OPNc-MC é de 1.2ng/mL. Assim utilizamos esta mesma concentração de VEGF recombinante no meio de cultivo para estes ensaios de proliferação. As células HUVEC cultivadas na presença de VEGF recombinante, assim como aquelas cultivadas na presença do meio OPNc-CM aumentam significativamente a taxa de proliferação, quando comparadas com aquelas cultivadas com o meio de cultura usual no tempo de 48hs ($p < 0.05$) (Figura 07). Notadamente, as células HUVEC cultivadas em meio OPNc-MC apresentam maior potencial proliferativo do que as células cultivadas com meio de cultura contendo a VEGF recombinante no tempo de 48hs ($p < 0.05$). Quando o OPNc-MC foi pré-tratado com o anticorpo neutralizante anti-OPNc, a ativação da proliferação promovida pelo meio OPN-MC retornou à níveis similares aos observados para as células cultivadas em meio de cultura usual. O pré-tratamento com um anticorpo não relacionado anti-IgY não promoveu qualquer alteração neste perfil de proliferação promovido pelo OPNc-MC. Por outro lado, quando o meio OPNc-MC foi pré-tratado com o anticorpo neutralizante anti-VEGF, embora tenha sido observada uma diminuição da proliferação de células HUVEC, o potencial proliferativo destas células ainda permaneceu maior que o observado quando as células foram cultivadas em meio de cultura contendo a VEGF recombinante. Em conjunto, estes dados indicam que a OPNc secretada estimula a secreção do VEGF-A, além de

induzir a expressão de seu receptor e a formação de vasos sanguíneos. Além disto, estes resultados indicam que a OPNc contida no meio condicionado OPNc-MC contribui de forma mais significativa que o VEGF na indução da proliferação de células HUVEC.

Formatado

4.2. Vias de sinalização relacionadas com o efeito da OPNc secretada na angiogênese no CO: o envolvimento de AP-1 e da via de PI3K/Akt

No CO, a via de PI3K/Akt demonstrou ser essencial na ativação dos diversos efeitos pró-tumorigênicos da OPNc em células OvCar-3 (Tilli *et al.*, 2011). Com base nestes dados anteriores e dados da literatura que indicam o envolvimento dessa via de sinalização em etapas relacionadas a angiogênese (Hu *et al.*, 2005), investigamos se essa mesma via de sinalização poderia estar envolvida nos efeitos da OPNc na indução de expressão de VEGF-A em células OvCar-3. Um dos fatores de transcrição que são capazes de induzir a expressão de VEGF é o AP-1 (Pagès *et al.*, 2005). Além disto, a via de sinalização PI3K/Akt tem sido descrita como capaz de estimular a expressão das proteínas que compõem o fator de transcrição AP-1 (Chen *et al*., 2012; Li *et al*., 2004). De forma a investigar os mecanismos moleculares pelos quais a OPNc ativa a expressão de VEGF-A em células OvCar-3, avaliamos se o OPNc-MC é capaz ativar o fator de transcrição AP-1 e a via de sinalização PI3k/Akt. Por meio de ensaios de imunoblot, avaliamos a expressão de c-Jun e c-Fos, assim como a fosforilação de c-Jun, a partir de extratos celulares totais de células cultivadas com o MC de células superexpressando as três isoformas da OPN e o VV.

O cultivo das células OvCar-3 na presença do meio OPNc-MC, mas não o MC das demais isoformas e VV, promove aumento significativo na expressão de c-Jun, c-Fos e fosforilação de c-Jun (Figura 08).

De forma a avaliar se a indução da expressão de VEGF-A, c-Fos, c-Jun e de fosforilação de c-Jun está associado especificamente com a OPNc, realizamos o cultivo das células OvCar-3 em meio OPNc-MC pré-tratado com o

anticorpo neutralizante anti-OPNc ou o anticorpo não relacionado (anti-IgY). As células OvCar-3 ao serem cultivadas com o OPNc-MC em presença do anticorpo anti-OPNc apresentaram níveis de expressão de VEGF-A, c-Fos, c-Jun e fosforilação de c-Jun significativamente menores em comparação com as células cultivadas apenas com o meio OPNc-MC (Figura 09A e B). Quando cultivadas na presença do OPNc-MC pré-tratado com o anticorpo não relacionado anti-IgY não foram observadas alterações na expressão destas proteínas. Resultados similares foram observados nas duas séries de clones estudadas. Em conjunto, estes resultados indicam que a OPNc secretada, presente no meio OPNc-MC, medeia a ativação de expressão de VEGF-A, c-Fos, c-Jun e de fosforilação de c-Jun.

Corroborando com dados anteriores gerados por nosso grupo (Tilli *et al.*, 2011), células OvCar-3 não transfectadas cultivadas na presença do OPNc-MC, mas não o MC das demais isoformas e do VV, apresentaram aumento de Akt fosforilado nas duas séries de clones isolados analisados (Figura 10A). Nenhuma alteração foi observada no perfil de fosforilação de ERK1/2. Na presença do inibidor específico da via PI3K (LY-294002), não observamos ativação da expressão de VEGF-A, c-Fos e de c-Jun fosforilado em resposta ao cultivo com o meio OPNc-MC (Figura 10B e C). Estes resultados indicam que a OPNc secretada presente no meio OPNc-MC ativa a expressão de VEGF-A, c-Fos e de fosforilação de c-Jun através da via de sinalização da PI3K/Akt.

4.3. O meio condicionado da OPNc promove a fosforilação de c-Jun por meio dos receptores do tipo integrinas de forma RGD-dependente

As regiões N-terminal e C-terminal da OPN apresentam domínio de ligação com diferentes integrinas e CD44. O principal domínio funcional da OPN é o domínio de ligação à integrina arginina-glicina-aspartato (RGD). Entretanto, a ligação da OPN às integrinas depende não somente do motivo RGD, mas também de motivos protéicos independentes de RGD (Bellachène *et al.*, 2008). Neste contexto, investigamos o envolvimento dos receptores do tipo

integrinas e suas interações com as isoformas de '*splicing'* da OPN e ainda o envolvimento destes receptores nas vias que induzem a fosforilação de c-Jun em células OvCar-3.

Investigamos se a indução da fosforilação de c-Jun, em resposta ao meio OPNc-MC está associado com o domínio RGD desta isoforma. Células OvCar-3 não transfectadas cultivadas no meio OPNc-MC na presença do peptídeo RGD, mas não no MC das demais isoformas e VV, apresentaram reversão da fosforilação de c-Jun induzida pelo meio OPNc-MC (Figura 11). Estes resultados indicam que a OPNc secretada presente no meio OPNc-MC medeia a fosforilação de c-Jun por meio do receptor de integrina, de forma dependente da sequência RGD.

5. Discussão

Nosso estudo consiste na primeira descrição das possíveis vias de sinalização capazes de mediar o efeito da OPNc na indução da expressão de VEGF-A, contribuindo para uma melhor compreensão dos mecanismos moleculares associados com a angiogênese em tumores ovarianos. Dados da literatura indicam que a OPN (isoforma completa, denominada da OPNa) induz a formação de novos vasos sanguíneos, sustentando a progressão tumoral e a formação de metástases em distintos modelos tumorais (Wang *et al*., 2011; Dai *et al.*, 2009). Shijubo *et al.* (1999) mostraram a correlação positiva entre a expressão de VEGF e a OPN em pacientes com estadiamento I de câncer de pulmão. Adicionalmente, Chakraborty *et al.* (2008) mostraram em tumores de mama que a OPN induz a expressão do VEGF, ativando a via de sinalização Brk/NF-kB/ATF-4. Com base nos dados da literatura que mostram o importante papel funcional da OPN no contexto angiogênico, Kou *et al.* (2010) desenvolveram um anticorpo neutralizante que reconhece o VEGF e a OPN de forma simultânea (VEGF/OPN-BsAb). Animais imunodeficientes com carcinoma hepatocelular foram tratados com esse anticorpo e os resultados indicam que esta molécula é capaz de inibir a angiogênese tumoral. Os trabalhos da literatura que investigam o envolvimento da OPN na angiogênese, utilizam estratégias experimentais que englobam as isoformas da OPN em conjunto. Entretanto, a contribuição de cada isoforma de '*splicing*' da OPN neste processo apenas foi caracterizada nos tumores de pulmão não pequenas células (Blasberg *et al*., 2010). Contudo, até o presente momento nenhuma via de sinalização foi descrita. O presente estudo envolve a investigação do efeito da isoforma OPNc, tanto *in vitro* quanto *in vivo,* sobre as vias de sinalização e mecanismos moleculares possivelmente envolvidos com a indução de expressão de VEGF-A no carcinoma de ovário.

O processo de angiogênese é uma das etapas críticas para a expansão das células do tumor primário e dos focos metastáticos. Ao longo deste processo, as células tumorais interagem com diversos elementos do hospedeiro, que atuam facilitando o processo de metastatização (Nguyen *et al.,*

2009; Horak *et al.*, 2008). Este processo resulta do balanço entre fatores estimulatórios (VEGF, PDGF, TGF-α e β, FGF-2 e citocinas pró-inflamatórias) e inibitórios da angiogênese (interferons, angiostatina, endostatina, trombospondina e inibidores de MMPs). O primeiro passo na caracterização das isoformas da OPN e de suas potenciais funções em eventos associados com a angiogênese foi identificar que a OPNc induz a superexpressão do RNA mensageiro do *Vegf in vitro* e *in vivo* (Tilli *et al*., 2011). Essa expressão de *Vegf* diferencialmente induzida pela OPNc justifica a avaliação das vias de sinalização ativadas neste evento. Escolhemos no presente estudo a estratégia de superexpressão das isoformas da OPN na linhagem celular tumoral OvCar-3, a qual é uma linhagem bem estabelecida como modelo de estudos funcionais do CO (Luo *et al*., 2009).

Demonstramos recentemente que a OPNc encontra-se especificamente expressa no CO e que esta isoforma parece ser a principal ativadora de eventos relacionados à progressão dos tumores de CO, induzindo o aumento na proliferação celular, migração, invasão celular, crescimento celular independente de ancoragem e crescimento de tumores *in vivo*. Adicionalmente, demonstramos que a superexpressão da OPNc induz a expressão do RNA mensageiro de *Mmp-2*, *Mmp-9* e *Vefg*, indicando a potencial ação desta isoforma na estimulação da formação de novos vasos, essencial na progressão tumoral (Tilli *et al.*, 2011). Em adição a estes dados, identificamos que a OPNc potencialmente controla a superexpressão de fatores pró-angiogênicos, como *Epdr-1*, *Pdgf-a, Tgfbr1, Tnf, Fgfr-2* e *Vegfa* (Capítulo V desta tese de doutorado). A superexpressão de VEGF em pacientes com carcinoma de ovário está associada com a tumorigênese e progressão tumoral. Além disto, o VEGF estimula a formação de ascite e está correlacionado com pior prognóstico dos pacientes com CO (Zhang *et al*., 2003; Schumacher *et al*., 2007; Belotti *et al*., 2003, Gadducci *et al.*, 1999; Li *et al*., 2004). Adicionalmente, o tratamento com o anticorpo monoclonal anti-VEGF de pacientes com CO, apresenta resultados promissores em estudos de fase clínica (Monk *et al.*, 2005; Burger *et al.*, 2007). Assim uma melhor compreensão dos mecanismos envolvidos na regulação de expressão do VEGF pode ser importante no

desenho de novos estudos de fase clínica. Dentre os fatores pró-angiogênicos induzidos pela OPNc nos tumores ovarianos, investigamos as possíveis vias e mecanismos moleculares pelos quais a OPNc controla a indução de expressão do VEGF-A nas células OvCar-3.

Demonstramos que OPNc ativa a expressão das proteínas que compõem o fator de transcrição AP-1 e do VEGF-A. Dados da literatura indicam que o fator de transcrição AP-1 é capaz de ativar a transcrição do VEGF em células OvCar-3, mas também em outros modelos de células tumorais (Hu *et al*., 2001; Swenson *et al*., 2011; Dong *et al.*, 2011). Nesse contexto, levantamos a hipótese de que a OPNc modularia a expressão de c-Fos, c-Jun e fosforilação de c-Jun de forma a induzir a expressão de VEGF. Estudos adicionais devem ser realizados com o objetivo de confirmar se no nosso modelo essa indução de expressão do fator de transcrição AP-1 pela OPNc leva a uma maior atividade do promotor do VEGF.

O mecanismo molecular pelo qual as isoformas da OPN modulam os efeitos funcionais pró-angiogênicos não está esclarecido. Até o presente momento, não existem dados na literatura que elucidem as vias de sinalização mediadas por estas isoformas. A via de sinalização PI3K é a via classicamente ativada na angiogênese dos tumores ovarianos (Hu *et al*., 2005). Nossos dados demonstraram que a OPNc secretada ativa a via de sinalização PI3K/Akt e que os efeitos na indução de expressão de VEGF-A, c-Fos e fosforilação de c-Jun são mediados por esta via de sinalização. O efeito da OPNc é bloqueado com o inibidor da PI3K, o LY294002. Em conclusão, estes resultados sugerem que a OPNc estimula a indução de VEGF-A através da ativação da via PI3K/Akt. Em concordância com nossos achados, Fang *et al*., (2005) mostraram em células tumorais de ovário que a apigenina, um flavonóide não tóxico, é capaz de inibir a expressão de VEGF em nível transcricional, através da via de sinalização PI3K/AKT/p70S6K1.

A OPN é uma proteína secretada (sOPN) e exerce suas principais funções na matriz extracelular, atuando como fator autócrino e parácrino em relação à interação com os receptores de membrana (Buback *et al*, 2009). Diversos trabalhos mostraram a tradução alternativa do mRNA da OPN

gerando a isoforma intracelular (iOPN) em células do sistema imune, fibroblastos, queratinócitos e células endoteliais (Shinohara *et al*., 2008; Mazière *et al*., 2010; Cantor *et al*., 2009). No presente estudo, através da utilização do meio condicionado das células que superexpressam as isoformas da OPN, nós mostramos que os efeitos moleculares da expressão de VEGF-A, c-Fos, c-Jun, fosforilação de c-Jun e Akt estão relacionados com a isoforma secretada da OPNc. Segundo os dados gerados neste estudo, a ação da OPNc sobre o estímulo de expressão das proteínas do fator de transcrição AP-1 e VEGF-A parece ser mediado majoritariamente pela proteína secretada, pois a depleção da OPNc secretada pela incubação com anticorpo anti-OPNc inibe a expressão destas proteínas. Até o presente momento não foi descrito em nenhum tipo tumoral isoformas intracelulares da OPN. Novos estudos devem esclarecer a presença das isoformas intracelulares da OPN em células tumorais, a regulação, o papel funcional, e especialmente a combinação destas com as isoformas secretadas da OPN.

A OPN apresenta um amplo espectro funcional, assim múltiplos domínios funcionais foram identificados. O principal domínio funcional da OPN é o domínio de ligação à integrina arginina-glicina-aspartato (RGD), o qual é responsável pela migração e sobrevivência celulares. A sequência RGD está localizada no éxon 6 da OPN humana e este é o domínio que se liga às integrinas. A trombina cliva a OPN e produz dois fragmentos de tamanho aproximadamente equivalente, os quais apresentam os domínios de ligação à integrina e CD44. Outro domínio funcional, denominado de WLNPDP é capaz de promover migração e sobrevivência de leucócitos e neutrófilos através destes sítios alternativos de uma maneira independente de RGD (Dai *et al*., 2009). A clivagem da OPN humana pela trombina também expõe a sequência de adesão SVVYGLR, que é um ligante de integrinas. Este domínio está envolvido na adesão celular e migração. Além disto, as sequências ELVTDFPTDLPAT e SVVYGLR foram definidas como domínios de ligação. A ligação da OPN à diferentes integrinas resulta em distintas consequências funcionais, que pode depender tanto do tipo de integrina específica quanto do tipo celular. A ligação à integrina depende do motivo RGD, mas também de sítios da OPN independentes de RGD, como descritos acima. Nesse estudo

mostramos que a interação da OPNc com as integrinas de forma RGD potencialmente medeia a fosforilação de c-Jun. Em concordância com nossos dados, a literatura mostra que a relação da OPNa com a angiogênese está relacionada predominantemente com a interação desta proteína com a integrina do tipo αvβ3, sendo essa interação dependente da sequência RGD. Brooks *et al.* (1994) demonstraram através de experimentos de angiogênese *in vivo* utilizando membrana corio-alantóica de embrião de galinha, que a expressão da integrina αvβ3 aumenta durante a angiogênese.

Dados da literatura sobre o papel funcional das isoformas da OPN em distintos modelos tumorais mostram que os efeitos das isoformas da OPN são tumor e tecido específicos (Mirza *et al*., 2008; He *et al*., 2006; Ivanov *et al*., 2009; Blasberg *et al.,* 2009; Couter *et al*., 2010; Tilli *et al*., 2011; Tilli *et al*., 2012). Em contraste aos dados encontrados em nosso estudo, encontra-se descrito apenas em câncer de pulmão, que a superexpressão da OPNc está associada com a diminuição da angiogênese, enquanto que a OPNa e OPNb apresentam papéis funcionais opostos (Blasberg *et al*., 2009). Nesse contexto, é preciso melhor compreender os mecanismos moleculares pelos quais estas isoformas apresentam funções tecido e tumor-específicas no processo angiogênico. Especial destaque tem sido dado à necessidade de caracterização dos éxons 4 na função da proteína OPN e das consequências de sua deleção na função que exercem nos distintos tumores. Assim, levantamos algumas hipóteses que poderiam explicar as funções tecido específicas da OPNc. Recentes trabalhos revelaram que as modificações pós tradução (PTMs) da OPN são célula-específicas, refletindo as diversas funções desta proteína em diferentes sistemas fisiológicos (Anborgh *et al*., 2011; Christensen *et al.,* 2010; Hunter *et al*., 1994). Adicionalmente, as funções da OPN refletem a habilidade desta proteína interagir com os receptores. A interação da OPN com as integrinas depende não somente da sequência RGD, mas também de sítios específicos de fosforilação da OPN (Weber *et al.,* 2002). Christensen *et al.,* (2007) demonstraram que as funções fisiológicas da OPN são dependentes das PTMs e que a interação da OPN com as integrinas é dependente destas modificações pós tradução. Neste contexto, levantamos a hipótese que a deleção da sequência do éxon 4 da OPN poderia gerar perda

de sítios de PTMs, e ainda, gerar a aproximação destes sítios. Uma vez deletados este éxon, é possível que haja uma modificação neste padrão e/ou espaçamento entre os sítios de PTMs, potencialmente afetando a função da OPN, conforme observado em outros modelos (Chen-Izu *et al*., 2007). Como consequência, estas deleções poderiam alterar a estrutura protéica das isoformas da OPN, suas interações com diferentes tipos de receptores ou a afinidade com os mesmos, e ainda, a interação com parceiros protéicos.

Mostramos que a superexpressão da OPNc potencialmente regula o microambiente angiogênico, através da ativação de proliferação das células endoteliais. Através de abordagens *in vivo*, demonstramos que os tumores formados pela superexpressão da OPNc são mais vascularizados e superexpressam o receptor de VEGF-A, indicando que a OPNc é potencialmente um fator importante na indução de neovascularização, assim como outras proteínas com conhecido papel na progressão tumoral de tumores ovarianos. Como por exemplo, a endotelina-1 que também é capaz de induzir a expressão do VEGF via a ativação do fator induzido por hipoxia-1 alfa (HIF-1alpha) (Spinella *et al.*, 2002).

A proliferação de células endoteliais consiste em um dos eventos associados com a angiogênese tumoral. Observamos que o meio OPNc-MC é capaz de estimular a proliferação das células endoteliais HUVEC. A capacidade estimulatória deste meio condicionado na proliferação celular poderia ser mediada pela OPNc, pelo VEGF-A e ainda, outros fatores pró-angiogênicos estimulados pela OPNc. Através de ensaios com anticorpos bloqueadores e o VEGF recombinante, mostramos que majoritariamente a OPNc é responsável pela estimulação da proliferação das células endoteliais. Adicionalmente, levantamos a hipótese de que a OPNc em sinergismo com o VEGF poderiam estimular esses efeitos proliferativos, como demonstrado para VEGF/bFGF e o estrogênio/VEGF (Goto *et al*., 1993; Xiao *et al*., 2004). Uma vez que o pré-tratamento do meio OPNc-MC com o anticorpo neutralizante anti-VEGF, embora tenha sido observada uma diminuição da proliferação de células HUVEC, o potencial proliferativo destas células ainda permaneceu maior que o observado quando as células foram cultivadas em meio de cultura

contendo a VEGF recombinante. No contexto molecular, Somanath et al. (2009) mostraram a alça de regulação entre as integrinas do tipo αvβ3 e os receptores VEGFR2 nas células endoteliais. Esses autores mostraram que a ativação de ambos receptores regula distintas etapas relacionadas com a angiogênese, incluindo proliferação, migração, sobrevivência celular e formação de novos vasos. Novos estudos se fazem necessário com o objetivo de caracterizar os receptores e a regulação entre eles nos eventos relacionados com a angiogênese estimulados pela OPNc e o VEGF nas células endoteliais.

6. Conclusões

Em conclusão, nossos dados indicam que a OPNc parece ter importante papel na indução de eventos relacionados à angiogênese, quais sejam a ativação da expressão de VEGF-A, de seu receptor (VEGFR-2), assim como parece estimular a formação de vasos em resposta à superexpressão desta isoforma. O conjunto de dados gerados por este trabalho indica que estes efeitos são mediados de forma importante pela via de PI3K/Akt, pelos fatores de transcrição da família AP-1 e pelas integrinas de forma RGD-dependente (Figura 12). Os resultados gerados por este estudo contribuem para o melhor entendimento dos mecanismos moleculares relacionados ao processo angiogênico ativados por células tumorais de ovário que superexpressam a OPNc.

Manuscrito em preparação: 'Osteopontin-c is more effective than OPNa and OPNb isoforms to promote VEGF expression and tumor-associated angiogenesis in ovarian cancer'.

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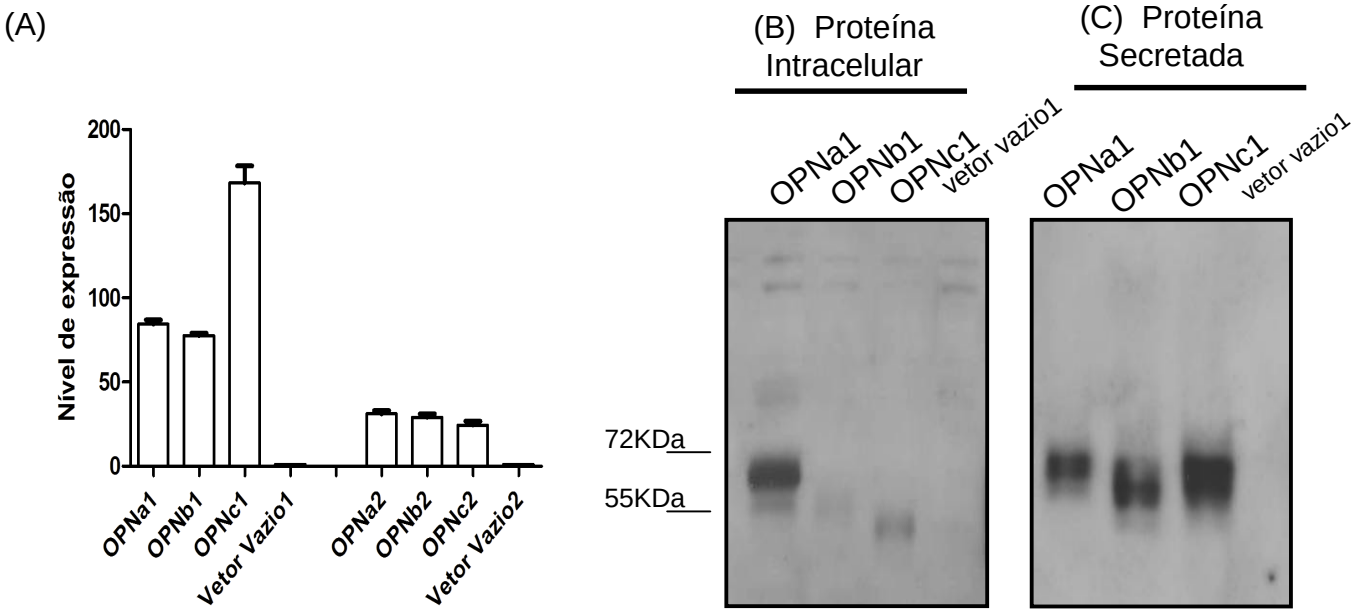


Figura 01. Caracterização da expressão das isoformas da OPN em células OvCar-3 transfectadas. (A) O nível de expressão de cada isoforma da OPN foi determinado por qRT-PCR. Para o cálculo do nível de expressão relativo de cada clone nas linhagens celulares utilizamos como referência a linhagem transfectada com o vetor vazio. O valor relativo de expressão foi calculado pelo método de delta-delta CT, conforme descrito na metodologia. (B e C) A superexpressão das isoformas da OPN foi analisada por imunoblot utilizando anticorpo anti-OPN (R&D systems) em extrato total de proteínas (B) e meio condicionado (C). O peso molecular das isoformas da OPN apresentam variabilidade de acordo com as modificações pós tradução, que são célula-específicas.

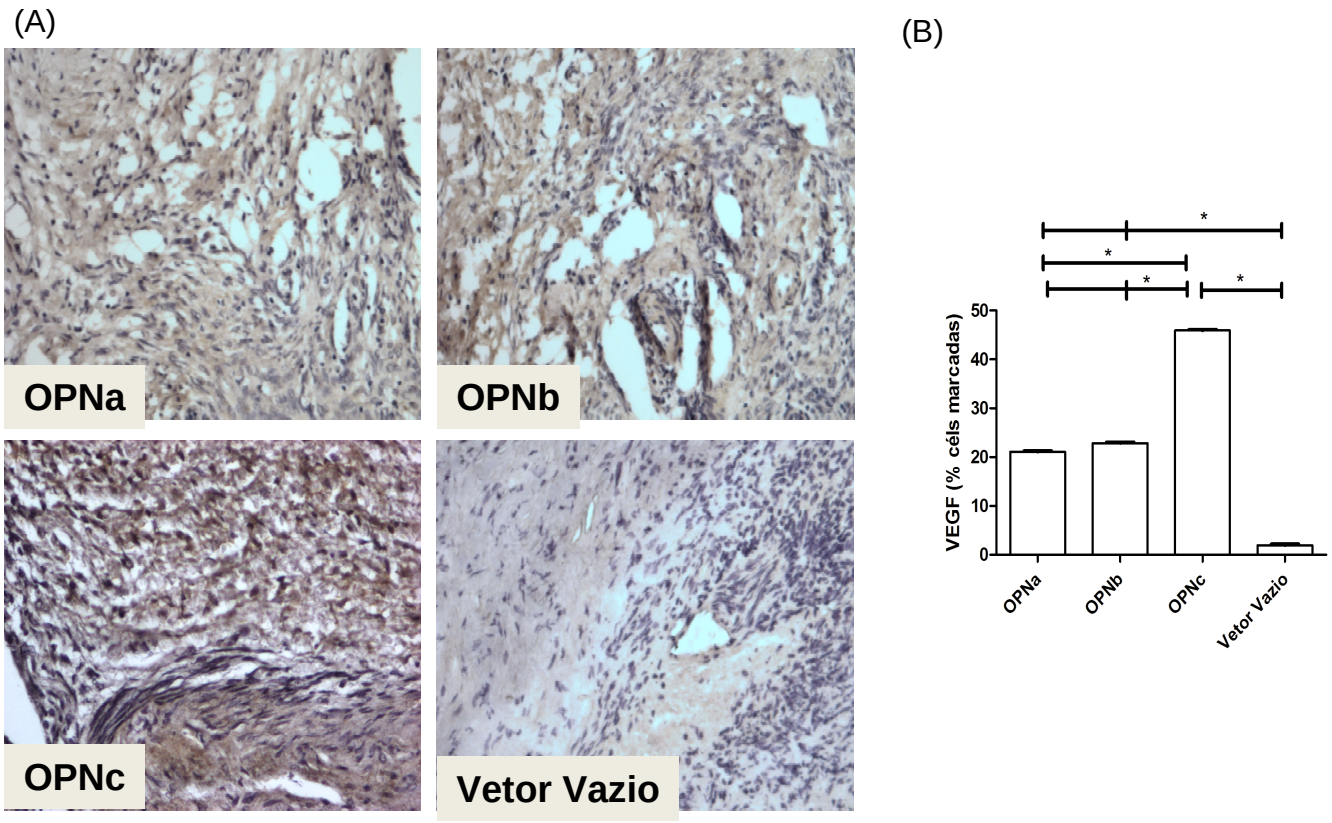
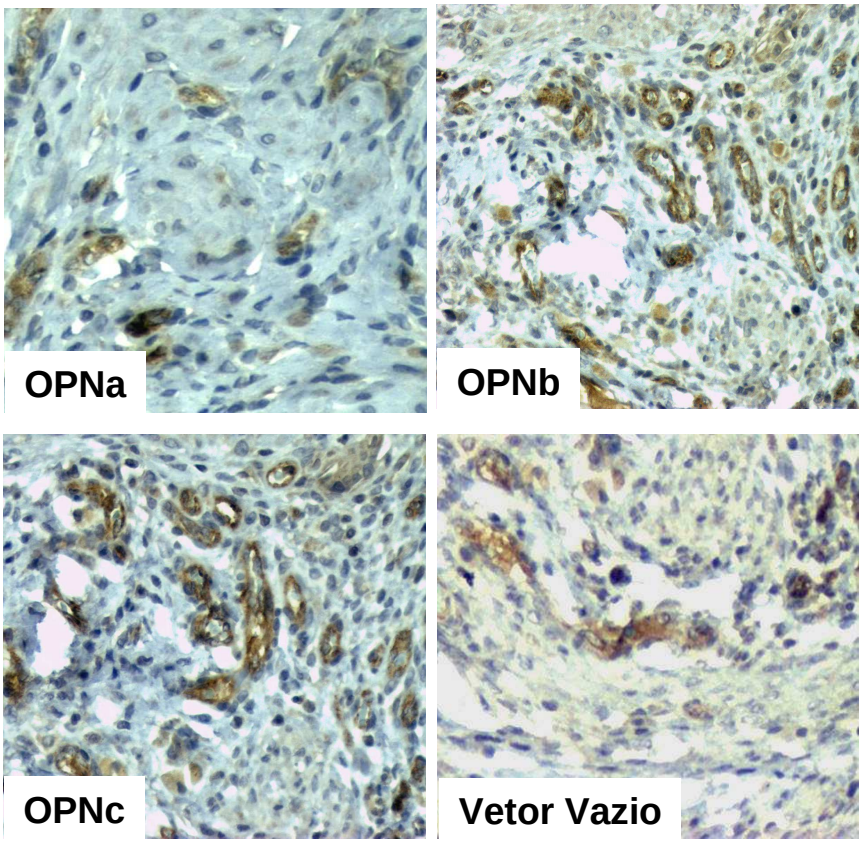


Figura 02. A superexpressão da OPNc induz a expressão de VEGF-A. (A) Imagens representativas da marcação com VEGF-A, (B) Percentual de células marcadas com VEGF-A em tumores resultantes da inoculação de células OvCar-3 transfectadas com as isoformas OPNa, OPNb, OPNc e vetor vazio em camundongos atímicos; *p < 0.0001, em relação a célula transfectada com o vetor vazio.

(A)



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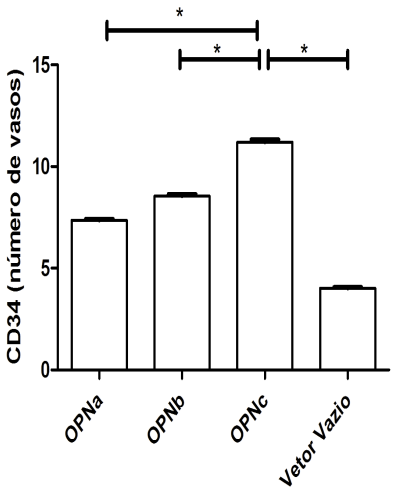

Figura 03. A superexpressão da OPNc induz formação de vasos. (A) Imagens representativas da marcação com CD34, (B) Número de vasos formados em tumores resultantes da inoculação de células OvCar-3 transfectadas com as isoformas OPNa, OPNb, OPNc e vetor vazio em camundongos atímicos; *p < 0.0001, em relação a célula transfectada com o vetor vazio.

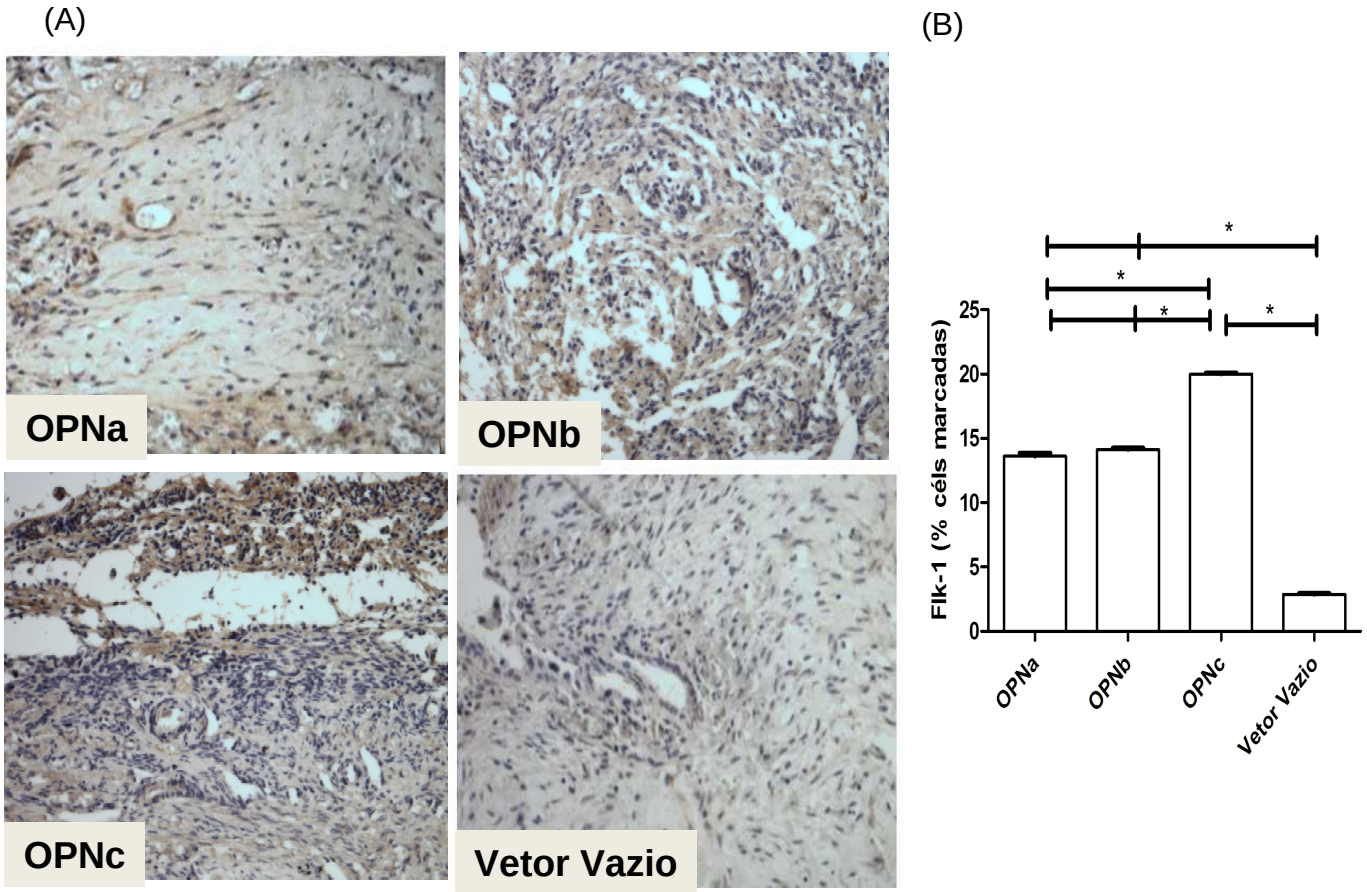

Figura 04. A superexpressão da OPNc induz a expressão do receptor de VEGF. (A) Imagens representativas da marcação com VEGFR-2 (Flk-1). (B) Percentual de células marcadas com VEGFR-2 em tumores resultantes da inoculação de células OvCar-3 transfectadas com as isoformas da OPN e vetor vazio em camundongos atímicos; *p < 0.0001, em relação a célula transfectada com o vetor vazio.

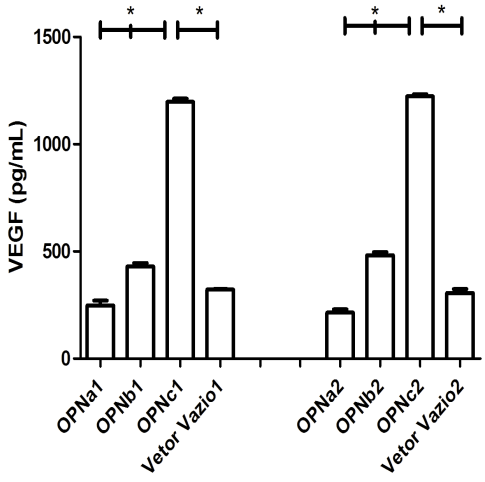

Figura 05. A superexpressão da OPNc induz a expressão de VEGF-A. (A) O nível de secreção de VEGF-A nas células OvCar-3 transfectadas com as isoformas da OPN e vetor vazio foi mensurado por ELISA. As diferenças significativas de secreção de VEGF-A estão representadas por * ($p < 0.0001$).

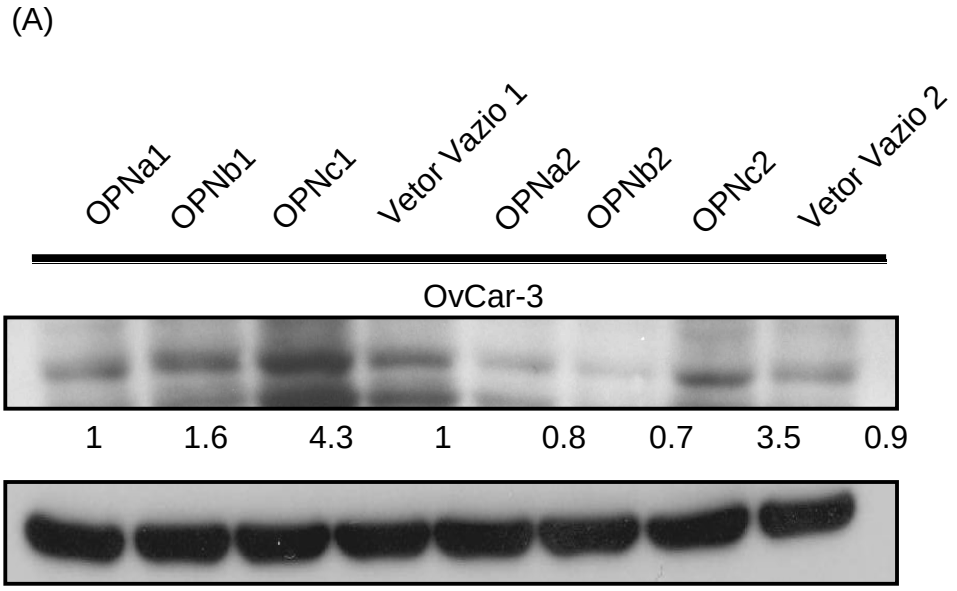

Figura 06. A superexpressão da OPNc induz a expressão de VEGF-A. (A) O nível de expressão de VEGF-A nas células OvCar-3 cultivadas com o meio condicionado secretado pelas células que superexpressam OPNa, OPNb, OPNc e vetor vazio foi mensurado por imunoblot. A intensidade de marcação para VEGF está indicada na figura. Utilizamos como normalizador a expressão de β-actina.

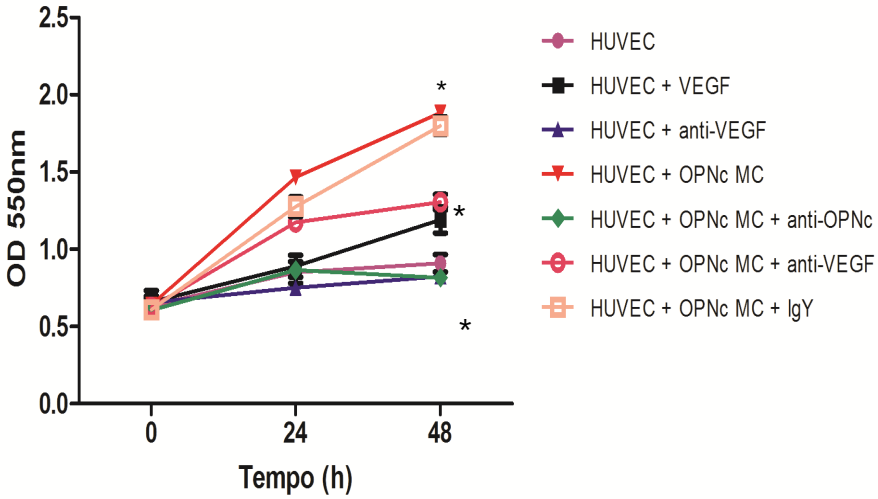

Figura 07. A OPNc e o VEGF recombinante estimulam a proliferação celular das células HUVEC. Análise da cinética da proliferação celular por coloração com cristal violeta. * $p < 0.05$ vs. clone transfectado com o vetor vazio. As legendas de cada uma das curvas estão representadas à direita do gráfico, conforme os símbolos específicos de cada clone. O tempo está representado em horas após o plaqueamento das células.

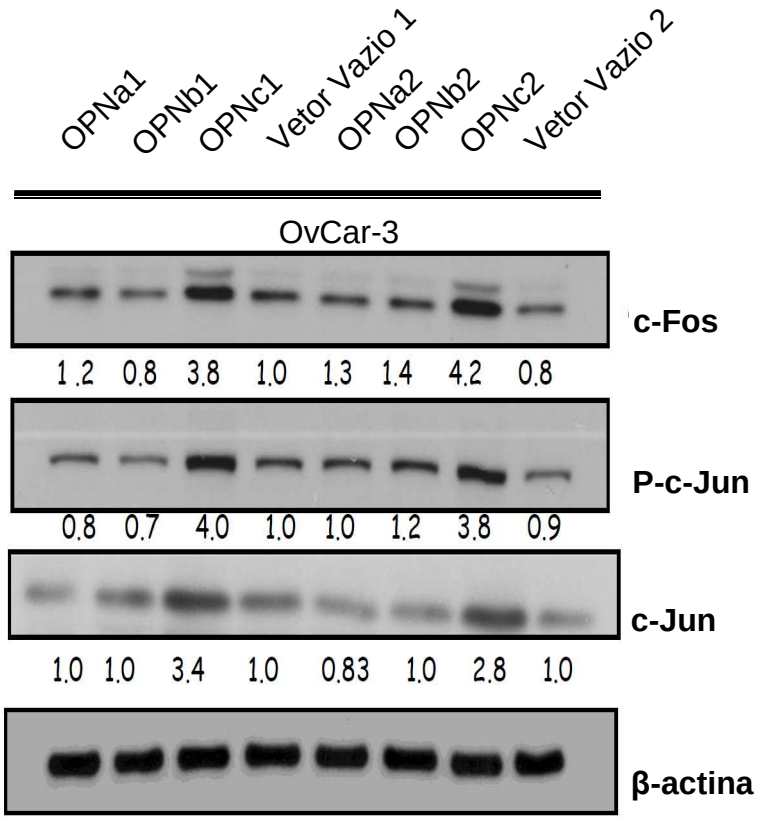

Figura 08. A superexpressão da OPNc estimula a expressão de c-Fos, P-c-Jun e c-Jun. O nível de expressão de c-Fos, c-Jun e fosforilação de c-Jun foi mensurado por imunoblot, nas células OvCar-3 cultivadas com o meio condicionado de cada isoforma e vetor vazio. A intensidade de marcação para essas proteínas está indicada na figura. Utilizamos como normalizador a expressão de β-actina.

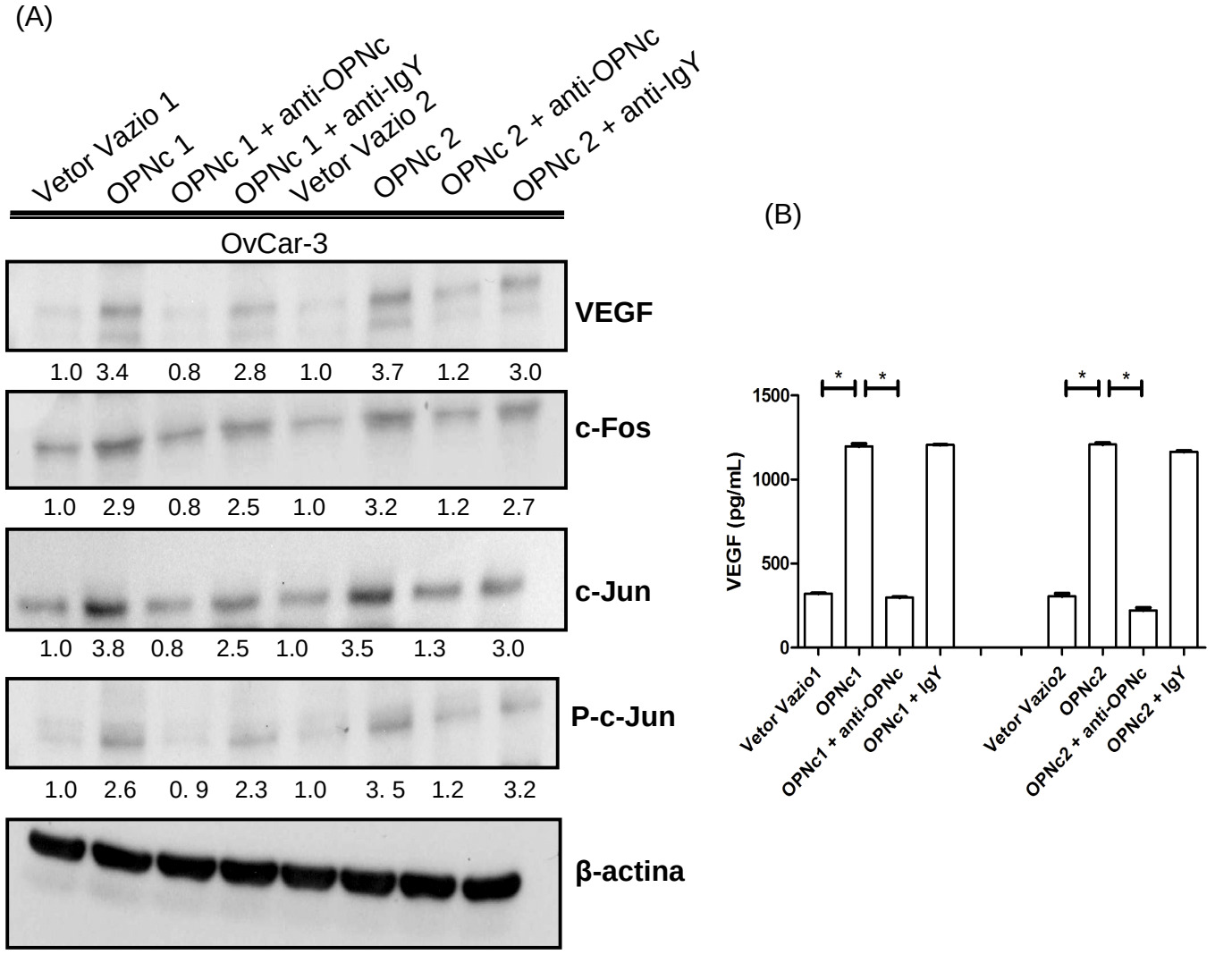

Figura 09. A neutralização da OPNc no meio condicionado inibe a indução de VEGF, c-Fos, c-Jun e fosforilação de c-Jun. (A) O nível de expressão de VEGF**,** c-Fos, c-Jun e fosforilação de c-Jun foram avaliadas na presença e ausência do anticorpo policlonal anti-OPNc ou na presença de um anticorpo não relacionado (anti-IgY). As células OvCar-3 foram cultivadas com o meio condicionado de células transfectadas com o vetor vazio e OPNc. A intensidade de marcação para essas proteínas está indicada na figura. (B) Avaliação da secreção de VEGF-A por ELISA na presença e ausência do anticorpo policlonal anti-OPNc ou na presença de um anticorpo não relacionado (anti-IgY). As diferenças significativas de secreção de VEGF estão representadas por * ($p < 0.0001$).

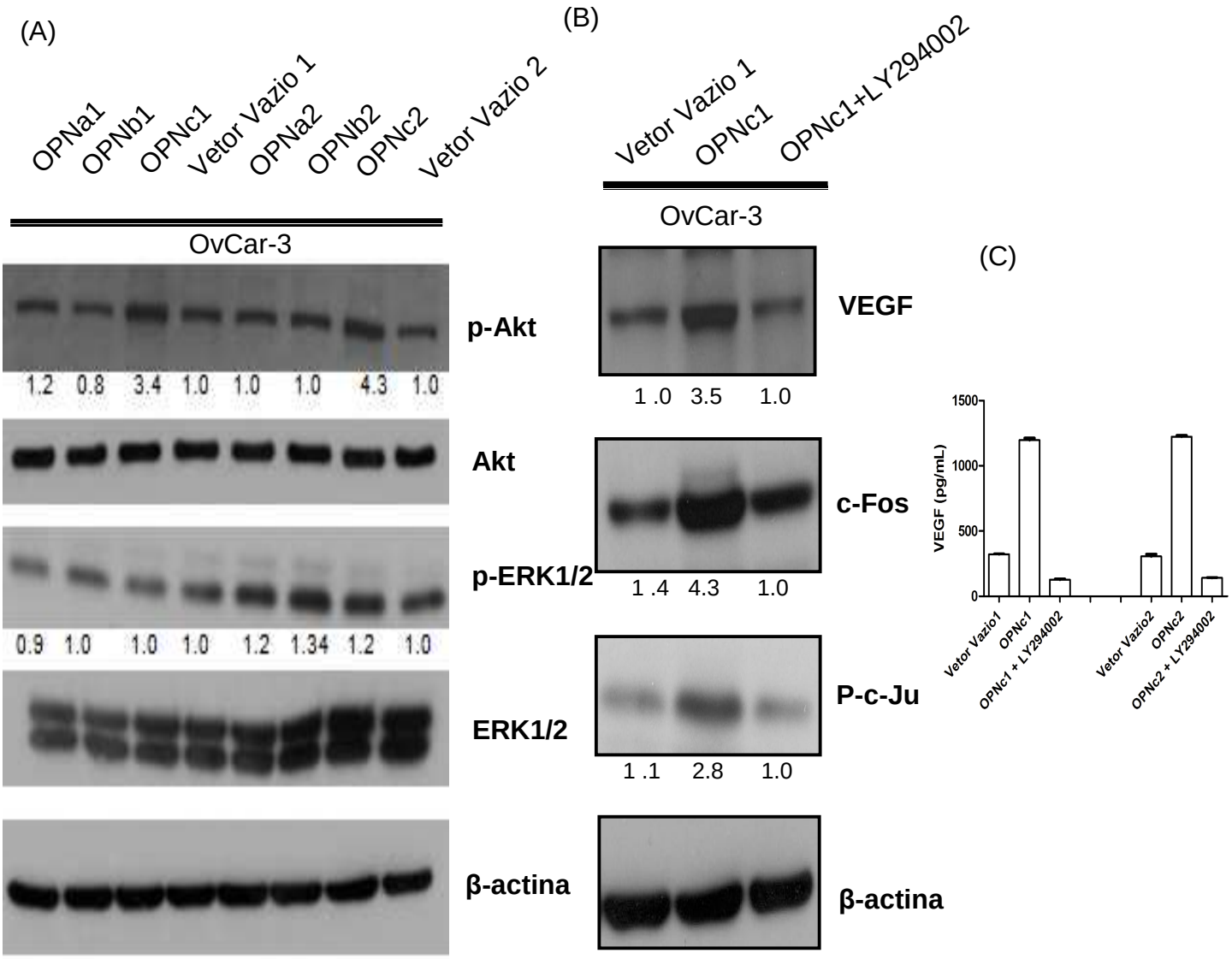

Figura 10. A via de sinalização PI3K/Akt medeia o efeito da OPNc na modulação de expressão de VEGF-A e o AP-1. (A) Avaliação das vias de sinalização PI3K/Akt e ERK1/2 nas células OvCar-3 cultivadas com o meio condicionado de cada isoforma da OPN e vetor vazio. O nível de expressão dessas proteínas foi mensurado por imunoblot nas duas séries de clones estudados. A intensidade de marcação para p-Akt e p-ERK1/2 está indicada na figura. Utilizamos como normalizador a expressão de β-actina. (B) O nível de expressão de VEGF-A**,** c-Fos e fosforilação de c-Jun foi avaliada na presença ou ausência do inibidor da PI3K - LY294002. As células OvCar-3 foram cultivadas com o meio condicionado de células transfectadas com o vetor vazio e OPNc. A intensidade de marcação para essas proteínas está indicada na figura. Utilizamos como normalizador a expressão de β-actina. (C) Avaliação da secreção de VEGF-A por ELISA. As diferenças significativas de secreção de VEGF-A estão representadas por * ($p < 0.0001$).

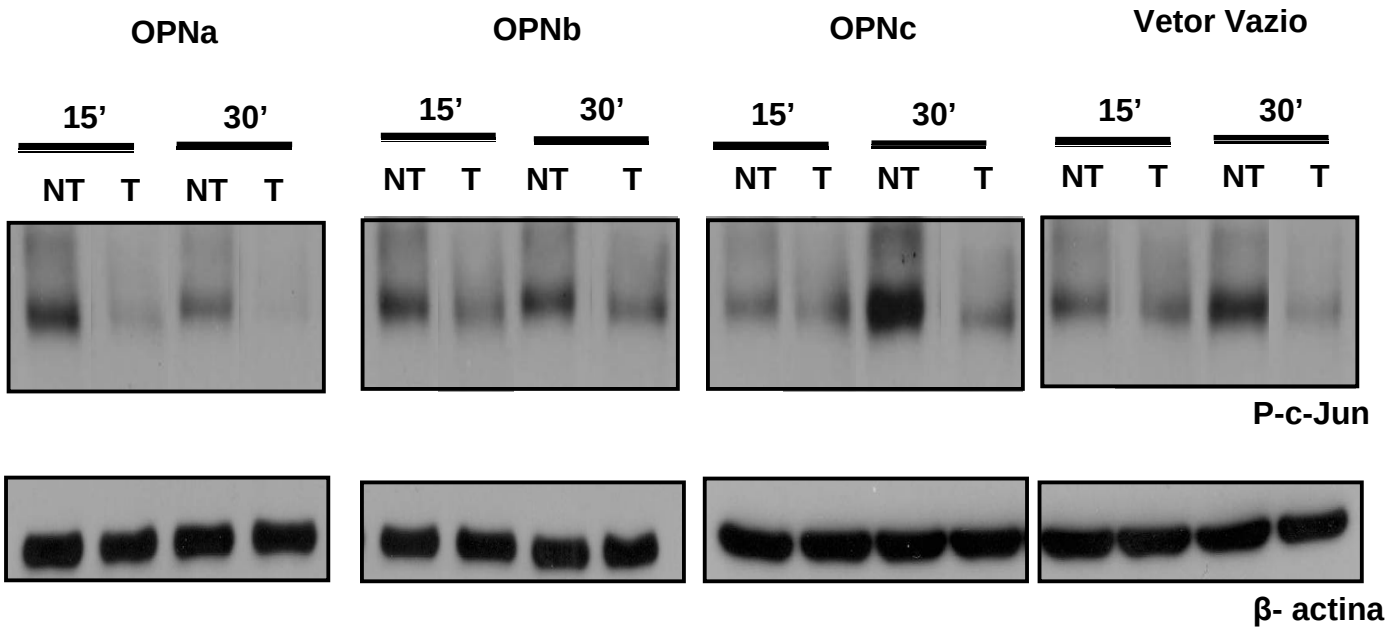

Figura 11. O bloqueio da sequência GRGDS inibe a indução de fosforilação de c-Jun pela OPNc. O nível de fosforilação de c-Jun foi avaliado na presença (T) ou ausência (NT) do peptídeo neutralizante da sequência GRGDS. As células OvCar-3 foram cultivadas, durante os tempos indicados na figura, com o meio condicionado de células transfectadas com o vetor vazio e as isoformas OPNa, OPNb e OPNc.

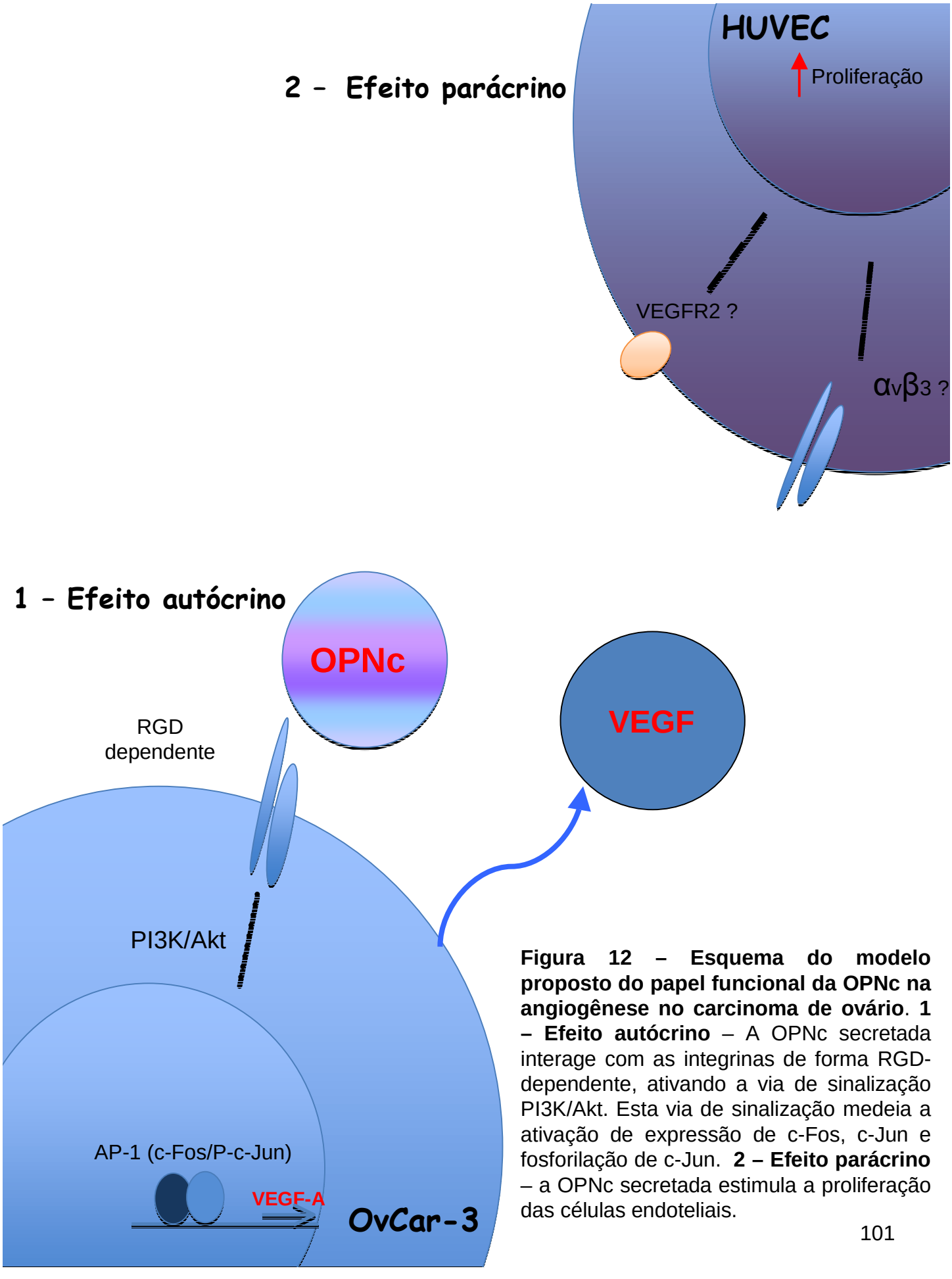

Figura 12 – Esquema do modelo proposto do papel funcional da OPNc na angiogênese no carcinoma de ovário. **1 – Efeito autócrino** – A OPNc secretada interage com as integrinas de forma RGD-dependente, ativando a via de sinalização PI3K/Akt. Esta via de sinalização medeia a ativação de expressão de c-Fos, c-Jun e fosforilação de c-Jun. **2 – Efeito parácrino** – a OPNc secretada estimula a proliferação das células endoteliais.

Capítulo III

Os resultados contidos neste terceiro capítulo da tese foram publicados no periódico *Experimental and Molecular Pathology* em 2012; 92:13–19.

O objetivo deste trabalho foi à caracterização do perfil de expressão das isoformas variantes de *splicing* da OPN em amostras de pacientes com câncer de próstata (CaP) em comparação à amostras de pacientes com tumor benigno da próstata (HPB). Observamos que a OPNa, OPNb e OPNc apresentam maior nível de expressão nas amostras de CaP quando comparado com o HPB. Adicionalmente, o nível de expressão das isoformas da OPN se correlaciona positivamente com o Escore de Gleason, indicando a potencial utilização destes biomarcadores também na avaliação prognóstica de pacientes com CaP. Dentre as isoformas da OPN, a OPNc apresenta maiores níveis de sensibilidade (100%), especificidade (90%), valor preditivo positivo (88%) e negativo (100%). Estes dados indicam que o nível de expressão da OPNc pode potencialmente ser utilizado como biomarcador para o diagnóstico e prognóstico do CaP e que supera os resultados obtidos para as demais isoformas e para a dosagem do antígeno específico da próstata (PSA).

Experimental and Molecular Pathology 92 (2012) 13–19

Contents lists available at SciVerse ScienceDirect

Experimental and Molecular Pathology

journal homepage: www.elsevier.com/locate/yexmp

Expression analysis of osteopontin mRNA splice variants in prostate cancer and benign prostatic hyperplasia

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ARTICLE INFO

Article history:
Received 1 August 2011
Available online 22 September 2011

Keywords:
Osteopontin
Splicing isoforms
Prostate cancer
Diagnosis

ABSTRACT

Osteopontin splicing isoforms (OPN-SI) present differential expression patterns and specific tumor roles. Our aims were to characterize OPN-SI expression in prostate cancer (PCa) and benign prostate hyperplasia (BPH) tissues, besides evaluating their potential as biomarkers for PCa diagnosis and prognostic implications. Prostatic tissue specimens were obtained from 40 PCa and 30 benign prostate hyperplasia (BPH) patients. Quantitative real time PCR (qRT-PCR) was used to measure OPN-SI mRNA expression. Immunohistochemical analysis was performed using an anti-OPNc polyclonal antibody. Biostatistical analyses evaluated the association of OPN-SI and total Prostate Specific Antigen (PSA) serum levels with clinical and pathological data. PCa tissue samples presented significantly higher levels of OPNa, OPNb and OPNc transcripts ($p<0.01$) than in BPH specimens. OPN-SI mRNA expression were positively correlated with Gleason Score ($p<0.01$). ROC curves and logistic regression analyses demonstrated that OPN-SI and PSA were able to distinguish PCa from BPH patients ($p<0.01$). The OPNc isoform was the most upregulated variant and the best marker to distinguish patients' groups, presenting sensitivity and specificity of 90% and 100%, respectively. Immunohistochemistry analysis also demonstrated OPNc upregulation in PCa samples as compared to BPH tissues. OPNcprotein was also strongly stained PCa tissues presenting High Gleason Score. Multivariate analysis indicated that OPNc expression levels above the cut-off value presented a chance 4-fold higher for PCa occurrence. We conclude that OPN-SI were overexpressed in PCa tissues, strongly associated with PCa occurrence and with tumor cell differentiation. Our results suggest OPNc splicing isoform as an important biomarker contributing to improve PCa diagnosis and prognosis, besides providing insights into early steps of PCa carcinogenesis.

Introduction

Prostate cancer (PCa) and benign prostatic hyperplasia (BPH) are the most common prostatic diseases in aging men, with an increasing number of affected men over the past two decades. Most prostate adenocarcinomas arise concomitantly to BPH, and PCa is found incidentally in a significant number of transurethral prostatectomy (TURP) specimens (10%). BPH arises in the transition zone or peri-urethral glands where stromal and epithelial nodules develop, whereas PCa arises in the peripheral zone of the prostate gland where epithelial cells undergo malignant transformation (McPherson et al., 2010). Currently, the most commonly used screening technique for early detection of PCa is the measurement of total Prostate Specific Antigen serum levels (PSA), with increasing numbers of PCa cases diagnosed every year. However, this marker presents low specificity, and has led to a large number of unnecessary biopsies of BPH patients, which means overdiagnosis and overtreatment of clinically insignificant hypertrophy. Moreover, many PCa tumors present PSA serum levels within normal limits. Hence, the differential diagnosis of PCa and BPH patients is still a major challenge for urological clinicians (Andrew et al., 2008; Cao and Ma, 2011). Therefore, additional biomarkers for PCa diagnosis and treatment follow-up

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doi:10.1016/j.yexmp.2011.09.014

are still necessary and must be developed. Although several biomarkers have been proposed for early detection of PCa (El Melegy et al., 2010; Kucur et al., 2008; Mearini et al., 2010; You et al., 2010), only serum PSA is considered as a marker for PCa diagnosis, which is often combined with digital rectal examination (DRE). The selection and evaluation of new markers might help overcoming the limits of the current assay and the clinical examination.

High-throughput technologies have enabled the identification of genes and their corresponding proteins that are differentially regulated in PCa (Brooks, 2002; Mackinnon et al., 2009; Mendes et al., 2008;). Many of these findings provided new insights into the biology of prostate carcinogenesis and have identified the next generation of candidate biomarkers and potential therapeutic targets. Among those targets, osteopontin (OPN) has been identified as overexpressed in localized and metastatic PCa tissues (Angelucci et al., 2002; Khodavirdi et al., 2006; Liu et al., 2011; Ramankulov et al., 2007; Zhang et al., 2011). OPN is one of the most upregulated proteins in PCa tissues when compared to BPH (Forootan et al., 2006). This glycophosphoprotein act as important autocrine and paracrine signal that affect growth and behavior of prostate carcinoma cells (Angelucci et al., 2004; Robertson et al., 2010). OPN-associated androgen independent growth of PCa cells commonly occurs in the advanced stage of the disease, and has been significantly associated with survival. Besides, OPN has also been described as an important marker for stage, grade and early tumor progression in PCa (Robertson et al., 2010; Weber et al., 2011).

Post-translational modifications regulate OPN's role in physiological and pathological processes (Anborgh et al., 2011). Besides, OPN mRNA is subject to alternative splicing, resulting in isoforms that are smaller than full-length OPN (Crawford et al., 1998; Kon et al., 2000). Alternative splicing leads to deletions in the N-terminal portion of OPN, located upstream from the central integrin and C-terminal CD44 binding domains (Kiefer et al., 1989). Osteopontin-a (OPNa) is the full-length isoform, while osteopontin-b (OPNb) lacks exon 5 and osteopontin-c (OPNc) lacks exon 4 (Young et al., 1990). Alternative splicing is an important mechanism to increase structural and functional diversity of proteins (Blencowe, 2006; Luco and Misteli, 2011). In addition, growing evidence indicates that splicing variants can provide diagnostic and/or therapeutic targets for several cancers (Pajares et al., 2007). Thus, it is reasonable to speculate that these OPN splice isoforms (OPN-SI) present differential expression between PCa and BPH specimens and whether these variants could be related to PCa carcinogenesis.

In an earlier report, we had shown higher expression of OPN-SI in PCa tissues as compared to prostate non-tumoral samples (Tilli et al., 2011b). In this report, in vitro and in vivo studies demonstrated that OPNc and OPNb isoforms are able to activate different aspects of PCa progression. Despite these data, the roles of each OPN-SI has been shown to be tumor and tissue specific (Blasberg et al., 2010; ; He et al., 2006; Mirza et al., 2008; Tilli et al., 2011a;). Also, since the overexpression of some of these OPN-SI has been associated with tumor progression (Ivanov et al., 2009; He et al., 2006; Mirza et al., 2008; Tilli et al., 2011a;;), one can speculate these may also function in the pathogenesis of BPH. Based on the differential expression of full-length OPN in prostate tissue in relation to BPH (Forootan et al., 2006) and on previous data demonstrating that OPN-SI differential expression behaves as potential new markers for cancer diagnosis (, He et al., 2006; Mirza et al., 2008; Tilli et al., 2011a), the aim of this study was to examine and quantify the expression of OPN-SI in human PCa and BPH tissues and evaluate their implications as diagnostic and prognostic markers.

By evaluating the expression pattern of each OPN-SI in these samples, we have demonstrated herein that OPNa, OPNb and OPNc splice variants are overexpressed in PCa in relation to BPH. Among these isoforms, the OPNc was the most upregulated isoform in PCa, and outperformed the remaining isoforms and PSA serum levels in PCa diagnostic accuracy. We here present evidence that OPNc could be an auxiliary additional biomarker for improvement of PCa diagnosis.

Material and methods

Patients

Tissue samples were obtained from biopsy-proven clinically localized PCa patients which were collected during prostatectomy. All BPH cases corresponded to patients that were submitted to surgical treatment by open adenomectomy. BPH diagnosis was done by clinical symptoms and by analyzing the prostate volume, and all the cases included presented strong or moderate bladder outlet obstruction designated by the IPSS score (International Prostate Symptom Score) (Barry et al., 1992). Tissue samples were obtained from 40 PCa patients (ages 41–74 years, median 67) and 30 patients with benign prostate hyperplasia (BPH) (ages 58–87 years, median 69.5) and soon after collected, were stored in RNA Later (Ambion) at −20 °C until processing. Samples were collected from February, 2003 to August, 2005 at Universidade Estadual do Rio de Janeiro (UERJ), Instituto Nacional de Câncer (INCa) and Universidade Federal de Uberlândia (UFU) hospitals. Patients were classified by clinical stage and pathological Gleason Score. Prostatectomy specimens were graded according to Gleason system as determined by histological examination. Prior to surgery, the patients had not received any chemotherapy or hormonal therapy. The local Ethics Committee approved this study and all patients provided informed consent for this study. Some features of PCa patients included in this study, such as age at diagnosis, preoperative level of prostate-specific antigen (PSA), Gleason score and tumor staging (pT) are shown on Tables 1 and 5.

RNA preparation, cDNA synthesis and quantitative real-time RT-PCR

RNA was extracted from tumor tissues using the RNeasy kit (Qiagen) and RNA mass was determined on a NanoDrop™ 1000 spectrophotometer (Thermo Scientific). cDNA synthesis was carried out using SuperScript II First-Strand Synthesis System for RT-PCR using oligo(dT) primer (Invitrogen) using 1 μg of total RNA. The amplification of fragments corresponding to each OPN isoform was performed using the following oligonucleotides pairs: OPNa:5′-ATC TCC TAG CCC CAC AGA AT-3′ (forward) and 5′-CAT CAG ACT GGT GAG AAT CAT C-3′ (reverse); OPNb: 5′-CTC CTA GCC CCA CAG ACC CT-3′ (forward) and 5′-TAT CAC CTC GGC CAT CAT ATG-3′ (reverse); OPNc: 5′-CTG AGG AAA AGC AGA ATG-3′ (forward) and 5′-AAT GGA GTC CTG GCT GT-3′ (reverse). GAPDH was

Table 1
Descriptive statistics of continuous variables in tissue and serum from PCa and BPH patients.

	Median	Range	Percentile				
			10th	25th	50th (median)	75th	90th
PCa (n = 40)							
OPNa[a]	62.43	0.00–331.34	0.00	0.07	62.43	120.18	302.69
OPNb[a]	43.47	0.00–122.16	0.00	1.46	43.47	81.94	114.21
OPNc[a]	200.36	0.00–900.54	9,10	110.90	200.36	601.60	811.93
PSA ng/mL	7.51	0.30–21.10	4.24	5.58	7.51	11.87	18.38
Age, years	67.0	41.00–74.00	58.0	60.5	67.0	71.0	73.0
BPH (n = 30)							
OPNa[a]	0.20	0.00–55.52	0.00	0.00	0.20	2.85	17.56
OPNb[a]	0.50	0.00–42.66	0.00	0.06	0.50	3.50	35.86
OPNc[a]	0.00	0.00–9.00	0.00	0.00	0.00	0.00	7.7
PSA ng/mL	4.15	0.20–24.28	0.86	2.75	4.15	7.90	14.61
Age, years	69.50	58.0–87.0	59.2	63.00	69.5	73.0	81.7

[a] OPN isoforms tissue expression levels are indicated by fold-change in relation to the BPH tissue sample presenting the lowest expression level. Serum PSA levels are represented in ng/mL.

amplified with primers 5′-TGA CCC CTT CAT TGA CCT CA-3′ (forward) and 5′-AGT CCT TCC ACG ATA CCA AA-3′ (reverse) and served as an internal control to normalize expression data and to verify integrity of the cDNA. In order to evaluate similar PCR amplification efficiencies of OPN-SI and GAPDH genes, a serial dilution analysis was performed using cDNA synthesized from total RNA from PC3 prostate tumor cells. PC3 cell line was supplied by ATCC and cultured in a humidified environment containing 5% CO2 at 37 °C. Cells were cultured in RPMI medium supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin and 100 units/mL streptomycin.

All quantitative real time PCR (qRT-PCR) reactions were conducted using the SYBR Green detection reagent (Applied Biosystems). Conditions for OPN-SI PCR amplification were: 50 °C for 2 min and 94 °C for 5 min, followed by 10 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 90 s. At the end of each cycle the temperature decreased 0.5 °C, followed by 30 cycles of 94 °C for 30 s, 50 °C for 30 s and 72 °C for 90 s, ending the reaction with 72 °C for 15 min. A melting curve analysis was generated to determine amplification efficiency and specificity (60–90 °C with a heating rate of 0.2 °C/s and continuous fluorescence measurement). Product purity, size and absence of primer dimers were confirmed by the DNA melting curve analysis and by agarose gel electrophoresis. Relative gene expression of the target gene was calculated by using the ΔΔCT method. GAPDH amplification was used as normalization control for OPN isoforms transcription level evaluation.

Immunohistochemistry

Ten representative tissue samples from PCa and BPH confirmed by the examining pathologist were stained for OPNc. Paraffin sections from PCa and BPH tumors were deparaffinized in xilol and rehydrated in alcohol according to standard procedures. Antigen retrieval for OPNc was not required. The slides were incubated with an affinity-purified anti-OPN-c chicken IgY (produced by Gallus Immunotech from the peptide ac-SEEKQNAVSC), at 1:160 dilution for 32 min at room temperature. The secondary antibody, HRP-conjugated goat anti-chicken IgY secondary antibody (Pierce) diluted to 1:80 in PBS (phosphate buffered saline) containing 1% bovine serum albumin, was applied for 16 min at room temperature. Antibody binding was detected using the ChromoMapTM DAB detection kit (Dako). Negative control slides were incubated in absence of primary antibody only. All slides were counterstained with Hematoxylin for 2 min. DAB staining intensity was compared between PCa and BPH. The staining results were evaluated by 2 researchers, with the density of immunolabeling scored as weakly (+), moderately (++), or strongly (+++) positive.

Statistical analysis

All the statistical analyses were performed by using the SPSS software version 18.0 (SPSS Inc, Chicago, IL, USA). Descriptive statistics were calculated. The OPN-SI expression levels for BPH and PCa patients did not follow the Gaussian distribution, and variables were logarithmically transformed [Log_n (OPNc + 0.5)] for analyses purpose. The median comparisons between the two groups were performed by using the non-parametric Mann–Whitney 2-tailed *U*-test. Sensitivity, specificity, accuracy and predictive values were estimated based on the gold-standard pathological diagnosis. ROC curves were constructed for both OPN-SI expression and pre-surgical PSA serum concentration by plotting sensitivity × 1-specificity, and cut-off values were calculated based on the largest area under the curve. The ability of OPNc isoform to predict PCa occurrence was verified by univariate and multivariate logistic regression analyses. The logistic regression models using the "Enter" method with 95% confidence interval were adjusted for PSA serum concentrations. *P* value <0.05 was considered statistically significant.

Results

Relative quantification of OPN-SI expression in prostatic tissues

Quantitative RT-PCR assays were performed to estimate the mRNA expression levels of OPNa, OPNb and OPNc splicing isoforms in prostate tissue samples, employing GAPDH as an endogenous control. The three known OPN-SI were detected in both PCa (n = 40) and BPH (n = 30) samples (Fig. 1A). We then compared the expression levels of OPNa, OPNb and OPNc isoforms in these prostatic tissues (Fig. 1 and Table 1). Descriptive analysis of continuous variables for serum and tissue samples in both patients' groups is shown in Table 1. OPNa, OPNb and OPNc isoforms expression levels in PCa presented median relative expression levels of 62.43; 43.47 and 200.36, respectively. On the other hand, for BPH specimens, OPNa, OPNb and OPNc isoforms presented median relative expression levels of 0.20; 0.50 and 0.00, respectively. The median differences for all OPN-SI variants between the two groups were statistically significant ($p<0.01$). OPN-SI expression profiles of prostate tissues demonstrated that OPNa, OPNb and OPNc isoforms were upregulated and consistently overexpressed in PCa specimens as compared

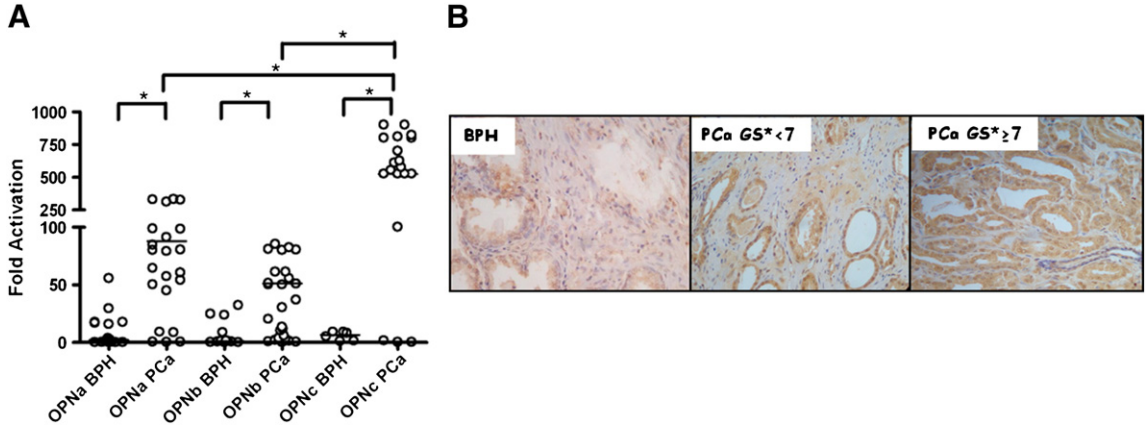

Fig. 1. OPN-SI are overexpressed in PCa samples. (A) The relative expression level of OPNa, OPNb and OPNc isoforms was analyzed by qRT-PCR approach using splicing isoform specific oligonucleotides, as described in methods. The expression level of each OPN isoforms was analyzed in PCa (40 samples) and BPH (30 samples). (A) The horizontal bold lines represent the median relative mRNA expression value (50th percentile) for each patient. Relative expression of each tissue sample (fold activation) was calculated in relation to a BPH samples presenting the lowest expression level of one OPN isoform. GAPDH gene was used as a normalization constitutive gene. (B) Immunohistochemistry of OPNc isoform in benign and malignant prostate tissues. Representative tissue samples tested for OPNc expression in BPH tumors , malignant prostate tumors presenting Gleason Score (GS) below 7 or above 7 are shown. Immunostaining was weak in BPH samples, moderate to strong in PCa tumors with low GS and a strong expression in high GS PCa samples. Magnification 40×.

to BPH samples (Fig. 1B). Interestingly, there was a striking upregulation of OPNc expression in PCa samples in relation to BPH specimens in all cases, including the 10th percentile (Table 1). The median (50th percentile) value of OPNc isoform expression was found approximately 200-fold higher in PCa (median relative expression level: 200.36) compared to BPH (median relative expression level: 0.00) tissue specimens. OPNc mRNA overexpression in relation to BPH samples has been validated at the protein level when testing samples from these tissues by immunohistochemistry using an anti-OPNc antibody. Staining intensity for PCa samples was consistently stronger than BPH tissue samples (Fig. 1B and Table 2).

Evaluation of OPN isoforms mRNA expression in the differential diagnosis of PCa and BPH

Based on OPN-SI differential expression in PCa in relation to BPH samples, ROC curve analyses were generated to estimate the potential of OPN-SI expression in tissue samples and pre-surgical PSA serum levels to distinguish PCa from BPH patients. Fig. 2 shows that OPNa, OPNb, OPNc and PSA levels can significantly distinguish these two groups of patients. However, OPNc isoform expression presented the best performance and significant discriminatory value for the patient's population (AUC 0.97; 95% CI 0.90–0.99, $p<0.01$) when compared to tissue expression of OPNa (AUC 0.74; 95% CI 0.63–0.84, $p<0.01$), OPNb (AUC 0.77; 95% CI 0.65–0.86, $p<0.01$) and PSA serum levels (AUC 0.72; 95% CI 0.59–0.81, $p<0.01$).

A cut-off value was calculated for each OPN-SI and serum PSA based on the highest value of the slope region in the ROC curves (Fig. 2 and Table 3). The cut-off values were estimated in order to obtain the best discriminatory power between PCa and BPH patients. The sensitivity, specificity, accuracy and positive and negative predictive values for the OPN isoforms expression in tissue samples and PSA serum levels are shown in Table 3. The OPNc mRNA transcript expression level had the best discriminatory power in distinguishing between PCa and BPH tissues, with sensitivity and specificity of 90% and 100%, respectively. The OPNc outperformed OPNa, OPNb and PSA serum levels in PCa diagnosis. When performing a combined analysis of positivity for OPNa, OPNb and OPNc isoforms, specificity was improved (100%) in relation to positivity for PSA, OPNb and OPNa alone, but maintained in relation to OPNc. However, using this panel of markers, there was a huge decrease in sensitivity properties (42.3%), as compared to tests using positivity for any individual marker. When adding positivity for serum PSA to this combined analysis, diagnostic properties are quite similar to the test positive for the 3 OPN isoforms. Additionally, we also tested whether considering positivity for both OPNc and PSA above cut-off points could improve diagnostic properties. When compared to positivity for OPNc alone, this evaluation decreased sensitivity and specificity in distinguishing PCa from BPH samples. In summary, these results indicate that among the markers tested herein, the evaluation of OPNc expression levels alone in PCa tissues provide the better sensitivity and specificity results in predicting PCa.

Considering that the most accurate PCa diagnostic properties were achieved by testing the upregulation of the OPNc isoform in tissue samples in relation to the OPNa, OPNb and PSA markers, we have further tested logistic regression models to evaluate the value of OPNc isoform expression levels to predict the chances of PCa occurrence (Table 4). Multivariate analyses demonstrated that patients with high OPNc transcript levels presented significant chances of having PCa, ranging almost three to four-fold higher when compared to PSA ($p<0.05$). Although measurement of PSA promote a 1.31-fold increase in the prediction of PCa ($p<0.05$), this individual risk is considerably lower as compared the risk promoted by OPNc tissue expression level. Our data suggest that OPNc isoform expression analysis significantly added to the diagnostic power of this multivariate model, indicating that this splicing isoform is an independent factor for the differential diagnosis of PCa. Besides, these data suggest that more emphasis should be placed on evaluation of OPNc tissue expression level to better contribute to the prediction of PCa.

Table 2
Immunohistochemical analyses of OPNc staining in BPH and PCa.

Histology	OPNc Staining			
	n	+	++	+++
BPH	10	10	–	–
PCa	10			
Gleason score<7	5	–	5	–
Gleason score≥7	5	–	–	5

Weakly (+), moderately (++), or strongly (+++) positive staining.

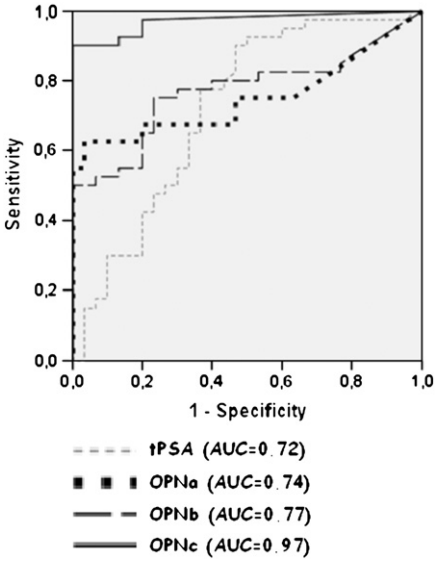

Fig. 2. Receiver-operating characteristic (ROC) curves analyses for evaluating diagnostic properties of OPN-SI as compared to serum PSA. Individual markers comprising tissue OPNa, OPNb and OPNc and serum PSA from men with PCa (n = 40) and with BPH (n = 30) were tested. OPN-SI expression levels were analyzed by qRT-PCR reaction using GAPDH as the internal control. Serum PSA was measured and represented as ng/mL on serum samples from these patients. The AUC for each marker are shown on the left with $p<0.05$.

Relationship of OPN isoforms expression with other clinicopathological features

Median comparisons for the OPN-SI expression and serum PSA levels were also evaluated according to Gleason Score (GS) of tissue specimens and TNM tumor stage classification. The GS of tumor specimens were classified into high (≥7) and low (<7) grades. All three isoforms presented significant higher transcript levels in the high GS samples as compared to low GS specimens ($p<0.01$). The same was not observed when correlating PSA levels to GS (Table 5). Once OPNc transcript presented the most significant differences in mRNA expression level between high and low GS samples, we further tested OPNc protein expression by immunohistochemistry using an anti-OPNc antibody in these two groups of PCa tumors. Tumor samples presenting high GS also presented a stronger staining pattern for OPNc protein as compared to samples presenting low GS, as well to BPH samples (Fig. 1B). As far as the splice variants of the OPN gene are concerned, no statistically significant correlation was observed between their levels of expression and TNM stage. Specimens were classified into early (T2) and advanced (T3) stages, but neither the three splice variants nor PSA levels presented significant differences between tumor stages ($p>0.01$). These data indicate that OPN-SI, especially OPNc, expression levels are correlated to PCa tumor cell differentiation and could have implications as prognostic markers.

Table 3
Diagnostic parameters for OPN isoforms and PSA serum levels.

	OPNa	OPNb	OPNc	PSA	Positive test: [OPNa + OPNb + OPNc]	Positive test: [OPNa + OPNb + OPNc + PSA]	Positive test: [OPNc + PSA]
Cut-off[a]	29.34	1.75	9.0	4.3	29.34/1.75/9.0[b]	29.34/1.75/9.0 /4.3 [c]	9.0 /4.3 [d]
Sensitivity	62.5	75.0	90.0	90.0	42.3	42.3	47.5
Specificity (%)	96.7	76.7	100.0	53.3	100.0	100.0	96.0
Predictive Value Positive (%)	96.2	81.1	100.0	72.0	100.0	100.0	95.0
Predictive Value Negative (%)	65.9	69.7	88.2	80.0	56.0	56.0	58.0
Accuracy (%)	77.1	75.7	94.3	74.3	67.0	67.0	68.5

[a] The cut-off values were selected for each OPN isoform and PSA serum levels based on the highest value for the slope region in each ROC curve. Samples presenting values above the cut-off value were considered positive for an individual marker.
[b] : Values indicate respectively the cut-off levels for OPNa, OPNb and OPNc isoforms.
[c] : Values indicate respectively the cut-off levels for OPNa, OPNb and OPNc isoforms and PSA.
[d] : Value indicate respectively the cut-off level for OPNc isoform and PSA.

Discussion

In the present study, for the first time, it is pinpointed the differential expression of OPN-SI between PCa and BPH tissue specimens. Taking a step forward, we investigated the value of OPN-SI expression pattern in the case of discrimination between CaP and BPH tissue specimens. We demonstrated that an important improvement in PCa diagnostic features can be achieved by using the OPNc isoform expression as an auxiliary tissue marker to better distinguish these two prostatic diseases. Moreover, OPNc expression level seems to add prognostic information related to PCa cell differentiation. Although the expression of OPN-SI is present in both benign hyperplastic and cancerous lesions of the prostate, OPN-SI overexpression, especially OPNc, might be enhancing a transcriptional pathway that is critical to the pathogenesis of prostate malignant tumors. Functional assays previously performed by our group corroborate to these findings, once PCa cells overexpressing OPNc, and to a lesser extent OPNb, activated different aspects of PCa progression, such as cell proliferation, migration, invasion, soft agar colony formation and tumor formation in vivo. Besides, we also demonstrated that secreted OPNc can stimulate the proliferation of prostate non-tumoral cells. Further, we showed that when overexpressed, OPNc better activated PI3K signaling, an important pro-survival pathway involved in tumor formation and progression (Tilli et al., 2011b). Similar data were obtained for ovarian carcinoma cells (Tilli et al., 2011a). As a whole, these data give support to the notion that during the pathogenesis of PCa, OPNc overexpression activates import pathways promoting malignant tumor formation.

A number of alternatively spliced genes have been reported in PCa, some of them being employed for diagnosis and prognosis prediction in PCa, such as kallikreins, the spliced variant of fibroblast growth factor receptor (FGF-R2) and truncated form of VEGF receptor flt-4 (VEGFR-3) (Cho et al., 2010). Other reports also indicate the use of alternatively spliced genes for these purposes other tumor models (Arafat et al., 2011; Goodison et al., 1999; Pajares et al., 2007);. Besides, some authors also suggested that the full length OPN and its splicing isoforms may be potential biomarkers in cancer (Anborgh et al., 2011). We have shown herein that the three OPN-SI present features of PCa tissue biomarkers and demonstrated that all three isoforms can efficiently distinguish PCa from BPH samples. Nevertheless, we identified that among tested OPN isoforms, OPNc expression levels as the most important marker associated with PCa occurrence and cancer progression, which is in accordance to pro-tumorigenic roles of OPNc, previously described by our group (Tilli et al., 2011b).

The OPNc expression levels have significantly distinguished PCa from BPH with a very high accuracy and the odds for PCa occurrence was four-fold higher when OPNc levels were highly expressed in tissues. These results corroborate previous findings, in which the OPNc isoform expression has been associated with tumor progression in ovarian, breast and pancreatic cancer tumor models (, He et al., 2006; Mirza et al., 2008; Sullivan et al., 2009; Tilli et al., 2011a). Hence, the potential use of OPNc expression in PCa diagnosis is in accordance to its biological role in PCa and in other tumor models, as aforementioned. Contrarywise, in other reports the role of OPNc is less clear, once OPNa and OPNb are the most predominant isoforms expressed in other tumor types, such as liver cancer, malignant mesothelioma, glioma and lung cancer (Chae et al., 2009; Courter et al., 2010; Goparaju et al., 2010; Ivanov et al., 2009; Yan et al., 2010;). Furthermore, in mesothelioma, non-small-cell lung cancer (NSCLC), and head and neck squamous cell carcinoma (HNSCC), the OPNa and OPNb isoforms are able to stimulate pro-tumorigenic behaviors, but not the OPNc (Blasberg et al., 2010; Courter et al., 2010; Ivanov et al., 2009;). In lung cancer, OPNc overexpression was associated with decreased angiogenic properties, whereas OPNa and OPNb presented opposite roles (Blasberg et al., 2010). These data reinforces the notion that OPN-SI present tumor and tissue specific expression profiles and roles. Therefore, OPNc may not function as a biomarker on tumors in which this isoforms is not upregulated when compared to non-malignant tumor samples, or in cancer types where it does not behave as a pro-tumorigenic factor, such as in mesothelioma and glioma (Ivanov et al., 2009; Yan et al., 2010).

We have provided evidence that the OPNc isoform could be part of a panel of tissue biomarkers developed to increase the specificity of PCa diagnosis, which corroborate findings published elsewhere (Avgeris et al., 2011; Day et al., 2011; Korbakis et al., 2009;; Mlcochová et al., 2009; Rigau et al., 2010; Steiner et al., 2010). However, the ability of OPNc expression to discriminate between PCa and BPH patients in biopsies should be validated in a different and large population cohort in order to demonstrate the usefulness of tissue OPNc expression evaluation as a routine marker for clinical use in PCa patient management.

Table 4
Odds ratios estimates for PCa occurrence by logistic regression analyses of PCa and BPH patients.

Markers	Univariate			Multivariate		
	Odds ratio	95% CI	P value	Odds ratio	95% CI	P Value
L_n (OPNc level + 0,5)	2.84	1.84–4.39	p<0.05	3.97	1.95–8.06	p<0.05
PSA	1.13	1.01–1.26	p<0.05	1.31	1.06–1.64	p<0.05

CI = confidence interval; Ln = neperian logarithm.

Table 5
Relationships between OPN isoforms and PSA levels in clinical–pathological parameters.

Parameters	n	Median expression level			
		OPNa	OPNb	OPNc	tPSA
Gleason Score					
<7	15	0.35	3.54	112.06	7.1
≥7	25	84.54	61.19	526.39	7.9
P value		0.006	0.003	0.001	0.192
Tumor Stage					
T2	28	62.43	33.6	200.35	7.42
T3	12	69.31	50.54	175.26	7.68
P value		0.457	0.493	1.0	0.493

Serum PSA, the most used biomarker for PCa primary screening, was incorporated in our investigation for comparison purposes. However, when combined to OPNc expression levels, although its specificity was improved, sensitivity values decreased. These lower diagnostic properties for the combination of OPNc and PSA compared to the OPNc alone indicate that the PSA may present contradictory levels within PCa and BPH samples. In this study, the PSA assay was able to accurately distinguish 53.3% of patients, but interestingly, among patients that presented PSA>4 ng/mL, the OPNc was able to correctly detect 96% of PCa, while the PSA alone contributed to the very low specificity, suggesting that almost 50% of patients may suffer unnecessary biopsy procedures. It is important to emphasize that the histopathological analysis presents an accuracy of 80% (Wang et al., 2008), probably because morphological changes may have not occurred and cannot be detected in early tumor development. However, in initiating tumor cells, the expression levels have been already altered, explaining the high accuracy achieved by the OPNc expression (94%), further evidencing its utility as an auxiliary tool in tissue analysis to improve PCa diagnosis. This result is reinforced by the lack of improvement in diagnosis with the combination of the three OPN-SI, with or without PSA. Based on our data, it is possible that PCa diagnosis could be improved even further if OPNc expression can be combined with other tumor tissue biomarkers, as has been proposed elsewhere (Rigau et al., in 2011; Troyer et al., 2004). Our findings would be undoubtedly reinforced if the prostate specimens were tested for OPNc in addition to the expression of other important PCa biomarkers, such as *PCA3* (Durand et al., 2011), *CRISP3* (Kosari et al., 2002), *AMACR* (Ouyang et al., 2011) and *HEPSIN* (Kelly et al., 2008), which are known to be upregulated. Additionally, due to the great utility of OPNc evaluation in PCa diagnosis shown here, and because OPNc isoform is secreted (Tilli et al., 2011a), it is possible that this isoform can be found in the circulation. Currently, we are investigating this hypothesis in a large clinical setting.

OPN-SI was also scanned for any potential prognostic valude which could arise from any relationship with clinicopathological data of the patients examined. As a result, OPNa, OPNb and OPNc isoforms expression levels appeared remarkably increased in high GS tumor tissues, as compared to tissue samples presenting GS below 7. Thus, less differentiated PCa cells displayed higher OPN-SI expression levels. Among the three isoforms, OPNc presented the most significant correlation to GS. These data was further reinforced by demonstrating that OPNc protein is overexpressed in high GS PCa tissues, as compared to low GS samples. Aberrant cell differentiation is considered to be a key mechanism in the onset of PCa and BPH. In normal prostatic epithelium, cells coexist in many stages in a continuum of differentiated phenotypes, progressing from stem cells to secretory mature luminal cells via a transient amplifying population. Deregulated differentiation and proliferation modify prostate epithelial homeostasis and are thus major causes of tumorigenesis (Bidaux et al., 2007). Together with our previous findings, our data provide evidence that OPNc by presenting its pro-tumorigenic roles activate important molecular pathways involved in aberrant PCa cell differentiation. The PCa cell differentiation, evaluated by GS, has been recognized as one of the most significant predictors of patient outcome and tumor progression (Bostwick, 1994; Humphrey, 2004). OPNc expression level correlation to PCa cell differentiation corroborates the potential roles of this splicing isoform on activating PCa tumor progression features. Consequently, these results are in accordance to those already published that propose full length OPN as an unfavorable prognostic marker for prostate cancer (Weber et al., 2011; Zhang et al., 2011). Our data has enriched this field by suggesting specific alternatively spliced transcripts can be used to monitor the progress of PCa.

Conclusions

The present study demonstrated that the three OPN-SI were detected in both PCa and BPH tissue specimens, and their expression levels were significantly higher in PCa malignant tissues. Our data propose that among isoforms, OPNc presented as a potential biomarkers for the discrimination between PCa and PBH. Besides, OPNc overexpression in PCa samples and its role in PCa progression give support to the potential use of this isoform as a potential target for PCa therapy. Further studies investigating OPNc expression levels in a large population cohort are warranted in order to better validate its applicability in a clinical setting.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgments

We thank to financial agencies Swiss Bridge Foundation; CAPES; FAPERJ (grants numbers E-26/111.496/2008; 103.124/2008; 110.506/2011); CNPq 573806/2008-0 and INCT for Cancer, who supported this work.

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Capítulo IV

Os resultados contidos neste quarto capítulo da tese foram publicados no seguinte periódico *The Prostate* em 2012; 72(15):1688-99.

Baseado no perfil de expressão diferencial das isoformas da OPN nas amostras de CaP (Tilli *et al*., 2012a), o objetivo deste trabalho foi avaliar o papel funcional das isoformas da OPN em distintos aspectos e características relacionadas à progressão de células de tumor de próstata. Observamos que a OPNc e a OPNb apresentam efeitos estimulatórios sobre a proliferação, migração e a invasão da linhagem celular de CaP, PC-3. Além disto, esta isoforma é capaz de induzir a formação de colônias de células PC-3 de forma independente de ancoragem e crescimento de tumores xenotransplantados a partir de células PC-3 que superexpressam esta isoforma. A partir dos tumores formados *in vivo* pela superexpressão da OPNc e OPNb, observamos um significativo aumento de expressão do mRNA de *Vegf*, *Mmp-2* e *Mmp-9* quando comparado com os tumores formados pela superexpressão da OPNa e vetor vazio. Adicionalmente, mostramos que a OPNc estimula a proliferação celular da RWPE-1 (linhagem não tumoral de próstata), indicando que a OPNc apresenta características pró-tumorigênicas. Os ensaios funcionais utilizando o meio condicionado e o anticorpo anti-OPNc demonstraram que os efeitos pró-tumorigênicos são estimulados pela OPNc secretada. Descrevemos também que os efeitos da OPNb e OPNc são mediados pela via de sinalização PI3K/Akt. Os resultados gerados por este estudo contribuem para o melhor entendimento da biologia e dos mecanismos moleculares dos tumores de próstata. Esses resultados gerados no presente estudo reforçam que o papel funcional das isoformas da OPN é tumor e tecido específico. O papel crucial destas isoformas em distintas etapas da progressão destes tumores indicam a OPNb e OPNc como potenciais alvos para futuras estratégias terapêuticas para o CaP.

Both Osteopontin-c and Osteopontin-b Splicing Isoforms Exert Pro-Tumorigenic Roles in Prostate Cancer Cells

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BACKGROUND. Alternative splicing of the osteopontin (*opn*, *spp*1) gene generates three protein splicing isoforms (OPN-SI), designated as OPNa, OPNb, and OPNc, which have demonstrated specific roles in different tumor models. This work aims to investigate the roles of each OPN-SI in prostate cancer (PCa) progression by using in vivo and in vitro functional assays.

METHODS. The expression levels of OPN-SI in prostate cell lines were analyzed by qRT-PCR. PC-3 was stably transfected with expression vectors containing OPNa, OPNb, and OPNc, as well as empty vector controls. PC-3 cells overexpressing each construct were analyzed for in vivo tumor growth and in relation to different aspects mimicking tumor progression, such as cell proliferation, migration, invasion, and soft agar colony formation.

RESULTS. OPN-SI are overexpressed in PCa as compared to non-tumoral prostate cell lines. OPNc and OPNb overexpressing cells significantly activated enhanced xenograft tumor growth and PC-3 proliferation, migration, invasion, and soft agar colony formation, as well as the expression of MMP-2, MMP-9, and VEGF. These isoforms also support sustained proliferative survival. We found that both OPNc and OPNb pro-tumorigenic roles are mainly mediated through PI3K signaling. Inhibition of this pathway by using LY294002 specifically inhibited tumor progression features evoked by OPNc and OPNb overexpression.

CONCLUSIONS. Our data provide evidence that both OPNc and OPNb splicing isoforms promote distinct aspects of PCa progression by inducing PI3K signaling. These data give support to strategies aiming to downregulate OPNc and OPNb expression as an approach to inhibit PCa progression. *Prostate* © 2012 Wiley Periodicals, Inc.

KEY WORDS: **osteopontin; splicing isoforms; alternative splicing; prostate cancer**

Additional supporting information may be found in the online version of this article.

Grant sponsor: Swiss Bridge Foundation; Grant number: E-26/111.496/2008; Grant sponsor: FAPERJ; Grant numbers: 103.124/2008; 110.506/2011; Grant sponsor: CNPq; Grant number: 573806/2008-0; Grant sponsor: INCT.

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Received 5 November 2011; Accepted 5 March 2012
DOI 10.1002/pros.22523
Published online in Wiley Online Library (wileyonlinelibrary.com).

INTRODUCTION

Prostate cancer (PCa) comprises a heterogeneous group of tumors with a broad spectrum of pathological, molecular features and clinical behaviors. This diversity confounds the development of therapy strategies and of specific biomarkers, especially due to differences in genetic composition. An improved understanding of the molecular basis of prostate carcinogenesis and tumor progression could establish new PCa markers and also help to identify new targets for improved treatment options.

In PCa, previous studies suggest a role for increased osteopontin (OPN, SPP1) tissue expression both in the malignant transformation of prostate epithelial cells and also as an important determinant of tumor progression and patient survival [1–5]. This glycophosphoprotein act as important autocrine and paracrine signal that affect growth and behavior of prostate carcinoma cells [2,3]. Besides, OPN has also been described as an important marker for stage, grade, and early tumor progression in PCa [6,7].

Variations in splicing enhance proteome diversity and modulate cancer-associated proteins. OPN mRNA is subject to alternative splicing, resulting in OPN splicing isoforms (OPN-SI) that are shorter than full-length OPN [8–11]. Cancer-specific splicing variants potentially provide diagnostic, prognostic, and predictive biomarkers, in addition to potential targeted therapies [12]. Osteopontin-a (OPNa) is the full-length isoform, while osteopontin-b (OPNb) lacks exon 5 and osteopontin-c (OPNc) lacks exon 4 [9]. The roles of OPN-SI have been demonstrated to be tumor specific, although the mechanisms controlling this are currently unknown [8–11,13]. We recently published the first report about OPN isoforms in PCa, by demonstrating the expression pattern of each OPN-SI in PCa and benign prostate hyperplasia (BPH) samples [14]. We showed that OPNa, OPNb, and OPNc splice variants are overexpressed in PCa in relation to BPH. Besides, we observed that among these isoforms, the OPNc variant was the most upregulated in PCa, and outperformed the remaining isoforms and PSA serum levels in PCa diagnostic accuracy. Additionaly, immunohistochemistry analysis also demonstrated OPNc protein upregulation in PCa samples as compared to BPH tissues. OPNc protein was also strongly stained in PCa tissues presenting high Gleason Score. Although presenting this differential expression pattern, data about the roles of each OPN-SI in PCa progression are lacking, once all data regarding the functional evaluation of OPN in PCa relates to the full length isoform.

In the current study, we further identified OPN-SI transcript overexpression in PCa in relation to non-tumoral prostate cell lines. Based on this evidence and on our previous data [14] of a potential role for OPN-SI in PCa progression, we addressed the function of PCa-derived OPN-SI by examining the effect of their ectopic overexpression in PC-3 cells. PC-3 cells overexpressing each of the OPN-SI were evaluated for features mimicking different steps of PCa tumor progression by in vivo and in vitro approaches. Our data demonstrated by the first time that the overexpression of OPNc and OPNb increases PC-3 cell growth, sustained proliferative survival, migration, invasion, anchorage-independence, and tumor formation in vivo, suggesting a possible functional role for OPNc and OPNb in PCa progression and survival. Additionally, we demonstrated herein that these tumor promoting roles could be mediated through the activation of the Phosphatidylinositol-3 Kinase (PI3K)/Akt signaling pathway.

MATERIALS AND METHODS

Cell Culture, OPN Plasmids, and Transfection

We used three PCa cell lines: PC-3, DU-145, and LNCaP (supplied from ATCC) and two non-tumoral prostate cell lines, RWPE-1 and PrEC, which were cultured in standard conditions. RWPE-1 and PrEC cell line were a gift from Dr. Carlos Moreno (Emory University, EUA). All cell lines were cultured in culture medium (CM) supplemented with 10% fetal bovine serum (FBS), 100 IU/ml penicillin and 100 mg/ml streptomycin in a humidified environment containing 5% CO_2 at 37°C.

The open reading frame of OPN splice variants, OPNa, OPNb, and OPNc were cloned into pCR3.1 mammalian expression vector as previously described [9]. OPN-SI expression vectors were kindly provided by Dr. George Weber (Cincinnati University) and these DNA constructs were used for transfection into PC3. Transfections were performed using Lipofectamine^{TM} 2000 (Invitrogen, CA). Expression plasmids were transfected into PC-3 cells and the stably expressing cell clones were selected with 800 μg/ml of G418. Six OPN isoform overexpressing clones (designated OPNa1, OPNa4, OPNb2, OPNb4, OPNc1, and OPNc4) and one empty vector control clone (empty vector) were selected.

Quantitative Real-Time RT-PCR (qRT-PCR)

RNA was extracted using the RNeasy kit (Qiagen). cDNA synthesis was carried out using SuperScript II First-Strand Synthesis System for RT-PCR using oligo(dT) primer (Invitrogen) and 1 μg of total RNA. Conditions for MMP-2 and MMP-9 PCR amplification by qRT-PCR were 50°C for 2 min, 94°C for 5 min

followed by 40 cycles of 94°C for 30 sec, 58°C for 30 sec, and 72°C for 90 sec; 72°C for 15 min and finally a melting curve analysis (60–90°C with a heating rate of 0.2°C and continuous fluorescence measurement). Conditions for VEGF PCR amplification were 50°C for 2 min, 94°C for 5 min followed by 40 cycles of 94°C for 30 sec, 60°C for 30 sec, and 72°C for 90 sec; 72°C for 15 min and finally a melting curve analysis (60–90°C with a heating rate of 0.2°C and continuous fluorescence measurement).

The amplification of fragments corresponding to each OPN isoform, MMP-2, MMP-9, and VEGF was performed using the following oligonucleotide pairs: OPNa:5′-ATC TCC TAG CCC CAC AGA AT-3′ (forward) and 5′-CAT CAG ACT GGT GAG AAT CAT C-3′ (reverse); OPNb: 5′-CTC CTA GCC CCA CAG ACC CT-3′ (forward) and 5′-TAT CAC CTC GGC CAT CAT ATG-3′ (reverse); OPNc: 5′-CTG AGG AAA AGC AGA ATG-3′ (forward) and 5′-AAT GGA GTC CTG GCT GT-3′ (reverse); MMP-2: 5′-AAA ATG GAT CCT GGC TTC CC-3′ (forward) and 5′-TAG CCA GTC GGA TTT GAT GC-3′ (reverse); MMP-9: 5′-TGA CAG CGA CAA GAA GTG-3′ (forward) and 5′-CAG TGA AGC GGT ACA TAG G-3′ (reverse); VEGF: 5′-CTT GCC TTG CTG CTC TAC C-3′ (forward) and 5′-CAC ACA GGA TGG CTT GAA G-3′ (reverse). GAPDH was amplified with primers 5′- TGA CCC CTT CAT TGA CCT CA -3′ (forward) and 5′-AGT CCT TCC ACG ATA CCA AA-3′ (reverse), and Actin was amplified with primers 5′-TGA CCC AGA TCA TGT TTG AGA-3′ (forward) and 5′-ACT CCA TGC CCA GGA AGG A -3′ (reverse), which served as an internal control to normalize expression data and to verify integrity of the cDNA. All qRT-PCR reactions were conducted using the SYBR Green (Applied Biosystems). Relative gene expression of the target gene was calculated by using the ΔΔCT method.

Ki-67 Staining

The expression of Ki-67 was accomplished via immunohistochemical techniques on serial paraffin sections of xenograft tumors overexpressing each OPN-SI and controls. Tumors were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer. Samples were paraffin embedded and tissue sections were processed using standard protocols. The mouse monoclonal Ki-67 antibody (clone MIB-1, Dako) was used at a dilution of 1:50. The next incubation step employed the streptavidin-biotin-peroxidase complex (LSAB, Dako) and color development was obtained with diaminobenzidine solution (Kit DAB, Dako). The positive immunoreactions were manually counted using a 40× magnification at Olympus BH-2 microscope coupled to a digital camera.

Cell Proliferation, Death, Migration, Invasion, and Soft Agar Growth Assays

Cell proliferation was analyzed by crystal violet. Cell death analyses were conducted by using trypan blue exclusion assays. For the analysis by crystal violet and Trypan blue, wild-type PC-3, PC-3 cells transfected either with OPNa, OPNb or OPNc plasmid constructs, or pCR3.1 empty vector were plated in triplicate in 24-well microtiter plates. For these assays, 5×10^4 cells were plated per well. For crystal violet incorporation assays, cells were washed twice with PBS and fixed in glutharaldeyde for 10 min, followed by staining with 0.1% crystal violet and solubilization with 0.2% Triton X-100. Microtiter plates were read on a spectrophotometer at 550 nm. Viable and non-viable cells were counted using a Neubauer's chamber. Total number of cells/well for each cell clone group was calculated and plotted against each time point. Cell proliferation assays for OPNc depletion using anti-OPNc antibody were evaluated either in the absence or in the presence of anti-OPNc IgY antibody (Gallus Immunotech), added every other day at 4 μg/ml. This antibody was produced by immunizing a chicken with a peptide representing the splice junction of osteopontin-c (Ac-SEEKQNAVSC-COOH). Specific binding to OPNc has been has been demonstrated by the providers. Proliferation assays were also performed using non-specific goat IgG and chicken IgY antibodies (Gallus Immunotech) as immunoglobulin negative controls (4 μg/ml). In order to test whether secreted OPN isoforms were performing the observed cell behavior, wild type PC-3 and RWPE-1 cells were incubated with conditioned media (CM) obtained from PC-3 cells transfected with OPNa, OPNb, OPNc or empty vector, and cell proliferation was measured by crystal violet incorporation. These assays were performed in serum-free conditions, in order to eliminate skewing of the results by cell activation promoted by growth factors. Forty two hours before starting the experiment, cells were cultured in 0.2% FBS in order to synchronize cells on cell cycle. In order to prepare CM, cell number was normalized by plating the same cell amount of cells in each assayed culture plate for individual OPN-SI overexpressing clone.

Cell migration assays were evaluated by in vitro wound closure and transwell assays, as described by others [15]. Wild-type PC-3 or PC-3 cells transfected with either OPNa, OPNb, OPNc, or empty vector were grown in six-well microtiter plates to near-confluent level in RPMI medium containing 20% FBS. Mitomycin C (5 μg/ml; Sigma) was added to inhibit cell proliferation, and the cells were incubated for additional 3 hr. Multiple uniform and constant streaks were

made on the monolayer culture with 10 μl pipette tips. Plate dishes were immediately washed with PBS to remove detached cells. Cell migration was monitored for 72 hr, and pictures were taken at 0, 18, 24, 48, and 72 hr time points with a digital camera attached to an inverted Nikon phase contrast microscope.

To determine the effect of OPN isoforms on the invasion potential of PC-3 cells, transwell invasion in vitro assays were performed as reported [16]. Cell culture inserts (24-well, pore size 8 μm; BD Biosciences) were seeded with 25,000 wild-type PC-3 cells, PC-3 cells transfected either with OPNa, OPNb or OPNc plasmid constructs, or pCR3.1 empty vector in 500 μl of medium with 0.1% FBS. Pre-coated inserts with growth factor reduced Matrigel (40 μl, 1 mg/ml; BD Biosciences) were used. As chemotactic agent added at the lower chamber, 750 μl of CM containing 20% FBSs has been used. After 48 hr, non-invading cells were wiped from the upper side of the membrane and cells on the lower side were fixed in glutharaldeyde followed by staining with 0.1% crystal violet. The lower side of the filter was photographed by using a phase-contrast microscope at 10-fold magnification and the number of cells was manually counted. Each individual experiment had triplicate inserts and six microscopic fields were counted per insert.

Anchorage independent growth was analyzed in soft agar medium. Six-well plates were coated with 1.2% agarose-supplemented growth medium to resist cell adhesion. PC-3 wild-type or PC-3 cells transfected with either OPNa, OPNb, OPNc, or empty vector were then trypsinized, and 5×10^2 cells were resuspended in 3 ml of growth medium containing 0.6% agarose. After being plated, colonies were allowed to grow for 4 weeks. Representative colonies were visualized by both phase contrast microscopy and the total number of colonies was determined.

Preparation of Cell Lysates and Immunoblot for Detecting OPN Isoforms

Cells were harvested and rinsed twice with PBS. Cell extracts were prepared using cell lysis solution containing SDS 1% and phosphatase and protease inhibitors. Cells were scraped and total protein extract was purified using 20 mm mini spin Receiver Columns (Macherey-Nagel) by centrifuging 1,400 rpm for 1 min and then cell extracts collected. Total protein concentration was measured using the BCA assay kit (BioRad), according to the manufacturer's instructions. Immunoblot assays for detecting protein isoforms were performed using 25 μg of purified protein extracts, which were subjected to a 7.5% SDS/PAGE and the resolved proteins were transferred electrophoretically to PVDF (Millipore) membranes. Membrane was blocked for 1 hr using 5% non-fat dry milk diluted in Tris buffered saline (5%/TBS). Then, membranes were incubated O/N with the human anti-OPNc primary antibody (R & D systems) diluted 1:500 in 5%/TBS. After three washes for 10 min in TBS buffer, membranes were incubated for 1 hr with the anti-goat IgG-HRP secondary antibody. Chemiluminescence detection was performed using ECL PLUS (Amersham Biosciences) in accordance with the manufacturer's instructions. As positive controls for staining the three OPN isoforms with anti-OPNc antibody, we used whole cell extracts from MDA-MB-435 (breast cancer human cell lines) and OPN recombinant protein.

Treatment of Cells With LY294002 and Immunoblotting

LY294002, a PI3K inhibitor, was obtained from Cell Signaling Technology (Maryland). Untransfected PC-3 cells and those overexpressing either OPN-SI or empty vector control were cultured as described above for cell proliferation, migration, and soft agar colony assays and then treated with 50 μM of LY294002. Immunoblot was performed using 50 μg of protein extracts. Cells were harvested and rinsed twice with PBS. Cell extracts were prepared with Cell Lysis Buffer (Cell Signaling Technology), sonicated and cleared by centrifugation at 15.000*g*. Total protein concentration was measured using the BCA assay kit (BioRad), according to the manufacturer's instructions. PI3-Kinase activation was analyzed by the levels of Akt Ser^{473} phosphorylation. Membranes were incubated with anti-total Akt and anti-phospho-Akt antibodies (Cell Signaling Technology). The horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (Pierce, Rockford, IL) were diluted 1:1,000 in PBST containing 5% bovine serum albumin. Chemiluminescence detection (Amersham Biosciences) was performed in accordance with the manufacturer's instructions.

In vivo Tumorigenicity of OPN-SI Transfectants

For the analysis of tumor progression in vivo we used 6 weeks athymic $BALB/c^{nude/nude}$ mice. Mice were injected subcutaneously (s.c.) into the left flank with 5×10^6 of PC-3 cell clones. Tumor volumes (V) were analyzed every 2 days using the following formula: V (mm^3) = width × $length^2$ × 0.52. A total of 25 mice were randomly assigned to five groups (five mice/group) corresponding to five transfectant clones. Pictures were taken 24 days after injection.

Data and Statistical Analysis

All results are presented as mean ± standard error of at least three independent experiments. For in vitro and in vivo data assays, statistical comparisons

among groups were performed by the Student's *t*-test or ANOVA. $P < 0.05$ was considered significant. *P*-values are indicated in the Figure legends.

RESULTS

Overexpression of OPN-SI in PCa Cells Potentially Promotes Tumor Growth and Cell Proliferation

We previously demonstrated that all OPN-SI are overexpressed in PCa in relation to BPH samples and that among these isoforms, OPNc was the most upregulated in PCa samples. Additionally, we also demonstrated that OPNc protein is overexpressed in high Gleason Score (GS) tumor samples, as compared to low GS samples [14]. To further validate these data and compare OPN-SI expression profiling between tumor and non-tumoral prostate samples, we used herein OPN-SI specific oligonucleotides and qRT-PCR to evaluate the expression level of these three splice variants in prostate tumoral and non-tumoral cell lines (Fig. 1A). We found that the three OPN-SI are overexpressed in PCa cell lines, as compared to non-tumoral samples. These data, in association with our previous results [14], indicated that OPN-SI expression levels could be involved in PCa progression. Based on these data, we then assessed the functional significance of elevated OPN-SI expression during the development of PCa by investigating whether the stable overexpression of each OPN-SI in a human PCa cell line could alter tumor growth in vivo and in vitro.

PC-3 cell line was stably transfected with expression plasmids containing the three OPN-SI. The analysis of isolated clones demonstrated that each OPN-SI overexpression clone present higher mRNA levels of the corresponding isoform in relation their endogenous levels in empty vector transfected cells (Supplementary Fig. 1A). This was further demonstrated at the protein level (Fig. 1B). Immunoblot analysis of total protein

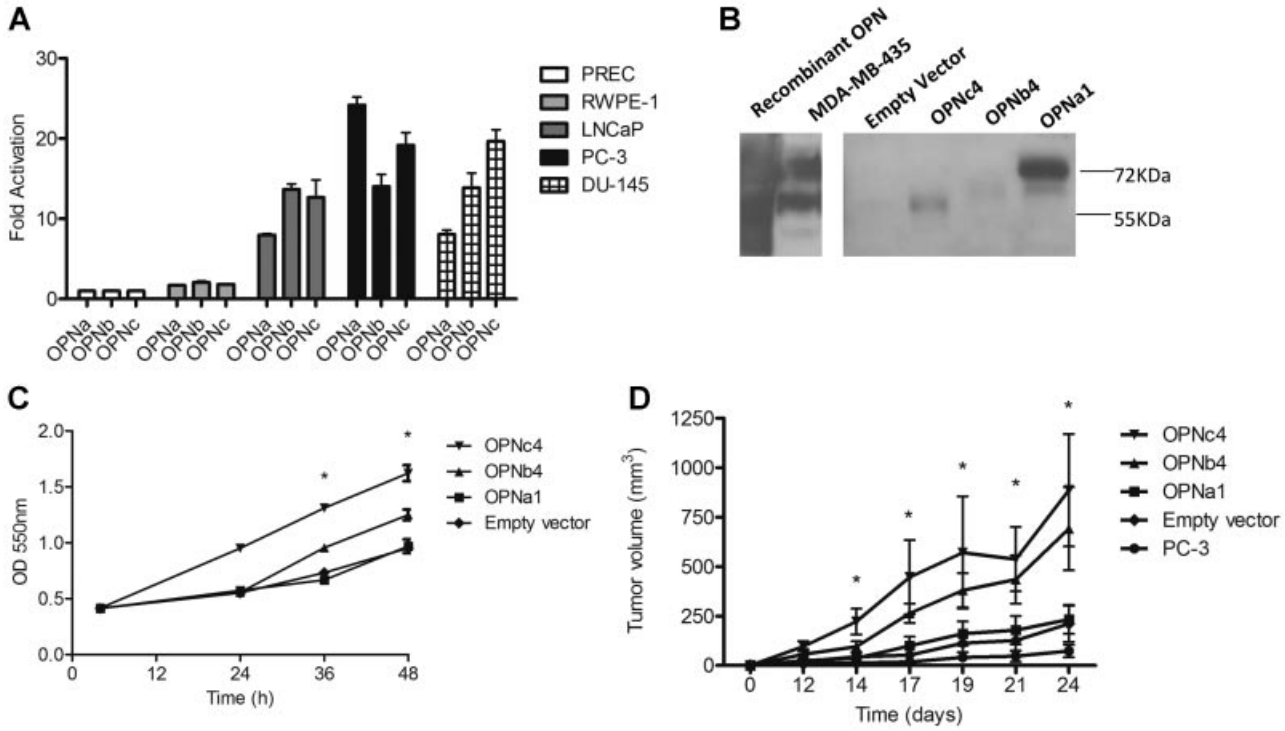

Fig. 1. OPN-SI are overexpressed in PCa and support tumor growth. OPN-SI expression levels were analyzed by qRT-PCR and were represented by fold activation in relation to the sample presenting the lowest expressing levels. GAPDH was used as a normalization control. **A**: OPN-SI mRNA expression levels was analyzed in different prostate cell lines. Results are representative of three independent experiments. Horizontal bars represent standard deviations (S.D.). **B**: Immunoblot analysis using the human anti-OPN antibody and total protein extracts from OPN-SI overexpression clones demonstrating the overexpression of OPNc (around 55 KDa), OPNb (around 60 KDa), and OPNa (around 72 KDa) protein isoforms in each corresponding overexpression clone, respectively. OPN-SI molecular weights presents variability according to post-translational modifications which are cell type dependent. Recombinant OPN and MDA-MB-435 total protein extracts were used as positive controls for OPN-SI detection and image on the right demonstrates the X-ray film exposed in a shorter time to better demonstrate the bands corresponding to the three isoforms. **C**: PC-3 transfected cells with either OPNa, OPNb or OPNc, or empty vector control and proliferation kinetics analysis was evaluated by crystal violet staining. $^{*}P < 0.05$ versus empty vector control clone. **D**: Tumor growth rate of mice s.c implanted with OPN-SI overexpression cells and empty vector controls. PC3 non-transfected cells were also implanted. $^{*}P < 0.05$ versus empty vector cells.

extracts from each OPN-SI isolated clone was probed with a human anti-OPN antibody (R & D systems), which recognizes three OPN isoforms. This antibody recognizes predominantly OPNc (around 55 KDa), OPNb (around 60 KDa), and OPNa (around 72 KDa) isoforms on OPNc, OPNb, and OPNa overexpression clones, respectively. These data demonstrated that each overexpression clone present higher levels of the corresponding protein OPN splicing isoform (Fig. 1B). We firstly asked whether the proliferation rates of PC-3 cells overexpressing OPNa, OPNb, and OPNc were altered as compared to an empty vector control clone. As shown in Figure 1C, OPNc overexpressing cells significantly activated proliferation rates as compared to OPNa and empty vector control clone in the range of 4–48 hr of cell culture ($P < 0.05$). OPNb also stimulated PC-3 cell proliferation, however, presenting no statistical significance ($P = 0.07$), as compared to controls. Similar results were obtained when analyzing proliferation rates by trypan blue assays (Supplementary Fig. 2A). The same proliferation behavior was observed when testing stably expressing clones presenting different OPN isoform expression levels, as demonstrated on Supplementary Fig. 2B–D. To further assess the functional significance of elevated expression of OPN-SI during the development of PCa, we then investigated whether stable overexpression of each OPN-SI in PC-3 cell line could alter the tumor growth in vivo. Tumors overexpressing OPNc, and to a lesser extent OPNb, grew faster and produced tumors with higher volume as compared to controls (Fig. 1D and Supplementary Fig. 3). Implantation of cells overexpressing OPNc resulted in extremely rapid tumor growth and formation of larger ($\geq$500 mm^3) tumors between 17 and 19 days after s.c. injection. In contrast, tumors resulting from OPNa or empty vector overexpressing clones were of comparable size (<200 mm^3) to those generated with empty vector control cells. Taken together, these results indicated that overexpression of OPNc and OPNb are able to enhance xenograft prostate tumor growth rates, suggesting that these isoforms accelerate tumor progression.

Tissue sections from xenografts expressing the three OPN-SI constructs were stained for Ki-67 to assess proliferation. As shown in Figure 2A,B, the percentage of Ki-67 positive nuclei in OPNc and OPNb overexpressing tumors was 3.5 and 2.5-fold higher, as compared to cells overexpressing OPNa and empty vector control, respectively. Ki-67 staining, in addition to cell proliferation in vitro assays (Fig. 1C), indicated that OPNc and OPNb overexpression were associated

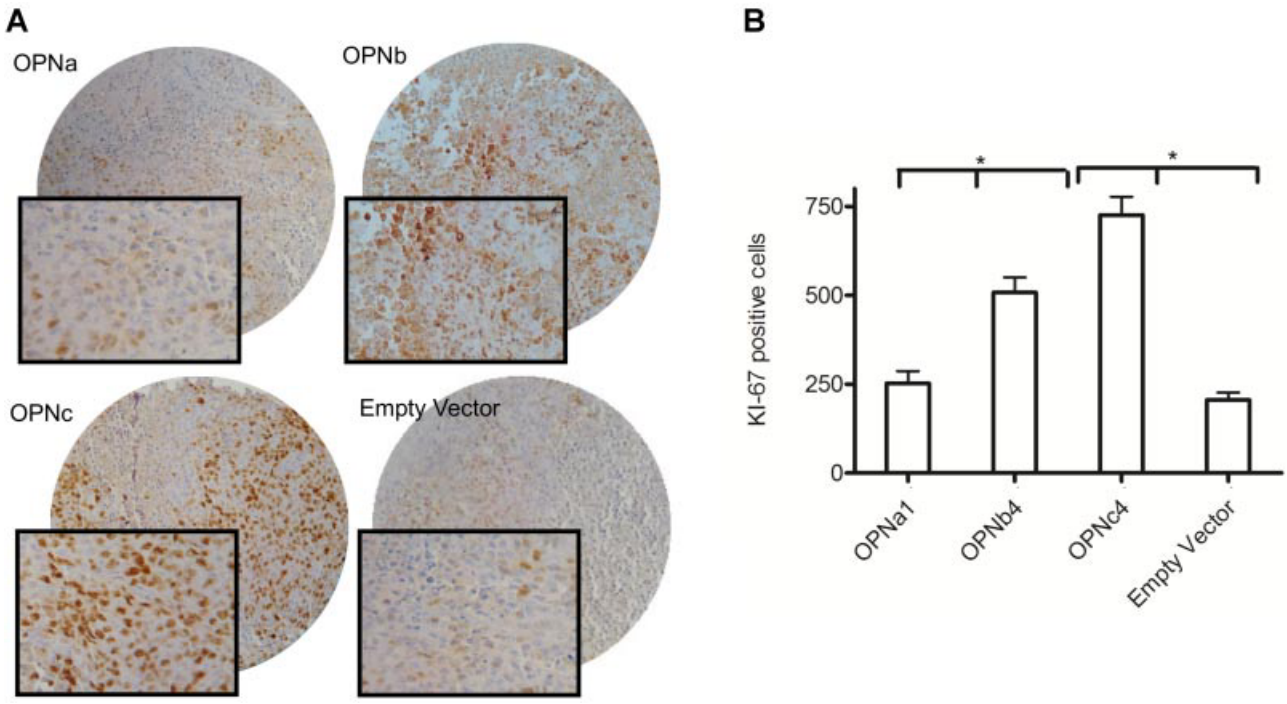

Fig. 2. OPNc and OPNb activate tumor formation by inducing proliferation. Tumors formed by each OPN-SI PC-3 overexpressing cells, as well as empty vector were analyzed for Ki-67 positive staining. **A**: Representative images of xenograft tumor spots showing Ki-67 expression as determined by nuclear staining, characterized by a dark brown reaction in the nucleus of tumor cells. 10× objectives on an Olympus BH-2 microscope with the inset showing a 40× magnified view of the same field. **B**: Quantification of immunohistochemical Ki-67 positive nuclei staining as tested in (A).

with increased PCa tumor growth. Real time PCR analysis of total RNA obtained from xenograft tumors resulting from OPNa, OPNb, and OPNc overexpression clones demonstrated that these tumor tissues maintain each OPN-SI overexpression as compared to tumors formed by empty vector transfected cells (Supplementary Fig. 1B).

To further explore the role of each tumor derived OPN-SI on the regulation of downstream molecules involved on PCa tumor progression, we examined whether tumor xenografts overexpressing OPN-SI could induce MMP-2, MMP-9, and VEGF mRNA expression. In PCa, a functional interplay and an increased expression of these gene products is associated with invasive and metastatic potential. As shown in Figure 3A–C, the expression levels of these transcripts were up-regulated in OPNc and OPNb overexpressing xenografts, as compared to those tumors resulting from empty vector control ($P < 0.01$).

We then explored the functional mechanisms by which OPN variants promote tumor growth. In order to achieve this objective, we examined the biological effects of OPN-SI overexpression in PC-3 cells under in vitro conditions that mimic important steps of tumor progression.

Secreted OPNc and OPNb Activate Sustained PC-3 Proliferative Signaling

In serum-starving conditions, cells overexpressing OPNc and to a lesser extent, OPNb, can significantly enhance cell proliferation (Fig. 4A). These results suggest that OPNc and OPNb modulates the growth of PCa cells independently of growth factors, typical

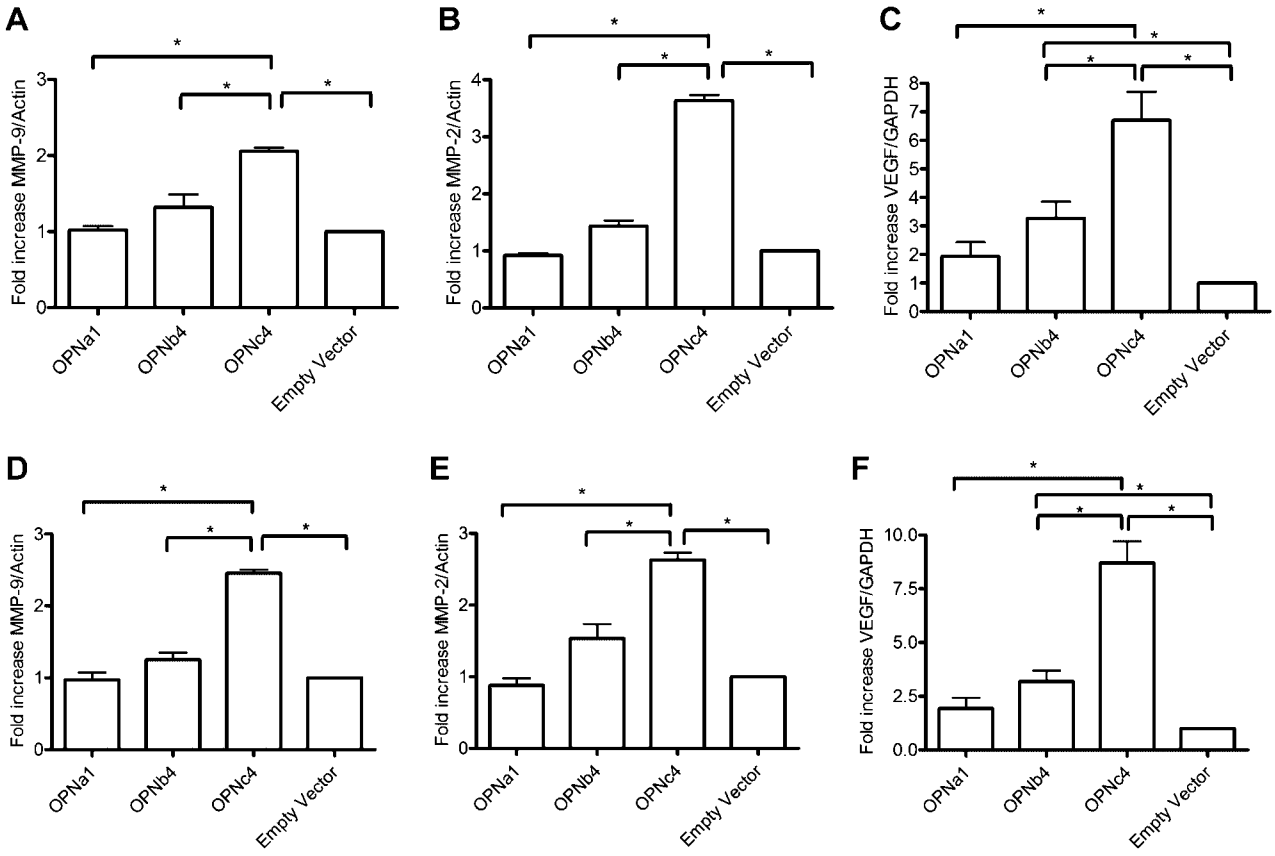

Fig. 3. MMPs and VEGF are overexpressed in PCa cells overexpressing OPNb and OPNc isoforms. **A**–**C**: Induction of MMP2 (A), MMP9 (B), and VEGF (C) mRNA expression in xenograft tumors formed by cells overexpressing the three OPN-SI, as compared to tumors formed by PC3 cells transfected with empty vector controls. **D**–**E**: Induction of MMP2 (D), MMP9 (E), and VEGF (F) mRNA expression in PC3 cells overexpressing the three OPN-SI as compared to PC3 cells transfected with empty vector controls. Total RNA from cells overexpressing OPNa, OPNb, OPNc, and empty vector control was prepared to conduct quantitative real-time PCR (qRT-PCR) analysis using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) or actin as internal controls. D–E: The amount of targets was analyzed using the comparative CT method, where the threshold cycle (CT) values of each target sequence are given by the 2DDCT formula. We present the data as log n-fold change in gene expression normalized to the endogenous reference genes (GAPDH or actin) relative to the expression of cells overexpressing empty vector control. $^{*}P < 0.002$.

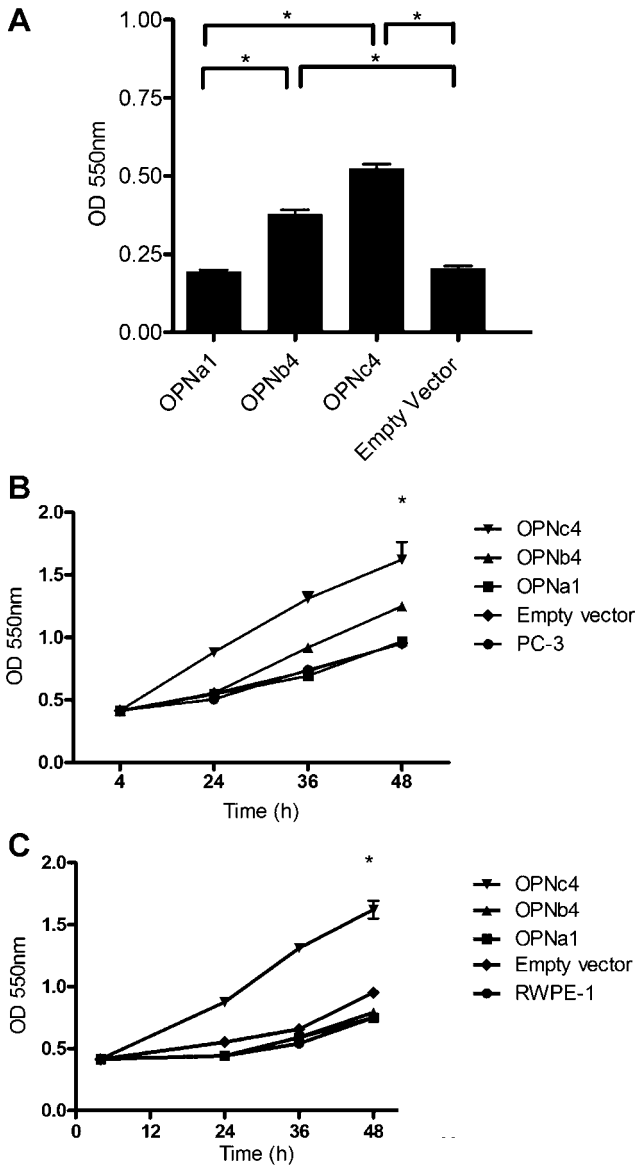

Fig. 4. OPNc and OPNb stimulate cell proliferation under serum starving conditions by stimulating cell survival. **A**: Cells were grown in 0.2% FBS and cell numbers were counted by crystal violet staining at 96 hr after plating. O.D., optical density measured at 550 nm. $^{*}P < 0.0001$ versus empty vector control clone. All results are representative of at least three independent experiments. PC-3 (**B**) and RWPE-1 (**C**) non-transfected cells were assayed for cell proliferation rates by crystal violet staining after incubation with conditioned medium (CM) secreted from OPN-SI and empty vector overexpressing cells. Legend on the right indicate from which cell clones the CM was obtained. Results are representative of at least three independent experiments. O.D., optical density at 550 nm. The standard deviations (error bars) indicate the variability within each experiment. $^{*}P < 0.05$ versus PC-3 or RWPE-1 non-transfected cells.

features of oncoproteins [17]. We then hypothesized that the higher proliferation rates observed for OPNc and OPNb overexpressing clones could be explained by induction of cell death promoted by OPNa overexpression. All three OPN-SI cell clones presented similar proportions of cell death, as shown by exclusion of trypan blue analysis (data not shown). These results further evidence the potential role of OPNc and OPNb on favoring PCa cell proliferation and survival, which is not associated with stimulated cell death promoted by OPNa or control cells.

We also evaluated proliferation-kinetics of untransfected PC-3 and RWPE-1 cells in the presence of conditioned CM produced by each OPN-SI and empty vector overexpressing cell lines (Fig. 4B,C). We found that untransfected PC-3 cells cultured in OPNc-CM and OPNb-CM displayed higher proliferation rates as compared to controls. Of note, OPNc-CM, but not OPNb-CM, stimulated the proliferation of RWPE-1 non-tumoral prostate epithelial cells (Fig. 4C). These changes in PC-3 cell proliferation indicates that cell growth were directly dependent on the secreted OPNc protein, as the addition of a polyclonal anti-OPNc antibody, but not control anti-rabbit goat IgG immunoglobulin, significantly suppressed proliferation rates promoted by PC-3 overexpressing OPNc (Supplementary Fig. 4A). Similar results were obtained when performing these assays using an IgY non-specific antibody as a negative control (data not shown). Accordingly, these cells also presented an increase in cell death, providing additional evidence for a survival effect for secreted OPNc in this prostate tumor cell line (Supplementary Fig. 4B). The same effect was not observed when anti-OPNc antibody was added to OPNa overexpression clones (Supplementary Fig. 4C,D). When the same procedure was performed on wild type PC3 non-transfected cells, although presenting no significant effect on cell proliferation, anti-OPNc antibody increased cell death rates in these cells, indicating that even basal OPNc levels in PC3 cells could potentially also exert pro-survival roles (Supplementary Fig. 4E,F). These data indicated that this antibody could specifically inhibit the effect of OPNc protein isoform on cell proliferation and survival. Altogether, these data indicated that secreted OPNc, in addition to enhancing proliferation of PCa cells, also stimulates proliferation of normal prostate epithelial cells, further suggesting a role of this isoform in prostate tumorigenesis and cell survival. Conversely, the OPNb isoform likely exerts its main effects on cell proliferation at later stages of PCa progression. A serial dilution analysis of OPNc and OPNb-CM promoted a proportional decrease on PC3 cell proliferation (Supplementary Fig. 5A,C). On the other hand, OPNb-CM serial dilution analysis only promoted a proportional decrease on PC3 cell proliferation (Supplementary Fig. 5B). On RWPE1 cells, proliferation rates were maintained, even according to OPNb CM serial dilution analysis (Supplementary Fig. 5D).

PC-3 Cells Overexpressing OPNc and OPNb Stimulate Prometastatic Processes

We then elucidated whether OPN-SI overexpressed in PC-3 cells are able to induce altered phenotypes associated with additional processes favoring tumor progression, such as migration, invasion, and anchorage independent cell growth.

PC-3 cells overexpressing OPN-SI or empty vector were subjected to an in vitro wound closure migration assay (Fig. 5A). Migration of OPNc and OPNb expressing cells were enhanced as compared to OPNa and empty vector-expressing cells. Eighteen hours after the scratch assay was initiated, OPNc and OPNb enhanced PC-3 cell motility into the wound. After 24 hr, OPNc and OPNb overexpressing cells completely closed the wound edges (data not shown).

OPNc and OPNb overexpression also stimulated PC-3 invasion capacity by at least 5.0-fold and 2.5-fold ($P < 0.0001$), as compared to cells overexpressing OPNa and empty vector control (Fig. 5B). These results suggest that OPNc and OPNb isoforms increased cell migration and invasion of PC-3 cells in vitro.

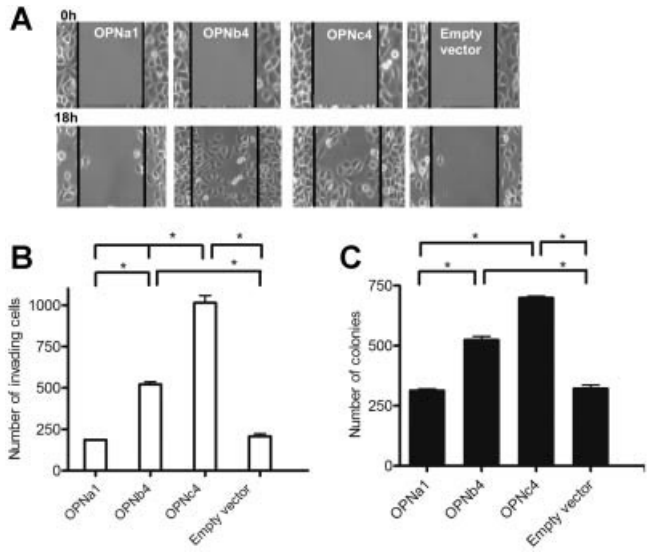

Fig. 5. OPNc and OPNb stimulate prometastatic processes in PCa cells. Cell clones of OPN-SI and empty vector control cells were analyzed for cell migration (**A**), invasion (**B**), and anchorage independent growth (**C**). A: Phase-contrast micrographs photographs were taken at 0 and 18 hr after clones subjected to migration by wound closure assays are shown. Samples were tested in triplicates. **B**: Invading cells tested by transwell invasion assays were stained with crystal violet and the number of cells manually counted. Data are reported as average of number of invading cells ± S.D. of three independent experiments. $^{*}P < 0.001$. C: Quantification of the number of PC-3 cell colonies grown in semisolid agarose medium transfected with different constructs, as indicated. Results are representative of at least three independent experiments.$^{*}P < 0.01$ vesrsus empty vector cells.

Similarly to in vivo assays (Fig. 3A–C), there was a significant increase in MMP-2, MMP-9, and VEGF mRNA levels ($P < 0.01$) in PC-3 cells overexpressing OPNc and OPNb, as compared to OPNa and empty vector overexpressing clones (Fig. 3D–F). These results indicated that the upregulation of these transcripts could be one of the molecular mechanisms by which these OPN variants can activate PC-3 cell migration and invasion capacity. OPNc-CM and OPNb-CM also significantly activated PC-3 cell migration and invasion (data not shown), reinforcing the notion that the secreted isoforms of these OPN variants acts as stimulatory factors for PC-3 cell proliferation, migration, invasion, and metastatic potential.

To examine whether OPN-SI overexpression also alters the ability for anchorage-independent growth as a measure for tumorigenic potential, soft agar assays were performed. OPNc and OPNb overexpressing PC-3 cells significantly increased the number of colonies formed, while OPNa isoform promoted no significant effects (Fig. 5C). The three OPN-SI overexpression clones also promoted an increase in the size of colonies formed, although OPNc and OPNb displayed a more pronounced effect (data not shown). These data suggest that OPNc and OPNb can act as activating factors to enhance anchorage independent growth, indicative of their roles on regulating metastatic potential.

OPNc and OPNb Mediate PCa Progression Features Through the PI3K/Akt Pathway

During PCa progression, tumor cells undergo molecular alterations that lead to the acquisition of uncontrolled growth properties. One such set of molecular alterations may be mediated by the PI3K-Akt signaling pathway [18]. To explore the molecular mechanisms by which OPN-SI mediate their effects on PCa progression, we examined if the overexpression of each OPN-SI would modulate PI3K activity and Akt phosphorylation (Ser^{473}).

As shown in Figure 6A, an increased phosphorylation level on Ser^{473} was readily detected in protein extracts of OPNc and OPNb overexpression cells (2.1-fold and 1.46-fold increase, respectively), but not in the vector-controls or OPNa overexpressing cells. Differential ERK 1/2 hyperphosphorylation was not observed among cell extracts overexpressing all OPN-SI (data not shown). The activation of PC-3 cell proliferation, migration and soft agar colony formation mediated by OPNc and OPNb overexpression were significantly inhibited when these cells were treated with LY294002 (Fig. 6B–D). PC-3 cells overexpressing OPNc and OPNb could better overcome the effects of LY29400 on inhibiting the number of soft agar

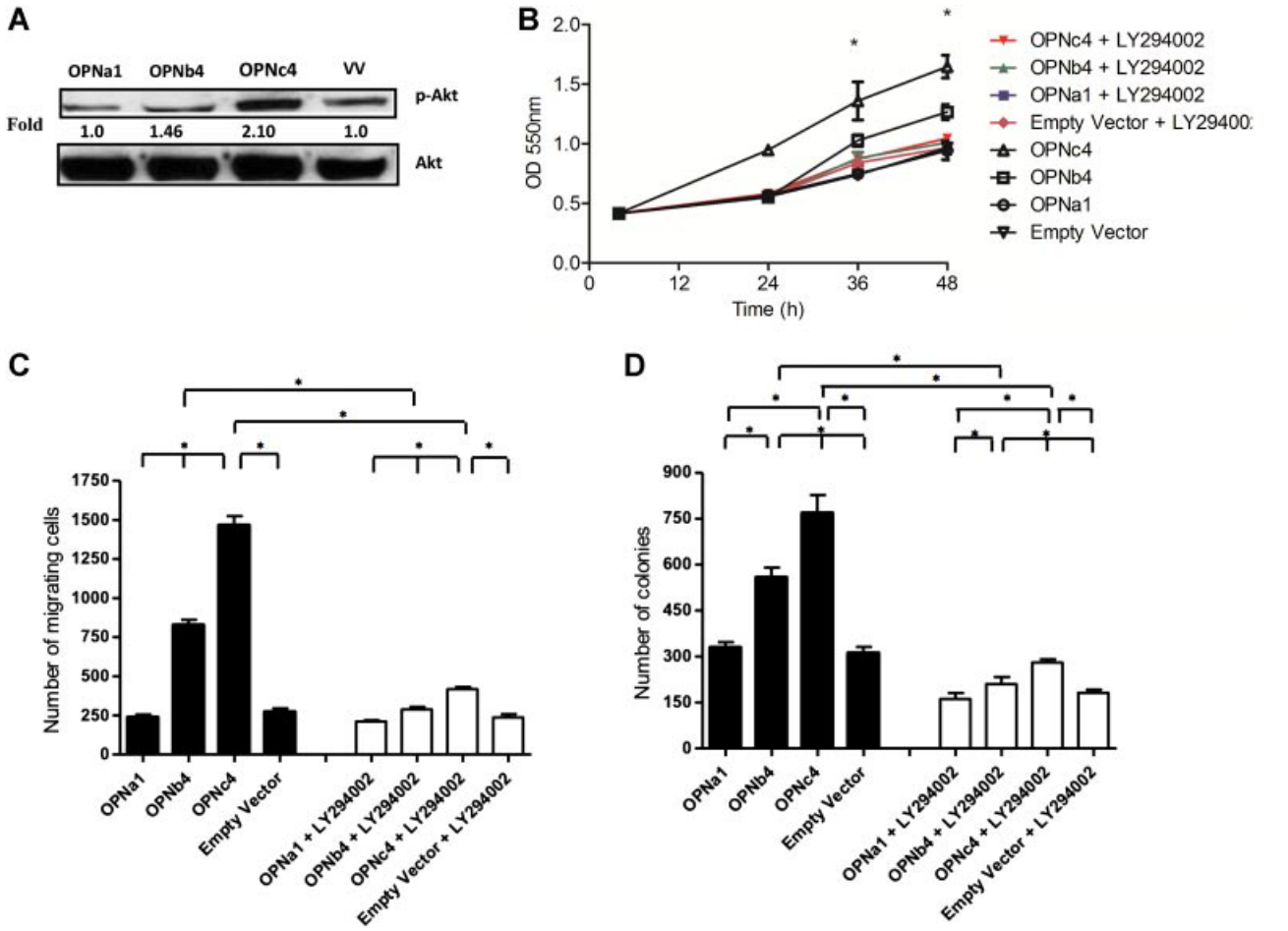

Fig. 6. PI3K/Akt signaling pathway mediate ONPc and OPNb roles on activating tumor progression features. **A**: Total Akt and p-Akt (60 KDa) levels in PC-3 cells overexpressing OPN-SI and empty vector control were measured by immunoblot using their specific antibodies. Non-phospho Akt was used as a loading control. Fold changes were calculated based on densitometric quantification. **B**: Growth curves of PC-3 overexpressing cell clones OPNc, OPNb, OPNa, and empty vectors cultured with or without LY294002 are plotted, as indicated at the legend on the right. $^{*}P < 0.05$ vesrsus empty vector control clone. **C** PC-3 cells overexpressing each OPN-SI, as compared to empty vector control were evaluated for cells migration capacity by transwell assays after LY294002 treatment at the 48 hr time point. **D**: The number colonies of anchorage independent growth formed by PC-3 cells overexpressing each OPN-SI as compared to empty vector control cells was analyzed in the presence or absence of LY294002 inhibitor. $^{*}P < 0.002$.

colonies formed (Fig. 6D). These results indicate that PI3K signaling is mediating tumor progression features evoked by OPNc and OPNb overexpression.

DISCUSSION

Current data debate that OPN splice variants present different tumor and tissue specific roles. While in some tumor models an individual OPN-SI behaves as a tumor stimulating factor, in other tumor contexts the same variant presents just the opposite effect, acting individually or in concert with the remaining isoforms to modulate tumor progression [8–11,13]. Although total OPN has been a key biomarker for detecting PCa progression, no reports until now have described the roles of each OPN-SI in prostate tumorigenesis [4–7]. Herein, we attempted to characterize the involvement of each OPN-SI as an important approach to understand some of the aberrant and deregulated genetic control in PCa.

We previously demonstrated that OPN-SI are overexpressed in PCa tissues in relation to BPH samples and that OPNc protein was up-regulated in high gleason score tumor samples [14]. Data provided herein reinforced OPN-SI transcript overexpression in PCa cell lines as compared to non-tumoral ones. Based on this, we then hypothesized whether this OPN-SI overexpression in PCa could play important role in PCa progression.

We found that cells overexpressing OPNc and OPNb, but not the OPNa splice variant, are able to enhance PCa tumor growth in vivo, mainly mediated

by inducing cell proliferation. In vitro, although OPNb overexpressing cells promoted an increase at proliferation rates, the results were not significant. These controversial findings indicate that the role of OPNb on activating tumor growth and cell proliferation in vivo could be potentially mediated by paracrine signals secreted by the tumor microenvironment, as has been intensely investigated [19,20]. It remains to be determined whether OPNb acts independently or in concert with OPNc to activate signaling pathways that promote PCa progression.

In serum starvation conditions, we showed that cell overexpressing OPNc, and to a lesser extent OPNb, still enhanced PC-3 proliferation. These data indicated that OPN-SI can promote tumor progression in a manner that is independent of growth factors, a typical feature of oncogenic gene products [17]. The data presented here provide evidence that cells overexpressing OPNc and OPNb have enhanced cell proliferation due to the promotion of cell survival by these isoforms. First, cells overexpressing OPNc and OPNb stimulated PI3K pro-survival signaling, which could be one of the molecular mechanisms of activating pathways activating cell proliferation and/or rescuing cells from apoptotic stimuli, such as serum starvation [18,21]. Second, PC-3 cells overexpressing OPNc had decreased proliferation and were induced to die, when treated with an anti-OPNc antibody. Finally, cells overexpressing OPNc and OPNb treated with LY294002 had improved survival, overcoming apoptosis typically induced by this drug treatment. Previous reports found that total OPN is able to prevent cell death in response to diverse stress stimuli, including serum starvation and also inhibit apoptosis in several systems [21], but no reports have described previously the individual role of each OPN-SI. Based on published reports and our present results, we propose that OPNc and OPNb splicing isoforms may enhance PC-3 survival due to enhanced cell proliferation. Further studies should be carried out to determine the cellular and mechanisms able to stimulate cell growth evoked by OPNb and OPNc overexpression and whether apoptosis or other mechanisms of cell death inhibition are also involved in mediating the survival roles for OPNc and OPNb in PCa. Besides promoting higher proliferating rates in PC-3 tumor cells, we have also showed that secreted OPNc also stimulates RWPE-1 (a normal prostate epithelia cell line) proliferation. Therefore, these data indicated that OPNc is not only involved in PCa progression and survival, but may also contribute to early steps of PCa tumorigenesis. OPNb, on the other hand, appears to exert its main effects on cell proliferation at later stages of tumor progression. Our data also demonstrated that OPNc and OPNb stimulated PC-3 cell motility and invasion and soft agar colony formation, inferring the roles of these isoforms on metastatic potential. Corroborating to these observations, cells overexpressing OPNc and OPNb isoforms significantly activated in vitro and in vivo the expression of MMP-2, MMP-9, and VEGF, molecules critical to PCa invasion and metastatic potential [22]. Some reports detailed the signaling pathways mediating each one of the OPN-SI roles. For instance, in hepatocarcinoma cell lines, OPN-SI differentially activated migration-associated signaling pathways. OPNa and OPNb, but not OPNc, increased the expression of urokinase type plasminogen activator and the phosphorylation of p42/p44 MAP kinase [11]. These same isoforms promoted pancreatic and fibrosarcoma tumors mainly by protecting cells against stress-induced apoptosis, by inducing NF-kappaB activation and FAK phosphorylation [13]. Our group recently demonstrated that OPNc activated ovarian tumor progression features by inducing the PI3K signaling pathway [8]. The present work adds a mechanistic understanding of the roles of OPN-SI in tumor progression by demonstrating that both OPNc and OPNb can activate distinct aspects of PCa progression through activation of PI3K signaling. Hence, specific knowledge of which signaling pathway and specific OPN-SI are mediating tumor progression opens possibilities to apply anti-splicing isoforms strategies in addition to signaling pathways inhibitors as therapeutic approaches to inhibit tumor progression [12,23]. Therefore, we propose that OPNc and OPNb-mediated roles on activating PCa progression features may be linked to the activation of PI3K signaling to promote PCa progression in both localized and advanced PCa. In support of our findings, previous studies have demonstrated that treatment of PCa cells with LY294002 resulted in cell cycle-mediated arrest, induction of apoptosis and inhibition on cellular invasion [24,25]. Although LY294002 can inhibit other gene products [26], in our study, PC-3 proliferation and migration capacity were specifically inhibited in OPNc and OPNb overexpressing cells, indicating that at least the effect on these cell features are mainly mediated by PI3K signaling and upstream signals activated by these splicing isoforms. However, cells overexpressing OPNc and OPNb overcame soft agar colony formation inhibition, indicating that these OPN-SI could be inducing PI3K signaling, which is classically involved on mediating soft colony formation, cell survival and anoikis, as described [24–26]. Moreover, PI3K/Akt signals have been reported to directly involve the upregulation of MMP-2 and MMP-9 [22,27]. Therefore, it would appear from the present data, that the OPNc and OPNb-mediated signals could possibly operate through a cell receptor/PI3K/Akt/MMP-2/-9

pathway. Further experiments will focus on verifying which specific receptor and downstream signals are being activated by OPNc and OPNb to modulate PI3K signaling and promote PCa progression.

CONCLUSIONS

We provide strong evidence that overexpression of OPNc and OPNb splice variants may be involved in distinct steps of PCa progression. Additionally, we demonstrated that the secreted forms of these OPN-SI are mainly mediating OPN effects on tumor progression by activating at least the PI3K pro-survival pathway. Therefore, a better understanding of the molecular mechanisms by which OPNc and OPNb activate PI3K signaling and its main targets will open up possibilities for developing improved PCa therapeutic approaches that specifically downregulate these isoforms and their effects, inhibiting PCa progression.

ACKNOWLEDGMENTS

We thank Dr. Marxa L. Figueiredo (Department of Pharmacology and Toxicology, University of Texas Medical Branch) and Luciana Bueno Ferreira for reviewing this manuscript.

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Supplementary Figure legends:

Supplementary Figure 1: Osteopontin isoforms overexpression in PC3 cells and derived tumor xenografts. (A) PC3 cell clones overexpressing OPNa, OPNb and OPNc (left to right graphs , respectively) overexpress the corresponding mRNA transcript, as compared to endogenous levels of these isoforms in PC3 cells transfected with the empty vector control clones. (B) Tumor xenografts resulting from PC3 cell clones overexpressing OPNa, OPNb and OPNc (left to right graphs , respectively) maintain the overexpression of OPNa, OPNb and OPNc isoforms, respectively, as compared formed by empty vetor transfected cell clones. Total RNA from whole cells extracts or tissue derived from tumor xenografts were purified and cDNA was prepared to use as templates for qRT-PCR assays using OPN-SI specific nucleotides. Results represent fold activation of relative mRNA expression of each OPN-SI in relation to the corresponding OPN isoform endogenous levels in empty vector transfected cell clones. GAPDH was used as a normalization control. Results are representative of three independent experiments.

Supplementary Figure 2: OPNc and OPNb overexpression supports cell proliferation. (A) Stably transfected cells with either empty vector, OPNa, OPNb or OPNc were plated as indicated in Material and Methods. On every consecutive day, three wells per cell line were harvested and the total number of cells was measured. Proliferation kinetics analysis was evaluated by exclusion of trypan blue. (B) Proliferation kinetics analysis as measured by crystal violet staining during a 48 h time course of PC3 overexpressing clones named OPNc1, OPNb2 and OPNa4, as compared to empty vevtor control cells**.** * $p < 0.05$ vs. empty vector control clone. These PC3 cell clones present differential OPN-SI mRNA expression levels, as demonstrated in (D). (C) OPN-SI expression levels were analyzed by qRT-PCR using isoform specific primers as presented in Material and Methods. Fold increase represents the augment of expression level of each OPN isoform in relation to empty vector control clone. Transfectant clones OPNa1, OPNb4 and OPNc4 expressed similar levels of OPNa, OPNb and OPNc isoforms, respectively, as compared to the empty vector control clone; (D) Transfectant clones OPNa4, OPNb2 and OPNc1 exhibit differential expression levels of OPNa, OPNb and OPNc isoforms, respectively, as compared to the empty

vector control clone. Different cell clones for each OPN isoform was tested isolatedly and presented similar proliferation behavior. The standard deviations (error bars) indicate the variability within each experiment.

Supplementary Figure 3: Representative pictures of tumors grown in nude mice. Tumor growth rate of mice s.c implanted with OPN-SI overexpression cells and controls as described on Figure 1D. * $p< 0.05$ vs. empty vector cells.

Supplementary Figure 4: Depletion of OPNc isoform using a anti-OPNc specific antibody inhibit PC3 cell proliferation and survival mediated OPNc overexpression. 5 X 10^4 cells PC3 cells either overexpressing OPNc (clone OPNc4) (A, B); OPNa (clone OPNa1) (C, E) and PC3 non-transfected cells (E, F) were plated and the total number of cells as indicative of cell proliferation (A, C, E) or cell death (B, D, F) was measured by trypan blue exclusion staining. Cell number was measured at 0 hour time point (bar OPNc4 control 0 h) and after 96 of cell culture (bar OPNc4 control 96 h). Cells were treated with anti-OPNc antibody (bar OPNc4 anti-OPNc) or with rabbit anti-goat IgG (bar OPNc4 IgG) at the concentration of 4 mg/mL for 96 hours. Significant differences in cell number are represented by asterisk. *, $P < 0.0001$ vs. OPNc4 control 96 h.

Supplementary Figure 5: OPNc and OPNb mediated proliferation is dependent on protein concentration on conditioned medium secreted by PC3 overexpressing cells.

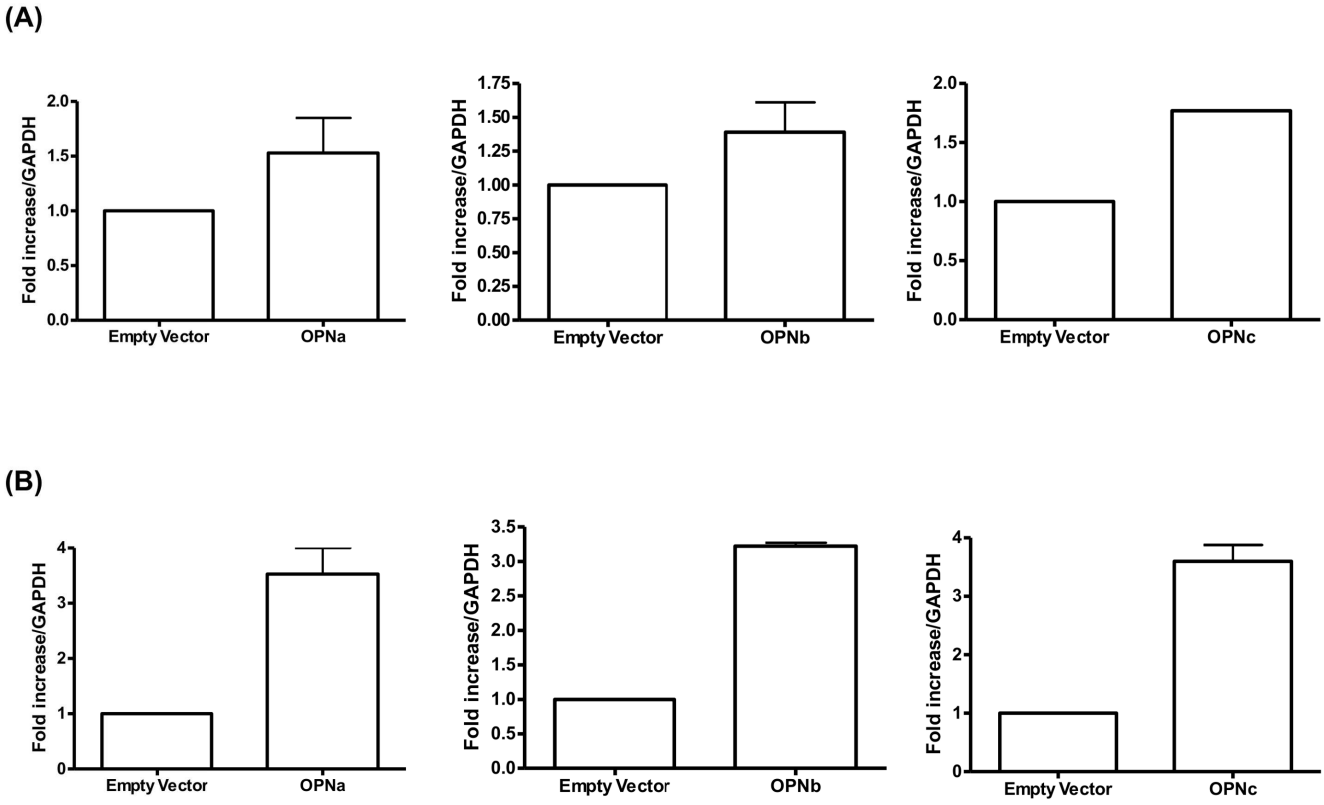
Conditioned medium (CM) secreted by OPNc (OPNc-CM) or OPNb (OPNb-CM) overexpressing cells was serially diluted in culture media from 100 to 25% and PC3 or RWPE-1 cell proliferation was measured at OD 550 nm by crystal violet staining, as described in Material and Methods. Standard culture media containing 0,2% FBS was used as a control for cell proliferation analysis, which was represented here as 0% at the *x* axis. PC3 (A and C) or RWPE-1 (B and D) cells were cultured in different dilutions of OPNc-CM (A, B) and OPNb-CM (C, D). Proliferation kinetics analysis as measured by crystal violet staining during a 48 h time cours * $p< 0.05$ vs. cells cultured in control culture media.

Supplementary Figure 6: Data sheet reporting detailed information regarding OPNc specific antibody from Gallus Immunotech (for review only)

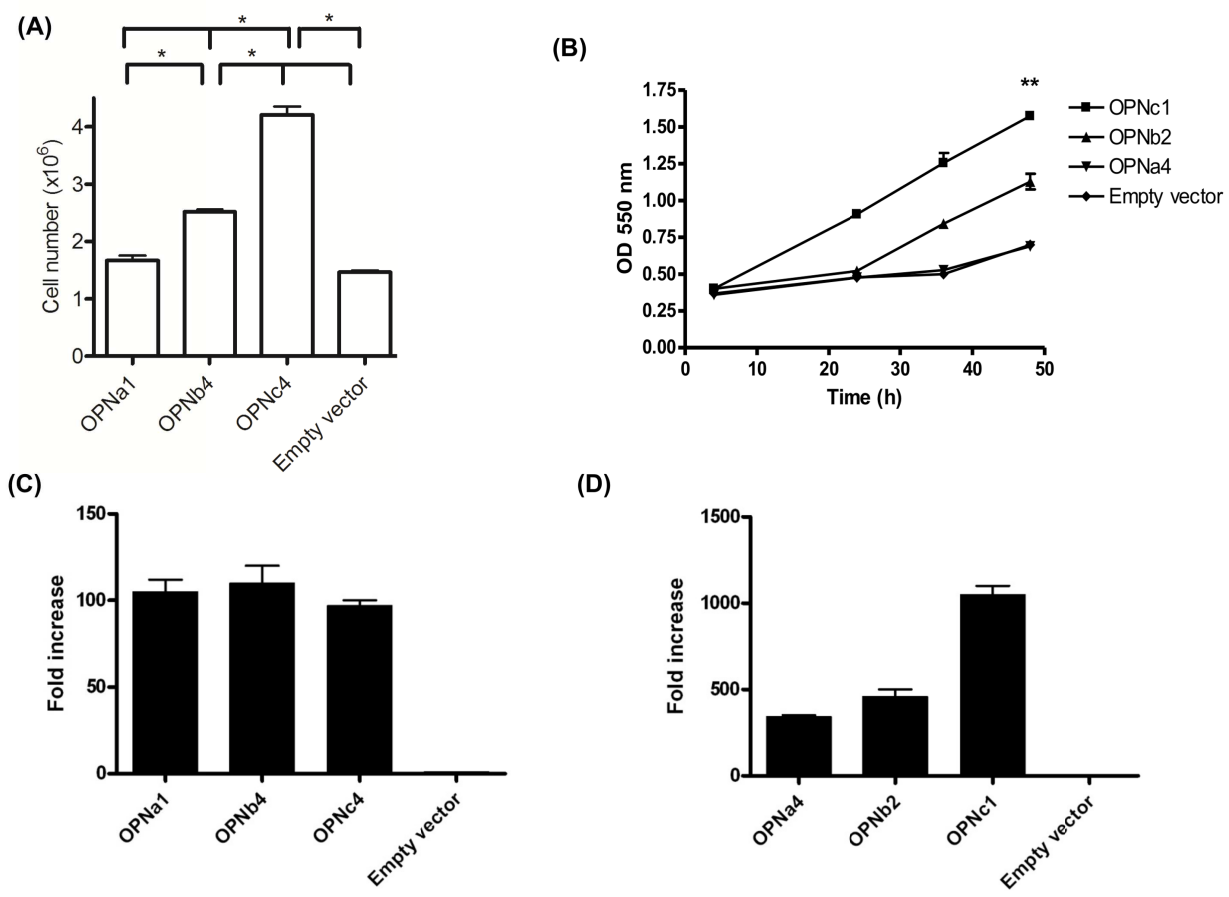
Supplementary Figure 7: Cell proliferation analysis of OPNc overexpression clone and PC3 non-transfected cells upon treatment with a non-specific IgY (only for review). 5 X 10^4 cells PC3 cells either overexpressing OPNc (clone OPNc4) (A) and PC3 non-transfected cells (B) were plated and the total number of cells as indicative of cell proliferation was measured by trypan blue exclusion staining. Cell number was measured at 0 hour time point (bar OPNc4 or PC3 control 0 h) and after 96 of cell culture (bar OPNc4 or PC3 control 96 h). Cells were treated with anti-OPNc antibody (bar OPNc4 + anti-OPNc or PC3 + anti-OPNc) or with a non-specific IgY (bar OPNc4 + IgY or PC3 + IgY) at the concentration of 4 mg/mL for 96 hours.

Supplementary Figure 8: Mitomycin C efficiently inhibit the proliferation of PC3 cells overexpressing OPNc and OPNb isoforms (only for review). Cells proliferation analysis was performed using crystal violet staining for 48 h after adding or not mytomycin C at a concentration of 5 μg/ml in standard culture media. PC3 cell clones overexpressing OPNa, OPNb and OPNc, as well empty vector control cells wer

Supplementary Figure 1



Supplementary Figure 2



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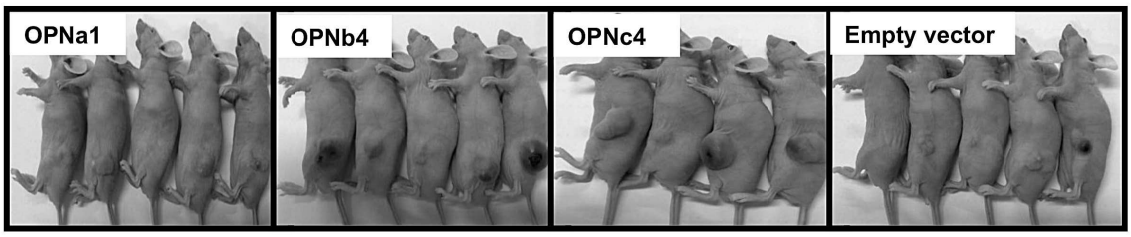
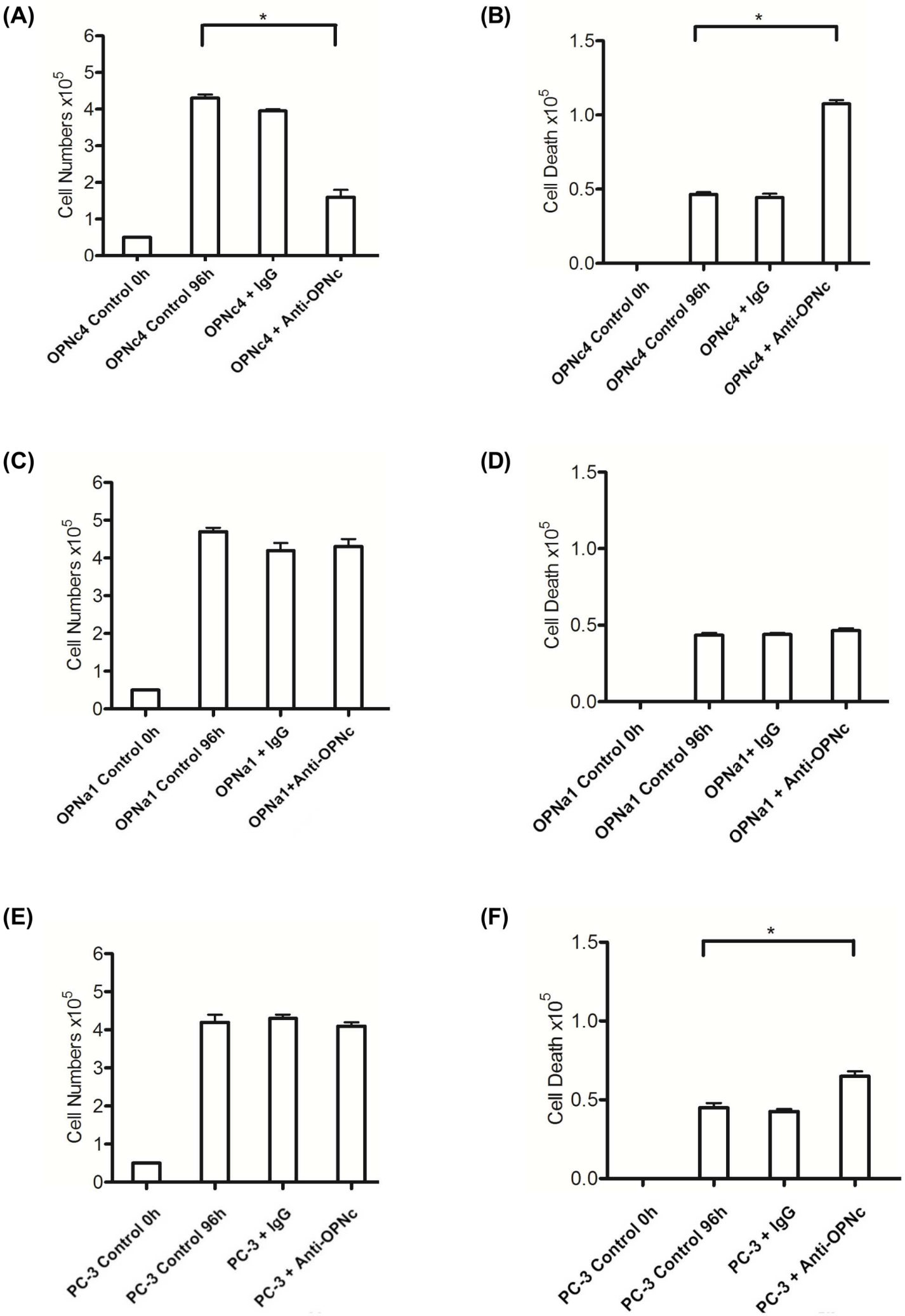
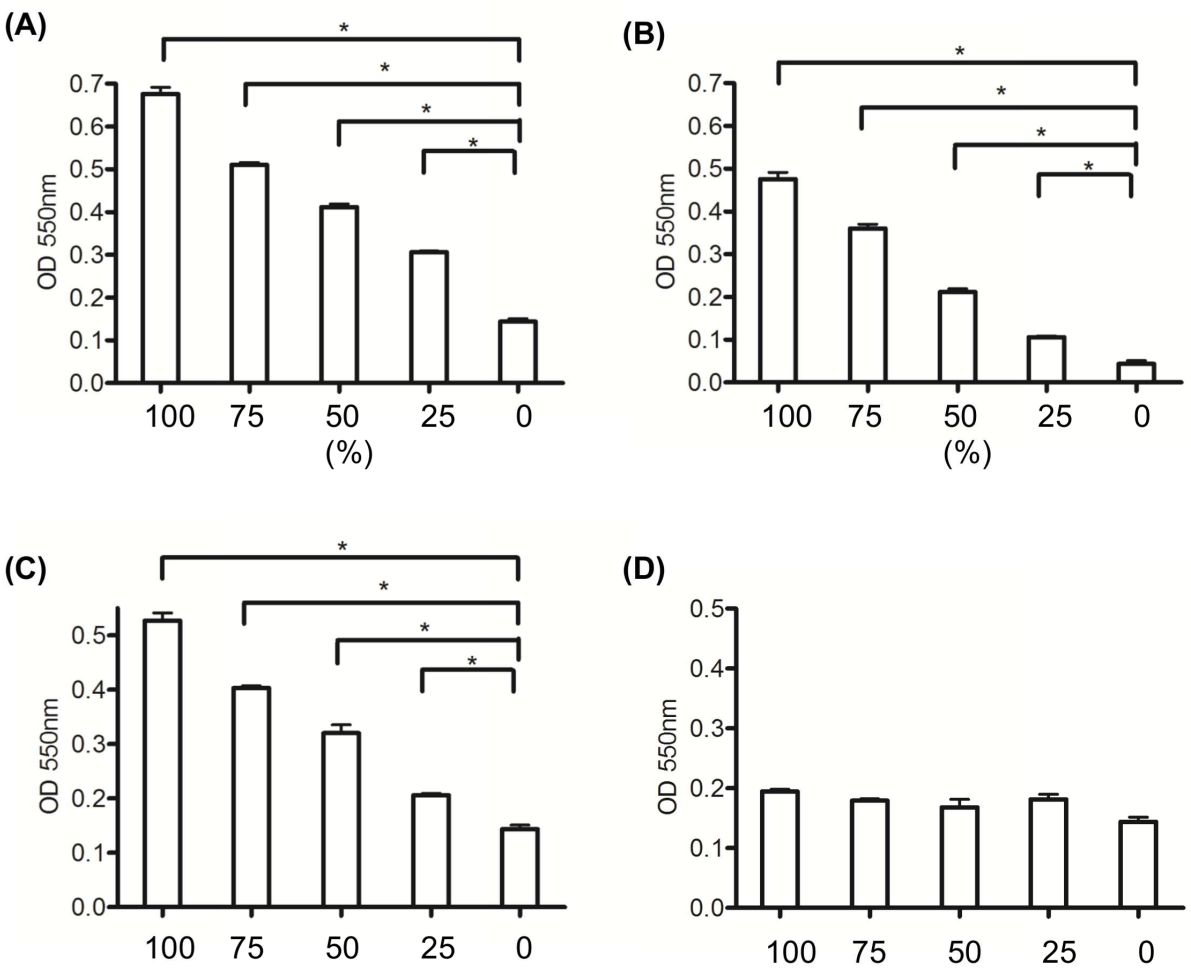


Supplementary Figure 4

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Capítulo V

Os resultados contidos nesse capítulo da tese serão submetidos, para a revista *Cell Communication and Signaling*.

Nas publicações anteriores, caracterizamos a OPNc como um importante produto gênico na indução da tumorigênese e progressão tumoral no CO e CaP. No entanto, pouco se sabe sobre os mecanismos moleculares capazes de mediar os efeitos da OPNc. Dentro deste contexto, o objetivo deste estudo foi melhor compreender os mecanismos moleculares relacionados à função desta isoforma nestes modelos tumorais. Através de PCR em tempo real, caracterizamos neste capítulo que a OPNc altera a expressão de 34 genes, no modelo de CO, e 16 genes, no CaP, essenciais para a transformação e progressão tumoral destas neoplasias.

Identificamos que a OPNc controla a superexpressão de fatores pró-angiogênicos, como EPDR-1, PDGF-A, TGFBR1, TNF, FGFR-2 e VEGF-A; assim avaliamos funcionalmente se o meio condicionado das células OvCar-3 e PC-3 transfectadas com a OPNc é capaz de estimular as células endoteliais. Mostramos que o meio condicionado destas células transfectadas com a OPNc induz a proliferação, migração e adesão das células endoteliais HUVEC, de forma a contribuir para a neovascularização. Os resultados gerados evidenciam a contribuição de distintos produtos gênicos nos mecanismos moleculares relacionados à progressão dos tumores de CO e CaP em resposta à superexpressão da OPNc. Apresentamos as figuras, legendas e tabelas ao final do capítulo.

Title: Osteopontin-c splicing isoform activates ovarian and prostate tumor progression features by modulating the expression of key cancer-related genes.

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Abstract: Alternative splicing of the osteopontin gene generates three splicing isoforms (OPN-SI), designated OPNa, OPNb and OPNc, which have specific roles in different tumor models. Previously, we showed that the OPNc isoform promotes different aspects of ovarian (OC) and prostate (PCa) tumor progression. This study investigated the signaling pathways which are modulated in OC and PCa cell lines overexpressing OPNc in comparison with empty-vector (EV) transfected cells. Here we studied genes regulated by OPNc in a model of human ovarian (OvCar-3 cells) and prostate cancer (PC-3 cells) using a PCR array approach, by comparing the gene-expression profile of OvCar-3 and PC-3 cells overexpressing OPNc versus EV-transfected cells. Among 84 cancer pathway focused genes tested, we identified, respectively, 34 and 16 genes that were differentially expressed between OvCar-3 and PC-3 OPNc-overexpressing cells and those transfected with the EV control. Functional classification of these genes into the hallmarks–of-cancer categories showed that OPNc can affect the expression of genes involved in these cancer pathways in these tumor models. Furthermore, we were able to validate part of the data obtained from this by further investigating the effects of OPNc-

conditioned medium (CM) on the steps of angiogenesis process. OPNc-CM induces endothelial cell adhesion, and the proliferation and migration of endothelial cells, further supporting our array findings. This study provides the first evidence that OPNc can lead to numerous changes in gene expression, which influence multiple aspects of OC and PCa tumor progression and malignant growth.

INTRODUCTION

Osteopontin (OPN) is a secreted, integrin-binding phosphoprotein that has been clinically and functionally associated with cancer. Abundant data have demonstrated that OPN is expressed in a variety of cancers, including carcinomas of breast, prostate, colon, ovary, stomach, liver, lung, mesotheliomas, squamous cell carcinomas, sarcomas and multiple myeloma (1). OPN expression has also been observed in the tumor microenvironment of different cancer types, such as macrophages and stromal cells (2). In particular, several studies on ovarian and prostate carcinomas have demonstrated increased OPN expression, which has been associated with advanced tumor stage and poor patient prognosis (3,4). In addition to clinical findings, experimental studies have shown that OPN can functionally contribute to malignancy (1).

OPN functional diversity has been associated to post-translational modifications, such as phosphorylation, glycosylation, sulfation, enzymatic cleavage and protein crosslinking, causing OPN proteins to bind differentially to seven types of integrins and specific CD44 splice variants (2,5). Another mechanism underlying the functional diversity of OPN is the existence of splice variants (OPNa, OPNb and OPNc). OPNa is a full-length isoform, while OPNb and OPNc lack exons 5 and 4, respectively (2).

We recently published the first reports about OPN splicing isoforms (OPN-SI) in ovarian carcinoma (OC) and prostate cancer (PCa), by demonstrating the expression patterns and functional roles of each OPN-SI in these tumor models (6-8). We showed that OPNc was specifically expressed in ovarian tumors, compared

to benign and non-tumoral ovarian samples (6). We also observed that among OPN isoforms, OPNc was the most up-regulated splice variant in PCa samples, and outperformed the remaining isoforms and PSA serum levels in PCa diagnostic accuracy (7). Based on these data, we addressed the function of each OPN-SI in OC and PCa by examining the effect of their ectopic overexpression in OvCar-3 and PC-3 cells, respectively. Cells overexpressing each OPN-SI were evaluated for features mimicking different steps of tumor progression by using *in vivo* and *in vitro* approaches. Our data demonstrated that OPNc overexpression increased OvCar-3 and PC-3 cell growth, sustained proliferative survival, migration, invasion, anchorage-independence and tumor formation *in vivo*, suggesting a possible role for OPNc in OC and PCa progression and survival. Additionally, we demonstrated that these tumor-promoting effects were mediated mainly through the activation of the Phosphatidylinositol-3 Kinase (PI3K)/Akt signaling pathway. In the PCa model, we demonstrated that OPNb also stimulated all these tumor-progression features, althought to a lesser extent than OPNc (8).

It has been demonstrated that the role of each OPN-SI is tumor-specific, although the mechanisms controlling these patterns are currently unknown (6,8-13). Although we have described some of the roles of OPNc in OC and PCa progression, detailed characterization of the molecular mechanisms mediating these pro-tumorigenic features is currently lacking. An in-depth characterization of the genes and signaling pathways that modulate the roles of OPNc in these tumor models might improve understanding of the functions of this splice variant in each of these malignancies. In addition, this characterization could indicate additional roles of OPNc in different aspects of tumor progression. In the present report, we used a PCR Cancer Array representing genes involved in the main hallmarks of cancer, as an experimental approach to identify signaling pathways that are modulated in OC and PCa cell lines that overexpress OPNc, in comparison with empty vector transfected cells. Our data indicate that OPNc-overexpressing cells have specific transcriptional patterns in OC and PCa cell line tumor models. Based on these results, we suggest that these patterns could be an auxiliary tool to improve understanding of the molecular pathways modulating the specific roles of

ONPc in these tumors. As an approach to further validate the data obtained herein and based on marked up-regulation of *Vegfa* in response to OPNc overexpression, we demonstrated functionally that OPNc conditioned medium secreted by OvCar-3 and PC-3 cells overexpressing this splicing isoform, stimulates proliferation, migration and adhesion of endothelial cells.

MATERIALS AND METHODS

Cell Culture, OPN Plasmids and Transfection

As a model to examine the signaling pathways modulated by OPNc overexpression in ovarian and prostate carcinoma, we used OvCar-3 and PC-3 cell lines provided by the ATCC. All cell lines were cultured in medium supplemented with 20% (OvCar-3) or 10% (PC-3) fetal bovine serum (FBS), 100 IU/ml penicillin and 100 mg/ml streptomycin in a humidified environment containing 5% CO_2 at 37ºC. The open reading frame of the OPNc splice variant was cloned into the pCR3.1 mammalian expression vector, as previously described (6,8). Transfections were performed using Lipofectamine^{TM} 2000 (Invitrogen, CA). The expression plasmid was transfected into OvCar-3 and PC-3 cells, and stably expressing cell clones were selected with 600 µg/ml or 800 µg/ml of G418, respectively. OvCar-3 and PC-3 cells transfected with empty vector (EV) were used as a negative control in these assays. HUVEC cells were isolated as described previously (14). These cells were cultured in MCDB131 medium (GIBCO) supplemented with 20% fetal bovine serum (FBS), 2 mM L-glutamine, 50 µg/mL of heparin, and 50 µg/mL of endothelium cell growth supplement (ECGS), hereafter referred to as complete medium.

Human Cancer Pathway Finder PCR Array

The Human Cancer Pathway Finder SuperArray (PAHS-033A; Qiagen) was used to analyze mRNA levels of 84 genes related to cell cycle control, apoptosis,

signal transduction molecules, adhesion, angiogenesis, invasion and metastasis. Quality control of RNA samples, synthesis of cDNA and real-time RT-PCR arrays were performed as described by the manufacturer (Qiagen). In brief, cDNA was prepared from 1 µg of total RNA by using a RT2 PCR Array first strand kit. A total volume of 25 µl of PCR mixture, which included 12.5 µl of RT2 Real-Time SYBR Green PCR Mastermix (containing HotStart DNA polymerase and SYBR Green dye), 11.5 µl of RNase-free water and 1 µl of cDNA synthesis reaction, was loaded in each well of the PCR Array. PCR amplification was conducted with an initial 10-min step at 95℃ followed by 40 cycles of 95℃ for 15 s and 60℃ for 1 min. The fluorescent signal from SYBR Green was detected immediately after the extension step of each cycle, and the cycle at which the product was first detectable was recorded as the cycle threshold. All genes represented in the array showed a single peak in the melting-curve analysis of the specific amplification products. Fold-changes in gene expression were calculated using the ΔΔCT method, and five stably expressed housekeeping genes (β2 microglobulin, hypoxanthine phosphoribosyltransferase 1, ribosomal protein L13a, GAPDH and β-actin) were used for normalization of the results. Only genes showing a 1.5-fold or greater change were considered for further analysis. Data analysis of gene expression was performed using Excel-based PCR Array Data Analysis Software provided by the manufacturer (Qiagen).

In order to prepare the CM, cell number was normalized by plating the same numbers of OvCar-3 and PC-3 at a density of 5 X 10^5 cells in each assayed culture plate for individual OPNc and the EV-overexpressing cell clone. After reaching 80% cell confluency, cells were washed twice with PBS (phosphate buffered saline) and cultured with RPMI in serum-free conditions for 48 h. Collected CM was clarified by centrifuging at 1500 rpm for 5 min. All functional assays were performed using freshly prepared CM. Total protein concentration of this CM was measured using the BCA assay kit (BioRad) with bovine serum albumin as a standard, according to the manufacturer's instructions. CM produced by OPNc-overexpressing cells or those transfected with EV controls, which were termed

OPNc-CM and EV-CM, respectively, were used as a substrate for HUVEC endothelial adhesion, proliferation and migration, as described below.

Cell adhesion assays

Bacteriological 96-well plates (Greiner Bio-One) were coated with OPNc-CM and EV-CM, which were used as coating substrates for HUVEC adhesion. HUVEC cells were seeded at a density of 2 X 10^4 cells and incubated at 37℃ for 2 h in either the OPNc-CM or EV-CM pre-coated wells. Attached cells were stained with crystal violet, and the cell-incorporated dye was quantified by measuring absorbance at 550 nm with a SPECTRAmax GEMINI-XS using SoftMax Pro software Version 3.1.1. In order to test the involvement of specific cell receptors in HUVEC cell adhesion capacity, anti-αvβ3, anti-αvβ5 (Chemicon), CD44 (Ancell) or mouse purified IgG (10 µg/mL, Serotec) antibodies were pre-incubated with HUVEC cells at 37℃ for 2 h. Then, HUVEC cells were plated on OPNc-CM or EV-CM pre-coated plates, and the HUVEC adhesion to these substrates was measured.

Migration assays

HUVEC cell-migration assays were evaluated by *in vitro* wound closure assay, as described by others (15). Wild-type HUVEC cells were grown in six-well microtiter plates to near total confluency in complete culture medium. Multiple uniform and constant streaks were made on the monolayer culture with 10 µl pipette tips. The plates were immediately washed with PBS to remove detached cells. HUVEC cells were incubated with CM obtained from OvCar-3 and PC-3 cells transfected with OPNc or empty vector. Cell migration was monitored for 6 h and photographs were taken at the 0 and 6 h time points.

Proliferation assays

HUVEC cells (2 X 10^4, 24-well plates) were cultured with OPNc-CM or EV-CM and cell proliferation was analyzed by crystal violet assays. For the crystal

violet incorporation assays, cells were washed twice with PBS and fixed in glutaraldehyde for 10 min, followed by staining with 0.1% crystal violet and solubilization with 0.2% Triton X-100. Microtiter plates were read on a SPECTRAmax GEMINI-XS using SoftMax Pro software Version 3.1.1.

Transcriptome–interactome analysis

In order to prepare a protein interaction map of genes differentially expressed in OvCar-3 and PC-3 OPNc-overexpressing cells, putative protein-protein interaction networks were investigated through functional protein association networks using the STRING software (http://string.embl.de/newstring_cgi/show_input_page.pl).

Statistical analyses

In all experiments, unless otherwise indicated, data are reported as mean ± SEM. Data were analyzed by comparisons using a two-tailed t test, and a p value <0.05 was considered significant.

Results

OPNc modulates the expression of key cancer-related genes in OvCar-3 and PC-3 cells overexpressing this isoform

In order to describe the cancer gene pathways modulated by OPNc overexpression in OvCar-3 and PC-3 cell lines, we performed a qPCR Array analysis of total mRNA obtained from OPNc-overexpressing cells, compared to samples from the EV control clone. Using this *in vitro* model, we assessed the impact of OPNc overexpression on different hallmarks of cancer by using the Cancer Pathway Finder Array. This array consisted of 84 genes representing the major biological pathways involved in tumorigenesis: 1) cell cycle control and DNA damage repair, 2) apoptosis and cell senescence, 3) signal transduction molecules

and transcription factors, 4) adhesion, 5) angiogenesis and 6) invasion and metastasis.

A complete list of genes whose expression is significantly modulated by OPNc overexpression in OvCar-3 and PC-3 is shown in Table I and Table II. Among the 84 cancer pathway-focused genes tested, we identified, respectively, 34 and 16 genes that were differentially expressed between OvCar-3 and PC-3 OPNc-overexpressing cells and those transfected with the EV control ($p < 0.05$ with at least 1.5 fold up- or down-regulation). Differentially expressed genes as a consequence of OPNc overexpression in OvCar-3 and PC-3 cells are included in all major biological pathways involved in the tumorigenesis investigated here.

In OvCar-3 OPNc-overexpressing cells, the transcript levels of several factors involved in cell cycle control, including *Rb1*, *Cdk2*, *Cdkn1a*, *Ccne1*, *S100a4* and *Cdc25a* were up-regulated ($p < 0.05$; Table I). Correspondingly, overexpression of OPNc induced the transcriptional levels of some anti-apoptotic factors, such as *Bcl2l1*, *Bad* and *Casp8*. In this OvCar-3 OPNc-overexpressing cell line model, the most significant up-regulated genes were those related to invasion and metastasis (*Mmp2* and *Serpine1*), cell adhesion (*Itgb3*) and angiogenesis (*Vegfa*). Only 4 genes are down-regulated in OvCar-3 OPNc-overexpressing cells when compared to EV transfected cells. These genes are related to DNA damage repair (*Atm*), act as transcription factors (*Fos* and *Myc*), or perform an important role in cancer-cell invasion (*Mmp1*).

PC-3 OPNc-overexpressing cells demonstrated a distinct transcriptional pattern, compared to OvCar-3 overexpressing this isoform (Table II). The most significant up-regulated genes identified here are related to chromosome-end replication (*Tert*), invasion and metastasis (*Plau*, *Serpine1*, *Mmp9* and *Mmp1*), cell adhesion (*Itgb3* and *Itgav*) and angiogenesis (*Angpt1* and *Vegfa*). Transcriptional suppression in PC-3 cells as a result of OPNc overexpression was observed for *Htatip2* (a gene related to apoptosis control) and *Fos*, a transcription factor-coding gene. The specific transcriptional patterns observed for both OvCar-3 and PC-3 OPNc-overexpressing cells revealed the potential of this isoform to contribute to all the main acquired capabilities required for tumor progression, although each of

these tumor cells evokes different signaling pathways. Among the differentially expressed genes, 7 (*Bcl2l1*, *Bad*, *Fos*, *Itgav*, *Itgb3*, *Vegfa* and *Serpine1*) were commonly modulated as a result of OPNc overexpression in both OvCar-3 and PC-3 cells (Table III), and are presumably required for shared functions related to cancer progression in these two tumor models. As a whole, these data indicate that OvCar-3 and PC-3 OPNc-overexpressing cells modulate specific transcriptional patterns related to key steps of cancer progression, although part of these signaling pathways is commonly regulated in both cell-line tumor models.

OPNc-CM induces endothelial cell adhesion, proliferation and migration

The data obtained here demonstrated that *Vegfa* is one of the most upregulated transcripts in response to OPNc overexpression in both OvCar-3 and PC-3 cells (Table III). VEGFA is one of the earliest and a key mediator of angiogenesis (16). Also, OvCar-3 and PC-3 OPNc-overexpressing cells and xenograft tumors up-regulate the expression of *Vegf* transcript (6,8) and protein (data not shown). Based on these data, we aimed to further validate the data obtained from this Cancer Gene Array by testing the effect of OPNc-CM on different aspects of angiogenesis.

Angiogenesis is a multistep process that activates the migration and proliferation of endothelial cells in the perivascular stroma in order to form new capillary vessels. During this process, these sprouting cells stop proliferating and then adhere, align, form tubes, and finally yield new operational vessels (17). We then investigated whether OPC-CM is involved in this multistep angiogenic process by studying its effect on the adhesion, proliferation and migration of human umbilical vein endothelial cells (HUVECs).

A higher proportion of HUVEC adhered cells was observed when using OPNc-CM secreted from OvCar-3 and PC-3 cells as a coating substrate, compared to EV-CM ($p < 0.0001$) (Figure 1A). In order to investigate which putative cell receptors mediated HUVEC adhesion to OvCar-3 and PC-3 OPNc-CM, HUVEC cells were pre-incubated with anti-αvβ3, anti-αvβ5, CD44 or mouse

purified non-specific IgG antibodies. An HUVEC pre-incubation with an anti-αvβ3 blocking antibody significantly inhibited the adhesion of these cells to OvCar-3 OPNc-CM ($p < 0.0001$) but not to PC-3 OPNc-CM (Figure 1A). Adhesion of HUVEC cells to PC-3 OPNc-CM was only reversed by an HUVEC pre-incubation with an anti-CD44 blocking antibody ($p < 0.0001$). Anti-αvβ5 or IgG non-specific antibody produced no significant effects on HUVEC adhesion to both OvCar-3 and PC-3 OPNc-CM. These data indicate that HUVEC adhesion to OvCar-3 OPNc-CM is mediated by αvβ3, while its binding to PC-3 OPNc-CM is mediated by CD44 receptors.

We next asked whether the proliferation rates of HUVEC cells cultured with OPNc-CM were altered compared to EV-CM secreted by OvCar-3 and PC-3 cells. As shown in Figure 1B, OPNc-CM secreted from both OvCar-3 and PC-3 cells significantly activated HUVEC proliferation rates, compared to EV-CM in the range of 0 – 48 h of cell culture ($p < 0.05$).

The effect of OPNc-CM on modulating the migration of endothelial cells was also tested, by evaluating the migration of HUVEC cells when cultured in OPNc-CM or EV-CM secreted by OvCar-3 and PC-3 cells. HUVEC cells cultured in OPNc-CM produced by both OvCar-3 and PC-3 cells showed a higher migration rate than HUVEC cells cultured in EV-CM (Figures 1C and 1D).

In summary, these data indicate that OPNc-CM could act as a pro-angiogenic factor for HUVEC cells, and that this CM can significantly interfere with different key aspects of the early angiogenic process. In addition, these data further validate the gene expression patterns obtained by these gene array data.

DISCUSSION

The main finding of this study is that OPNc overexpression can modulate key cancer molecular pathways associated with ovarian and prostate tumor progression. The array data revealed that OPNc overexpression modulates the expression of genes related to cell cycle control, apoptosis, signal transduction molecules, adhesion, angiogenesis, invasion and metastasis. Among the

differentially expressed genes identified, we could partially validate data obtained here, by demonstrating that OPNc-overexpressing cells secrete factors that are able to activate early angiogenic processes, as evidenced by upregulation of transcripts with pro-angiogenic properties in response to OPNc overexpression. Three main factors prompted us to specifically investigate genes that are differentially expressed between OPNc-overexpressing cells and those transfected with EV control. First, OPNc is recognized as an important contributor to several aspects of OC and PCa progression. Second, in the OC model, OPNa and OPNb-overexpressing cells show similar behaviors in comparison to EV-transfected and OvCar-3 wild-type cells. Due to these similar properties, investigating OPNc in relation to EV vector control would, in principle, represent general gene expression patterns modulated by OPNc in comparison to the remaining two other isoforms. Third, in the PCa model, OPNb isoform also stimulated several progression features. However, this isoform is not able to alter pro-tumorigenic effects, as evidenced for OPNc (6,8).

The roles of OPNc in OC and PCa progression are related to the absence of the protein sequence contained in exon 4. Some evidence about the functional properties of this region suggests that it may play a critical role in OPN solubility, making soluble OPN available for receptor ligation (10). Another hypothesis relates to OPN post-translational modifications (PTMs) (10,18-20). Recent reports have also shown that the pattern of phosphorylation of OPN isoforms produced by different tumor cell types can regulate its roles and receptor interactions (13). Possibly, the deletion of exon 4 could alter the pattern of phosphorylation, causing functional modifications. Additionally, the OPN protein domain coded by exon 4 is highly enriched in proline residues. Proline-rich motifs do not merely act as structural spacers, but frequently also have an important role in protein-protein interactions that are essential for cellular processes. Further work is required to elucidate these hypotheses regarding the OPNc isoform and their impact on functional roles in ovarian and prostate cancer cells.

A plethora of data shows that human cancer is a multistage genetic and epigenetic disease, which requires the acquisition of certain properties common to

all tumors. These alterations, while having the potential to produce a wide array of different cancer-cell genotypes, have been suggested to affect main aspects of cell physiology involved in cancer development, which are termed 'Hallmarks of Cancer' (21). The OPNc-induced genes, as evidenced by the cancer array performed here, and their relationships to the main acquired capabilities of cancer progression are discussed below.

Cell cycle and DNA damage repair

Previous studies from our group demonstrated that OPNc favors OC and PCa cell proliferation (6,8). Here, we identified several cell proliferation-related genes in OC and PCa as a result of OPNc overexpression.

In OvCar-3 OPNc-overexpressing cells, we observed significant downregulation of ataxia telangiectasia mutated gene (*Atm*/-4.72), and upregulation of *Rb1* (+1.8), *Cdk2* (+1.85), *Cdkn1a* (+2.07), *Ccne1* (+2.3), *S100a4* (+2.66) and *Cdc25a* (+2.8). *Atm* and *Rb1* are classical tumor-suppressor genes (22-24). However, a growing body of evidence shows that *Atm* mutations confer increased susceptibility to OC (25). These data indicate that down-regulation of *Atm* mediated by OPNc overexpression could be involved in OC progression. More recently, the literature has shown that SMYD2 is methylated by RB1, stimulating tha cell cycle progression (26). Siu et al. (2010) (27) have demonstrated that P21(*Cdkn1a)*-activated kinase 4 (Pak4) overexpression promotes OC cell migration, invasion and cell proliferation through the Pak4/c-Src/EGFR pathway. Cyclin E (*Ccne1*) amplification and overexpression are related to mechanisms of primary treatment failure in serous OC and stimulation of cell proliferation (28,29). In addition, *Cdk2* gene amplification, overexpression and activation have also been observed in OC samples (30). In particular, S100A4 protein has been reported to function in cell cycle progression, motility, adhesion and invasive properties. *S100a4* gene overexpression has been related to the malignant potential of some tumors and has been closely associated with metastasis, including OC (31,32).

Broggini et al. (2000) (33) reported that high expression of *Cdc25a* is related to a poorer prognosis in OC patients.

In PC-3 OPNc-overexpressing cells, we found upregulation of *E2f1* (+2.04) and *Brca1* (+2.38). *E2f1* expression level is low in benign and localized PCa samples, and is significantly upregulated in metastatic tissues from hormone-resistant PCa patients. Ectopic expression of *E2f1* significantly inhibited androgen receptor (AR) mRNA and protein levels in prostate epithelial cells, contributing to the progression of hormone-independent PCa (34). In addition, Zheng et al. (2009) (35) showed that *E2f1* activated the expression of the transcription factor *Egr1,* which in turn activated the PI3K/Akt pathway to resist drug-induced apoptosis. Gao et al. (2001) (36) reported that overexpression of *Brca1* in PCa cells can cause activation of STAT3, JAK1 and JAK2, which may provide a new critical survival signal for PCa growth in the presence of normal *Brca1* (36).

The functional relationship between downregulation of *Atm* and upregulation of *Rb1*, *Cdk2, Cdkn1a, Ccne1, S100a4* and *Cdc25a* possibly regulates OvCar-3 cell proliferation signaling. On the other hand, in PC-3 cells, the overexpression of *E2f1* and *Brca1* could stimulate cell proliferation in response to OPNc overexpression.

Apoptosis and Cell Senescence

Evasion of apoptosis plays a key role in cancer development, drug resistance and recurrence (21). Previously, we demonstrated that OPNc and OvCar-3 and PC-3-overexpressing cells that were treated with the anti-OPNc antibody decreased their proliferation rates and were induced to die, further evidencing a survival role for OPNc in these tumor cell lines (6,8).

We found that *Bcl2l1* and *Bad*, two anti-apoptotic genes, were upregulated in OvCar-3 and PC-3 OPNc-overexpressing cells compared to EV-transfected cells. Bcl2-like 1 (*Bcl2l1*) inhibits apoptosis by blocking the translocation of BAX to the mitochondrial outer membrane (37). *Bad* is a death-promoting BH3-only member of the BCL-2 family, and its proapoptotic activity is regulated primarily by phosphorylation in response to survival factors (38). Page et al. (2000) (39)

showed that OC cells overexpressing constitutively active Akt/AKT1, which are highly resistant to paclitaxel, contained high levels of phospho-BAD. Other investigators have shown that increased expression of BAD provided a proliferative advantage to prostate tumors, while BAD dephosphorylation increased sensitivity of PCa cells to apoptosis (40).

We also observed a significant up-regulation of *Casp8* and *Apaf1* in OPNc-overexpressing OvCar-3 cells. The cascade initiated by caspase-8 is involved in death-receptor-mediated apoptosis (41). In OC, the *Apaf1* gene seems to be active. Additionally, dysfunction in the apoptosome assembly process has been correlated with chemoresistance (42).

In PC-3, we also identified *Htatip2* (-5.77) and *Tert* (+15.65) de-regulated expression in response to OPNc overexpression. Zhang et al. (2008) (43) demonstrated that *Htatip2* (TIP30) nuclear expression is associated with PCa progression and metastasis. Knockdown of TIP30 suppresses PC-3 and LNCaP cell growth. Recently, Sabaliauskaite et al. (2012) (44) suggest that *Tert* expression can be a valuable tool for early prediction of PCa biochemical recurrence after radical prostatectomy.

The up-regulation and down-regulation of all these transcripts may therefore represent mechanisms by which OPNc may mediate evasion of apoptosis in OC and PCa.

Signal transduction molecules and transcription factors

Among signaling pathways that are typically activated in ovarian and prostate tumor progression, PI3K/Akt has an important pro-survival role and mediates several pro-tumorigenic features evoked by OPNc overexpression in OvCar-3 and PC-3 cells (6,8). To further explore the molecular mechanisms by which OPNc may affect tumor progression features, we also analyzed expression profiles of additional signal-transduction molecules and transcription factors.

We demonstrated here, the existence of different deregulated signal transduction pathways in OvCar-3 and PC-3 cells as a result of OPNc overexpression. The *Fos* gene was found to be downregulated in response to OPNc overexpression in OvCar-3 (-2.12) and PC-3 (-5.76) cells.

In OvCar-3 OPNc-overexpressing cells, there was a downregulation of *Myc* (-1.82) and upregulation of the *Pik3r1* (+1.65), *Raf1* (+1.73), *Erbb2* (+2.46) and *Akt1* (+2.56) genes. Okuyama et al. (2010) (45) showed that the tumor environment downregulates c-MYC protein levels, and that might be a strategy for cancer cells to survive under conditions of limited energy sources. However, to date, there is no evidence of c-MYC down-regulation in OC cells. PI3K p85 subunit (*Pik3r1)* is highly expressed and positively correlated with OC progression (46). Increased expression of *Raf-1* has been associated with OC cell survival, by stimulating cell proliferation and inhibiting apoptosis (47). The *Erbb2* (*Her2/Neu*) receptor is amplified and overexpressed in 9-30% of ovarian cancers, and has also been correlated with poor prognosis and chemoresistance (48).

In PCa, we report here that OPNc is able to stimulate *Ets2* transcription factor overexpression. Carbone et al., (2004) (49) showed that downregulation of *Ets2* in PCa cells inhibited of anchorage-dependent and independent growth, cell cycle alterations, and induction of apoptotic cell death.

The ability of OPNc to down- or upregulate all these genes may therefore provide new insight into the mechanisms by which ovarian and prostate cancer cells may regulate the expression of these genes and stimulate several pro-tumorigenic features.

Adhesion

Integrins directly bind components of the extracellular matrix, providing the necessary traction for cell motility and invasion, and also regulate cell proliferation (50). Lössner et al. (2009) (51) showed that integrin alphavbeta3 overexpression and engagement by its ligand increased adhesion, motility and proliferation of human OC cells. In PCa, McCabe et al. (2007) (52) showed that functional

modulation of the alphavbeta3 integrin receptor in PCa cells is required for tumor progression within bone and determines tumor-induced bone tissue transformation.

In OC, in addition to upregulation of integrins in response to OPNc overexpression, we found an upregulation of Pinin (*Pnn*/+1.85). Mary et al. (2011) (53) showed *Pnn* was overexpressed in OC specimens, compared to benign tissues. These authors suggested that PNN may modify the co-repressor function of C-terminal binding protein 2 (CtBP2), which is an OC oncogene that modulates histone deacetylase (HDAC) activity and DNA repair.

The upregulation of a number of integrins and adhesion molecules in cells that constitutively overexpress OPNc may therefore represent mechanisms by which cells acquire global cell ability to adhere, promoting ovarian and prostate tumor progression.

Angiogenesis

Previously, we showed that OPNc significantly increases *Vegfa* mRNA (6,8) and protein levels (data not shown) in OC and PCa. Here, we also observed *Vegfa* upregulation in response to OPNc-overexpression in OvCar-3 and PC-3 cells. Published reports have indicated that in OC, VEGFA is overexpressed and is associated with increased tumor growth, ascites fluid accumulation, metastases, poor prognosis and reduced survival (54). In PCa, elevated circulating VEGFA has been demonstrated to be predictive of biochemical progression in men undergoing radical prostatectomy (55).

In addition to *Vegfa* overexpression, we showed here that OvCar-3 OPNc-overexpressing cells upregulate *Epdr1* (+1.5), *Pdgfa* (+1.54), *Tgfbr1* (+1.57), *Tnf* (+1.83) and *Fgfr2* (+2.34). *Pdgf* induces cell growth, survival, transformation, migration and vascular permeability (56) and has also been described as a useful marker for OC (57). Additionally, *Vegfa*, *Vegfc* and *Pdgfa* may promote lymphatic metastasis in epithelial OC through other mechanisms besides lymphangiogenesis (58). Activated TGFBR1 phosphorylates SMAD2, which dissociates from the receptor and interacts with SMAD4. The SMAD2-SMAD4 complex modulates the

transcription of the TGF-beta-regulated genes. Smad4 is correlated with the invasion and metastasis of OC (59). The cytokine TNF-α is an important component of the interplay between malignant cells and the immune system. Charles et al. (2009) (60) found that TNF-α maintained TNFR1-dependent IL-17 production by $CD4^+$ cells and this led to myeloid cell recruitment into the tumor microenvironment and enhanced ovarian tumor growth. Gene amplification or missense mutation of *Fgfr2* occurs in OC (61). Literature data have shown that combining *Fgfr2* inhibitors with platinum-containing cytotoxic agents for the treatment of epithelial OC may result in increased antitumor activity (62).

In PC3 OPNc-overexpressing cells, in addition to *Vegfa*, OPNc upregulates the expression of angiopoietin-1 (*Ang1*) (+12.62). *Ang1* bind to Tie2 promoting blood-vessel stability. These interactions enhance endothelial cell survival, leading to a more stable vasculature with decreased permeability (63).

Based on the highly significant upregulation of several pro-angiogenic transcripts in response to OPNc overexpression, we attempted to validate data obtained by using this array by investigating the effect of OPNc-CM on activating angiogenic properties. Our data clearly demonstrated that OPNc-CM activated different steps involved in the angiogenic process, such as endothelial cell proliferation, adhesion and migration. In the light of data previously published by our group and those presented here, we partially validated that OvCar-3 and PC-3 cells OPNc-overexpressing cells secrete specific proteins that create a permissive environment, which favors their own angiogenesis induction.

The multifunctional role of OPN in cancer progression and metastasis is notably exerted through its interaction with integrins and CD44 cell-surface receptors. Current data suggest that OPN splice variants have different tumor and tissue roles, and also specifically activated signaling pathways. While in some tumor models an individual OPN-SI behaves as a pro-tumorigenic factor, in other tumor contexts the same variant has the opposite effect, acting individually or in concert with the remaining isoforms to modulate tumor progression (6,13,36,65). In the present report, we again demonstrated the specific roles of OPNc in mediating OC and PCa angiogenic signaling pathways.

Regarding the HUVEC adhesion properties, we demonstrated that different cell receptors mediate HUVEC binding to OPNc-CM secreted either from OvCar-3 or PC-3 cells. In the OvCar-3 cell model, we found that putative pro-angiogenic factors present in OPNc-CM interact with integrin alphaV-β3. Conversely, in the PCa model, this interaction is mediated by CD44 receptors. These data comprise the first report about receptors that are able to mediate endothelial cell adhesion to OPNc-CM. These results suggest that OPNc promotes tumor angiogenesis in both ovarian and prostate cancer through different signaling pathways and the interaction of receptors, as showed for interleukin-8 (IL-8) (66,67). The proangiogenic activity of IL-8 from glioma cells occurs predominantly following binding to CXCR2, and from melanoma cells to CXCR1. Moreover, to date, only one published report has investigated the roles of different OPN-SI (12). These authors showed that in non–small cell lung cancer cells, OPNa overexpression significantly increased tubule length formation. In this same tumor model, OPNb had a similar, but less-pronounced effect, while OPNc significantly decreased tubule length, compared with controls in each cell line. Additionally, OPNa overexpression was associated with significant increases in VEGF secretion, whereas OPNb had no effect, and OPNc upregulation was associated with VEGF downregulation (12). In the light of literature reports and data previously published by our group and presented here, we suggest that OPNc modulates angiogenic processes in a tumor- and tissue-specific context.

Invasion and metastasis

Invasion and metastasis require the destruction of the extracellular matrix and basement membranes to facilitate growth or migration of tumor cells into vascular and lymphatic spaces. Cell migration, a component of cellular invasion, contributes to several important steps in tumor progression. The ability to form colonies in semisolid medium correlates well with tumor malignancy and metastasis formation. Previously, we showed that OPNc overexpression stimulated OvCar-3 and PC-3 migration, invasion and colonies formed in semisolid medium.

In addition, OvCar-3 and PC-3 cells overexpressing OPNc resulted in extremely rapid tumor growth *in vivo*. In these tumors, well-known markers of tumor progression such as *Mmp2* and *Mmp9* were consistently upregulated (6,8).

Transcriptional levels of pro-metastatic genes such as *Mmp1* and *Serpine1* in OC and PCa; *Mta1, Mta2* and *Mmp2,* in OC; and *Plau* and *Mmp9,* in PCa were significantly modulated in OPNc-overexpressing cells.

Overexpression of *Mmp1* in PCa cells increase cell invasion, migration, prostate-tumor growth, and the incidence of lung metastasis (68). However, our work provides the first indication that OPNc might downregulate *Mmp1* in OC. Elevated tumor *Serpine1* level is associated with a poor prognosis and reduced disease-free survival in patients with ovarian and prostate carcinomas (69,70). This SERPIN maintains an angiogenic 'scaffold', stabilizes nascent capillary structure, and facilitates tumor stromal penetration through precise control of the proteolytic microenvironment, suggesting that it has an important function in tumor dissemination (69).

Some published reports have shown that *Mta1* expression was significantly enhanced in tissue from patients in advanced stages of OC, indicating an important role of *Mta1* in the progression of OC. *In vitro*, overexpression of exogenous *Mta1* in OvCar-3 cells enhanced the ability of anchorage-independent growth in semisolid medium. Additionally, *Mta2* overexpression was associated with more aggressive behaviors of epithelial ovarian cancers (71).

Here, we report that OPNc-overexpressing cells stimulate *Mmp2* overexpression in OC and *Mmp9* in PCa. Schmalfeldt et al., (2001) (72) showed that protein expression of pro-MMP-2 was low in benign ovarian tumors, but increased significantly from low-malignant potential tumors to advanced OC. Matrix metallaprotinase-9 (MMP-9) is zinc-containing proteinase that plays crucial roles in prostate tumor invasion and metastasis (73). High plasma or serum levels of uPA correlate with the PCa tumor progression, in particular as a marker of a poor prognosis, suggesting that the uPA/uPAR axis is a PCa therapeutic target (74).

The ability of OPNc to upregulate all these genes, as shown, may therefore reveal a novel mechanism by which ovarian and prostate-cancer cells may regulate the expression of these genes and enhance their invasive and metastatic potential.

Conclusion:

Specifically, we have shown here that an ovarian and prostate cancer cell lines that constitutively overexpress OPNc have a significantly altered gene expression profile, which reflects the main acquired capabilities of cancer. Figures 3 and 4 show a summary of an interaction network for genes induced by OPNc overexpression in OC and PCa, respectively. We have also shown, for the first time, the molecular mechanisms of OPNc-induced pro-angiogenic expression factors and their potential role in regulating angiogenesis in OC and PCa tumor models. Taken together, these data not only support our previously characterized OPNc pro-tumorigenic cellular functions, but also suggest the existence of novel potential functions and pathways by which OPNc may contribute to OC and PCa tumor progression and malignant growth. The use of the hallmarks of cancer as a method of functional classification of regulated genes has led to a more global mechanistic view of how OPNc may influence tumor-cell behavior to promote ovarian and prostate tumor progression and metastasis. Further work is required to functionally validate the cancer molecular mechanisms stimulated by OPNc in OC and PCa cells. Finally, given the diversity of genes for which OPNc is able to regulate expression, it is possible that OPNc signaling may be a key regulatory circuit which dictates cellular physiology during OC and PCa tumor progression. This study provides a framework for the identification of key contributors to malignancy, and may lead to new insights useful in the development of therapeutic interventions for cancer treatment and prevention.

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Table I. OPNc-modulated genes in OvCar-3 cells.

			Ovarian Carcinoma	
Function	**Refseq**	**Gene name**	**Fold change (OPNc X empty vector)**	**p value**
Cell Cycle Control and DNA Damage Repair				
	NM_000051	*Atm*	-4,72	0,001434
	NM_000321	*Rb1*	+1,8	0,024487
	NM_001798	*Cdk2*	+1,85	0,034487
	NM_000389	*Cdkn1a*	+2,07	0,019299
	NM_001238	*Ccne1*	+2,3	0,003111
	NM_002961	*S100a4*	+2,66	0,011249
	NM_001789	*Cdc25a*	+2,8	0,000906
Apoptosis and Cell Senescence				
	NM_138578	*Bcl2l1*	+2,17	0,020561
	NM_004322	*Bad*	+2,44	0,003939
	NM_001228	*Casp8*	+2,86	0,022621
	NM_001160	*Apaf1*	+3,84	0,013762
Signal Transduction Molecules and Transcription Factors				
	NM_005252	*Fos*	-2,12	0,001039
	NM_002467	*Myc*	-1,82	0,008713
	NM_181504	*Pik3r1*	+1,65	0,003498
	NM_002880	*Raf1*	+1,73	0,008429
	NM_004448	*Erbb2*	+2,46	0,00272
	NM_005163	*Akt1*	+2,56	0,002432
Adhesion				
	NM_002204	*Itga3*	+1,74	0,046677
	NM_002687	*Pnn*	+1,85	0,028181
	NM_002210	*Itgav*	+1,91	0,002056
	NM_002213	*Itgb5*	+2,85	0,007166
	NM_002203	*Itga2*	+3,44	0,000838
	NM_000212	*Itgb3*	+5,15	0,047081
Angiogenesis				
	NM_017549	*Epdr1*	+1,5	0,008205
	NM_002607	*Pdgfa*	+1,54	0,022463
	NM_004612	*Tgfbr1*	+1,57	0,009081
	NM_000594	*Tnf*	+1,83	0,032322
	NM_000141	*Fgfr2*	+2,34	0,001965
	NM_003376	*Vegfa*	+51,53	0,005972
Invasion and Metastasis				
	NM_002421	*Mmp1*	-2,18	0,045249
	NM_004689	*Mta1*	+1,56	0,021296
	NM_004739	*Mta2*	+2,33	0,038178
	NM_000602	*Serpine1*	+6,44	0,010252
	NM_004530	*Mmp2*	+611,98	0,001808

Multiple genes related to cell apoptosis, cell cycle, angiogenesis, invasion and metastasis were evaluated for expression using RT2 Profiler PCR Array system. This table list indicate genes that present significant delta CT ($p < 0.05$) values and genes with at least 1.5-fold change in gene expression levels in OPNc-overexpressing cells relative to EV OvCar-3 transfected cells. Positive values indicate up-regulation of individual genes; negative values indicate down-regulation. RefSeq is a stable reference for genome annotation. The data were evaluated by two-tailed Student's t test.

Table II. OPNc-modulated genes in PC-3 cells.

			Prostate Carcinoma	
Function	**Refseq**	**Gene name**	**Fold change (OPNc X empty vector)**	**p value**
Cell Cycle Control and DNA Damage Repair				
	NM_005225	*E2f1*	+2,04	0,034428
	NM_007294	*Brca1*	+2,38	0,042483
Apoptosis and Cell Senescence				
	NM_006410	*Htatip2*	-5,77	0,00973
	NM_004322	*Bad*	+2,03	0,030344
	NM_138578	*Bcl2l1*	+2,5	0,043878
	NM_198253	*Tert*	+15,65	0,046841
Signal Transduction Molecules and Transcription Factors				
	NM_005252	*Fos*	-5,76	0,029691
	NM_005239	*Ets2*	+2,21	0,009982
Adhesion				
	NM_000212	*Itgb3*	+4,86	0,015519
	NM_002210	*Itgav*	+8,7	0,019915
Angiogenesis				
	NM_001146	*Angpt1*	+12,25	0,000213
	NM_003376	*Vegfa*	+21,43	0,000005
Invasion and Metastasis				
	NM_002658	*Plau*	+7,63	0,000021
	NM_000602	*Serpine1*	+9,9	0,000601
	NM_004994	*Mmp9*	+12,62	0,004373
	NM_002421	*Mmp1*	+19,45	0,00162

Multiple genes related to cell apoptosis, cell cycle, angiogenesis, invasion and metastasis were evaluated for expression using RT2 Profiler PCR Array system. This table list indicate genes that present significant delta CT ($p < 0.05$) values and genes with at least 1.5-fold change in gene expression levels in OPNc-overexpressing cells relative to EV PC-3 transfected cells. Positive values indicate up-regulation of individual genes; negative values indicate down-regulation. RefSeq is a stable reference for genome annotation. The data were evaluated by two-tailed Student's t test.

Table III. OPNc-commonly modulated genes in both OvCar-3 and PC-3 carcinoma models.

			Ovarian Carcinoma		Prostate Carcinoma	
Function	**Refseq**	**Gene Name**	**Fold change (OPNc X empty vector)**	**p value**	**Fold change (OPNc X empty vector)**	**p value**
Apoptosis and Cell Senescence						
	NM_138578	*Bcl2l1*	+2,17	0,020561	+2,5	0,043878
	NM_004322	*Bad*	+2,44	0,003939	+2,03	0,030344
Signal Transduction Molecules and Transcription Factors						
	NM_005252	*Fos*	-2,12	0,001039	-5,76	0,029691
Adhesion						
	NM_002210	*Itgav*	+1,91	0,002056	+8,7	0,019915
	NM_000212	*Itgb3*	+5,15	0,047081	+4,86	0,015519
Angiogenesis						
	NM_003376	*Vegfa*	+51,53	0,005972	+21,43	0,000005
Invasion and Metastasis						
	NM_000602	*Serpine1*	+6,44	0,010252	+9,9	0,000601

Multiple genes related to cell apoptosis, cell cycle, angiogenesis, invasion and metastasis were evaluated for expression using RT2 Profiler PCR Array system. This table list indicate genes that present significant delta CT ($p < 0.05$) values and genes with at least 1.5-fold change in gene expression levels in OPNc-overexpressing cells relative to EV OvCar-3 and PC-3 transfected cells. Positive values indicate up-regulation of individual genes; negative values indicate down-regulation. RefSeq is a stable reference for genome annotation. The data were evaluated by two-tailed Student's t test.

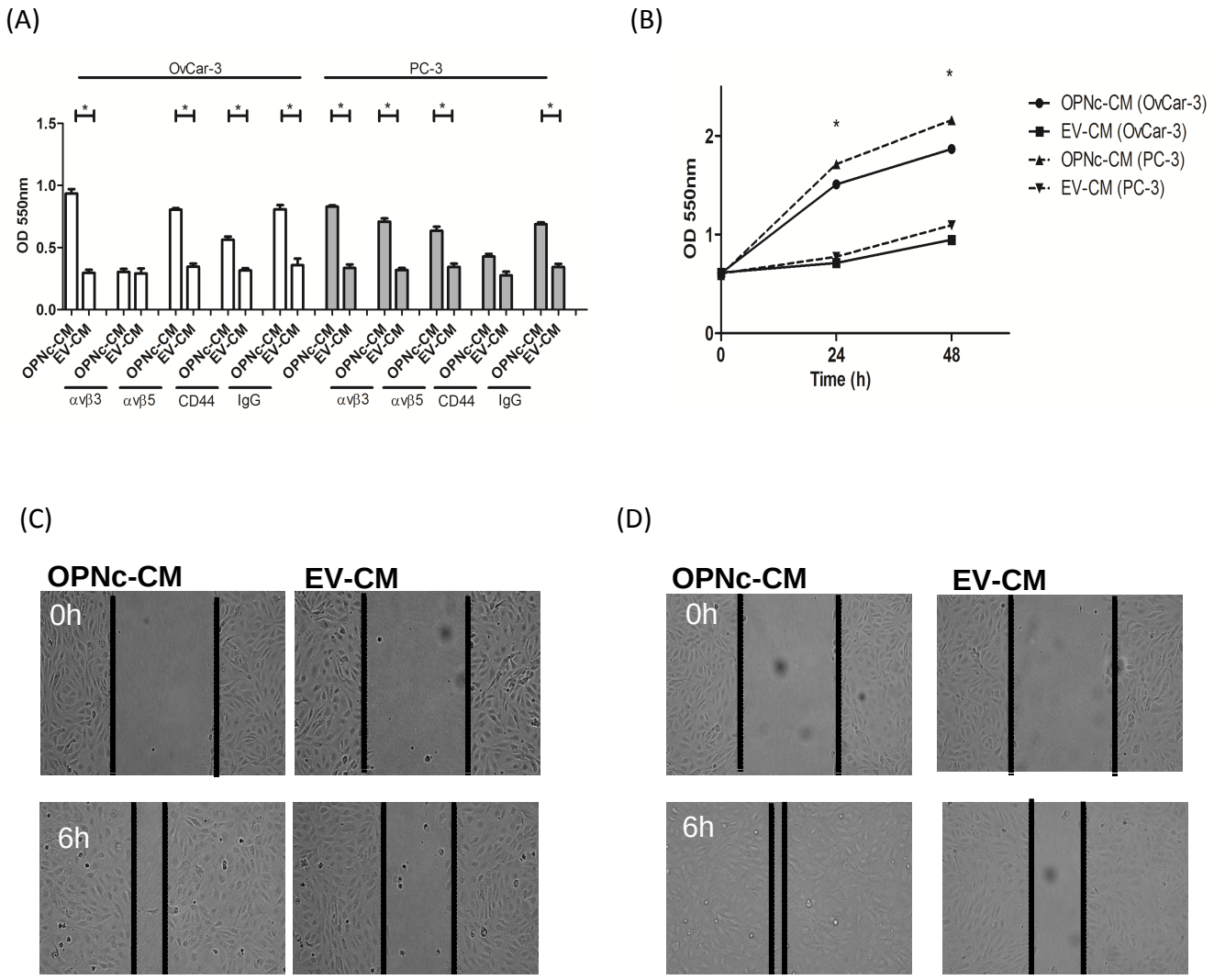

Figure 1. OPNc-CM from OvCar-3 and PC-3 cells induces adhesion, proliferation and migration of HUVEC cells. (A) Endothelial cells were plated onto OPNc and EV-conditioned medium to evaluate cell adhesion (CM). Cells were allowed to adhere for 2 hours at 37°C and were quantified as described in "Adhesion assays". For blocking assays, HUVECs cells were incubated for 1 hour in the presence of antibody anti-αvβ3, anti-αvβ5 e anti-CD44 (10μg/ml). Error bars represent the means ± SD of 3 independent experiments. O.D., optical density measured at 550 nm * $p < 0.0001$. (B) HUVECs were cultured with OPNc and EV-CM and proliferation was followed for 24 and 48 hours. Proliferation kinetics analysis was evaluated by crystal violet staining. Error bars represent the means ± SD of 3 independent experiments. * $p < 0.005$. O.D., optical density measured at 550 nm. (C) OPNc-CM activates HUVEC cell migration. HUVEC cells were plated as indicated in "Migration assays" and analyzed for cell migration by wound closure assay. Phase-contrast micrographs photographs were taken at 0 and 6 hours after subjected to migration are shown. For each conditioned medium, 3 plates were used per experiment.

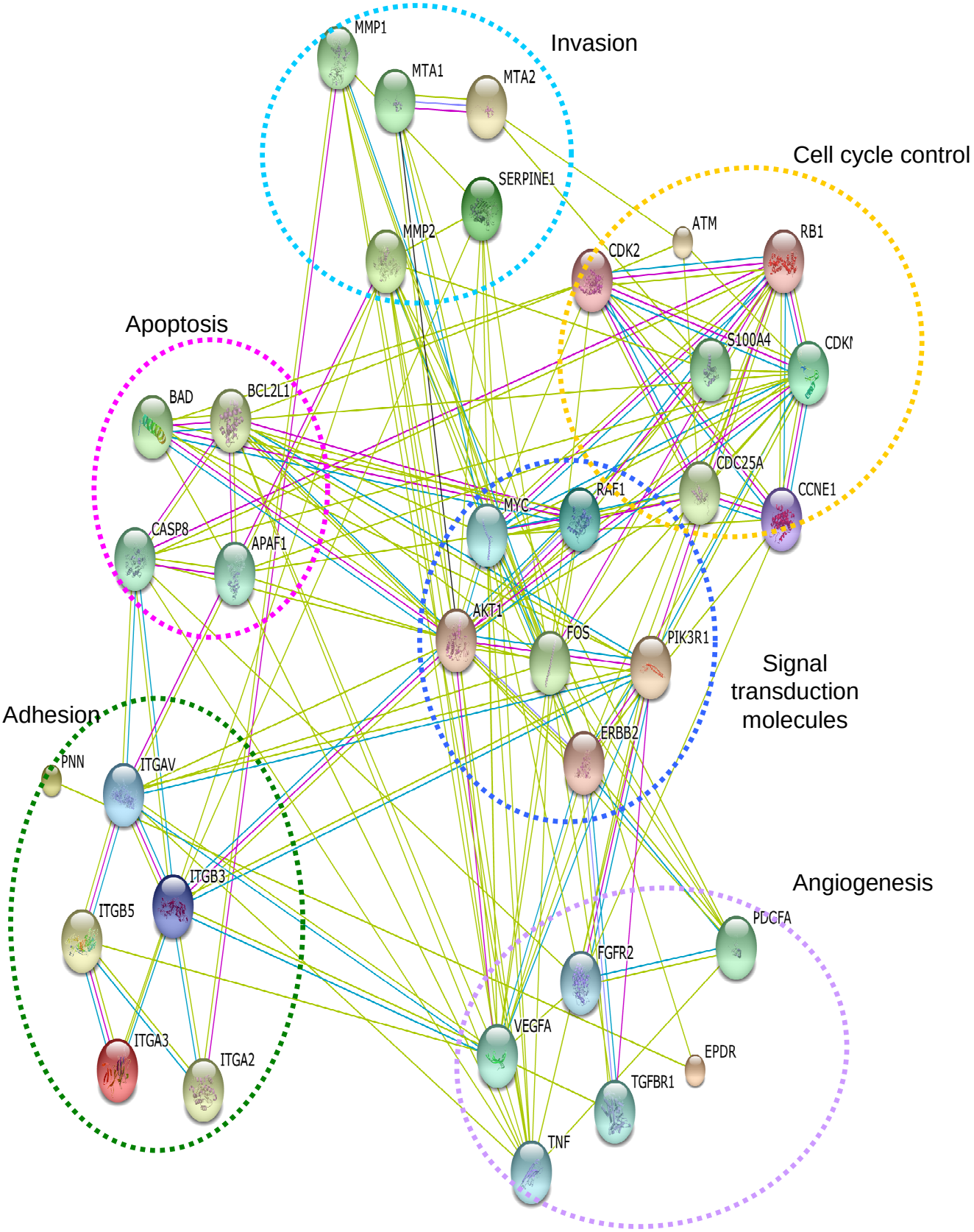

Figure 2. OPNc-overexpression induced main acquired capabilities of cancer interactome in ovarian carcinoma. Circuit representation of an interactome network. To model an interaction network STRING 9.0 software was used, where a node represents a protein and a line represents an interaction.

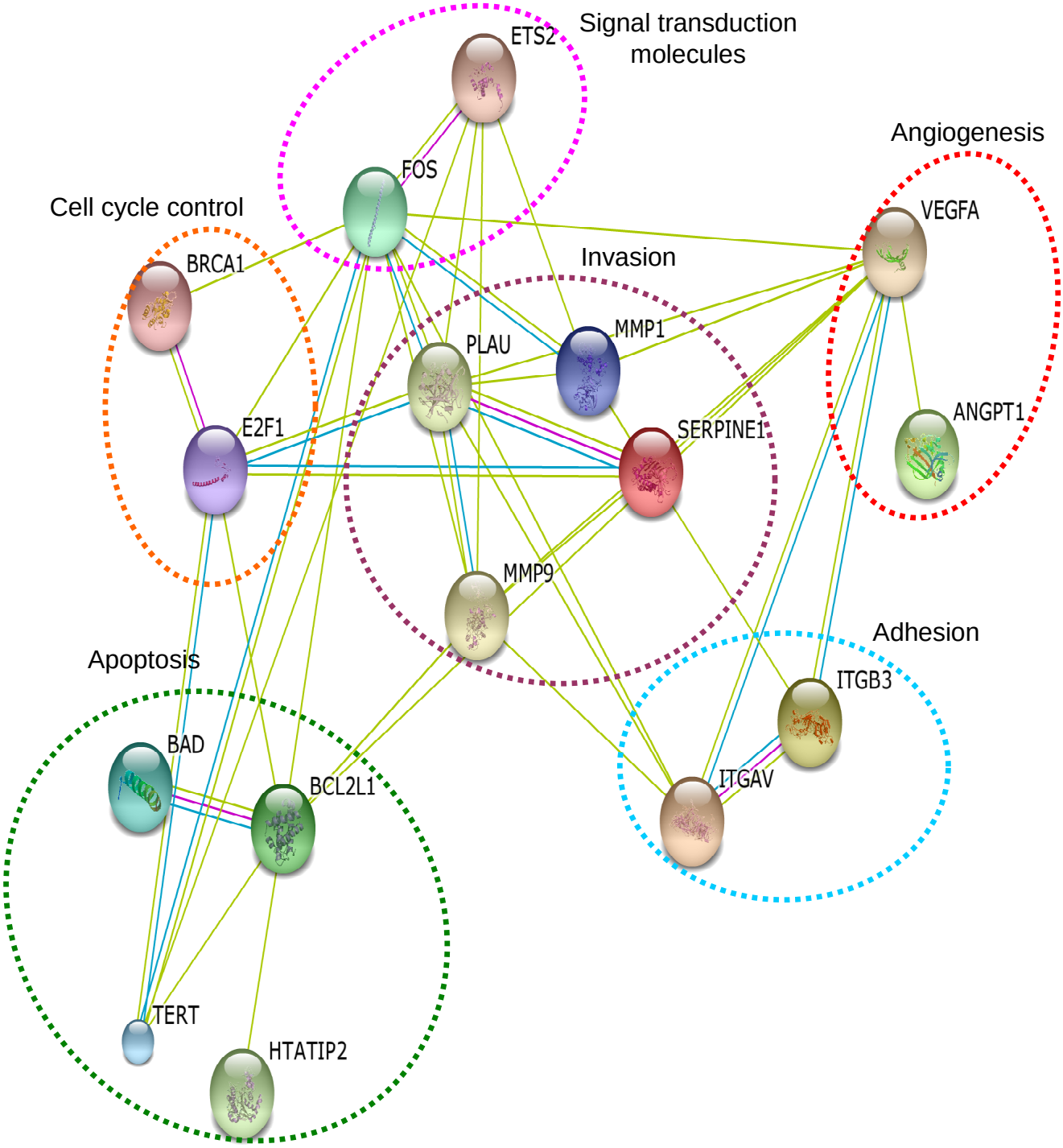

Figure 3. OPNc-overexpression induced main acquired capabilities of cancer interactome in prostate carcinoma. Circuit representation of an interactome network. To model an interaction network STRING 9.0 software was used, where a node represents a protein and a line represents an interaction.

8. DISCUSSÃO

Nosso estudo consiste na primeira descrição do papel funcional e de vias de sinalização capazes de mediar as ações das isoformas da OPN nos modelos de CO e CaP. Adicionalmente, este estudo, diferentemente dos demais, envolve uma investigação mais abrangente, tanto *in vitro* quanto *in vivo,* de distintas etapas e mecanismos moleculares envolvidos nas características relacionadas à progressão de células tumoriais de CO e CaP promovidas pelas isoformas da OPN.

O primeiro passo na caracterização das isoformas da OPN e de suas potenciais funções nestas neoplasias foi avaliar o perfil de expressão destas isoformas nestes tumores. Nas amostras de ovário, nossos resultados demonstram que a OPNc encontra-se expressa somente nas amostras de carcinoma de ovário e tumor '*borderline*'. Estes resultados indicaram que a OPNc é encontra-se especificamente expressa nas amostras tumorais de ovário, assim com descrito para o tumor de mama (Mirza *et al.,* 2008; He *et al*., 2006). Entretanto, não foi possível neste estudo correlacionar o perfil de expressão destas isoformas com os dados clínicos das pacientes, devido ao pequeno número de amostras de tumores ovarianos, especialmente considerando a categorização dos subtipos histológicos. Nas amostras de próstata, observamos que as isoformas da OPN encontram-se superexpressas nas amostras teciduais de CaP em comparação com o tumor benigno da próstata (HPB). Nesse trabalho mostramos que a avaliação da expressão tecidual das isoformas da OPN é mais sensível e específica do que o tPSA para o diagnóstico do CaP. Demonstramos também que o nível de expressão das isoformas é mais eficiente que a avaliação da concentração de tPSA em distinguir os pacientes com CaP em relação àqueles com HPB. Dentre as isoformas da OPN, a OPNc apresenta os mais altos níveis de sensibilidade (100%), especificidade (90%), valor preditivo positivo (88%) e negativo (100%) no diagnóstico do CaP. Adicionalmente, o nível de expressão das isoformas da OPN se correlaciona positivamente com o Escore de Gleason, indicando a potencial utilização destes biomarcadores também na avaliação prognóstica de pacientes com CaP. Este estudo, diferentemente dos demais, envolve a investigação do potencial uso das isoformas de '*splicing*' da OPN como

biomarcadores, sendo nosso estudo em CaP o primeiro a fazer estas investigações. Nossos resultados indicam a potencial inclusão da OPNc em um painel de biomarcadores teciduais de forma a aumentar a sensibilidade e especificidade do diagnóstico dos tumores de próstata. Contudo, existe a necessidade de validação desses resultados em um maior número de amostras, incluindo amostras de variadas etnias. Em contraste aos dados encontrados em nosso estudo, no qual observamos modulação da expressão da OPNc nas amostras tumorais malignas de CO e de CaP, a OPNc apresenta baixos níveis de expressão em mesotelioma e tumores de pulmão, enquanto a OPNa e OPNb encontram-se superexpressas nestes modelos (Ivanov *et al*., 2009; Blasberg *et al*., 2009). Nossos dados constituem-se em evidências adicionais de que o perfil de expressão das isoformas da OPN é tumor e tecido específico (Tilli *et al*., 2011; Tilli *et al*., 2012a).

A expressão diferencial destas isoformas justifica a avaliação funcional das mesmas e a investigação de suas funções na progressão destes tumores. Escolhemos no presente estudo a estratégia de superexpressão ectópica das isoformas da OPN em linhagens celulares tumorais bem estabelecidas como modelos *in vitro* e *in vivo* de estudos funcionais do Co e CaP (Luo *et al*., 2009; Khodavirdi *et al*., 2006).

No modelo de CO, observamos que a linhagem tumoral OvCar-3 que superexpressa a OPNc ativa diversas características relacionadas à progressão tumoral e tumorigênese. Mostramos que a OPNc promove aumento da proliferação, migração, invasão celular, formação de colônias, expressão de *Vegf*, *Mmp-2* e *Mmp-9* e ainda, formação de tumores em camundongos atímicos, o mesmo não tendo sido observado para as isoformas OPNa e OPNb. Já no modelo de CaP, observamos que a linhagem tumoral PC-3 que superexpressa tanto a OPNb quanto a OPNc promovem o aumento destas características relacionadas à progressão tumoral testadas no presente estudo.

Dados da literatura sobre o papel funcional da OPNb e OPNc em outros modelos tumorais mostram que os efeitos das isoformas da OPN são distintos e tecido específicos. No câncer de mama, a OPNc não altera a proliferação celular, mas estimula o crescimento independente de ancoragem (He *et al*., 2006). Em contraste, no mesotelioma e no câncer de pulmão (HNSCC), a OPNa e a OPNb, mas não a OPNc, são capazes de estimular efeitos pró-

tumorigênicos (Mirza *et al.,* 2008; He *et al*., 2006; Ivanov *et al*., 2009; Blasberg *et al*., 2009; Courter *et al.,* 2010). No câncer de pulmão, a superexpressão da OPNc está associada com a diminuição da angiogênese, enquanto que a OPNa e OPNb apresentam papéis funcionais opostos (Blasberg *et al*., 2009).

Apesar de ter sido demonstrado o papel funcional destas isoformas em distintos modelos tumorais (Mirza *et al*., 2008; He *et al*., 2006; Ivanov *et al*., 2009; Blasberg *et al.,* 2009; Couter *et al*., 2010; Tilli *et al*., 2011), poucos destes estudos caracterizam as vias envolvidas nas ações das OPNs (Chae S *et al*., 2009; Courter *et al*., 2010). Também não foi ainda descrito como estas isoformas são capazes de mediar os efeitos pró-tumorigênicos em cada modelo tumoral. Especial destaque tem sido dado à necessidade de caracterização dos éxons 4 e 5 na função da proteína OPN e das consequências de sua deleção na função que exercem nos distintos tumores. Neste sentido, torna-se fundamental também a caracterização dos receptores celulares e vias de sinalização capazes de mediar a função destas isoformas.

Dados previamente publicados indicam que diferentes isoformas de uma mesma proteína podem apresentar características funcionais distintas e que essas características são mediadas por alterações nas modificações pós-tradução (PTMs) (Meng *et al.,* 2003; Wu *et al.*, 2005). O proto-oncogene HMGA1 apresenta duas isoformas variantes de '*splicing*', HMGA1a e HMGA1b. Estas isoformas quando superexpressas causam transformação celular. Entretanto, apresentam papéis funcionais distintos no contexto tumoral, especialmente devido às diferenças quanto à perda de sítios de fosforilação (Edberg *et al.*, 2005). Outro exemplo da literatura consiste nas variantes de '*splicing*' da caspase-9, caspase-9a e caspase-9b. No tumor de pulmão, a caspase-9a é pró-apoptótica, enquanto que a caspase-9b é anti-apoptótica. A ativação ou inibição da via de sobrevivência celular é mediada pela razão entre a expressão da caspase-9a/caspase-9b. Goehe *et al*. em 2010, caracterizaram a presença ou ausência da fosforilação da Ser^{52} como o regulador dessa razão de expressão entre as caspase-9a/caspase-9b. Diversos trabalhos revelam diferentes tipos de PTMs na proteína c-Myc, incluindo a fosforilação, ubiquitinação, O-glicosilação, e acetilação. A fosforilação está correlacionada com a regulação das atividades biológicas mediadas por c-Myc. Estudos *in vitro* mostram que mutações específicas em sítios de fosforilação na região N-

terminal estão relacionadas com a capacidade de induzir a transformação celular (Hann, 2006). Estes trabalhos indicam que as PTMs apresentam um importante papel funcional e biológico na modulação da função destas proteínas. A OPN apresenta múltiplas funções biológicas bem descritas e caracterizadas, especialmente como citocina inflamatória, na calcificação e remodelamento de tecidos mineralizados, na adesão celular, na angiogênese e carcinogênese (Beausoleil *et al*., 2011; Weber *et al*., 2010). Recentes trabalhos revelam que as PTMs da OPN são célula-específicas, refletindo as diversas funções desta proteína em diferentes sistemas fisiológicos (Anborgh *et al*., 2010; Christensen *et al.,* 2010; Hunter *et al*., 1994). Adicionalmente, as funções da OPN refletem a habilidade desta proteína interagir com os receptores. A interação da OPN com as integrinas depende não somente da sequência RGD, mas também de sítios específicos de fosforilação da OPN (Weber *et al.,* 2002). Sorensen *et al.,* (2007) demonstraram que as funções fisiológicas da OPN são dependentes das PTMs e que a interação da OPN com as integrinas é dependente destas modificações pós tradução. A OPN pode estimular a reabsorção óssea somente se estiver fosforilada (Sorensen *et al*., 2005). Neste contexto, levantamos a hipótese que a deleção da sequência do éxon 4 e 5 da OPN poderia gerar perda de sítios de PTMs, e ainda, gerar a aproximação destes sítios. Análises preliminares *in silico* realizadas por nosso grupo demonstram que estes éxons contêm alguns potenciais sítios de fosforilação e também foram observados sítios de fosforilação adjacentes a estes éxons. Uma vez deletados estes éxons, é possível que haja uma modificação neste padrão e/ou espaçamento entre os sítios de PTMs, potencialmente afetando a função da OPN, conforme observado em outros modelos (Chen-Izu *et al*., 2007). Como consequência, estas deleções poderiam alterar a estrutura protéica das isoformas da OPN, suas interações com diferentes tipos de receptores ou a afinidade com os mesmos, e ainda, a interação com parceiros protéicos.

A OPN apresenta regiões intrínsecas desordenadas, por esta razão essa proteína assume sua estrutura conformacional de forma dependente da interação com o receptor (Yamaguchi *et al.,* 2010; Kazanecki *et al.*, 2007). Yamaguchi *et al*. (2010), demonstraram que a OPN murina apresenta interações intramoleculares dependente de sequências específicas na

molécula, as quais ainda não foram caracterizadas. Os autores demonstraram que a interação intramolecular da OPN influencia diretamente a interação com os receptores, especialmente as integrinas. Estes dados reforçam nossa hipótese de que a deleção dos éxons 4 e 5 potencialmente alterariam a interação com os receptores e a estrutura protéica das isoformas da OPN. Estudos futuros de avaliação da estrutura da OPN completa e de suas isoformas poderiam decifrar em parte estes questionamentos.

A progressão tumoral é caracterizada pela habilidade da célula em superar o controle do microambiente sobre a proliferação celular, inibição da morte celular por apoptose, migração, invasão tecidual, de capilares e vasos linfáticos e invasão e proliferação no sítio tumoral secundário. Estes eventos são denominados de '*Hallmarks of Cancer*', descritos e caracterizados por Weinberg & Hanahan, 2011.

A proliferação celular consiste em um dos eventos associados com a progressão tumoral. A via de sinalização PI3K/Akt é uma das principais vias que controlam o crescimento/sobrevivência celular. Desde a descoberta dos papéis funcionais da via de sinalização PI3K/Akt, numerosos membros desta via foram caracterizados apresentando função aberrante ou silenciados em neoplasias, incluindo o CO e CaP (Carracedo *et al.*, 2008; Song *et al.,* 2008; Hua *et al*., 2008; Kosaka *et al.,* 2011). Como consequência das alterações nesta via de sinalização, durante a progressão tumoral, a célula comporta-se de maneira independente do ambiente externo. Observamos que a OPNc, no modelo de ovário e próstata, e a OPNb, no modelo de próstata, são capazes de estimular a proliferação celular da OvCar-3 e PC-3 na presença e ausência de fatores de crescimento, que são características típicas de proteínas envolvidas na progressão tumoral (Hanahan *et al*., 2000). A capacidade estimulatória da autonomia de fatores de crescimento exercida por estas isoformas na proliferação celular poderia ser mediada pela alteração dos sinais de crescimento extracelulares, nos receptores destes sinais ou ainda alteração nas vias de sinalização (Lee *et al*., 2007). Dados na literatura indicam que a OPN altera a expressão de genes relacionados com múltiplos aspectos da progressão tumoral, incluindo os genes envolvidos na auto-suficiência aos fatores de crescimento (Cook *et al*., 2005; Johnston *et al*., 2008). Como demonstrado no presente trabalho, a superexpressão da OPNb e OPNc

estimulam a via de sinalização PI3K/Akt nos modelos de CO e CaP. A ativação desta via de sinalização poderia ser um dos mecanismos moleculares pelo qual estas isoformas estimulam a proliferação celular e a proliferação na ausência de fatores de crescimento. Inge *et al*. (2011), demonstraram que a via de sinalização PI3K/Akt medeia o efeito da E-caderina em pacientes com câncer, mesmo em condições limitantes de fatores de crescimento. Courter *et al*. (2010), demonstraram que a OPN é capaz de prevenir a morte celular em resposta ao stress, incluindo a ausência de fatores de crescimento. Além de nosso trabalho, apenas os dados de Courter *et al*., (2010), estudando os tumores de pâncreas e fibrosarcoma, determinaram o envolvimento da via de FAK e NF-kB mediada pelas isoformas a e b da OPN. No capítulo V dessa tese, identificamos uma série de genes relacionados com as vias de proliferação celular que são regulados pela superexpressão da OPNc em células OvCar-3 e PC-3. No modelo do CO, identificamos a diminuição de expressão do mRNA do *Atm* (-4.72), e por outro lado superexpressão de *Rb1* (+1.8), *Cdk2* (+1.85), *Cdkn1a* (+2.07), *Ccne1* (+2.3), *S100a4* (+2.66), *Cdc25a* (+2.8) e a via de sinalização *Raf1* (+1.73). Por outro lado, no modelo de CaP, identificamos a superexpressão de *E2f1* (+2.04) e *Brca1* (+2.38). A regulação de expressão destes genes encontra-se descrita na literatura nesses modelos tumorais. Contudo, no presente trabalho, mostramos que essa regulação poderia ser mediada pela OPNc. Se por um lado a OPNc regula a expressão de genes envolvidos no processo de proliferação celular, essa isoforma também regula a expressão de genes envolvidas na apoptose. O mRNA de *Bcl2l1* e *Bad*, clássicos inibidores da apoptose, encontram-se superexpressos nas células OvCar-3 e PC-3 transfectadas com a OPNc. Adicionalmente, identificamos em CO superexpressão de caspase-8 e *Apaf1.* No modelo de CaP, identificamos a superexpressão de *Tert* e inibição de expressão de *Htatip2*. Estes achados resultam em uma melhor compreensão das vias de sinalização que são ativadas mediante a ação da OPNc nos modelos de progressão celular de CO e CaP. Estes achados são de especial importância para o futuro delineamento de potenciais alvos terapêuticos para estas neoplasias, já que correspondem às vias que estimulam de fato as características celulares relacionadas à progressão destes tumores.

A metástase é um processo complexo, constituído de variadas etapas, e que resulta das interações entre as células tumorais e o microambiente tecidual onde estas células se encontram. Durante a disseminação do tumor, as células tumorais devem ser capazes de se soltar do tumor primário (perda de interação célula-célula) e escapar do tecido de origem. Precisam também invadir a matriz extracelular, migrar ativamente pelo estroma intersticial, induzir a formação de novos vasos sanguíneos e/ou linfáticos (angiogênese), essenciais para a expansão da massa tumoral. Por estes mesmos vasos, as células podem alcançar a corrente sanguínea ou linfática, após atravessar a membrana basal e o endotélio dos vasos, sobreviver na circulação, interagir com o endotélio vascular, extravassar e ainda, proliferar no parênquima do órgão-alvo (Anisimov *et al*., 2009; Laconi *et al.,* 2008; Fedarko *et al*., 2008). Observamos nos ensaios de '*wound healing*', que as células que superexpressam a OPNc, nos modelo de ovário e próstata, e a OPNb, no modelo de CaP, apresentaram altas taxas de migração celular, demonstrando que estas isoformas são importantes na migração, um dos eventos inicias da invasão tumoral. A resposta inicial da célula à migração é a polarização celular e emissão de protusões. Sabe-se que no processo de migração celular, ocorre ativação da proteína Cdc42 e de membros da família de Rho GTPases, importantes reguladores da polarização e emissão de protusões, respectivamente (Mack *et al.,* 2010; Baker *et al*., 2009; Friedl *et al*., 2003). Embora diversos receptores celulares estejam relacionados com o processo de migração celular, as integrinas são os principais receptores associados com este evento (Schneider *et al.,* 2010; Baker *et al*., 2009; Hood *et al.,* 2003; Schwartz *et al*., 2006). Estes receptores atuam na adesão da célula à matriz extracelular ou a células vizinhas, facilitando a emissão das protusões celulares. As integrinas ativam as principais vias de sinalização intracelular culminando com a ativação da migração celular (Baker *et al*., 2009; Schneider *et al*., 2010). Sabe-se que a OPN interage com diferentes integrinas, incluindo as formas αvβ3, αvβ1, αvβ5, α4β1, α9β1 e α8β1 (Fedarko *et al.,* 2008). Estas interações não dependem somente da sequência RGD, mas também de alguns sítios específicos de fosforilação da OPN (He *et al.,* 2006). Através de ensaio de PCR em tempo real, identificamos que a superexpressão da OPNc pode modular a expressão das integrinas nas células OvCar-3 e PC-3. Em ambos modelos tumorais

observamos superexpressão do mRNA das subunidades αv e β3; e no modelo de CO, α3, α2 e β5. Com base nestes dados, é provável que o papel funcional diferenciado da OPNb e OPNc, não somente em relação à migração celular, mas também em relação aos outros eventos celulares analisados, poderia estar associado à interação diferencial destas isoformas com diferentes subunidades das integrinas ou alteração de afinidade com estes receptores, principalmente devido a ausência do éxon 4 e 5, como descrito anteriormente.

Para que haja a migração de células tumorais através da matriz extracelular dos diferentes tecidos, é necessário que haja degradação da mesma. As MMPs medeiam esta degradação durante fases precoces da tumorigênese, contribuindo para a formação do microambiente que promove o crescimento dos tumores (Overall *et al.,* 2006; Egeblad *et al.,* 2002). As MMPs também participam em estágios mais tardios do desenvolvimento do câncer, promovendo o crescimento sustentado tanto de tumores primários como metastáticos na ativação de fatores de crescimento, inativação de proteínas de ligação a fatores de crescimento ou pela liberação de moléculas mitogênicas residentes na matriz (Klein *et al.,* 2009). Observamos que a OPNc, no modelo de CO e CaP, e a OPNb, no modelo de CaP, estimulam a expressão do mRNA *Mmp-2* e *Mmp-9*. A clivagem de laminina-5 por MMP-2 revela um sítio críptico da molécula de laminina que desencadeia a motilidade celular (Egeblad *et al*., 2002). A ativação das MMPs também se relaciona com os mecanismos que as células utilizam para escapar da vigilância imunológica. A proliferação de linfócitos T, que é regulada pela sinalização de citocinas pelo receptor α da interleucina-2, poder ser inibida pela clivagem deste receptor pela MMP-9 (Muller *et al*., 2006). Assim, a ação das isoformas OPNb e OPNc ativando as MMPs nos tumores de CO e CaP contribui para as etapas iniciais e mais tardias da progressão tumoral, cujos mecanismos são destas proteases.

O processo de angiogênese é também crítico para a expansão das células do tumor primário e dos focos metastáticos. Ao longo deste processo, as células tumorais interagem com diversos elementos do hospedeiro, que atuam facilitando o processo de metastatização (Nguyen *et al.,* 2009; Horak *et al.*, 2008). Este processo resulta do balanço entre fatores estimulatórios (VEGF, PDGF, TGF-α e β, FGF-2 e citocinas pró-inflamatórias) e inibitórios da angiogênese (interferons, angiostatina, endostatina, trombospondina e

inibidores de MMPs). Observamos neste estudo que a OPNc, nos dois modelos tumorais, e a OPNb, no modelo de CaP, estimulam também a expressão do VEGF, indicando também a ação destas isoformas na estimulação da formação de novos vasos, essencial na progressão tumoral. Dados da literatura têm demonstrado que as MMPs-2, 9 e 14 regulam diretamente a angiogênese (Geiger *et al.,* 2001). Há evidências também de que a inibição da MMP-2 em células tumorais está associada à angiogênese deficiente (Geiger *et al*., 2001; Egeblad *et al*., 2002; Stafford *et al.*, 2008). Assim, o fato das isoformas OPNb e OPNc ativarem a expressão não somente das MMPs, mas também de VEGF reforçam a ação destas isoformas nestas importantes etapas da progressão tumoral, assim como demonstrado por Belotti *et al*., (2003). Esse estudo consiste na primeira descrição das possíveis vias de sinalização capazes de mediar o efeito da OPNc na indução da expressão de VEGF-A, contribuindo para uma melhor compreensão dos mecanismos moleculares associados com a angiogênese em tumores ovarianos. Identificamos que OPNc ativa a expressão das proteínas que compõem o fator de transcrição AP-1 e do VEGF-A. Dados da literatura indicam que o fator de transcrição AP-1 é capaz de ativar a transcrição do VEGF em células OvCar-3, assim como em outras linhagens tumorais (Hu *et al*., 2001; Swenson *et al*., 2011; Dong *et al.*, 2011). Assim, levantamos a hipótese de que a OPNc modularia a expressão de c-Fos, c-Jun e fosforilação de c-Jun de forma a induzir a expressão de VEGF. Contudo, estudos funcionais devem ser realizados com o objetivo de confirmar se no nosso modelo essa indução de expressão do fator de transcrição AP-1 pela OPNc leva a uma maior atividade do promotor do VEGF.

Por outro lado, nesse estudo avaliamos o efeito parácrino do meio condicionado de células que superexpressam a OPNc em células endoteliais HUVEC. Mostramos que o meio condicionado de células OvCar-3 e PC-3 transfectadas com a OPNc estimula a proliferação, adesão e migração das células endoteliais, indicando o importante papel desta isoforma no envolvimento de etapas da angiogênese. No capítulo V identificamos os prováveis fatores pró-angiogênicos presentes no meio condicionado da OPNc no modelo de CO, *Epdr-1*, *Pdgf-a, Tgfbr1, Tnf, Fgfr-2* e *Vegfa;* já no modelo de CaP, a OPNc regula a expressão de Angiopoietina-1 e *Vegf*. Todos estes fatores encontram-se descritos na literatura com importante função pró-

angiogênica nos tumores de próstata e ovário (Shao *et al*., 2011; Versnel *et al*., 1994; Zhao *et al*., 2009; Leemans *et al*., 2011; Charles *et al*., 2009; Katoh, 2008; Cascone & Heymach, 2012).

À medida que as células tumorais se locomovem pela matriz extracelular distinta daquela encontrada em seu extrato tecidual de origem, os sinais externos de proliferação e sobrevivência vão sendo também progressivamente alterados (Joyce *et al*., 2009). De fato, quando as células epiteliais e endoteliais normais são desalojadas de seu tecido de origem, estas células iniciam a processo de morte celular programada (He *et al.,* 2006). A apoptose, por sua vez, frequentemente envolve a geração de radicais livres (Weber *et al.,* 1995) e o crescimento independente de ancoragem reflete essa resistência à '*anoikis*'. Há evidências de que as integrinas sejam moléculas sinalizadoras do desalojamento (Joyce *et al.,* 2009; Schneider *et al.,* 2010). Conforme observado no presente estudo, a OPNc, no modelo de ovário, aumenta significativamente o número e o tamanho de colônias que cresceram independente de ancoragem. Interessantemente, a OPNb inibe o número de colônias formadas neste modelo tumoral, sugerindo uma potencial regulação negativa desta isoforma na progressão do tumor de ovário e formação de metástase. Recentes trabalhos demonstram que as células tumorais expressam isoformas variantes de '*splicing*' que estão envolvidas na progressão tumoral e em diferentes etapas da formação de metástase. As isoformas variantes de '*splicing*' de HIF1a (Song *et al*., 2008), Syk (Huang *et al*., 2008), CD44 (Gao *et al*., 2004) e S100A4 (Gross *et al.,* 2010) são exemplos de isoformas que apresentam papel pró-metastáticos, enquanto outras atuam como inibidores. No modelo de CaP, a OPNb e OPNc apresentam propriedades relacionadas com o maior crescimento independente de ancoragem, sugerindo a ativação de um maior potencial metastático destas células. Segundo Mirza *et al.* (2006), a OPN induz a expressão de genes de oxido-redutases. Com isso, a indução de genes codificadores de oxido-redutases pela OPN poderia causar um efeito anti-oxidante e anti-'*anoikis*'. Esta propriedade diferencial da OPNb e OPNc no potencial de formação de colônias de crescimento independente de ancoragem, pode estar relacionado com a expressão gênica diferencial induzida por cada uma das isoformas, em especial na indução de genes relacionados aos efeitos anti-oxidante e anti-'*anoikis'*, assim como foi

demonstrado para a OPNc no modelo de câncer de mama (He *et al*., 2006). Nesse estudo identificamos que a regulação de expressão de *Mta1*, no modelo de CO, e *Ets2*, no CaP, pela superexpressão da OPNc poderiam modular o crescimento independente de ancoragem (Ji *et al.*, 2006; Carbone *et al.*, 2004).

Na tentativa de melhor definir o fenótipo tumorigênico das isoformas da OPN, avaliamos a capacidade das isoformas em induzir a formação de tumores em camundongos atímicos. Nos modelos de CO e CaP, os clones que superexpressam a OPNc induziram a formação de tumores significativamente maiores do que aqueles que superexpressam as isoformas da OPNa, OPNb e os controles. No CaP, a OPNb induziu a formação de tumores maiores do que a OPNa e os controles. Os tumores formados pela superexpressão da OPNc, nos dois modelos tumorais, e OPNb, no modelo de CaP, apresentam alta taxa proliferativa como evidenciado pela marcação de Ki-67. Esses resultados corroboram nossos dados *in vitro* que indicam o importante papel funcional desta isoformas no estímulo à proliferação celular. No modelo de CO, os tumores formados pela superexpressão da OPNc são mais vascularizados e superexpressam VEGF e VEGFR-2, indicando o papel importante dessa isoforma na modulação do microambiente pró-angiogênico. De acordo com nossos achados, outras proteínas superexpressas em tumores, como por exemplo, a endotelina-1, é capaz de induzir a expressão do VEGF via a ativação do fator induzido por hipoxia-1 alfa (HIF-1alpha) (Spinella *et al.*, 2002). Esse conjunto de resultados reforçam o importante papel da OPNb e OPNc não somente nas etapas inicias do estabelecimento do tumor, tais como a proliferação, migração e invasão, mas também no crescimento e estabelecimento dos tumores primários e na ativação do potencial metastático dos tumores de ovário e próstata.

A OPN é uma proteína secretada (sOPN) e exerce suas principais funções na matriz extracelular, atuando como fator autócrino e parácrino em relação à interação com os receptores de membrana (Buback *et al*, 2009). Contudo, Shinohara *et al*., (2008) descreveram em células do sistema imune a isoforma intracelular da OPN (iOPN), resultante de tradução alternativa do mRNA da OPN. Os autores descreveram que a iOPN controla a migração celular dos linfócitos. Adicionalmente, a expressão da iOPN foi caracterizada em fibroblastos, queratinócitos e células endoteliais (Mazière *et al*., 2010;

Cantor *et al*., 2009). No presente estudo, através da utilização do meio condicionado das células que superexpressam as isoformas da OPN, nós mostramos que os efeitos celulares na proliferação, migração e invasão celular estão relacionados com as isoformas secretadas da OPN. Além da superexpressão da OPNc secretada estimular a proliferação das células OvCar-3 e PC-3, nós também demonstramos que a forma secretada desta isoforma altera a proliferação da IOSE e RWPE-1. Estes dados indicam que a OPNc secretada não somente estimula a progressão tumoral, mas também contribui com eventos precoces da tumorigênese em CO e CaP. Em contraste, no modelo de CaP, a OPNb não estimula a proliferação celular da linhagem normal imortalizada de próstata. Estes resultados indicam que a OPNb apresenta um importante papel funcional na progressão do tumor de próstata e não nos eventos iniciais da tumorigênese. Segundo os dados gerados neste estudo, a ação da OPNc sobre o estímulo na proliferação celular parece ser mediado majoritariamente pela proteína secretada, pois a depleção da OPNc secretada pela incubação com anticorpo anti-OPNc inibe a proliferação celular estimulada pela superepxressão desta isoforma. Adicionalmente, observamos que as isoformas secretadas da OPNc, nos modelos de ovário e próstata, e a OPNb, no modelo de próstata, também estimulam a migração e invasão celulares. Apesar de nossos dados demonstrarem que grande parte dos efeitos ativadores de distintas etapas da progressão dos tumores de CO e CaP são mediados pela isoformas OPNb (somente no modelo de CaP) e OPNc secretadas, não podemos excluir a possibilidade que uma isoforma intracelular destas isoformas contribua para seus efeitos. Cabe ressaltar que até o presente momento não foi descrito em nenhum tipo tumoral isoformas intracelulares da OPN. Os estudos funcionais previamente publicados que descrevem o papel funcional das isoformas da OPN, não consideram o papel isolado da isoforma sOPN e iOPN, uma vez que os ensaios não são realizados com o meio condicionado (Fedarko *et al*., 2008; Mirza *et al*., 2008; Rayhman *et al*., 2008; Asai-Sato *et al*., 2005; Lee *et al*., 2007). Entretanto, baseado nos nossos resultados, não podemos excluir o potencial papel da iOPN. A iOPN poderia apresentar um efeito aditivo a proteína secretada, e desta forma, contribuir para os efeitos funcionais descritos. Considerando os diferentes efeitos funcionais das isoformas da OPN nos distintos modelos tumorais, nossa

hipótese é que as PTMs em combinação com a clivagem proteolítica (Christensen *et al.,* 2007; Kazanecki *et al*., 2007) e o balanço entre as isoformas secretadas e intracelulares da OPN poderiam modular os efeitos funcionais das isoformas da OPN tecido e tumor específico (Shinohara *et al*., 2008; Cantor *et al.*, 2009). Novos estudos devem esclarecer a presença das isoformas intracelulares da OPN em células tumorais, a regulação, o papel funcional, e especialmente a combinação destas com as isoformas secretadas da OPN.

O mecanismo molecular pelo qual as isoformas da OPN modulam os efeitos funcionais pró-tumorigênicos não está esclarecido e poucos dados na literatura elucidam algumas vias de sinalização. Em uma linhagem celular de carcinoma hepatocelular, a OPNa e OPNb estimulam a migração celular pelo aumento da expressão do fator ativador do plasminogênio do tipo uroquinase e pela fosforilação de p42/p44 MAP cinase, mas estas vias não são ativadas pela OPNc (Chae *et al*., 2009). Outros trabalhos demonstraram que a superexpressão da OPNa e OPNb em linhagem de fibrosarcoma e tumor pancreático protegem da morte celular por apoptose, sendo este efeito mediado por FAK e NF-kB (Courter *et al.,* 2010). Quanto à interação com os receptores, a OPN pode interagir com as diferentes subunidades de integrinas e específicas isoformas variantes de '*splicing*' do CD44 (Lee *et al*., 2007; Chae *et al*., 2009). A ativação da integrina $\alpha v/\beta 3$ pela OPN aumenta a fosforilação de EGFR mediada por c-Src, ativando as vias de sinalização PI3K/ Akt e ERK1/2. Em contraste, a interação com CD44 ativam a via da PLC-γ-dependente Akt (Chae *et al*., 2009). No entanto, já foi descrito o intercruzamento destas vias de sinalização (Bellahcene *et al*., 2008). Nossos dados demonstraram que a OPNc, no modelo de ovário e próstata, e a OPNb, no modelo de próstata, ativam a via de sinalização PI3K/Akt e que os efeitos funcionais na proliferação, migração e formação colônias são mediados por esta via de sinalização. Os efeitos da OPNb e OPNc são bloqueados com o inibidor da PI3K, o LY294002. Em conclusão, estes resultados sugerem que a OPNb e OPNc estimulam os efeitos funcionais pró-tumorigênicos através da ativação da via PI3K/Akt. No capítulo II, investigamos as possíveis vias e mecanismos moleculares pelos quais a OPNc controla a indução de expressão

do VEGF-A nas células OvCar-3. Observamos que a OPNc secretada ativa a via de sinalização PI3K/Akt e que os efeitos na indução de expressão de VEGF-A, c-Fos e fosforilação de c-Jun são mediados por esta via de sinalização. Estes resultados sugerem que a OPNc estimula a indução de VEGF-A através da ativação da via PI3K/Akt.

No capítulo V, mostramos que as células OvCar-3 e PC-3 que superexpressam a OPNc apresentam alterações de expressão gênica que se correlaciona com as principais alterações descritas por Hanahan & Weinberg (2011), caracterizadas como '*Hallmarks of Cancer*'. Nosso estudo consiste na primeira descrição do mecanismo molecular pelo qual a OPNc regula os efeitos funcionais descritos nesta tese nos modelos de progressão tumoral das linhagens Ovcar-3 e PC-3. Ainda, identificamos potenciais novas funções e vias de sinalização estimuladas pela superexpressão da OPNc nesses modelos tumorais. Contudo, são necessários novos estudos que confirmem funcionalmente esses mecanismos moleculares estimulado pela OPNc nas linhagens.

9. Conclusão

Em conclusão, caracterizamos o '*splicing*' alternativo das isoformas da OPN em amostras teciduais de ovário e próstata. No modelo de CO, a OPNc encontra-se especificamente expressa nas amostras tumorais de CO. Para o carcinoma de próstata, as isoformas da OPN encontram-se superexpressas e apresentam também potencial aplicação como biomarcadores de prognóstico. Observamos que a OPNc, no modelo de ovário e próstata, e a OPNb, no modelo de próstata, apresentam efeitos estimulatórios sobre a proliferação, migração, invasão, formação de colônia e crescimento de tumores *in vivo*. Estes resultados indicam que estas isoformas apresentam características pró-tumorigênicas. Descrevemos também que os efeitos da OPNb e OPNc são mediados pela via de sinalização PI3K/Akt. Observamos que a OPNc nos dois modelos tumorais é capaz de modular a expressão de genes essenciais para a transformação e progressão tumoral. Com base na observação de que tumores que superexpressam a OPNc apresentam altos níveis de expressão de marcadores típicos de angiogênese, tais como VEGF-A, Flk-1 e CD34, realizamos também uma investigação do papel funcional e do mecanismo molecular pelo qual a OPNc estimula a angiogênese no CO. A OPNc secretada interage com receptores do tipo integrinas RGD-dependente, ativando a via de sinalização PI3K/Akt. Observamos também que esta via de sinalização ativa a expressão c-Fos e c-Jun e a fosforilação de c-Jun. Adicionalmente, observamos que de forma parácrina, o meio condicionado da OPNc induz a proliferação, migração e adesão das células endoteliais HUVEC, de forma a contribuir para a neovascularização. Os resultados gerados por este estudo contribuem para o melhor entendimento da biologia e dos mecanismos moleculares dos tumores de ovário e próstata. Em conjunto com os dados da literatura, os resultados gerados no presente estudo reforçam que o papel funcional das isoformas da OPN é tumor e tecido específico. O papel crucial destas isoformas em distintas etapas da progressão destes tumores indicam as isoformas OPNb e OPNc como potenciais alvos terapêuticos para o CO e CaP.

10. Perspectivas

1 . Identicar o perfil de expressão das isoformas da OPN em um maior número de amostras de CO, tumores '*borderline*', tumores benignos e tecido não-tumoral. Adicionalmente, avaliar a correlação entre a expressão das isoformas da OPN com os parâmetros clínicos e patológicos das pacientes.

2. Caracterizar os receptores envolvidos nas ações das distintas isoformas da OPN nos modelos tumorais de CO e CaP.

3. Validar os dados do PCR em tempo real para os dois modelos de estudo, através de abordagem proteômica e ensaios funcionais.

4. Realizar ensaios de análise de atividade do promotor do VEGF em células OvCar-3 quando cultivadas com o meio condicionado das células transfectadas com a OPNc.

5. Realizar ensaios *in vivo* com o meio condicionado das células que superexpressam a OPNc, como o ensaio com membrana corioalantóica de galinha (ensaio de CAM), com o objetivo de avaliar a formação de vasos *in vivo*.

6. Caracterizar as modificações pós tradução da OPNc e validar funcionalmente a importância das fosforilações no efeito funcional desta isoforma no CO e CaP.

7. Avaliar a OPNc, nos modelos de CO e CaP, e a OPNb, no modelo de CaP, como alvo terapêutico para estas neoplasias.

Anexo I

Atividades extras desenvolvidas no estágio de doutorado sanduíche

1. Introdução

Os resultados apresentados neste item referem-se à atividades acrescidas à proposta inicial do projeto de doutorado sanduíche. O grupo belga, no qual realizei meu estágio de doutorado sanduíche, recentemente demonstrou a importância da expressão da OPN completa no desenvolvimento do glioma anaplástico e do glioblastoma (Lamour *et al.,* 2009). Um dos mecanismos relacionados à alta taxa de recorrência destes tumores é a existência de um pequeno subgrupo de células tronco tumorais (CSCs) no glioblastoma. Estas CSCs retêm muitas das propriedades das células tronco normais, incluindo a habilidade de se auto-renovar e de se diferenciar em células heterogêneas presentes nos tumores. Uma vez que estas células apresentam uma alta resistência à quimioterapia e radioterapia, seu estudo também representa um grande desafio na pesquisa do câncer. Até o presente momento, nenhuma avaliação do perfil de expressão e estudo funcional das isoformas da OPN foi realizado em células tronco tumorais de gliomas.

2. Objetivos

Dentro deste contexto, apresentamos os seguintes objetivos:

1 - Investigar o perfil de expressão das isoformas da OPN em células tronco de glioma.

2 - Caracterizar o papel funcional das isoformas da OPN em células tronco de glioma.

3. Resultados

3.1. Perfil de expressão das isoformas da OPN nas linhagens de glioblastoma

O primeiro passo na caracterização do papel funcional das 3 diferentes isoformas da OPN em glioblastoma foi avaliar o perfil de expressão das isoformas da OPN em linhagens celulares (U87-MG, ZEMA, BALGI, COMI) e em linhagens primárias de pacientes (GBMa e GBMc).

Avaliamos o perfil de expressão nas linhagens de glioblastoma por PCR em tempo real. Observamos que o nível de expressão da OPNa e OPNb apresentam-se similares (Figura 1). Por outro lado, a isoforma OPNc apresenta menor nível de expressão quando comparado com a OPNa e OPNb.

3.2. A superexpressão da OPNb estimula a formação de neuro-esferóides.

O cultivo das células U87-MG com meio de cultura sem soro enriquecido com EGF e bFGF induz a formação de neuro-esferóides. Esse meio de cultura seleciona as células tronco tumorais de glioma. Avaliamos se os clones transfectados com as isoformas da OPN alteram a formação de neuro-esferóides. Observamos que a superexpressão da OPNb, e não das demais isoformas da OPN, estimula o tamanho (Figura 2) e número dos esferóides (Figura 3).

Como descrito anteriormente, a OPN é uma proteína secretada e exerce suas funções na matriz extracelular. Investigamos se os efeitos observados na formação de esferóides estão associados com as isoformas secretadas da OPN. Assim, avaliamos a formação de esferóides na linhagem U87-MG e GBMa não transfectada na presença do meio condicionado (MC) das células que superexpressam a OPNa, OPNb, OPNc, o vetor vazio e células não transfectadas. Demonstramos que a isoforma OPNb secretada é capaz de ativar significativamente a formação de esferóides (Figura 4) em comparação às células que foram cultivadas com o MC das isoformas OPNa, OPNb e vetor vazio.

Este estudo consistiu na primeira descrição do papel funcional das isoformas da OPN em células tronco tumorais de glioma. O primeiro passo na caracterização das isoformas da OPN e de suas potenciais funções nesta neoplasia foi avaliar o perfil de expressão destas isoformas em linhagem celular deste tumor. Nossos resultados demonstram que a OPNa e OPNb são expressas e apresentam semelhante nível de expressão na linhagens de glioma. Por outro lado, a OPNc encontra-se menos expressa. Estes resultados em conjunto, sugerem um importante papel funcional da OPNb nas células tronco tumorais de glioma. Nesse contexto, a OPNb poderia ser utilizada como potencial alvo terapêutico para tratamento destes tumores.

3.3. Caracterização do papel funcional das isoformas da OPN na angiogênese do glioblastoma

3.3.1. A superexpressão da OPNb e OPNc estimulam a angiogênese em glioblastoma

Com o objetivo de caracterizar o papel das isoformas da OPN na angiogênese, realizamos ensaios *in vitro* com as células endoteliais HUVEC. Estas células foram tratadas com o MC das células U87-MG transfectadas com as isoformas da OPN e o vetor vazio. As células HUVEC foram testadas quanto ao potencial proliferativo, de adesão e de migração, como eventos principais associados à angiogênese. O MC da OPNb e OPNc resulta no aumento de adesão, proliferação e migração das células HUVEC (Figura 5). Demonstramos que o aumento da adesão celular promovido pela OPNb e OPNc é revertido pelo bloqueio do receptor CD44. Estes dados sugerem o envolvimento importante da OPNb e OPNc na angiogênese e dos receptores CD44 na indução da adesão celular.

4. Perspectivas:

1 - Identificar as vias de sinalização ativadas pela OPNb na formação dos neuro-esferóides.

2 - Caracterizar o(s) receptor(es) ativados pela OPNb na formação dos neuro-esferóides.

3 - Avaliar *in vivo* o papel da OPNb na progressão dos neuro-esferóides, através do modelo de implante ortotópico dos neuro-esferóides em camundongos.

4 – Caracterizar as proteínas pró-angiogênicas presentes no MC da OPNb e OPNc de células U87-MG transfectadas com estas isoformas.

5 - Avaliar os mecanismos moleculares estimulados pela OPNb e OPNc nas etapas relacionadas com a angiogênese.

5. Referência:

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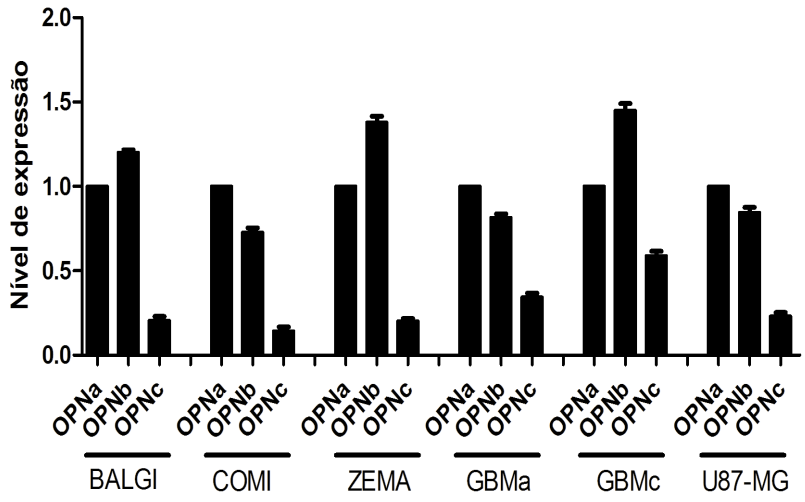

Figura 1. Caracterização do perfil de expressão das isoformas da OPN nas linhagens celulares de glioblastoma. (A) Perfil de expressão das isoformas variantes de '*splicing*' da OPN nas linhagens celulares de glioblastoma. O nível de expressão das isoformas da OPN foi analisado por PCR em tempo real utilizando oligonucleotídeos específicos para cada isoforma da OPN. Utilizamos a expressão do GAPDH como gene normalizador. Para o cálculo do nível de expressão relativo das isoformas nas linhagens celulares utilizamos como referência a expressão da OPNa. O valor relativo de expressão foi calculado pelo método de delta-delta CT.

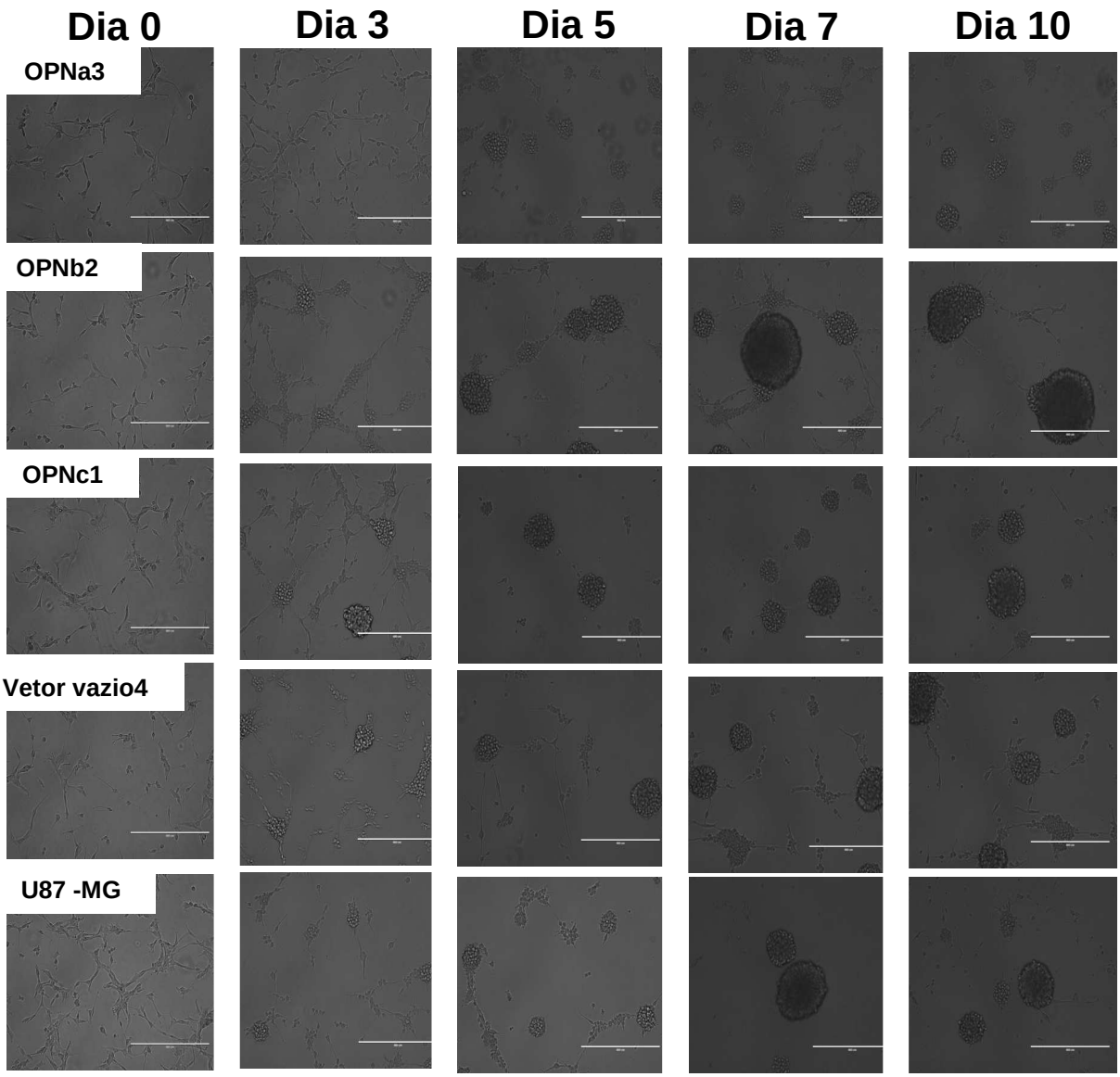

Figura 2. A superexpressão da OPNb estimula o tamanho dos neuro-esferoides. Fotografias representativas do modelo de formação de neuro-esferoides nas células que superexpressam as isoformas da OPN, células transfectadas com vetor vazio e células não transfectadas. As células foram cultivadas em meio de cultura sem soro enriquecido com EGF e bFGF induz a formação de neuro-esferoides. Os intervalos de tempo estão representados em dias e indicado na figura. Ensaio representativo de três experimentos independentes.

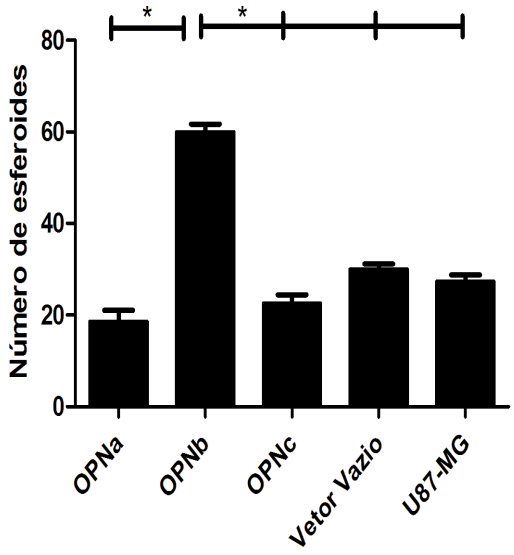

Figura 3. A superexpressão da OPNb estimula o número dos neuro-esferoides. As células foram plaqueadas em baixa densidade em placas de 96-poços em meio de cultura sem soro enriquecido com EGF e bFGF. O número de esferoides foi mensurado após 7 dias. As diferenças significativas no número de células estão representadas por * ($p < 0.0001$).

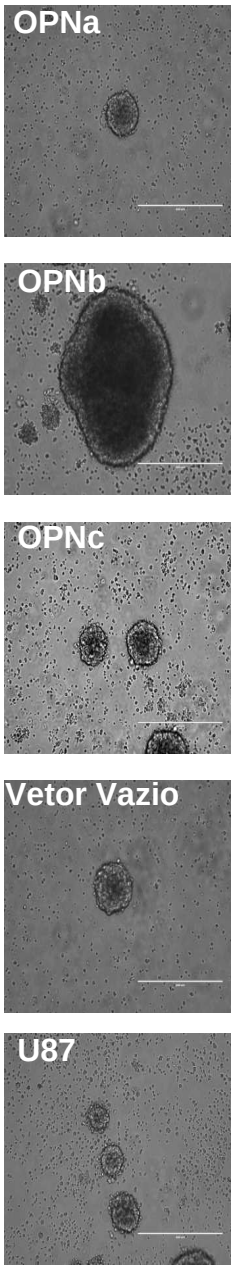

Figura 4. A OPNb secretada estimula o tamanho dos neuro-esferoides. Fotografias representativas do modelo de formação de neuro-esferoides na linhagem primária de glioblastoma (GBMa) cultivada com o meio condicionado das células transfectadas com as isoformas da OPN, células transfectadas com vetor vazio e células não transfectadas. As células foram cultivadas em meio de cultura sem soro enriquecido com EGF e bFGF que induz a formação de neuro-esferoides. Imagens dos esferoides após 10 dias de cultura. Ensaio representativo de três experimentos independentes.

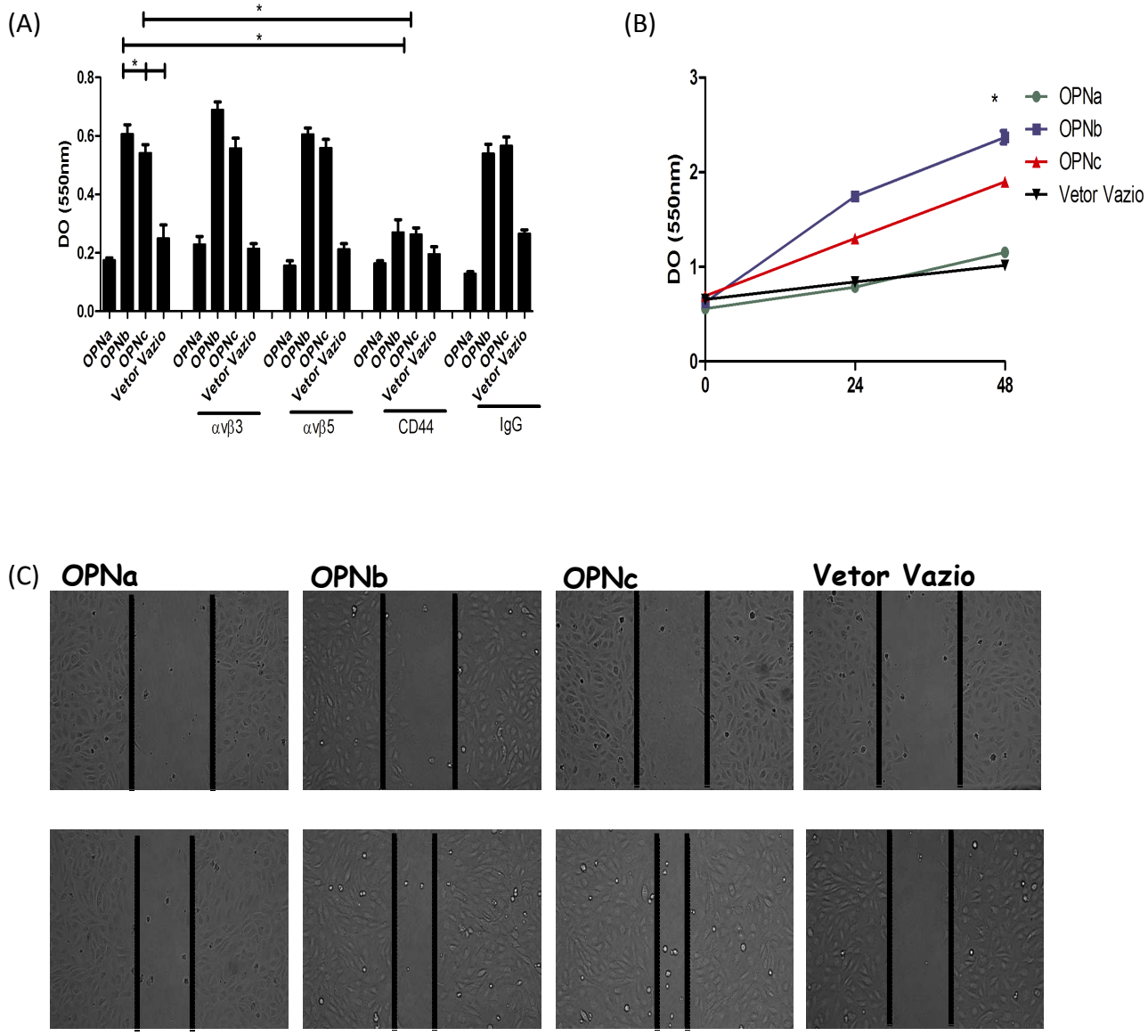

Figura 5. O meio condicionado da OPNb e OPNc induz a adesão, proliferação e migração celular em HUVEC. (A) Ensaio de adesão celular. O meio condicionado das células U87-MG que superexpressam as isoformas da OPN e o vetor vazio foram utilizados como substrato para adesão celular da linhagem HUVEC. Para o bloqueio dos receptores, as células foram incubadas com anticorpos bloqueadores anti-αvβ3, anti-αvβ5 e anti-CD44 (10µg/mL). * $p < 0.0001$. (B) Análise de proliferação celular na linhagem HUVEC cultivada na presença do meio condicionado de células que superexpressam as isoformas da OPN e o vetor vazio. Análise da cinética de proliferação celular foi realizada por coloração com cristal violeta. As legendas de cada uma das curvas estão representadas à direita do gráfico, conforme os símbolos e cores específicos para cada isoforma. O tempo está representado em horas após o plaqueamento celular. OD = densidade ótica em 550nm. * $p < 0.005$. (C) Fotografias representativas do ensaio de migração das células HUVEC cultivadas na presença do meio condicionado de células que superexpressam as isoformas da OPN e o controle pelo método de 'wound healing'. Os intervalos de tempo após a ranhura da placa estão representados em horas e indicado na figura. Ensaio representativo de três experimentos independentes.

Anexo II

Revisão submetida para a Revista *Cancer Letters*

Osteopontin splicing isoforms: known roles, potential clinical applications and activated signaling pathways

Elsevier Editorial System(tm) for Cancer Letters

Manuscript Draft

Manuscript Number: CAN-D-12-01811

Title: Osteopontin splicing isoforms: known roles, potential clinical applications and activated signaling pathways

Article Type: Mini Review

Keywords: osteopontin, alternative splicing, functional roles, clinical application

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September 20, 2012

To Editor-in-Chief

Professor Dr. Manfred Schwab

German Cancer Research Center (DKFZ), Heidelberg, Germany

Title: Osteopontin splicing isoforms: known roles, potential clinical applications and activated signaling pathways

Cover letter:

Dear Editor-in-Chief

Please find enclosed the manuscript entitled "*Osteopontin splicing isoforms: known roles, potential clinical applications and activated signaling pathways*" by Etel RP Gimba & Tilli TM, submitted for consideration for publication as a Minireview Article in the journal Cancer Letters.

The manuscript presents an overview of osteopontin splicing isoforms (OPN-SI), their expression patterns, functional roles in different cell contexts, and also the known signaling pathways by which these variants modulate several pathophysiological conditions. We also discuss and present some hypotheses to explain their tissue-specific expression and roles. Based on these data, we also discuss possible clinical applications of OPN-SI, especially as biomarkers and potential therapeutic targets.

Given the importance of alternative splicing in diverse pathological conditions, including cancer, and the reported impact of osteopontin on tumor progression and several other physiological conditions, we consider that this Minireview will provide an updated collation of current published data for those interested in this specific issue, and also for those interested in translational research and transcriptional regulation. Given this broad spectrum of interests, we think this Minireview is appropriate for publication in Cancer Letters. Both authors have seen and agree with the contents of this manuscript, and there is no competing interest to report. We certify that the submission is original and is not under review at any other publication.

Yours sincerely,

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Abstract

Osteopontin is subject to alternative splicing, which generates three isoforms, termed OPNa, OPNb and OPNc. These variants show specific expression and roles in different cell contexts. We present an overview of current knowledge of the expression profile of OPN splicing isoforms (OPN-SI), their tissue-specific roles, and the pathways mediating their functional properties in different pathophysiological conditions. We also describe their putative application as biomarkers, and their potential use as therapeutic targets by using antibodies, oligonucleotides or siRNA molecules. This synthesis provides new clues for a better understanding of OPN splice variants, their roles in normal and pathological conditions, and their possible clinical applications.

Title Page

Minireview

Title:

Osteopontin splicing isoforms: known roles, potential clinical applications and activated signaling pathways

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Abstract

Osteopontin is subject to alternative splicing, which generates three isoforms, termed OPNa, OPNb and OPNc. These variants show specific expression and roles in different cell contexts. We present an overview of current knowledge of the expression profile of OPN splicing isoforms (OPN-SI), their tissue-specific roles, and the pathways mediating their functional properties in different pathophysiological conditions. We also describe their putative application as biomarkers, and their potential use as therapeutic targets by using antibodies, oligonucleotides or siRNA molecules. This synthesis provides new clues for a better understanding of OPN splice variants, their roles in normal and pathological conditions, and their possible clinical applications.

Keywords: osteopontin, alternative splicing, functional roles, clinical application

1. Introduction

Alternative splicing is the process whereby identical pre-mRNA molecules are spliced in different ways, and is important in both normal development and disease processes [1]. Aberrant splicing in pathological conditions is caused by errors in RNA splicing or its regulation. Disruption of splicing patterns can produce splice variants that have different functions [2]. Several kinds of disease-related genes can undergo alternative splicing, contributing to genetic diversity and pathological processes [3]. Among these gene products, osteopontin (OPN), a matricellular phosphoprotein expressed in several tissues, has been described as involved in several physiopathological processes including inflammation, bone calcification, immune response, and cancer [4]. OPN has several isoforms, as a result of post-translational modifications (PTMs) [5,6], alternative translation

[7] and alternative splicing [8-10]. Of these, splicing variants have been studied in different cell contexts, and their role in cell physiology, especially in cancer cells, has been investigated [9,11-17].

The OPN transcript is subject to alternative splicing, generating three splice variants. OPNa is the full-length isoform, OPNb lacks exon 5, and OPNc has a deleted exon 4. The full-length isoform has been broadly characterized in different cellular processes, and its role in cancer biology has been widely investigated [4]. Studies of the functional role and expression profiling of OPN splicing isoforms (OPN-SI) are more recent and mainly focused on tumor cells [9,11-17]. Due to OPN-SI tissue-specific roles and peculiar expression profiling, available data may provide the first clues about the usefulness of OPN-SI for clinical purposes. As for other splice variant products [3], studies of OPN-SI will not only provide new tools for improved understanding of disease-associated genome complexity and diversity, but also establish new approaches to describe specific biomarkers and novel strategies for splicing variants-targeted therapies. Here, we review current knowledge about OPN-SI expression profiling, their tumor and cell type-specific roles, some hypotheses to explain these, and signaling pathways by which these isoforms produce their effects. We also discuss data regarding the potential use of OPN splice variants as biomarkers, and future directions to specifically target OPN-SI for treatment purposes.

2. OPN splice variants, their expression patterns, and putative roles in pathological processes

Since the first description of OPN splice variants in glioma cells seventeen years ago [18], several reports have described the expression patterns and the roles of OPN-SI in different cell contexts, mainly in tumors. The main feature of these OPN variants is their tissue-specific expression and functional roles. In human glioma cells, for example, OPNa and OPNc activate invasion [19]; while in prostate cancer cells, OPNb and OPNc have several pro-tumorigenic roles, by activating different steps of tumor progression [17]. In ovarian and breast tumor cells, only OPNc activates tumor-progression features [16]. In other tumor types, OPN-SI show opposing roles, or each individual OPN variant activates different aspects of tumor progression. In lung cancer cells, OPNb affected proliferation, while OPNc expression was correlated with tumor-cell invasion [20]. Other studies have demonstrated that OPNa is overexpressed in lung cancer tumors and stimulates several tumorigenic features, whereas OPNb has fewer effects than OPNa. In this tumor type, OPNc inhibits proliferation and other important cancer hallmarks, such as angiogenesis and VEGF secretion [21,13]. In mesothelioma cells, OPNa is markedly up-regulated and significantly activates cell proliferation, wound closure, and invasion [11]. In hepatocellular carcinoma cells (HCC), tumor tissues predominantly expressed OPNa and OPNb, whereas normal liver tissues mainly expressed OPNc. In this tumor model, the effects of these isoforms depend on the cell line tested [22]. Other investigators have demonstrated that in HCC cells, increased expression of OPNc splice variant is associated with cellular invasion and appears to correlate with metastatic potential [23].

More recent data demonstrated that OPN splice variants may be involved in the response of normal cells to carcinogenic stimuli, such as nicotine and smoking. It has been demonstrated that pancreatic ductal adenocarcinoma (PDA) cells, upon nicotine treatment,

selectively induced *de novo* expression of OPNc and increased α7-nAChR expression levels. In these cells, OPNc was found in 87% of pancreatic lesions, of which 73% were from smokers and showed a significant correlation with invasive PDA tumor samples. In this tumor model, OPNc supports anchorage-independent growth and can modulate metastatic behavior [14,15].

In addition to their role in cancer cells, OPN-SI are beginning to be studied in other physiopathological conditions. A recent report investigated the expression of OPN-SI in tissue samples of calcific aortic valve disease (CAVD). The splice variants OPNa, OPNb, and OPNc are differentially expressed during CAVD progression, and are able to inhibit biomineralization. Also, these isoforms are potential biomarkers of early and late stages of CAVD [24]. Further studies on the expression patterns of each OPN-SI and their roles in different tissue and pathological contexts should reveal key signaling pathways by which these conditions are specifically modulated by the splice variants, such as in immune-system activation, inflammatory responses, and autoimmune diseases [25-27].

The reasons why these isoforms have these cell-type specific patterns and roles remain unknown. One hypothesis states that PTMs impact OPN-SI structural and interaction properties, as well their roles in different cell contexts [8,28-30]. Recent reports have also suggested that the degree of phosphorylation of splicing isoforms produced by different tumor-cell types can regulate their roles and receptor interactions [12,31,32]. The proto-oncogene HMGA1 has two splice variants, termed HMGA1a and HMGA1b. These isoforms have different functions in specific tumor contexts, especially due to differences caused by loss of phosphorylation sites [33]. Another example is caspase-9 splice variants, In lung cancer cells, caspase-9a is pro-apoptotic, while caspase-9b is anti-apoptotic. The

modulation of the lung cancer cell-survival pathway is mediated by the ratio between these isoform expression levels, which is defined by their Ser^{52} phosphorylation patterns [34]. This suggests that the deletion of exons 4 or 5 could alter the pattern of PTMs, resulting in functional modifications. This hypothesis is further reinforced by data showing that the N-terminal region of OPN contains conserved amino-acid sequence motifs that mediate lymphocyte migration and survival [10,35]. Furthermore, sequences corresponding to exons 4 and 5 contain PTM sites and also glutamine residues essential for transglutaminase crosslinking. As a consequence, OPNa and OPNb, but not OPNc, are able to form polymeric OPN complexes with altered functional properties [10,36]. Possibly, these OPN polymerization patterns might be changed according to each cell type and developmental conditions, hence modifying the roles of OPN-SI in specific cell contexts. Also in support of this presumption, in some types of tumors, OPNc plays antagonistic roles, such as in ovarian and breast tumors, as opposed to hepatocellular carcinoma cells (8,16,22). Further work is required to elucidate PTM patterns of OPN-SI and their impact on their roles in tumor cells.

Besides phosphorylation, additional PTMs, such as glycosylation and tyrosine sulfation, which can also be cell type-specific and depend on physiological and pathological factors, could also impact on OPN-SI structure and specific roles [30,36]. Additionally, OPN-SI structural domains, associated receptors, and regulator/binding proteins could also differentially modulate their roles in specific tissues. It is widely reported that the roles of OPN reflect its ability to interact with the corresponding receptors. OPN interaction with integrin receptors depends not only on the RGD domain, but also on several OPN phosphorylation sites [28]. OPN is an intrinsically disordered protein with

cryptic integrin-binding motifs. For this reason, it has a conformational structure which is dependent on receptor interaction, especially with integrins [37,38]. Future studies to more thoroughly evaluate the structure and PTM patterns of the full-length and OPN splice variants could help to better delineate these issues.

The OPN protein domain coded by exon 4 is highly enriched in proline residues. These amino acids have a special role in protein function due to its unique side structure and effects on overall protein conformation. Proline-rich domains tend to disrupt both α-helical and β-sheet structures [39]. Proline-rich motifs do not merely act as structural spacers, but frequently have an important role in protein-protein interactions that are essential for cellular processes. These properties could be among the mechanisms by which OPNc shows cell type-specific roles. Because in this isoform, exon 4 is deleted, the absence of these specific proline-rich sequences could impair the affinity of OPNc for other proteins, providing a mechanism for enhancing the plasticity and dynamics of OPN-SI-mediated signal transduction [40].

Tissue-specific alternative splicing is greatly affected by splicing factors present in the particular tissue. These may be ubiquitously expressed, but be present at different levels or preferentially expressed in certain tissues. Therefore, the predominant OPN-SI produced in a specific pathological condition or tissue and its specific role, could not only result from structural modifications and their binding properties, but could also depend on the expression patterns of splicing factors in a cell type-specific context [41].

3. The signaling pathways mediating OPN-SI roles

The signaling pathways and the receptors mediating the actions of these OPN-SI are just beginning to be investigated. A few papers have described the main signaling pathways mediating OPN-SI roles in activating different aspects of tumor progression. In glioma cells, for example, it has been demonstrated that cellular invasion induced by OPNa and OPNc is mediated by the PI-3K /AKT/ NF-kB pathway, via ligation of αvβ3 integrin [42]. In ovarian and prostate cancer tumor cells, several aspects of tumor progression which are activated by OPNc overexpression, such as proliferation, migration, invasion and anchorage-independent growth, are also being modulated by the PI-3K/AKT signaling pathway [16,17]. Specifically in the prostate cancer model, OPNb also activated these processes through this same pathway, although to a lesser degree [17]. In HCC cells, OPN-SI can differentially modulate the migratory activity of HCC cell lines, probably by differentially activating signaling pathways involved in tumor metastasis, such as urokinase-type plasminogen activator (uPA) and p42/p44 MAP kinase. In these cells, the uPA level was significantly increased by expression of either OPNa or OPNb, whereas it was weakly increased by OPNc. The phosphorylation of ERK1/2 was also significantly increased by OPNa- or OPNb-conditioned medium, but OPNc-conditioned medium had no significant effects. These results suggested that OPNa and OPNb are involved in the activation of integrin signaling, but that OPNc cannot activate this pathway [22]. Finally, in head and neck cancer cells, the RGD region of OPNa and OPNb isoforms inhibited apoptosis by inducing NF-kappaB activation and FAK phosphorylation [12]. Hence, describing the pathways by which each OPN-SI mediates their roles could provide insights into how they specifically modulate the phenotype in different cell contexts in response to

the overexpression of OPN-SI variants. In addition, understanding the signaling activated by these isoforms can help to improve the design of approaches attempting to use OPN-SI as targets for treatment options, as discussed below.

4. OPN-SI: diagnostic and prognostic implications and putative application as therapeutic targets

Some published data regarding the expression patterns of OPN splice variants have provided evidence suggesting that some of these variants could be used as diagnostic and prognostic biomarkers. Especially in cases where tumors contain an OPN-SI that is specifically expressed in tumor cells, or whose expression pattern is correlated to tumor progression, these isoforms can be used as biomarkers to improve the accuracy of diagnostic approaches and also to evaluate patient outcomes.

In soft tissue sarcoma (STS) samples, high mRNA expression levels of OPN-SI are significantly associated with poor prognoses. In addition, women with high mRNA expression levels of OPNa and OPNb have an especially high risk of STS tumor-related death. These features imply that these OPN splice variants are negative prognostic and predictive markers for STS patients [43]. As described above, OPN-SI OPNa, OPNb, and OPNc are differentially expressed during CAVD progression. Based on these data, these isoforms may help in developing diagnostic and risk-stratification tools to follow the progression of asymptomatic aortic valve degeneration [44]. In prostate cancer tumor tissues, the OPNc isoform has been described as the most upregulated splice variant, and is efficient to distinguish this neoplastic disease from benign prostate hyperplasia (BPH),

showing sensitivity and specificity of 90% and 100%, respectively. In these tumors, OPNc protein was also strongly stained in prostate cancer tissues showing a high Gleason score, hence also showing prognostic implications [45]. In breast cancer samples, OPNc showed similar properties, also behaving as a diagnostic and prognostic marker that may have value in a diagnostic panel together with conventional breast-cancer markers [9]. In addition, OPNc shows potential for application as a specific biomarker for ovarian tumors, because it is specifically expressed in ovarian malignant and borderline tumors, but not in ovarian non-tumor samples [16]. Patani N et al. (2008) [46] also demonstrated that the expression levels of OPN-SI in breast-cancer tissues predicted local recurrence, disease-free survival, and bone metastases. In breast cancer samples, OPNc expression was particularly associated with tumor grade and poor prognosis. In lung cancer patients, a mass spectrometric method has been developed to quantify OPN isoforms in human plasma. In this study, OPNa was substantially elevated in non-small cell lung cancer (NSCLC) patients, showing diagnostic potential [47]. Also in these tumors, OPNc showed a significant correlation with invasion, suggesting a potential application as a biomarker for invasive lung cancer [48].

These important data, demonstrating diagnostic and prognostic applications of OPN-SI in several tumor types, provide evidence for new approaches that can be developed for molecular-targeted therapy for these different kinds of cancers. Conventional treatments such as chemotherapy and radiotherapy cause several off-target effects on patients with different kinds of cancer. The possibility to develop strategies to target OPN isoforms that are specifically expressed in tumor samples or play a key role in tumor progression is an opportunity to define specific targets for new approaches to cancer treatment.

Full-length OPN plays an important role in determining the oncogenic potential of various cancers [4]. However, as demonstrated above, some of the OPN splice variants particularly activate the tumorigenic process. Therefore, optimized cancer treatment strategies should consider targeting those isoforms with higher tumorigenic potential. OPN-SI-based anticancer therapy may provide new insights for the effective management of cancer, creating opportunities for more-effective and targeted therapies with reduced adverse effects.

Similar to transcription, splicing is a ubiquitous cellular process. Targeted splicing machinery may induce global effects on splicing events in cells. Therefore, developing drugs that target splicing is difficult. Tumor-specific OPN-SI splice variants whose expression patterns and activities have been successfully characterized, such as OPNc in ovarian carcinoma and breast cancer [8,16], may become attractive targets for ablation or splicing modification. The extreme specificity of their expression suggests that a variant-specific treatment may allow targeting of cancerous cells, with minimal impact on healthy tissues [49]. Different compounds, such as antibodies, chemically modified oligonucleotides, and siRNA molecules have been proposed to regulate the expression of splice-variant products [3,41,49,50]. The selectivity of these approaches has been demonstrated, and some clinical trials are currently ongoing for other alternative splicing products, especially for targeting mutated survival motor neuron (SMN1) and Duchenne muscular dystrophy (DMD) genes, related to Spinal muscular atrophy (SMA) and Duchenne muscular dystrophy diseases [51,52], respectively.

Given the importance of each OPN-SI in the progression of different pathological conditions, these observations indicate that inhibition of OPN splice variants with some of these developed targeting tools seems a promising approach for treatment of different kinds of cancer, considering their tissue-specific functional roles.

5. Conclusions

Based on OPN-SI expression patterns and their described functional roles in different contexts, these isoforms have been proposed as additional diagnostic and prognostic markers for several pathological conditions, especially cancer. The ongoing characterization of the signaling pathways and the receptors mediating their role in tumor progression is providing new opportunities to better understand their effects on physiological and pathological conditions. In addition, recent approaches to target splice variants have provided new opportunities to treat OPN-SI disease-related pathological conditions, preventing off-target effects. Future studies to describe the involvement of OPN-SI in other tissues and diseases should amplify the development and application of these proposed approaches.

6. Acknowledgements

Our grateful thanks to all those researchers who have been contributing to the knowledge of OPN biology. We also thank Janet Reid for revising the English text of this manuscript.

7. Conflict of Interest Statement:

Nothing to declare

8. References

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Conflict of Interest Statement

We have not conflict of interest to declare