DÉBORA CAMPANELLA BASTOS

"EVALUATION OF THE BIOLOGICAL ROLE OF FATTY ACID SYNTHESE (FASN) ENZYME IN LYMPHATIC ENDOTHELIAL CELLS STIMULATED OR NOT BY MELANOMA CELLS"

"AVALIAÇÃO DO PAPEL BIOLÓGICO DA ENZIMA ÁCIDO GRAXO SINTASE (FASN) EM CÉLULAS ENDOTELIAIS LINFÁTICAS ESTIMULADAS OU NÃO POR CÉLULAS DE MELANOMA"

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UNIVERSIDADE ESTADUAL DE CAMPINAS
FACULDADE DE ODONTOLOGIA DE PIRACICABA

DÉBORA CAMPANELLA BASTOS

"Evaluation of the biological role of fatty acid synthese (FASN) enzyme in lymphatic endothelial cells stimulated or not by melanoma cells"

Orientador: Prof. Dr. Edgard Graner

"Avaliação do papel biológico da enzima ácido graxo sintase (FASN) em células endoteliais linfáticas estimuladas ou não por células de melanoma"

Tese de doutorado apresentada à Faculdade de Odontologia de Piracicaba, Universidade Estadual de Campinas, para obtenção do Título de Doutora em Estomatopatologia, na área de Patologia.

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Este exemplar corresponde à versão final da Tese defendida pela aluna, e orientada pelo Prof.Dr. Edgard Graner.

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Prof. Dr. EDGARD GRANER

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"Mantenha seus pensamentos positivos, porque seus pensamentos tornam-se suas palavras, suas palavras tornam-se suas atitudes, suas atitudes tornam-se seus hábitos, seus hábitos tornam-se seus valores e seus valores, tornam-se seu destino."

M. Gandhi

RESUMO

A enzima ácido graxo sintase (FASN) é responsável pela síntese endógena de ácidos graxos e tem alta expressão em diversas neoplasias malignas. Cerulenina e orlistat são inibidores farmacológicos da atividade de FASN e têm sido descritos como potenciais agentes anti-tumorais. O sistema linfático constitui a via primária de metástases de diversas neoplasias malignas, dentre elas o melanoma. Em estudos prévios, realizados no Laboratório de Patologia Bucal da FOP-UNICAMP, a inibição da FASN com orlistat foi capaz de reduzir significantemente as metástases linfonodais em modelo murino de melanoma e no modelo ortotópico de carcinoma espinocelular oral. Além disso, o meio condicionado por células de melanoma humano tratadas com inibidores da FASN inibiu a formação de capilares *in vitro* em matrigel, efeito este mediado pela isoforma anti-angiogênica VEGF-A_{165b}. A correlação entre a FASN e a linfangiogênese ainda não está descrita na literatura, neste trabalho investigamos os efeitos da inibição farmacológica da FASN sobre os fatores de crescimento endotelial vascular (VEGF-C e -D) em células de melanoma murino (B16-F10) e humano (SK-Mel-25) através de RT-PCR quantitativo e ELISA e verificamos os efeitos do bloqueio de FASN sobre a linfangiogênese por meio de culturas tridimensionais de células endoteliais linfáticas humanas (HDLEC) e culturas primárias de endotélio linfático de camundongo. Avaliamos também, o efeito da exposição de células SK-Mel-25 a inibidores da FASN sobre a proliferação de

células HDLEC. O tratamento de células B16-F10 e SK-Mel-25 com cerulenina ou orlistat modulou de forma diferencial a expressão de VEGF-C e -D, inibindo o primeiro ao mesmo tempo em que estimulou o último. Além disto, estas mesmas drogas reduziram em células endoteliais linfáticas a viabilidade, a proliferação, migração e formação de capilares em ensaio *ex vivo,* além de provocar apoptose. O tratamento com inibidores farmacológicos de FASN em células SK-Mel-25 também induziu um fenótipo anti-linfangiogênico, observado em experimentos com meio condicionado. Em conjunto, nossos resultados sugerem que a inibição farmacológica da FASN inibe a linfangiogênese por atuar ao mesmo tempo nas células dos vasos linfáticos e de melanoma. Esses resultados explicam, pelo menos em parte, a redução do número de metástases linfonodais em modelos animais tratados com orlistat e reforçam a teoria de que o bloqueio da FASN é um alvo terapêutico em potencial para os melanomas.

ABSTRACT

Fatty acid synthase (FASN) is responsible for *de novo* synthesis of long-chain fatty. FASN has emerged as a potential therapeutic target for human cancers since its overexpression is associated with depth of invasion and poor prognosis. Cerulenin and orlistat are pharmacological inhibitors of FASN and have been described as potential anti-tumor agents. Lymphatic vessels are the primary route of metastasis in several maliginancies including melanomas. In previous studies performed in our laboratory, we demonstrated that FASN activity is essential for melanoma and oral squamous cell carcinoma progression, as its pharmacological inhibition with orlistat reduces lymphatic metastasis in both experimental intraperitoneal and subcutaneous melanomas and orthotopic tongue carcinomas. We also reported that cell culture medium previously conditioned by human melanoma cells in the presence of orlistat induces an anti-angiogenic phenotype mediated by $VEGFA_{165b}$. Therefore, in order to understand a possible correlation between FASN and lymphangiogenesis, here we investigate the effects FASN inhibitors on the secretion of vascular endothelial growth factors (VEGF) -C and -D by murine (B16-F10) and human (SK-Mel-25) melanoma cells with the aid of qRT-PCR and ELISA assays. We also analyzed the effect of FASN blockage on tridimensional cultures of human lymphatic endothelial cells (HDLEC) and primary cultures of murine lymphatic endothelium. In addition, we also evaluated an indirect effect of the treatment with cerulenin and orlistat in SK-Mel-25 cells on HDLEC

proliferation. The incubation of B16-F10 or SK-Mel-25 cells with cerulenin or orlistat modulated VEGF-C and -D expression by significantly inhibiting the former and increasing the latter. In addition, these drugs reduced the viability, proliferation, and migration as well as promoted apoptosis in human lymphatic endothelial cells (HDLEC). These compounds also inhibited lymphatic capillary formation in a murine *ex vivo* assay. Finally, conditioned media from orlistat-treated human melanoma cells resulted in an anti-lymphangiogenic phenotype. These data suggest that FASN inhibitiors reduce lymphangiogenesis by acting simultaneously in the lymphatic endothelium and melanoma cells. Taken together, the studies here presented explain, at least in part, the anti-metastatic effect of orlistat observed in experimental tumors and further suggest that FASN is a potential therapeutic target for melanomas.

SUMÁRIO

INTRODUÇÃO

Ácido graxo sintase (FASN)

As primeiras observações sobre alterações metabólicas durante o desenvolvimento tumoral e aquisição do fenótipo maligno foram feitas por Otto Warburg, que detectou um aumento da glicólise aeróbica em células neoplásicas, que foi então chamado de “efeito Warburg” (Warburg, 1956). De fato, os níveis elevados de consumo de carbono através da glicólise aeróbica são essenciais na aquisição do fenótipo maligno, pois gera vantagens seletivas para as células tumorais em seu microambiente (Garber, 2004; Garber, 2006; Shaw, 2006; Bui & Thompson, 2006). Uma segunda característica metabólica importante no desenvolvimento tumoral é a alta taxa de consumo energético nos processos de síntese de proteínas e DNA (Clemens, 2004; Averous, 2006; Voeller *et al.,* 2004; Rahman *et al.,* 2004). Além disto, alterações na via lipogênica, que resultam em aumento na síntese de ácidos graxos, ocorrem com frequência em células tumorais (Kuhajda, 2000). Os ácidos graxos e seus derivados fazem parte da estrutura das membranas celulares, atuam como hormônios e mensageiros intracelulares e são importantes substratos para o metabolismo energético (Liu *et al.,* 2010). Os ácidos graxos livres podem ser obtidos da dieta ou derivados de síntese *de novo* pela enzima ácido graxo sintase (FASN) no fígado, tecido

adiposo, na mama durante a lactação e no endométrio durante a fase proliferativa (Menendez *et al.,* 2007; Liu *et al.,* 2010).

No entanto, a alteração lipogênica em neoplasias malignas não foi foco de grande interesse científico até 1994, quando Kuhjada e colaboradores identificaram o antígeno oncogênico-519 (OA-519), molécula encontrada nas células tumorais de pacientes com câncer de mama com prognóstico ruim, posteriormente identificada como sendo a ácido graxo sintase (FASN) (Kuhajda *et al.,* 1994). FASN (E.C.2.3.1.85) é uma enzima multifuncional responsável pela síntese de ácidos graxos de cadeia longa a partir da condensação do acetil-CoA e malonil-CoA (Kuhajda, 2000; Brink *et al*., 2002; Baron *et al*., 2004; Menendez & Lupu, 2007). Estruturalmente, FASN é um homodímero formado por duas cadeias polipeptídicas (~270 kDa cada) que contém sete diferentes sítios catalíticos seqüencialmente organizados, além de um sítio para a proteína carregadora de acil (Brink *et al*., 2002).

Em condições normais, FASN é importante para inúmeros processos biológicos, como armazenamento de energia na forma de triglicerídeos, produção de ácidos graxos durante a lactação, síntese de membranas celulares, manutenção do ciclo endometrial em fase proliferativa e produção da substância surfactante nos pulmões em desenvolvimento (Kuhajda *et al*., 2000; Lu *et al*., 2001). Sua expressão, entretanto, é baixa ou até mesmo ausente na maioria dos tecidos humanos adultos normais, uma vez que a maior parte dos ácidos graxos

usados por suas células provém da dieta (Kuhajda, 2000; Kuhajda *et al*. 2000; Menendez *et al.,* 2005a). Em contraste, FASN está presente em quantidades elevadas em diversos tipos de neoplasias malignas, como as de mama (Wang *et al*., 2001a; Li *et al*., 2008), cólon (Visca *et al*., 1999; Ogino *et al*., 2008, Dowling *et al*., 2009), próstata (Epstein *et al*., 1995; Swinnen *et al*., 2000a; Swinnen *et al.,* 2002; Rossi *et al*., 2003; Van de Sande *et al.,* 2002; Shah *et al.,* 2006), ovário (Alo *et al*., 2000; Wang *et al.,* 2001a; Ueda *et al*., 2010), endométrio (Pizer *et al*., 1998a; Tsuji *et al*., 2004), carcinoma de células renais (Horiguchi *et al.,* 2008), estômago (Kusakabe *et al*., 2002), esôfago (Nemoto *et al*., 2001), pulmão (Piyathilake *et al*., 2000; Wang *et al*., 2002; Orita *et al*., 2007; Orita *et al*., 2008; Cerne *et al*., 2010), bexiga (Visca *et al*., 2003), pâncreas (Walter *et al.,* 2009), mieloma múltiplo (Okawa *et al*., 2008; Wang *et al*., 2008); gliomas (Zhao *et al*., 2006); carcinomas espinocelulares bucais (CECs) (Krontiras *et al*., 1999; Silva *et al*., 2004; Agostini *et al*., 2004; Silva *et al*., 2008a; Silva *et al*., 2008b; Silva *et al*., 2009) e melanomas cutâneos (Innocenzi *et al*., 2003; Kapur, 2005a; Kapur, 2005b) e orais (Andrade *et al*., 2011), além de sarcomas de tecidos moles (Rossi *et al.,* 2006). Em alguns destes tumores, a alta expressão desta enzima está associada a uma maior taxa de recorrência e desenvolvimento de metástases, e conseqüentemente pior prognóstico (Epstein *et al.,* 1995; Pizer *et al*., 1996b; Gansler *et al*., 1997; Vlad *et al.,* 1999; Visca *et al.,* 1999; Alo *et al.,* 2000; Swinnen *et al.,* 2002; Innocenzi *et al.,* 2003; Rossi *et al*., 2003; Takahiro *et al.,* 2003; Silva *et al*., 2004; Visca *et al.,* 2004; Kapur *et al.,* 2005b; Van de Sande 2005; Rossi *et al.,*

2006; Ogino *et al.,* 2008; Silva *et al.,* 2009; Walter *et al.,* 2009; Ueda *et al.,* 2010; Cerne *et al.,* 2010). Estudos com soro de pacientes com câncer de mama, próstata, cólon, e ovário têm demonstrado valores elevados de FASN, em comparação ao soro de indivíduos sadios (Wang *et al.,* 2004). Em estudo clinico de pacientes com câncer de mama foi demonstrado que os valores sorológicos de FASN aumentam com o estádio tumoral (Alo *et al.,* 1999). No entanto, mais pesquisas são necessárias para determinar o papel prognóstico e o valor preditivo dos valores de FASN no soro de pacientes com câncer.

Swinnen *et al.* (2003) demonstraram experimentalmente que a atividade de FASN é necessária para a produção dos fosfolipídios que compõe as membranas das células LNCaP (linhagem derivada de um adenocarcinoma de próstata metastático). Isto pode causar, além de um aumento na velocidade da produção das membranas celulares, alterações na sua composição lipídica, com profundos efeitos em várias vias de transdução de sinais (Baron *et al.,* 2004). Portanto, FASN não somente participa das vias de transdução de sinais, do metabolismo, regula a proliferação e favorece a sobrevivência de células tumorais, mas também modula o desenvolvimento, manutenção e progressão metastática das neoplasias malignas humanas (Menendez & Lupu, 2007).

Innocenzi *et al*. (2003) demonstraram, por meio de reações imunohistoquímicas, que FASN é altamente expressa em melanomas, observando também uma correlação positiva entre a sua expressão e o índice Breslow

(distância em milímetros desde a camada nucleada mais superficial até o nível mais profundo de invasão). Além disso, houve uma correlação inversa entre a quantidade de FASN e tempo de sobrevida total, indicando que uma alta produção de FASN pode estar associada a uma maior taxa de recorrência, maior risco de desenvolvimento de metástases e conseqüentemente pior prognóstico para os pacientes. Kapur *et al*. (2005a) avaliaram a expressão imunohistoquímica de FASN em 155 lesões melanocíticas cutâneas, verificando uma crescente expressão de FASN em nevos, melanomas primários e metastáticos. Mostraram também uma correlação positiva entre expressão de FASN e os índices de Breslow e de Clark, indicadores de comportamento biológico agressivo.

Inibidores específicos de FASN

A cerulenina ([2R, 3S] –2,3 epoxi-1-oxo-7, 10 trans*,* transdodecadienamida) é um produto natural do fungo *Cephalosporium Ceruleans*, capaz de inibir a síntese de ácidos graxos por bloquear irreversivelmente a atividade de FASN (Omura, 1976; Pizer *et al*., 1996) por meio de uma ligação covalente no sítio catalítico β-cetoacil sintase (Kuhajda *et al*., 2000). No entanto, a aplicação clínica da cerulenina é limitada, devido a sua instabilidade, causada pela presença de um grupo epóxi extremamente reativo (Lupu & Menendez, 2006). c75 é uma pequena molécula sintética com cadeia de 7 carbonos (α-metileno-γ-butirolactona) que possui efeitos inibitórios sobre a atividade da FASN comparáveis aos da cerulenina, sendo mais estável do que esta última e apresentando, portanto,

melhor efeito em experimentos *in vivo* (Kuhajda *et al*., 2000; Li *et al*., 2001). Tanto a cerulenina como o c75 produzem significativa inibição da progressão do ciclo celular, bloqueando a passagem das fases G0/G1 para a fase S em linhagens celulares derivadas de neoplasias malignas humanas (Pizer *et al*., 1996; Furuya *et al*., 1997; Pizer *et al*., 1998a; Kuhajda, 2000; Li *et al*., 2001; De Schrijver *et al*., 2003). Alli *et al.* (2005) observaram que camundongos transgênicos *neu*-N, que super-expressam ErbB2 e desenvolvem câncer de mama de forma espontânea, tiveram uma significante diminuição no aparecimento dos tumores quando tratados com c75. Apesar do c75 ter fornecido a primeira evidência *in vivo* da redução tumoral mediante a inibição de FASN, o tratamento com este composto causa perda de peso e anorexia devido a ação no sistema nervoso central (Loftus *et al.,* 2000; Aja *et al*., 2008).

Triclosan (5-cloro-2-[2,4-diclorofenoxifenol) é agente anti-séptico utilizado em sabonetes, dentifrícios e enxaguatórios bucais, capaz de bloquear a atividade de FASN pela inibição do domínio enoil redutase (Liu *et al*., 2002). A substância EGCG (epigalocatequina-3-galato) é um componente do chá verde que tem demonstrado atividades comparáveis as da cerulenina e do c75 por bloquear FASN no sítio β-cetoacil redutase e induzir apoptose em várias linhagens tumorais humanas (Puig *et al*., 2007; Vergote *et al*., 2000; Yeh *et al.,* 2003; Brusselmans *et al*., 2005; Wang & Tian, 2001). Apesar da sua aparente especificidade, o EGCG apresenta características que podem limitar seu uso terapêutico, como a elevada

dosagem para obter atividade anti-tumoral e baixa estabilidade química (Puig *et al*., 2009).

Orlistat (tetrahidrolipstatin, comercializado pela Roche como Xenical®) é um fármaco aprovado pela "Food and Drug Administration" (FDA), derivado semi-sintético da lipstatina, utilizado para o tratamento da obesidade por inibir irreversivelmente a ação de lipases gástricas e pancreáticas (Guerciolini *et al.,* 1997). Orlistat é capaz de bloquear também a função tioesterase da FASN, responsável pela liberação do palmitato (Kridel *et al*., 2004). Foi demonstrado que esta droga possui efeito anti-proliferativo e anti-tumoral em células de câncer de mama e de próstata e também em modelos xenográficos (Menendez *et al*., 2004; Knowles *et al.,* 2004). Menendez *et al.* (2004, 2005a) demonstraram que o tratamento de células derivadas de carcinoma de mama (SK-Br3) e de estômago (NCI-N87) com orlistat causa, além da inibição da síntese de ácidos graxos, um bloqueio do ciclo celular nas fases G0/G1, evitando a entrada na fase S, além de aumentar o índice apoptótico. Menendez *et al*. (2004) observaram que inibição de FASN com orlistat ou com siRNA diminui a expressão do receptor de superfície ErbB2 em linhagens celulares de câncer de mama e de ovário, além de aumentar os níveis de $p27^{Kip1}$, uma proteína regulatória do ciclo celular que interage com o complexo ciclina-CDK2 e -CDK4, inibindo a progressão do ciclo celular para a fase G1. Em estudo realizado por nosso grupo, células B16-F10 foram injetadas na cavidade peritoneal de camundongos C57BL6 e resultaram no desenvolvimento

de tumor primário com formação de metástases nos linfonodos mediastínicos em 100% dos casos. O tratamento destes animais com orlistat reduziu em cerca de 50% o número de linfonodos metastáticos. Neste mesmo trabalho, experimentos de citometria de fluxo mostraram que o tratamento da linhagem celular B16-F10 com orlistat inibe a proliferação, pois há acúmulo de células em G0/G1 e bloqueio da passagem para a fase S, que foi acompanhado por um aumento na quantidade da proteína $p27^{Kip1}$ e concomitante redução de Skp2, responsável pela ubiquitinação de $p27^{Kip1}$ (Carvalho *et al*., 2008).

Efeitos biológicos da inibição da expressão e atividade de FASN

A inibição da atividade de FASN causa um aumento na taxa de morte por apoptose em células malignas (Li *et al*., 2001; Knowles *et al*., 2008). A maneira pela qual isso ocorre ainda não está clara, embora alguns mecanismos tenham sido propostos. O acúmulo de malonil-CoA após o tratamento com inibidores de FASN pode mediar, ao menos em parte, a citotoxicidade resultante da inibição da FASN, seja pelo seu próprio efeito tóxico ou por bloquear a oxidação dos ácidos graxos (Pizer *et al*., 2000; Li *et al*., 2001; Thupari *et al*., 2001; De Schrijver *et al*., 2003). Segundo Pizer *et al*. (1998a), o bloqueio de FASN não tem uma ação direta no DNA ou na maquinaria de replicação e envolve p53, molécula que foi relacionada a perturbações na síntese de ácidos graxos (Li *et al.,* 2001). Entretanto, o efeito citotóxico decorrente do bloqueio de FASN pode ocorrer de maneira independente de p53, através da ativação direta de sinais apoptóticos

como a alta expressão de Bax, pois a inibição de FASN resulta na liberação do citocromo C e ativação de caspases (Heiligtag *et al*., 2002). Bandyopadhyay *et al.* (2006) inibiram a expressão de FASN através de siRNA em células de câncer de mama, o que levou a um acúmulo de malonil-CoA. Esta molécula, por sua vez, é capaz de inibir a enzima de membrana mitocondrial CPT-1, responsável pela transesterificação de ácidos graxos de cadeia longa (na forma de acil-CoAs) em acil-carnitina, o que faz com que estes entrem na mitocôndria para serem oxidados. Também foi verificado um aumento do lipídio ceramida, que está relacionado a respostas apoptóticas celulares mediadas por indutores de apoptose, como FAS/FAS ligante, TNF-α, fatores de crescimento, hipóxia e danos ao DNA. Foi observado ainda, que a inibição de FASN promove aumento na expressão de genes pró-apoptóticos como BNIP3, TRAIL e DAPK2. Estudos realizados em nosso laboratório mostraram o que a inibição de FASN com orlistat ou cerulenina em células de melanoma murino B16-F10 inibe a proliferação e induz morte por apoptose, com liberação de citocromo c e ativação das caspases -9 e -3. A morte induzida por orlistat é precedida por estresse oxidativo (EROs e [Ca2+]cit) e, em ambos os casos, a apoptose é independente da participação de p53, calcineurina ou abertura de poro de transição de permeabilidade mitocondrial (Zecchin *et al*., 2011).

Apesar de promissores, os estudos que visam a inibição farmacológica de FASN como uma forma de quimioterapia para o tratamento de neoplasias

malignas devem ser interpretados com cautela. Está demonstrado que o bloqueio de FASN *in vivo* pode causar efeitos colaterais como anorexia e perda de peso (Clegg *et al*., 2002), além de apresentar potencial efeito teratogênico (Chirala *et al*., 2003). A respeito da expressão diferencial de FASN por células normais e células tumorais, o papel da atividade de FASN em células não-malignas ainda é incerto. Em 2005, Almeida *et al.* mostraram que a inibição de FASN por cerulenina também é capaz de reduzir o crescimento de culturas primárias de fibroblastos gengivais normais. Em trabalho publicado recentemente por nosso grupo (Seguin *et al*, 2012), o tratamento das células RAEC, derivadas do endotélio da aorta de coelho, com orlistat provocou inibição significativa do crescimento e da progressão do ciclo celular, caracterizado pelo acúmulo de células nas fases G0/G1 e redução da porcentagem de células na fase S. Além disso, Browne *et al.* (2006) demonstraram que a inibição de FASN com esta droga é capaz de reduzir significativamente a proliferação de células endoteliais HUVEC de maneira dose-dependente, além de reduzir a proliferação de vasos sanguíneos em ensaios *ex vivo*, sugerindo um possível papel anti-angiogênico para inibidores de FASN.

Linfangiogênese

O sistema linfático é um importante componente da circulação e tem como função primária manter o equilíbrio osmótico e hidrostático entre o espaço intersticial e os capilares sanguíneos (Swartz *et al.*, 2001). Os vasos linfáticos também têm grande importância no sistema imunológico, pois participam do

transporte de células inflamatórias e antígenos. A linfangiogênese é o processo pelo qual os vasos linfáticos são formados. Em um tecido adulto normal, o processo de linfangiogênese é mínimo (Björndahl *et al*., 2005), no entanto em certas circunstâncias como cicatrização de feridas, crescimento tumoral e geração de metástases, há participação tanto da angiogênese como da linfangiogênese (Carmeliet & Jain, 2000; Skobe *et al*., 2001a e b; Stacker *et al*., 2002; Leiter *et al*., 2004; Hirakawa *et al*., 2007).

Em neoplasias malignas, os vasos linfáticos também podem atuar como barreira contra a disseminação de células tumorais, drenando-as para os linfonodos e evitando que estas caiam diretamente na corrente sanguínea. Porém, muitas vezes, ao invés de serem eliminadas pelo sistema imunológico, as células neoplásicas conseguem proliferar nos linfonodos ou mesmo atingir outros órgãos por disseminação linfática, formando as metástases. Em muitas neoplasias malignas como as de mama, boca, as de colo de útero, próstata, pulmão e melanoma, a principal via de metástase é a linfática, inicialmente para linfonodos regionais (Fidler, 2001; Alitalo *et al.,* 2011). A presença de células neoplásicas em linfonodos regionais é um fator importante para o estadiamento destes tumores e planejamento do tratamento cirúrgico, radioterápico ou quimioterápico (Taipale *et al*., 1999; Fidler, 2001). Interações entre células tumorais e vasos linfáticos são de primordial importância para a progressão tumoral, entretanto, os mecanismos moleculares são muito pouco estudados. Dadras *et al*. (2001) verificaram que

ocorre maior formação de vasos linfáticos dentro e ao redor de melanomas metastáticos do que nos respectivos tumores primários.

Fator de crescimento endotelial vascular (VEGF)

A regulação do crescimento de vasos sanguíneos e linfáticos depende da produção de diversos fatores de crescimento. Os membros da família VEGF são considerados elementos importantes no processo de angiogênese e linfangiogênese, pois são mitógenos específicos para células endoteliais, atuando em condições fisiológicas e patológicas, como progressão tumoral (Hirorata & Sakakibara, 1999; Falanga *et al*., 2004; Cho *et al*., 2006; Franket *et al*., 2004; Carmeliet & Jain, 2000; Skobe *et al*., 2001a e b; Dadras *et al*., 2003; Hirakawa *et al*., 2007).

A família VEGF é composta por 7 membros (VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, VEGF-F e PIGF) com diferentes propriedades biológicas. VEGF-A, VEGF-B, VEGF-E, VEGF-F e PIGF têm grande importância no processo de angiogênese e vasculogênese, enquanto que o VEGF-C e VEGF-D estão ligados ao processo de linfangiogênese e metástase linfática (Skobe *et al*., 2001a; Stacker *et al*., 2001; Padera *et al*., 2002; Jain *et al*., 2002; Karkkainen, 2004). Esses fatores são produzidos e secretados por células do estroma e tumorais e seus receptores (VEGFR-1, VEGFR-2 e VEGFR-3), que possuem atividade de tirosina-quinase, são expressos na superfície de células endoteliais. VEGF-A, VEGF-B e PIGF são capazes de se ligar em VEGFR-1, enquanto que o VEGFR-2

é ativado por VEGF-A, VEGF-E, VEGF-C (em sua forma clivada de 21kDa) e por VEGF-D. VEGFR-3, por sua vez, induz a linfangiogênese através da ligação com o VEGF-C (forma clivada de 31kDa) ou VEGF-D. VEGFR-1 e VEGFR-2 são expressos em células endoteliais vasculares e células tumorais, ao passo que a expressão de VEGFR-3 é aparentemente restrita as células endoteliais linfáticas (Kowanetz & Ferrara, 2006). Em ambas as formas de angiogênese, fisiológica ou patológica, a hipóxia é fator determinante. Situações de hipóxia induzem a expressão de *VEGF* e seu receptor, através do fator-1 induzido por hipóxia (HIF-1α), que ao mesmo tempo torna o ambiente atrativo para macrófagos. Em um tumor, o fenótipo angiogênico pode ser adquirido pela hipóxia resultante do aumento da distância entre células tumorais em crescimento e os capilares ou da ineficiência dos vasos recém-formados em nutri-lo (Denko, 2008). A expressão de VEGF pode ser induzida pela exposição das células tumorais à hipóxia ou fatores de crescimento e, em ambos os casos, ocorre pelo aumento da transcrição do gene *VEGF* via HIF-1α. Este fator tem um papel importante na progressão tumoral por regular a angiogênese, sobrevivência celular e resistência a drogas, sendo também recentemente relacionado com linfangiogênese tumoral (Schoppmann *et al.,* 2006). A hipóxia aumenta a disponibilidade intracelular da proteína HIF-1α, que por sua vez aumenta a transcrição de VEGF, o qual difunde-se através dos tecidos e atua seletivamente sobre as células endoteliais, controlando a produção de proteases, migração, proliferação e diferenciação (Carmeliet *et al*., 1998). Quando novos vasos se formam, a concentração de oxigênio é reestabelecida e a

atividade de HIF-1α diminui, o que bloqueia a produção de VEGF. Em um tecido normal, bem oxigenado, a degradação de HIF-1α é constante e depende da sua ubiquitinação pelo produto do gene *VHL* (Iliopoulos *et al*., 1996). Mutações neste gene podem levar ao aumento na produção de HIF-1α e produção excessiva de VEGF, resultando no desenvolvimento de hemangioblastomas (síndrome de Von Hippel-Lindau) (Maxwell *et al*., 1999).

VEGF-C estimula a migração de células endoteliais linfáticas aumentando também a permeabilidade vascular e a proliferação e sua expressão não parece ser regulada por hipóxia (Leu *et al*., 2005). VEGF-D ou FIGF (c-*fos-induced growth factor*) possui 61% de homologia com o VEGF-C e se liga, em camundongos, exclusivamente em VEGFR-3. Pouco se sabe sobre o VEGF-D em condições fisiológicas, porém seus RNAs mensageiros já foram observados em melanócitos, fibroblastos, no mesênquima pulmonar e na parede vascular. Em adultos, VEGF-C e VEGF-D regulam a linfangiogênese em processos inflamatórios, afetam a dinâmica de fluidos nos vasos linfáticos, a formação de válvulas vasculares e recrutam células de músculo liso para o desenvolvimento de vasos linfáticos coletores, além de estarem relacionados a progressão tumoral e formação de metástases (revisado em Jussila *et al*., 2002).

A expressão de VEGFR-3 é também um fator importante na determinação da resposta linfangiogênica, pois já foi mostrada a co-expressão de VEGF-C, VEGF-D e VEGFR-3 em melanomas (Achen *et al*., 2001) e carcinomas de pulmão

de células não-pequenas (Kajita *et al*., 2001). *In vitro*, a co-expressão de VEGF-C e VEGFR-3 promove a sobrevivência, proliferação e migração de células endoteliais linfáticas induzidas por diversas vias de sinalização, como a via PKC, MAPK, PI3K, e Akt (Oh *et al.,* 1997). Há também uma correlação inversa entre a expressão imunohistoquímica de VEGF-C e sobrevida livre de doença em pacientes com câncer de mama (Kinoshita *et al*., 2001). Schietroma *et al*. (2003) avaliaram a expressão de VEGF-C em cultura de células de melanoma humano obtidas de tumores primários e linfonodos metastáticos e sugeriram que a expressão deste fator nos melanomas primários pode participar do processo de disseminação metastática. Os fatores VEGF-C e VEGF-D são responsáveis pela linfangiogênese ou hiperplasia linfática intra e peritumoral, invasão e metástases linfáticas (Isaka *et al*., 2004; Da *et al*., 2008). Níveis elevados de VEGF-C têm sido associados a metástases linfonodais e prognóstico em diversas neoplasias malignas de esôfago (Kitadai *et al.,* 2001), tireóide (Bunone *et al.,* 1999); próstata (Tsurusaki *et al*., 1999; Zeng *et al.,* 2004); ovário (Yokoyama *et al*., 2003), bexiga (Nakashima *et al.,* 2003), pâncreas (Tang *et al.,* 2001; Sipos *et al.,* 2004), pulmão (Arinaga *et al.,* 2003; Kajita *et al.,* 2001), cabeça e pescoço (Charoenrat *et al*., 2001; Benke *et al.,* 2010), cavidade oral (Wen *et al.,* 2001; Yu *et al*., 2002; Tanigaki *et al.,* 2004), estômago (Yonemura *et al.,* 1999; Ichikura *et al.,* 2001; Yan *et al.,* 2004; Liu *et al*., 2004), intestino (Akaji *et al.,* 2000; Kawakami *et al.,* 2003; Kaio *et al.,* 2003; Furudoi *et al.,* 2002; George *et al.,* 2001; Onogawa *et al.,* 2004; Kasama *et al*., 2004), colo uterino (Hashimoto *et al*., 2001; Van Trappen *et al*.,

2002; Ueda *et al*., 2002), mama (Kinoshita *et al.,* 2001), mesotelioma (Ohta *et al.,* 1999) e melanoma (Straume *et al*., 2003; Schietroma *et al*., 2003; Dadras *et al*., 2005; Rinderknecht & Detmar 2008). Em menores proporções, níveis elevados de VEGF-D foram associados a metástases linfonodais em cânceres de intestino, ovário e mama (George *et al.,* 2001; White *et al,* 2002; Nakamura *et al.,* 2003) e pobre prognóstico em neoplasias de intestino (White *et al,* 2002) e ovário (Yokoyama *et al.,* 2003). No entanto, O- charoenrat *et al.* (2001) mostraram que níveis aumentados de VEGF-C e VEGF-A associados com níveis diminuídos de VEGF-D são características comuns em diferentes linhagens de células de carcinomas espinocelulares de cabeça e pescoço, quando comparadas aos níveis apresentados por células epiteliais normais. Esses mesmos autores mostraram que níveis aumentados de RNA mensageiro para VEGF-A e VEGF-C e níveis diminuídos de VEGF-D foram significantemente encontrados em amostras de carcinomas de cabeça e pescoço quando comparados ao epitélio normal. Além disso, um padrão de invasão infiltrativo e a presença de metástases linfonodais foram associados com a expressão das isoformas angiogênicas VEGF-A_{121} e -A_{165} e VEGF-C nestas amostras. Niki *et al.* (2001) também estudaram a expressão dos fatores VEGF-A, VEGF-C e VEGF-D em adenocarcinomas de pulmão e investigaram a associação destes com a presença de metástases linfonodais. Estes autores mostraram que VEGF-A e VEGF-C promovem metástases por mecanismos distintos, enquanto o último facilita a entrada das células tumorais na vasculatura linfática o primeiro estimula o crescimento tumoral através da

angiogênese. Entretanto, os níveis de VEGF-D foram inversamente associados com a presença de metástases linfonodais, sugerindo que baixos níveis de VEGF-D possa ter um importante papel na disseminação metastática.

Conforme dito anteriormente, a enzima lipogênica FASN encontra-se super-expressa em uma grande variedade de tumores humanos e é considerada um oncogene em potencial. Segundo Menendez & Lupu (2007), FASN não somente participa de vias de transdução de sinais, do metabolismo, regula a proliferação e favorece a sobrevivência de células tumorais, mas também modula o desenvolvimento, manutenção e progressão metastática das neoplasias malignas humanas. Browne *et al*. (2006) demonstraram que orlistat inibe FASN em células endoteliais de vasos sanguíneos humanas (HUVEC), bloqueando eficientemente a síntese de ácidos graxos e reduzindo a proliferação. Além disso, os mesmos autores observaram que o orlistat inibe a neovascularização em ensaio *ex vivo*, o que sugere fortemente uma atuação como droga anti-angiogênica, por prevenir a expressão do VEGFR-2 na superfície das células endoteliais. Por outro lado, em estudo realizado por Menendez *et al*. (2005a), a inibição específica da atividade de FASN com c75 ocasionou um aumento dose-dependente na produção de VEGF-A (maior do que 500%) em células de câncer de mama com alta expressão de ErbB2. Ao mesmo tempo, o bloqueio da atividade de FASN ativou substancialmente a via MAPK, causando um acúmulo de HIF-1α nestas mesmas células. O aumento da quantidade de VEGF, a ativação de MAPK e o acúmulo de

HIF-1α também foram observados quando o gene que codifica a FASN foi silenciado através de siRNA. Portanto, estes autores sugerem que a inibição do metabolismo endógeno de ácidos graxos nas células tumorais desencadeia uma reação semelhante ao que ocorre em condições de hipóxia, que ativamente recupera a cascata MAPK-HIF-1α -VEGF dependente de ErbB2.

Em trabalho realizado por nosso grupo (Carvalho *et al*., 2008), o tratamento de camundongos portadores de melanomas intraperitoneais (causados pela injeção de células B16-F10) com orlistat reduziu em 50% o número de metástases espontâneas para os linfonodos mediastínicos. Estes resultados indicam que a inibição da atividade de FAS tem um importante papel na disseminação metastática de melanomas de camundongos. Além disso, em trabalho recente realizado por nosso grupo foi observado que meios condicionados por células de melanoma humano tratadas com inibidores da FASN inibiu a formação de capilares *in vitro* em matrigel, efeito este mediado pela isoforma anti-angiogênica VEGF-A_{165b} (Seguin *et al*., 2012). Neste mesmo trabalho foi demonstrado que a inibição da FASN com cerulenina ou orlistat aumenta a expressão de RNA mensageiro de VEGF total e modula diferencialmente as isoformas angiogênicas e anti-angiogênicas deste fator. Como não há informações na literatura sobre uma possível relação entre FASN e o processo de linfangiogênese, o objetivo deste trabalho foi avaliar o papel biológico desta enzima em células de endotélio linfático estimuladas ou não por células de melanoma.

Objetivo

Avaliar o efeito da inibição farmacológica da FASN sobre a linfangiogênese através de modelos animais de melanoma e cultura de células endoteliais estimuladas ou não por células de melanoma.

Objetivos iniciais

1. Avaliar a expressão de RNAs mensageiro para VEGF-C e –D em células B16-F10 tratadas com cerulenina ou orlistat.
2. Avaliar os níveis da proteína VEGF-D secretadas por células B16-F10 tratadas com cerulenina, orlistat ou siRNA para FASN.
3. Verificar o efeito da inibição farmacológica da FASN com cerulenina ou orlistat sobre a viabilidade, proliferação e apoptose das céluals HDLEC.
4. Verificar os efeitos da inibição da FASN sobre o crescimento de capilares linfáticos através de culturas primárias tridimensionais *ex vivo*.
5. Avaliar os níveis das proteínas VEGF-C e -D secretadas por células de melanoma humano (SK-Mel-25) tratadas com cerulenina ou orlistat.
6. Estudar o efeito do meio condicionado por células SK-Mel-25 tratadas com cerulenina ou orlistat sobre a proliferação de células HDLEC.

Fatty acid synthase inhibitors alter lymphatic vessel architecture and modulate lymphangiogenic response of melanoma cells

Abstract

Fatty acid synthase (FASN) is overexpressed and associated with poor prognosis in several human cancers, including melanomas. We have previously shown that FASN inhibition with orlistat significantly reduces spontaneous lymph node metastasis following the intraperitoneal implantation of B16-F10 mouse melanoma cells in C57BL/6 mice. Here we sought to investigate the effects of FASN inhibitors on lymphatic vessels, the primary route of metastasis in melanomas. B16-F10 cells were injected intradermally in the ears of C57BL/6 mice and peritumoral lymphatics were evaluated by fluorescent microlymphangiography. The density of positive lymphatic vessels was significantly higher in orlistat-treated mice with and, at lesser extent, without melanomas, in comparison with their respective controls. Accordingly, the production of LYVE-1, PROX-1, and VEGFR-3 was enhanced in the tumor tissues from orlistat-treated animals. The volumes of metastatic lymph nodes were reduced in 39.25% in orlistat-treated mice as well as the amount of VEGF-C mRNAs. Similarly, inhibition of VEGF-C expression was observed in cultured B16-F10 cells treated with both cerulenin and orlistat. In contrast, VEGF-D expression and secretion was increased in B16-F10 cells treated with either drugs

or transfected with siRNAs specific for FASN. Finally, a significant reduction of the lymphatic cell extensions was observed in the spheroid *in vitro* assay with conditioned medium from both cerulenin- and orlistat-treated B16-F10 cells. Taken together, these results show that orlistat reduced the volume of lymph node metastases and affects lymphatic vessel architecture. Moreover, FASN inhibitors differentially modulate VEGF-C and D expression in mouse melanoma cells.

Abbreviations

FASN: Fatty acid synthase

LYVE-1: Lymphatic vessel endothelial hyaluronan receptor-1

PROX-1: Prospero homeobox protein-1

VEGFR-3: Vascular endothelial growth factor receptor-3

VEGF: Vascular endothelial growth factor receptor

siRNA: small interfering RNA

Keywords

Fatty acid synthase, lymphatic vessels, lymphangiogenesis, B16-F10, melanoma, orlistat.

Introduction

Human fatty acid synthase (FASN, EC2.3.1.85, 270-kDa) is the cytosolic enzyme that produces long-chain fatty acids from acetyl-CoA and malonyl-CoA [1, 2]. Physiologically, FASN is primarily expressed in hormone-sensitive cells and cells with high lipid metabolism [3]. In most normal cells *de novo* fatty acid synthesis is rarely needed, and FASN protein levels are usually low. In contrast in several human malignancies and some pre-neoplastic lesions, FASN expression has been described as up-regulated [4] and suggested as a prognostic marker [4-24]. FASN was originally identified as the oncogenic antigen-519 (OA-519) in breast cancer patients with markedly poor prognosis [25, 26]. The first experimental evidences of an oncogenic role for FASN came from its over-expression in HBL100 immortalized breast epithelial cells, which showed increased proliferation and survival, and anchorage-independent growth [27]. Similarly, human prostate epithelial cells (iPrECs) overexpressing both androgen receptor and FASN became tumorigenic, suggesting that the later acts as a prostate cancer oncoprotein by inhibiting apoptosis [28]. Interestingly, FASN pharmacological inhibition decreased tumor cell proliferation and elicit tumor cell death, preventing tumor growth in animal models [29]. Orlistat is a pancreatic lipase inhibitor originally developed as an anti-obesity drug that also irreversibly blocks FASN activity and shows both cytostatic and cytotoxic properties on malignant cells [30]. We have previously shown that FASN inhibition with orlistat significantly reduces the number of

spontaneous mediastinal lymph node metastasis from intraperitoneal B16-F10 mouse melanomas [31]. We also demonstrated that orlistat reduces the proliferation and promotes apoptosis through activation of the intrinsic pathway in B16-F10 cells, independent of p53 or mitochondrial permeability transition [31, 32]. Furthermore, FASN inhibition with orlistat reduces both the proliferation of human blood vessel endothelial cells and neovascularization in an *ex vivo* assay, suggesting anti-angiogenic abilities for this drug [33].

Lymphatic vessels regulate tissue fluid homeostasis, immune cell trafficking, and the absorption of dietary fats [34, 35]. In adult tissues, lymphangiogenesis is observed during wound healing, tumor growth and metastasis [36]. The main route for metastasis in several malignancies, such as those from breast, pancreas, stomach, colon, head and neck, prostate, lung, and melanomas, is via lymphatic drainage to regional lymph nodes [37, 38]. VEGF family members have a central role in both angiogenesis and lymphangiogenesis in physiological and pathological conditions such as tumor progression and metastasis [39]. VEGF-C and VEGF-D regulate lymphangiogenesis in inflammation and metastasis by affecting fluid dynamics, valve formation, and migration of vascular smooth muscle cells to the lymphatic collectors [40]. In fact, high expression of both factors was detected in several human malignancies, such as melanoma, adenocarcinoma of the lung, breast, colon, head and neck, and gastric cancers [37]. VEGF-C and VEGF-D are also clearly associated with lymphatic hyperplasia and intra and peritumoral

lymphatic formation, which may affect metastasis [41, 42]. Indeed, the expression of VEGF-C in cutaneous melanoma is positively associated with lymph node metastasis [43]. The tyrosine kinase receptor VEGFR-3 is also important for the lymphangiogenic response and its co-expression with VEGF-C or VEGF-D in melanomas and lung carcinomas was already described [44, 45]. The presence of lymph node metastasis is important for tumor staging and therapeutic decisions and the density of tumor-associated lymphatic vessels seems to correlate with the incidence of lymph node metastasis and poor prognosis in a variety of human cancers including melanomas [36, 37, 46, 47, 48].

As the role of FASN on the lymphatic network and tumor-induced lymphangiogenesis is still unknown, here we report that orlistat reduces the size of lymph node metastasis from experimental melanomas and affects the lymphatic vessel structure. Moreover, FASN inhibitors differentially modulate VEGF-C and D expression in mouse melanoma cells.

Materials and methods

Cell culture

B16-F10 cells were obtained from ATCC (American Type Culture Collection, Manassas, VA) and cultured in RPMI (Invitrogen, Carlsbad, CA) supplemented with 1 or 10% of fetal bovine serum (FBS, Cultilab, Campinas, SP, Brazil) and 1:100 antibiotic/antimycotic solution (Invitrogen) at 37^{o}C in a humidified atmosphere with 5% CO_2. hTERT-HDLEC human lymphatic endothelial cells [49]

were cultured in Endothelial Basal Medium-2 (EBM™-2, Lonza, USA) supplemented with single quots (Lonza), 5% of FBS, and 1:500 penicillin-streptomycin (Invitrogen). For the cell culture experiments, orlistat (Xenical, Roche, Switzerland) was extracted from capsules according to Knowles et al. [50] and used to inhibit FASN. The antibiotic cerulenin (Sigma-Aldrich, St Louis, MO, USA), a natural product that irreversibly inhibits FASN [51] was used to confirm the effects of orlistat.

Animal models for the study of lymphatic vessels

The studies were previously approved by the Committee for Ethics in Animal Research of the State University of Campinas (Protocol 1934-1). For the hole punch assay, Balb-C mice were anesthetized with ketamine/xylazine (10/100 mg/kg i.p.) and a hole (1.5 mm of diameter) was made in the center of both ears by using a metal ear punch (Golgran, Brazil). The animals were treated for 25 days with i.p. injections of orlistat (Roche, 240 mg/kg) or the equivalent amount of vehicle (33% ethanol in PBS) according to Kridel et al. [30] and sacrificed by cervical dislocation. For the analysis of peritumoral lymphatic vessels in primary tumors, and lymph nodes metastases, 10^5 B16-F10 cells resuspended in 5 µl of PBS were injected intradermally in each ear of C57BL/6 mice with 5-8 weeks of age. C57BL/6 mice without surgical wounds or melanomas were also treated and sacrificed as described above. The number of animals and experimental groups are shown in the Table 1. Functional lymphatics were evaluated by fluorescent

microlymphangiography as described below. Metastatic lymph nodes were blackened and therefore easily detected and collected. The volumes of the primary tumors and cervical metastatic lymph nodes were calculated by using the formula volume=width (mm) x length (mm)/2. Samples of the primary tumors, lymph nodes, and livers were collected and immediately frozen in liquid nitrogen for total RNA and protein extractions or Oil red O staining [52]. Experiments were made, at least, 3 times independently.

Fluorescent microlymphangiography

Functional lymphatics were visualized as described elsewhere [53], after the injection of 2 µl of lysine-fixable FITC-dextran (2,000 kDa, Molecular Probes, Eugene, OR, USA). Photomicrographies were performed in a Leica DMR microscope with epifluorescence (Leica Microsystems, Germany) and images with identical conditions of light, contrast, and magnification were analized with the Scion Image software (Scion Corporation, USA).

Western blotting

Western blotting reactions were performed as previously described [31] with anti FASN (Transduction Laboratories, Lexington, KY), LYVE-1, PROX-1, and VEGFR-3 (Reliatech, Germany) primary antibodies diluted at 1:3,000, 1:200, 1:1,000 and 1:200 respectively. Antibodies against mouse β-actin (1:60,000, Sigma-Aldrich, USA) were used as the loading control.

Quantitative RT-PCR (qRT-PCR)

Total RNA was extracted from tumor tissues or cell pellets using the Trizol reagent (Invitrogen) according to the manufacturer's instructions. All RNA samples were treated with 1 U of DNAse I (amplification grade, Invitrogen) for 10 min at room temperature in order to eliminate genomic DNA contamination. cDNAs were synthesized from 1 - 3 µg of total RNA using the First-Strand cDNA Synthesis SuperScripT II RT (Invitrogen). qRT-PCR was conducted using SYBR® Green Real-time PCR Master Mix (Applied Biosystems, Warrington, UK). The cycling conditions were 95 ^{o}C for 10 min, and 40 cycles of 95 ^{o}C for 15 s and 57 ^{o}C for 1 min in a StepOne Plus Real-Time PCR System (Applied Biosystems). Primer sequences are listed in Table 2 and the relative gene expression determined by the $2^{-\Delta\Delta}$CT method with the aid of the SDS software version 2.0.

siRNA-silencing of FASN expression

The 25-mer RNA molecules were synthesized, annealed, and purified by the manufacturer (Stealth RNAi, Invitrogen). The following sequences targeting *Mus musculus* FASN (NM_00798) were used: 5' CAA TGA TGG CCA ACC GGC TCT CTT T 3'), 5' TGG GAA GAC CCG AAC TCC AAG TTA T 3', and 5' CCT CTG GGC ATG GCT ATC TTC TTG A 3'. B16-F10 cells grown to 50% confluence were transfected with 200 nM of a mixture containing equal parts of specific siRNAs using a liposome method according to manufacturer's instructions (Lipofectamine 2000, 3 µl/ml, Invitrogen). As negative controls, cells were transfected with

equimolar concentrations of a nonspecific control oligo (Stealth RNAi Negative Control Duplexes, Medium GC Duplex, Invitrogen). FASN knockdown was assessed by western blotting after 48 hours.

Spheroid assay

The *in vitro* lymphangiogenesis assay with spheroids was performed as described by Hammer et al. [54]. Briefly, spheroids were generated by seeding 2 X 10^3 hTERT-HDLEC cells in each well of 96-well nonadherent culture plates with round botton (Greiner, Frickenhausen, Germany) in reduced EBM-2 medium (without growth factors) containing 1% FBS and 0.24% high viscosity methylcellulose (Sigma). After 24 hours at 37°C and 5% CO_2, cells from each well formed spheroids that were collected and embedded into 600 µl of collagen gels. The collagen (BD Biosciences, DK) stock solution was prepared according the manufacturer, mixed 1:1 with 1.2% methylcellulose dissolved in reduced medium. Spheroids were them seeded in 24 well plates and incubated at 37°C in 5% CO_2 in the presence of conditioned medium obtained from B16-F10 cells treated or not with cerulenin or orlistat. After 24 hours, the spheroid sprouting/migration was visualized and photographed in a phase-contrast microscope (Leica Microsystems, Germany). The total number of cells that migrate from each spheroid was counted with the aid of the Scion Image software (Scion Corporation, USA). Filopodia-like cell extensions (0, 1, 2, 3 or more) were counted in cells sprouting out of the spheroids.

Conditioned media and ELISA assays

Conditioned media were obtained by seeding 30 X 10^4 B16-F10 cells in 100 mm culture dishes with complete growth medium. After 24 hours, medium was changed by fresh medium containing 50, 100, or 300 µM of orlistat supplemented with 1% FBS, wich was collected after additional 24 hours. Cells and debris were removed by centrifugation at 1,100 Xg for 3 min, and conditioned medium (30% conditioned medium from B16-F10 cells and 70% fresh medium supplemented with 1% of FBS) used to treat the spheroids (8-10 spheroids/well, 2 wells for each condition). Migration of endothelial cells was evaluated after 24 hours. The concentration of VEGF-D in the conditioned media was determined by the VEGF-D enzyme-linked immunosorbent assay (Mouse VEGF-D DuoSet ELISA, R&D Systems, Minneapolis, MN) following the manufacturer's instructions.

Statisical Analysis

Statistical significance between the control and experimental groups was determined by Student's t-test. Error bars for all graphs represent standard deviation.

Results

FASN inhibition reduces the size of lymph node metastases in experimental melanomas

Once injected intradermally in the C57BL/6 mice ears, B16-F10 cells develop primary tumors and lymph node metastases in approximately one week and 20 days, respectively (Fig. 1a, b). The most frequent pattern of metastatic spread was to the cervical lymph nodes, bilaterally (Fig. 1b). Periauricular lymph nodes were also detected in both groups, and two of the control animals showed axillary lymph node metastasis. The volumes of primary tumors or the number of lymph node metastasis were calculated and no differences between the control and orlistat-treated groups detected (data not shown). Importantly, the cervical lymph node metastases from the orlistat-treated mice were 39.25% smaller than from the control animals ($p<0.05$, Student's *t*-test) (Fig. 1c). Histologically, the primary tumors from the both groups were indistinguishable and characterized by malignant melanocytes with hyperchromatic nuclei, multiple nucleoli, cytoplasmic melanin accumulation, and atypical mitoses, generally adjacent to the ear cartilage (Fig. 1d). The lymph node metastases were microscopically confirmed and characterized by the peripheral infiltration of B16-F10 cells (Fig. 1e). As depicted in Fig. 1f and g, the effectiveness of the systemic FASN inhibition with orlistat was demonstrated in liver section stained with Oil red O, in which the hepatocytes from control mice were rich in large lipid droplets, in contrast with the weakly stained cells from the orlistat-treated group.

Orlistat affects lymphatic vessel architecture

In order to check whether the treatment with orlistat affects the lymphatic vasculature, we first examined the lymphatic network at the periphery of the surgical wounds performed in Balb-C mice ears. Twenty five days after the beginning of the treatment with orlistat, fluorescent dextran microlymphangiographies revealed more positive lymphatic vessels in the treated than in control mice (Fig. 2a, b). Similar observations were made in B16-F10 peritumoral lymphatics, that after the treatment with orlistat were clearly more branched and thick than the controls (Fig. 2d, e). Although in a lesser extent, the lymphatic vessel density was also higher in the ears of orlistat-treated mice without surgical wounds or experimental melanomas, suggesting that the drug systemically changes lymphatic vessel architecture (Fig. 2g, h). The analysis of lymphatic vessel densities with the image software clearly showed higher lymphatic vessel density in the orlistat-treated mice from the three experimental models (Fig. 2c, f, i). The studied animals showed no significant physical or behavioral alterations during the period of treatment.

Effects of orlistat on FASN, LYVE-1, PROX-1, and VEGFR-3 production by experimental melanomas

The association between FASN expression and/or activity and lymphatic molecular markers is still largely unknown. As depicted in Fig. 3a and b, the intensities of FASN, LYVE-1, PROX-1, and VEGFR-3 protein bands were increased in the primary tumors of orlistat-treated mice, which was confirmed by densitometric

analysis (Fig. 3c-f). No significant differences were observed in the metastatic lymph node tissues (data not shown). Taken together, these results suggest an increase in the intratumoral lymphatic vasculature.

Effects of FASN inhibitors on the expression of VEGF-C and VEGF-D

In order to verify a possible role of FASN inhibitors on melanoma-induced lymphangiogenesis, we next analyzed the expression of VEGF-C and VEGF-D in both experimental models and cultured B16-F10 cells treated or not with cerulenin or orlistat. As depicted in Fig. 4a and b, orlistat did not affect the expression of VEGF-D, but decreased VEGF-C levels in experimental lymph node metastasis. Interestingly, the transcripts for both growth factors in the primary tumors were not affected by this drug (data not shown). Cerulenin increased VEGF-D expression by B16-F10 cells in all tested concentrations (Fig. 4c) and orlistat reduced VEGF-C at 100 and 300 μM after 24 and 48 hours, respectively (Fig. 4d, e). A slight but not statistically significant increase of VEGF-D was observed with orlistat at 24 hours. Enhanced secretion of VEGF-D by cerulenin- and orlistat-treated B16-F10 cells was also verified by ELISA (Fig. 4f, g). These observations were further confirmed by the FASN knockdown with specific siRNAs (Fig. 4h, i).

Conditioned media from B16-F10 melanoma cells treated with cerulenin or orlistat reduce the filopodia-like cell extensions by cultured lymphatic endothelial cells

In order to better understand the effects of FASN inhibitors on the lymphatic vasculature, we next used conditioned medium from B16-F10 cells to incubate

hTERT-HDLEC cells in the spheroid assay. These studies showed that conditioned medium from cerulenin- (Fig. 5a, c, e) or orlistat-treated (Fig. 5b, d, f) B16-F10 cells does not affect lymphatic endothelial cell migration in comparison with the respective controls. Importantly therefore, we found that the number of cells without filopodia-like cell extensions was significantly higher in the presence of cerulenin or orlistat while the number of cells with 3 or more of these structures was higher in the controls (Fig. 5g, h).

Discussion

FASN has been suggested as a metabolic oncoprotein and a potential therapeutic target for human cancers. Normal cells utilize dietary fat for their metabolism and FASN activity is low or absent in most normal adult human tissues [55, 56]. In contrast, high FASN levels have been found in a variety of human cancers, which are associated with poor prognosis in several of these malignancies [4].

FASN inhibitors induce apoptosis in several cell cancer lines and restrain tumor growth in xenografic models [4], however, the biological mechanisms responsible for these abilities requires further investigation. Previous studies from our laboratory suggest that FASN activity is essential for melanoma and oral cancer progression, since its pharmacological inhibition reduces metastasis in both experimental intraperitoneal melanomas and orthotopic tongue squamous cell carcinomas [31, unpublished data]. Here we show that the size of lymph node metastases from experimental cutaneous melanomas is significantly reduced by

the treatment with orlistat. It is possible to speculate from these results that FASN may play a role in tumor angiogenesis and/or lymphangiogenesis. In fact, Browne et al. [33] demonstrated that orlistat reduces the proliferation of human endothelial cells (HUVEC) and inhibits neovascularization in an *ex vivo* assay. Seguin et al. [57] recently showed that FASN inhibitors reduced the viability, proliferation, and the formation of capillary-like structures in matrigel by rabbit aortic endothelial cells (RAEC), as well as the tumor-cell mediated development of human umbilical vein endothelial cells (HUVEC) capillary-like structures.

Since lymphatic vessels are the primary route of metastasis in melanoma [37], here we investigated the effects of FASN inhibition on lymphatic vessels and tumor-induced lymphangiogenesis. Our results provide the first demonstration that systemic FASN inhibition with orlistat affects lymphatic architecture, as the number of lymphatic endothelial cell extensions was clearly decreased following cell incubation with conditioned medium from orlistat- or cerulenin-treated B16-F10 melanoma cells. Filopodia are essential for endothelial cell migration and formation of the lymphatic lumen [58]. Indeed, filopodial outgrowth depends on actin polymerization at specific glycerophospholipids composed by cell membrane fatty acids with 16-20 carbons (PtdIns (4,5)P2 and (3,4,5)P3), that are critical for the membrane-cytoskeleton interactions via activation of the PI3K pathway [59, 60]. Moreover, it is well established that FASN affects the synthesis of phospholipids incorporated into the lipids rafts, detergent-resistant microdomains which act as

platforms for signal transduction, including via PI3K [61]. Despite the fact that the involvement of FASN in filopodia outgrowth is largely unknown, our results suggest that FASN inhibitors may change lymphatic endothelial cell-to-cell or cell-to-matrix contacts and probably increase lymphatic permeability.

The treatment with orlistat did not affect VEGF-D expression in our primary melanomas and their metastases, however, decreased VEGF-C mRNAs were found in the latter and in orlistat-treated B16-F10 cells. VEGF-C stimulates the proliferation and initial migration of lymphatic endothelial cells through VEGFR-2 and/or VEGFR-3 stimulation [41, 63] and changes lymphatic structure by inducing filopodial sprouting and outgrowth [62]. In addition, lymphatic vessel integrity and filopodial outgrowth seem to be dependent of VEGFR-3 activation, which is found in the filopodial extensions on the tip of the sprouting front [36, 58, 63, 64]. Interestingly, here we observed that LYVE-1, PROX-1, and VEGFR-3 protein levels were enhanced in the primary tumor tissues from orlistat-treated mice, which may reflect intratumoral lymphangiogenesis. In fact, Leu et al. [65] observed no functional lymphatic vessels in murine sarcomas induced by fibrosarcoma cells (FSaII) that express VEGF-C and VEGFR-3, possibly due to the collapse induced by the continuously growing cancer cells. On the other hand, our studies show that FASN inhibition with cerulenin or orlistat and its knockdown with specific siRNAs increase VEGF-D expression and secretion by B16-F10 cells. Indeed, we have previously observed that FASN inhibition stimulates the production of total VEGFA

in both B16-F10 and SK-MEL-25 human melanoma cells, as well as of the anti-angiogenic isoform $VEGF_{165b}$ in the latter [57]. Accordingly, Menendez et al. [66] showed that the inhibition of FASN with C75 results in HIF-1α accumulation and a dose-dependent increase in the VEGF-A production in breast cancer cells with high expression of ErbB2, in a hypoxia-like response. In fact, hypoxia enhances VEGF-D and VEGFR-3 mRNAs in vein endothelial cells and regulates VEGF-D activity in smooth muscle cells [67, 68]. In breast cancer, VEGF-D expression was associated with HIF-1α [69, 70] and hypoxia related with lymphangiogenesis, lymphatic differentiation, and VEGF-A, VEGF-C, and VEGF-D expression [71]. Moreover, antibodies against VEGFR-3 inhibit hypoxia-induced lymph node metastases in orthotopic model of cervix cancer [71].

In summary, the studies here presented show that orlistat reduces the size of lymph node metastasis from B16-F10 experimental melanomas. FASN inhibitors also modify lymphatic vessel architecture and decreasing in the number of filopodia-like cell extensions in endothelial lymphatic cells and decrease VEGF-C expression in both metastatic lymph nodes and cultured melanoma cells. Finally, FASN inhibitors differentially modulate VEGF-C and -D expression in cultured mouse melanoma cells.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Table 1 Number of animals in each experimental groups

	Number of animals (# of experiments)	
	Control groups	Experimental groups
Balb-C (hole punch assay)	45 (3)	45 (3)
C57Bl/6 (peritumoral FM)	15 (3)	15 (3)
C57Bl/6 (FM)	15 (3)	15 (3)
C57Bl/6 (T/ LN volumes)	40 (4)	40 (4)

FM: Fluorescent Microlymphangiography; T: primary tumor; LN: lymph node metastasis

Table 2 Primers sequences for the amplification of mouse VEGF-C, VEGF-D, VEGFR-3, and GAPDH

Gene	Sequence	Accession number
Mouse VEGF-C	Forward: 5' AGG CCC CAA ACC AGT AAC AA 3' Reverse: 5' AGA CAT GCA TCG GCA GGA A 3'	NM_009506
Mouse VEGF-D	Forward: 5' GCC AGC CTG TGG AAA GCA 3' Reverse: 5' AGT CCC TGG GCC CTT GTC 3'	NM_010216
Mouse GAPDH	Forward: 5' CAT GGC CTT CCG TGT TCC TA 3' Reverse: 5' GCG GCA CGT CAG ATC CA 3'	NM_008084.2

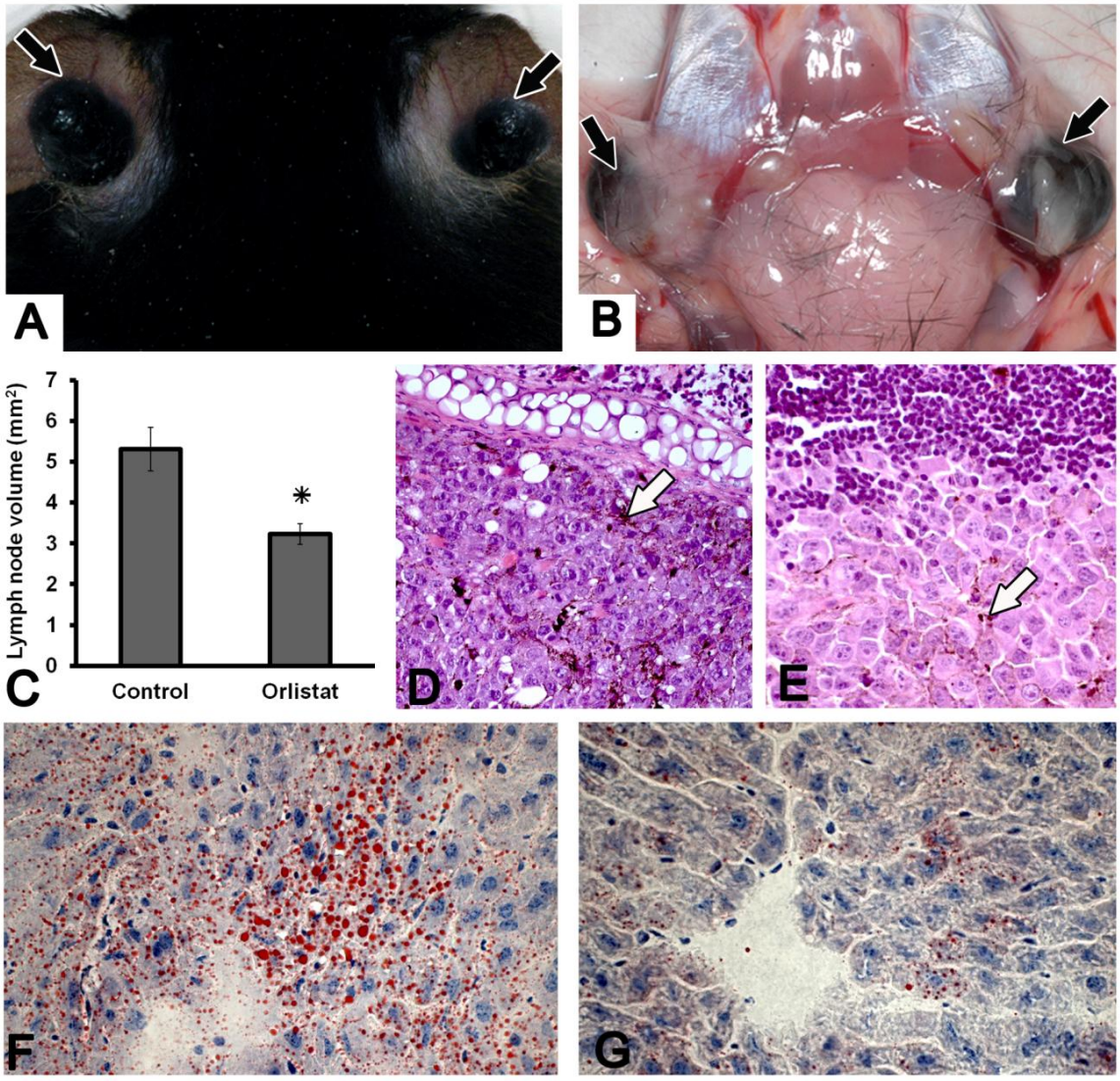

Fig. 1 Experimental B16-F10 melanomas. **(a)** Representative tumors *(arrows)* grown after bilateral intradermal inoculation of B16-F10 cells in the ears of C57BL/6 mice. **(b)** Bilateral metastases in the cervical lymph nodes *(arrows)* of the same animal shown in **(a)**. **(c)** The treatment with orlistat significantly reduced the size of lymph node metastases in the experimental groups. Hematoxylin-eosin stained sections from one of the primary tumors shown in **(a)** demonstrate highly pleomorphic cells and intense melanin accumulation *(arrow)* adjacent to the ear cartilage **(d)**. Melanin-rich metastatic cells *(arrow)* were observed at the periphery of the affected lymph nodes **(e)**. Representative Oil red O staining of a frozen section from the liver of a control animal showing many large lipid droplets (red) **(f)** in contrast with the sample from an orlistat-treated mice **(g)** (*d-g original magnification 400X*; Student's t-test)

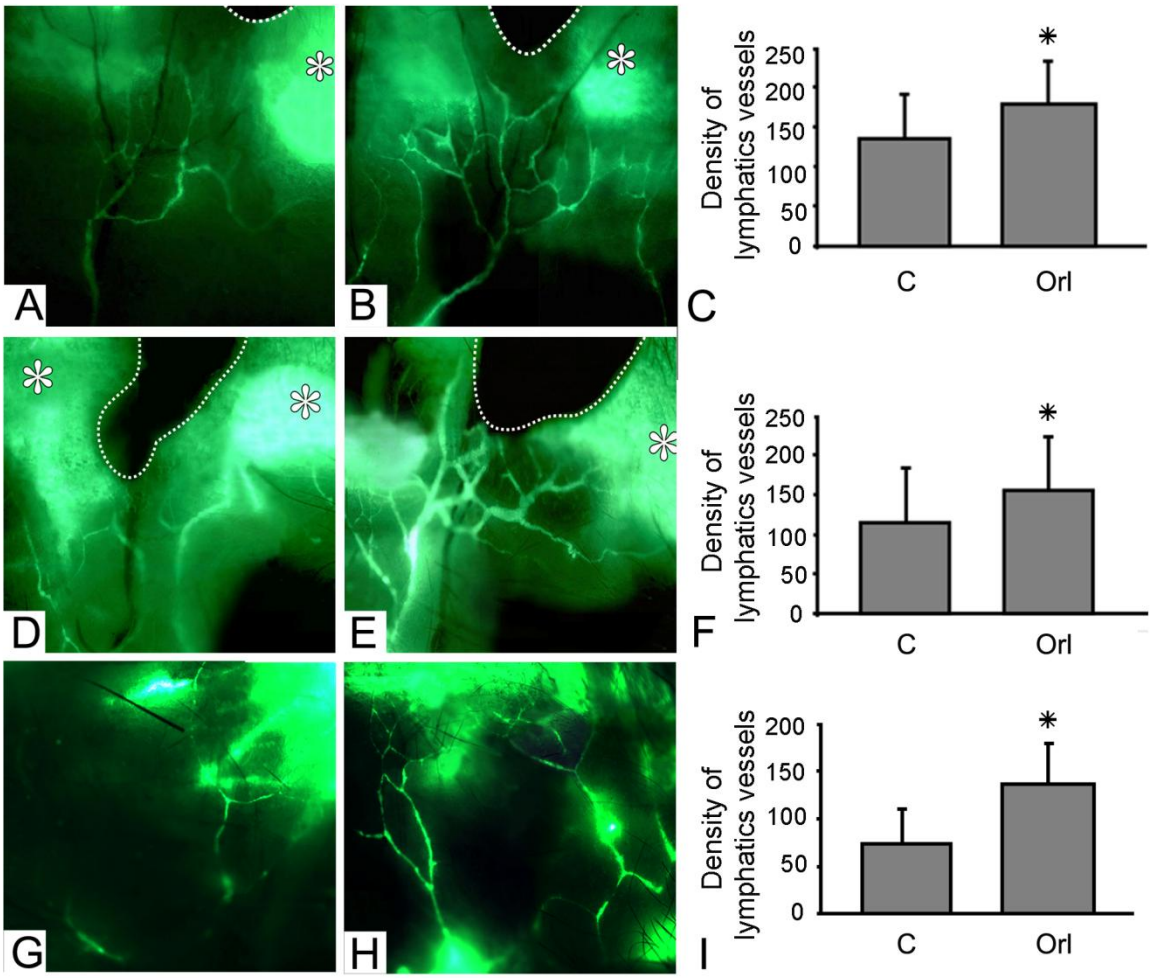

Fig. 2 After the systemic treatment with orlistat, fluorescent dextran microlymphangiography was performed in 3 distinct experiments: 25 days after the preparation of a 1.5 mm surgical wound in the central portion of the ears of Balb-C mice **(a, b)**, 20 days following the inoculation of B16-F10 melanoma cells in the ears of C57BL/6 mice **(d, e)**, and in the ears of mice without surgical wounds or experimental tumors **(g, h)**. The lymphatic vessel networks were clearly more branched and dense in orlistat-treated mice **(b, e, h)** than in control animals **(a, d, g**). Lymphatic vessel densities were calculated with the aid of an image software in predeterminated circular areas and confirmed the significant increased in treated animals **(c, f, i)**. *Asterisks* show the site of FITC-dextran injections; *doted lines* delimit the area of the surgical wounds or experimental tumors; * *p<0.05*, Student's t-test; C: ethanol control; Orl: orlistat

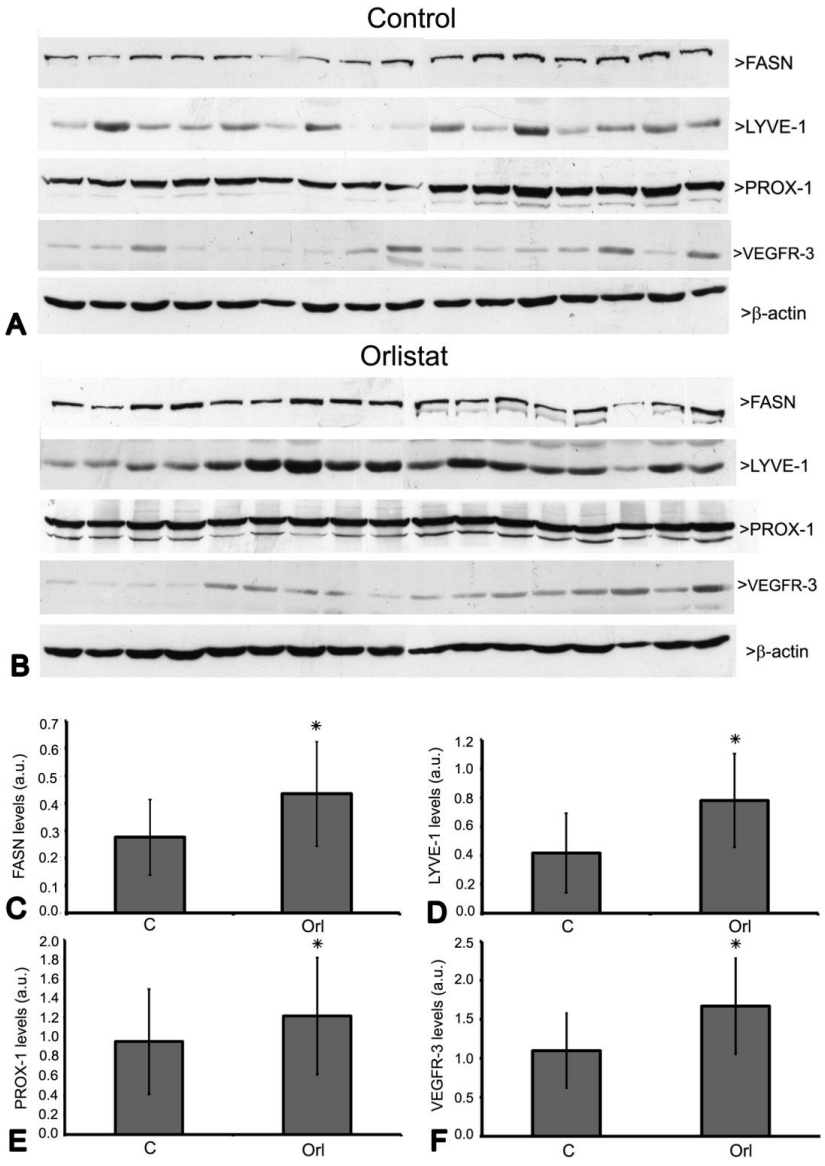

Fig. 3 Representative western blotting reactions for FASN, LYVE-1, PROX-1, and VEGFR-3 in protein lysates obtained from the experimental primary melanomas **(a, b)**. Anti-β-actin antibodies were used as the loading controls. Densitometric analyses demonstrate an increase in the amount of these proteins within the tumor tissues **(c-f)** (*$^{*}p<0.05$*, Student's t-test; C: ethanol control; Orl: orlistat)

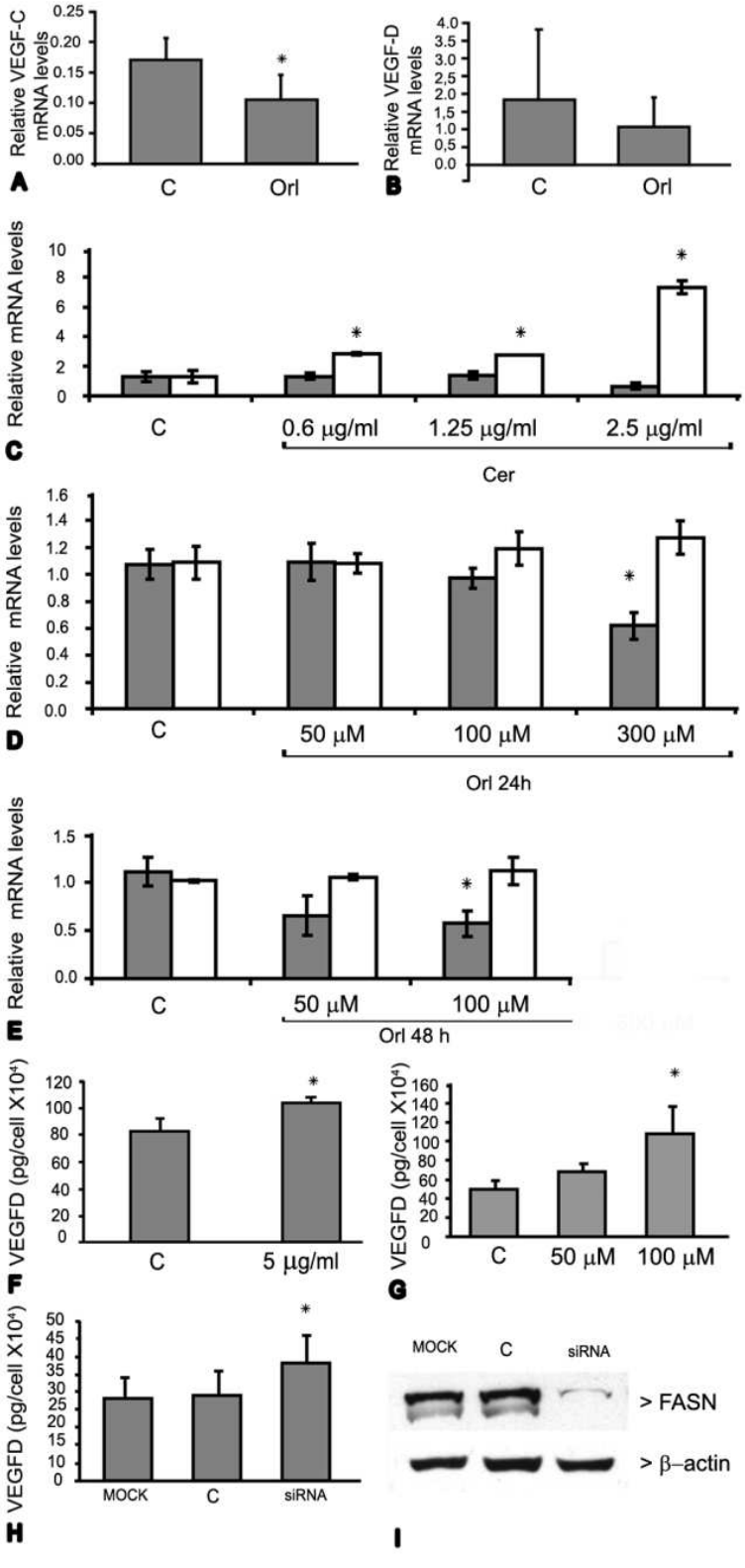

Fig. 4 qRT-PCR analysis of VEGF-C **(a)** and VEGF-D **(b)** in metastatic lymph nodes shows that VEGF-C is significantly decreased by the treatment with orlistat. **(c-e)** Relative expression of VEGF-C *(gray bars)* and VEGF-D *(white bars)* in B16-F10 mouse melanoma cells treated with cerulenin **(c)** or orlistat for 24 **(d)** and 48 hours **(e)**. Cerulenin increases VEGF-D expression in all tested concentrations while orlistat reduced VEGF-C at 300 µM after 24 hours and at 100 and 300 µM after 48 hours. A slight increase of VEGF-D is observed with orlistat at 24 hours. The highest concentration of orlistat for the 48 hours period was cytotoxic. VEGF-D concentration was significantly increased in the conditioned medium from both cerulenin or orlistat-treated B16-F10 cells as well as after transfection with siRNAs specific for FASN **(f, g, h)**. **(i)** Representative western blotting reaction confirming FASN knockdown in the siRNA-transfected B16-F10 cells lysates. (C: ethanol or DMSO controls; Orl: orlistat; Cer: cerulenin; **p<0.05*, Student's t-test)

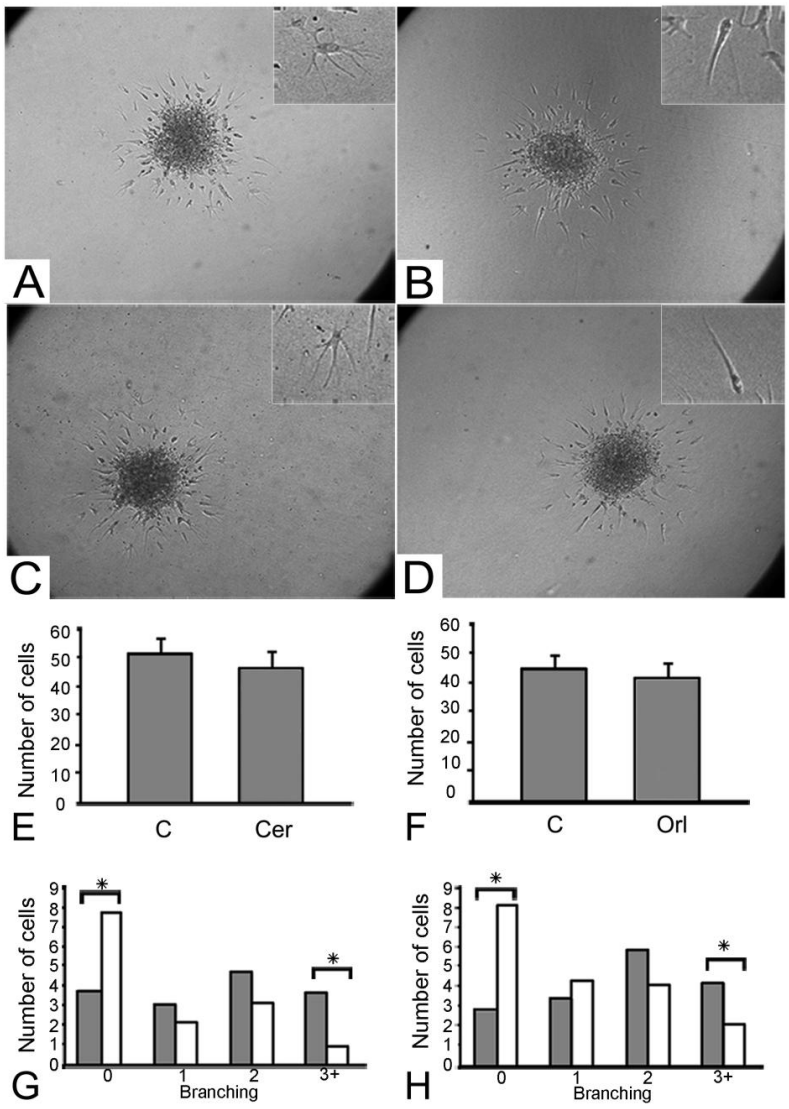

Fig. 5 Conditioned medium from B16-F10 melanoma cells treated with cerulenin or orlistat does not inhibit the lateral endothelial cell migration **(a-f)**, however, reduces the growth of filopodia-like cell extension in the spheroid migration assay with hTert-HDLEC cells **(g, h)**. Representative phase contrast photomicrographies of the spheroids in the presence of control media **(a, b)** or conditioned media from B16-F10 cells treated with cerulenin **(c)** or orlistat **(d)**. The number of cells without filopodia-like cell extension (*detail* **a, c**) is significantly enhanced in the presence of conditioned media from B16-F10 cells treated with both FASN inhibitors *(white bars)* while the number of cells with 3 or more cell extension (*detail* **b, d**) is higher in the presence of the control media **(g, h)** (*gray bars:* control media; $^{*}p<0.05$, Student's t-test)

Fatty acid synthase inhibitors reduce lymphatic endothelial cell proliferation, migration, and lymphangiogenesis in an ex vivo model.

Abstract

Fatty acid synthase (FASN) activity is essential for endogenous lipogenesis in cancer cells. Human malignancies, such as melanoma, highly express FASN which is associated with tumor invasion and poor prognosis. Cerulenin and orlistat inhibit FASN activity and have been described as potential anti-cancer agents. We have previously shown that FASN activity is required for melanoma and oral squamous cell carcinoma progression, as its pharmacological inhibition reduces lymphatic metastasis in experimental intraperitoneal and subcutaneous melanomas and orthotopic tongue squamous cell carcinomas. In addition, we demonstrated that orlistat reduces the proliferation and promotes apoptosis through activation of the intrinsic pathway in B16-F10 mouse melanoma cells. The role of FASN in the lymphatic vasculature is largely unknown. Here we show that both cerulenin and orlistat decrease the viability, proliferation, and migration of human lymphatic endothelial cells (HDLEC), as well as lymphatic capillary formation in a murine ex vivo assay. Our present studies that conditioned media from orlistat-treated human melanoma cells significantly decrease HDLEC proliferation. Taken together, the results here presented suggest that FASN

inhibitors act directly on lymphatic endothelial cells and through melanoma cell stimulation in order to provide an anti-lymphangiogenic phenotype.

Key words

Fatty acid synthase; metastasis; lymphangiogenesis; orlistat; cerulenin, melanoma.

Introduction

Human fatty acid synthase (FASN, EC2.3.1.85) is a 270-kDa cytosolic enzyme that endogenously synthesizes long-chain fatty acids from acetyl-CoA and malonyl-CoA [1,2]. FASN is minimally expressed in most normal human tissues, except in the liver, lactating breast, fetal lung and adipose tissue [3,4]. Importantly, this enzyme has been considered a metabolic oncoprotein highly expressed in malignant cells and a potential target for the treatment of several human cancers [5,6]. FASN is associated with poor prognosis in several types of human cancers, including melanomas [7-27]. Cancer cells seem to be dependent on the de novo fatty acid synthesis for proliferation and survival, as these molecules are essential for both cell membrane construction and generation of lipid signaling molecules. Pharmacological inhibition of endogenous lipogenesis through FASN inactivation is able to block cell cycle progression and to increase apoptosis in cells derived from prostate, breast, stomach, colon, endometrial, oral cavity, and ovarian cancers, as well as melanoma [28-36]. To date, several compounds are known to inhibit FASN, as cerulenin, C75, orlistat, C93, and epigallocatechin-3-gallate (EGCG) [37]. Cerulenin induces apoptosis in breast cancer cell lines [28, 38-39], delays disease

progression in an ovarian cancer xenograft model [9], as well as suppresses liver metastasis in a colon cancer xenograft model [40]. Orlistat (tetrahydrolipstatin) shows cytostatic and cytotoxic effects on tumor cells, as demonstrated by its antiproliferative activity in prostate and breast cancer cell lines and growth inhibitory and pro-apoptotic effects on xenograft prostate tumors [41]. Moreover, orlistat increases the survival rates of gastric tumor-bearing mice [42].

Previous studies from our laboratory suggest that FASN activity is essential for melanoma and oral cancer progression, since its pharmacological inhibition reduces metastasis in experimental intraperitoneal and subcutaneous melanomas and orthotopic tongue squamous cell carcinomas [43-45]. Despite the apparent marginal role of FASN in normal cells, orlistat is able to inhibit blood vessel endothelial cell proliferation and angiogenesis [46,47].

Lymphatic vessels are the main route for metastasis in several malignancies, such as those from breast, pancreas, stomach, colon, head and neck, prostate, lung, and melanomas [48,49]. Vascular endothelial growth factor (VEGF) family members have a central role in both angiogenesis and lymphangiogenesis under physiological and pathological conditions [50]. VEGF-C and VEGF-D regulate lymphangiogenesis and are overexpressed in several human malignancies, such as melanoma, adenocarcinomas of the lung, and breast, colon, head and neck, and gastric cancers [48]. The expression of VEGF-C in cutaneous melanoma is associated with lymph node metastases [51] and vascular endothelial growth factor

receptor-3 (VEGFR-3) co-expressed with VEGF-C and VEGF-D in melanomas and non-small cell lung carcinomas [52,53]. Moreover, the density of tumor-associated lymphatic vessels is also associated with lymph node metastasis and poor prognosis in a variety of human cancers, including melanomas [48, 54-56].

As the role of FASN on the lymphatic network and tumor-induced lymphangiogenesis is still unknown, here we show that FASN inhibitors decrease the viability, proliferation, and the migration of cultured human lymphatic endothelial cells (HDLEC) as well as lymphangiogenesis in an ex vivo assay. Moreover, conditioned media from orlistat-treated human melanoma cells significantly decrease HDLEC proliferation, further suggesting that this drug induces an anti-lymphangiogenic phenotype in cancer cells.

Materials and Methods

Cell culture

B16-F10 murine melanoma cells and SK-Mel-25 human melanoma cells (ATCC, VA, USA) were maintained in RPMI (Invitrogen, CA, USA) with 10% of FBS (Cultilab, Brazil). Human oral squamous carcinoma cells (SCC-9, ATCC) were grown in DMEM/F-12 (Invitrogen) with 10% FBS and 400 ng/ml hydrocortisone. hTERT-HDLEC human lymphatic endothelial cells [57] were cultured in Endothelial Basal Medium-2 (EBM™-2, Lonza, MD, USA) supplemented with single quots (Lonza), 5% of FBS, and 1:500 penicillin-streptomycin (Invitrogen). Primary human dermal lymphatic endothelial cells (HDLEC, Promocell, Heidelberg, Germany) were

cultured in endothelial cell growth medium MV2 (Promocell). Cell lines were cultured with or without antibiotic/antimycotic solution (Invitrogen) at 37°C in a humidified atmosphere with 5% CO_2. Orlistat (Roche, Switzerland), prepared as described elsewhere [58], or cerulenin (Sigma-Aldrich, MO, USA) were added to the cell culture medium at the concentrations described in the figure legends.

Cell viability, proliferation, and apoptosis

Cell viability was determined by seeding of 2 X 10^5 HDLEC cells in 6-well culture plates with 3(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT, Sigma) according to the manufacturer's instructions. For the cell cycle and apoptosis analyses, HDLEC cells were seeded in T-25 culture flasks (4 X 10^5cells) and serum starvation for 24 h performed for the cell cycle analysis. The medium was replaced by fresh medium containing FASN inhibitors or medium previously conditioned by SK-Mel-25 cells and HDLEC cells incubated for additional 24 h. Cells were then collected, and cell cycle or apoptosis analyzed as previously described [59]. All experiments were performed 3 times independently.

Spheroid assay

The in vitro lymphangiogenesis assay with spheroids was performed as described by Hammer et al. [60]. Briefly, spheroids were generated by seeding 2 X 10^3 hTERT-HDLEC cells in each well of 96-well nonadherent culture plates with round botton (Greiner, Frickenhausen, Germany) in reduced EBM-2 medium (without growth factors) containing 1% of FBS and 0.24% of high viscosity methylcellulose

(Sigma). After 24 h at 37°C and 5% CO_2, cells from each well formed spheroids that were collected and embedded into 600 µl of type I collagen gels and seeded in 24 well plates. The type I collagen from rat tail (BD Biosciences, DK) was prepared according the manufacturer and mixed 1:1 with 1.2% methylcellulose dissolved in reduced medium. FASN inhibitors were added in both collagen gels and the cell culture medium. After 24 h, the spheroid sprouting/migration was visualized and photographed in a phase-contrast microscope (Leica Microsystems, Germany). The total number of cells that migrate from each spheroid was counted with the aid of the Scion Image software (Scion Corporation, USA).

Lymphatic ring assay

Tridimensional primary cultures of lymphatic mouse endothelial cells were performed according Bruyère et al. [61]. Briefly, thoracic ducts were removed from female C57Bl/6 mice with 4-8 weeks of age by microsurgery and cut into 1-mm pieces. The explants were embedded between two layers of type I collagen (2 mg/ml; Serva Electrophoresis, Germany) and cultured for 11 days in MCDB-131 (Invitrogen) supplemented with 4% Ultroser G (BioSepra, USA), 25 mM of NaHCO3, 1% of glutamine, 100 U/ml of penicillin and 100mg/ml of streptomycin. Cultures were maintained at 37°C in a humidified incubator (HERAcell 150; Heraeus, Germany) under reduced oxygen condition (5% O_2, 5% CO_2, and 90% N_2). The rings were photographed each 2 days and the image analysis performed at day 10. For quantification, a grid of concentric circles was generated with the aid

of the Scion Image software (Scion Corporation, USA) by successive increments at fixed intervals from the thoracic duct boundary and the number of microvessel-grid intersections counted and plotted according to the distance from the ring. At least 5 images per experimental condition were used. To determine significant differences between experimental conditions, values obtained from 0.15 to 0.75 mm were compared. On day 11, lymphatic rings were harvested from the agar cylinder, washed for 30 min in PBS, fixed for 30 min with 70 % ethanol and used for alpha smooth muscle actin (α-SMA) immunostaining [61]. All experiments were approved by the Committee for Ethics in Animal Research of the State University of Campinas (Protocol 1934-1).

Protein extraction and western blots

Protein lysates and western blotting reactions were done as previously described [43] with antibodies against FASN (Transduction Laboratories, Lexington, KY, USA) diluted at 1:3,000. Antibodies against mouse β-actin (1:30,000, Sigma-Aldrich, USA) were used as the loading control.

Effects of FASN inhibitors on the expression of VEGFR-3

Orlistat- or cerulenin-treated HDLEC cells were immunostained with anti-VEGFR-3 antibodies (Reliatech, Germany). Culture dishes (150 mm) received 1 x 106 HDLEC cells that were treated 24 h latter with orlistat or cerulenin for additional 24 h. Then, the cells were collected by scraping with cell-spatula (TPP, Switzerland) in ice-cold PBS. The cell suspension was fixed in ETOH 70% for 15 minutes, washed

with PBS, blocked with 1% PBS/BSA and incubated with primary antibodies against VEGFR-3 (1:100) for 1 h at room temperature. After another washing step, cells were incubated with FITC-anti-rat antibodies (1:250, Vector Laboratories, USA) for 1 h at room temperature. After further washing, the cell suspension was analyzed in a FACSCalibur flow cytometer equipped with an argon laser and CellQuest software (Becton Dickinson).

Conditioned media and ELISA

To verify the effect of medium conditioned by orlistat- or cerilenin- treated SK-Mel-25 cells on HDLEC proliferation, 4 X 10^5 SK-Mel-25 cells were seeded in T-25 flasks with growth medium without antibiotics containing 10% of FBS. After 24 h, cells were serum-starved for additional 24 h and the medium changed by fresh medium containing cerulenin or orlistat, which was collected after 48 h. Cells and debris were removed by centrifugation at 1,100 Xg for 3 min, and conditioned medium (30% conditioned medium and 70% fresh complete medium) used to incubate the HDLEC cells previously seeded in T-25 flasks (4 X 10^5 cells) and serum-starved for 24 h. Cell cycle progression was evaluated after 24 h as described above. The concentrations of VEGF-C and VEGF-D in the conditioned media were determined by ELISA (Human VEGF-C Quantikine ELISA/Human VEGF-D DuoSet ELISA, R&D Systems, Minneapolis, MN) following the manufacturer's instructions.

Results

FASN inhibitors reduce cell proliferation and promote apoptosis in HDLEC cells.

As depicted in Fig. 1a, cultured HDLEC cells produce the FASN protein at levels comparable to cancer-derived cell lines such as SCC-9, SK-Mel-25 and B16-F10. The viability of HDLEC cells was reduced by approximately 20-30% with all tested concentrations of cerulenin (Fig. 1b) or orlistat (Fig. 1c) in comparison with the respective controls. Cell cycle analysis by flow cytometry showed that the incubation of HDLEC cells with 2.5 µg/ml of cerulenin (Fig. 1d) or 100 µM of orlistat (Fig. 1e) for 24 h enhances the G0-G1 population and reduces the percentage of cells in S phase. The same concentrations of the drugs also significantly increased the percentage of apoptotic HDLEC cells (Fig. 1f, g). These studies show that primary cultures of nonmalignant lymphatic endothelial cells actively synthesize FASN and are sensitive to FASN inhibitors.

FASN inhibitors decrease migration of lymphatic endothelial cells in the spheroid model.

In order to better understand the effects of FASN inhibitors on the lymphatic vasculature, we next used cerulenin or orlistat to incubate hTERT-HDLEC cells in the spheroid migration assay. These studies showed that all tested concentrations of cerulenin (Fig. 2a-d) or orlistat (Fig. 2e, f) reduced lymphatic endothelial cell migration, which was measured by counting the number of cells isolated from the spheroid after 24 h (Fig. 2g, H).

Cerulenin and orlistat inhibit the lymphatic capillary growth.

The treatment with 2 or 2.5 µg/ml (Fig. 3b, c) of cerulenin promoted an anti-lymphangiogenic effect in cultured explants from mice lymphatic duct when compared with the DMSO controls (Fig. 3a). Orlistat inhibited capillary formation at 100 and 300 µM (Fig. 3e, f) when compared with the ETOH controls (Fig. 3d). After the quantitative analyses, we observed that the growth of lymphatic structures in the DMSO controls reached the maximal distance of 1.15 mm (Fig. 3g, h), which was reduced to 0.75 mm (35%) in the cerulenin-treated explants. Similarly, the maximum distance reached by lymphatic structures in ETOH was 1.0 mm and did not exceed 0.7 mm (30% of reduction) in the presence of orlistat. Immunofluorescence reactions for α-SMA in whole mounts showed that most collagen gels were completely negative or weakly positive in areas not related to the structure of vascular networks (data not show).

FASN inhibitors modulate VEGF-C and VEGF-D release by melanoma cells.

In order to verify a possible role for FASN inhibitors on the melanoma-induced lymphatic cell response, we next analyzed the production of VEGF-C and VEGF-D in cultured SK-Mel-25 treated or not with cerulenin or orlistat. Both drugs stimulated the secretion of VEGF-D (Fig. 4a, b) and, in contrast, reduced the production of VEGF-C (Fig. 4c, d). In addition, the immunopositivity for VEGFR-3 was significantly reduced in HDLEC cells treated with cerulenin or orlistat (Fig. 4e, f).

Conditioned media from orlistat-treated melanoma cells inhibit lymphatic endothelial cell proliferation.

In order to verify whether the conditioned media from cerulenin- or orlistat-treated SK-Mel-25 cells affect HDLEC cell proliferation, we next used flow cytometry. As depicted in Fig. 5a and b, HDLEC cell proliferation was significantly inhibited by conditioned media from cerulenin- or orlistat-treated SK-Mel-25 cells, effect that was probably not due a consequence of residual active FASN inhibitors, as cell culture media containing cerulenin or orlistat previously incubated for 48 h at 37°C did not affect HDLEC cell viability (Fig. 5c, d).

Discussion

The association between FASN expression and activity with tumor growth, metastasis and prognosis has been clearly demonstrated in several types of human malignancies [7]. In fact, fatty acid biosynthesis within tumor tissues occurs at high rates regardlessis nutritional supply [3, 62]. In fact, FASN pharmacological blockage or knockdown inhibit cell cycle progression and promote apoptosis in cultured cancer cell lines [6,29,30,41,43,59,63-64].

Normal human tissues preferentially use dietary fat for the synthesis of new structural lipids and de novo fatty-acid synthesis maintained at low levels [3,64]. Here we show that FASN is critical for both proliferation and survival of HDLEC cells, as cerulenin and orlistat significantly reduced their proliferation and viability, and induced apoptosis. Indeed, we have previously demonstrated that the growth

of normal gingival fibroblasts in primary cultures is reduced by cerulenin [65] and Browne et al. [46] reported that orlistat inhibits proliferation and promotes apoptosis in VEGFA-stimulated HUVECs. In addition, recent studies from our group demonstrated that orlistat reduces the viability and proliferation of rabbit aortic endothelial cells (RAEC) and impairs the development of capillary-like structures by the same cells in matrigel [47]. We also found that the formation of HUVEC capillary-like structures is decreased by culture medium previously conditioned by orlistat-treated SK-Mel-25 cells, which is, at least in part, a result of the enhanced production of VEGFA165b by melanoma cells [47]. Therefore, the results here presented further corroborate the growing body of evidence indicating that FASN is needed for the growth and maintenance of nonmalignant cells.

Our present studies also show that both cerulenin and orlistat inhibit HDLEC lateral migration in the spheroid assay and decrease the growth of murine lymphatic capillaries in an *ex vivo* model. These findings are similar of those from Browne et al. [46] showing that pharmacological inhibition of FASN with orlistat decreases ex vivo human neovascularization and provides the first evidence of the anti-lymphangiogenic properties of this drug. The primary physiological roles of lymphatic vessels are leukocyte trafficking and fluid homeostasis, however, lymphatics also essential for metastatic dissemination [66-68]. VEGF family members orchestrate both angiogenic and lymphangiogenenic processes in physiological and pathological conditions. VEGF-A, VEGF-B, VEGF-E, VEGF-F

and PIGF act in angiogenesis and vasculogenesis while full-length VEGF-C and VEGF-D preferentially bind to VEGFR-3 in order to promote lymphangiogenesis [69,70]. However, proteolytic processing of VEGF-C enables it to induce angiogenesis through the activation of VEGFR-2 [71]. Increased VEGF-C levels have been associated with lymph node metastasis and poor prognosis in cancers of the esophagus [72], thyroid [73], prostate [74,75], ovary [76], blader [77], pancreas [78,79], lung [53,80], head and neck [81,82], oral cavity [83-85], stomach [86-89], intestine [90-95], uterine cervix [96-98], breast [99], as well as melanomas [51,100-102] and mesotheliomas [103]. High levels of VEGF-D were also associated with lymph node metastasis in colorectal, ovarian, and breast cancers (94,104,105) and poor prognosis in colorectal and ovary tumors [104,105]. Moreover, the co-expression of VEGFR-3 with VEGF-C or VEGF-D in melanomas and non-small cell lung carcinomas was already described [52,53]. In vitro, the interaction between VEGF-C and VEGFR-3 is important for survival, proliferation, and migration of lymphatic endothelial cells due to the activation of signaling cascades that include PKC, MAP kinase, PI3K, and Akt [106]. The participation of VEGF-C in regulating tumor-driven lymphangiogenesis in humans is not completely understood, however, increased VEGF-C-mediated lymphangiogenesis and metastases were observed in xenograft models of pancreas and breast cancers as well as melanomas [107-111].

Here, we show increased VEGF-D and down-regulation of VEGF-C in SK-Mel-25 cells treated with both cerulenin and orlistat. Similar results were found in B16-F10 mouse melanoma cells [44]. Furthermore, HDLEC cell proliferation was significantly inhibited by conditioned media from cerulenin- or orlistat-treated SK-Mel-25 cells, suggesting that FASN inhibitors produce an anti-lymphangiogenic phenotype in melanoma cells. Accordingly, O-charoenrat et. al. [81] demonstrated that the infiltrative growth of head and neck cancer is associated with both enhanced expression of VEGF-C and decreased levels of VEGF-D, which was also predictive for the presence of lymphatic invasion and cervical metastases. Likewise, the balance between VEGF-C and VEGF-D was suggested as more important than VEGF-C alone and low VEGF-D levels have been associated with lymphatic invasion and lymph node metastases in lung carcinomas [112]. Previous results from our group show that orlistat reduces experimental melanoma and squamous cell carcinoma metastasis [43-45], which may be a result of the anti-lymphangiogenic phenotype and differential VEGF-C and VEGF-D regulation here described. In addition, Menendez et al. [113] demonstrated that FASN inhibition with C75 increases the production of VEGF-A and accumulates HIF-1 a in breast cancer cells suggesting an hypoxia-like response. The relationship between hypoxia, VEGF-C and VEGF-D production and FASN is still unknown, however, it is possible to speculate according to our results that VEGF-C availability is not estimulated by hypoxia [114,115].

In summary, we reported that cerulenin and orlistat decrease the proliferation, migration and the expression of VEGFR-3 while promote apoptosis in HDLEC cells. These compounds also increase VEGF-D and inhibited VEGF-C in human melanoma cells resulting in an anti-lymphangiogenic response.

Acknowledgments

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Conflict of interest

The authors declare that they have no conflict of interest.

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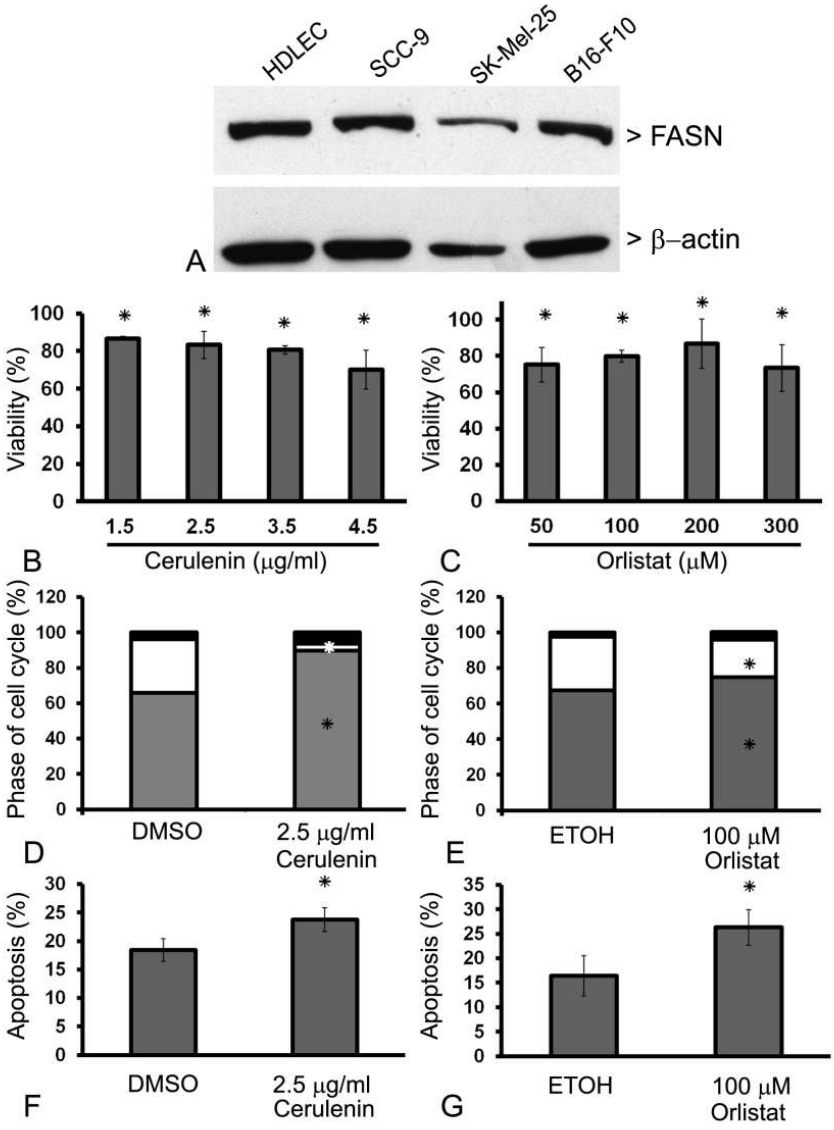

Fig 1 *FASN inhibitors reduce proliferation and promote apoptosis in HDLEC cells*. **(a)** Western blotting reaction showing that HDLEC cells are high FASN producers in comparison with melanoma (SK-Mel-25 and B16-F10) and oral carcinoma cells (SCC-9). The viability of HDLEC cells was reduced in all tested concentrations of cerulenin **(b)** or orlistat **(c)**, when compared with the respective controls (DMSO or ETOH). Cell cycle analysis by flow cytometry showed that the incubation of HDLEC cells with 2.5 μg/ml of cerulenin **(d)** or 100 μM of orlistat **(e)** for 24 h enhances the G0-G1 population and reduces the percentage of cells in S phase (▪G0/G1, □S, ■G2/M). The treatment with 2.5 μg/ml of cerulenin **(f)** or 100 μM of orlistat **(g)** for the same period of time also significantly increases the percentage of HDLEC cells in apoptosis (*p <0.05, Student's t-test)

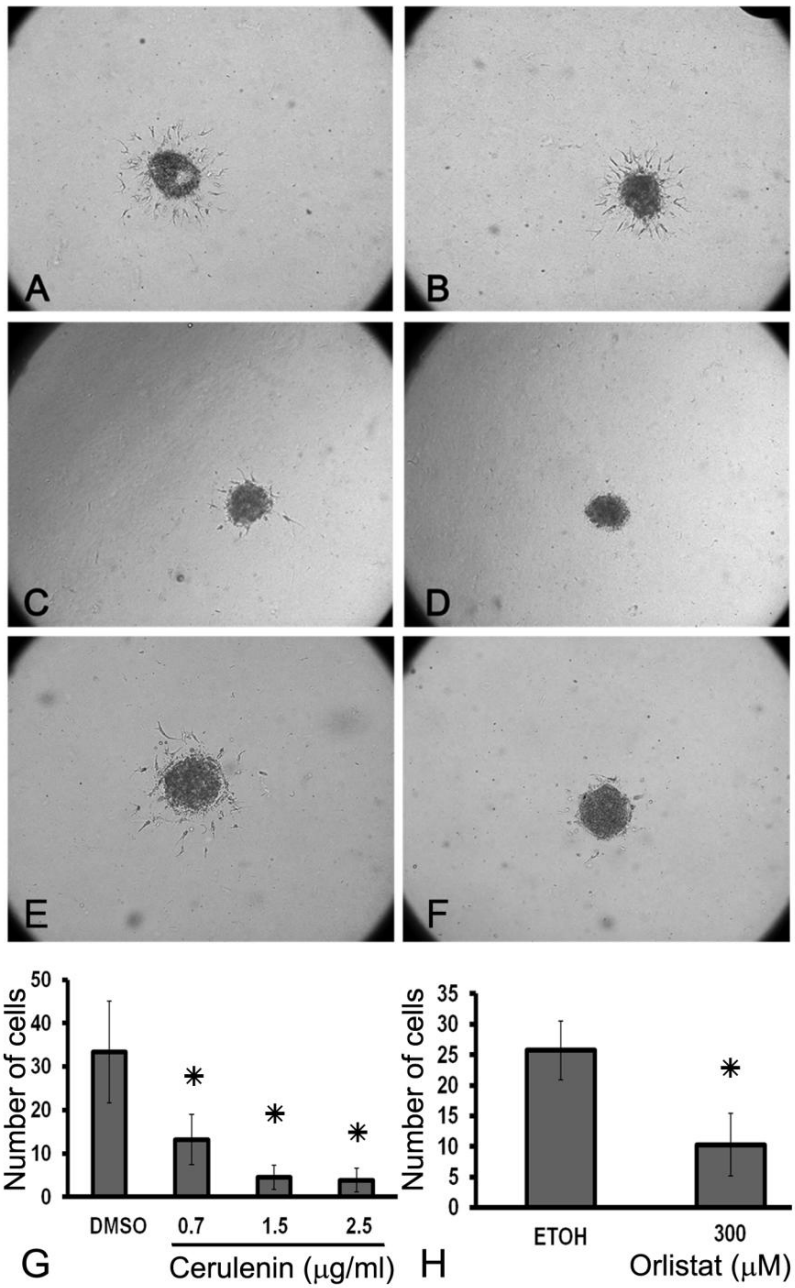

Fig 2 *FASN inhibitors decrease migration of lymphatic endothelial cells in the spheroid model.* **(a-f)** Representative phase-contrast microscopic aspects of hTert-HDLEC spheroids in the presence of DMSO **(a)**, 0.7, 1.5, and 2.5 µg/ml of cerulenin **(b-d)** or ETOH **(e)** and 300 µM of orlistat **(f)**. The number of cells isolated from the spheroids after 24 h was significantly higher in the controls than in cells treated with cerulenin **(g)** or orlistat **(h)**. ($^{*}p<0.05$, Student's t-test)

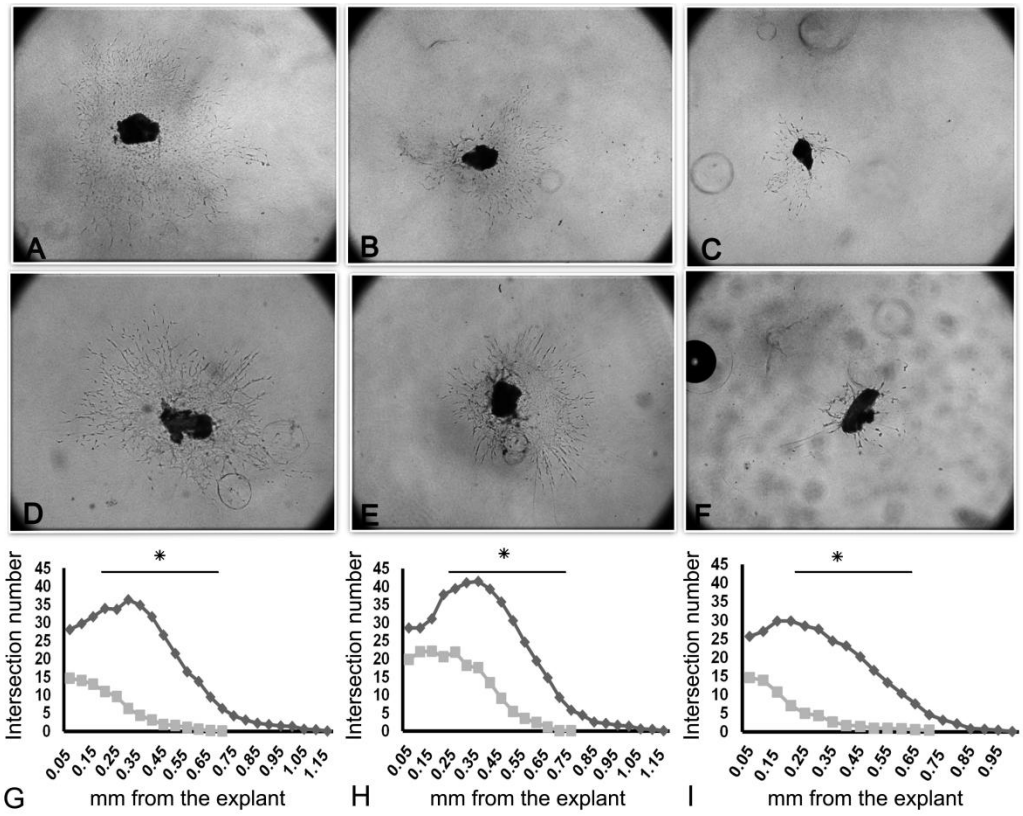

Fig 3 *Cerulenin and orlistat inhibit the ex vivo lymphatic capillary growth.* The treatment with 2.0 **(b)** or 2.5 µg/ml **(c)** of cerulenin promoted an anti-lymphangiogenic effect on the explants from mice lymphatic ducts when compared with the DMSO controls **(a)**. Orlistat also reduced capillary formation at 100 **(e)** and 300 µM **(f)** when compared with the ETOH controls **(d)**. The growth of lymphatic structures in the DMSO controls (♦) reached the maximal distance of 1.15 mm, while the treatment with 2.0 or 2.5 µg/ml of cerulenin (■) reduced this distance to 0.75 mm (35%) **(g, h)**. The higher distance reached by lymphatic structures in ETOH (♦) was 1.0 mm, which did not exceed 0.7 mm **(i)** in presence of 100 µM (■) (*p< 0.05, teste t de Student)

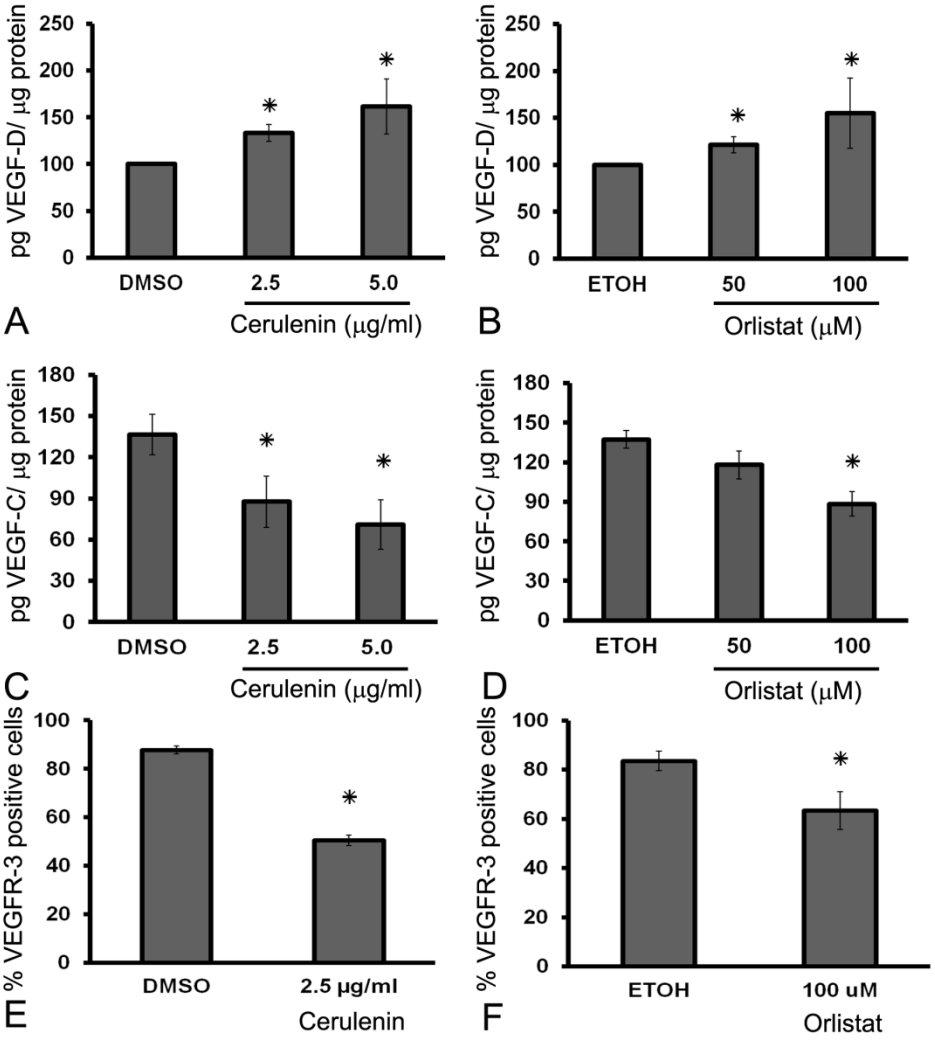

Fig 4 *Effects of FASN inhibitors on VEGF-C and VEGF-D production by melanoma cells.* VEGF-D concentration was significantly increased in the conditioned medium from cerulenin or orlistat-treated SK-Mel-25 cells **(a, b)**. In contrast, the production of VEGF-C by these cells was significantly reduced in the same experimental conditions **(c, d)**. The number of VEGFR-3 positive HDLEC cells was also reduced by cerulenin **(e)** and orlistat **(f)** (*$p<0.05$, Student's t-test)

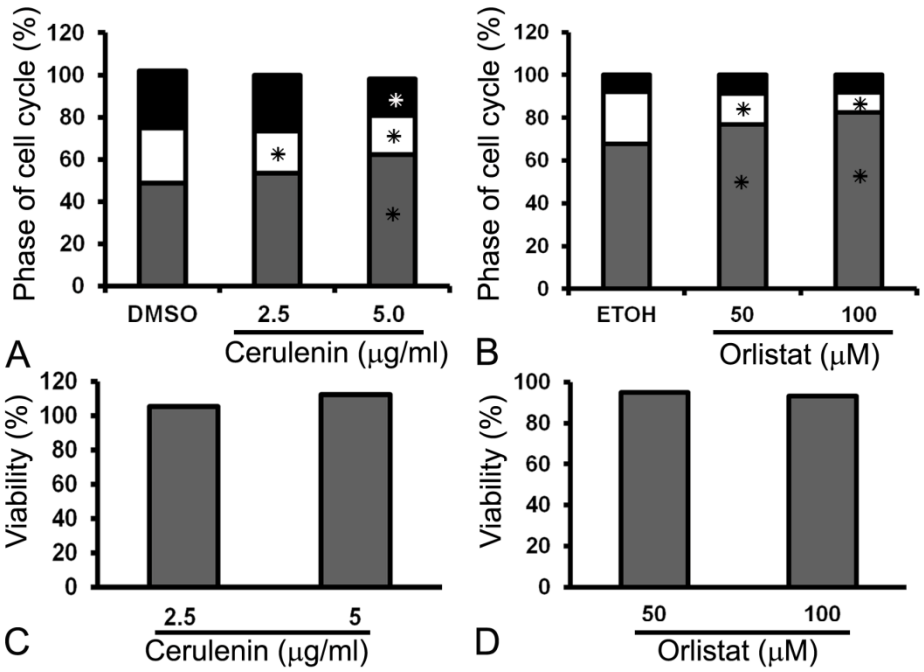

Fig 5 HDLEC cell proliferation was significantly inhibited by conditioned media from both cerulenin- **(a)** and orlistat-treated **(b)** SK-Mel-25 cells. Culture media containing cerulenin **(c)** or orlistat **(d)** and incubated without cells for 48 h at 37°C did not modify the viability of HDLEC cells. (■G0/G1, □S, ■G2/M; *$p<0.05$, Student's t-test)

Conclusão geral

Inibidores de FASN atuam nas células endoteliais linfáticas reduzindo sua viabilidade, proliferação e migração, além de promover apoptose. Nas células de melanoma, esses compostos modulam a expressão de fatores linfangiogênicos, os quais inibem a proliferação das células endoteliais linfáticas e diminuem a quantidade de extensões celulares "filopodia-like".

Conclusões específicas

1. O tratamento de células de melanoma murino B16-F10 com cerulenina ou orlistat tem efeitos opostos sobre a expressão de RNAs mensageiros para VEGF-D e VEGF-C, aumentando o primeiro e reduzindo o último.
2. O tratamento de células B16-F10 com orlistat ou cerulenina e a inibição da FASN com siRNA aumenta a secreção da proteína VEGF-D.
3. Meios de cultura previamente condicionados por células B16-F10 tratadas com cerulenina ou orlistat não modificam a migração de células endoteliais hTert-HDLEC, porém reduzem seu número de extensões celulares filopodio-*like.*
4. O tratamento de células endoteliais linfáticas HDLEC com cerulenina ou orlistat reduz a viabilidade, proliferação ao mesmo tempo em que causa apoptose.

5. Cerulenina ou orlistat inibem a migração de células endoteliais linfáticas hTert-HDLEC em experimento com esferoides.

6. Cerulenina ou orlistat inibem a formação de capilares linfáticos a partir do ducto torácico de camundongos em ensaio ex vivo.

7. VEGF-C e VEGF-D são diferencialmente modulados pela inibição da FASN em células de melanoma humano SK-Mel-25, reduzindo o primeiro e aumentando o último.

8. Meios de cultura condicionados por células de melanoma humano SK-Mel-25 tratadas com cerulenina ou orlistat reduzem a proliferação de células endoteliais linfáticas HDLEC.

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C E R T I F I C A D O

Certificamos que o Protocolo nº **1934-1**, sobre **"Avaliação do papel biológico do ácido graxo sintase (FASN) em células endoteliais linfáticas"**, sob a responsabilidade de **Prof. Dr. Edgard Graner / Débora Campanella Bastos**, está de acordo com os Princípios Éticos na Experimentação Animal adotados pelo Colégio Brasileiro de Experimentação Animal (COBEA), tendo sido aprovado pela Comissão de Ética na Experimentação Animal – CEEA/Unicamp em **31 de agosto de 2009**.

C E R T I F I C A T E

We certify that the protocol nº **1934-1**, entitled **"Evaluation of biological role of fatty acid synthase (FASN) in lymphatic endothelial cells"**, is in agreement with the Ethical Principles for Animal Research established by the Brazilian College for Animal Experimentation (COBEA). This project was approved by the institutional Committee for Ethics in Animal Research (State University of Campinas - Unicamp) on **August 31, 2009**.

Campinas, 31 de agosto de 2009.

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