UNIVERSIDADE DE SÃO PAULO
INSTITUTO DE BIOCÊNCIAS

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**Análise do secretoma de carcinoma de cabeça e pescoço e de
seu efeito no microambiente tumoral**

**Analysis of the head and neck carcinoma secretome and its
effect on the tumor microenvironment**

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BIANCA RODRIGUES DA CUNHA

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Orientadora: Eloiza Helena Tajara da Silva

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Dedico

*À minha filha e ao meu marido
que dão sentido a minha vida*

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RESUMO

CUNHA, BR. **Análise do secretoma de carcinoma de cabeça e pescoço e de seu efeito no microambiente tumoral.** 2017 216 f. Tese (Doutorado) – Instituto de Biociências, Universidade de São Paulo, São Paulo, 2017.

Ao longo dos últimos anos, tornou-se evidente que o início e a progressão do câncer dependem de vários componentes do microambiente tumoral, incluindo células imunes e inflamatórias, fibroblastos, células endoteliais, adipócitos e matriz extracelular. Estes componentes e as células neoplásicas interagem entre si e trocam sinais pró e antitumor. O presente estudo teve como objetivo analisar o secretoma de células neoplásicas sob estresse e seu efeito no microambiente tumoral. Para este fim, duas linhagens celulares de carcinoma epidermóide de cabeça e pescoço foram cultivadas em duas condições de estresse: hipóxia e radiação. Os meios condicionados por estas células (secretoma 1) e o seu controle foram utilizados para cultivar células neoplásicas e fibroblastos humanos normais da cavidade oral. Os resultados sugerem que os sinais derivados das células neoplásicas em resposta a estresse dirigem a expressão gênica e proteica, bem como o comportamento celular das células vizinhas. Foram identificadas 38 proteínas celulares e nove proteínas secretadas com expressão aumentada e 61 proteínas celulares e 70 secretadas com expressão reduzida em células neoplásicas sob estresse hipóxico. Também foram identificadas 59 proteínas celulares e 29 proteínas secretadas com expressão aumentada e 59 proteínas celulares e 19 secretadas com expressão reduzida em células neoplásicas e fibroblastos humanos normais tratados com o meio condicionado por células sob estresse hipóxico. O secretome de células sob estresse não foi capaz de induzir proliferação de células neoplásicas e fibroblastos humanos normais, mas promoveu migração e invasão. Os resultados podem contribuir para o melhor entendimento do efeito dos fatores parácrinos liberados pelas células neoplásicas sobre a expressão gênica, bem como sobre o comportamento das células tumorais e estromais.

Palavras-chave: secretoma, microambiente tumoral, hipóxia, radiação, câncer de cabeça e pescoço.

ABSTRACT

CUNHA, BR. **Analysis of the head and neck carcinoma secretome and its effect on the tumor microenvironment.** 2017. 216 f Tese (Doutorado) – Instituto de Biociências, Universidade de São Paulo, São Paulo, 2017.

Over the past years, it has become evident that cancer initiation and progression depends on several components of the tumor microenvironment, including inflammatory and immune cells, fibroblasts, endothelial cells, adipocytes, and extracellular matrix. These components and the neoplastic cells interact with each other providing pro and antitumor signals. The present study aimed to analyze the secretome of cancer cells under stress and their effect on the tumor microenvironment. For this purpose, two cell lines from head and neck carcinomas were cultured in two stress conditions – hypoxia and radiation. The medium conditioned by these cells (secretome 1) and their control were used to grow untreated neoplastic cells and normal human fibroblasts from oral cavity. Our results showed that signals derived from cancer cells in response to stress drive gene and protein expression and cell behavior. Thirty-eight overexpressed cellular and 9 secreted proteins, and 61 underexpressed cellular and 70 secreted proteins were identified in neoplastic cells under hypoxic stress. Fifty-nine overexpressed cellular and 29 secreted proteins, and 59 underexpressed cellular and 19 secreted proteins were identified in neoplastic cells and normal human fibroblasts treated with the medium conditioned by cells under hypoxic stress. The secretome of cells under stress was not able to induce proliferation of cancer cells and normal human fibroblasts, but promoted migration and invasion. The results may contribute to understand the effect of paracrine factors released by neoplastic cell on gene expression as well as on stromal and tumor cells behavior.

Keywords: secretome, tumour microenvironment, hypoxia, radiation, head and neck cancer.

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1 INTRODUÇÃO

1.1 SECRETOMA

O termo secretoma foi utilizado pela primeira vez no estudo de Tjalsma et al., 2000 para designar o conjunto de proteínas secretadas pelo *Bacillus subtilis* por diferentes mecanismos de secreção. A definição de secretoma foi posteriormente ampliada para referir o conjunto de proteínas secretadas, liberadas ou desprendidas no espaço extracelular por uma célula, tecido ou organismo, sob determinadas condições e em um determinado momento (Agrawal et al., 2010). Tais proteínas compreendem uma grande variedade de moléculas bioativas, que desempenham importantes papéis na regulação de interações célula-célula e célula-matriz extracelular. Podem atuar de forma autócrina ou parácrina, influenciando positiva ou negativamente a capacidade das células de sobreviver, proliferar e se diferenciar (Pocsfalvi et al., 2011).

A secreção de proteínas pode ser realizada pela via clássica, a mais frequente, ou pela via não-clássica. Na via clássica, as proteínas são sintetizadas no retículo endoplasmático rugoso (ER) e, graças a seus peptídeos-sinais localizados no N-terminal (Schatz e Dobberstein, 1996), são transportadas para o aparelho de Golgi em vesículas revestidas pelo complexo COP-II e posteriormente distribuídas em vesículas secretórias, que se fundem à membrana plasmática liberando seu conteúdo o meio extracelular (Mellman e Warren, 2000; Lee et al., 2004). Durante a passagem pelo Golgi, as proteínas podem ser modificadas por diferentes processos, tais como glicosilação (Nickel e Wieland, 1998), fosforilação (Yellaturu et al., 2009) e palmitoilação (Linder e Deschenes, 2007; Greaves et al., 2009).

As proteínas, mesmo contendo o peptídeo-sinal, também podem ser exportadas do citoplasma ou núcleo por vias independentes do ER/Golgi, a chamada via secretora não clássica. Esta via corresponde ao transporte de proteínas para o compartimento extracelular, com sua ligação à superfície da célula ou com liberação em forma solúvel. Os exemplos incluem a secreção do fator de crescimento de fibroblastos 2 (FGF2) e de citocinas envolvidas em processos inflamatórios e angiogênese (Nickel, 2010). A exportação não-clássica utiliza mecanismos de transporte vesicular e não vesicular. As vias não vesiculares realizam a translocação direta auto-sustentada de proteínas através da membrana

plasmática por meio de oligomerização induzida por lipídios, como ocorre no caso de FGF2, ou a secreção mediada por transportadores ABC. As vias vesiculares dependem de intermediários carreadores, como corpos multivesiculares, exossomos, autofagossomos e lisossomos secretores, que se fundem à membrana plasmática para liberar sua carga (Rabouille *et al.*, 2012; Zhang e Schekman, 2013).

Depois de secretadas, as proteínas participam de importantes processos fisiológicos normais, tais como defesa imune, coagulação do sangue, digestão, remodelação da matriz extracelular, sinalização e comunicação célula-célula. Entre estas proteínas, estão hormônios, fatores de crescimento, proteases e enzimas digestivas, citocinas/quimiocinas imunorreguladoras (Mukherjee e Mani, 2013) e metabólitos. Mais recentemente, têm sido incluídos neste grupo, proteínas da matriz extracelular, vesículas derivadas de membrana plasmática e pequenos exossomos, que podem conter compostos não proteicos como RNAs e lipídeos (Stastna e Van Eyk, 2012; Paltridge *et al.*, 2013). Além dos processos normais, o secretoma também está envolvido na patogênese de muitas doenças, como o câncer, promovendo invasão de tecidos vizinhos, evasão de defesas imunes e colonização de órgãos distantes (Pocsfalvi *et al.*, 2011). Seu estudo, portanto, pode levar ao entendimento dos mecanismos que geram alterações fisiológicas importantes, bem como ao desenvolvimento de terapias específicas focando o microambiente tecidual.

1.1.1 O câncer como modelo de estudo do secretoma

O câncer é uma das doenças mais prevalente, com estimativas acima de 14 milhões de novos casos e 8 milhões de mortes em 2012 (Torre *et al.*, 2015). Infelizmente, os biomarcadores disponíveis para esta doença não possuem alta especificidade e sensibilidade. Por este motivo, é urgente a necessidade de novos marcadores, tanto para avaliação de risco e diagnóstico precoce, como para prognóstico e resposta à terapia. Nesse aspecto, o estudo do secretoma pode identificar compostos de importância na área.

Por muitos anos, o câncer foi considerado um processo de célula autônoma. O conceito era centrado nas células geneticamente transformadas e na sua progressão para uma condição maligna. No entanto, o estudo do microambiente tumoral mostrou que a progressão neoplásica depende de uma densa rede de

interações entre as células tumorais e as células do estroma circundante (revisto por Mueller e Fusenig, 2004). Como participantes destas interações, estão proteínas solúveis, antígenos, restos celulares derivados de apoptose e necrose, RNA e DNA, procedentes tanto da célula neoplásica como de suas vizinhas.

Neste microambiente, o estroma fornece não apenas a estrutura de apoio, mas uma parceria de sucesso para o tumor e para seus objetivos de proliferação e disseminação. Esta estrutura inclui a matriz extracelular (ECM), uma rede macromolecular não celular rica em proteínas estruturais fibrilares (como colágeno e fibronectina), proteínas estruturais não fibrilares (como proteoglicanos/glicosaminoglicanos) e proteínas matricelulares que participam das interações célula-célula e célula-ECM (Theocharis *et al.*, 2016). Os componentes celulares do estroma são representados pelos fibroblastos, células endoteliais, inflamatórias e do sistema imune, entre outros (Mueller e Fusenig, 2004), que geram sinais determinantes para o comportamento biológico do tumor. A comunicação estroma-tumor ocorre diretamente entre células ou por uma variedade de moléculas secretadas, como fatores de crescimento, citocinas, quimiocinas e microRNAs (Kohlhapp *et al.*, 2015; Nasser *et al.*, 2015), e é influenciada também por forças mecânicas (Mierke, 2014). Este secretoma heterotípico, que não deriva apenas das células tumorais mas também de células estromais associadas, representa uma fonte importante de marcadores e reguladores-chave do processo tumorigênico (Karagiannis *et al.*, 2010; Zeng *et al.*, 2013).

O microambiente tumoral, portanto, é muito mais amplo que a unidade central formada pelo neoplasma. Sua complexidade é decorrente dos elementos residentes ou recrutados e das interações multidirecionais e dinâmicas entre eles, que acabam gerando uma perda da arquitetura normal dos tecidos, locais de inflamação, hipóxia e, conseqüentemente, de neoangiogênese para contemplar as necessidades elevadas de oxigênio e nutrientes (Paltridge *et al.*, 2013; Zeng *et al.*, 2013) (Figura 1).

Figura 1. Secretoma do microambiente tumoral. Citocinas, quimiocinas, microRNAs e fatores de crescimento secretados por células neoplásicas e estromais, incluindo fibroblastos, células epiteliais, endoteliais e inflamatórias, podem exercer efeitos pró-tumorigênicos e antitumorigênicos. Realmente, estes fatores, expressos de forma autócrina e parácrina, podem promover proliferação celular, angiogênese e transição epitelial-mesenquimal, e consequentemente a progressão e a capacidade invasiva do tumor. Ao contrário, eles também podem ativar células imunes contra células neoplásicas, modulando suas respostas e seu comportamento biológico.

1.1.2 O *cross-talk* entre células neoplásicas e estroma

Um dos primeiros processos no desenvolvimento do câncer é a transição epitélio-mesenquima (EMT), um processo celular caracterizado pela mudança do fenótipo epitelial para mesenquimal, que aumenta a capacidade de migração, invasão e resistência a apoptose. Este processo também ocorre durante a embriogênese e durante a regeneração de tecidos após injúria geralmente associada a inflamação (Kalluri e Weinberg, 2009).

No processo neoplásico, a remodelagem da matriz extracelular acompanha a transição epitélio-mesenquimal. A análise do secretoma de células nesta fase revelou, como esperado, a expressão elevada de proteases, componentes da ECM e fatores que promovem migração e expressão reduzida de proteínas de adesão (Mathias *et al.*, 2009; Mathias *et al.*, 2010).

Mudanças no secretoma levando a alterações da ECM e indução parácrina de proliferação celular devem ocorrer desde os primeiros estágios do desenvolvimento do câncer. Realmente, o secretoma de células não transformadas, mas com expressão sustentada do oncogene c-Myc, mostrou uma elevada capacidade de induzir proliferação, proteger do processo de senescência, modular a composição e a função da ECM e da membrana basal, e atrair fibroblastos. A redução de componentes da matriz neste secretoma pode ser vista como um evento potencialmente capaz de interromper a organização tecidual normal, enquanto a habilidade de atrair fibroblastos sugere que o secretoma na fase inicial da tumorigênese pode alterar o *cross-talk* entre células epiteliais e estromais (Pocsfalvi *et al.*, 2011).

Os fibroblastos representam as mais abundantes células do estroma tumoral, conhecidas por atuar em crescimento, sobrevivência, proliferação, migração, invasão e metástase (Shimoda *et al.*, 2010; Lin *et al.*, 2012). Em situações normais, os fibroblastos estão em quiescência e são ativados quando os tecidos precisam ser remodelados durante a cicatrização após injúria, dando origem a miofibroblastos. No microambiente tumoral, a ativação de fibroblastos é induzida por estímulos derivados de células neoplásicas ou do sistema imune, especialmente fatores de crescimento e interleucinas, que provocam uma mudança em sua morfologia e função (Han *et al.*, 2015). Também parece ser autoinduzida por mutações levando à

perda do gene *TIMP*, que está relacionado com o controle da matriz por meio da inibição de metaloproteinases (Shimoda *et al.*, 2014).

Os fibroblastos ativados são frequentemente chamados de fibroblastos associados a carcinoma (CAFs), e compreendem uma população heterogênea identificada por marcadores específicos (Sugimoto *et al.*, 2006), sendo o mais importante a actina de músculo liso alfa (α -SMA). Esta proteína também está presente em fibroblastos residentes e em miofibroblastos (De Wever *et al.*, 2008; Shiga *et al.*, 2015).

Os CAFs podem se originar de tipos diferentes de precursores. O primeiro deles é o fibroblasto residente submetido, por exemplo, a estresse oxidativo crônico (Toullec *et al.*, 2010). Em função do seu perfil de expressão ser significativamente similar ao do fenótipo secretor associado a senescência, podem também ser derivados de células senescentes. A evidência para uma terceira origem de CAFs vem do fato de células-tronco mesenquimais de medula óssea exibirem expressão elevada de α -SMA e vimentina, após tratamento com secretoma de células neoplásicas. Este resultado aparentemente envolve mecanismos dependentes de TGF-beta solúvel ou presente em vesículas exossomais, que levam a diferentes caminhos de diferenciação, com fibroblastos portadores de fenótipo *in vivo* indutor de tumores (Peng *et al.*, 2014), ou pró-invasivo/pró-angiogênico e promotor de proliferação tumoral, respectivamente (Chowdhury *et al.*, 2015; Webber *et al.*, 2015).

O TGF-beta é uma citocina secretada em forma latente (Horiguchi *et al.*, 2012) que, após ativação, torna-se um potente indutor de transição epitélio-mesenquimal (EMT) (Zarzynska, 2014). Células mesenquimais com alterações cromossômicas idênticas às do carcinoma adjacente (portanto, previamente com fenótipo epitelial) expressam α -SMA e vimentina, e não expressam queratina (Petersen *et al.*, 2003). Estes achados sugerem que os CAFs podem também ser diretamente originados de células neoplásicas que sofreram transição epitélio-mesenquimal desencadeada por TGF-beta.

Outras citocinas também participam da ativação de fibroblastos. A interleucina 6 estromal, por exemplo, atua neste processo reduzindo a expressão das proteínas supressoras de tumor p16^{INK4A}, p21 e p53 em fibroblastos pela via JACK2/STAT3 dependente do regulador negativo AUF1 (hnRNP D0) e promovendo a expressão de marcadores de miofibroblastos, como a α -SMA (Hendrayani *et al.*, 2014).

Os CAFs formam uma população heterogênea com grandes diferenças em relação ao transcrito e ao secretoma, com subtipos mostrando um perfil semelhante ao de fibroblastos normais ou um padrão divergente, com alta secreção de TGF- β . Esta heterogeneidade inclui também fenótipos com mobilidade ou com baixo potencial migratório, este último provavelmente responsável pela síntese de fatores de crescimento, citocinas metaloproteinases e componentes da matriz, enquanto outros respondem a estes fatores (Costea *et al.*, 2013).

A comunicação no microambiente tumoral inclui sinais bidirecionais, que podem ter início na célula neoplásica, ativando e posteriormente induzindo a célula estromal a secretar promotores de proliferação, migração e invasão, importantes para a progressão neoplásica (Mishra *et al.*, 2011; Slany *et al.*, 2013). Por outro lado, os sinais podem partir de fibroblastos normais e CAFs, inibindo por exemplo a proliferação das células do tumor, conforme sugerem alguns ensaios *in vitro* e *in vivo* (Chen, S. X. *et al.*, 2014).

Os efeitos negativos sobre a tumorigênese podem depender do contexto ou da fase da transformação neoplásica. Muitos estudos mostram um efeito inverso, como o observado pela análise de secretoma de miofibroblastos derivados de câncer gástrico comparados aos de tecidos adjacentes. Entre os primeiros, ocorre síntese e ativação mais elevada das proteases MMP1, MMP2 e MMP3, que podem estimular a migração de células tumorais e contribuir para as propriedades quimiotáticas do meio condicionado por CAFs (Holmberg *et al.*, 2013).

Em carcinoma nasofaríngeo, o secretoma de CAFs também mostra proteínas relacionadas com invasão e metástase, bem como proteínas que atuam na proteção contra injúria causada por espécies reativas de oxigênio, influenciando deste modo o destino e a sobrevivência dos elementos celulares do microambiente (Ge *et al.*, 2012). Não apenas fatores solúveis, mas exossomos ricos em metaloproteinases derivados de CAFs podem igualmente promover motilidade e ativar padrões de sinalização nas células neoplásicas ligados a invasão e metástase (via RhoA/ROCK) e diferenciação (via Notch) (Shimoda *et al.*, 2014).

1.1.3 O secretoma inflamatório e o câncer

É estimado que 25% das neoplasias estão associadas a inflamação (Mantovani *et al.*, 2008). As primeiras observações desta associação foram feitas pelo médico alemão Rudolf Virchow no século XIX, que descreveu infiltrados de leucócitos em tumores, inicialmente ligados apenas a respostas imunes (Elinav *et al.*, 2013). Hoje é amplamente reconhecido que a inflamação exerce tanto efeitos supressores quanto promotores de tumor. As características do câncer propostas e atualizadas por Hanahan e Weinberg (2011) incluem agora dois novos traços que levam em conta a inflamação – o ambiente inflamatório e a evasão ao ataque imune.

Existem muitos exemplos de inflamação crônica que aumentam o risco de desenvolver câncer. Entre elas, estão a predisposição de pacientes com doença de Crohn para câncer colorretal (Cannon, 2015) e as infecções pelo *Helicobacter pylori* em câncer gástrico (Polk e Peek, 2010) e pelo vírus do papiloma humano (HPV) em câncer cervical (Groves e Coleman, 2015) e de cabeça e pescoço (Herrero *et al.*, 2003; Human papillomaviruses, 2007). Da mesma forma, drogas anti-inflamatórias não esteroidais diminuem o risco e a consequente mortalidade em vários tipos de tumor (Chan *et al.*, 2007; Flossmann e Rothwell, 2007).

A inflamação pode contribuir para múltiplas etapas do desenvolvimento do câncer, gerando fatores de crescimento, de sobrevivência e pró-angiogênicos, sinais para a transição epitélio-mesenquimal, enzimas que modificam a matriz extracelular e facilitam invasão e metástase, bem como espécies reativas de oxigênio, que são mutagênicas e aceleram a progressão neoplásica (Hanahan e Weinberg, 2011).

Segundo Mantovani e cols. (2008), a relação inflamação-câncer pode ser interpretada como tendo duas vias: a via extrínseca, determinada por processos inflamatórios desencadeados por agentes externos, e a via intrínseca, iniciada por alterações genéticas em oncogenes e supressores de tumor, que ativam a expressão de programas relacionados a inflamação. As duas vias convergem, resultando na ativação de fatores de transcrição, principalmente o fator nuclear kappa B (NFkB), o tradutor de sinal e ativador de transcrição 3 (STAT3) e o fator indutor de hipóxia 1α (HIF1α). Estes fatores de transcrição recrutam e ativam vários leucócitos para produção de mediadores inflamatórios, incluindo citocinas, quimiocinas, ciclo-oxigenase 2 (COX-2) e prostaglandinas, os quais vão desencadear cascatas de sinais similares em outras células inflamatórias, estromais

e tumorais. O resultado é um quadro amplo de inflamação no microambiente tumoral com consequente proliferação celular, mudanças de características da célula epitelial para mesenquimal, indução de formação de vasos sanguíneos e linfáticos e inibição da resposta imune.

No infiltrado de leucócitos presente na maioria, se não todas, as neoplasias estão os macrófagos associados a tumor (TAMs), mastócitos e células T. Os macrófagos derivam geralmente de monócitos recrutados pelo tumor via quimiocinas e, dependendo dos sinais do microambiente, podem ter tanto um papel pró- quanto anti-inflamatório, e exercer uma atuação promotora ou supressora de tumor. Em resposta a infecções ou irritações crônicas, sintetizam citocinas inflamatórias que recrutam outras células do sistema imune, criando condições propícias ao desenvolvimento de uma neoplasia (Balkwill e Mantovani, 2012). Após esta fase, os tumores inibem o estado imune ativo dos macrófagos e estimulam um fenótipo trófico que promove a progressão neoplásica (Qian e Pollard, 2010). Existem evidências de que este segundo tipo de atuação ocorre em resposta ao fator 1 estimulador de colônia de macrófagos e a interleucinas presentes no microambiente tumoral (Condeelis e Pollard, 2006), com consequente produção pelos macrófagos de fatores de crescimento, angiogênicos e metaloproteases, que facilitam invasão e metástase (Bogels *et al.*, 2012).

A disseminação destas metástases para determinados sítios anatômicos parece ser em parte influenciada por macrófagos. Os tumores primários geram fatores solúveis ou exossomos, que atraem macrófagos para nichos pré-metastáticos e promovem sua reprogramação, por exemplo por transferência de oncoproteínas pelos exossomos. A aquisição de um fenótipo pró-vasculogênico contribui para o desenvolvimento de um ambiente nutritivo nestes nichos e maiores chances de sobrevivência de células tumorais (Peinado *et al.*, 2012; Wynn *et al.*, 2013).

A discrepância nos sinais enviados pelas células tumorais para ativação dos macrófagos pode explicar prognósticos tão contraditórios como os observados em diferentes tumores. Por exemplo, a presença de macrófagos em carcinoma de mama tem sido associada com sobrevida reduzida (Tsutsui *et al.*, 2005), enquanto em câncer colorretal indica prognóstico favorável (Forssell *et al.*, 2007). Na comparação de proteínas secretadas por estes dois tipos de carcinomas que poderiam influenciar o fenótipo de macrófagos, foi identificado o proteoglicano

versican exclusivamente em linhagens de carcinoma de cólon. Embora a presença de versican no estroma não tenha sido associada com sobrevida maior neste tipo de câncer, sua expressão em células epiteliais periféricas foi ligada a melhor prognóstico, sugerindo que os fatores do microambiente podem influenciar o fenótipo dos macrófagos (Bogels *et al.*, 2012).

Além dos macrófagos, outras células que atuam na resposta inflamatória apresentam papel importante no crescimento e na progressão do tumor. Entre elas, estão os neutrófilos que, de forma similar aos macrófagos, exibem duas funções – pró- e anti-tumoral (Galdiero *et al.*, 2013). Os neutrófilos são atraídos pela interleucina 8 liberada pelo tumor e, como resposta, secretam óxido nítrico, espécies reativas de oxigênio e metaloproteinases (De Larco *et al.*, 2004). Dependendo do contexto, podem liberar elastase favorecendo proliferação celular (Houghton *et al.*, 2010) e promover transição epitélio-mesenquimal (Grosse-Steffen *et al.*, 2012), ou mesmo modular lise de células tumorais via linfócitos T (Mittendorf *et al.*, 2012).

As células dendríticas (CD) são outros importantes reguladores de inflamação, principalmente em função de sua capacidade de secretar diferentes citocinas e quimiocinas. São capazes de iniciar, prolongar e regular a resposta imune. Embora as CDs atuem no silenciamento do desenvolvimento do tumor, os tumores, por sua vez podem explorar as CDs para evasão do ataque imune (Chaput *et al.*, 2008). O perfil do secretoma destas células, embora menos rico que o proteoma citoplasmático, mostrou-se muito mais específico e mais sensível a alterações funcionais, indicando ser este secretoma uma fonte interessante de biomarcadores potenciais envolvidos na regulação dos processos inflamatórios (Gundacker *et al.*, 2009).

1.1.4 Componentes do secretoma e angiogênese

A angiogênese é definida como a formação de novos vasos a partir de uma rede vascular pré-existente, um processo complexo motivado pela necessidade de suprir os tecidos com nutrientes, oxigênio e componentes do sistema imune, que requer a interação entre diferentes tipos de células, matriz extracelular, citocinas e fatores de crescimento. Apesar de importante para desenvolvimento, regeneração

tecidual e sobrevida, a angiogênese desempenha papel central na progressão tumoral, invasão e metástase e no processo de metastatização (Chung *et al.*, 2010)

Várias condições desencadeiam o sinal angiogênico, entre eles estresse metabólico (hipóxia, acidez, hipoglicemia), estresse mecânico (compressão gerada por proliferação celular elevada), resposta inflamatória (infiltração de células imunes no tecido) e mutações genéticas (ativação de oncogenes ou inibição de genes supressores de tumor) (Carmeliet, 2000). Entre os principais efetores deste processo estão ativadores de proliferação e migração de células endoteliais, como os vários fatores de crescimento ligantes de receptores tirosina quinases (VEGF, FGFs, PDGF, EGF), e o ácido lisofosfático (LPA), um fosfolípido que interage com receptores de superfície acoplados a proteína G (Hu *et al.*, 2001; Bergers e Benjamin, 2003). Entre os inibidores, estão a trombospondina-1 e as estatinas derivadas de plasminogênio e colágeno (angiostatina, endostatina, tunstatina e canstatin) (Bergers e Benjamin, 2003). Estas moléculas pró- e anti-angiogênicas podem ser liberadas por células tumorais ou derivadas do microambiente tumoral (Bergers e Benjamin, 2003).

O papel do secretoma estromal na angiogênese pode ser avaliado em experimentos de cocultura. Por exemplo, Zhong L *et al.* (Zhong *et al.*, 2008) observaram que células de câncer de pulmão e estromais, cultivadas em câmaras separadas que permitem difusão bidirecional de fatores solúveis, mostraram aumento de migração e proliferação celular, indução de formação de tubos endoteliais e secreção elevada de proteínas envolvidas em angiogênese, inflamação, proliferação e transição epitélio-mesenquimal. Entre estas proteínas, foram identificadas citocinas e quimiocinas, como CCL2, VEGF, IL-18, TNF-alfa e GM-CSF.

Muitos estudos focando o papel do secretoma na promoção da angiogênese revelaram a participação de outras proteínas, como a interleucina-8 liberada por células de glioblastoma (Dwyer *et al.*, 2012), e a CYR61 (Estrada *et al.*, 2009; Park *et al.*, 2015) e o fator de crescimento derivado de hepatócito (HDGF) (Thirant *et al.*, 2012) liberados por células tronco de medula óssea e de glioblastoma, respectivamente.

Não apenas fatores solúveis, mas vesículas secretadas por células tumorais promovem migração, invasão, formação de tubos e neovascularização *in vivo* (Kim *et al.*, 2002). O perfil proteico destas vesículas, incluindo microvesículas e

exossomos, mostrou muitas diferenças na comparação de câncer colorretal primário e metastático (Choi *et al.*, 2012), com várias proteínas derivadas de tumor metastático que atuam na progressão neoplásica. O antígeno CD276, em especial, é um marcador endotelial de superfície e um imunoregulador que exibiu expressão elevada em vasos sanguíneos de diferentes tumores, bem como na própria célula tumoral (Seaman *et al.*, 2007). Outros ensaios mostraram resultados similares. Por exemplo, exossomos liberados de células de glioma em condições hipóxicas revelaram-se potentes indutores de angiogênese *in vivo* e *ex vivo* (Kucharzewska *et al.*, 2013).

Em função dos inúmeros dados da literatura, a angiogênese é muito estudada para o desenvolvimento de novas terapias. Um dos alvos recentemente identificado é a RalBP1, uma GTPase multifuncional que regula a secreção do fator angiogênico VEGF pelo tumor e cujo bloqueio reduz significativamente a formação de novos vasos (Lee *et al.*, 2012; Lee e Goldfinger, 2014).

Inibidores da angiogênese visando o VEGF estão produzindo benefícios clínicos comprovados para um número crescente de tipos de câncer. No entanto, estas terapias produzem uma resposta inicial seguida, quase que inevitavelmente, por resistência à terapia (Bergers e Hanahan, 2008). A explicação para este resultado sugere que mediadores alternativos estão envolvidos no processo, tais como HGF, IGFBP-7 e catepsina L (Winiarski *et al.*, 2013).

Como citado previamente, drogas anti-inflamatórias não esteroidais têm se revelado eficazes na prevenção de câncer. Seus principais mecanismos de ação são efeito pró-apoptótico e inibição da angiogênese, dois processos que atuam na supressão da proliferação e na transformação maligna (Thun *et al.*, 2002). Uma dessas drogas, sulindac, utilizada em prevenção de câncer colorretal, promove uma redução expressiva de moduladores de apoptose e angiogênese secretados, dentre eles a interleucina 8 (Ji *et al.*, 2009).

1.1.4.1 Angiogênese e hipóxia

A hipóxia é caracterizada por níveis reduzidos de oxigênio (abaixo de 2%) e suas consequências incluem instabilidade genética, maiores taxas de mutação e apoptose (Nelson *et al.*, 2004), expressão de genes associados a angiogênese, invasividade, fenótipo metastático, resistência a radioterapia e quimioterapia

(Semenza, 2003; Brown e Wilson, 2004; Bertout *et al.*, 2008). Por meio da indução de expressão de fatores quimiotáticos, a hipóxia também promove o recrutamento de células T reguladoras (Treg) que, por sua vez, modulam a tolerância imune e cooperam para o crescimento neoplásico (Facciabene *et al.*, 2011). Em um microambiente tridimensional, as células tumorais podem ser expostas a condições de hipóxia aguda e crônica, que induzem e selecionam alterações metabólicas (Bristow e Hill, 2008). Enquanto a exposição à hipóxia cíclica induz o aumento dos níveis de ROS na célula, causando danos ao DNA e ativando vias de transdução de sinais relacionadas com proliferação celular, a hipóxia crônica aumenta a instabilidade genômica por meio da redução de reparo no DNA (Hussain *et al.*, 2003; Bristow e Hill, 2008). Além disso, tanto a hipóxia crônica como a intermitente podem levar a célula a expressar um fenótipo glicolítico mesmo em ambientes oxigenados (um fenômeno conhecido como efeito de Warburg) que, se acoplado a uma perfusão deficiente, resulta em acidificação do microambiente. Wojtkowiak e cols. (2012) sugerem que a hipóxia e a acidose contribuem para a seleção de fenótipos que permitem à célula neoplásica sobreviver, proliferar e eventualmente metastatizar.

1.1.5 Formas de estudo do secretoma

Dependendo dos alvos de interesse, o tipo de amostra a ser utilizada e as metodologias para estudo do secretoma variam bastante, cada uma delas com vantagens e limitações (Mantione *et al.*, 2014). A partir de biofluidos, que refletem diretamente a condição *in vivo*, e de meios condicionados obtidos por cultura de células, podem ser extraídos ácidos nucleicos, proteínas, metabólitos, lipídeos e carboidratos, que exigem diferentes métodos de análise.

1.1.5.1 Biofluidos

O plasma e o soro estão entre os biofluidos mais utilizados para diagnóstico, principalmente pela sua característica minimamente invasiva de coleta e porque representam um reservatório de moléculas liberadas pelos tecidos. Entretanto, a identificação de biomarcadores no sangue não é simples em função das variações

de concentração, dos seus níveis relativamente reduzidos e influenciados por fatores pré-analíticos e clínicos, e de sua degradação ou sequestro que podem ocorrer durante seu transporte pela corrente sanguínea. Além disso, o sangue contém não somente moléculas secretadas pelo tumor, mas também por outros órgãos. A presença de proteínas de alta abundância, tais como albumina e imunoglobulinas, igualmente dificulta a detecção das proteínas de baixa abundância. Pelos motivos acima, muitos estudos têm focado outros fluidos biológicos e linhagens celulares (Lin *et al.*, 2013).

Para análise do secretoma, o sistema *in vivo* é a situação ideal porque reflete a verdadeira condição fisiológica mas, por outro lado, é muito complexo, principalmente pelas dificuldades de obtenção de amostras puras e pela ausência de uma tecnologia de coleta apropriada (Chen *et al.*, 2008; Xue *et al.*, 2008). Os poucos estudos que investigaram o secretoma *in vivo* analisaram biofluidos, tais como saliva (Winck *et al.*, 2015), fluido intersticial que perfunde o tecido (Celis *et al.*, 2004), secreção aspirada de mamilo (Varnum *et al.*, 2003), efusão pleural (Yu *et al.*, 2011) e fluido de ascite (Choi *et al.*, 2011). Estes e outros fluidos, como bile, suco pancreático e gástrico (Farina, 2014), são promissores para a descoberta de biomarcadores, uma vez que representam um reservatório de proteínas secretadas sem a complexidade do plasma ou do soro.

Alguns métodos têm sido empregados para amostragem do secretoma *in vivo*, como o de microdiálise e ultrafiltração. O método de microdiálise depende da difusão passiva de substâncias por uma membrana semipermeável graças a um gradiente de concentração, mas ainda oferece muitos desafios para recuperação de peptídeos e proteínas. A técnica de ultrafiltração também utiliza membranas semipermeáveis e possui algumas vantagens em relação à de microdiálise. A utilização de uma sonda de ultrafiltração capilar (CUF), que extrai fluidos aplicando vácuo a membranas com diferentes poros e cargas superficiais, facilita muito a coleta de longa duração, é pouco invasiva e não altera a concentração original do biofluido (Chen *et al.*, 2008; Stastna e Van Eyk, 2012).

1.1.5.2 Meio condicionado

Estudos utilizando o meio condicionado (CM) por culturas celulares tem sido amplamente utilizado para identificar proteínas secretadas e biomarcadores de câncer (Mukherjee e Mani, 2013; Schaaij-Visser *et al.*, 2013). Embora linhagens de células neoplásicas, mesmo que cocultivadas com outras células, não reconstituam o tecido tumoral e seu microambiente, o secretoma liberado no meio de cultura tem a vantagem de ser menos complexo que biofluidos, facilita a identificação de proteínas de baixa abundância, permite a repetição de experimentos e o controle de variáveis. Além disso, compreende uma boa opção para avaliar a concentração dos componentes e fornecer informações sobre possíveis processos biológicos envolvidos (Stastna e Van Eyk, 2012; Lin *et al.*, 2013).

Contudo, a descoberta de biomarcadores em meios condicionados também tem seus desafios. Por exemplo, as proteínas secretadas no CM podem ser mascaradas por proteínas do soro adicionado para enriquecer o meio de cultura. Por este motivo, as células são inicialmente cultivadas em meio com soro e posteriormente lavadas com solução tampão para retiradas de proteínas séricas. O experimento é seguido por uma fase de incubação em meio sem soro, que é então coletado após um período de um ou dois dias. A deprivação de soro pode resultar em alteração do metabolismo, diminuição de proliferação e aumento de morte celular (Lin *et al.*, 2013), uma condição que em parte mimetiza a deprivação de nutrientes quando o tumor primário atinge um determinado volume. Como esta condição *in vitro* modifica a taxa de síntese proteica, e consequentemente o secretoma, e eleva a concentração de proteínas intracelulares liberadas por autólise, ela pode ser contornada por introdução ao meio de cultura de tags isobáricos, aminoácidos análogos ou marcados com isótopos, o que permite a captura ou a diferenciação entre as proteínas do soro e as secretadas (Colzani *et al.*, 2009; Eichelbaum *et al.*, 2012; Lin *et al.*, 2013). A otimização do tempo de incubação e da taxa de confluência também parece minimizar a contaminação por proteínas intracelulares (Mbeunkui *et al.*, 2006).

Uma das dificuldades da utilização de meios condicionados para análise do secretoma é o fato das proteínas secretadas normalmente estarem presentes em níveis baixos, o que exige uma etapa de concentração, com precipitação ou ultrafiltração, antes da análise. Este processamento pode introduzir outras

limitações, como perda de proteínas, geralmente de baixo peso molecular (Xue *et al.*, 2008).

Apesar dos desafios mencionados acima, o estudo do meio condicionado tem se mostrado útil no delineamento do perfil secretório de diferentes tumores e na identificação de proteínas e vesículas secretadas, bem como de biomarcadores potenciais (Dowling e Clynes, 2011).

1.1.6 Metodologias

Para a análise de transcritos presentes em exossomos, as escolhas mais comuns são *microarrays* e seqüenciamento em larga escala de cDNA, que são de certa forma complementares (Mantione *et al.*, 2014). As proteínas secretadas, por sua vez, podem ser identificadas por espectrometria de massa associada a cromatografia líquida e por técnicas que utilizam anticorpos para captura e detecção de alvos (Mukherjee e Mani, 2013). Metabólitos também são identificados por espectrometria de massas ou por ressonância magnética (Patel e Ahmed, 2015).

As ferramentas de bioinformática também são relevantes na compreensão do secretoma. Como referido previamente, as proteínas secretadas pela via clássica possuem peptídeos-sinais localizados no N-terminal, que podem ser previstos por *softwares* como o SignalP. Aquelas desprovidas de peptídeos-sinais e secretadas pela via não clássica podem ser previstas pelo *software* SecretomeP por meio da análise de características específicas de sua sequência primária, tais como modificações traducionais, carga e tamanho. As limitações que restringem essas abordagens computacionais são principalmente relacionadas com os falsos positivos e os falsos negativos selecionados (Mantione *et al.*, 2014). Por outro lado, as ferramentas de bioinformática permitem distinguir as proteínas genuinamente secretadas dos contaminantes intracelulares que surgem por lise. A simples determinação da localização celular onde os produtos são ativos utilizando os termos do projeto Gene Ontology (Ashburner *et al.*, 2000) pode auxiliar na identificação de falsos-positivos. Da mesma forma, os bancos de dados Secreted Protein Database (Chen *et al.*, 2005), ExoCarta (Mathivanan *et al.*, 2012), Plasma Proteome (Muthusamy *et al.*, 2005) e PeptideAtlas (Desiere *et al.*, 2006) fornecem

importantes informações sobre os componentes do secretoma em geral, de exossomas, do plasma e do soro (Lin *et al.*, 2013).

1.2 CÂNCER DE CABEÇA E PESCOÇO

O câncer é uma das principais causas de morbidade e mortalidade em todo o mundo. No Brasil, está entre as três primeiras causas de morte, juntamente com doenças do aparelho circulatório e respiratório. Com o aumento da expectativa de vida, sua prevalência provavelmente aumentará (Kulasingam e Diamandis, 2008; Idb, 2010), uma situação que caracteriza essa doença como um problema de saúde pública e justifica a mobilização em torno de uma estratégia para prevenção, controle e assistência aos pacientes (Ramos *et al.*, 2007). O perfil de incidência mostra que os tipos mais freqüentes de câncer no Brasil, como em outros países, são os de próstata no sexo masculino e os de mama no sexo feminino, excetuando-se os de câncer de pele não-melanona, que apresentam os índices mais elevados (Inca, 2015). Outros tumores são também muito freqüentes na população brasileira e devem refletir a atuação de fatores ambientais e de estilo de vida. Entre eles estão os de vias aéreas superiores e intestino, e os de colo de útero entre as mulheres.

Entre as neoplasias de vias aéreas superiores, está o carcinoma epidermóide de cabeça e pescoço (CECP), que atinge sítios anatômicos próximos - cavidade oral, faringe e laringe – e representa o sexto tipo mais freqüente de câncer no mundo (Thompson, 2006). Há uma forte associação desse grupo de carcinomas com o abuso de fumo, álcool e exposição a agentes virais (Leemans *et al.*, 2011), mas os mecanismos exatos pelos quais esses fatores induzem transformação e progressão maligna nas células epiteliais não são completamente entendidos. Apesar da lenta diminuição nas taxas de incidência (~4% desde 1980) e do progresso em ferramentas para diagnóstico precoce e para terapia de CECP, não ocorreram mudanças significativas nas taxas de sobrevivência nos últimos 30 anos, que permanecem em torno de 50%. Infelizmente, a maioria dos casos é diagnosticada em estágios avançados (60% nos estágios III e IV), nos quais a doença é quase sempre fatal (Soulieres *et al.*, 2004; Weber *et al.*, 2007)

O quadro de prognóstico desfavorável é agravado pelo fato das vias aéreas superiores serem os locais com maior freqüência de tumores secundários causados

pela contínua exposição aos mesmos carcinógenos que provocaram o tumor primário (Azad *et al.*, 2012). A terapia convencional de CECP é baseada em três tipos de tratamentos: cirurgia, radioterapia e quimioterapia. Os procedimentos cirúrgicos geralmente são invasivos e podem causar lesões estéticas irreversíveis, com um significativo comprometimento funcional (perda da voz, movimentos dos ombros limitados, perda do sentido olfativo, do paladar e da capacidade de engolir líquidos (Barbosa, *et al.*, 2004). A radioterapia ocupa um lugar de destaque no tratamento de CECP, tanto na forma isolada para lesões pequenas como combinada com quimioterapia para tumores em estágios avançados. É comum a ocorrência de efeitos adversos, que incluem edema, dor e dificuldade de deglutição, entre outros. Três características do tumor determinam a resposta à radioterapia: propriedades intrínsecas das células tumorais, extensão da hipóxia e capacidade de proliferação celular entre os intervalos da radioterapia (Begg, 2012). A recorrência é uma das principais preocupações entre os oncologistas que tratam CECP e diferentes abordagens têm sido desenvolvidas para contornar esse problema e aumentar a sobrevida dos pacientes como, por exemplo, a utilização de radioterapia fracionada (Bourhis *et al.*, 2006). Apesar das novas abordagens, o sucesso do tratamento nem sempre é atingido.

A atividade proliferativa das células de CECP e seu potencial de invasão e formação de metástases dependem não somente das alterações genéticas e epigenéticas que ocorrem no epitélio, mas também do microambiente tumoral, cujo papel no prognóstico é provavelmente função das características específicas dos sub-sítios anatômicos atingidos e das interações moleculares entre as células neoplásicas e os elementos vizinhos (Vered *et al.*, 2011).

2 OBJETIVOS

O presente projeto teve como objetivo geral analisar o secretoma de células de carcinoma epidermóide de cabeça e pescoço (CECP) em condições de estresse e investigar o seu efeito nas interações tumor – microambiente. Os objetivos específicos compreenderam:

a) investigar proteínas e metabólitos do secretoma de células de carcinoma epidermóide de cabeça e pescoço (CECP) submetidas a hipóxia.

b) Analisar, em células epiteliais neoplásicas de CECP não tratadas, alterações de morfologia, índice de proliferação, migração, invasão e composição do secretoma, causadas por fatores parácrinos induzidos por hipóxia e radiação em células de CECP.

c) Analisar, em fibroblastos humanos normais procedentes da cavidade oral, alterações de morfologia, índice de proliferação migração, invasão e composição do secretoma, causadas por fatores parácrinos induzidos por hipóxia e radiação em células de CECP.

d) Comparar os resultados obtidos por tratamento com hipóxia a resultados obtidos por indução de radiação, outro fator de estresse, em relação à aquisição de características mais agressivas, incluindo proliferação, migração e invasão.

3 MATERIAIS E MÉTODOS

3.1 MATERIAIS

3.1.1 Linhagens celulares de cabeça e pescoço

No presente estudo, foram analisadas as linhagens celulares *HEp-2* e *SCC-25* de carcinomas epidermóides de cabeça e pescoço. A linhagem *HEp-2* foi originalmente descrita como procedente de carcinoma de laringe (American Type Culture Collection / ATCC, E.U.A - CCL-23) e foi gentilmente cedida pela Profa. Dra. Otávia Luisa Damas de Cabellero, Instituto Ludwig, NY, e estocada em nitrogênio líquido no laboratório de Marcadores Moleculares e Bioinformática Médica da Faculdade de Medicina de São José do Rio Preto/FAMERP. A linhagem *SCC-25* é procedente de carcinoma epidermóide de língua (American Type Culture Collection / ATCC, E.U.A - CRL-1628) e foi gentilmente cedida pelo Prof. Dr. Fábio Daumas Nunes da Faculdade de Odontologia da Universidade de São Paulo/USP, São Paulo. As características gerais dessas células estão apresentadas na Tabela 1.

Tabela 1. Características das linhagens utilizadas e dados dos pacientes.

Linhagem	Características	Dados dos pacientes	
		Idade (anos)	Sexo
<i>HEp-2</i>	linhagem celular (ATCC: CCL-23) originalmente descrita como proveniente de carcinoma epidermóide de laringe, com contaminação por células HeLa.	Não consta	Não consta
<i>SCC-25</i>	linhagem celular (ATCC: CRL-1628) proveniente de carcinoma epidermóide de língua	70	masculino

3.1.2 Fibroblastos humanos de cavidade oral

Os fibroblastos humanos normais provenientes de gengiva foram gentilmente cedidos por Lucas Offenbecker Guerra, diretor de pesquisa do Grupo Investiga (LTDA), Campinas/SP.

3.2 MÉTODOS

3.2.1 Cultivo celular de linhagens

A linhagem celular SCC-25 foi cultivada em frascos de 75 cm² contendo 8 mL de meio Dulbecco's Modified Eagle Medium: Ham's F12 Nutrient (meio DMEM/F12) (1:1) (Cutilab, Campinas, SP) com bicarbonato de sódio 1.2 g/L, L-glutamina 2,5 mM, HEPES 15 mM e piruvato de sódio 0.5 mM. A linhagem HEp-2 foi cultivada em frascos de 75 cm² contendo 8 mL de meio essencial mínimo (MEM) (Cutilab) com L-glutamina 2,0 mM, piruvato de sódio 1 mM e bicarbonato de sódio 1500 mg/L. Nos experimentos de indução de hipóxia com CoCl₂, as células SCC-25 e HEp-2 foram também cultivadas em meio RPMI com L-glutamina 2 mM, HEPES 10 mM, piruvato de sódio 1 mM e bicarbonato de sódio 1500 mg/L. Os fibroblastos humanos normais foram cultivados em frascos de 75 cm² contendo meio Dulbecco's Modified Eagle Medium - DMEM (Cutilab) com L-glutamina 4 mM, piruvato de sódio 1 mM e bicarbonato de sódio 1500 mg/L. Todos os meios foram suplementados com 10% de soro fetal bovino (SFB) (Cutilab), aminoácidos não essenciais 10 mM (Sigma-Aldrich, St. Louis, MO, USA) e solução de antibiótico/antimicótico 0,1% (Sigma-Aldrich).

O crescimento e a morfologia celular foram monitorados a cada 24h em microscópio invertido e os meios de cultura dos frascos foram trocados a cada três dias. Ao atingirem uma subconfluência de cerca de 70%, as células foram subcultivadas por tripsinização. Para isto, o meio de cultura foi aspirado e as células foram lavadas com 5 mL de solução tampão fosfato-salina sem cálcio e magnésio pH 7,2 (PBS). O meio de lavagem foi aspirado e as células foram tripsinizadas com 2 mL de solução de tripsina 0,05% - EDTA 0,02% e incubadas a 37°C por 2min para que a tripsina pudesse agir e induzir o descolamento das células. Após esse período, os frascos foram examinados ao microscópio para averiguar se as células estavam soltas e, caso ainda estivessem aderidas, foram deixados por mais 1 min na estufa. Em seguida, a tripsina foi inativada com a adição de 3 mL de meio DMEM:F12, MEM ou DMEM (dependendo da linhagem celular) com 10% de SFB. O material foi aspirado e dividido em dois tubos de ensaio, que foram centrifugados a 2500g por 5 min. O *pellet* com células foi ressuspensionado em 1 mL de meio e recultivado. Periodicamente, um dos *pellets* foi ressuspensionado em SFB e solução de

dimetilsulfóxido (DMSO) (Sigma-Aldrich) 10:1 e congelado para manutenção do estoque de linhagens.

Para avaliação do número de células a serem semeadas na fase de recultivo ou no início dos experimentos, foi utilizado o contador automático *Countess® Cell Counter* (Invitrogen, Foster City, CA, USA). Uma alíquota de 5 µl de meio contendo as células tripsinizadas foi adicionado a um tubo *ependorf* com 5 µL do corante azul de tripano (Sigma-Aldrich) e os 10 µL totais foram aplicados em lâmina própria para contagem. O aparelho utiliza um algoritmo para análise de imagens e para cálculo do número de células vivas e mortas. As primeiras brilham devido à ausência do corante em seu interior, pois a membrana celular íntegra impede sua entrada. As células mortas, ao contrário, ficam opacas por causa da presença do azul de tripano em seu citoplasma, uma vez que a membrana celular não é mais seletiva. O número de células viáveis/mL identificadas pelo aparelho foi utilizado para calcular o volume de meio com células a ser semeado.

Para obtenção de réplicas, as células foram semeadas com densidade de 1×10^6 para frascos de 75 cm², 1×10^5 em frascos de 25 cm², 2×10^4 em placas de 12 poços ou 2×10^3 em placas de 96 poços, e cultivadas a 37°C em atmosfera de 5% de CO₂ por 24 horas. Em todos os experimentos, após adesão das células ao frasco, o meio de cultura foi trocado por meio sem soro para facilitar a sincronização do ciclo celular. As células foram mantidas nesse meio por 24 horas, tempo suficiente para que ingressassem na fase G₀. Todos os procedimentos de cultivo celular foram realizados em fluxo laminar bidirecional, específico para esse fim, para manutenção da esterilidade dos materiais bem como para evitar a contaminação cruzada de linhagens.

3.2.2 Indução de estresse

As células HEp-2 e SCC-25 foram submetidas a duas condições de estresse - hipóxia e radiação.

3.2.2.1 Indução de Hipóxia

Para indução de hipóxia, as células foram tratadas com CoCl_2 (Sigma–Aldrich) que é um agente mimetizador de hipóxia e atua na inibição de enzimas sensoras de oxigênio pela troca de ferro por cobalto, tornando essas enzimas incapazes de marcar a subunidade alfa do fator de transcrição 1 induzível por hipóxia (HIF1 α) para degradação. O tratamento com o composto CoCl_2 mimetiza o acúmulo de HIF1 α observado em condições de hipóxia (Schofield e Ratcliffe, 2004).

3.2.2.2 Irradiação celular

As células foram irradiadas com raio-X em equipamento específico para pesquisas biológicas (RS 2000, Rad Fonte Technologies, Inc., Suwanee, GA), com energia de 200 kVp e 25 mA e filtro padrão de Cu 0.3 mm, disponível no Serviço de Radioterapia do Hospital das Clínicas da Faculdade de Medicina de Ribeirão Preto, USP. Os raios-X gerados sob essa condição possuem espectro com energia mínima de 95 kV e máxima de 200 kV, e a metade do valor do feixe com 0,62 mm de Cu. O gradiente de dose desse raio-X em tecido-equivalente é de cerca de 10% a 0,5 cm de profundidade. As placas foram alinhadas de forma equidistante do centro do feixe e dentro do cone para garantir uma taxa de dose uniforme (aproximadamente 1,55 Gy/min) e entrega total da dose por fração. O controle de qualidade foi realizado utilizando dosímetros Nanodot (Landauer, Inc., Glenwood, IL).

3.2.3 Seleção da dosagem de CoCl_2 e radiação

Curvas de proliferação e ensaio de citotoxicidade (MTS) (CellTiter 96® Aqueous, Promega, Madison, WI, USA) foram realizados para identificação das condições mais adequadas de tratamento com CoCl_2 e radiação.

Para avaliação das taxas de proliferação, foram realizadas curvas de crescimento com contagem de células cultivadas em placas de 12 poços, semeadas em concentração de 2×10^4 . Para os ensaios de citotoxicidade, as células foram cultivadas em placas de 96 poços, semeadas em concentração de 2×10^3 . Após a

adesão das células ao frasco, o meio de cultura foi trocado por meio sem soro para facilitar a sincronização do ciclo celular. As células foram mantidas nesse meio por 24 horas, tempo suficiente para que ingressassem na fase G_0 . As células foram então submetidas aos diferentes tratamentos ($CoCl_2$ ou radiação). Após os tempos determinados para cada condição de tratamento, as células foram tripsinizadas e contadas utilizando-se o equipamento Countess® Automated Cell Counter (Invitrogen, Forster City, CA, USA) nos ensaios de proliferação. Para os ensaios de MTS, após os tempos determinados para cada condição de tratamento, o meio foi removido e substituído por 100 μ L de meio sem SFB e 20 μ L do composto MTS (Promega). As placas foram então incubadas por 1 a 4 horas e a leitura da absorbância no comprimento de onda de 490 nm foi realizada com o leitor de placas (Thermo Plate).

3.2.3.1 Curvas de proliferação e ensaio de citotoxicidade (MTS) para seleção do melhor tempo e dosagem nos experimentos de indução de hipóxia

Para curva de crescimento e ensaio de citotoxicidade (MTS), com a linhagem HEP-2 nas condições controle (meio sem $CoCl_2$) e tratadas com $CoCl_2$, as células foram contadas após 6 h, 12 h, 24 h, 48 h e 72 h a partir do início do experimento (0 h), ou seja, após 24 h de cultivo sem SFB. Para teste da concentração final de $CoCl_2$, foram utilizadas as unidades 50 μ M, 100 μ M, 150 μ M, 200 μ M, 250 μ M e 300 μ M. Para os experimentos com a linhagem SCC-25 nas condições controle e após tratamento com $CoCl_2$, as células foram contadas após 24 h, 48 h, 72 h e 96 h a partir do início do experimento (0 h), ou seja, após 24 h de cultivo sem SFB. Em função dos dados obtidos nos experimentos com a linhagem HEP-2, foram retirados os tempos de 6 h e 12 h e acrescentado o tempo de 96 h. Para teste da concentração final de $CoCl_2$, foram utilizadas as concentrações 50 μ M, 100 μ M, 150 μ M e 200 μ M. Todos os experimentos foram realizados em triplicata.

3.2.3.2 Curvas de proliferação e ensaio de citotoxicidade (MTS) para seleção de melhor tempo e dosagem nos experimentos de radiação

Para curva de crescimento e ensaio de citotoxicidade (MTS), a linhagem HEP-2 foi cultivada por 24 h em meio sem SFB e, em seguida, cultivada em meio com SFB 10% e submetida a radiação de 2 Gy, 4 Gy, 8 Gy e 16 Gy. As células foram contadas após 24 h, 48 h, 72 h e 96 h. Todos os experimentos foram realizados em quadruplicata.

3.2.4 Obtenção de meio condicionado MC1_{Controle} e MC1_{Tratado} (secretoma 1)

3.2.4.1 Experimentos com Hipóxia

As células HEP-2 e SCC-25 foram cultivadas em meio RPMI e não em meio EMEM e DMEM:F12, respectivamente, como é indicado pela ATCC, em função da interação do composto CoCl₂ com a histidina presente em grandes quantidades nesses meios e do consequente efeito reduzido na indução de hipóxia (Torii *et al.*, 2011)

As células foram mantidas em cultura até atingirem uma confluência de aproximadamente 70%. Em seguida, foram submetidas a condições de hipóxia com CoCl₂ 100 µM por 24 h, exceto o controle. Após duas lavagens com PBS para retirada de restos de soro, o meio foi substituído por meio de cultura com SFB 0%. Os meios de cultura coletados após o período de 24 h contêm o secretoma gerado após a resposta a estresse hipóxico (secretoma 1) e foram denominados meios condicionados MC1 (MC1_{Hipo} e MC1_{Controle}) (Figura 2). Após este período, as células foram imediatamente retiradas com *scraper* e o *pellet* foi armazenado a -80°C. Os meios condicionados (ou secretomas 1) foram coletados, centrifugados a 15000 g por 10 min e filtrados com filtros de PVDF de 0,22 µm para retirada de restos celulares e possíveis contaminantes. Em seguida, foram armazenados a -80°C para posterior análise.

Figura 2. Obtenção dos secretomas MC1_{Hipo} e MC1_{Controle} após tratamento das células com CoCl₂ por 24 h para indução de hipóxia ou após o mesmo período sem tratamento, respectivamente.

3.2.4.2 Experimentos com Radiação

As células HEp-2 foram mantidas em cultura até atingirem uma confluência de aproximadamente 70%. Em seguida, foram irradiadas com a dosagem selecionada de 4 Gy (raios-X), exceto o controle. Após 24 h, foram realizadas duas lavagens com PBS para retirada de restos celulares e de soro. O meio foi substituído por meio com SFB 0% e as células foram cultivadas por mais 24 h. Após este período, as células foram imediatamente retiradas com *scraper* e o *pellet* foi armazenado a -80°C . Os meios condicionados (ou secretomas 1) foram coletados e designados MC1: MC1_{Rad} e MC1_{Controle} (Figura 3). Foram centrifugados a 15000 g por 10 min e filtrados com filtros de PVDF de 0,22 μm para retirada de restos celulares e possíveis contaminantes. Em seguida foram armazenados a -80°C para posterior análise.

Figura 3. Obtenção dos secretomas MC1_{Rad} e MC1_{Controle} após tratamento das células com raios-X (4Gy) ou ausência de tratamento, respectivamente.

3.2.5 Tratamento de células com o secretoma 1 e efeito do secretoma na fisiologia e no comportamento celular

O efeito do secretoma1 obtido nos ensaios de hipóxia e radiação na fisiologia e no comportamento celular foi avaliado por curvas de proliferação e ensaios de citotoxicidade (MTS), migração e invasão (Figura 4).

Figura 4. Experimento para avaliação do efeito do secretoma 1 na fisiologia e no comportamento celular realizado com curvas de proliferação e ensaios de citotoxicidade (MTS), migração e invasão

3.2.5.1 Curvas de proliferação e ensaios de citotoxicidade

As células foram semeadas em placas de 12 e 96 poços para os ensaios de curva de proliferação e MTS, respectivamente. Após sua adesão ao frasco, as células foram cultivadas por 24 h em meio sem SFB e então cultivadas em meio com SFB 10% (controle negativo), em MC1_{Controle} (controle) ou em MC1_{Tratado} (MC1_{Hipo} ou MC1_{Rad}) e foram contadas ou mensuradas as absorbâncias após 24 h, 48 h, 72 h, 96 h e 120 h a partir do início do experimento. Os experimentos foram realizados em triplicata e os meios foram trocados diariamente. O MC1_{Hipo} foi utilizado no tratamento de HEp-2, SCC-25 e fibroblastos humanos normais e o MC1_{Rad} no tratamento de HEp-2 e fibroblastos humanos normais.

3.2.5.2 Ensaios de Migração e Invasão Celular

As células foram cultivadas em triplicatas em placas de 24 poços com insertos de 6,5 mm de diâmetro contendo uma membrana de 8 µm de porosidade (*BD BioCoat™ Migration/Invasion Chambers – MIC*, Franklin Lakes, NJ, USA). Um total de 5×10^4 células/300 µL de meio MEM, DMEM:F12 e DMEM (para HEP-2, SCC-25 e fibroblastos, respectivamente) sem SFB foram semeadas no compartimento superior do inserto. No compartimento inferior (correspondente ao poço da placa de cultivo), foram adicionados 500 µL de meio condicionado (MC1_{Hipo}, MC1_{Rad} ou MC1_{Controle}) com 10% de SFB. Em seguida, as placas foram incubadas por um período de 6 ou 9 h a 37°C e CO₂ 5%, durante o qual as células, atraídas pelo soro e pelos componentes presentes nos meios condicionados, migraram pela membrana em direção ao compartimento inferior da câmara. As células presentes no inserto foram fixadas com paraformaldeído 0,4% à temperatura ambiente por 20min. Aquelas presentes na superfície superior da membrana (e que, portanto, não migraram) foram retiradas com o auxílio de uma haste de algodão, enquanto as células presentes na face inferior foram coradas por 5min com o composto fluorescente 4',6-diamidino-2-fenilindol/DAPI (Uniscience, São Paulo, SP, Brasil) e submetidas a um passo de lavagem com PBS 1X. Cinco campos da superfície inferior da membrana, selecionados casualmente, foram fotografados em microscópio de fluorescência invertido utilizando o programa *Axiovision Release 4.8* e as células presentes foram contadas nas fotografias obtidas.

A capacidade de invasão de células tratadas com o secretoma 1 foi avaliada em câmaras de invasão com matrigel, contendo insertos de 6,5 mm de diâmetro com membranas de 8 µm de porosidade (*BD BioCoat™ Migration/Invasion Chambers – MIC*). Este ensaio baseia-se no fato de que células com capacidade invasiva são capazes de degradar o matrigel, que faz a função da matriz extracelular no tecido vivo, e migrar em direção ao meio com soro. As células foram inoculadas no inserto, na mesma concentração utilizada no ensaio de migração e, após 44 ou 48 h a 37°C e CO₂ 5%, foram fixadas com 0,4% de formaldeído à temperatura ambiente por 20 min. As células remanescentes que não degradaram o matrigel (presentes, portanto, na superfície superior da membrana) foram igualmente retiradas com o auxílio de uma haste de algodão. Os passos seguintes seguiram o mesmo protocolo do ensaio de migração (Figura 5).

Da mesma forma que nos experimentos para obtenção de curvas de proliferação, o MC1_{Hipo} foi utilizado no tratamento de HEp-2, SCC-25 e fibroblastos humanos normais e o MC1_{Rad} no tratamento de HEp-2 e fibroblastos humanos normais.

Figura 5. Esquema dos ensaios de migração e invasão. Os ensaios diferenciam-se pela presença de uma camada de matrigel sobre a membrana de 8 µm de porosidade na câmara de invasão. A câmara de migração contém apenas a membrana de 8 µm de porosidade. O matrigel faz a função da matriz extracelular que as células devem degradar para atingir o meio com soro e com os meios condicionados. As células são depositadas na parte superior do inserto e aquelas que não atravessam a membrana após um período específico são retiradas com auxílio de uma haste de algodão

3.2.6 Tratamento de células com os meios condicionados MC1_{Controle} e MC1_{Tratado} (secretoma1) e obtenção de meio condicionado MC2_{Controle} e MC2_{Tratado} (secretoma2)

Para avaliação do efeito do secretoma 1 obtido de células em hipóxia no perfil de proteínas celulares e secretadas das linhagens SCC-25, HEp-2 e de fibroblastos humanos normais, os meios condicionados MC1_{Controle} e MC1_{Hipo} em triplicata foram acrescidos de SFB até atingirem 10% e adicionados a 6 réplicas de culturas não tratadas, cultivadas em garrafas de 75 cm² com confluência de 70%. Nos experimentos de hipóxia três garrafas receberam MC1_{Hipo} e três garrafas receberam MC1_{Controle}. Após 24 h, o meio foi substituído por MEM, DMEM:F12 e DMEM (HEp-2, SCC-25 e fibroblastos, respectivamente) com SFB 0% e as células foram cultivadas por mais 24 h. As células da cultura foram imediatamente retiradas com scrapper e o *pellet* de células e os novos meios condicionados (com o secretoma 2) (Figura 6) foram coletados e designados MC2: MC2_{Hipo} e MC2_{Controle} e seguiram para os experimentos de espectrometria de massas e ressonância magnética nuclear

Figura 6. Obtenção dos secretomas MC2_{Hipo} e MC2_{Controle} após tratamento das células com MC1_{Hipo} e MC1_{Controle}, respectivamente.

3.2.7 Análise Proteômica

As proteínas celulares e provenientes dos meios condicionados foram analisadas por espectrometria de massas (Figura 7).

Figura 7. Proteínas e metabólitos analisados por espectrometria de massas e ressonância magnética nuclear, respectivamente.

3.2.7.1 Extração direta de proteínas

As proteínas celulares das linhagens e dos fibroblastos foram extraídas com solução de uréia 7 M. As células foram sonicadas em gelo por 10 s na potência 3 e em seguida deixadas em gelo por 50 s. Este procedimento foi repetido 4 vezes.

3.2.7.2 Concentração e quantificação de proteínas

As proteínas de meios condicionados e as proteínas celulares foram concentradas logo após a extração e lavadas com tampão de bicarbonato de amônio 50 mM em filtros Amicon Ultra-4.0 (3K), Centrifugal Filter Devices (Millipore Corporation, Bedford, MA, USA), segundo protocolo do fabricante. Os metabólitos foram separados das proteínas nesta etapa: as proteínas concentradas ficaram na parte superior do filtro e os metabólitos por serem menores que os poros do filtro passaram e foram recuperados. As proteínas foram quantificadas pelo método Bradford com kit Coomassie (Bradford) Protein Assay (Pierce Biotechnology Inc., Rockford, IL, USA) em leitor de placas (Thermo Plate). Esta abordagem foi escolhida

por causa da compatibilidade do tampão bicarbonato de amônia 50 mM utilizado durante a concentração de proteínas com os reagentes do kit. A quantificação pelo método Bradford requer a construção de uma curva padrão sempre que for realizada. A curva padrão é feita a partir de diluições de concentrações conhecidas da proteína albumina sérica bovina (BSA) fornecida com o kit. Para a quantificação, 300 µL do corante foram adicionados a cada um dos poços de uma placa de Elisa, que continham 10 µL da amostra de proteína a ser quantificada ou de cada uma das diluições de BSA. A placa foi colocada sob agitação por 2 min e as amostras foram então quantificadas em leitor de placas (Thermo Plate), no comprimento de onda de 595nm.

3.2.7.3 Digestão de proteínas em solução

Foram utilizadas 15 a 100 µg de proteína em um volume máximo de 200 µL. Cada amostra recebeu a solução de DTT (ditiotreitól) em concentração final de 5 mM e foi incubada durante 25 min a 56°C (etapa de redução). Foi adicionada uma solução de iodoacetamida (IAA) em concentração final de 14 mM e o material foi incubado durante 30 min à temperatura ambiente na ausência de luz (etapa de alquilação). Para efetuar o *quench* da IAA livre, foi adicionado DTT em concentração final de 5 mM e o material foi novamente incubado por 15 min à temperatura ambiente protegido da luz. Posteriormente, foi adicionada uma solução de tripsina 20 ng/µL, em proporção de 1:50 de enzima:substrato e o material permaneceu durante 16 horas a 37°C. Após esse período, foi adicionado TFA (ácido trifluoroacético) na concentração final de 0.4% de modo a bloquear a reação da enzima. No caso de pH acima de 2.0, foi adicionado mais TFA. Em seguida, as amostras foram centrifugadas a 2500 g por 10 min à temperatura ambiente e o *pellet* foi descartado.

3.2.7.4 Dessalinização de proteínas

As proteínas foram dessalinizadas em coluna C18 - SEP-Pak 1 cc-100 mg (Waters, Milford, MA, USA). As colunas foram ativadas com 3 mL de acetonitrila

(ACN) 100%, equilibradas com 1 mL de 50/50 ACN/água com 0,1% de ácido fórmico (AF) e também com 3 mL de TFA. Após o equilíbrio das colunas, as proteínas foram aplicadas e a coluna foi lavada com 3 mL de TFA 0,1% para retirada do sal da amostra e equilibrada com 1 mL de ácido fórmico 0,1%. Finalmente, as proteínas foram eluídas em 2 mL de 50/50 ACN/água com AF 0,1% e em 1 mL de 80/20 ACN/água com AF 0,1%.

As proteínas foram secas em *SpeedVac (Eppendorf Vacufuge)* e congeladas em freezer -20°C até os experimentos de espectrometria de massas.

3.2.7.5 Identificação de proteínas

Os peptídeos dos meios condicionados e das células foram analisados por cromatografia líquida acoplada a espectrometria de massas no equipamento LTQ Orbitrap Velos (Thermo Scientific, Wilmington, DE, USA) conectado ao sistema de cromatografia líquida EASY-nLC II (Proxeon Thermo Scientific) pela fonte nanoeletrospray (Proxeon). Os espectros foram adquiridos utilizando o *Software MassLynx V.4.1* e os arquivos foram convertidos para o formato mgf pelo *Software Mascot Distiller v.2.2.1.0, 2008* (Matrix Science Ltd.). Para identificação das proteínas, os dados foram analisados no banco de dados não-redundante de proteínas de *Homo sapiens* (NCBI nr) utilizando o *MASCOT v.2.0* (Matrix Science Ltd.).

Os resultados foram exportados para o *Software Scaffold Q+* (versão 3_00_05) e visualizados utilizando os parâmetros: probabilidade de identificação de proteínas >95%, um peptídeo, 95% de probabilidade de peptídeo. A quantificação relativa foi obtida a partir da contagem de espectros de uma proteína dividida pela contagem total de espectros da amostra. A identificação de peptídeos e proteínas foi filtrada para um máximo de 1% de taxa de falsas descobertas (*false discovery rate – FDR*). As proteínas identificadas e comuns nos diferentes tratamentos foram demonstradas utilizando a ferramenta *InteractiVenn* (Heberle *et al*, 2015).

3.2.7.6 Western Blotting

Para validação das proteínas identificadas nos experimentos de espectrometria de massas, foi realizada a técnica de Western blotting. Após a extração, 12 µg de proteínas de cada amostra a serem validadas foram misturadas ao tampão de amostra *leamml* (Tris-HCl 62,5 mM pH6.8, SDS 2%, Glicerol 10%, Azul de bromofenol 0,1%, DTT 50 mM) na proporção 1:1, desnaturadas a 96°C por 10 min e separadas por eletroforese em gel de poliacrilamida com SDS-PAGE (*sodium dodecyl sulfate polyacrylamide gel electrophoresis*) unidimensional. O gel unidimensional utilizado é constituído por um gel de empacotamento 5% e um gel de resolução 12%. As amostras foram aplicadas nos géis e em cada gel foram aplicados 5 µL do marcador de peso molecular PageRuler Prestained Protein Ladder (Thermo Scientific, Wilmington, DE, USA).

A eletroforese foi realizada a 110 V no gel de empacotamento e 130 V no gel de resolução com tampão de corrida (Tris-base 25 mM, Glicina 192 mM, 0,1% SDS). Após a corrida as proteínas foram transferidas para uma membrana de PVDF (Millipore) utilizando o tampão de transferência (Tris-base 25 mM, Glicina 200 mM pH 8.5+ 20% de metanol na hora do uso), corrente de 325 mA por 70 min.

As membranas foram bloqueadas com 5% de leite desnatado em solução de TBST (Tris-base 20mM, NaCl 137mM, 0,1% Tween-20) por 60 min à temperatura ambiente. Os anticorpos primários foram diluídos em TBST com 5% de leite desnatado ou com 5% de BSA, seguindo as especificações de cada anticorpo. As membranas foram incubadas com os anticorpos primários durante a noite a 4°C.

A β -actina foi utilizada como controle endógeno e normalizador do experimento. O anticorpo primário utilizado foi o anticorpo monoclonal produzido em camundongo anti- β -actina (Sigma-Aldrich, Missouri, EUA) na diluição 1:5000. Para as proteínas de interesse TXN1, PHB2, NEDD8, TPT1 e fosfoTPT1 (denominadas com as siglas dos genes que as codificam), foram utilizados anticorpos monoclonais produzidos em coelho (Cell Signaling, Danvers, MA, EUA) na diluição 1:1000.

Após três lavagens com TBST, as membranas foram incubadas com anticorpos secundários conjugados a peroxidases na diluição 1:1000 por 60 min sob agitação. Estes anticorpos foram produzidos em camundongo (Sigma-Aldrich, Missouri, EUA) ou coelho (Cell Signaling, Danvers, MA, EUA) e foram diluídos em

TBST com 5% de leite desnatado. Após a incubação, as membranas foram lavadas três vezes por 5 min com TBST.

Para revelação foi utilizado o kit ECL Select Western Blotting Detection Reagent (sigma-Aldrich) e a imagem foi obtida com o fotodocumentador Fuison Fx (Vilber Loumart) e pelo software FusionCapt Advance Solo 4. O programa ImageJ foi utilizado para quantificar os níveis de expressão das bandas das proteínas de interesse.

3.2.8 Análise de Metabólitos

Os perfis metabólicos dos diferentes meios condicionados (Figura 7) foram avaliados por Ressonância Magnética Nuclear (RMN) no espectrômetro Varian Unit Inova 500MHz (Agilent Technologies, Santa clara, CA, USA). A RMN é uma técnica quantitativa não destrutiva, não-invasiva, que permite obter informações de estruturas moleculares em solução com base em propriedades dos átomos, centradas em interações nucleares. É uma técnica robusta e viável para aplicações na análise de dados de metaboloma, possui alta reprodutibilidade e permite a detecção de uma ampla gama de metabólitos estruturalmente diversos.

As amostras foram filtradas em filtros *Amicon Ultra-0.5 (3K)*, *Centrifugal Filter Devices* (Millipore). Cada filtrado foi diluído em solução salina e injetado no *probe* do equipamento. Para quantificação dos metabólitos, foi adicionado à amostra 2,2-dimetil-2-silapentano-5-sulfonato de sódio 0.5 mM (DSS), um padrão interno, que é indicador do desvio químico e corresponde ao padrão de frequência zero. Na fase de preparação, foi confeccionado um mix com 480 µL da amostra, 60 µL de tampão fosfato (pH 7,4) e 60 µL de água deuterada (D₂O), de acordo com os protocolos descritos por (Beckonert *et al.*, 2007).

Os espectros de hidrogênio foram analisados pelo software *Chenomx NMR Suite*, que permite avaliação da composição da amostra pela sua comparação com mais de 200 espectros de metabólitos na base de dados do próprio programa. Para identificação, foram também consultados bancos de dados e repositórios de dados de RMN, tais como o site do Projeto Metaboloma Humano (Wishart *et al.*, 2007; Wishart *et al.*, 2009) e a Base de Dados de RMN, BMRB (Doreleijers *et al.*, 2003).

3.2.9 Análise de expressão gênica

Para avaliação do efeito do secretoma 1 na expressão de genes da via de ciclo celular e proliferação, os meios condicionados dos experimentos de hipóxia (MC1_{Controle} e MC1_{Hipo}) e dos experimentos de radiação (MC1_{Controle} e MC1_{Rad}) em triplicata foram acrescidos de SFB até concentração 10% e adicionados a 12 réplicas de culturas não tratadas, cultivadas em garrafas de 25 cm² com confluência de 70%. Três garrafas receberam MC1_{Hipo} e três garrafas receberam MC1_{Controle}, três garrafas receberam MC1_{Rad} e três garrafas receberam MC1_{Controle}. Após 24 h de tratamento, as culturas foram imediatamente bloqueadas com trizol e os RNAs foram extraídos e armazenados a -80°C para uso posterior.

3.2.9.1 Extração seqüencial de RNA

O RNA das linhagens celulares HEp-2 e SCC-25 e de fibroblastos foi extraído pelo método do trizol (Life Technologies, Grand Island, NY, USA), segundo protocolo já estabelecido no laboratório (Sambrook e Russel, 2001).

O meio das placas de cultura foi retirado e as células foram lavadas com 5 mL de PBS. O PBS foi aspirado e foram adicionados 2 mL de trizol (Sigma-Aldrich), uma solução monofásica de fenol e tiocianato de guanidina que mantém a integridade do RNA enquanto promove a lise celular. O material foi, então, distribuído em dois tubos de 1,5 mL e mantido à temperatura ambiente por 5 min. Foram adicionados 200 µL de clorofórmio gelado para cada mL de amostra em trizol, os tubos foram homogeneizados por 1 min e incubados por 10 min à temperatura ambiente. As amostras foram centrifugadas a 12000 g, por 10 min a 4°C, para separar a fase aquosa, que contém RNA, da interfase branca e leitosa que contém principalmente proteínas, e da fase orgânica, com DNA e proteínas. A fase aquosa com RNA foi transferida para um novo tubo com 500 µL de isopropanol gelado e 10 µg de glicogênio e mantida por 20 min à temperatura ambiente. Após essa etapa de precipitação, as amostras foram centrifugadas a 14000 g por 15 min a 4°C e o sobrenadante foi descartado por inversão. O sedimento foi lavado três vezes com 1 mL de etanol 70% gelado, agitado até se soltar do tubo e centrifugado a 14.000 g por 2 min a 4°C. O excesso de etanol foi retirado por pipetagem e os tubos foram

colocados em banho seco a 42°C por 1 min para secagem total do precipitado. O material foi ressuspendido em 20 µL de água livre de nucleases. As amostras foram colocadas em banho seco a 55°C por 10min para melhor eluição do RNA. Os RNAs foram armazenados a -80°C.

3.2.9.2 Reação em Cadeia da Polimerase pela transcriptase reversa (RT-PCR)

De acordo com o resultado da quantificação no equipamento *NanoDrop*, foi preparado um volume de RNA com concentração de 5 µg. O RNA de foi tratado com RQ1 *RNase-Free DNase* (Promega, Madison, WI, USA) para eliminação de DNA genômico, em reação com volume final de 25 µL.

O cDNA (DNA complementar) foi confeccionado pela reação de transcrição reversa com o sistema *High Capacity cDNA Reverse Transcription Kit* (Applied Biosystems, Foster City, CA, USA). Esse sistema utiliza a enzima transcriptase reversa *MultiScribe* e a inicialização da síntese da molécula complementar por iniciadores aleatórios – *random primers*.

Os volumes e as concentrações dos reagentes utilizados na reação de transcrição reversa foram: 5 µL de *random primers* 10X, 2 µL de dNTPs 100 mM, 5 µL de RT buffer 10X, 2,5 µL de enzima *MultiScribe* 50 U/µL. Os reagentes foram adicionados aos 25 µL provenientes do tratamento com DNase. O volume da reação foi completado para 50 µL com água tratada com DEPC. As condições da reação de RT-PCR foram: 25°C por 10 min, 37°C por 120 min, 4°C ∞. No final da reação, foram acrescentados 50 µL de água tratada com DEPC, sendo o volume final igual a 100 µL e a concentração de cDNA igual a 50 ng/µL. As amostras foram então armazenadas em freezer -20°C.

3.2.9.3 PCR array

Foram utilizadas placas de PCR Array - TaqMan® Array, Human Cyclins & Cell Cycle Regulation, Fast 96-well (Applied Biosystems, Forster City, CA, USA) (Figura 8) para analisar, em linhagens tratadas com o secretoma 1, o perfil de expressão de genes envolvidos no ciclo celular e proliferação. Estas placas são pré-desenhadas com 44 sondas de genes de interesse e 4 sondas de controles endógenos (*18S*, *GAPDH*, *HPRT1* e *GUSB*), e utilizam o sistema Taqman.

	1	2	3	4	5	6	7	8	9	10	11	12
A	18S	GAPDH	HPRT1	GUSB	ATM	ATR	18S	GAPDH	HPRT1	GUSB	ATM	ATR
B	CCNA1	CCNA2	CCNB1	CCNB2	CCND1	CCND2	CCNA1	CCNA2	CCNB1	CCNB2	CCND1	CCND2
C	CCND3	CCNE1	CCNE2	CCNH	CDC2	CDC25A	CCND3	CCNE1	CCNE2	CCNH	CDC2	CDC25A
D	CDK2	CDK4	CDK6	CDK7	CDKN1A	CDKN1B	CDK2	CDK4	CDK6	CDK7	CDKN1A	CDKN1B
E	CDKN2A	CDKN2B	CDKN2C	CDKN2D	E2F1	E2F2	CDKN2A	CDKN2B	CDKN2C	CDKN2D	E2F1	E2F2
F	E2F3	E2F4	GSK3B	HDAC1	HDAC2	HDAC3	E2F3	E2F4	GSK3B	HDAC1	HDAC2	HDAC3
G	HDAC4	HDAC5	HDAC6	HDAC7	HDAC9	PPP2CA	HDAC4	HDAC5	HDAC6	HDAC7	HDAC9	PPP2CA
H	RAF1	RB1	TGFB1	TGFB2	TGFB3	TP53	RAF1	RB1	TGFB1	TGFB2	TGFB3	TP53

Figura 8. Desenho da placa utilizada em experimentos de PCR Array (FAST Human Cyclins and Cell Cycle Regulation, Applied Biosystems).

3.2.9.4 Análise de PCR em tempo real

A PCR em tempo real foi realizada no equipamento *ABI Prism 7500 Fast Sequence Detection System* (Applied Biosystems) com o sistema *TaqMan* (Applied Biosystems) para os genes da via de ciclo celular. O *Sistema TaqMan* utiliza sondas marcadas com fluorescência com comprimentos de onda diferentes e, desta forma, permite amplificação de dois genes (gene de interesse e constitutivo) na mesma reação. Todas as sondas foram desenhadas pela própria empresa. Para amplificação, foi utilizado o *Taqman Universal PCR Master Mix* (Applied Biosystems), que contém os reagentes necessários para a reação. A fluorescência é capturada e coletada continuamente para cada amostra entre 520 e 660 nm, e a intensidade de fluorescência detectada é diretamente proporcional à quantidade de cDNA da amostra. Os dados são traduzidos por *software* específico e plotados em um gráfico que mostra a intensidade de fluorescência versus o número de ciclos.

Quanto maior a quantidade de cDNA molde presente no início da reação, menor é o número de ciclos para detecção de uma intensidade de fluorescência estatisticamente significativa. Esse ponto é definido como limiar ou C_t e ocorre durante a fase exponencial de amplificação. As reações foram feitas com um volume final de 10 μ l, que incluiu 5 μ l de *Taqman Universal PCR Master Mix*, uma sonda específica liofilizada para cada gene, 1 μ l de cDNA da amostra a ser analisada na concentração de 50 ng/ μ L e 4 μ l de água para completar o volume final da reação. Os ciclos foram de 2 min a 50°C; 20 s a 95°C e 40 ciclos de 95°C por 3 s e 60°C por 30 s.

A fórmula utilizada para avaliação da expressão dos genes foi:

<p>Taxa relativa de expressão = $2^{-(\Delta C_t \text{ do gene alvo} - \Delta C_t \text{ do calibrador})}$ = $2^{-\Delta \Delta C_t}$</p> <p>O ΔC_t foi obtido pela subtração dos C_ts dos genes alvos pelos C_ts dos genes endógenos.</p>

Foram considerados diferencialmente expressos os transcritos que exibiram expressão relativa ≥ 2.0 ou ≤ -2.0 em relação ao controle, após a transformação dos valores para logaritmo de base 2. Dessa forma, um valor de expressão relativa igual a +1 equivale a um aumento de expressão de 2 vezes, e um valor de -1 equivale a uma diminuição de expressão de 2 vezes.

3.2.10 Análise estatística

Os dados obtidos nos ensaios de curva de crescimento e citotoxicidade (MTS) foram analisados pelo teste ANOVA multifatorial seguido do teste de Tukey (utilizando $p < 0,05$) para comparação de células tratadas com CoCl_2 e controle nos tempos de tratamento (0 h, 6 h, 12 h, 24 h, 48 h, 72 h e 96 h), para células irradiadas e controle nos tempos de tratamento (0 h, 24 h, 48 h, 72 h e 96 h) e também para comparação de células tratadas com o secretoma 1 nos tempos de tratamento (0 h, 24 h, 48 h, 72 h, 96 h e 120 h). As análises das concentrações de proteínas e metabólitos e dos ensaios de migração e invasão foram feitas pelo teste t (utilizando $p < 0,05$).

4 RESULTADOS

4.1 INDUÇÃO DE ESTRESSE - HIPÓXIA

As células HEP-2 e SCC-25 foram submetidas a estresse por hipóxia para obtenção dos respectivos secretomas a serem utilizados em ensaios de meio condicionado (MC).

Indução de Hipóxia

Seleção de tempo de tratamento e dosagem para indução de hipóxia

Curvas de proliferação

Inicialmente, foram realizadas curvas de proliferação para seleção do melhor tempo de tratamento e dosagem de indutor de hipóxia (CoCl_2). Os resultados obtidos nesses ensaios utilizando as linhagens HEP-2 e SCC-25 tratadas com CoCl_2 (Figura 9) mostraram redução de proliferação em relação ao controle não tratado em todas as concentrações do composto. A análise estatística por ANOVA multifatorial pelo *GraphPrism* indicou diferença significativa de taxas de proliferação entre diferentes concentrações de CoCl_2 e períodos de tratamento ($p < 0,0001$) nas duas linhagens. A análise por ANOVA seguida do teste de Tukey foram aplicados para os seguintes grupos: fator tempo para cada concentração de CoCl_2 nas linhagens HEP-2 (50 μM , 100 μM , 150 μM , 200 μM , 250 μM e 300 μM) e SCC-25 (50 μM , 100 μM , 150 μM e 200 μM); fator concentração para os diferentes tempos de cultivo das linhagens HEP-2 (0 h, 6 h, 12 h, 24 h, 48 h e 72 h) e SCC-25 (0 h, 24 h, 48 h, 72 h e 96 h). O tempo 0 h corresponde ao momento em que o composto foi adicionado às culturas. Os resultados mostraram diferenças significativas em quase todas as concentrações da droga. Na linhagem HEP-2, não foi observada diferença significativa entre controle versus tratado apenas no tempo 6 h e nas concentrações de 50 μM e 100 μM . Na linhagem SCC-25, foram observadas diferenças no tempo 24 h e concentrações 150 μM ($p < 0,05$) e 200 μM ($p < 0,01$). Nos demais tempos, todos os tratamentos nas várias concentrações tiveram diferenças em relação ao controle.

Figura 9. Curvas de proliferação realizadas em seis tempos (0h, 6h, 12h, 24h, 48h, 72h ou 96h) para as células (A) HEP-2 e (B) SCC-25: controle (linha azul escuro) ou tratadas com CoCl_2 em diferentes concentrações (50µM, 100µM, 150µM, 200µM, 250µM e 300µM nas linhas verde, vermelha, roxa, azul turquesa, alaranjada e azul claro, respectivamente). Cada ponto representa a média de 3 réplicas.

Ensaio de citotoxicidade (MTS)

Também foram realizados ensaios de citotoxicidade para seleção do melhor tempo de tratamento e dose de CoCl_2 . No ensaio com a linhagem SCC-25, a análise estatística por ANOVA multifatorial pelo *GraphPrism* indicou diferença significativa entre as concentrações de CoCl_2 e os períodos de tratamento ($p < 0,0001$). A análise por ANOVA seguida do teste de Tukey também mostrou diferença significativa entre as doses de tratamento e o controle na maior parte dos tempos. Pode ser observado na Figura 10 que as células tratadas continuaram a proliferar, embora com eficiência menor que as não tratadas. O tempo e a concentração selecionados para os ensaios posteriores foram de 100uM por 24h porque nessas condições, além da redução na proliferação e, portanto, do efeito do CoCl_2 , não ocorreu prejuízo drástico do potencial proliferativo até 48h.

Figura 10. Ensaio de citotoxicidade (MTS) realizado em quatro tempos (24horas, 48horas, 72horas e 96horas) com as células SCC-25 controle (linha azul escuro) ou tratadas com CoCl_2 em diferentes concentrações (50uM, 100uM, 150uM e 200uM nas linhas verde, vermelha, roxa e azul claro, respectivamente). Cada ponto representa a média de 4 réplicas.

4.1.1 Análise proteômica de proteínas celulares e secretadas em ensaios de hipóxia

As células HEP-2 e SCC-25 tratadas com o indutor de hipóxia no tempo e dose selecionados e os respectivos meios condicionados com seu secretoma, bem como as células HEP-2, SCC-25 e fibroblastos normais tratadas com esses secretomas foram submetidas a protocolos de extração e análise por espectrometria de massas. Os dados obtidos permitiram a identificação de um grande número de proteínas com expressão diferencial, tanto celulares como secretadas. Para facilitar a citação dessas proteínas no texto que se segue, seus nomes (conferidos pelo *Universal Protein Resource* (UniProt: a hub for protein information, 2015) foram substituídos pelos símbolos oficiais dos genes que as codificam (Database Resources of the National Center for Biotechnology Information, 2017).

4.1.1.1 Proteínas celulares

Proteínas celulares de linhagem HEP-2 submetida a condições de hipóxia. Nas análises por espectrometria de massas do lisado de células HEP-2 tratadas com CoCl_2 e controle, foi identificado um total de 56 proteínas com expressão diferencial (Tabela 2) entre 910 identificadas: 17 mostraram expressão elevada nas células tratadas (ATP5B, BAG1, CCT4, CNBP, CUL4A, CUTA, DDOST, DYNC1LI1, HSPE1, KHSRP, MRPL12, NCL, PPP1R12A, PRDX5, PSMC6, SKP1 e SQSTM1) e 39 expressão reduzida (ANXA5, CCT6A, CKAP4, COX4I1, ENO1, FASN, FUBP1, HNRNPA1, HSPA1A, HUWE1, ILF3, LARP1, LMNB1, MST4, NDUFS8, PDIA3, PNP, PSMA2, PSMD2, PUF60, RARS, RBMX, RPL12, RPL5, RPS13, RPS15A, RPS19, RPS5, SAR1A, STAT1, TAGLN, TALDO1, TECR, TUBB, TUBB3, TUFM, UQCRB, VAPA e YWHAЕ).

Proteínas celulares de linhagem HEP-2 tratada com secretoma 1 de células HEP-2 em hipóxia. No lisado de células HEP-2 tratadas com o secretoma 1 (MC1_{Hipo} e $\text{MC1}_{\text{Controle}}$) de células HEP-2 submetidas ou não a hipóxia, foram identificadas por espectrometria de massas 34 proteínas com expressão diferencial (Tabela 3) entre 880 identificadas, 18 com expressão elevada (ACLY, ATP5A1, ATP5H, CD97,

DYNC1H1, PHB2, PPP2R1A, PRDX2, RBM39, RPLP2, RPS23, RPS4X, RPS8, SFN, SLC25A3, THRAP3, TXN1 e XPO1) e 16 com expressão reduzida (AIMP1, C14orf166, DPY30, FTSJ3, GLO1, LARS, LGALS3BP, MCM6, PDIA3, PGM1, RPS6, SARS, SRSF2, SSB, SUMF2 e UQCRH) nas células tratadas com MC1_{Hipo}.

Proteínas celulares de fibroblastos tratados com secretoma 1 de HEp-2 em hipóxia. Nas análises por espectrometria de massas de proteínas celulares obtidas de fibroblastos tratados com o secretoma 1 (MC1_{Hipo} e MC1_{Controle}) de células HEp-2 submetidas ou não a hipóxia, foram identificadas 502 proteínas. Destas, 22 apresentaram expressão diferencial (Tabela 4), 13 com expressão elevada (ANXA5, CTTN, ENO1, HDLBP, ILF3, MYDGF, PARK7, RPS11, S100A11, SYNCRIP, TUBB4B, TUBB6 e VIM) e nove com expressão reduzida (CDC37, HNRNPC, HSPE1, HSPG2, MVP, NPM1, NT5E, PRDX1 e THY1) nas células tratadas com o meio condicionado MC1_{Hipo}.

Proteínas celulares de linhagem SCC-25 submetida a condições de hipóxia. Nas células SCC-25 submetidas a condições de hipóxia, foram identificadas 43 proteínas com expressão diferencial (Tabela 5) entre 923 identificadas, 21 com expressão elevada (CCT6A, CTSD, DBNL, DSG2, FLNB, G3BP1, GCLM, GMPS, GPI, HNRNPDL, MRPL12, NCSTN, NUP160, PGK1, RBBP7, RBM14-RBM4, SH3BGRL3, SRSF6, THRAP3, UBE2N e VPS13D) e 22 com expressão reduzida (ACBD3, ADAR, CALU, CAT, CCAR2, CKAP4, CKMT1B, FSCN1, GNB2L1, HMBS, HSPA5, IFI16, IFIT1, LAP3, MCM4, MSN, PA2G4, PTBP3, RPS14, SEC22B, TNC e VBP1) nas células em hipóxia.

Proteínas celulares de linhagem SCC-25 tratada com secretoma 1 de células SCC-25 em hipóxia. Nas células SCC-25 tratadas com o secretoma 1 (MC1_{Hipo} e MC1_{Controle}), foram identificadas 35 proteínas com expressão diferencial (Tabela 6) entre 966 identificadas, 16 com expressão elevada (AKR1C1, CACYBP, CALR, CDH1, COX5A, KRT18, MCM5, NEDD8, PARP9, PNP, RAB14, SDHA, TMED2, TNKS1BP1, TPD52L2 e TPT1) e 19 com expressão reduzida (BCAP31, C14orf166, CAPN2, CBX3, CNPY2, DNAJC14, DYNC1LI2, FASN, FBL, GLUD1, HMBS, HNRNPF, HPRT1, HSP90AB1, IMMT, KPNA6, PSMC3, SEPT2 e SRP72) nas células tratadas com MC1_{Hipo}.

Proteínas celulares de fibroblastos tratados com secretoma 1 de SCC-25 em hipóxia. Nas análises por espectrometria de massas de proteínas celulares obtidas de fibroblastos tratados com o secretoma 1 (MC1_{Hipo} e MC1_{Controle}) de célula SCC-25, foram identificadas 483 proteínas. Destas, 27 apresentaram expressão diferencial (Tabela 7), 12 com expressão elevada (CCT2, CKAP4, COL6A1, DPP4, ERO1A, FAM129B, GAPDH, GNS, MT1E, MYOF, PGK1 e PKM) e 15 com expressão reduzida (CALU, CAPZA2, DBN1, DBNL, ECHS1, GLO1, HDLBP, HMGB1, HNRNPU, HSPA4, HSPA9, NACA, SUB1, TLN1 e UBQLN1) nos fibroblastos tratados com MC1_{Hipo}.

Diagrama de Venn. O diagrama de Venn foi utilizado para visualizar as proteínas compartilhadas por células HEp-2 (Figura 13) e SCC-25 (Figura 14) controles e após tratamento com CoCl₂, MC1_{Controle} e MC1_{Hipo}. A maior fração de proteínas comuns a todas as condições atingiu 403 em HEp-2 e 397 em SCC-25. Em HEp-2 e SCC-25, foram detectadas, respectivamente, 96 e 82 proteínas exclusivas de células em condições de hipóxia, 85 e 94 exclusivas de células controles, 74 e 94 exclusivas de células tratadas com MC1_{Hipo}, 72 e 103 exclusivas de células tratadas com MC1_{Controle}. Também foi observado que 26 e 35 proteínas são comuns a dois controles (controle de condição de hipóxia e controle de tratamento com secretoma) e 20 e 30 são comuns aos tratamentos com indução de hipóxia e com o secretoma MC1_{Hipo}, respectivamente para HEp-2 e SCC-25.

Validação por Western blot. Entre as proteínas com expressão diferencial, foram selecionadas quatro (PHB2, TXN1, NEDD8 e TPT1) para experimentos de validação por Western blot em função de sua relevância apontada por dados de literatura. Os resultados confirmaram níveis aumentados de PHB2 em células HEp-2 tratadas com o secretoma de HEp-2 hipóxica em relação a células tratadas com o secretoma controle. TXN1, por outro lado, mostrou níveis reduzidos em células HEp-2 tratadas, o inverso do observado nas análises por espectrometria de massas (Figura 11).

O mesmo resultado de PHB2 foi obtido para NEDD8 e para a forma fosforilada (mas não para a forma não-fosforilada) de TPT1 em células SCC-25 tratadas com o secretoma de SCC-25 hipóxica (Figura 12).

Tabela 2. Números de peptídeos únicos de proteínas celulares em três réplicas de linhagem HEp-2 controle ou tratada com CoCl_2 , obtidas em três experimentos independentes. Valor de p (teste t) atribuído pelo programa *Scaffold*. $p < 0,05$. As proteínas são representadas pelos símbolos dos genes correspondentes. Rosa e verde - menor e maior expressão, respectivamente, nas células tratadas com CoCl_2 em relação ao controle.

Gene (NCBI)	Células HEp-2 controles			Células HEp-2 tratadas com CoCl ₂			Valor de p
	Réplicas						
	1	2	3	1	2	3	
ANXA5	83	88	95	17	13	13	0.014
CCT6A	31	59	38	23	1	0	0.041
CKAP4	72	78	57	35	47	1	0.037
COX4I1	10	1	1	0	0	0	0.0000018
ENO1	56	61	50	35	42	32	0.013
FASN	83	98	12	0	66	1	0.033
FUBP1	4	49	38	23	28	19	0.0091
HNRNPA1	52	29	38	6	8	77	0.031
HSPA1A	83	59	57	93	94	10	0.028
HUWE1	31	49	38	0	19	19	0.032
ILF3	31	19	19	0	1	0	0.016
LARP1	2	19	19	0	0	0	0.0000018
LMNB1	4	49	38	0	0	19	0.0072
MST4	2	19	1	0	0	0	0.0093
NDUFS8	10	19	1	0	0	0	0.015
PDIA3	11	68	57	13	13	16	0.042
PNP	2	1	19	0	0	1	0.047
PSMA2	2	1	29	0	0	0	0.023
PSMD2	2	19	29	0	0	1	0.010
PUF60	2	1	1	0	0	0	0.022
RARS	4	49	19	0	0	0	0.015
RBMX	10	1	19	0	0	0	0.012
RPL12	31	29	29	0	19	0	0.021
RPL5	2	29	19	12	1	0	0.028
RPS13	2	1	19	0	1	0	0.044
RPS15A	2	19	19	0	0	0	0.0000018
RPS19	31	19	29	0	1	1	0.013
RPS5	31	19	19	12	1	1	0.030
SAR1A	10	19	1	0	0	0	0.015
STAT1	2	39	19	12	0	0	0.040
TAGLN	2	19	19	12	1	0	0.023
TALDO1	31	39	48	12	19	1	0.0097
TECR	10	1	1	0	0	0	0.0000018
TUBB	20	23	23	18	17	16	0.026
TUBB3	52	68	67	0	0	29	0.0089
TUFM	31	49	29	12	19	19	0.046
UQCRB	10	1	1	0	0	0	0.0000018
VAPA	10	1	19	0	0	0	0.012
YWHAE	83	78	76	13	13	11	0.0058
ATP5B	19	19	14	22	22	25	0.037
BAG1	0	0	0	12	1	1	0.00012
CCT4	10	19	19	35	38	29	0.011
CNBP	0	0	0	12	1	19	0.011
CUL4A	0	0	0	12	1	1	0.00012
CUTA	10	1	0	23	28	19	0.016
DDOST	0	0	0	12	1	1	0.00012
DYNC1LI1	0	0	0	12	1	19	0.011
HSPE1	10	0	1	23	47	48	0.020
KHSRP	31	29	48	81	66	58	0.023
MRPL12	0	1	1	23	19	19	0.016
NCL	16	15	10	21	19	19	0.037
PPP1R12A	0	0	0	23	1	1	0.036
PRDX5	62	39	38	69	75	77	0.027
PSMC6	0	0	0	23	1	1	0.036
SKP1	0	0	0	12	19	19	0.0027
SQSTM1	0	0	0	23	1	1	0.036

Tabela 3. Números de peptídeos únicos de proteínas celulares em três réplicas de linhagem HEp-2 tratadas com o secretoma 1 (MC1_{Hipo} ou MC1_{Controle}) de linhagem HEp-2 submetida ou não a condições de hipóxia. Valor de p (teste t) atribuídos pelo programa *Scaffold*. $p < 0,05$. Rosa e verde - menor e maior expressão, respectivamente, nas células tratadas com MC1_{Hipo} em relação ao controle tratado com MC1_{Controle}.

Genes (NCBI)	Células HEp-2 tratadas com o secretoma MC1 _{Controle}			Células HEp-2 tratadas com MC1 _{Hipo}			Valor de p
	Réplicas						
	1	2	3	1	2	3	
AIMP1	1	20	10	0	0	0	0.015
C14orf166	29	39	41	1	1	0	0.0039
DPY30	19	1	21	0	1	0	0.048
FTSJ3	1	1	10	0	0	0	0.00000043
GLO1	1	1	10	0	0	0	0.00000043
LARS	19	20	10	0	0	10	0.047
LGALS3BP	29	30	31	20	20	20	0.000048
MCM6	39	30	21	1	1	10	0.021
PDIA3	78	59	62	90	89	10	0.019
PGM1	19	1	21	1	0	0	0.049
RPS6	29	20	21	1	1	10	0.012
SARS	1	1	10	0	0	0	0.00000043
SRSF2	1	1	10	0	0	0	0.00000043
SSB	1	20	21	0	0	0	0.0087
SUMF2	1	1	21	0	0	0	0.020
UQCRH	19	20	10	0	1	0	0.044
ACLY	1	1	10	50	50	20	0.037
ATP5A1	11	11	10	16	13	13	0.025
ATP5H	0	0	0	1	20	10	0.015
CD97	0	0	0	20	20	20	0.0020
DYNC1H1	1	1	10	20	30	20	0.014
PHB2	0	39	41	70	59	72	0.045
PPP2R1A	29	20	31	40	40	41	0.017
PRDX2	0	0	0	30	1	31	0.026
RBM39	0	0	0	1	1	20	0.018
RPLP2	1	20	21	50	40	41	0.0048
RPS23	0	0	0	1	1	10	0.00000041
RPS4X	1	1	10	30	30	41	0.0030
RPS8	0	1	10	20	30	31	0.0023
SFN	0	49	41	70	79	92	0.039
SLC25A3	0	0	0	1	1	20	0.018
THRAP3	1	1	10	20	20	20	0.0000020
TXN	29	49	41	60	59	72	0.028
XPO1	1	0	0	1	20	20	0.045

Tabela 4. Números de peptídeos únicos de proteínas celulares em três réplicas de fibroblastos humanos normais tratados com o secretoma 1 (MC1_{Hipo} ou MC1_{Controle}) de linhagem HEP-2 submetida ou não a condições de hipóxia. Valor de p (teste t) atribuído pelo programa *Scaffold*. $p < 0,05$. Rosa e verde - menor e maior expressão, respectivamente, nas células tratadas com MC1_{Hipo} em relação ao controle tratado com MC1_{Controle}.

Gene (NCBI)	Fibroblastos tratados com o secretoma MC1 _{Controle} de HEP-2			Fibroblastos tratados com o secretoma MC1 _{Hipo} de HEP-2			Valor de p
	Réplicas						
	1	2	3	1	2	3	
CDC37	2	2	3	1	1	1	0,016
HNRNPC	4	3	3	2	2	1	0,024
HSPE1	3	2	2	0	1	1	0,024
HSPG2	8	7	9	3	5	3	0,008
MVP	5	6	6	3	2	4	0,016
NPM1	6	8	7	2	4	4	0,014
NT5E	1	2	2	0	0	1	0,047
PRDX1	3	2	4	1	1	1	0,026
THY1	1	2	2	1	0	0	0,047
ANXA5	16	15	18	28	23	24	0,008
CTTN	4	4	4	5	6	7	0,026
ENO1	7	5	11	18	17	14	0,015
HDLBP	0	0	1	2	1	2	0,047
ILF3	0	0	0	1	1	2	0,016
MYDGF	2	1	1	2	3	3	0,047
PARK7	3	1	4	6	5	6	0,033
RPS11	0	1	1	2	2	2	0,016
S100A11	5	6	7	13	11	11	0,003
SYNCRIP	0	0	2	4	4	4	0,0075
TUBB4B	8	8	9	10	10	12	0,035
TUBB6	4	5	4	7	6	6	0,013
VIM	221	207	224	251	249	238	0,012

Tabela 5. Números de peptídeos únicos de proteínas celulares em três réplicas de linhagem SCC-25 controles ou tratadas com CoCl_2 , obtidas em três experimentos independentes. Valor de p (teste t) atribuído pelo programa *Scaffold*. $p < 0,05$. As proteínas são representadas pelos símbolos dos genes correspondentes. Rosa e verde - menor e maior expressão, respectivamente, nas células tratadas com CoCl_2 em relação ao controle.

Gene (NCBI)	Células SCC-25 controles			Células SCC-25 tratadas com CoCl ₂			Valor de p
	Réplicas						
	1	2	3	1	2	3	
ACBD3	11	1	17	0	0	0	0.0040
ADAR	11	20	17	0	0	0	0.0031
CALU	22	1	1	0	0	0	0.038
CAT	22	1	1	0	0	0	0.038
CCAR2	22	20	1	0	0	0	0.017
CKAP4	12	89	84	41	56	57	0.024
CKMT1B	45	30	34	0	1	23	0.035
FSCN1	78	11	67	41	37	57	0.045
GNB2L1	45	20	42	10	0	11	0.032
HMBS	11	1	1	0	0	0	0.00026
HSPA5	28	33	31	20	21	27	0.046
IFI16	22	39	25	10	0	0	0.015
IFIT1	11	1	17	0	0	0	0.0040
LAP3	11	30	17	0	0	0	0.024
MCM4	11	20	25	0	0	0	0.010
MSN	45	39	34	0	0	11	0.0021
PA2G4	22	30	25	10	1	11	0.0021
PTBP3	11	20	25	10	0	0	0.046
RPS14	11	1	17	0	0	0	0.0040
SEC22B	22	1	1	0	0	0	0.038
TNC	45	59	76	31	28	23	0.024
VBP1	22	1	1	0	0	0	0.038
CCT6A	22	30	17	31	47	46	0.045
CTSD	22	30	1	72	47	46	0.031
DBNL	22	1	17	41	28	46	0.026
DSG2	11	1	1	52	28	46	0.011
FLNB	45	59	59	93	84	91	0.0029
G3BP1	11	0	1	31	19	34	0.021
GCLM	0	1	0	31	19	23	0.013
GMPS	0	0	1	21	1	23	0.042
GPI	34	20	17	52	47	34	0.046
HNRNPDL	0	20	17	31	28	34	0.042
MRPL12	0	0	0	21	19	11	0.0038
NCSTN	0	0	0	10	1	23	0.031
NUP160	0	1	0	21	19	23	0.0075
PGK1	45	20	34	10	10	81	0.0041
RBBP7	0	20	1	31	28	34	0.022
RBM14	0	0	0	10	1	11	0.000063
SH3BGRL3	0	0	1	10	19	11	0.050
SRSF6	11	0	0	21	19	11	0.048
THRAP3	0	1	17	21	28	23	0.049
UBE2N	0	0	0	21	19	11	0.0038
VPS13D	0	0	0	10	1	11	0.000063

Tabela 6. Números de peptídeos únicos de proteínas celulares em três réplicas de linhagem SCC-25 tratadas com o secretoma 1 (MC1_{Hipo} ou MC1_{Controle}) de linhagem SCC-25 submetida ou não a condições de hipóxia. Valor de p (teste t) atribuídos pelo programa *Scaffold*. p<0,05. Rosa e verde - menor e maior expressão, respectivamente, nas células tratadas com MC1_{Hipo} em relação ao controle.

Gene (NCBI)	SCC-25 tratadas com o secretoma MC1 _{Controle} de SCC-25			SCC-25 tratadas com o secretoma MC1 _{Hipo} de SCC-25			Valor de p
	Réplicas						
	1	2	3	1	2	3	
BCAP31	20	10	20	0	0	0	0.0068
C14orf166	20	30	30	10	1	0	0.013
CAPN2	10	10	1	0	0	0	0.000000022
CBX3	30	51	40	20	20	20	0.028
CNPY2	10	10	20	0	0	0	0.015
DNAJC14	20	20	1	10	0	0	0.048
DYNC1LI2	10	20	1	0	0	0	0.018
FASN	20	20	30	0	0	1	0.012
FBL	10	10	1	0	0	0	0.000000022
GLUD1	50	61	60	40	20	40	0.035
HMBS	10	20	1	0	0	0	0.018
HNRNPF	30	41	40	30	20	20	0.047
HPRT1	10	20	20	10	0	0	0.047
HSP90AB1	14	14	14	10	11	11	0.00044
IMMT	10	10	1	0	0	0	0.000000022
KPNA6	20	20	20	10	1	0	0.015
PSMC3	20	10	20	0	1	0	0.044
SEPT2	20	10	1	0	0	0	0.016
SRP72	10	20	1	0	0	0	0.018
AKR1C1	0	0	1	30	20	20	0.014
CACYBP	0	0	0	20	1	1	0.017
CALR	10	12	11	15	16	16	0.0031
CDH1	20	20	20	50	30	40	0.029
COX5A	0	0	0	10	1	1	0.000000021
KRT18	0	20	20	30	39	50	0.041
MCM5	0	0	0	10	1	1	0.000000021
NEDD8	10	10	1	20	20	20	0.00000011
PARP9	0	0	0	10	1	20	0.017
PNP	0	10	1	30	20	20	0.026
RAB14	10	0	0	20	20	1	0.048
SDHA	20	20	20	40	30	30	0.019
TMED2	10	0	1	20	30	20	0.023
TNKS1BP1	10	10	1	50	39	60	0.0025
TPD52L2	0	0	1	20	20	30	0.013
TPT1	10	10	1	20	20	30	0.017

Tabela 7. Números de peptídeos únicos de proteínas celulares em três réplicas de fibroblastos humanos normais tratados com o secretoma 1 (MC1_{Hipo} ou MC1_{Controle}) de linhagem SCC-25 submetida ou não a condições de hipóxia. Valor de p (teste t) atribuído pelo programa *Scaffold*. $p < 0,05$. Rosa e verde - menor e maior expressão, respectivamente, nas células tratadas com MC1_{Hipo} em relação ao controle.

Gene (NCBI)	Fibroblastos tratados com o secreteroma MC1 _{Controle} de SCC-25			Fibroblastos tratados com o secretoma MC1 _{Hipo} de SCC-25			Valor de p
	Réplicas						
	1	2	3	1	2	3	
CALU	5	5	4	2	3	1	0,026
CAPZA2	1	1	1	0	0	0	< 0.00010
DBN1	3	5	3	0	0	1	0,0095
DBNL	2	4	3	1	1	1	0,022
ECHS1	3	3	4	2	2	2	0,016
GLO1	2	2	1	0	0	0	0,0083
HDLBP	1	1	1	0	0	0	< 0.00010
HMGB1	1	1	1	0	0	0	< 0.00010
HNRNPU	3	3	3	1	0	2	0,029
HSPA4	1	1	1	0	0	0	< 0.00010
HSPA9	4	4	4	2	1	3	0,03
NACA	2	2	2	1	1	1	< 0.00010
SUB1	1	1	1	0	0	0	< 0.00010
TLN1	19	16	15	12	12	14	0,033
UBQLN1	1	1	1	0	0	0	< 0.00010
CCT2	0	0	0	2	2	1	0,0085
CKAP4	7	5	8	10	8	11	0,041
COL6A1	6	7	5	8	7	8	0,036
DPP4	1	1	1	2	3	2	0,017
ERO1A	1	0	0	2	2	3	0,012
FAM129B	0	0	0	1	1	1	< 0.00010
GAPDH	9	5	13	33	15	26	0,05
GNS	0	0	0	1	2	2	0,0072
MT1E	2	1	1	3	4	3	0,015
MYOF	0	1	0	4	2	2	0,034
PGK1	4	2	6	7	8	7	0,04
PKM	18	16	18	21	20	22	0,025

Figura 11. O secretoma_{1Hipo} promoveu elevação da expressão de PHB2 e diminuição da expressão de TXN1. Western blot para (A) PHB2 e (B) TXN1 de extratos proteicos de HEp-2 tratada com MC1_{Controle} (C1, C2, C3) e com MC1_{Hipo} (H1, H2). Quantificação dos níveis de expressão das proteínas de interesse (C) PHB2 e (D) TXN1 normalizados pelos níveis de expressão do controle endógeno β-actina (pelo programa ImageJ).

Figura 12. O secretome 1_{Hipo} promoveu aumento de expressão de NEDD8, mas não alterou os níveis de TPT1. Western blot para (A) NEDD8, (B) TPT1 e TPT1 fosforilada de extratos proteicos de SCC-25 tratada com MC1_{Controle} (C1, C2) e com MC1_{Hipo} (H1, H2, H3). Quantificação dos níveis de expressão das proteínas de interesse (C) NEDD8, (D) TPT1 e (E) TPT1 fosforilada, normalizados pelos níveis de expressão do controle endógeno β-actina (pelo programa ImageJ).

Localização subcelular e processos biológicos associados a proteínas celulares

Na distribuição de todas as proteínas celulares das linhagens HEp-2, SCC-25 e fibroblastos por localização subcelular (Figura 15 A-F), pode ser observado que a maior parte (50 a 96%) está localizada na célula (citoplasma, membrana e núcleo) e uma pequena parte (2%) principalmente no espaço extracelular. Estas últimas podem ser decorrentes de contaminação com o meio de cultura durante o processamento do material ou possuem tanto localização celular como extracelular. Uma outra parcela pequena (2 a 3%) não tem localização definida (Uniprot).

De acordo com a busca realizada no Gene Ontology (Ashburner *et al.*, 2000), as proteínas celulares diferencialmente expressas após tratamento com indutor de hipóxia e processos biológicos relacionados estão apresentadas nas Tabelas 8 e 9. As proteínas celulares diferencialmente expressas após tratamento com MC1_{Hipo} e MC1_{Controle} (apresentadas nas Tabelas 10 a 13) estão relacionadas com resposta a estresse, transcrição, transporte de proteínas, transdução de sinal e adesão, proliferação e diferenciação.

Figura 15. Localização subcelular de proteínas celulares identificadas em (A) linhagem HEP-2 e (B) SCC-25 após tratamento com CoCl_2 *versus* controle; (C) linhagem HEP-2 e (D) SCC-25 após tratamento com MC1_{Hipo} *versus* $\text{MC1}_{\text{Controle}}$; fibroblastos após tratamento com o secretoma MC1_{Hipo} *versus* $\text{MC1}_{\text{Controle}}$ de (E) HEP-2 e (F) SCC-25.

Tabela 8. Processos e atividades atribuídos às proteínas celulares de HEP-2 e SCC-25 com expressão aumentada após tratamento com CoCl₂ em relação aos controles (segundo Gene Ontology).

Processo biológico	HEp-2 tratadas com CoCl ₂ X controle	SCC-25 tratadas com CoCl ₂ X controle
	proteínas	
Angiogênese	<i>ATP5B, NCL</i>	<i>GPI</i>
Apoptose	<i>BAG1, CUL4A, SQSTM1</i>	<i>NCSTN</i>
regulação negativa	<i>PRDX5</i>	
regulação	<i>PSMC6</i>	<i>CTSD, DBNL, DSG2</i>
Autofagia	<i>SQSTM1</i>	
Comunicação celular		
transdução de sinal	<i>BAG1, PPP1R12A, SKP1, SQSTM1</i>	<i>DBNL, FLNB, G3BP1, NCSTN, NUP160, UBE2N</i>
Ciclo celular		
replicação do DNA		<i>RBBP7, RBM14</i>
ciclo celular mitótico	<i>CUL4A, DYNC1LI1, PPP1R12A, PSMC6, SKP1</i>	<i>NUP160</i>
regulação negativa	<i>CUL4A</i>	
Proliferação celular		
regulação negativa	<i>CUL4A</i>	
regulação positiva	<i>CNBP</i>	
regulação		<i>NCSTN, RBBP7</i>
Adesão celular	<i>ATP5B, PPP1R12A</i>	<i>DSG2</i>
Migração celular		<i>SH3BGRL3</i>
Processo de desenvolvimento celular		
diferenciação celular	<i>SQSTM1</i>	<i>FLNB</i>
Homeostase celular		
Organização de componente celular		
crescimento celular		<i>RBBP7</i>
Expressão gênica	<i>DDOST, KHSRP, PSMC6</i>	<i>SRSF6</i>
Processo metabólico		<i>GCLM, GPI, SH3BGRL3</i>
processo metabólico de ATP	<i>ATP5B</i>	
processo metabólico de carboidrato		<i>GMPS</i>
processo metabólico de ácido orgânico		<i>GMPS</i>
processo de oxidação-redução		
processo metabólico de proteína	<i>CCT4, DDOST</i>	<i>CCT6A, UBE2N</i>
processamento de RNA	<i>PSMC6</i>	<i>PGK1</i>
Resposta a estímulo		
resposta imune	<i>DDOST</i>	<i>FLNB, GPI, UBE2N</i>
resposta inflamatória	<i>PRDX5</i>	
resposta a estresse oxidativo	<i>PRDX5</i>	<i>GCLM</i>
resposta a estresse	<i>ATP5B, BAG1, PSMC6, SQSTM1</i>	<i>UBE2N</i>
Transcrição	<i>CNBP, KHSRP</i>	<i>HNRNPDL, MRPL12, RBBP7, RBM14, SRSF6, THRAP3</i>
Tradução	<i>DDOST, HSPE1, MRPL12</i>	<i>MRPL12, NCSTN</i>
Transporte		
transporte de proteína	<i>DYNC1LI1</i>	<i>G3BP1</i>
transporte de RNA	<i>CUTA, SQSTM1</i>	<i>VPS13D</i>
transporte transmembrana		<i>NUP160</i>

Tabela 9. Processos e atividades atribuídos às proteínas celulares de HEP-2 e SCC-25 com expressão diminuída após tratamento com CoCl₂ em relação aos controles (segundo Gene Ontology).

Processo biológico	HEp-2 tratadas com CoCl ₂ X controle	SCC-25 tratadas com CoCl ₂ X controle
	proteínas	
Angiogênese	STAT1	
Apoptose	ANXA5, HSPA1A, LARP1, LMNB1, MST4, PSMA2, PSMD2, STAT1, YWHAE, PDIA3, PUF60	ADAR, CAT, HSPA5, CCAR2, GNB2L1
Autofagia	LARP1	IFI16
Comunicação celular transdução de sinal	ANXA5, HSPA1A, LARP1, PDIA3, STAT1, VAPA, YWHAE	ADAR, CAT, GNB2L1, HSPA5, SEC22B, TNC
Ciclo celular replicação do DNA ciclo celular mitótico	RPS15A, ILF3, MST4, PSMA2, PSMD2, TUBB, TUBB3, YWHAE	GNB2L1, CAT, PA2G4 MCM4 MCM4
Proliferação celular regulação negativa regulação positiva regulação	HSPA1A, STAT1 RPS15A PNP	TNC FSCN1, IFI16, PA2G4
Adesão celular	ENO1, HSPA1A, LARP1, VAPA	MSN, SEC22B, TNC
Migração celular	STAT1	FSCN1, GNB2L1, MSN
Processo de desenvolvimento celular diferenciação celular	HUWE1, MST4, STAT1, TAGLN	PTBP3
Organização de componente celular crescimento celular	ENO1	GNB2L1
Expressão gênica	FUBP1, HNRNPA1, HSPA1A, PSMA2, PSMD2, RARS, RBMX, RPL12, RPL5, RPS13, RPS15A, RPS5	ADAR, RPS14, TNC
Processo metabólico processo metabólico de carboidrato processo metabólico de ácido orgânico processo de oxidação-redução processo metabólico de proteína processo de modificação proteica processo metabólico de RNA processamento de RNA	COX4I1, FASN TALDO1 PSMD2 ENO1, TECR, UQCRB CCT6A, PDIA3, RPL12, RPL5, RPS13, RPS15A, RPS19, RPS5, TUBB3 CCT6A, MST4, PDIA3, TUBB3, YWHAE HSPA1A, PSMA2, PSMD2, RPL5, RPS13, RPS15A, RPS19, RPS5 HNRNPA1, PUF60, RBMX, RPS19	CKMT1B, HMBS, ACBD3 VBP1, LAP3 CALU, IFIT1, VBP1 RPS14, SEC22B ADAR, CCAR2, PTBP3
Resposta a estímulo resposta imune resposta inflamatória resposta a estímulo externo resposta a hipóxia resposta a estresse oxidativo resposta a estresse	HSPA1A PNP, RBMX, STAT1, TUBB COX4I1, ENO1, RPS19 NDUFS8 ANXA5, HSPA1A, PSMA2	ADAR, IFI16, IFIT1 IFI16 CAT, HMBS
Transcrição	ENO1, FUBP1, ILF3, LARP1, LMNB1, STAT1	IFI16, PA2G4
Tradução	LARP1, LMNB1, RPL12, RPL5, RPS13, RPS15A, RPS19, RPS5, TUFM	GNB2L1, PA2G4, RPS14
Transporte transporte de proteína transporte de RNA transporte transmembrana transporte mediado a vesícula	PDIA3, SAR1A HNRNPA1 COX4I1 VAPA	ACBD3 ADAR, SEC22B SEC22B

Tabela 10. Processos biológicos atribuídos às proteínas celulares de HEp-2 e SCC-25 com expressão elevada após tratamento com MC1_{Hipo} em relação a controles (segundo Gene Ontology).

Processo biológico	HEp-2 tratadas com MC1 _{Hipo} x MC1 _{Controle} de HEp-2	SCC-25 tratadas com MC1 _{Hipo} x MC1 _{Controle} de SCC-25
	proteínas	
Apoptose anti-apoptose regulação negativa regulação	<i>PRDX2</i> <i>PHB2, PPP2R1A</i>	 <i>CACYBP, TPT1</i> <i>KRT18</i>
Comunicação celular sinalização célula-célula transdução de sinal	<i>CD97, TXN</i> <i>CD97, PHB2, PPP2R1A, SFN,</i> <i>THRAP3, TXN</i>	 <i>CALR, KRT18, NEDD8, RAB14</i>
Ciclo celular replicação do DNA ciclo celular mitótico regulação positiva	<i>PPP2R1A</i> <i>DYNC1H1, PPP2R1A</i> <i>PHB2</i>	 <i>CACYBP, KRT18, MCM5</i> <i>MCM5</i> <i>COX5A</i>
Proliferação celular proliferação de queratinócitos regulação positiva regulação	<i>SFN</i> <i>RPS4X</i> <i>ATP5A1, PHB2, TXN</i>	 <i>KRT18</i> <i>CALR, PNP, TPD52L2</i>
Adesão celular	<i>PPP2R1A, SFN</i>	<i>CDH1, KRT18, PNP, TNKS1BP1</i>
Migração celular		<i>PARP9</i>
Processo de desenvolvimento celular diferenciação celular	<i>PPP2R1A</i>	<i>AKR1C1, CACYBP, CALR, COX5A, TPT1</i>
Homeostase celular	<i>TXN</i>	<i>TPT1</i>
Organização de componente celular crescimento celular organização da mitocôndria organização da membrana	<i>PPP2R1A, SFN</i> <i>PHB2</i> <i>SFN</i>	 <i>RAB14, TMED2</i>
Organização de matriz extracelular		<i>CDH1</i>
Expressão gênica	<i>XPO1</i>	
Processo metabólico processo metabólico de ATP processo glicolítico processo metabólico de ácido orgânico processo de oxidação-redução processo metabólico de proteína	<i>ATP5A1, ATP5H</i> <i>SLC25A3</i> <i>ACLY</i> <i>TXN</i>	 <i>AKR1C1, COX5A, PNP, SDHA</i> <i>NEDD8</i>
Resposta a estímulo resposta de defesa resposta imune resposta a estímulo externo resposta a estresse oxidativo resposta a estresse	 <i>CD97</i> <i>PHB2</i> <i>PRDX2, TXN</i>	 <i>PARP9</i> <i>PNP</i> <i>PARP9, RAB14, TNKS1BP1, TPT1</i>
Senescência		<i>CALR</i>
Transcrição	<i>PHB2, RBM39, RPLP2, RPS23,</i> <i>RPS4X, RPS8, THRAP3, TXN</i>	<i>CDH1, NEDD8</i>
Tradução	<i>RPLP2, RPS23, RPS4X, RPS8</i>	
Transporte transporte de proteína transporte de RNA transporte transmembrana transporte mediado por vesícula	 <i>RPLP2, RPS23, RPS4X, RPS8, XPO1</i> <i>XPO1</i> <i>SLC25A3</i> <i>DYNC1H1</i>	 <i>NEDD8, PNP, RAB14, TMED2</i> <i>RAB14</i>

Tabela 11. Processos biológicos atribuídos às proteínas celulares de HEp-2 e SCC-25 com expressão reduzida após tratamento com MC1_{Hipo}, em relação a controles (segundo Gene Ontology).

Processo biológico	HEp-2 tratadas com MC1 _{Hipo} x MC1 _{Controle} de HEp-2	SCC-25 tratadas com MC1 _{Hipo} x MC1 _{Controle} de SCC-25
	proteínas	
Angiogênese	<i>AIMP1</i>	
Apoptose		
Ligação	<i>GLO1, AIMP1, RPS6</i>	<i>BCAP31, HSP90AB1</i>
ligação de proteína		
Comunicação celular		<i>HSP90AB1</i>
sinalização célula-célula		
transdução de sinal	<i>AIMP1</i>	
Ciclo celular	<i>AIMP1, LGALS3BP, PDIA3, RPS6</i>	<i>BCAP31, CNPY2, HNRNPF, PSMC3, SEPT2</i>
replicação do DNA		
ciclo celular mitótico	<i>MCM6</i>	
Proliferação celular	<i>MCM6, RPS6, SRSF2</i>	<i>PSMC3, SEPT2</i>
regulação negativa		
Adesão celular		<i>HPRT1</i>
Processo de desenvolvimento celular	<i>AIMP1, LGALS3BP</i>	<i>FASN, HSP90AB1, SEPT2</i>
diferenciação celular		
Homeostase celular	<i>GLO1</i>	<i>FASN, HPRT1, HSP90AB1</i>
Organização de componente celular		<i>IMMT</i>
organização do citoesqueleto		
organização da membrana		<i>DYNC1LI2</i>
Organização de matriz extracelular		<i>IMMT</i>
Expressão gênica		<i>CAPN2</i>
Processo metabólico	<i>GLO1, FTSJ3, RPS6, SARS, SRSF2, SSB</i>	<i>CNPY2, HNRNPF, KPNA6, PSMC3</i>
processo metabólico de carboidrato		
processo metabólico de ácido orgânico	<i>PGM1</i>	
processo de oxidação-redução	<i>GLO1</i>	<i>GLUD1</i>
processo metabólico de proteína	<i>PDIA3, UQCRH</i>	<i>FASN, GLUD1</i>
processo metabólico de RNA	<i>SUMF2</i>	<i>CAPN2, CNPY2, FBL, HMBS, PSMC3</i>
Resposta a estímulo	<i>LARS, SRSF2, SSB</i>	<i>FBL, PSMC3</i>
resposta de defesa		
resposta imune		<i>CAPN2</i>
resposta a estímulo externo	<i>AIMP1, PDIA3</i>	<i>PSMC3</i>
resposta a hipóxia	<i>C14orf166, DPY30</i>	<i>C14orf166, CBX3</i>
resposta a estresse	<i>LARS, RPS6, SARS, SSB</i>	
Senescência		
Transcrição		<i>BCAP31, DNAJC14, KPNA6, SRP72</i>
Tradução	<i>C14orf166</i>	<i>C14orf166</i>
Transporte		<i>DYNC1LI2</i>

Tabela 12. Processos biológicos atribuídos às proteínas celulares com expressão aumentada em fibroblastos após tratamento com MC1_{Hipo} em relação aos controles tratados com MC1_{Controle} de HEP-2 e SCC-25 (segundo Gene Ontology).

Processo biológico	Fibroblastos tratados com MC1 _{Hipo} x MC1 _{Controle} de HEP-2	Fibroblastos tratados com MC1 _{Hipo} x MC1 _{Controle} de SCC-25
	proteínas	
Angiogênese	MYDGF	
Apoptose regulação	ANXA5, MYDGF	GAPDH, FAM129B
Autofagia	PARK7	
Comunicação celular transdução de sinal	S100A11, ANXA5, MYDGF, VIM, CTTN	MYOF
Ciclo celular ciclo celular mitótico regulação negativa	TUBB4B S100A11	
Proliferação celular regulação negativa regulação positiva	S100A11 MYDGF	DPP4
Adesão celular	S100A11, ENO1, CTTN, PARK7, HDLBP	COL6A1, DPP4, PKM, FAM129B
Migração celular	CTTN	DPP4
Processo de desenvolvimento celular diferenciação celular	SYNCRIP, VIM	ERO1A, COL6A1, PGK1
Organização de componente celular crescimento celular organização do citoesqueleto organização da mitocôndria organização da membrana	ENO1 TUBB6, TUBB4B PARK7 CTTN	MT1E
Organização de matriz extracelular		ERO1A, COL6A1
Expressão gênica	VIM, RPS11	
Processo metabólico processo metabólico de ATP processo metabólico de ácido orgânico processo metabólico de proteína processo de modificação proteica processo metabólico de RNA	ILF3 SYNCRIP, RPS11, MYDGF	PKM, GAPDH GNS, PGK1 CKAP4 CCT2, ERO1A
Resposta a estímulo resposta imune resposta inflamatória resposta a estímulo externo resposta a hipóxia resposta a estresse oxidativo	TUBB4B PARK7 SYNCRIP, ANXA5, ILF3, TUBB4B	GNS, CCT2, CKAP4 ERO1A, DPP4, PKM, MYOF ERO1A
resposta a estresse	ILF3, ENO1	
Senescência	SYNCRIP, RPS11, ILF3	GAPDH
Transcrição		
Tradução	HDLBP	
Transporte	RPS11, CTTN	

Tabela 13. Processos biológicos atribuídos às proteínas celulares com expressão reduzida em fibroblastos após tratamento com MC1_{Hipo} em relação aos controles tratados com MC1_{Controle} de HEP-2 e SCC-25 (segundo Gene Ontology).

Processo biológico	Fibroblastos tratados com MC1 _{Hipo} x MC1 _{Controle} de HEP-2	Fibroblastos tratados com MC1 _{Hipo} x MC1 _{Controle} de SCC-25
	proteínas	
Angiogênese	<i>HSPG2</i>	
Apoptose		
regulação positiva	<i>HSPE1</i>	
regulação		GLO1, HSPA9
Ligação		
ligação de proteína	<i>CDC37, HNRNPC</i>	
ligação de RNA	<i>HNRNPC</i>	
Comunicação celular		
transdução de sinal	<i>MVP</i>	SUB1, UBQLN1
Proliferação celular		
regulação	<i>NPM1, PRDX1</i>	<i>CALU</i>
Adesão celular	<i>PRDX1, NT5E, THY1</i>	DBN1, DBNL, CALU, HDLBP
Migração celular		
regulação negativa	<i>THY1</i>	CALU, CAPZA2
Processo de desenvolvimento celular		
diferenciação celular	<i>HSPE1, THY1</i>	GLO1, HNRNPU, HSPA9
Organização de componente celular		
organização do citoesqueleto		<i>DBN1, TLN1</i>
Organização de matriz extracelular	<i>HSPG2</i>	
Expressão gênica	<i>NPM1</i>	GLO1, HNRNPU, NACA, SUB1
Processo metabólico		
processo metabólico de carboidrato	<i>NT5E</i>	
processo metabólico de DNA		HNRNPU
processo metabólico de ácido orgânico		GLO1, ECHS1
processo de oxidação-redução	<i>PRDX1</i>	
processo metabólico de proteína	<i>HSPG2, CDC37</i>	UBQLN1
processo metabólico de RNA		<i>HNRNPU</i>
Resposta a estímulo		
resposta imune	<i>MVP, PRDX1</i>	
resposta a hipóxia		UBQLN1
resposta a estresse oxidativo	<i>PRDX1</i>	
resposta a estresse	<i>NPM1, HSPE1</i>	DBNL, HMGB1, CAPZA2, HSPA4
Senescência	<i>NPM1</i>	
Transporte		
transporte de lipídio		<i>HDLBP</i>
transporte de proteína	<i>NPM1, MVP</i>	HSPA9, HSPA4
transporte de RNA	<i>MVP</i>	

4.1.1.2 Proteínas Secretadas

Secretoma 1 de células HEp-2 submetidas a condições de hipóxia. No secretoma 1, ou seja, no meio condicionado por células em condições de hipóxia e seu controle, foram identificadas 1084 proteínas. Destas, 46 apresentaram expressão diferencial, das quais 34 são extracelulares (Tabela 14), quatro com expressão elevada (FSTL1, GAPDH, HSPG2 e NDRG1) e 30 com expressão reduzida (ACLY, AGRN, CCT8, CFH, COL3A1, CTSL, EFEMP1, FBLN1, FBN2, GSTP1, HDGF, HINT1, HSPA1A/HSPA1B, IGFBP2, IGFBP4, LAMA3, LAMA5, LTBP1, NPC2, NPNT, PCSK9, PGK1, PSMD2, PXDN, QSOX1, SEMA3B, TFPI, TGFBI, TIMP2 e YWHAZ) no meio condicionado MC1_{Hipo}.

Secretoma 2 de células HEp-2 tratadas com secretoma 1 de células HEp-2 em hipóxia. No secretoma 2 de células HEp-2 tratadas com o secretoma 1 (MC1_{Hipo} e MC1_{Controle}), foram identificadas 653 proteínas. Destas, 24 apresentaram expressão diferencial, das quais 15 são extracelulares (Tabela 15), nove com expressão elevada (ACTN4, ARPC2, C1R, CALU, CTSZ, GGH, HSPA8, LPL e SLC39A10) e seis com expressão reduzida (CLTC, DSTN, FBLN1, FKBP4, PSAP e TALDO1) no meio condicionado por células tratadas com MC1_{Hipo} (MC2_{Hipo}).

Secretoma 2 de fibroblastos tratados com secretoma 1 de HEp-2 em hipóxia. Nas análises por espectrometria de massas do secretoma 2 de fibroblastos tratados com o secretoma 1 (MC1_{Hipo} e MC1_{Controle}) de HEp-2 submetidas a hipóxia, foram identificadas 149 proteínas. Destas, 10 apresentaram expressão diferencial (Tabela 16), sendo todas extracelulares, quatro mostraram expressão elevada (AHSG, IGFBP4, MMP1 e YWHAB) e seis expressão reduzida (APP, ECM1, FBN1, ITGBL1, LAMB1 e LTBP2) no meio condicionado por células tratadas com MC1_{Hipo} (MC2_{Hipo}).

Secretoma 1 de células SCC-25 submetidas a condições de hipóxia. No secretoma 1, ou seja, no meio condicionado por células em condições de hipóxia e seu controle, foram identificadas 493 proteínas. Destas 66 apresentaram expressão diferencial, das quais 45 são extracelulares (Tabela 17), cinco com expressão elevada (CBR1, DBNL, HMGB1, HSPB1 e TPM3) e 40 com expressão reduzida (AARS, APP, ARHGDIB, BLVRA, CA2, CCT4, CLIC1, CLTC, CMPK1, CNDP2,

DKK3, DNAJB1, EIF4A1, EIF6, FAM49B, FLNB, GANAB, GAPDH, GDI2, GNPDA1, HNRNPA1, IQGAP1, MVP, OLA1, OTUB1, PAICS, PDCD5, PDIA6, PGD, PPA1, PPP1CA, PSMA2, PSMA5, RPLP2, S100A11, SERPINB5, TCP1, TFRC, TNC e YWHAB) no meio condicionado MC1_{Hipo}.

Secretoma 2 de células SCC-25 tratadas com secretoma 1 de células SCC-25 em hipóxia. No secretoma 2 de células SCC-25 tratadas com o secretoma 1 (MC1_{Hipo} e MC1_{Controle}), foram identificadas 813 proteínas. Destas, 26 apresentaram expressão diferencial, 14 são extracelulares (Tabela 18), 11 com expressão elevada (AHCY, FABP5, GFPT1, IQGAP1, LGALS1, MYL6, PKM, PPA1, PRDX5, SDC1 e UBA1) e 3 com expressão reduzida (GAA, HNRNPL e PPL) no meio condicionado por células tratadas com MC1_{Hipo} (MC2_{Hipo}).

Secretoma 2 de fibroblastos tratados com secretoma 1 de SCC-25 em hipóxia. Nas análises por espectrometria de massas do secretoma 2 de fibroblastos tratados com o secretoma 1 (MC1_{Hipo} e MC1_{Controle}) de SCC-25, foram identificadas 116 proteínas. Destas, nove apresentaram expressão diferencial (Tabela 19), sendo todas extracelulares, cinco mostraram expressão elevada (COL6A2, COL6A3, MDH1, TPI1 e YWHAB) e quatro expressão reduzida (COL1A2, FBN1, HSPB1 e NID1) no meio condicionado por células tratadas com MC1_{Hipo} (MC2_{Hipo}).

Diagrama de Venn. As proteínas identificadas nos secretomas 1 (MC1_{Controle} e MC1_{Hipo}) e secretoma 2 (MC2_{Controle} e MC2_{Hipo}) foram utilizadas para construir o diagrama de Venn (Figuras 16 e 17). Uma fração significativa de proteínas comuns a todas as condições incluiu 110 proteínas em HEP-2 e 175 em SCC-25. O número de proteínas exclusivas de cada condição foi bastante expressivo, com 319 e 11 exclusivas do MC1_{Hipo}, 365 e 59 exclusivas MC1_{Controle}, 80 e 107 exclusivas do MC2_{Hipo}, 74 e 120 exclusivas do MC2_{Controle} no secretoma de células HEP-2 e SCC-25, respectivamente. No secretomas da linhagem HEP-2 estão presentes 6 proteínas exclusivas dos meios MC1_{Hipo} e MC2_{Hipo} e 27 exclusivas dos MC1_{Controle} e MC2_{Controle}, enquanto em SCC-25 estão presentes 3 e 30 proteínas, respectivamente (Figuras 16 e 17).

Tabela 14. Números de peptídeos únicos em três réplicas do secretoma 1 de células HEp-2 controles ou tratadas com CoCl_2 , obtidas em três experimentos independentes. Valor de p (teste t) atribuído pelo programa *Scaffold*. $p < 0,05$. As proteínas são representadas pelos símbolos dos genes correspondentes. Rosa e verde - menor e maior expressão, respectivamente, nas células tratadas com CoCl_2 em relação aos controles.

Gene (NCBI)	Secretoma 1 de células HEp-2 controles (MC1 _{Controle})			Secretoma 1 de células HEp-2 tratadas com CoCl2 (MC1 _{Hipo})			Valor de p
	Réplicas						
	1	2	3	1	2	3	
ACLY	26	36	23	0	16	0	0.024
AGRN	52	81	100	13	0	18	0.011
CCT8	13	20	23	0	0	0	0.0033
CFH	10	71	12	13	16	36	0.0094
COL3A1	65	51	46	0	0	18	0.0043
CTSL	26	20	23	13	0	0	0.016
EFEMP1	14	66	11	13	32	0	0.019
FBLN1	22	86	11	0	0	0	0.026
FBN2	91	71	92	0	0	0	0.00023
GSTP1	13	25	23	13	0	0	0.050
HDGF	13	20	15	0	0	0	0.0017
HINT1	13	0.5	1	0	0	0	0.021
HSPA1A/1B	65	56	46	10	13	89	0.014
IGFBP2	65	66	54	39	47	36	0.017
IGFBP4	39	30	38	0	16	0	0.0066
LAMA3	39	10	23	0	0	0	0.045
LAMA5	18	86	11	0	0	0	0.012
LTBP1	13	0.5	15	0	0	0	0.023
NPC2	13	15	1	0	0	0	0.0061
NPNT	13	0.5	15	0	0	0	0.023
PCSK9	65	76	15	13	16	0	0.035
PGK1	65	76	77	17	21	25	0.0044
PSMD2	13	15	1	0	0	0	0.0061
PXDN	78	18	25	26	16	0	0.038
QSOX1	26	20	15	13	0	0	0.038
SEMA3B	26	10	38	0	0	0	0.038
TFPI	39	20	15	0	0	0	0.026
TGFBI	26	36	23	0	0	18	0.034
TIMP2	52	36	38	13	16	0	0.0100
YWHAZ	26	25	23	0	0	0	0.000011
FSTL1	16	11	14	39	16	71	0.013
GAPDH	13	18	21	27	24	27	0.037
HSPG2	25	11	23	13	32	18	0.015
NDRG1	0	0	0	26	16	36	0.011

Tabela 15. Números de peptídeos únicos em três réplicas do secretoma 2 de células HEp-2 tratadas com o secretoma 1 (MC1_{Hipo} ou MC1_{Controle}) de linhagem HEp-2 submetida ou não a condições de hipóxia. Valor de p (teste t) atribuído pelo programa *Scaffold*. p<0,05. Rosa e verde - menor e maior expressão, respectivamente.

Gene (NCBI)	Secretoma 2 de HEp-2 tratadas com MC1 _{Controle} de células HEp-2			Secretoma 2 de HEp-2 tratadas com MC1 _{Hipo} de células HEp-2			Valor de p
	Réplicas						
	1	2	3	1	2	3	
CLTC	97	10	71	60	60	40	0.036
DSTN	19	20	30	10	10	10	0.020
FBLN1	78	13	13	18	18	18	0.018
FKBP4	19	10	20	0	10	0	0.047
PSAP	78	10	12	16	13	15	0.036
TALDO1	29	40	30	0	20	10	0.026
ACTN4	12	17	13	23	19	20	0.027
ARPC2	0	10	0	20	20	10	0.046
C1R	12	12	11	15	15	13	0.015
CALU	0	0	0	10	30	10	0.025
CTSZ	19	20	30	40	40	51	0.015
GGH	29	20	40	70	70	91	0.0066
HSPA8	78	70	60	80	90	91	0.044
LPL	49	50	40	70	10	91	0.012
SLC39A10	0	0	0	10	10	10	0.0000000079

Tabela 16. Números de peptídeos únicos em três réplicas do secretoma 2 de fibroblastos tratados com o secretoma 1 (MC1_{Hipo} ou MC1_{Controle}) de linhagem HEp-2 submetida ou não a condições de hipóxia. Valor de p (teste t) atribuído pelo programa *Scaffold*. p<0,05. Rosa e verde - menor e maior expressão, respectivamente.

Gene (NCBI)	Secretoma 2 de fibroblastos tratados com MC1 _{Controle} de células HEp-2			Secretoma 2 de fibroblastos tratados com MC1 _{Hipo} de células HEp-2			Valor de p
	Réplicas						
	1	2	3	1	2	3	
APP	1	2	1	0	0	0	0,011
ECM1	6	7	6	3	5	4	0,013
FBN1	7	7	4	2	2	4	0,0089
ITGBL1	3	2	1	0	0	0	0,031
LAMB1	5	2	2	0	0	0	0,037
LTBP2	4	3	5	1	0	0	0,004
AHSG	0	0	0	2	1	2	0,017
IGFBP4	3	2	3	4	4	4	0,035
MMP1	2	0	1	3	3	2	0,024
YWHAB	1	0	0	1	2	2	0,013

Tabela 17. Números de peptídeos únicos em três réplicas do secretoma 1 de células SCC-25 controles ou tratadas com CoCl_2 , obtidas em três experimentos independentes. Valor de p (teste t) atribuído pelo programa *Scaffold*. $p < 0,05$. As proteínas são representadas pelos símbolos dos genes correspondentes. Rosa e verde - menor e maior expressão, respectivamente, nas células tratadas com CoCl_2 em relação as controles.

Gene (NCBI)	Secretoma 1 de células SCC-25 controles (MC1 _{Controle})			Secretoma 1 de células SCC-25 tratadas com CoCl ₂ (MC1 _{Hipo})			Valor de p
	Réplicas						
	1	2	3	1	2	3	
AARS	21	12	16	1	0	0	0.033
APP	1	12	1	0	0	0	0.0035
ARHGDIB	1	1	1	0	0	0	0.00036
BLVRA	14	1	1	0	0	0	0.018
CA2	1	1	16	0	0	0	0.040
CCT4	21	12	1	0	0	0	0.024
CLIC1	21	12	32	0	0	0	0.021
CLTC	1	1	1	0	0	0	0.00036
CMPK1	14	24	16	0	0	0	0.0035
CNDP2	1	1	16	0	0	0	0.040
DKK3	1	1	1	0	0	0	0.00036
DNAJB1	1	12	1	0	0	0	0.0035
EIF4A1	49	41	65	1	0	35	0.041
EIF6	1	12	16	0	0	0	0.012
FAM49B	14	1	16	0	0	0	0.018
FLNB	21	29	1	0	0	0	0.035
GANAB	35	35	16	1	0	0	0.022
GAPDH	63	10	65	16	14	10	0.045
GDI2	42	59	48	38	0	0	0.050
GNPDA1	14	18	1	0	0	0	0.0092
HNRNPA1	35	18	40	1	0	0	0.021
IQGAP1	42	35	40	1	0	0	0.00062
MVP	14	12	16	0	0	0	0.00036
OLA1	1	1	1	0	0	0	0.00036
OTUB1	14	1	1	0	0	0	0.018
PAICS	14	18	24	0	0	0	0.0033
PDCD5	1	12	24	0	0	0	0.049
PDIA6	21	18	24	1	0	0	0.0081
PGD	1	18	24	0	0	0	0.031
PPA1	21	29	24	1	0	0	0.0055
PPP1CA	1	12	1	0	0	0	0.0035
PSMA2	28	24	16	0	0	0	0.0029
PSMA5	14	18	32	0	0	0	0.019
RPLP2	14	12	16	1	0	0	0.032
S100A11	28	53	48	19	0	0	0.020
SERPINB5	42	35	32	19	0	0	0.012
TCP1	14	1	24	0	0	0	0.050
TFRC	14	1	1	0	0	0	0.018
TNC	1	12	1	0	0	0	0.0035
YWHAB	49	41	48	10	10	70	0.016
CBR1	21	1	0	37	34	35	0.014
DBNL	0	12	1	28	34	35	0.0032
HMGB1	1	1	0	19	69	70	0.047
HSPB1	0	0	16	19	34	35	0.034
TPM3	28	23	40	47	70	70	0.025

Tabela 18. Números de peptídeos únicos em três réplicas do secretoma 2 de células SCC-25 tratadas com o secretoma 1 (MC1_{Hipo} ou MC1_{Controle}) de linhagem SCC-25 submetida ou não a condições de hipóxia. Valor de p (teste t) atribuído pelo programa *Scaffold*. $p < 0,05$. Rosa e verde - menor e maior expressão, respectivamente.

Gene (NCBI)	Secretoma 2 de SCC-25 tratadas com MC1 _{Controle} de células SCC-25			Secretoma 2 de SCC-25 tratadas com MC1 _{Hipo} de células SCC-25			Valor de p
	Réplicas						
	1	2	3	1	2	3	
GAA	19	31	20	0	0	0	0.0039
HNRNPL	29	31	40	20	1	1	0.016
PPL	19	21	10	0	1	0	0.047
AHCY	1	21	20	31	40	39	0.014
FABP5	0	0	0	20	30	20	0.0021
GFPT1	0	0	10	20	1	20	0.049
IQGAP1	10	94	11	81	89	78	0.028
LGALS1	1	0	0	41	20	29	0.017
MYL6	1	0	10	20	20	20	0.015
PKM	37	32	29	39	43	43	0.033
PPA1	38	10	30	61	50	69	0.028
PRDX5	57	31	30	92	60	78	0.044
SDC1	1	0	0	10	20	20	0.041
UBA1	1	31	0	61	50	39	0.032

Tabela 19. Números de peptídeos únicos em três réplicas do secretoma 2 de fibroblastos tratados com o secretoma 1 (MC1_{Hipo} ou MC1_{Controle}) de linhagem SCC-25 submetida ou não a condições de hipóxia. Valor de p (teste t) atribuído pelo programa *Scaffold*. $p < 0,05$. Rosa e verde - menor e maior expressão, respectivamente

Genes (NCBI)	Secretoma 2 de fibroblastos tratados com MC1 _{Controle} de células SCC-25			Secretoma 2 de fibroblastos tratados com MC1 _{Hipo} de células SCC-25			Valor de p
	Réplicas						
	1	2	3	1	2	3	
COL1A2	7	8	12	2	4	3	0,02
FBN1	2	2	2	0	0	0	0,0001
HSPB1	2	2	2	0	1	1	0,05
NID1	2	2	2	0	1	1	0,05
COL6A2	1	0	0	2	1	2	0,012
COL6A3	1	0	0	1	3	2	0,05
MDH1	1	0	0	1	1	2	0,034
TPH1	0	0	0	1	1	2	0,0033
YWHAB	0	0	0	1	1	1	0,0027

Figura 16. Diagrama de Venn evidenciando o número de proteínas comuns aos secretomas 1 e 2 da linhagem HEP-2 nas condições: controle (MC1_{Controle}) tratada com CoCl₂ (MC1_{Hipo}), tratada com MC1_{Controle} (MC2_{Controle}) e tratada com MC1_{Hipo} (MC2_{Hipo}).

Figura 17. Diagrama de Venn evidenciando o número de proteínas comuns aos secretomas 1 e 2 da linhagem SCC-25 nas condições: controle (MC1_{Controle}) tratada com CoCl₂ (MC1_{Hipo}), tratada com MC1_{Controle} (MC2_{Controle}) e tratada com MC1_{Hipo} (MC2_{Hipo}).

Localização subcelular e processos biológicos associados a proteínas secretadas. Na distribuição de todas as proteínas secretadas pelas linhagens HEp-2, SCC-25 e fibroblastos por localização subcelular (Figura 18 A-F), pode ser observado que grande parte está localizada no espaço extracelular celular (38% a 65%) e as demais (34 a 56%) no citoplasma, na membrana plasmática e no núcleo. Este achado confere credibilidade ao resultado, considerando que as proteínas foram extraídas de meio condicionado (que pode ter contaminação com proteínas celulares derivadas de lise). Uma parcela pequena de proteínas (1 a 6%) não tem localização definida (Uniprot).

De acordo com a busca realizada no Gene Ontology (Ashburner *et al.*, 2000), as proteínas diferencialmente expressas nos secretoma 1 e no secretoma 2 estão relacionadas com organização da matriz extracelular, adesão célula-matriz, resposta a estresse, transdução de sinal, angiogênese e resposta inflamatória. (Tabelas 20-23).

Figura 18. Localização subcelular de proteínas identificadas no secretoma 1 de (A) HEP-2 e (B) tratadas com CoCl_2 *versus* controle; secretoma 2 de (C) HEP-2 e (D) SCC-25 após tratamento com MC1_{Hipo} de HEP-2 e SCC-25, respectivamente *versus* controle; secretoma 2 de fibroblastos humanos normais após tratamento com MC1_{Hipo} *versus* $\text{MC1}_{\text{Controle}}$ obtidos de (E) HEP-2 e (F) m SCC-25.

Tabela 20. Processos e atividades atribuídos às proteínas com expressão aumentada nos meios condicionados por células tratadas com CoCl₂ (MC1_{Hipo}) em relação ao secretoma 1 controle (MC1_{Controle}) (segundo Gene Ontology).

Processo biológico	MC1 _{Hipo} x MC1 _{Controle} de HEP-2	MC1 _{Hipo} x MC1 _{Controle} de SCC-25
	proteínas	
Angiogênese		
regulação	<i>HSPG2</i> <i>GAPDH</i> , <i>NDRG1</i>	<i>HSPB1</i> <i>DBNL</i> , <i>HMGB1</i>
Autofagia	<i>GAPDH</i>	
Comunicação celular		
transdução de sinal	<i>FSTL1</i>	<i>DBNL</i> , <i>HSPB1</i>
Proliferação celular		
regulação	<i>NDRG1</i>	
Adesão celular	<i>NDRG1</i>	
Migração celular		<i>HMGB1</i> , <i>HSPB1</i>
Processo de desenvolvimento celular		
diferenciação celular		<i>HMGB1</i>
Organização da matriz extracelular	<i>HSPG2</i>	
Expressão gênica		<i>HSPB1</i>
Processo metabólico		
processo metabólico de carboidrato	<i>GAPDH</i> , <i>HSPG2</i>	
processo de oxidação-redução	<i>GAPDH</i>	<i>CBR1</i>
processo metabólico de proteína	<i>HSPG2</i>	<i>HSPB1</i>
processo metabólico de RNA		
Resposta a estímulo		
resposta imune		<i>HMGB1</i>
resposta inflamatória		<i>HMGB1</i>
resposta a hipóxia	<i>NDRG1</i>	
resposta a estresse	<i>FSTL1</i> , <i>GAPDH</i>	
Transcrição		<i>HMGB1</i>
Tradução	<i>GAPDH</i>	<i>HSPB1</i>
Transporte		
transporte de proteína	<i>GAPDH</i>	<i>TPM3</i>

Tabela 21. Processos e atividades atribuídos às proteínas com expressão reduzida nos meios condicionados por células tratadas com CoCl₂ (MC1_{Hipo}) em relação ao secretoma 1 controle (MC1_{Controle}) (segundo Gene Ontology).

Processo biológico	MC1 _{Hipo} X MC1 _{Controle} de HEP-2	MC1 _{Hipo} X MC1 _{Controle} de SCC-25
	proteínas	
Angiogênese	LAMA5, TGFB1	
Apoptose	CTSL, GSTP1, HSPA1A/HSPA1B, HINT1, PCSK9, YWHAZ	APP, GAPDH, PDCD5, PSMA2, PSMA5, PDIA6, YWHAB
Autofagia	QSOX1	GAPDH
Comunicação celular		
transdução de sinal	AGRN, IGFBP2, COL3A1, EFEMP1, FBN2, GSTP1, FBLN1, HDGF, HDGF, HINT1, IGFBP4, LTBP1, NPNT, YWHAZ, PSMD2, SEMA3B, TIMP2	APP, CLIC1, CLTC, DKK3, FLNB, GDI2, IQGAP1, PPP1CA, S100A11, TNC, YWHAB
Ciclo celular		
replicação do DNA		PPP1CA
ciclo celular mitótico	PSMD2, TIMP2	S100A11 APP, PSMA2, PSMA5
Proliferação celular	GSTP1, FBLN1, HDGF, HSPA1A/HSPA1B, HDGF, LAMA5, IGFBP4, TGFB1, TIMP2	S100A11, SERPINB5, TNC
Adesão celular	COL3A1, FBLN1, LAMA5, LAMA3, NPNT, SEMA3B, TGFB1	APP, ARHGDIB, DNAJB1, IQGAP1, PAICS, TNC
Migração celular	FBLN1, LAMA5, LAMA3, SEMA3B	
Processo de desenvolvimento celular		
diferenciação celular	FBN2, LAMA5, LAMA3, NPNT, PCSK9, PGK1, TIMP2	FLNB, EIF6
Organização de componente celular		
crescimento celular	IGFBP2, HSPA1A/HSPA1B	
organização da membrana	AGRN	CLTC
Organização da matriz extracelular	COL3A1, EFEMP1, FBLN1, FBN2, LAMA5, LAMA3, NPNT, PXDN, TGFB1, TIMP2	APP, SERPINB5
Expressão gênica	HSPA1A/HSPA1B, YWHAZ	AARS, EIF4A1, HNRNPA1, PPA1, TNC, PSMA2, PSMA5, RPLP2, YWHAB
Processo metabólico		
processo metabólico de ATP	AGRN, NPC2, IGFBP4	CMPK1, GNPDA1, PSMA5
processo metabólico de carboidrato	ACLY	OLA1
processo de oxidação-redução	PGK1	GAPDH, GNPDA1, PGD
processo metabólico de proteína	PXDN, QSOX1	BLVRA, GAPDH, PGD
processo de modificação proteica	CCT8, CTSL, PCSK9	CCT4, CNBP2, EIF4A1, GANAB, RPLP2, TCP1
processo metabólico de RNA	CCT8	AARS, CCT4, GANAB, TCP1
processamento de RNA	HSPA1A/HSPA1B, YWHAZ	EIF4A1, PSMA2, PSMA5, RPLP2, YWHAB
Resposta a estímulo		
resposta imune	CFH, COL3A1, CTSL, PXDN	AARS, HNRNPA1
resposta inflamatória	GSTP1, IGFBP4	APP, ARHGDIB
resposta a estresse	IGFBP2, PCSK9, TFPI, GSTP1	CA2, DNAJB1, GAPDH, OTUB1, PSMA2, PSMA5, SERPINB5, PDIA6, TFRC, APP
Transcrição	AGRN, EFEMP1, HDGF, HINT1, PSMD2	DKK3, DNAJB1
Tradução		EIF4A1, GAPDH, EIF6, PPA1, RPLP2
Transporte	NPC2, PCSK9, PSMD2	HNRNPA1, TFRC, GAPDH, GDI2, MVP

Tabela 22. Processos e atividades atribuídos às proteínas com expressão aumentada nos secretomas 2 (MC2_{Hipo}), ou seja, meios condicionados por células tratadas com MC1_{Hipo} em relação ao secretoma 2 controle (MC2_{Controle}) (segundo Gene Ontology).

Processo biológico	MC2 _{Hipo} X MC2 _{Controle} de HEp-2 tratadas com secretoma 1 de HEp-2	MC2 _{Hipo} X MC2 _{Controle} de SCC-25 tratadas com secretoma 1 de SCC-25
	proteínas	
Apoptose	<i>ACTN4, SLC39A10</i>	<i>LGALS1, PKM, PRDX5</i>
Autofagia	<i>HSPA8</i>	
Comunicação celular sinalização célula-célula transdução de sinal	<i>ARPC2, LPL, SLC39A10</i>	<i>GFPT1, IQGAP1, LGALS1, SDC1</i>
Ciclo celular	<i>HSPA8</i>	
Proliferação celular regulação	<i>SLC39A10</i>	
Adesão celular	<i>ARPC2, HSPA8</i>	<i>IQGAP1, LGALS1, PKM</i>
Migração celular		<i>IQGAP1, SDC1</i>
Processo de desenvolvimento celular diferenciação celular	<i>LPL</i>	<i>FABP5, LGALS1, MYL6</i>
Organização de componente celular crescimento celular organização da membrana	<i>ARPC2, HSPA8</i>	<i>MYL6</i>
Expressão gênica	<i>HSPA8</i>	<i>GFPT1, PPA1</i>
Processo metabólico processo metabólico de carboidrato processo metabólico de lipídio processo metabólico de ácido orgânico processo de oxidação-redução processo metabólico de proteína processo de modificação proteica processo metabólico de RNA	<i>CALU</i> <i>GGH</i> <i>CTSZ, GGH</i> <i>HSPA8</i>	<i>FABP5, GFPT1</i> <i>FABP5</i> <i>AHCY, PPA1</i> <i>PRDX5</i> <i>UBA1</i> <i>UBA1</i>
Resposta a estímulo resposta imune resposta inflamatória resposta a hipóxia resposta a estresse oxidativo resposta a estresse	<i>C1R</i> <i>LPL</i> <i>ACTN4</i> <i>C1R, CTSZ, LPL</i>	<i>AHCY, PRDX5, SDC1</i> <i>AHCY, PKM</i> <i>PRDX5, SDC1</i> <i>FABP5, LGALS1, UBA1</i>
Transcrição	<i>HSPA8</i>	<i>PRDX5</i>
Transporte transporte de proteína transporte transmembrana transporte mediado a vesícula	<i>ACTN4</i> <i>SLC39A10</i> <i>CTSZ</i>	

Tabela 23. Processos e atividades atribuídos às proteínas com expressão reduzida nos secretomas 2 (MC2_{Hipo}), ou seja, nos meios condicionados por células controles tratadas com MC1_{Hipo} em relação ao secretoma 2 controle (MC2_{Controle}) (segundo Gene Ontology).

Processo biológico	MC2 _{Hipo} X MC2 _{Controle} de HEp-2 tratadas com secretoma 1 de HEp-2	MC2 _{Hipo} X MC2 _{Controle} de SCC-25 tratadas com secretoma 1 de SCC-25
	proteínas	
Autofagia	<i>PSAP</i>	
Comunicação celular transdução de sinal	<i>CLTC, FBLN1, FKBP4, PSAP</i>	
Ciclo celular ciclo celular mitótico	<i>CLTC</i>	
Proliferação celular regulação	<i>FBLN1</i>	
Adesão celular	<i>FBLN1</i>	<i>PPL</i>
Processo de desenvolvimento celular diferenciação celular	<i>FKBP4, PSAP</i>	<i>PPL</i>
Organização de componente celular crescimento celular organização do citoesqueleto organização da membrana	<i>PSAP</i> <i>DSTN</i> <i>CLTC</i>	<i>PPL</i>
Organização da matriz extracelular	<i>FBLN1</i>	
Expressão gênica		<i>HNRNPL</i>
Processo metabólico processo metabólico de carboidrato processo metabólico de lipídio processamento de RNA	<i>TALDO1</i> <i>PSAP</i>	<i>GAA</i> <i>HNRNPL</i>
Transporte transporte de lipídio transporte de proteína	<i>PSAP</i> <i>CLTC</i>	

Tabela 24. Processos atribuídos às proteínas com expressão aumentada nos meios condicionados por fibroblastos tratados com MC1_{Hipo} em relação a controles tratados com MC1Controle das linhagens HEp-2 e SCC-25(segundo Gene Ontology).

Processo biológico	MC2 _{Hipo} X MC2 _{Controle} de fibroblastos tratados com secretoma 1 de HEp-2	MC2 _{Hipo} X MC2 _{Controle} de fibroblastos tratados com secretoma 1 de SCC-25
	proteínas	
Apoptose	<i>IGFBP4, YWHAB</i>	<i>YWHAB</i>
Comunicação celular transdução de sinal	<i>AHSG, IGFBP4, YWHAB</i>	<i>YWHAB</i>
Proliferação celular	<i>IGFBP4</i>	
Adesão celular	<i>YWHAB</i>	<i>YWHAB, COL6A2, COL6A3</i>
Migração celular	<i>MMP1</i>	
Organização de componente celular crescimento celular organização da membrana	<i>IGFBP4</i> <i>YWHAB</i>	<i>YWHAB</i>
Organização da matriz extracelular	<i>MMP1</i>	<i>COL6A2, COL6A3</i>
Expressão gênica		
Processo metabólico processo metabólico de DNA processo metabólico de ácido orgânico processo de oxidação-redução processo metabólico de proteína	<i>IGFBP4</i> <i>MMP1</i>	<i>MDH1</i> <i>MDH1, TPI1</i>
Resposta a estímulo resposta inflamatória	<i>AHSG</i>	
Transcrição	<i>YWHAB</i>	<i>YWHAB</i>
Transporte transporte mediado a vesícula	<i>AHSG</i>	

Tabela 25. Processos atribuídos às proteínas com expressão reduzida nos meios condicionados por fibroblastos tratados com MC1_{Hipo} em relação a controles tratados com MC1_{Controle} das linhagens HEp-2 e SCC-25(segundo Gene Ontology).

Processo biológico	MC2 _{Hipo} X MC2 _{Controle} de fibroblastos tratados com secretoma 1 de HEp-2	MC2 _{Hipo} X MC2 _{Controle} de fibroblastos tratados com secretoma 1 de SCC-25
	proteínas	
Angiogênese	<i>ECM1</i>	<i>HSPB1</i>
Autofagia		<i>HSPB1</i>
Comunicação celular transdução de sinal	<i>APP, ECM1, FBN1, LTBP2</i>	<i>COL1A2, FBN1, HSPB1</i>
Proliferação celular regulação	<i>LAMB1</i>	<i>HSPB1</i>
Adesão celular	<i>APP, ITGBL1, LAMB1</i>	<i>NID1</i>
Migração celular	<i>LAMB1</i>	<i>HSPB1</i>
Processo de desenvolvimento celular diferenciação celular	<i>LAMB1</i>	
Organização de componente celular organização da membrana		<i>NID1</i>
Organização da matriz extracelular	<i>APP, FBN1, LAMB1</i>	<i>COL1A2, FBN1, NID1</i>
Expressão gênica		
Processo metabólico processo metabólico de proteína	<i>APP</i>	
Resposta a estímulo resposta imune resposta inflamatória	<i>APP</i> <i>ECM1</i>	<i>COL1A2</i>
Senescência		
Transcrição	<i>APP</i>	<i>HSPB1</i>
Tradução		<i>HSPB1</i>
Transporte transporte de proteína	<i>LTBP2</i>	<i>HSPB1</i>

4.1.2 Análise de metabólitos em secretomas

As análises por ressonância magnética do secretoma 1 de células HEp-2 e SCC-25 em condições de hipóxia (MC1_{Hipo}) ou não (MC1_{Controle}), bem como o secretoma 2 (MC2_{Hipo} e MC2_{Controle}) de células HEp-2, SCC-25 e fibroblastos humanos normais tratados com o secretoma 1 permitiram a identificação de 29 metabólitos (Tabelas 26-31). Alguns espectros estão disponíveis nas Figuras 19-22.

Secretoma 1 de células HEp-2. Foi observada elevação estatisticamente significativa nas concentrações de 9 metabólitos do secretoma 1 (2-oxoisocaproato, 3-metil-oxovalerato, acetato, isoleucina, lactato, treonina, triptofano, tirosina e valina) de células tratadas com CoCl₂ (MC1_{Hipo}) em relação ao seu controle (Tabela 26).

Secretoma 2 de células HEp-2. Não foram observadas diferenças significantes na comparação dos secretomas 2 de HEp-2 (Tabela 27).

Secretoma 1 de células SCC-25. Nos secretomas 1 de células SCC-25, oito metabólitos mostraram concentração elevada (2-oxoisocaproato, 3-metil-2-oxovalerato, acetato, glicose, glutamato, glutamina, fenilalanina e valina) e três (formato, fumarato e lactato) concentração reduzida no meio condicionado por células em hipóxia em relação ao seu controle não tratado (Tabela 28).

Secretoma 2 de células SCC-25. Na comparação dos secretomas 2 de SCC-25, foram detectados 15 metabólitos (acetato, alanina, asparagina, aspartato, etanol, formato, glutamato, glutamina, glicina, lactato, metionina, mio-inositol, fenilalanina, piruvato e valina) com concentração elevada em MC2_{Hipo} em relação ao seu controle (MC2_{Controle}) (Tabela 29).

Secretoma 2 de fibroblastos. O secretoma 2 de fibroblastos mostraram concentrações significativamente reduzidas de aspartato, glutamato, fenilalanina e piruvato após tratamento com MC1_{Hipo} de SCC-25 (Tabela 30) e de 2-oxoisocaproato, formato, glicina e piruvato após tratamento com MC1_{Hipo} de HEp-2 (Tabela 31).

Tabela 26. Concentrações de metabólitos (mM) em três réplicas do secretoma 1 de células HEp-2 controles ou submetidas a condições de hipóxia com CoCl_2 . Valor de p (teste t, $p < 0,05$). Em cinza, os metabólitos com diferenças significantes entre os dois grupos.

Metabólito	Secretoma 1 de células HEp2 controles (MC1 _{Controle})			Secretoma 1 de células HEp2 tratadas com CoCl ₂ (MC1 _{Hipo})			Valor de p
	Réplicas						
	1	2	3	1	2	3	
2-Oxoisocaproato	0,0138	0,0129	0,014	0,0335	0,0386	0,0341	0.006
3-Metil-2-oxovalerato	0,0238	0,0217	0,0208	0,0461	0,0489	0,0462	<0.001
Acetato	0,0621	0,0677	0,0618	0,0887	0,079	0,0809	0.013
Alanina	0,2318	0,2333	0,1807	0,2076	0,2248	0,2166	0.958
Arginina	0,4167	0,5224	0,4627	0,758	0,4454	0,5504	0.349
Asparagina	0,3213	0,3198	0,3664	0,3708	0,3382	0,3456	0.452
Aspartato	0,1767	0,1615	0,1717	0,1646	0,1712	0,1695	0.784
Etanol	11,5919	9,4232	7,437	8,766	11,3322	11,1251	0.571
Formato	0,1921	0,1697	0,1143	0,0726	0,0877	0,076	0.077
Fumarato	0,0044	0,0043	0,003	0,0016	0,0023	0,0019	0.058
Glicose	2,6074	2,5935	2,4179	2,213	1,4212	2,1023	0.133
Glutamato	0,3615	0,3427	0,3147	0,2892	0,2879	0,28	0.060
Glutamina	0,895	1,1504	1,2659	1,8054	1,1479	1,5664	0.166
Glicerol	0,2494	0,2284	0,2856	0,1833	0,185	0,1857	0.053
Glicina	0,302	0,2865	0,259	0,2229	0,2356	0,2247	0.053
Histidina	0,0824	0,0646	0,0697	0,0761	0,0722	0,0784	0.612
Isoleucina	0,021	0,0612	0,0465	0,104	0,0834	0,0838	0.039
Lactato	4,9379	5,044	6,4405	9,4203	10,6453	9,9655	0.005
Leucina	0,0376	0,0141	0,0946	0,0757	0,0736	0,0541	0.524
Metionina	0,041	0,0494	0,0567	0,0458	0,0542	0,06	0.533
Mio-Inositol	0,1377	0,1334	0,118	0,1208	0,1026	0,118	0.149
Fenilalanina	0,0397	0,0239	0,0447	0,0725	0,0464	0,0557	0.111
Prolina	0,1584	0,1224	0,1089	0,1554	0,0973	0,1531	0.838
Piroglutamato	0,2944	0,3275	0,2835	0,3329	0,2825	0,3067	0.796
Piruvato	0,229	0,2225	0,2386	0,1711	0,2279	0,1864	0.186
Treonina	0,0854	0,0743	0,0887	0,1313	0,1171	0,128	0.006
Triptofano	0,0082	0,0074	0,0087	0,0163	0,0243	0,0178	0.045
Tirosina	0,0949	0,0778	0,0969	0,1175	0,1167	0,1275	0.022
Valina	0,053	0,0523	0,0703	0,0978	0,0993	0,0935	0.025

Tabela 27. Concentrações de metabólitos (mM) em três réplicas do secretoma 2 de células HEp-2 controles tratadas com MC1_{Controle} ou tratadas com MC1_{Hipo}. Valor de p (teste t, p<0,05).

Metabólito	Secretoma 2 de células HEp-2 tratadas com MC1 _{Controle} de células HEp-2			Secretoma 2 de células HEp-2 tratadas MC1 _{Hipo} de células HEp-2			Valor de p
	Réplicas						
	1	2	3	1	2	3	
2-Oxoisocaproato	0,014	0,0137	0,0147	0,017	0,0158	0,0131	0.430
3-Metil-2-oxovalerato	0,0208	0,0197	0,0225	0,0254	0,0249	0,0224	0.079
Acetato	0,0618	0,0624	0,0689	0,0768	0,0832	0,0632	0.253
Alanina	0,1807	0,1895	0,1886	0,1795	0,1736	0,2249	0.735
Arginina	0,4627	0,6118	0,5373	0,5229	0,5641	0,2421	0.482
Asparagina	0,3664	0,371	0,3946	0,3795	0,3857	0,3131	0.546
Aspartato	0,1717	0,1589	0,1655	0,1799	0,1764	0,1576	0.505
Etanol	7,437	6,833	7,0654	7,3941	7,3949	10,2221	0.330
Formato	0,1143	0,1211	0,1216	0,1192	0,1284	0,1765	0.338
Fumarato	0,003	0,0039	0,0027	0,0023	0,0026	0,0045	0.937
Glicose	2,4179	2,4402	2,8062	3,0072	3,8487	2,3513	0.373
Glutamato	0,3147	0,3592	0,3193	0,315	0,2958	0,3111	0.260
Glutamina	1,2659	1,4812	1,5134	1,6361	1,8241	0,8227	0.983
Glicerol	0,2856	0,2755	0,2167	0,2102	0,2302	0,2168	0.213
Glicina	0,259	0,2516	0,2578	0,2646	0,2776	0,2918	0.116
Histidina	0,0697	0,079	0,0758	0,0739	0,0804	0,0634	0.715
Isoleucina	0,0465	0,1884	0,0826	0,0398	0,0427	0,0605	0.309
Lactato	6,4405	6,2254	6,1348	6,2178	6,0068	5,249	0.287
Leucina	0,0946	0,112	0,1717	0,0348	0,0538	0,0191	0.071
Metionina	0,0567	0,0548	0,0575	0,0532	0,0502	0,0557	0.205
Mio-Inositol	0,118	0,1472	0,1372	0,1161	0,1514	0,1407	0.895
Fenilalanina	0,0447	0,0449	0,0457	0,0492	0,0413	0,0245	0.451
Prolina	0,1089	0,1818	0,1738	0,1542	0,1698	0,1236	0.847
Piroglutamato	0,2835	0,3201	0,2657	0,2735	0,2305	0,3473	0.888
Piruvato	0,2386	0,241	0,2278	0,2246	0,2131	0,2406	0.392
Treonina	0,0887	0,087	0,093	0,0919	0,0905	0,076	0.589
Triptofano	0,0087	0,0083	0,0092	0,0111	0,0112	0,0084	0.256
Tirosina	0,0969	0,11	0,1043	0,1022	0,0902	0,0891	0.178
Valina	0,0703	0,0711	0,0737	0,0715	0,0672	0,0602	0.258

Tabela 28. Concentrações de metabólitos (mM) em três réplicas do secretoma 1 de células SCC-25 controles ou submetidas a condições de hipóxia com CoCl_2 . Valor de p (teste t, $p < 0,05$). Em cinza, os metabólitos com diferenças significantes entre os dois grupos.

Metabólito	Secretoma 1 de células SCC-25 controles (MC1 _{Controle})			Secretoma 1 de células SCC-25 tratadas com CoCl ₂ (MC1 _{Hipo})			Valor de p
	Réplicas						
	1	2	3	1	2	3	
2-Oxoisocaproato	0,0021	0,002	0,0028	0,0065	0,0096	0,0091	0.0036
3-Metil-2-oxovalerato	0,004	0,0036	0,0033	0,0091	0,0113	0,0102	0.0006
Acetato	0,0212	0,0224	0,0199	0,0311	0,0334	0,0341	0.0005
Alanina	0,2153	0,2296	0,2155	0,1459	0,206	0,199	0,1354
Arginina	0,4747	0,4896	0,4245	0,4783	0,6101	0,6421	0,1019
Asparagina	0,3188	0,344	0,2949	0,3071	0,3945	0,4385	0,2135
Aspartato	0,1563	0,1722	0,1549	0,1561	0,198	0,1835	0,2512
Etanol	162,9394	177,8665	172,0219	202,7045	177,9883	163,0021	0,4521
Formato	0,0708	0,0728	0,0705	0,0444	0,0525	0,0472	0.0007
Fumarato	0,0027	0,0041	0,0037	0,0015	0,0016	0,0018	0,0118
Glicose	0,1469	0,1633	0,1109	2,679	3,0637	3,8933	0.001
Glutamato	0,5077	0,5991	0,5012	0,6988	0,8545	0,8197	0.0109
Glutamina	1,2224	1,3049	1,1187	1,4275	1,6649	1,7733	0.0244
Glicerol	0,1907	0,1917	0,4388	0,256	0,2665	0,2289	0,7938
Glicina	0,1552	0,1611	0,15	0,1505	0,1833	0,1861	0,2067
Histidina	0,0583	0,0627	0,0502	0,0685	0,0768	0,0952	0,0566
Isoleucina	0,1174	0,1643	0,0961	0,1103	0,1175	0,1313	0,782
Lactato	13,9846	13,6262	13,6026	8,9249	8,948	7,1555	0.0009
Leucina	0,1691	0,1815	0,1513	0,1757	0,2357	0,2505	0,095
Metionina	0,0496	0,0525	0,0466	0,0491	0,0682	0,0671	0,1375
Mio-Inositol	0,1197	0,1226	0,0964	0,1135	0,1446	0,1557	0,1729
Fenilalanina	0,0425	0,044	0,0387	0,0501	0,0681	0,0784	0.0475
Prolina	0,1594	0,1815	0,1429	0,151	0,1911	0,1888	0,4117
Piroglutamato	0,2697	0,2663	0,2592	0,2619	0,3824	0,3826	0,1279
Piruvate	0,013	0,0129	0,0129	0,0111	0,0156	0,016	0,4545
Treonina	0,0891	0,0836	0,0991	0,0945	0,1215	0,1171	0,0983
Triptofano	0,0084	0,0136	0,0099	0,0094	0,0134	0,0172	0,3788
Tirosina	0,0754	0,0852	0,0702	0,0784	0,1068	0,1131	0,1231
Valina	0,0643	0,0643	0,0603	0,0772	0,0902	0,1056	0.028

Tabela 29. Concentrações de metabólitos (mM) em três réplicas do secretoma 2 de células SCC-25 controles tratadas com MC1_{Controle} ou tratadas com MC1_{Hipo}. Valor de p (teste t, $p < 0,05$). E m cinza, os metabólitos com diferenças significantes entre os dois grupos.

Metabólito	Secretoma 2 de células SCC-25 tratadas com MC1 _{Controle} de células SCC25			Secretoma 2 de células SCC-25 tratadas com MC1 _{Hipo} de células SCC25			Valor de p
	Réplicas						
	1	2	3	1	2	3	
2-Oxoisocaproato	0,0044	0,0045	0,0065	0,0066	0,0068	0,0069	0,0769
3-Metil-2-oxovalerato	0,0059	0,0061	0,0079	0,0083	0,0085	0,0082	0,0571
Acetato	0,0214	0,0266	0,0303	0,0383	0,0327	0,0408	0.0338
Alanina	0,1926	0,2068	0,2457	0,3006	0,2698	0,2923	0.0168
Arginina	0,4444	0,4372	0,5176	0,6099	0,5773	0,4931	0,0962
Asparagina	0,3171	0,3086	0,3792	0,4697	0,4348	0,4507	0.0088
Aspartato	0,0882	0,0833	0,0964	0,1489	0,1294	0,1333	0.0025
Etanol	3,2074	2,9066	3,6578	4,7929	4,3951	4,5578	0,0058
Formato	0,0626	0,0601	0,0781	0,0949	0,0892	0,1003	0.0126
Fumarato	0,002	0,0019	0,0025	0,0042	0,0026	0,0023	0,2192
Glicose	2,6224	3,1358	3,4594	3,8011	3,6289	3,6861	0,0638
Glutamate	0,3127	0,3253	0,3735	0,4138	0,4386	0,4613	0.012
Glutamina	1,0484	0,9307	1,1876	1,6787	1,6806	1,4654	0.0058
Glicerol	0,0748	0,0781	0,0891	0,1369	0,1011	0,0948	0,0934
Glicina	0,1892	0,1915	0,2268	0,2552	0,2409	0,2635	0.0215
Histidina	0,0603	0,065	0,0684	0,0945	0,0863	0,0706	0,06
Isoleucina	0,0606	0,0743	0,1395	0,1337	0,1315	0,0899	0,3942
Lactato	5,8877	5,9392	7,1721	9,3706	8,6517	9,1257	0.0044
Leucina	0,1568	0,1739	0,1999	0,2292	0,2216	0,1664	0,2853
Metionina	0,0452	0,045	0,0557	0,0633	0,0635	0,0647	0,013
Mio-Inositol	0,112	0,1162	0,1268	0,1495	0,1369	0,1535	0,0132
Fenilalanina	0,046	0,0472	0,0524	0,0654	0,0621	0,0576	0,0117
Prolina	0,1105	0,129	0,1337	0,1692	0,1419	0,1947	0,0582
Piroglutamato	0,4098	0,4591	0,5279	0,5287	0,5442	0,6199	0,0901
Piruvate	0,0695	0,0606	0,0754	0,0955	0,0885	0,0905	0.0086
Treonina	0,0878	0,0853	0,1072	0,1311	0,094	0,1275	0,1532
Triptofano	0,008	0,0122	0,0128	0,0128	0,0124	0,0144	0,2482
Tirosina	0,0785	0,0825	0,0841	0,0801	0,0911	0,1055	0,235
Valina	0,0708	0,0726	0,0853	0,0904	0,0903	0,0933	0.0318

Tabela 30. Concentrações de metabólitos (mM) em três réplicas do secretoma 2 de fibroblastos humanos normais tratados com o secretoma 1 (MC1_{Hipo} ou MC1_{Controle}) de linhagem HEP-2 submetida ou não a condições de hipóxia. Valor de p (teste t, $p < 0,05$). Em cinza, os metabólitos com diferenças significantes entre os dois grupos.

Metabólito	Secretoma 2 de fibroblastos tratados com MC1 _{Controle} procedente de células HEP-2			Secretoma 2 de fibroblastos tratados com MC1 _{Hipo} procedente de células HEP-2			Valor de p
	Réplicas						
	1	2	3	1	2	3	
2-Oxoisocaproato	0,0143	0,0136	0,0117	0,0138	0,0163	0,0099	0,9505
3-Metil-2-oxovalerato	0,0157	0,0149	0,0138	0,0142	0,0149	0,0116	0,3421
Acetato	0,0497	0,0448	0,0529	0,045	0,0395	0,0394	0,0591
Alanina	0,1397	0,1384	0,1324	0,1375	0,1241	0,1113	0,1874
Arginina	0,6451	0,5873	0,6514	0,6604	0,6036	0,5365	0,5374
Asparagina	0,3021	0,3087	0,3044	0,319	0,2905	0,2683	0,447
Aspartato	0,1367	0,1399	0,1401	0,1281	0,133	0,1194	0.0431
Etanol	4,118	3,4943	3,2495	3,5666	3,0431	3,9291	0,7825
Formato	0,0282	0,0272	0,0293	0,0258	0,0247	0,0199	0,0671
Fumarato	0,0001	0,0001	0,0001	0,0009	0,0001	0,0003	0,2418
Glicose	5,1485	5,1093	5,5416	5,2727	4,6936	4,704	0,1859
Glutamato	0,3075	0,299	0,3054	0,2546	0,2519	0,2206	0.0053
Glutamina	1,8014	1,5181	1,6908	1,7902	1,5173	1,5862	0,7549
Glicerol	0,0747	0,068	0,0591	0,1101	0,0756	0,0475	0,6047
Glicina	0,2027	0,1976	0,1975	0,1959	0,1695	0,162	0,0875
Histidina	0,0914	0,0861	0,0958	0,0894	0,0964	0,0816	0,72
Isoleucina	0,1019	0,1299	0,1066	0,1939	0,129	0,1004	0,3842
Lactato	2,1821	2,2132	2,052	2,8574	2,4469	2,1583	0,1803
Leucina	0,2275	0,2127	0,2342	0,234	0,2095	0,2064	0,4915
Metionina	0,0616	0,0572	0,0631	0,065	0,0645	0,0567	0,6791
Mio-Inositol	0,1107	0,098	0,106	0,1075	0,0972	0,0992	0,5005
Fenilalanina	0,0846	0,0745	0,0827	0,0726	0,0676	0,0694	0.0351
Prolina	0,0954	0,128	0,1113	0,1245	0,127	0,1008	0,6652
Piroglutamato	0,5508	0,5487	0,6072	0,6143	0,5459	0,5455	0,9916
Piruvate	0,1106	0,1082	0,0897	0,0727	0,0626	0,0507	0.0112
Treonina	0,1443	0,1383	0,1487	0,1525	0,1374	0,14	0,937
Triptofano	0,0119	0,0142	0,0158	0,0164	0,0127	0,014	0,8111
Tirosina	0,112	0,1148	0,1155	0,1153	0,103	0,0983	0,1735
Valina	0,1094	0,1248	0,1206	0,1274	0,1184	0,1153	0,738

Tabela 31. Concentrações de metabólitos (mM) em três réplicas do secretoma 2 de fibroblastos humanos normais tratados com o secretoma 1 (MC1_{Hipo} ou MC1_{Controle}) de linhagem SCC-25 submetida ou não a condições de hipóxia. Valor de p (teste t, <0,05). E m cinza, os metabólitos com diferenças significantes entre os dois grupos.

Metabólito	Secretoma 2 de fibroblastos tratados com MC1 ^{Controle} procedente de células SCC-25			Secretoma 2 de fibroblastos tratados com MC1 ^{Hipo} procedente de células SCC-25			Valor de p
	Réplicas						
	1	2	3	1	2	3	
2-Oxoisocaproato	0,0178	0,0179	0,0164	0,0149	0,0146	0,0151	0.0078
3-Metil-2-oxovalerato	0,0147	0,0182	0,0193	0,016	0,0158	0,016	0,3504
Acetato	0,0527	0,0529	0,0443	0,0515	0,0554	0,052	0,3862
Alanina	0,152	0,1552	0,1455	0,1348	0,1478	0,1343	0,0859
Arginina	0,618	0,6974	0,6587	0,6897	0,7249	0,6152	0,6639
Asparagina	0,2972	0,3456	0,3196	0,3236	0,3448	0,2913	0,9677
Aspartato	0,1338	0,1401	0,1298	0,1372	0,1437	0,1326	0,499
Etanol	3,4851	5,5805	3,7788	3,4999	3,6225	4,9513	0,7651
Formato	0,0324	0,0342	0,0285	0,0263	0,0263	0,0278	0.0492
Fumarato	0,0001	0,0001	0,0001	0,0001	0,0001	0,0001	1
Glicose	4,9726	5,3082	4,7969	5,2236	5,3599	4,7786	0,7024
Glutamato	0,2882	0,3078	0,269	0,2818	0,269	0,2219	0,2236
Glutamina	1,676	1,7791	1,7887	1,7354	1,8835	1,7159	0,6604
Glicerol	0,0594	0,0552	0,0561	0,0315	0,1396	0,0738	0,4759
Glicina	0,2113	0,2092	0,2039	0,1853	0,1943	0,1867	0.0055
Histidina	0,0985	0,1054	0,1056	0,1088	0,1047	0,0897	0,754
Isoleucina	0,1628	0,1784	0,0955	0,2002	0,1907	0,2076	0,1058
Lactato	3,5662	3,6391	3,6574	3,5385	3,8224	3,4545	0,8973
Leucina	0,2316	0,2456	0,2258	0,2422	0,2571	0,2297	0,4291
Metionina	0,0656	0,0661	0,0678	0,0617	0,0659	0,0638	0,1227
Mio-Inositol	0,1011	0,1218	0,1111	0,1075	0,1102	0,1049	0,5713
Fenilalanina	0,0866	0,0909	0,0803	0,0903	0,0914	0,0809	0,7421
Prolina	0,1044	0,1137	0,1115	0,1486	0,1412	0,1168	0,0623
Piroglutamato	0,7331	0,6202	0,5988	0,5785	0,6184	0,5366	0,2028
Piruvate	0,1215	0,1201	0,1283	0,0338	0,0363	0,035	P<0.0001
Treonina	0,1631	0,1612	0,1517	0,1559	0,156	0,1552	0,4486
Triptofano	0,0159	0,0167	0,0158	0,0161	0,0157	0,0155	0,3352
Tirosina	0,1148	0,0968	0,1099	0,1149	0,1247	0,1084	0,285
Valina	0,1275	0,1377	0,128	0,1334	0,146	0,1327	0,312

Figura 19. Espectros do metabólito lactato mostrando expressão reduzida no secretoma 1 da linhagem SCC-25 (B,D) tratada com CoCl_2 em relação ao controle (A,C). Concentrações de lactato (mM) no secretoma das células controle=13.984 e das células tratadas=8.924 (réplica 1, Tabela 20). Linha azul=espectro obtido, linha preta=espectro do lactato, linha vermelha=somatória dos espectros obtidos.

Figura 20. Espectros do metabólito etanol mostrando expressão elevada no secretoma 2 da linhagem SCC-25 (B,D) tratada com CoCl_2 em relação ao controle (A,C). Concentrações de etanol (mM) em $\text{MC2}_{\text{Controle}}=3.2070$ e $\text{MC2}_{\text{Hipo}}=4.7929$ (réplica 1, Tabela 21). Linha azul=espectro obtido, linha preta=espectro do etanol, linha vermelha=somatória dos espectros obtidos.

Figura 21. Espectros do metabólito glutamato diferencialmente expresso no secretoma 2 de fibroblastos humanos normais tratados com MC1_{Controle} (A,B,C) e MC1_{Hipo} (D,E,F) procedentes da linhagem HEp-2. Concentrações de glutamato (mM) em MC2_{Controle}=0.3075 e do MC2_{Hipo}=0.2546 (réplica 1, Tabela 22). Linha azul=espectro obtido, linha preta=espectro do glutamato, linha vermelha=somatória dos espectros obtidos.

Figura 22. Espectros do metabólito piruvato mostrando expressão elevada no secretoma 2 de fibroblastos humanos normais tratados com MC1_{Controle} (A) e MC1_{Hipo} (B) procedentes da linhagem SCC-25. Concentrações de piruvato (mM) do MC2_{Controle}=0.1215 e do MC2_{Hipo}=0.0338 (réplica 1, Tabela 4). Linha azul=espectro obtido, linha preta=espectro do piruvato, linha vermelha=somatória dos espectros obtidos.

4.1.3 Tratamento de células com o secretoma 1 e efeito do secretoma na fisiologia e no comportamento celular

Utilizando as linhagens HEp-2 e SCC-25 em hipóxia, foi avaliado o efeito de seu secretoma 1 em proliferação, viabilidade, migração e invasividade de HEp-2, SCC-25 e fibroblastos humanos normais.

O teste estatístico utilizado para a análise dos resultados de proliferação e viabilidade foi ANOVA multifatorial seguida do teste de tukey (utilizando $p < 0,05$) e para análise de migração e invasão foi teste t (utilizando $p < 0,05$).

Curvas de proliferação e ensaios de viabilidade

Proliferação e viabilidade de linhagens HEp-2 e SCC-25 tratadas com o secretoma 1. Utilizando as linhagens HEp-2 e SCC-25 em hipóxia, foi avaliado o efeito de seu secretoma 1 (MC1_{Controle} e MC1_{Hipo}) na proliferação e na viabilidade de HEp-2 e SCC-25. Os resultados obtidos nos ensaios de curva de proliferação (Figura 23) e viabilidade (Figura 24) não mostraram diferenças significativas nas células HEp-2 e SCC-25 tratadas com MC1_{Controle} e MC1_{Hipo}.

Proliferação e viabilidade de fibroblastos tratados com o secretoma 1. Utilizando as linhagens HEp-2 e SCC-25 em hipóxia, foi avaliado o efeito de seu secretoma 1 (MC1_{Controle} e MC1_{Hipo}) na proliferação (Figura 25) e na viabilidade (Figura 26) de fibroblastos normais. A análise por ANOVA seguida do teste de Tukey mostrou diferença significativa nas curvas de proliferação após 72h ($p < 0,05$ – HEp-2 e $P < 0,001$ -SCC-25). O ensaio de MTS realizado com o secretoma 1 de SCC-25 não mostrou diferença significativa até 72h. Após esse período (96h), os fibroblastos tratados com MC1_{Hipo} mostraram viabilidade menor que os tratados com MC1_{Controle} ($p < 0,01$), de forma similar ao que foi observado nos ensaios de curva de crescimento.

Figura 23. Curva de crescimento estabelecida em seis tempos (0h, 24h, 48h, 72h, 96h e 120h) para as células (A) HEP-2 e (B) SCC-25 tratadas com MC1_{Controle} e MC1_{Hipo} (linha azul e vermelha, respectivamente). Cada ponto representa a média de três réplicas.

Figura 24. Ensaio de MTS estabelecido em cinco tempos (24h, 48h, 72h, 96h e 120h) para as células (A) HEP-2 e (B) SCC-25 tratadas com MC1_{Controle} e MC1_{Hipo} (linha verde e alaranjada, respectivamente). Cada ponto representa a média de quatro réplicas.

Figura 25. Curva de crescimento estabelecida em seis tempos (0h, 24h, 48h, 72h, 96h e 120h) para fibroblastos tratados com MC1_{Controle} e MC1_{Hipo} (linha azul e vermelha, respectivamente) obtidos a partir de (A) HEP-2 e (B) SCC-25. Cada ponto representa a média de três réplicas.

Figura 26. Ensaio de MTS estabelecido em cinco tempos (24h, 48h, 72h, 96h e 120h) para fibroblastos tratados com MC1_{Controle} e MC1_{Hipo} (linha verde e alaranjada, respectivamente) obtidos a partir de (A) HEP-2 e (B) SCC-25. Cada ponto representa a média de quatro réplicas.

Ensaio de migração e invasão

Células HEP-2 tratadas com o secretoma 1 de HEP-2 em hipóxia. Não houve alteração na capacidade migratória destas células em relação ao controle (Figura 27A-C), mas foi observado um aumento significativo ($p=0,0001$) em sua capacidade de invasão (Figura 28A-C).

Células SCC-25 tratadas com o secretoma 1 de SCC-25 em hipóxia. Do mesmo modo que HEP-2, não ocorreu alteração na capacidade migratória de células SCC-25 em relação ao controle (Figura 27D-F), mas sim um aumento ($p=0,0007$) em sua capacidade de invasão (Figura 28D-F).

Fibroblastos humanos normais tratados com o secretoma 1 de HEP-2 ou de SCC-25 em hipóxia. Para os fibroblastos, foi observado tanto um aumento significativo na migração ($p=0,0268$) (Figura 27G-I) como na capacidade invasiva ($p=0,0033$) (Figura 28G-I) em presença do secretoma de HEP-2. O mesmo resultado foi visto com o secretoma de SCC-25 ($p=0,0022$, $p=0,0142$, respectivamente).

Figure 27. Migração celular. Resultado de ensaios realizados em câmara de Boyden após 9h de incubação. Fotomicrografias de HEp-2 (A,B), SCC-25 (D,E) e fibroblastos (G,H,J,K) tratados com secretoma1_{Controle} (B, E, H, K) e com o secretoma1_{Hipo} de HEp-2 em hipóxia (B,H) ou de SCC-25 em hipóxia (E,K). Os valores de p obtidos pelo teste t de Student foram: p=0,129 (A versus B), p=0,207 (D versus E), p=0,0268 (G versus H), p=0,0022 (J versus K). C, F, I, L: Gráficos com o número total de células em 5 fotomicrografias de triplicatas.

Figure 28. Invasão celular. Resultado de ensaios realizados em câmara de Boyden após 48h de incubação. Fotomicrografias de HEp-2 (A,B), SCC-25 (D,E) e fibroblatos (G,H,J,K) tratados com secretoma1_{Controle} (B, E, H, K) e com o secretoma1_{Hipo} de HEp-2 em hipóxia (B,H) ou de SCC-25 em hipóxia (E,K). Os valores de p obtidos pelo teste t de Student foram: p=0,0001 (A versus B), p=0,0007 (D versus E), p=0,0033 (G versus H), p=0,0142 (J versus K). C, F, I, L: Gráficos com o número total de células em 5 fotomicrografias de triplicatas.

4.1.4 Pcr array

A avaliação da expressão de genes relacionados com ciclo celular e proliferação foi realizado com placas de PCR Arrays pré-desenhadas com duplicatas de sondas para 44 genes de interesse e 4 controles endógenos. A Figura 29 mostra alguns desses genes na via do ciclo celular do banco de dados Kegg (Kegg Pathway Database, http://www.kegg.jp/kegg/tool/map_pathway1.html).

Foram consideradas confiáveis as diferenças de expressão dos genes que tiveram o mesmo resultado em pelo menos duas normalizações com dois controles endógenos. Aqueles com expressão elevada ou reduzida na normalização com apenas um controle endógeno não foram considerados.

Células HEP-2 tratadas com o secretoma 1 de HEP-2 em hipóxia

Nos ensaios de PCR Array, sete de 44 genes relacionados ao ciclo celular e proliferação (Figura 29) apresentaram expressão diferencial, cinco com expressão elevada (*CCND3*, *CDK2*, *CDKN2B*, *HDAC4* e *HDAC5*) e dois com expressão reduzida (*CDC25A* e *TGFB2*) nas células tratadas com o secretoma MC1_{Hipo} em relação às tratadas com MC1_{Controle} procedente de linhagem HEP-2 em hipóxia.

Células SCC-25 tratadas com o secretoma 1 de SCC-25 em hipóxia

Nas análises de PCR Array, dos 44 genes relacionados a ciclo celular e proliferação apenas o *TGFB1* apresentou expressão diferencial, com níveis elevados de transcritos nas células tratadas com o secretoma MC1_{Hipo} em relação às tratadas com MC1_{Controle} procedente de linhagem SCC-25 em hipóxia.

Figura 29. Via do ciclo celular. As marcações em azul representam os genes com expressão reduzida e as marcações em vermelho representam os genes com expressão elevada detectados em células HEp-2 após tratamento com secretoma de células em hipóxia (Kegg Pathway Database, http://www.kegg.jp/kegg/tool/map_pathway1.html).

4. 2 INDUÇÃO DE ESTRESSE - RADIAÇÃO

Seleção do tempo de tratamento e dosagem de radiação

Curvas de proliferação

O presente trabalho também investigou o efeito de outro fator de estresse sobre o microambiente tumoral, a radiação X, para comparar os resultados com aqueles obtidos por tratamento com hipóxia em relação à aquisição de características mais agressivas, incluindo proliferação, migração e invasão.

Os resultados obtidos no ensaio de curva de crescimento utilizando a linhagem HEP-2 irradiada (Figura 30) mostraram redução de proliferação em relação ao controle não irradiado em todas as doses, exceto nas doses de 2Gy e 4Gy que só foram significativamente menor que o controle no tempo de 72h ($p < 0,001$). A redução de proliferação celular foi proporcional à dose irradiada, sendo a menor proliferação com 16G. Nessa concentração, foi observada diferença significativa a partir do tempo 24h ($p < 0,05$).

Figura 30. Curvas de proliferação estabelecidas em cinco tempos (0h, 24h, 48h, 72h e 96h) para as células HEP-2: controle (linha azul escuro) ou irradiadas com raios-X em diferentes doses (2Gy, 4Gy, 8Gy e 16Gy nas linhas verde, vermelha, roxo e azul claro, respectivamente).

Ensaio de citotoxicidade (MTS)

No ensaio de citotoxicidade (MTS) utilizando a linhagem HEP-2 irradiada (Figura 31), ocorreu aumento da toxicidade em relação ao controle em todas as doses e foi maior com o aumento da dose. Os resultados obtidos no MTS mostraram

que todas as doses de radiação em todos os tempos tiveram proliferação menor que o controle, exceto com 2Gy nas primeiras 24h.

Com base nestes resultados, a dose de radiação e o tempo selecionados para ensaios posteriores foram de 4Gy por 48h porque, nessas condições, além da redução de proliferação e, portanto, do efeito da radiação, não ocorreu prejuízo do potencial proliferativo até 72h, indicando que as células após 48h ainda apresentavam potencial proliferativo.

Figura 31. Ensaio de citotoxicidade (MTS) estabelecido em quatro tempos (24h, 48h, 72h e 96h) para as células HEP-2: controle (linha azul escura) ou irradiadas com raios-X em diferentes doses (2Gy, 4Gy, 8Gy e 16Gy nas linhas verde, vermelha, roxo e azul claro, respectivamente).

4.2.1 Efeito do secretoma na fisiologia e no comportamento celular

Utilizando a linhagem HEP-2 irradiada, foi avaliado o efeito de seu secretoma em migração, invasão, proliferação e viabilidade de células HEP-2 e fibroblastos humanos normais.

Curvas de proliferação de HEP-2 tratada com o secretoma de células irradiadas

Os resultados obtidos nos ensaios de proliferação utilizando a linhagem HEP-2 controle (cultivada com MC1_{Controle}) e tratada (cultivada com MC1_{Rad}) não mostraram diferença significativa entre os dois grupo (Figura 32).

Ensaio de citotoxicidade (MTS) em linhagem HEP-2 e fibroblastos tratados com o secretoma de células irradiadas

Utilizando a linhagem HEP-2 irradiada e ensaio de MTS, foi avaliado o efeito do secretoma 1 dessas células na viabilidade de células HEP-2 (Figura 33) e fibroblastos humanos normais (Figura 34). Os resultados obtidos mostraram aumento significativo de sobrevivência no tempo de 72h ($p < 0,0001$) e a partir de 96h ($p < 0,01$) para as células HEP-2 e fibroblastos, respectivamente, tratadas com MC1_{Rad}.

Figura 32. Curva de crescimento estabelecida em seis tempos (início, 24h, 48h, 72h, 96h e 120h) para linhagem HEP-2 tratada com MC1_{Controle} (linha azul) ou com MC1_{Rad} (linha vermelha).

Figura 33. Ensaio de citotoxicidade (MTS) estabelecido em cinco tempos (24h, 48h, 72h, 96h e 120h) para linhagem HEP-2 tratada com MC1_{Controle} (linha verde) ou com MC1_{Rad} (linha alaranjada).

Figura 34. Ensaio de citotoxicidade (MTS) estabelecido em cinco tempos (24h, 48h, 72h, 96h e 120h) para fibroblastos tratados com MC1_{Controle} (linha verde) ou com MC1_{Rad} (linha alaranjada).

Ensaio de migração e invasão em linhagem HEp-2 e fibroblastos tratados com o secretoma de células irradiadas

Foi observado um pequeno, mas significativo aumento na capacidade migratória ($p=0,0084$) (Figura 35 A-C) e invasiva ($p=0,0096$) (Figura 36 A-C) de células HEp-2 tratada com o secretoma 1 de células HEp-2 irradiadas em relação ao controle.

Nos ensaios com fibroblastos, também foi observado um aumento significativo na capacidade migratória ($P=0,0117$) (Figura 35 D-F) e invasiva ($P=0,0149$) (Figura 36 D-F) em presença do secretoma de HEp-2 irradiada.

Figure 35. Migração celular. Resultados de ensaios realizados em câmera de Boyden após 6h de incubação. Fotomicrografias de HEp-2 (A,B) e fibroblatos (D,E) tratados com secretoma1_{Controle} (A, D) e secretoma1_{Rad} (B, E). Os valores de p obtidos pelo teste t de Student foram: $p=0,0084$ (A versus B), $p=0,0117$ (D versus E). Gráficos com o número total de células em 5 fotomicrografias de triplicatas.

Figure 36. Invasão celular. Resultados de ensaios realizados em câmera de Boyden após 44h de incubação. Fotomicrografias de HEp-2 (A,B) e fibroblatos (D,E) tratados com secretoma1_{Controle} (A, D) e secretoma1_{Rad} (B, E). Os valores de p obtidos pelo teste t de Student foram: $p=0,0096$ (A versus B), $p=0,0149$ (D versus E). Gráficos com o número total de células em 5 fotomicrografias de triplicatas.

4.2.2 Pcr array

O screening de genes relacionados com ciclo celular e proliferação foi realizado com placas de PCR Arrays, conforme descrito previamente.

Células HEP-2 tratadas com secretoma 1 de HEP-2 irradiadas

Nas análises por PCR Array, 12 de 44 genes relacionados com ciclo celular e proliferação (Figura 37) apresentaram expressão diferencial, quatro com expressão elevada (*CCND2*, *CCNH*, *CDC2* e *CDC25A*) e oito com expressão reduzida (*CCND1*, *CCND3*, *CCNE1*, *CDKN2B*, *E2F2*, *HDAC4*, *HDAC5* e *TGFB1*) nas células tratadas com o meio condicionado MC1_{Rad} em relação às tratadas com MC1_{Controle} procedente de linhagem HEP-2 irradiada.

Figura 37. Via do ciclo celular. As marcações em azul representam os genes com expressão reduzida e as marcações em vermelho representam os genes com expressão elevada detectados em células HEP-2 após tratamento com secretoma de células irradiadas (Kegg Pathway Database, http://www.kegg.jp/kegg/tool/map_pathway1.html).

5 DISCUSSÃO

O presente trabalho teve como objetivo investigar componentes do secretoma de células neoplásicas submetidas a estresse que influenciam o comportamento de células tumorais e estromais não submetidas a estas condições. Considerando que os fatores de estresse selecionam um genótipo maligno mais agressivo, é razoável supor que o microambiente tumoral seja também modificado em função das novas exigências da célula neoplásica mutante e promova alterações nos elementos mais próximos.

Dentre os fatores do microambiente tumoral atuantes no processo seletivo de genótipos resistentes mais adaptados, está a hipóxia, uma condição resultante de um estado de baixa perfusão causado pela ausência de uma rede efetiva de vasos sanguíneos, em especial nos locais mais internos dos tecidos tumorais. Aumentando as demandas de perfusão, está a capacidade proliferativa ilimitada da célula neoplásica e, conseqüentemente, seu consumo elevado de energia e oxigênio (Gao *et al.*, 2016). No tecido tumoral, as células em hipóxia convivem com células em normóxia dispostas em locais não muito distantes. Portanto, fatores parácrinos podem estabelecer rapidamente uma sinalização entre elas.

A hipóxia, assim como outros fatores de estresse, pode selecionar alterações genéticas que resultam em supressão imune local, desdiferenciação para estágios imaturos, aquisição de propriedades de células-tronco (Noman *et al.*, 2015) e de características mais agressivas, como migração (Corley *et al.*, 2005) e invasividade (Brizel *et al.*, 1996), bem como resistência a terapias anticâncer (Brown e Wilson, 2004).

Os resultados obtidos no presente trabalho mostraram que esta seleção pode ser desencadeada não apenas pela hipóxia em si sobre as células neoplásicas, mas pelo secretoma das células em hipóxia sobre as vizinhas, presentes em locais com melhor perfusão. Realmente, foi observado que esse secretoma induziu fibroblastos normais e células tumorais cultivados em normóxia a migrarem ou invadirem outros locais e a alterarem a expressão de genes e proteínas relacionadas com adesão, resposta imune, proliferação, apoptose e diferenciação.

Um aspecto importante dos resultados, que foi evidenciado pelo diagrama de Venn, foi a vulnerabilidade reduzida das proteínas celulares às alterações do microambiente, com a grande maioria delas comum a vários experimentos. No

secretoma, ao contrário, o diagrama de Venn revelou uma grande quantidade de proteínas exclusivas em cada condição.

Uma comparação feita entre as proteínas celulares diferencialmente expressas em células hipóxicas e em células tratadas com o seu secretoma mostrou que algumas delas aparecem com padrão de expressão inverso. Por exemplo, ILF3, ANXA5, ENO1 tiveram níveis reduzidos em HEP-2 tratada com CoCl_2 em relação ao controle, mas foram encontradas com níveis altos em fibroblastos tratados com o secretoma de HEP-2 hipóxica. O mesmo foi observado em células SCC-25 hipóxicas, que exibiram níveis aumentados de DBNL, enquanto fibroblastos tratados com o seu secretoma tiveram níveis reduzidos.

As proteínas secretadas também apresentaram essa divergência de perfil. O secretoma de HEP-2 em hipóxia apresentou níveis reduzidos de IGFBP4, enquanto fibroblastos tratados com MC1_{Hipo} de HEP-2 secretaram níveis elevados de IGFBP4. O MC1_{Hipo} de SCC-25 em hipóxia, por sua vez, mostrou níveis reduzidos de PPA1 e IQGAP1, enquanto o secretoma de SCC-25 tratadas com seu próprio MC1_{Hipo} mostrou níveis aumentados de ambas.

Como o presente trabalho propôs avaliar o efeito do secretoma de células sob estresse no comportamento e na fisiologia de outras células, as proteínas para validação foram selecionadas entre aquelas com expressão elevada nas células tratadas com MC1_{Hipo} em relação ao controle. O critério inicial foi o número de espectros para peptídeos únicos nas triplicatas do experimento, com foco nos mais significativos. Após este primeiro passo, a literatura foi pesquisada para escolha de proteínas que estavam envolvidas em processos biológicos relacionados com câncer. Assim, duas proteínas (PHB2 e TXN1) foram avaliadas por Western blot em HEP-2 e duas (NEDD8 e TPT1) em SCC-25 tratadas com MC1_{Hipo} em relação ao controle.

A PHB2 ou proibitina-2 pertence a um grupo altamente conservado de proteínas localizadas na mitocôndria, no núcleo e na membrana plasmática que são expressas em muitos tipos de célula. A Proibitina-1 (PHB1) e a proibitina-2 (PHB2) são duas subunidades homólogas do complexo PHB mitocondrial eucariótico, e são interdependentes, o que significa que a perda de uma leva simultaneamente à perda da outra (Peng *et al.*, 2015). Ambas, portanto, possuem papel crucial na regulação da função das mitocôndrias, e de processos que incluem supressão do estresse oxidativo, inflamação, proliferação, sobrevivência celular, desenvolvimento,

sinalização e apoptose (Mishra *et al.*, 2006; Theiss *et al.*, 2007; Artal-Sanz e Tavernarakis, 2009; Mishra *et al.*, 2010; Thuaud *et al.*, 2013).

A ligação de PHB2 a IGFBP-6 é essencial para migração celular pela ativação da via MAPK e, na sua ausência, não ocorre migração (Fu *et al.*, 2013). A presença de PHB2 também está relacionada aos primeiros estágios de invasão celular, sendo perdida em estágios posteriores (Chen, R. X. *et al.*, 2014). Jun Cheng e cols. (2014) submetaram células de carcinoma hepatocelular a condições de hipóxia e observaram em análises por espectrometria de massas níveis elevados de PHB2 nessas células. Seu bloqueio resultou em diminuição de formação de colônia e da capacidade invasiva (Cheng *et al.*, 2014). Estes dados e aqueles do presente trabalho, mostrando níveis elevados de PHB2 após tratamento com o secretoma de células submetidas a hipóxia, sugerem uma sinalização da célula em hipóxia para suas vizinhas em normóxia, induzindo estas últimas a invadirem outros tecidos.

A proteína *ubiquitin-like* NEDD8 (*neural precursor cell expressed, developmentally down-regulated 8*) possui alta similaridade de sequência com as ubiquitinas (Kamitani *et al.*, 1997), uma família de polipeptídeos pequenos conservados que se ligam covalentemente a outras proteínas. As ubiquitinas normalmente têm como alvo proteínas para degradação pelo proteassoma (Hershko *et al.*, 2000), enquanto as *ubiquitin-like* podem afetar a localização subcelular, a conformação e a associação com outras proteínas (Jentsch e Pyrowolakis, 2000).

NEDD8 é ligada a seu substrato de forma similar ao que ocorre na ubiquitinação, resultando na sua ligação ao substrato. A cascata de eventos, denominada nedilação, inclui atividades enzimáticas conjugantes (E1, E2, E3) e desconjugantes, e tem início com a ativação de NEDD8 pela enzima ativadora E1 de NEDD8 (NAE). O processo segue com a ação das enzimas de conjugação E2 e múltiplas proteínas tipo ligase E3, que sucessivamente ativam e transferem NEDD8 para uma molécula alvo (Tanaka *et al.*, 2012; Abidi e Xirodimas, 2015) (Figura 38).

As principais proteínas alvo de NEDD8 são membros da família culina (Cul-1, -2, -3, -4A, -4B, -5, and -7). As culinas participam de complexos formados por vários componentes chamados CRLs, que controlam a estabilidade de muitas proteínas com diferentes funções, incluindo regulação do ciclo celular, sinalização, reparo de DNA e resposta a hipóxia. Vários estudos têm mostrado que a via NEDD8 responde a estresse, com elevação de nedilação protéica e conseqüente degradação dos substratos (revisto por Abidi e Xirodimas, 2015).

Altos níveis de NEDD8 conjugados a Cul-1 são necessários para proliferação de células tumorais (Chairatvit e Ngamkitidechakul, 2007) e já foram observados em uma variedade de tumores, como adenocarcinomas de pulmão e carcinomas epidermóides. Existem evidências recentes mostrando que seu bloqueio, por exemplo por *knockdown* de NAE, resulta em taxas de crescimento reduzidas em linhagem de carcinoma oral (revisto por Abidi e Xirodimas, 2015). Também foi observado que a inibição da nedilação com uma molécula inibidora de NEDD8, MLN4924, suprime proliferação, migração, invasão e induz apoptose (Kuo *et al.*, 2015; Lan *et al.*, 2016).

Os achados de Ryu e colaboradores (2011) indicaram que a expressão e a atividade do fator de transcrição induzível por hipóxia, HIF-1 α , são inibidas pelo bloqueio da enzima E1 necessária para a conjugação de NEDD8 ao seu alvo, mas aumentada por expressão ectópica de NEDD8. Este estudo também observou que NEDD8 estabiliza HIF-1 α mesmo em normóxia, e aumenta seu nível em hipóxia. Nesse aspecto, o presente trabalho acrescenta uma informação nova, de que o secretoma induzido por hipóxia pode conter sinais para as células vizinhas elevarem a expressão de NEDD8, o que teria consequências na modulação da via HIF.

Figura 38. Nedilação: ativação e transferência de NEDD8 para um subtrato. A presença de níveis elevados de NEDD-2 leva a características de um fenótipo mais agressivo, com estabilização do fator de inibição de hipóxia 1 α (HIF1 α), capacidade para migrar e invadir outros tecidos, proliferação e inibição de apoptose. Modificado de Enchev, *et al* 2015.

No nível de transcritos, os resultados do presente trabalho também sugerem que o secretoma induzido por hipóxia altera, nas células vizinhas, a expressão de genes que atuam no controle do ciclo celular e na regulação da transcrição. Este resultado foi validado em experimentos de indução de estresse por outro fator que não a hipóxia: a radiação por raios X. A diferença entre a atuação dos dois fatores se resume no padrão de expressão observado para alguns genes: nos experimentos de hipóxia, ocorreu redução de níveis de transcritos envolvidos em proliferação (como o *CDC25A* e *TGFB2*) e elevação de repressores de transcrição (*HDAC4* e *HDAC5*) e de inibidores de quinases do ciclo celular (*CDKN2B*). Por outro lado, o tratamento com o secretoma induzido por radiação resultou em expressão elevada de alguns dos genes que promovem progressão do ciclo celular (ciclina e *CDC25A*) e expressão reduzida de inibidores do ciclo (*CDKN2B*) bem como de repressores de transcrição (*HDAC4* e *HDAC5*).

Do ponto de vista biológico, células em hipóxia poderiam enviar sinais de quiescência para as vizinhas, um alerta de que os níveis de oxigênio estão baixos. Ao mesmo tempo, enviariam estímulos para migração e invasão de locais com melhores condições de sobrevivência. A radiação, ao contrário, deveria induzir sinais de proliferação para recompor o tecido lesado. Este quadro hipotético recebe apoio dos dados obtidos nos ensaios de invasão e migração. Células neoplásicas e fibroblastos tratados com o secretoma induzido por hipóxia, não mostraram diferenças significativas nas taxas de proliferação e viabilidade ou mostraram redução de proliferação, mas exibiram aumento da capacidade de invasão. Células neoplásicas e fibroblastos tratados com o secretoma induzido por radiação, por sua vez, exibiram aumento de viabilidade e de capacidade de migração e invasão. Este achado pode, em parte, explicar a eficácia limitada do tratamento radioterápico no caso de certos tumores.

A ativação e a inativação periódicas de quinases dependentes de ciclina (CDKs) conduzem à progressão do ciclo celular. Em mamíferos, as principais CDKs do ciclo celular (CDK1, CDK2, CDK4 e CDK6) associam-se com ciclina específicas para formar holoenzimas de quinase ativa (Hustedt e Durocher, 2016). A formação alterada desses complexos ciclina/CDK é comum em câncer e pode afetar a proliferação celular (Canavese *et al.*, 2012; Sperka *et al.*, 2012). Os complexos ciclina D-CDK4/6 e ciclina E-CDK2 são fatores críticos para a progressão através da fase G0/G1 do ciclo celular. Estes fatores são regulados negativamente por

inibidores de CDK, tais como o p21, p27 (codificados pelos genes *CDKN1A* e *CDKN1B*, respectivamente) (Dobashi *et al.*, 2003), p16INK4a e p14ARF (codificados pelos genes *CDKN2B* e *CDKN2A*) (Hannou *et al.*, 2015).

A fosfatase CDC25A é um dos reguladores do ciclo celular mais cruciais: controla as passagens G1/S e G2/M e é especificamente degradada em resposta a dano no DNA, o que previne a divisão de células anormais. Seus níveis são controlados por diversas quinases CHK1, CHK2, CK1a, CK1e e ERK (Shen e Huang, 2012; Sur e Agrawal, 2016). O complexo E-CDK2 fosforila e ativa a fosfatase CDC25A, que então pode desfosforilar e ativar CDKs requeridas para transição G1/S. A CDC25A é considerado um oncogene e sua expressão excessiva provoca tumores em uma variedade de tecidos (Shen e Huang, 2012; Sur e Agrawal, 2016).

As histonas desacetilases 4 e 5 (HDAC4 e HDAC5) pertencem à família alfa de histonas desacetilases classe II. No núcleo, interagem com fatores de transcrição tais como MEF2 e reprimem a transcrição (Yang e Gregoire, 2005). A HDAC4 já foi associada com ação de proteção contra estresse e defesa contra dano ao DNA induzido por radiação (Kao *et al.*, 2003).

Em relação às alterações metabólicas desencadeadas pelo microambiente, foi observado no presente estudo aumento na concentração de glicose e diminuição de lactato no secretoma de células em hipóxia. Aparentemente, as células nesta condição diminuíram a captação de glicose, o que resultou no aumento de sua concentração no meio de cultura. Com a glicólise reduzida, a síntese e a liberação de lactato no meio também foi reduzida. Esse meio de cultura, com altos níveis de glicose e baixos níveis de lactato, quando adicionado a cultura de células em normóxia, provocou um aumento na concentração de lactato no seu secretoma, o que sugere aumento de glicólise. Em resumo, o secretoma de células em hipóxia, como as que estão em regiões internas do tumor com pouca oxigenação, potencialmente induzem aumento de glicólise em células vizinhas.

A glicólise aeróbica é um processo também denominado Efeito de Warburg e consiste no maior consumo de glicose por células proliferativas em relação a células quiescentes. A glicose, neste processo, é reduzida a lactato e não fosforilada pelo processo oxidativo da cadeia respiratória, mesmo na presença de oxigênio (Vander Heiden *et al.*, 2009; Deberardinis e Cheng, 2010). Embora existam muitas evidências de que este metabolismo alterado da glicose esteja relacionado com invasão, migração e homeostase energética, suas vantagens para as células

tumorais ainda não estão completamente esclarecidas (Yang *et al.*, 2014) (revisto por (Choi *et al.*, 2013).

Os tumores são metabolicamente heterogêneos, com sub-populações que realizam fosforilação oxidativa (fenótipo oxidativo), glicólise aeróbica (fenótipo Warburg) ou glicólise anaeróbica. A manutenção de uma alta taxa de glicólise está sob o controle positivo do fator de transcrição HIF-1, sabidamente associado com agressividade do tumor e prognóstico desfavorável (Unruh *et al.*, 2003; Brahimi-Horn *et al.*, 2007). O lactato, por sua vez, ativa HIF-1 em células com fenótipo oxidativo, mas não com fenótipo Warburg (De Saedeleer *et al.*, 2012)

O lactato não é um subproduto metabólico, mas um combustível para células com fenótipo oxidativo (Dhup *et al.*, 2012) e uma molécula sinalizadora em vários tipos celulares (Pinheiro *et al.*, 2012). Em tumores, o lactato está no centro de uma “simbiose metabólica” na qual as células hipóxicas/glicolíticas produzem lactato a partir da glicose e as células oxidativas usam o lactato como combustível (Sonveaux *et al.*, 2008). Além disso, o lactato em si induz migração e parece contribuir para escape imune e angiogênese, e sua concentração nos tecidos tem sido associada com radorresistência, metástase e sobrevida de pacientes (Hirschhaeuser *et al.*, 2011).

Em muitos aspectos, os resultados do presente estudo são consistentes com os de Ohanna e colaboradores (Ohanna *et al.*, 2011), que mostraram a formação de um secretoma com propriedades pró-tumoral e pró-metastática, ativado durante a senescência em resposta a diferentes estímulos, incluindo quimioterápicos.

A senescência parece ser estimulada por dano direto ao DNA, como o provocado por hipóxia ou radiação ou mesmo por insultos endógenos (Wahl e Carr, 2001). As células de mamíferos empregam a proteína p53 para responder a danos no DNA provocados por estresse. As respostas para tais danos pode causar parada do ciclo celular, senescência ou ativação do programa de morte celular programada (Wahl e Carr, 2001).

A senescência é uma resposta celular complexa e é frequentemente associada à expressão da proteína supressora de tumor p16^{INK4A} e a um fenótipo secretor (*senescence-associated secretory phenotype* ou SASP) com perfil dependente do tipo de estresse (Braig e Schmitt, 2006; Wiley *et al.*, 2016). Na

senescência, as células secretam citocinas pró-inflamatórias, fatores de crescimento e angiogênicos que atuam de forma parácrina e têm efeitos benéficos na regeneração tecidual (Coppe *et al.*, 2006; Coppe *et al.*, 2008). Por outro lado, estes fatores também possuem um efeito deletério porque promovem proliferação de células pré-malignas *in vitro* e *in vivo* (Krtolica *et al.*, 2001; Bavik *et al.*, 2006), estimulam migração e invasão de células pré-neoplásicas e de células endoteliais (Coppe *et al.*, 2008; Malaquin *et al.*, 2013), favorecem a formação de tumores *in vivo* e conferem propriedades metastáticas (Liu e Hornsby, 2007; Ohanna *et al.*, 2011).

Em resumo, o presente trabalho observou que o secretoma de células em hipóxia é capaz de alterar o perfil de expressão gênica e protéica de outras células, bem como seu secretoma e metaboloma. Este secretoma induzido por hipóxia cria assim condições para seleção de características mais adequadas ao ambiente de estresse.

Além disso, os sinais enviados por um secretoma com propriedades pró-tumorais e pró-metastáticas, ativado por estresse, afetam não apenas a composição protéica e o metabolismo, mas também o comportamento de outras células. No caso de radiação, o aumento da capacidade migratória e da invasividade de células vizinhas não irradiadas, neoplásicas ou pré-neoplásicas, podem em parte explicar a eficácia limitada do tratamento radioterápico no caso de certos tumores.

Deve ser levado em conta também a ação cooperativa da hipóxia e da radiação *in vivo*. Em regiões hipóxicas do tumor, os níveis de oxigênio não são suficientes para impedir que os radicais livres ligados ao DNA, gerados pela radiação, sejam reparados quimicamente por grupos sulfidril ou outras moléculas redutoras. Como resultado, a ação tóxica da radiação sobre a célula tumoral não é completamente obtida.

Estas observações são uma clara demonstração do papel do microambiente tumoral na resposta à terapia anticâncer. A identificação e a caracterização do secretoma têm, portanto, importantes implicações clínicas e podem abrir novas perspectivas para o tratamento do câncer.

6 CONCLUSÕES

1. O secretoma induzido por hipóxia altera o perfil de proteínas celulares, o secretoma e o metoboloma de células neoplásicas e normais não submetidas a este fator de estresse. Portanto, o secretoma induzido por hipóxia possui elementos capazes de desencadear a seleção de células com novos genótipos.
2. O secretoma induzido por hipóxia ou radiação promove o aumento da capacidade de migração e invasão de células neoplásicas e normais não submetidas a este fator de estresse. Portanto, o secretoma induzido por hipóxia possui elementos capazes de desencadear a seleção de células com genótipos mais agressivos.
3. O secretoma induzido por hipóxia não altera as taxas de proliferação e viabilidade ou mesmo reduz estas taxas em células neoplásicas e normais não submetidas a este fator de estresse.
4. O secretoma induzido por radiação eleva as taxas de proliferação e viabilidade ou mesmo reduz estas taxas em células neoplásicas e normais não submetidas a este fator de estresse.
5. O secretoma induzido por hipóxia promove o aumento de expressão das proteínas *prohibitina 2* (PHB2) e *neural precursor cell expressed, developmentally down-regulated 8* (NEDD8), o que favorece invasão e modulação da via HIF.
6. O efeito no ciclo celular do secretoma induzido por hipóxia é diferente daquele induzido por radiação: o primeiro causa aumento da expressão de vários genes relacionados ao bloqueio do ciclo celular e redução da expressão de genes relacionados a ativação do ciclo, o segundo tem ação inversa.
7. A hipóxia provoca aumento na concentração de glicose e diminuição de lactato no secretoma de células submetidas a este fator de estresse. Este secretoma, por sua vez, adicionado a cultura de células em normóxia causa aumento na concentração de lactato no seu secretoma, o que sugere aumento de glicólise nessas células.
8. Em resumo, a hipóxia promove a formação de um secretoma com propriedades pró-tumorais e pró-metastáticas.

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O PAPEL DO MICROAMBIENTE TUMORAL NO SECRETOMA

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INTRODUCTION

The secretome consists of a subset of proteins and metabolites released by a cell, tissue or an organism, which plays an important role in the regulation of cell to cell interactions essential to their normal physiological functions. Secretome alterations are often associated with atypical cellular phenotypes that are indicative of diseases such as cancer.

Secretome analysis have identified biomarkers for different pathological conditions including chronic inflammation, neurodegenerative disorders, cancer, cardiovascular disease and diabetes. Moreover, secretome studies have contributed to better understand the molecular mechanisms involved in pathogenesis and to the development of novel treatment strategies. (Paltridge JL et al. *Biochim Biophys Acta*. 2013 Nov;1834(11):2233-41; Xue H et al. *J Transl Med*. 2008 Sep 17;6:52. doi: 10.1186/1479-5876-6-52). Specifically, mutations that underlie cancer progression can lead to altered protein expression patterns which can be detected in serum and other body fluids, allowing for early diagnosis and prediction to treatment response. (Schaaij-Visser TB et al. *Biochim Biophys Acta*. 2013 Nov;1834(11):2242-58).

THE SECRETOME

The term secretome was first used in a study by Tjalsma et al., in the year of 2000 (Tjalsma H et al *Microbiol Mol Biol Rev*. 2000 Sep;64(3):515-47.) to designate the whole secretory processes in the bacteria *Bacillus subtilis*. The definition of secretome was later expanded to denote the global group of proteins secreted, released or detached into the extracellular space by a cell, tissue, organ or organism at any given time (Agrawal GK et al. *Proteomics*. 2010 Feb;10(4):799-827). Such proteins comprise a large variety of bioactive molecules that play important roles in regulating cell-cell and cell-extracellular matrix interactions. They may act in an autocrine or paracrine fashion, positively or negatively influencing the ability of cells to survive, proliferate and differentiate (Pocsfalvi G et al., *J Proteome Res*. 2011 Dec 2;10(12):5326-37).

Proteins secreted via the classical pathway, are synthesized in the endoplasmic reticulum (ER) presenting a signal peptide at their N-terminus (Schatz e Dobberstein, *Science* 1996 Mar 15;271(5255):1519-26. 1996). They are transported to the Golgi apparatus in a coat protein complex II (COPII)-coated vesicles and then, within secretory vesicles, to the plasma membrane and cell exterior (Mellman and Warren, *Cell*. 2000 Jan 7;100(1):99-112; Lee MC et al *Annu Rev Cell Dev Biol*. 2004;20:87-123.). During their passage through the Golgi apparatus, proteins are modified by different processes such as glycosylation (Nickel W, Wieland FT, *Histochem Cell Biol*. 1998 May-Jun;109(5-6):477-86), phosphorylation (Yellaturu CR et al. *J Biol Chem*. 2009 Mar 20;284(12):7518-32) and

palmitoylation (Linder ME, Deschenes RJ. Nat Rev Mol Cell Biol. 2007 Jan;8(1):74; (Greaves J et al. Mol Membr Biol. 2009 Jan;26(1):67-79.

Proteins, can also be exported by the non-classical pathway involving endoplasmic reticulum/Golgi-independent secretion. This pathway corresponds to the translocation of proteins from the cytoplasm directly across the plasma membrane into the extracellular compartment. Examples include the fibroblast growth factor 2 (FGF2) and cytokines involved in inflammation and angiogenesis (Nickel W. Curr Opin Biotechnol. 2010 Oct;21(5):621-6). The non-classical pathway uses vesicular and non-vesicular transport mechanisms. The non-vesicular mechanisms encompass self-sustained protein translocation through plasma membranes and ABC transporter-mediated secretion. The vesicular mechanism depend on intermediate carriers such as multivesicular bodies (MVBs), exosomes, autophagosomes and secretory lysosomes that fuse to the plasma membrane to release their contents (Rabouille C et al. Cell Sci. 2012 Nov 15;125(Pt 22):5251-5; Zhang M, Schekman R. Science. 2013 May 3;340(6132):559-61).

Secreted proteins are involved in a variety of *physiological processes* such as immune response, blood coagulation, digestion, remodeling of extracellular matrix, cell signaling and cell communication. These proteins include hormones, proteases and digestive enzymes, immunoregulatory cytokines/chemokines, growth factors (Mukherjee P, Mani S. Biochim Biophys Acta. 2013 Nov;1834(11):2226-32) and metabolites. Also, proteins of the extracellular matrix, plasma membrane derived vesicles, small exosomes which may contain non-protein compounds such as RNA and lipids were also included as part of the secretome (Paltridge et al Biochim Biophys Acta. 2013 Nov;1834(11):2233-41); Stastna, Eyk. Proteomics. 2012 Feb;12(4-5):722-35).

The secretome is also involved in the pathogenesis of several diseases, such as cancer, promoting invasion of the surrounding tissues, evasion of immune defenses and distant organ colonization (Pocsfalvi G et al., J Proteome Res. 2011 Dec 2;10(12):5326-37). *Therefore, a better understanding of the mechanisms regulating the secretome can lead to the development of novel targeted therapies with a focus on the tumor microenvironment.*

SECRETOME IN CANCER

For many years, cancer was considered a stand-alone cell process. The concept focused on genetically transformed cells and their progression to a malignant condition. However, studies of the tumor microenvironment have shown that the neoplastic progression depends on a network of interactions between cancer cells and the surrounding tumor stroma (reviewed in Mueller MM, Fusenig NE. Nature Reviews Cancer 4, 839-849). As members of these interactions there are soluble proteins, antigens, cell debris derived from apoptosis and necrosis, RNA and DNA from both neoplastic cells and its neighbors.

In the microenvironment, the stroma provides the support structure and facilitates tumor proliferation and dissemination. This structure includes extracellular matrix (ECM), a non-cellular macromolecular network rich in fibrillar protein structures (such as collagen and fibronectin), non-structural fibrillar proteins (as proteoglycan/glycosaminoglycan), matricellular proteins that participate in cell-cell interactions and cell-ECM (Theocharis AD et al. *Adv Drug Deliv Rev.* 2016 Feb 1;97:4-27). The stromal cell components include fibroblasts, endothelial, inflammatory and immune system cells. (Mueller MM, Fusenig NE. *Nature Reviews Cancer* 4, 839-849). The tumor-stroma communication occurs directly between cells or via a variety of molecules secreted, such as growth factors, cytokines, chemokines, microRNAs (Nasser MW et al. *Cancer Lett.* 2015 Aug 28;365(1):11-22; Kohlhapp FJ *Oncogene* 2015 Nov 26;34(48):5857-68) and can be influenced by mechanical forces (Mierke CT. *Rep Prog Phys.* 2014 Jul;77(7):076602. doi:10.1088/0034-4885/77/7/076602). This heterotypic secretome, which derives not only from tumor cells but also from cancer-associated stromal cells is an important source of markers and key regulators of the tumorigenic process. (Karagiannis GS et al. *Mol Oncol.* 2010 Dec;4(6):496-510; Zeng X et al. *J Proteomics.* 2013 Aug 26;89:51-70).

The tumor microenvironment is therefore much broader than the central unit formed by the neoplasm. Its complexity is due to the resident or recruited elements and multidirectional and dynamic interactions between them, which end up generating a loss of the normal tissues architecture, sites of inflammation, hypoxia and consequently neoangiogenesis to contemplate the high needs for oxygen and nutrients (Paltridge et al *Biochim Biophys Acta.* 2013 Nov;1834(11):2233-41; Zeng X et al. *J Proteomics.* 2013 Aug 26;89:51-70) (Figure 1).

THE CROSSTALK BETWEEN CANCER CELLS AND STROMAL CELLS

An early process in cancer progression is the epithelial-mesenchymal transition (EMT), a cellular process characterized by changing from epithelial to mesenchymal phenotype, which increases the capacity of migration, invasion, apoptosis and resistance. This process also occurs during embryogenesis and in tissue regeneration after injury, usually associated with inflammation (Kalluri R, Weinberg RA. *J Clin Invest.* 2009 Jun;119(6):1420-8).

During the neoplastic process, remodeling of the extracellular matrix follows epithelial-mesenchymal transition. Analysis of the cell secretome at this stage has shown, as expected, increased expression of proteases, ECM components, factors that promote migration and reduced expression of adhesion proteins (Mathias RA et al. *J Proteome Res.* 2009 Jun;8(6):2827-37; Mathias RA. *J Proteome Res.* 2010 Feb 5;9(2):1007-19).

Changes in the secretome leading to changes in the ECM and paracrine induction of cell proliferation should occur at early stages of cancer development. Non-transformed cells within the secretome induced expression of c-Myc showed high ability to induce proliferation, overcome senescence, modulate the composition/function of ECM and basement membrane and to attract fibroblasts. The reduction of matrix components can be seen as an event that is able to interrupt normal tissue organization. Moreover, the ability to attract fibroblasts suggests that the secretome can change the cross-talk between epithelial and stromal cells in early stages of tumorigenesis. (Pocsfalvi G et al. *J Proteome Res.* 2011 Dec 2;10(12):5326-37).

The fibroblasts represent the most abundant cells of the tumor stroma, and play a critical role in tumor growth, survival, proliferation, migration, invasion and metastasis (Shimoda M et al. *Semin Cell Dev Biol.* 2010 Feb;21(1):19-25 Lin ZY et al. *Biomed Pharmacother.* 2012 Oct;66(7):525-9). Under normal conditions, the fibroblasts are quiescent and are activated during tissue regeneration after injury, originating myofibroblasts. In the tumor microenvironment, fibroblast activation is induced by stimuli derived from neoplastic cells or immune system cells, particularly growth factors and interleukins, which lead to changes in its morphology and function (Han Y et al. *Tumour Biol.* 2015 Mar;36(3):1385-94). It also seems to be self-induced by mutations leading to loss of the TIMP gene, which is associated with the control of the extracellular matrix through direct inhibition of metalloproteinases (Shimoda M et al. *Nat Cell Biol* 16(9):889-901).

The activated fibroblasts are often called cancer-associated fibroblasts (CAFs) and comprise a heterogeneous cell population that include the α -smooth muscle actin (α -SMA) protein (Sugimoto H et al. *Cancer Biol Ther.* 2006 Dec;5(12):1640-6). This protein is also present in resident fibroblasts and myofibroblasts (De Wever O et al. *Int. J. Cancer.* 2008;123:2229–2238; Shiga K et al. *Cancers (Basel).* 2015 Dec 11;7(4):2443-58).

CAFS may originate from different resident precursors such as fibroblasts chronically exposed to oxidative stress (Toullec A et al. *EMBO Mol Med.* 2010 Jun; 2(6): 211–230) or senescent cells with similar profiles to the senescence-associated secretory phenotype. Bone marrow–derived mesenchymal stem cells (BMSCs) can differentiate in CAFs. This conclusion is based on the observation that direct co-culture model of colon cancer cells and BMSCs resembles the CAFs-like phenotype showing an increased expression of α smooth muscle actin (α -SMA) and vimentin in vitro. Importantly, results also reveal that Notch signaling mediates transformation of BMSCs to CAFs through the downstream TGF- β /Smad signaling pathway. (Peng Y et al. *Biochem Biophys Res Commun.* 2014 Aug 15;451(1):68-73. doi: 10.1016/j.bbrc.2014.07.074), or with a pro-angiogenic and pro-invasive phenotype (Chowdhury R et al. *Oncotarget.* 2015 Jan 20;6(2):715-31; Webber JP et al. *Oncogene.* 2015 Jan 15;34(3):290-302. 10.1038/onc.2013.560).

The cytokine TGF β is secreted in a latent form (Horigushi M et al. J Biochem. 2012 Oct; 152(4): 321–329) that upon activation becomes a potent inducer of epithelial-mesenchymal transition (EMT) (Zarzynska JM. Mediators Inflamm. 2014; 2014: 141747). Mesenchymal cells with chromosomal abnormalities similar to those found in adjacent cancer cells (with epithelial phenotype) express α -SMA and vimentin and do not express keratin (Petersen OW et al. Am J Pathol. 2003 Feb; 162(2): 391–402). These findings suggest that the CAFs can also be directly derived from neoplastic cells that have undergone epithelial-mesenchymal transition by TGF-beta induction.

Other cytokines also participate in fibroblast activation including the interleukin 6 (IL-6) that reduces the expression of the tumor suppressor proteins p16^{INK4A}, p21 and p53 in fibroblasts. IL-6 acts on the Jak2/STAT3 pathway through negative control of AUF1 (hnRNP D0) and promotes the expression of myofibroblast markers such as α -SMA (Hendrayani SF et al. J Biol Chem. 2014 Nov 7;289(45):30962-76).

CAFS constitute a heterogeneous population with a divergent transcriptome and secretome. Two distinct CAF subtypes have been described: one with a transcriptome and secretome closer to normal fibroblasts (CAF-N), another with a more divergent expression profile (CAF-D), with high secretion of TGF- β . This heterogeneity also includes a subpopulation of stationary/low migratory fibroblasts that might be responsible for synthesis of various growth factors, cytokines and matrix metalloproteinases (MMP) (Costea DE et al. Cancer Res. 2013 Jul 1;73(13):3888-901).

Communications in the tumor microenvironment include bidirectional signals, which activate neoplastic cell and subsequently induce the stromal cells to secrete factors involved in proliferation, migration and invasion, which are important for neoplastic progression (Mishra P et al. J Leukoc Biol. 2011 Jan;89(1):31-9; Slany A et al. Electrophoresis. 2013 Dec;34(24):3315-25). Furthermore, the signals can also derive from normal fibroblasts and CAFs that can inhibit proliferation of tumor cells (Chen SX et al. J Proteomics. 2014 Oct 14;110:155-71. doi: 10.1016/j.jprot.2014.07.031).

The negative effects on tumorigenesis may depend on the context or stages of neoplastic transformation. Several studies showed an inverse effect, as observed by secretome analysis of cancer-derived myofibroblasts compared with myofibroblasts from adjacent tissue, which have shown an increase in the production and activation of MMP-1, -2, and -3, that may contribute to the remodeling of the cancer cell microenvironment (Holmberg C et al. J Proteome Res. 2013 Jul 5;12(7):3413-22). In nasopharyngeal carcinoma, the secretome of CAFs also shows proteins related to invasion and metastasis, as well as proteins that act in the protection of cells against injuries of reactive oxygen species. Therefore, CAFs play an important role in the regulation of tumor microenvironment (Ge S. Exp Ther Med. 2012 May;3(5):857-860). Moreover, both fibroblast derived soluble

factors and metalloproteinases-rich exosomes derivatives from CAFS induce cancer cell motility and activate signaling patterns in neoplastic cells which are connected to invasion and metastasis (via RhoA/ROCK) and differentiation (Notch) (Shimoda M et al. Nat Cell Biol 16(9):889-901).

Senescence is a defense mechanism that has great impact on the ability of cells to communicate, regardless of the cell type and source of the signal. This mechanism is a response to stress which blocks cell proliferation. Senescence is often associated with expression of the tumor suppressor protein p16INK4a and to a secretory phenotype (senescence-associated secretory phenotype - SASP) with stress-type-dependent profile (Braig M, Schmitt CA. Cancer Res. 2006 Mar 15;66(6):2881-4; Wiley CD et al. Cell Metab. 2016 Feb 9;23(2):303-14). Cells in a senescent state secrete pro-inflammatory cytokines, growth and angiogenic factors that act in a paracrine manner (Coppé JP et al. J. Biol. Chem. 2006, 281, 29568–29574; Coppé JP et al. PLoS Biol. 2008 Dec; 6(12): e301. 10.1371/journal.pbio.0060301).

Senescence is seen as a cancer suppressor mechanism with the inflammatory response to the SASP phenotype stimulating cells from the immune system that can act in tissue regeneration and in the removal of senescent cells. However, the secretomes of these cells also include factors that promote proliferation of premalignant cells *in vitro* and *in vivo* (Krtolica A et al. Proc Natl Acad Sci U S A. 2001 Oct 9;98(21):12072-7; Bavik C et al. Cancer Res. 2006 Jan 15; 66(2):794-802) and stimulate migration and invasion of a pre-neoplastic and endothelial cells. Furthermore these factors also (Malaquin N et al. PLoS One. 2013 May10;8(5):e63607; Coppé JP et al. PLoS Biol. 2008 Dec; 6(12): e301. doi:10.1371/journal.pbio.0060301) promote the formation of tumors *in vivo* and confer metastatic properties Liu D, Hornsby PJ. Cancer Res. 2007 Apr 1;67(7):3117-26; Ohanna M et al. Genes Dev 2011 Jun 15;25(12):1245-61). Interestingly, SASP also presents anti-apoptotic, anti-angiogenic and cell proliferation suppressors (reviewed by Rodier F, Campisi J. J Cell Biol. 2011 Feb 21; 192(4): 547–556).

As suggested by Muñoz-Espín D et al (Cell. 2013 Nov 21;155(5):1104-18) and Storer M et al (Cell. 2013 Nov 21;155(5):1119-30), senescence is a normal programmed mechanism, which operates similarly to apoptosis to remove cells at risk with abnormal cell growth. Senescence is found in embryonic tissue remodeling and was probably adapted during evolution to act in tissue regeneration in the adult organism inducing an immune response that recognizes and removes unsuitable cells. A downside of this response is that the clearance of senescent cells can also remove non-senescent cells. Moreover, the number of senescent cells increases with age and the immune system may not be able to remove them completely, which can lead to a rich senescence-associated microenvironment secretome with cytokines, growth and angiogenic factors, increasing the risk of malignant

transformation (Campisi J. *Nature*. 2014 Jan 2;505(7481):35-6). The physiological relevance and the consequences of senescence were explored by Baker et al (Baker DJ et al. *Nature* 2016, 530, 184–189) who showed that the induction of apoptosis in p16^{INK4A}-positive senescent cells promoted extension of median mice lifespan, delayed tumorigenesis and attenuated age-related deterioration of several organs.

THE INFLAMMATORY SECRETOME AND CANCER

It is estimated that 25% of cancers are inflammation-related cancers (Mantovani A et al. *Nature*. 2008 Jul 24;454(7203):436-44). The first observations on this association were carried out by a Rudolf Virchow, a German physician, in the 19th century. He described leukocyte infiltration in tumors linked to the immune responses. (Elinav E et al. *Nat Rev Cancer*. 2013 Nov;13(11):759-71). Today it is widely recognized that inflammation promotes carcinogenesis. The hallmarks of cancer proposed by Hanahan and Weinberg in 2011 (*Cell*. 2011 Mar 4;144(5):646-74) currently include two new features that take into account inflammation - the inflammatory environment and evasion of immune attack.

There are several examples of chronic inflammation that increase the risk of developing cancer. These include the predisposition of patients with Crohn's disease to colorectal cancer (Cannon J.. *Surg Clin North Am*. 2015 Dec;95(6):1261-9, vii), *Helicobacter pylori* infection to gastric cancer (Polk DB, Peek RM. *Nat Rev Cancer*. 2010;10:403–414) and the human papilloma virus (HPV) to cervical cancer (Groves IJ, Coleman N. *J Pathol*. 2015 Mar;235(4):527-38) head and neck cancer (Herrero R et al. *J Natl Cancer Inst*. 2003 Dec 3;95(23):1772-83; IARC Monographs. 2007;90:1-636). Likewise, non-steroidal anti-inflammatory drugs decrease the risk of cancer death in various tumor types (Chan AT et al. *N. Engl. J. Med*. 356, 2131–2142 (2007); Flossmann E & Rothwell PM. *Lancet* 369, 1603–1613 (2007).

The inflammatory processes may contribute to multiple stages of tumor development through growth factors, survival factors, proangiogenic factors, signals that activate the EMT, enzymes that modify the extracellular matrix and facilitate invasion and metastasis, as well as reactive oxygen species, that are actively mutagenic and promote neoplastic progression (Hanahan D, Weinberg RA. *Cell*. 2011Mar 4;144(5):646-74).

According to Mantovani et al (Mantovani A et al. *Nature*. 2008 Jul 24;454(7203):436-44), inflammation-cancer relationship may be interpreted as having two pathways: the extrinsic pathway, initiated by inflammation typically triggered by external agents and the intrinsic pathway initiated by genetic alterations in oncogenes and tumor suppressors, which activate the expression of inflammation-related programs. These two pathways converge, result in the activation of transcription factors, such as nuclear transcription factor kappa B (NF-κB), Signal transducer and activator of transcription 3 (STAT3) and Hypoxia-inducible

factor 1- α (HIF1 α). These factors recruit and activate various leukocytes to produce inflammatory mediators, including cytokines, chemokines, cyclooxygenase-2 (COX-2) and prostaglandins, which will trigger a cascade of similar signals in other inflammatory cells, stroma and tumor cells. The result is a broad inflammatory signature in the tumor microenvironment with consequent cell proliferation, changes in epithelial cells to transition into mesenchymal cells, enhance the formation of blood and lymphatic vessels and inhibit immune response.

In the tumor-infiltrating lymphocytes there are tumor-associated macrophages (TAMs), mast cells and T cells. Macrophages are derived from monocytes usually recruited by the tumor through chemokines and depending on the microenvironmental signals they can have both pro- and anti-inflammatory effects, promoting or suppressing tumor activity. In response to chronic irritation and inflammation, synthesize inflammatory cytokines that recruit other cells of the immune system, and therefore promoting cancer development. (Balkwill FR, Mantovani A.. *Seminars in Cancer Biology*. 2012;22:33–40). After this phase, the macrophages display M2/trophic phenotype that promotes the neoplastic progression (Qian BZ, Pollard JW. *Cell*. 2010 Apr 2; 141(1):39-51). There are evidences that this second type of action occurs in response to macrophage colony-stimulating factor and interleukins present in the tumor microenvironment (Condeelis J, Pollard JW. *Cell*. 2006;124:263–266), with consequent production of growth factors and metalloproteases by macrophages facilitating invasion and metastasis (reviewed by Bögers M et al *Oncoimmunology* 2012 Sep 1;1(6):798-809).

The spread of these metastases to specific anatomical sites appears to be partly influenced by macrophages. Primary tumors generate exosomes or soluble factors that attract macrophages to pre-metastatic niche and promote their reprogramming, for example by transferring oncoproteins through exosomes. The acquisition of a pro-vasculogenic phenotype contributes to development suitable microenvironment for tumour progression (Peinado H et al. *Nat Med* 2012 Jun;18(6):883-91; Winn TA et al. 2013 Apr 25;496(7446):445-55).

The discrepancy in the signals sent by tumor cells for macrophage activation could explain the contradictory prognostics observed in different tumors. For example, the presence of macrophages have been associated with reduced survival in breast carcinoma (Tsutsui S et al. *Oncol Rep*. 2005 Aug;14(2):425-31), while it is associated with favorable prognosis in colorectal cancer (Forssell J et al. *Clin Cancer Res* March 1, 2007 13; 1472). Analysis of proteins secreted by either colon or breast carcinoma cells that could influence macrophage phenotype identified the proteoglycan versican (VCAN) exclusively in colon carcinoma cell lines. Although, the presence of stromal VCAN was not associated with increased survival in colorectal cancer, its expression by epithelial cells in the periphery of

the tumor was correlated with an improved prognosis suggesting that the microenvironmental factors can influence the phenotype of macrophages (Bögels M et al *Oncoimmunology* 2012 Sep 1;1(6):798-809).

In addition to macrophages, other cells that act on the inflammatory response play an important role in tumor progression. Among them are the neutrophils, which similarly to the macrophages may have both pro-tumoral and anti-tumoral effects (Galdiero MR et al. *Immunobiology*. 2013 Nov;218(11):1402-10). Neutrophils are attracted in response of interleukin (IL)-8 release by tumor cells and release nitric oxide, reactive oxygen species and matrix metalloproteinases (De Larco JE et al. 2004 Aug 1;10(15):4895-900). Depending on the context, neutrophils can release elastase promoting cell proliferation (Houghton AM et al. *Nat Med*. 2010 Feb;16(2):219-23) and epithelial-mesenchymal transition (Grosse-Steffen T et al. *Clin Dev Immunol*. 2012;2012:720768), or modulate tumor cell lysis via T-lymphocytes (Mittendorf EA et al. *Cancer Res*. 2012 Jul 1;72(13):3153-62).

Dendritic cells (DC) also play an important role in regulation of inflammation, mainly due to their ability to secrete different cytokines and chemokines. DCs are able to initiate, expand and regulate the immune response. Although cyclodextrins could be harnessed to silence cancer development, tumors in turn can exploit DC to evade immunity (Chaput N et al. *Oncogene* 2008 Oct 6;27(45):5920-31. The secretome profile of these cells, although less rich than the cytoplasmic proteome, proved to be more specific and sensitive to functional changes, indicating that this secretome could be an interesting source of potential biomarkers involved in the regulation of inflammatory processes (Gundacker NC et al. *J Proteome Res*. 2009 Jun;8(6):2799-811

SECRETOME COMPONENTS AND ANGIOGENESIS

Angiogenesis is defined as the formation of new vessels from a pre-existing vascular network. This process is driven by the demand for oxygen, nutrients, and requires interactions between different cell types, the extracellular matrix and several cytokines and growth factors. Although fundamental in development, tissue maintenance and survival, angiogenesis plays a central role in tumor progression, invasion and metastasis (Chung AS et al. *Nat Rev Cancer*. 2010 Jul;10(7):505-14).

Several conditions influence angiogenesis, including metabolic stress (hypoxia, acidosis, hypoglycemia), mechanical stress (compression generated by high cell proliferation), inflammatory responses (tumor-infiltrating immune cells) and genetic mutations (oncogene activation or inhibition of tumor suppressor genes) (Carmeliet P. *Nat Med* 2000 Apr;6(4):389-95). The major effectors of this process are activators of proliferation and migration of endothelial cells, such as growth factors that bind to the tyrosine kinase receptors (VEGF, FGF, PDGF, EGF), and lysophosphatidic acid (LPA) that interacts with the

surface of G protein-coupled receptors (Bergers G, Benjamin LE. *Nat Ver Cancer*. 2003 Jun;3(6):401-10; Hu YL et al. *J Natl Cancer Inst*. 2001 May 16;93(10):762-8). Among the inhibitors are thrombospondin-1 and statins (angiostatin, endostatin, canstatin and tunstatina) (Bergers G, Benjamin LE. *Nat Ver Cancer*. 2003 Jun;3(6):401-10. These pro- and anti-angiogenic factors may be released by tumor cells or derived from the tumor microenvironment (Bergers G, Benjamin LE. *Nat Ver Cancer*. 2003 Jun;3(6):401-10.

Zhong G et al (*Cancer Res* 2008 Sep 1;68(17):7237-45) studied the secretome of stromal cells during angiogenesis in a co-culture with lung cancer cells. They observed that co-culturing enhanced stromal cell migration, induced endothelial tube formation, increased cell proliferation, and regulated the secretion of proteins involved in angiogenesis, inflammation, cell proliferation, and epithelial-to-mesenchymal transition. Among these proteins, cytokines and chemokines, such as CCL2, VEGF, IL-18, TNF-alpha and GM-CSF were identified.

Multiple studies focusing on the role of the secretome in promoting angiogenesis have shown the involvement of other proteins, such as interleukin-8 released by glioblastoma cells (Dwyer J et al. *PLoS One*. 2012;7(9):e45562), CYR61 (Estrada R et al. *J Cell Physiol*. 2009 Jun;219(3):563-71; Park YS et al. *J Cell Physiol*. 2015 Jan;230(1):140-9) and hepatoma-derived growth factor (HDGF) (Thirant C et al. *Stem Cells*. 2012 May;30(5):845-53) released by bone marrow stem cells and glioblastoma, respectively.

Tumor cells secrete not only a variety of soluble factors, but also extracellular vesicles (EVs) that promote migration, invasion, tube formation, and neovascularization *in vivo* (Kim CW et al. *Cancer Res*. 2002 Nov 1;62(21):6312-7). The comparative proteomic analysis of these EVs, including exosomes and microvesicles, showed many differences between primary and metastatic cancer-derived EVs (Choi DS et al. *J Extracell Vesicles*. 2012 Sep 11;1). Notably, CD276 antigen, which is an immunoregulator and cell surface tumor endothelial marker, exhibited high expression in blood vessels of different tumors, as well as within the tumor cell (Seaman S et al. *Cancer Cell*. 2007 Jun;11(6):539-54). Other experiments showed similar results. For example, exosomes released from glioma cells in hypoxic conditions proved to be potent inducers of angiogenesis *in vivo* and *ex vivo* (Kucharzewska P et al. *Proc Natl Acad Sci U S A*. 2013 Apr 30;110(18):7312-7).

Angiogenesis has been extensively studied to develop new therapies. A new target is RalBP1 a multifunctional GTPase which regulates the secretion of angiogenic factor VEGF by tumor and whose locking significantly reduces the formation of new vessels (Lee S et al. *Cancer Res*. 2012 Oct 15; 72(20):5165-73; Lee S, Goldfinger LE. *FASEB J*. 2014 Sep;28(9):4158-68).

Angiogenic inhibitors targeting VEGF have demonstrated therapeutic efficacy in multiple human cancers. However, the benefits of these therapies are at best temporary and

are followed by resistance to therapy (Bergers G, Hanahan D. *Nat Rev Cancer*. 2008 Aug;8(8):592-603). One explanation for this outcomes is the involvement of alternative mediators such as HGF, IGFBP-7 and cathepsin L. (Winiarski BK et al. *Transl Oncol*. 2013 Dec 1;6(6):703-14).

As previously mentioned, nonsteroidal anti-inflammatory drugs have been found to be effective in preventing cancer. Its main mechanisms of action are pro-apoptotic and anti-angiogenic inhibiting malignant transformation and suppressing proliferation (Thun MJ et al. *J Natl Cancer Inst*. 2002 Feb 20;94(4):252-66). One example is sulindac, used to prevent colorectal cancer, which reduces the secreted modulators of apoptosis and angiogenesis such as interleukin 8 (Ji H et al. *Proteomics Clin Appl*. 2009 Apr;3(4):433-51).

APPROACHES FOR CANCER SECRETOME STUDIES

The approaches for secretome studies are dependent on the target, the sample type and could vary widely, each of them with advantages and limitations. (Mantione KJ et al *Med Sci Monit Basic Res*. 2014 Aug 23;20:138-42). The analyses of biofluids, which directly reflect the conditions *in vivo*, and conditioned medium obtained from a cell culture allows to extract nucleic acids, proteins, metabolites, lipids and carbohydrates, which require different methods of detection.

BIOFLUIDS

The serum- or plasma-based assays are the most popular choice for clinical diagnosis, mainly due to their non-invasive characteristic and because they represent a reservoir of molecules released by the tissues. However, the characterization of biomarkers in the blood is not simple due to their variations in concentration at relative low levels. Also, their identification can be influenced by pre-analytical and clinical factors, and sequestration or degradation that can occur during their transport throughout the bloodstream.

In addition, blood contains multiple other molecules secreted by other organs including high abundance proteins such as albumin and immunoglobulins that hinders the detection of low abundance proteins. Therefore, biomarker discovery studies have been shifting towards proteomic analyses of other proximal biological fluids and cell lines (Lin Q. *Biochim Biophys Acta*. 2013 Nov;1834(11):2360-71. doi: 10.1016/j.bbapap.2013.01.030).

Analysis of the secretome *in vivo* would be ideal as it would reflect a true physiological condition. However, those studies are quite complex mainly due to the lack of appropriate collection technology and sample purity. (Chen ST et al. *J Proteome Res* 2008 Apr; 7(4): 1379–1387; Xue H et al. *J Transl Med*. 2008; 6: 52). Nevertheless, some studies have investigated the secretome *in vivo* analyzing biofluids such as saliva (Winck FV et al. *Sci Rep*. 2015 Nov 5;5:16305), interstitial fluid of tissues (Celis JE et al. *Mol Cell Proteomics*.

2004 Apr; 3(4):327-44), nipple aspirate fluid (Varnum SM et al. Breast Cancer Res Treat. 2003 Jul; 80(1):87-97), pleural effusion (Yu CJ et al. J. Proteome. Res., 10 (2011), pp. 4671–4682) and ascites fluid (Choi DS et al. Proteomics, 11 (2011), pp. 2745–2751). These and other fluids such as bile, pancreatic and digestive juices (Farina A. Biochim Biophys Acta. 2014 May;1844(5):988-1002. doi:10.1016/j.bbapap.2013.10.011), are promising sources of biomarkers, since they represent a reservoir of secreted proteins in a less complex matrix when compared to plasma or serum.

Some dynamic methods have been applied for analyzing the secretome *in vivo*, such as microdialysis and ultrafiltration. The microdialysis method relies on the passive diffusion of substances across a semi-permeable membrane driven by a concentration gradient. However, peptide and protein recovery is still challenging. The ultrafiltration technique also uses semi-permeable membranes to separate substances but has some advantages when compared to microdialysis. The use of a capillary ultrafiltration (CUF) probe, which draws fluid applying vacuum membranes with the different pore sizes and surface charges of membrane fibers, greatly facilitates long-term and dynamic sampling collection and does not alter the original concentration of the biofluid (Chen ST et al. J Proteome Res 2008 Apr; 7(4): 1379–1387; Stastna M, Van Eyk JE.. Proteomics. 2012 Feb;12(4-5):722-35).

CONDITIONED MEDIUM

Studies using cell-conditioned medium (CM) have been widely used to identify secreted proteins and cancer biomarkers (Mukherjee P, Mani S. Biochim Biophys Acta. 2013 Nov;1834(11):2226-32; Schaaij-Visser TB et al. Biochim Biophys Acta. 2013 Nov;1834(11):2242-58). Although cancer cell lines do not reconstitute tumor tissue and its microenvironment, the secretome released in the culture medium is less complex than biofluids, and facilitates the identification of low abundance proteins. In addition, it comprises a good option to assess the concentration of the components and provides information about biological processes (Lin Q. Biochim Biophys Acta. 2013 Nov;1834(11):2360-71. doi: 10.1016/j.bbapap.2013.01.030; Stastna M, Van Eyk JE.. Proteomics. 2012 Feb;12(4-5):722-35; Xue H et al. J Transl Med. 2008; 6: 52).

However, the discovery of biomarkers in CM also has its technological challenges. For example, secreted proteins in CM are often masked by high amounts of proteins that enrich the culture medium. Therefore, cells are initially incubated in serum-supplemented medium, followed by a period of incubation in serum-free medium (one or two days), then collected as the CM. Serum deprivation may result in altered metabolism, decreased proliferation and increased cell death, as well as alterations in protein synthesis and secretion patterns (Lin Q. Biochim Biophys Acta. 2013 Nov;1834(11):2360-71. doi: 10.1016/j.bbapap.2013.01.03). Since, these *in vitro* condition modify the protein synthesis

rate and increase the intracellular proteins released by autolysis, isotope-labeled amino acids can be introduced into the culture medium enabling the differentiation between serum proteins and secreted proteins (Colzani M et al. *J. Proteome Res.*, 8 (2009), pp. 4779–4788; Eichelbaum K et al. *Nat Biotechnol.* 2012 Oct;30(10):984-90; Lin Q. *Biochim Biophys Acta.* 2013 Nov;1834(11):2360-71. doi: 10.1016/j.bbapap.2013.01.03). Furthermore, the optimization of incubation time and cellular confluence also appears to minimize contamination with intracellular proteins (Mbeunkui F et al. *J Proteome Res.* 2006 Apr;5(4):899-906).

Despite the challenges presented above, the study of conditioned medium has proven to be useful in identification of a secretory profile in different tumors (Dowling P, Clynes M. *Proteomics.* 2011 Feb;11(4):794-804).

METHODOLOGIES

Microarrays and large scale cDNA sequencing are complementary technologies used to analyze transcripts present in the exosomes (Mantione KJ et al *Med Sci Monit Basic Res.* 2014 Aug 23;20:138-42). Secreted proteins can be identified through liquid chromatography–mass spectrometry and methods using antibodies for targeted capture and detection (Mukherjee P, Mani S. *Biochim Biophys Acta.* 2013 Nov;1834(11):2226-32). Metabolites are identified using mass spectrometry and nuclear magnetic resonance (Patel S, Ahmed S. *J Pharm Biomed Anal.* 2015 Mar 25;107:63-74).

Bioinformatics tools are also relevant for understanding the secretome. As previously mentioned the proteins secreted by the classical pathway contain signal peptides, located at the N-terminus, which may be identified using software such as SignalP. Those proteins secreted by the non-classical pathway are devoid of signal peptide and can be predicted by SecretomeP software through the analysis of specific features in primary structure analysis, such as translational modifications, charges and size. The restrictions of these computational approaches are mainly related to false positives and false negatives (Mantione KJ et al *Med Sci Monit Basic Res.* 2014 Aug 23;20:138-42).

Bioinformatic tools also allow to distinguish between the true secreted proteins from intracellular contaminants. A simple determination of cell location where the products are active using the Gene Ontology project (<http://www.geneontology.org>) can help identifying false positives. Similarly, the Secreted Protein Database (<http://spd.cbi.pku.edu.cn>), ExoCarta (<http://www.exocarta.org>), Plasma Proteome (<http://www.plasmaproteomedatabase.org>) and PeptideAtlas (<http://www.peptideatlas.org/hupo/hppp>) provide important information about the components of the secretome, exosomes, plasma and serum.

CONCLUSION

The secretome is a rich reservoir for cancer biomarkers. Decoding the secretome in the tumor microenvironment and understanding how it enables the exchange of signals between tumor cells and stromal cells. Also, it could help better understanding the biochemical pathways involved in tumorigenesis, from early to advanced stages and identify potential biomarkers for the development of new diagnostic and prognostic tools, therapies and prediction to therapeutic responses.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no conflict of interest.

Figure 1. Tumor microenvironment and its secretome. Cytokines, chemokines, microRNAs and growth factors secreted by neoplastic and stromal cells, including fibroblasts, epithelial, endothelial and inflammatory cells, can exert pro-tumorigenic and anti-tumorigenic effects. In fact, these factors, expressed in both autocrine and/or paracrine manners, may promote cell proliferation, angiogenesis, and epithelial–mesenchymal transition, and consequently tumor progression and invasiveness. Otherwise, they can also activate

immune cells against neoplastic cells, driving the biological behavior and responses of the tumor.

RESEARCH ARTICLE

Inflammation and Cancer: Role of Annexin A1 and FPR2/ALX in Proliferation and Metastasis in Human Laryngeal Squamous Cell Carcinoma

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Abstract

The anti-inflammatory protein annexin A1 (ANXA1) has been associated with cancer progression and metastasis, suggesting its role in regulating tumor cell proliferation. We investigated the mechanism of ANXA1 interaction with formylated peptide receptor 2 (FPR2/ALX) in control, peritumoral and tumor larynx tissue samples from 20 patients, to quantitate the neutrophils and mast cells, and to evaluate the protein expression and co-localization of ANXA1/FPR2 in these inflammatory cells and laryngeal squamous cells by immunocytochemistry. In addition, we performed in vitro experiments to further investigate the functional role of ANXA1/FPR2 in the proliferation and metastasis of Hep-2 cells, a cell line from larynx epidermoid carcinoma, after treatment with ANXA1_{2–26} (annexin A1 N-terminal-derived peptide), Boc2 (antagonist of FPR) and/or dexamethasone. Under these treatments, the level of Hep-2 cell proliferation, pro-inflammatory cytokines, ANXA1/FPR2 co-localization, and the prostaglandin signalling were analyzed using ELISA, immunocytochemistry and real-time PCR. An influx of neutrophils and degranulated mast cells was detected in tumor samples. In these inflammatory cells of peritumoral and tumor samples, ANXA1/FPR2 expression was markedly exacerbated, however, in laryngeal carcinoma cells, this expression was down-regulated. ANXA1_{2–26} treatment reduced the proliferation of the Hep-2 cells, an

effect that was blocked by Boc2, and up-regulated ANXA1/FPR2 expression. ANXA1_{2–26} treatment also reduced the levels of pro-inflammatory cytokines and affected the expression of metalloproteinases and EP receptors, which are involved in the prostaglandin signalling. Overall, this study identified potential roles for the molecular mechanism of the ANXA1/FPR2 interaction in laryngeal cancer, including its relationship with the prostaglandin pathway, providing promising starting points for future research. ANXA1 may contribute to the regulation of tumor growth and metastasis through paracrine mechanisms that are mediated by FPR2/ALX. These data may lead to new biological targets for therapeutic intervention in human laryngeal cancer.

Introduction

Laryngeal cancer is one of the most common types of head and neck tumors that has a high mortality rate and a poor prognosis [1]. More than 12,500 new cases of laryngeal cancer are diagnosed annually and 3,560 annual deaths occur [2]. The development of better treatment as well as better diagnostic and preventive approaches requires an improved understanding of the complex process of laryngeal tumorigenesis.

Only 5% to 10% of all cancers are caused by the inheritance of mutated genes, whereas the remaining 90% to 95% of cases have been linked to genetic and epigenetic alterations caused by lifestyle and environmental factors, such as cigarette smoking and alcohol use [3, 4]. It is now well recognized that inflammation is a risk factor for most types of cancer, including laryngeal carcinomas [5, 6]. Chronic inflammation has been linked to various steps involved in tumorigenesis, including cellular transformation, promotion, proliferation, invasion, angiogenesis, and metastasis [7, 8]. Hanahan and Weinberg, in their recent review [9], recognized inflammation as a new hallmark of cancer that promotes multiple tumor features.

Inflammatory cells secrete numerous cytokines, chemokines and growth factors that can stimulate proliferation, inhibit apoptosis, induce morphogenesis and generate DNA-damaging reactive oxygen species [7], facilitating genomic instability [10]. Furthermore, these cells synthesize vascular endothelial growth factor (VEGF), angiopoietin, metalloproteinases and other proteins that can stimulate vascular endothelial cell mitosis and extracellular matrix remodeling [11]. Therefore, inflammation generates not only changes in the microenvironment to promote cancer but also changes that stimulate neoplastic progression.

It has been demonstrated that mast cells and neutrophils can be recruited by tumor cells. Increased numbers of mast cells have been reported in mammary [12] and lung carcinomas [13], among other tumors. Experimental evidence has indicated that activated mast cells release angiogenic growth factors and regulators, prostaglandin, metalloproteinases and cytokines [14], and may play a

dual role in promoting or inhibiting tumor growth depending on the local stromal conditions [15, 16]. Of the cell types residing in the tumor microenvironment, tumor-associated neutrophils (TANs) have received little attention [17]. Although there is evidence suggesting a role for neutrophils in enhanced disease progression in specific human tumors, the relationship between neutrophil infiltration and prognosis in human cancer has not been systematically investigated [18]. The role of neutrophils in tumor progression remains controversial [19] largely because these cells have both tumor-promoting and tumoricidal functions depending on the presence of transforming growth factor beta (TGF- β) [20, 21].

From these studies, it is clear that various inflammatory mediators are differentially expressed in several cancers and can stimulate disease progression [22]. Thus, agents that suppress these inflammatory mediators represent potential targets for the treatment of cancer. The anti-inflammatory protein annexin 1 (ANXA1), a 37-kDa member of the annexin superfamily, is a steroid-regulated protein that is implicated in mediating some of the beneficial activities of glucocorticoids [23], including the regulation of cell proliferation [24], differentiation [25] and metastasis [26].

Dysregulation of ANXA1 has been reported in multiple neoplasms, suggesting that this protein may play important roles in tumor development and progression [27]. ANXA1 is overexpressed in breast cancer [28] and hepatocellular carcinoma [29] but is markedly down-regulated in esophageal [30, 31] and head and neck carcinomas [32]. However, the molecular mechanisms by which ANXA1 modulates cellular responses have not been fully determined.

Advances in this area have shown a functional link between the anti-inflammatory properties of ANXA1 and receptor for formyl-Met-Leu-Phe (fMLP) FPR [33], a specific class of G protein-coupled 7-transmembrane receptors [34]. In humans, three family members have been identified, FPR1, FPR2/ALX (FPRL1) and FPR3 (FPRL2) [35]. Some of the effects of exogenous ANXA1 can be attenuated by Boc2, an antagonist of both FPR1 and FPR2 [33]. Recently, it has been shown that the ANXA1 N-terminal peptide, ANXA1_{2–26}, promotes the migration of WS1 human skin fibroblasts [36], and the invasiveness of SKCO-15 colorectal adenocarcinoma cells [24] through interaction with FPRs. However, no data are available regarding the role of ANXA1 and FPRs in laryngeal squamous cell carcinoma.

Several signaling pathways mediate inflammation-associated tumorigenesis. For example, cyclooxygenase-2 (COX-2), an enzyme involved in the conversion of arachidonic acid to prostaglandins, is considered to play important roles in cancer development by modulating cell proliferation and other important biological processes via their metabolites, G protein-coupled receptors and downstream effectors [37]. COX-2 is up-regulated in several malignancies [38–40], including head and neck carcinomas [41]. Actually, Abrahao et al. (2010) have shown that the pro-inflammatory COX-2 metabolite prostaglandin E2 and its EP3 receptor can activate head and neck carcinoma cells to proliferate and is a potential target for preventive approaches. Considering that glucocorticoids are highly effective

inhibitors of COX-2 expression [42], the steroid-regulated protein annexin A1, which is implicated in mediating some of the activities of glucocorticoids, may also be involved in the inflammatory network linked to COX-2.

Given the potential role for ANXA1 in multiple neoplasms such as the regulation of cell proliferation, differentiation and metastasis, we focused on the molecular mechanisms by which ANXA1 modulates these cellular responses. In this report, we showed that ANXA1 protein is down-regulated in human laryngeal carcinoma and can regulate tumor growth in a paracrine manner that is mediated by the receptor FPR2/ALX. This investigation demonstrates the potential significance of this ANXA1/FPR2 interaction in tumorigenesis. Overall, this study identified potential roles for the molecular mechanism of this protein interaction in laryngeal cancer, including its relationship with the prostaglandin pathway, providing evidence for the potential of ANXA1 as a therapeutic target and promising starting points for future research.

Materials and Methods

Tissue Samples

Human invasive laryngeal cancer tissues were obtained from 20 patients with laryngeal squamous cell carcinoma who were treated at the Hospital de Base in São José do Rio Preto, Brazil. The patients were male, alcoholic smokers, their age ranged from 50 to 80 years, and none of them had received radiotherapy or chemotherapy before intervention. In each patient (n=20), the surgical tumor samples were collected from the center of the laryngeal tumor, the peritumoral samples were collected from the tumor periphery, and the control samples were collected from the region free of tumor cells. Because of the high heterogeneity of laryngeal carcinoma, the tumor tissues were dissected and reviewed by a senior pathologist and were characterized as moderately differentiated, invasive carcinomas. These tissue samples were used in a previous study that was conducted in our laboratory, and the number of patients was sufficient to obtain statistically significant data [32]. The study protocol was approved by the Committee of Ethics in Research of the Federal University of São Paulo, School of Medicine, Brazil (CEP-0300/08), and written informed consent was obtained from all of the patients involved.

Cell Culture

The Hep-2 cell line, which was originally established from an epidermoid carcinoma of the larynx, was used in the present study (ATCC, Rockville, Maryland, USA). Cell line authentication was performed using the AmpFLSTR Identifier PCR Amplification Kit (Life Technologies) at the Special Techniques Laboratory, Hospital Israelita Albert Einstein (LATE -HIAE), São Paulo. We found 100% identity of our cell line compared with the American Type Culture Collection (ATCC) database. The cells were seeded at a density of 2×10^6 cells in a

75-cm² culture flask (Corning, NY, USA) and incubated in MEM-Earle medium (Cultilab, Campinas, SP, Brazil), pH 7.5, supplemented with 20% fetal calf serum (Cultilab), 1% non-essential amino acids, and 0.1% antibiotic/antimycotic solution (Invitrogen Corporation, Carlsbad, CA, USA), at 37°C in a humid atmosphere of 5% CO₂ [43].

Drug Treatment

For the pharmacological experiments, Hep-2 cells were seeded as previously described. Twenty-four hours later, after the cells had already adhered, they were incubated in serum-free medium to synchronize the cell cycle. After an additional 24 hours, the medium was replaced with complete growth medium containing the following: (a) 1 μM of ANXA1_{2–26} peptide (Ac-AMVSEFLKQAWFIENEEQEYVQTVK) (Invitrogen); (b) 1 μM of ANXA1_{2–26} peptide and 10 μM Boc2, a nonselective FPR antagonist (N-t-BOC-MET-LEU-PHE) (MP Biomedicals, Aurora, OH); (c) 0.01 μM dexamethasone (a glucocorticoid; Sigma-Aldrich, St. Louis, MO, USA); (d) 0.01 μM dexamethasone and 10 μM Boc2; or (e) 10 μM Boc2 alone. The drugs were first dissolved in small amounts of dimethyl sulfoxide (DMSO) and then diluted in medium (the final concentration of DMSO never exceeded 1%). The concentrations of the drugs were selected based on preliminary experiments and literature data [32, 44]. The control cells were treated with complete medium with the same concentration of DMSO used to dilute the drugs. Every 2 days, the medium was replaced with fresh medium containing the drugs at the same dose; the cell morphology was observed every day [43]. After 6, 24, 48, 72 and 96 hours, control and treated cells were harvested and counted using the Countess Automated Cell Counter (Invitrogen).

Fixation, Processing, and Embedding for Light and Electron Microscopy

Laryngeal samples and Hep-2 cells were fixed in 4% paraformaldehyde, 0.5% glutaraldehyde, and 0.1 mol/L sodium cacodylate buffer (pH 7.4) for 24 hours at 4°C, washed in sodium cacodylate, dehydrated through graded percentages of ethanol, and embedded in LRGold (London Resin Co., Reading, UK). For histopathological and morphological analyses, tissue sections (0.5-μm thick) were cut on an ultramicrotome (Reichert Ultracut; Leica, Austria), stained with 1% toluidine blue in 1% borax solution (TAAB Laboratories, Aldermaston, UK) and examined using an AXIOSKOP 2-Mot Plus ZEISS microscope (Carl Zeiss, Jena, Germany). For electron microscopy, sections (~90 nm) were cut on an ultramicrotome and placed on nickel grids for immunogold labeling [45].

Immunogold Labeling of Post-embedded tissues

Immunocytochemistry reaction (double labeling) was used to detect the expression and co-localization of ANXA1 and FPR2/ALX in mast cells, neutrophils, and control and tumor cells from laryngeal tissues and Hep-2 cells.

The Hep-2 cells were incubated with control medium, ANXA1_{2–26} and ANXA1_{2–26}+Boc2. After 48 hours, the culture was harvested, and the cells were embedded in LRGold resin for immunocytochemical analysis. Ultrathin sections of laryngeal tissues and Hep-2 cells were incubated in a step-by-step manner using the following reagents and/or conditions at room temperature: 1) distilled water; 2) 0.1 mol/L phosphate buffer containing 1% egg albumin (PBEA); 3) 0.1 mol/L PBS containing 5% PBEA for 30 minutes; 4) sheep polyclonal antibody LCPS1, raised against the N-terminal segment of ANXA1 (1:500 in PBEA) and rabbit polyclonal antibody FPR2/ALX (Abcam, Cambridge, UK) (1:500 in PBEA) for 2 hours; normal sheep and rabbit sera were used as the control (1:500); 5) three washes in PBEA containing 0.01% Tween 20; 6), donkey anti-sheep IgG (Fc fragment-specific) antibody (1:100 in PBEA) conjugated to 15-nm colloidal gold (British Biocell, UK) was added to detect ANXA1, and goat anti-rabbit IgG (Fc fragment-specific) antibody (1:100 in PBEA) conjugated to 10-nm colloidal gold (British Biocell) was added to detect FPR2/ALX. After 1 hour, the sections were washed in PBEA containing 0.01% Tween 20, and then in distilled water [46]. Sections were stained with uranyl acetate and lead citrate before examination on a ZEISS LEO 906 electron microscope (Carl Zeiss, Jena, Germany).

Multiplex Assays

To quantify the pro-inflammatory cytokines interleukins 6 (IL-6) and IL-8 as well as monocyte chemoattractant protein-1 (MCP-1), in the Hep-2 cell culture supernatants, we used the multiplex instrument LUMINEX xMAP MAGPIX (Millipore Corporation, Billerica, MA, USA). The cells were incubated with control medium, ANXA1_{2–26}, ANXA1_{2–26}+Boc2, Dexamethasone, Dexamethasone+Boc2 or Boc2. After 48 hours of treatment, the culture supernatants were removed, centrifuged and stored at -20°C . Antibody beads, controls, wash buffer, serum matrix and standards were prepared following the manufacturer's instructions (MILLIPLEX HCYTOMAG-60K kit). Next, 200 μL of wash buffer was added to each well of a magnetic 96-well plate and mixed on a shaker for 10 min. The wash buffer was decanted, and 25 μL of standards, controls and samples were added to the wells. Next, 25 μL of assay buffer was added to the samples, and 25 μL of serum matrix was added to the standards. Finally, 25 μL of magnetic beads (coated with a specific capture antibody) was added to all wells and incubated overnight at 4°C on a shaker. The next day, the plate was washed with wash buffer and incubated with 25 μL of detection antibodies for 1 hour on a shaker. Next, 25 μL of streptavidin-phycoerythrin was added to each well and incubated for 30 minutes on a shaker. The plate was washed and incubated with 150 μL of drive fluid for 5 minutes on a shaker. Finally, the plate was analyzed using MAGPIX with xPONENT software [47].

Real-time PCR

The Hep-2 cells were incubated with control medium, ANXA1_{2–26} or ANXA1_{2–26}+Boc2 and harvested after 48 hours. Total RNA was extracted using TRIzol Reagent (Invitrogen) according to the manufacturer's protocol. The genomic DNA was removed by DNase treatment according to the manufacturer's description (Promega). Aliquots (2 µg) of total RNA from control and treated cells were used for double-stranded cDNA synthesis using a High Capacity cDNA Archive kit (Applied Biosystems) according to the manufacturer's instructions. Four differentially expressed genes (*EP3* or *PTGER3*, *EP4* or *PTGER4*, *MMP2* and *MMP9*) were selected for quantitative real-time PCR experiments according to their direct or indirect involvement in inflammatory and metastatic processes based on the Gene Ontology database (<http://www.geneontology.org/>). Real-time PCR was performed in triplicate using a 7500 Fast Real-Time PCR System (Applied Biosystems). The reaction mixture consisted of a 20-µl total volume solution containing 10 µl of Power SYBR Green PCR Master Mix (Applied Biosystems), a 1 µM solution of each primer and 20 ng of cDNA. The PCR conditions were 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Melting curve analysis was conducted for each gene to check the specificity and identity of the RT-PCR products. For each primer set, the real-time PCR efficiencies (E) were measured in triplicate on serial dilutions of the same cDNA sample using the formula $E = [10^{(-1/\text{slope})}]$. The resulting values ranged from 1.96 to 2.02. Each transcript level was normalized to the internal standard *GAPDH*, and the values were Log2-transformed (y-axis) so that all of the values below -1 indicated down-regulation in gene expression, while values above 1 represented up-regulation compared with control cells [43].

Statistical Analysis

The values concerning the quantification of mast cells and neutrophils of the in vivo tissue samples were expressed as the mean $\pm$ SEM of the number of cells per mm² in three sections of 0.5 µm (leaving a space of 40 µm between each section) for each patient (n=20 patients).

Hep-2 cells were counted using the Countess Automated Cell Counter (Invitrogen). The concentration of the cytokines was determined using MAGPIX Xponent software (Millipore Corporation). The in vitro analyses were repeated a minimum of three times.

The ultrastructural and immunocytochemistry expression of ANXA1 and FPR2 was determined using ten cells for each group investigated. The area of each cell compartment was determined using Axiovision imaging software (Zeiss). In the nucleus and cytoplasm, the density of colloidal gold particles was calculated as the mean $\pm$ SEM of the number of particles per µm². In the plasma membrane, the density of colloidal gold particles was calculated as the mean $\pm$ SEM of the number of particles per µm.

The significant differences between the means were determined using analysis of variance. This test was followed by the Bonferroni post-hoc test on select

experimental groups using Graph-Pad Prism 4.0 (Graph-Pad, San Diego, CA, USA). A probability value less than 0.05 was considered to be statistically significant.

Results

The Presence of Degranulated Mast Cells and an Influx of Neutrophils Indicates Inflammation in the Tumor Microenvironment

To verify the interaction of inflammatory cells with laryngeal tumor cells, we performed histological analyses in larynx tissue samples to quantify mast cells and neutrophils, important cells of the inflammatory response. In control samples, we observed intact mast cells with metachromatic granules in close vicinity to blood vessels (Fig. 1A). In the peritumoral and tumor stroma, we verified the presence of degranulated mast cells (Fig. 1, B and C). In addition, we observed neutrophils that had transmigrated into tumor sections (Fig. 1D).

Statistical analysis showed that there were high numbers of mast cells in the control sections of the larynx and significantly fewer mast cells in the peritumoral and tumor regions ($P < 0.001$; Fig. 1E). Furthermore, intact mast cells were more abundant in control samples and significantly lower in peritumoral and tumor sections ($P < 0.001$; Fig. 1F). Using quantitative analysis of the neutrophils, we verified that there was a significant increase in these cells in the tumor tissues ($P < 0.001$) compared with control and peritumoral tissues (Fig. 1G). Examination of the neutrophils in control and peritumoral sections showed that most of these cells were localized in the blood vessels (Fig. 1H). By contrast, in the tumor samples, we observed a significant number of transmigrated neutrophils in the stroma ($P < 0.001$ compared with control and peritumoral samples) (Fig. 1H).

Up-Regulation of ANXA1/FPR2 in the Inflammatory Cells of Human Laryngeal Tumors

Ultrastructural immunocytochemistry showed for the first time the expression of FPR2/ALX and its co-localization with ANXA1 in mast cells (Fig. 2, A–C). The mast cells and neutrophils displayed ANXA1 and FPR2/ALX immunoreactivity in the plasma membrane, cytoplasm and nucleus (Fig. 2, A–F). No immunogold labeling was detected in sections that were incubated with nonimmune serum (data not shown). In these inflammatory cells, we verified a significant up-regulation of ANXA1 and FPR2/ALX expression in the peritumoral and tumor samples compared with the corresponding control tissues (Fig. 2, G–I).

Quantitative analysis revealed that there is higher expression of ANXA1 and its receptor in the cytoplasm and nucleus compared with that in the plasma membrane (Fig. 2, G–I). Co-localization of ANXA1 and FPR2/ALX was observed in the plasma membrane, cytoplasm and nucleus of mast cells and neutrophils (Fig. 2, G–I); however there was significant difference only in plasma membrane of peritumoral neutrophils (Fig. 2J).

Fig. 1. Influx of inflammatory cells in tumor samples from human laryngeal. (A–D) Representative images (original magnification, $\times 63$) of Toluidine Blue-stained tissue sections from laryngeal samples. (A) Control laryngeal sample showing intact mast cells (curve arrows) near the blood vessels. Peritumoral (B) and tumor (C) samples with degranulated mast cells (open curve arrows). (D) Tumor cells with mitotic activity (open arrow) and inflammatory cells, as extravascular neutrophils (arrows). (E) Total number of mast cells. (F) Intact and degranulated mast cells. (G) Total number of neutrophils. (H) Extra and intravascular neutrophils. Data are expressed as the mean $\pm$ SEM of the cell number per mm² ($n=20$ patients per group). One-way ANOVA followed by Bonferroni's test revealed a significant difference among the groups. *** $P<0.001$ vs. control, ### $P<0.001$ vs. peritumoral. Scale bars: 5 μ m.

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Fig. 2. Immunoreactivity to ANXA1 and FPR2/ALX in mast cells and neutrophils of laryngeal sections. Immunolabeling with 10-nm (FPR2/ALX) and 15-nm (ANXA1) colloidal gold particles. Mast cells (A–C) and neutrophils (D–F) showing subcellular localization of ANXA1 (arrows) and FPR2/ALX (arrowheads), and co-localization (curve arrows) in the plasma membrane, cytoplasm and nucleus (N) of control, peritumoral and tumor tissues. Density of

gold particles conjugated with ANXA1 and FPR2/ALX in mast cells (G–I) and neutrophils (J–L). Data are expressed as the mean $\pm$ SEM of colloidal gold particles per μm in the plasma membrane and per μm^2 in the cytoplasm and nucleus of cells ($n=10$ cells per group). One-way ANOVA followed by Bonferroni's test revealed a significant difference among the groups. * $P<0.05$, ** $P<0.01$, *** $P<0.001$ vs. control, ## $P<0.01$, ### $P<0.001$ vs. peritumoral. Stained with uranyl acetate and lead citrate. Scale bars: 1 μm .

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ANXA1_{2–26} Inhibits FPR2/ALX Receptor-Mediated Proliferation of Hep-2 Laryngeal Cancer Cells

Approximately 87% to 97% of the Hep-2 cells were viable under the experimental conditions (control, ANXA1_{2–26}, ANXA1_{2–26}+Boc2, Dexa, Dexa+Boc2 and Boc2) (S1 Figure). However, the growth curve of these cells showed significant differences among the conditions studied (Fig. 3).

Treatment with the peptide ANXA1_{2–26} significantly reduced the proliferation of Hep-2 laryngeal cancer cells compared with the control cells ($P<0.001$ at 48 and 96 hours, and $P<0.01$ at 72 hours). Similar results were obtained after treatment with Dexa and Dexa+Boc2 (S2 Figure). However, combination treatment with ANXA1_{2–26} and Boc2 (ANXA1_{2–26}+Boc) significantly augmented cell growth compared with ANXA1_{2–26}-treated cells ($P<0.01$ at 48 and 72 hours, and $P<0.001$ at 96 hours), demonstrating that Boc2 attenuated the antiproliferative activity of ANXA1_{2–26}. Boc2-treated cells had a proliferative rate that was similar to that of control cells (Fig. 3).

Loss of ANXA1/FPR2 Protein in Laryngeal Tumor Tissues and Up-regulation after *In Vitro* Treatment with the ANXA1_{2–26} Peptide

The epithelial cells of control, peritumoral and tumor laryngeal tissues displayed immunoreactivity to ANXA1 and FPR2/ALX, as well as co-localizations of the proteins, in the plasma membrane, cytoplasm and nucleus (Fig. 4, A–C). In the peritumoral and tumor samples, we observed a marked reduction of ANXA1 and FPR2/ALX protein expression compared with the corresponding control tissues (Fig. 4, H and I). Quantitative analysis revealed that there was higher expression of ANXA1 and its receptor in the cytoplasm and nucleus and lower expression in the plasma membrane (Fig. 4, G–I). Differences in the degree of ANXA1/FPR2 co-localization did not reach statistical significance among the laryngeal samples (Fig. 4, G–I).

Hep-2 cells that were treated with ANXA1_{2–26} or ANXA1_{2–26} plus Boc2 showed immunoreactivity for ANXA1 and FPR2/ALX (Fig. 4, D and E). Co-localization of the two proteins was detected in the plasma membrane, cytoplasm and nucleus of these cells but not in Boc2-treated cells (Fig. 4, D and E). No gold labeling was detected in cells that were incubated with nonimmune serum (Fig. 4 F). ANXA1/FPR2 expression was down-regulated in the plasma membrane but was increased in the cytoplasm and nucleus of Hep-2 cells (Fig. 4, J–L). Treatment with ANXA1_{2–26} displayed up-regulation of ANXA1 and FPR2 in the cytoplasm and nucleus compared with the corresponding control cells (Fig. 4, J–L). Furthermore, we detected marked reduction of ANXA1 protein and its receptor after

Fig. 3. Effect of the peptide ANXA1₂₋₂₆ and antagonist Boc2 on the proliferation of Hep-2 cells. Treatment with ANXA1₂₋₂₆ reduced the cellular growth. The antagonist Boc2 partially inhibited the anti-proliferative effect of ANXA1₂₋₂₆. The cells treated with Boc2 alone showed a level of proliferation similar to that of the control. The Hep-2 cells were seeded in MEM-Earle medium at a density of 2×10^6 cells in 75-cm² culture flask, and then were incubated with serum-free medium 24 hours prior to the addition of ANXA1₂₋₂₆ (1 μ M), ANXA1₂₋₂₆ (1 μ M)+Boc (10 μ M) or Boc (10 μ M) alone. All of the experiments were performed in triplicate to confirm the results. Data are expressed as the mean $\pm$ SEM of the cell number $\times 10^6$. ** $P < 0.01$ and *** $P < 0.001$ vs. control, ## $P < 0.01$ and ### $P < 0.001$ vs. ANXA1₂₋₂₆+Boc.

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ANXA1₂₋₂₆+Boc2 treatment compared with ANXA1₂₋₂₆-treated cells (Fig. 4, J–L). Treatment with ANXA1₂₋₂₆ significantly augmented the degree of ANXA1/FPR2 co-localization in the plasma membrane of Hep-2 cells (Fig. 4 J).

Decreased Pro-inflammatory Cytokine Levels Characterizes the Anti-inflammatory Effect of ANXA1 on Laryngeal Cancer Cells

The expression pattern of IL-6, IL-8 and MCP-1 was similar among the experimental conditions (Fig. 5, A–C). The Hep-2 cells that were incubated with ANXA1₂₋₂₆, ANXA1₂₋₂₆+Boc2, Dexa and Dexa+Boc2 showed a marked reduction in cytokine levels. However, the Boc2-treated cells showed a high level of cytokine expression that was similar to that observed under control conditions (Fig. 5, A–C).

EP3- and EP4-Mediated Metalloproteinase Signaling Can Be Regulated by ANXA1₂₋₂₆ in Tumor Cells

To investigate the possible effect of ANXA1 on the expression of genes that are involved in the prostaglandin pathway and, therefore, are related to tumorigenesis and inflammation, we carried out real time PCR in Hep-2 cells that were incubated with ANXA1₂₋₂₆ and Boc2. Prior to RT-PCR, we evaluated the quality of mRNA and cDNA obtained from the samples (S3 Figure, A and B).

Fig. 4. Ultrastructural immunocytochemistry detection of ANXA1 and FPR2/ALX co-localization in laryngeal cells by electron microscopy. Immunolabeling with 10-nm (FPR2/ALX) and 15-nm (ANXA1) colloidal gold particles. (A–C) Epithelial control, peritumoral and tumor cells from laryngeal tissues. (D–E) Hep-2 cells showing immunoreactivity to ANXA1 (arrows), FPR2 (arrowheads), and co-localizations (curve arrows) in the plasma membrane, cytoplasm and nucleus (N). Desmosomes (open curve arrows) are detected between these cells. (F) The absence of immunoreactivity to ANXA1 and FPR2/ALX in cells incubated with nonimmune serum. Density of gold particles conjugated with ANXA1 and FPR2/ALX in epithelial cells (G–I) and Hep-2 cells (J–L). Hep-2 cells were seeded in MEM-Earle medium at a density of 2×10^6 cells in 75-cm² culture flasks, and then were incubated with serum-free medium 24 hours prior to the addition of ANXA1₂₋₂₆ (1 μ M) and ANXA1₂₋₂₆ (1 μ M)+Boc2 (10 μ M). Data are expressed as the mean $\pm$ SEM of colloidal gold

particles per μm in the plasma membrane and per μm^2 in the cytoplasm and nucleus of cells ($n=10$ cells per group). One-way ANOVA followed by Bonferroni's test revealed a significant difference among the groups. * $P<0.05$, ** $P<0.01$, *** $P<0.001$ vs. control, # $P<0.05$, ## $P<0.01$, ### $P<0.001$ vs. ANXA1₂₋₂₆. Stained with uranyl acetate and lead citrate. Scale bars: 1 μm .

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Hep-2 cells that were treated with ANXA1₂₋₂₆ showed a significant decrease ($\text{Log}_2 \leq -1.0$) in *MMP2* expression and an increase ($\text{Log}_2 \geq 1.0$) in *MMP9* expression compared with control cells (Fig. 6). However, incubation of Hep-2 cells with ANXA1₂₋₂₆ plus Boc mediated up-regulation ($\text{Log}_2 \geq 1.0$) of *EP4*, *MMP2* and *MMP9* gene expression and down-regulation ($\text{Log}_2 \leq -1.0$) of *EP3* expression compared with control cells (Fig. 6).

Discussion

Although advances in new diagnostic tools and treatments have led to declining mortality rates, cancer remains a leading cause of death in industrialized countries. A better understanding of the interactions between tumors and inflammation may lead to new biological targets for therapeutic intervention in

Fig. 5. Effect of the peptide ANXA1₂₋₂₆ on proinflammatory cytokine expression. Low expression of IL-6 (A), IL-8 (B) and MCP-1 (C) after treatment with ANXA1₂₋₂₆ and dexamethasone. Hep-2 cells were seeded in MEM-Earle medium at a density of 2×10^5 cells in 75-cm² culture flasks, and then were incubated with serum-free medium 24 hours prior to the addition of ANXA1₂₋₂₆ (1 μM), ANXA1₂₋₂₆ (1 μM)+Boc2 (10 μM), Dexa (0.01 μM), Dexa (0.01 μM)+Boc2 (10 μM) or Boc2 (10 μM) alone. All of the experiments were performed in triplicate to confirm the results. Data are expressed as the mean $\pm$ SEM of the analyte concentration (pg/mL), determined using MAGPIX xPONENT software. * $P<0.05$, ** $P<0.01$ and *** $P<0.001$ vs. control, ## $P<0.01$ and ### $P<0.001$ vs. ANXA1₂₋₂₆, §§ $P<0.01$ and §§§ $P<0.001$ vs. ANXA1₂₋₂₆+Boc2.

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Fig. 6. Gene expression in Hep-2 cells using real-time PCR. (A) Down- and up-regulated expression of *MMP2* and *MMP9*, respectively, after ANXA1₂₋₂₆ treatment. (B) Overexpression of *EP4*, *MMP2* and *MMP9*, and down-regulation of *EP3* after ANXA1₂₋₂₆+Boc. Hep-2 cells were seeded in MEM-Earle medium at a density of 2×10^5 cells in 75-cm² culture flasks, and then were incubated with serum-free medium, 24 hours prior to the addition of ANXA1₂₋₂₆ (1 μ M) and ANXA1₂₋₂₆ (1 μ M)+Boc2 (10 μ M). All of the experiments are representative of three separate experiments. *GAPDH* was used as the internal reference and the control sample was used as the calibrator. Values were Log2 transformed (y-axis) so that all values below -1 indicated down-regulation in gene expression while values above 1 represented up-regulation compared with control cells.

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different neoplasms within the context of chronic inflammation, such as laryngeal carcinoma. We showed that ANXA1 protein may control tumor growth in a paracrine manner that is mediated by the receptor FPR2/ALX. In the inflammatory cells of human laryngeal carcinoma tissue samples, ANXA1/FPR2 expression was markedly exacerbated; however, in laryngeal carcinoma cells, this expression was down-regulated. Treatment with ANXA1₂₋₂₆ reduced the proliferation of Hep-2 cells and up-regulated ANXA1/FPR2 expression in these cells. Our results established, for the first time, the role of FPR2/ALX in laryngeal carcinoma cells as demonstrated by ultrastructural analyses showing the co-localization of ANXA1/FPR2.

The inflammatory components in a developing neoplasm may include several types of immune cells, including mast cells and neutrophils, which can produce an array of cytokines, such as the cell-killing mediators TNF- α and interleukins (ILs) [48, 49]. The presence of degranulated mast cells in the peritumoral and tumor laryngeal tissues suggests that neoplastic cells may recruit and activate mast cells to release mediators that could either be detrimental to the tumor or contribute to its development [50]. Intriguingly, most reports show the localization of mast cells predominantly at the tumor periphery [51] and associated with the vasculature surrounding malignant tissues, suggesting that these cells play a pro-angiogenic role [52]. The scarcity of mast cells within the tumor core may be a histology artifact that results from mast cell degranulation, leading to “ghosts” following staining [53]. Our data indicate that mast cells may represent a possible target for therapeutic intervention in controlling tumor growth and angiogenesis in laryngeal carcinoma.

Mast cells may be effective in inducing neutrophil migration to inflammatory sites [54], numerous transmigrated neutrophils were observed in the larynx tumor samples. Despite their known function as phagocytes, the role of tumor-associated neutrophils in neoplastic progression has been controversial [19, 55]. Neutrophils can induce DNA damage in proliferating cells through the generation of reactive oxygen and nitrogen species, which are normally produced by these cells to fight infection [55]. However, neutrophils can have both tumor-promoting and tumoricidal functions, depending on the presence of TGF- β [20]. The recognition of neutrophils as a key component of tumor growth opens an avenue for novel approaches to cancer therapies to decrease laryngeal carcinoma cell proliferation and metastasis in patients.

It is now evident that inflammatory cells have powerful effects on tumor development. Early in the neoplastic process, these cells are malignant promoters, producing an attractive environment for tumor growth, facilitating genomic instability and promoting angiogenesis [21]. Inflammatory cells can regulate the growth, migration and differentiation of all cell types in the tumor microenvironment [49]. Thus, it is clear that anti-inflammatory therapy is efficacious toward preventing early neoplastic progression.

In this anti-inflammatory and anti-proliferative study, we have unveiled whether ANXA1 protein could be mediated by receptors for formylated peptides (FPRs) in larynx cancer. The functions of FPRs in cancer are still not well established.

We analyzed the role of ANXA1/FPR2 interactions in the proliferation of Hep-2 cells after treatment with ANXA1_{2–26}, dexamethasone and/or Boc2. The addition of ANXA1_{2–26} and Dexa to the cell culture reduced cell growth, showing the antiproliferative effects of these anti-inflammatory drugs. However, the pharmacological antagonist Boc2 attenuated the antiproliferative activity of ANXA1, suggesting that this receptor plays central roles in the proliferation response in larynx cancer. These data corroborate the results of a recent study [56], which showed that the effects of ANXA1 on breast tumor cell proliferation were prevented by Boc2.

To uncover the subcellular localization and possible interaction of ANXA1/FPR2, we performed immunocytochemistry reactions in the inflammatory and epithelial cells of laryngeal tissue samples and in Hep-2 cells. Co-localization of ANXA1/FPR2 was detected in the mast cells, neutrophils, and tumor cells. These data suggest that the anti-inflammatory/antiproliferative activity of ANXA1 may be mediated by FPR2/ALX and show, for the first time, the presence of the receptor FPR2/ALX and its interaction with ANXA1 in mast cells and laryngeal tumor cells. Recently, we also detected the co-localization of ANXA1/Fpr2 in murine neutrophils, suggesting that the anti-inflammatory ANXA1 effects may be mediated through this receptor [57].

The mast cells and neutrophils in the peritumoral and tumor sections revealed high expression of ANXA1/FPR2. The tumor-activated inflammatory cells appear to increase the synthesis of ANXA1/FPR2 proteins as an anti-inflammatory response mechanism to resolve the inflammation and proliferation in laryngeal

cancer. The cellular differentiation and, in some cases, activation are stimuli for the synthesis of ANXA1, although the molecular processes are not yet fully understood [58].

Loss of ANXA1 and FPR2/ALX expression was detected in laryngeal carcinoma cells. However, Hep-2 cells had up-regulated expression of ANXA1, FPR2/ALX and co-localization after treatment with ANXA1₂₋₂₆ peptide. These results could signify the regulatory role of the ANXA1/FPR2 interaction in cancer development and, corroborate the previous investigation performed in our laboratory [32] demonstrating that ANXA1₂₋₂₆-treated Hep-2 cells exhibited increased ANXA1 expression as shown by real time PCR. In vitro treatment with ANXA1₂₋₂₆+Boc2 markedly reduced the expression of ANXA1/FPR2 in Hep-2 cells. This result confirms that the antagonist Boc2 blocks the antiproliferative FPR2-mediated activity of ANXA1. Overexpression of ANXA1 protein is observed in hepatocellular carcinoma [29] and pancreatic cancer [59]. By contrast, reduced levels of ANXA1 protein expression have been reported in esophageal cancer [30] and laryngeal cancer [32]. The discrepancy in these studies indicates that the effects of ANXA1 on cancer cells may be cell-type specific and that factors other than the expression level may affect its function.

The electron microscopy data demonstrated that ANXA1/FPR2 expression was high in the cytoplasm and nucleus and low in the plasma membrane. ANXA1, which is usually located in the cytoplasm, can translocate to the nucleus after stimulation. Recently, Lin and colleagues [60] analyzed 115 patients with oral carcinoma and observed high expression of ANXA1 in the nucleus of epithelial tumor cells that correlated with poor prognosis. Although these authors [60] have shown that ANXA1 translocation to the nucleus correlates with the regulation of epithelial cell proliferation and may represent a candidate prognostic marker [43], the precise physiological role of ANXA1 in the nucleus remains poorly understood.

Because of the importance of cytokine control of the inflammatory microenvironment favoring or inhibiting tumor progression [48, 61], we investigated the level of IL-6, IL-8 and MCP-1 released by Hep-2 cells. IL-6 is produced by tumor and stromal cells and contributes to tumorigenesis by various mechanisms, including angiogenesis, cell survival and metastasis [62]. IL-6 signaling leads to the increased production of inflammatory cytokines, proteases and growth factors, such as IL-1 β , IL-8, MCP-1, MMP9, MMP2 and VEGF [61, 63]. Our data showed that ANXA1 and dexamethasone inhibit the release of inflammatory cytokines by Hep-2 cells, which may prevent the development of laryngeal squamous cell carcinoma.

Additionally, we have begun to outline the anti-inflammatory pathway of ANXA1 in Hep-2 cells which may result in the inhibition of angiogenic and invasive phenotypes. Thus, we investigated the effect of ANXA1 on the expression of genes that are involved in prostaglandin signaling and whose role in head and neck cancer deserves evaluation [41]: *EP3* and *EP4* and their downstream effectors *MMP2* and *MMP9*. Prostaglandins are lipid mediators of inflammation released in response to different stimuli, including cigarette smoking [64]. They are

synthesized from arachidonic acid by cyclooxygenase (COX) in many cells, such as mast cells, and exert their signaling effects through G-protein-coupled receptors (reviewed by Aoki and Narumiya) [65].

Prostaglandin E2 is the most important COX metabolite present in human tumors and that bind to its receptors (EP1–4) on the plasma membrane of neoplastic, stromal or immune cells. The EP receptors may have distinct functions depending on the cell and tissue type [66]. For example, EP4 is linked to cAMP/PKA and PI3K/Akt pathways whereas the multiple isoforms of EP3 may negatively or positively regulate cAMP [67]. They can transactivate the epidermal growth factor receptor (EGFR), a biomarker that is virtually upregulated in all head and neck tumors, inducing the expression of several proteins, including COX 2, matrix metalloproteinases 2 and 9 and vascular endothelial growth factor [68]. Otherwise, EP signaling may stimulate EGFR ligand release via matrix metalloproteinases [69]. These molecular signaling cascades promote tumor cell proliferation, angiogenesis and extracellular matrix degradation and may be targets for the treatment and prevention of cancer. Indeed, several epidemiological studies have shown an inverse correlation between cancer risk and non-steroidal anti-inflammatory drugs, which are potent inhibitors of cyclooxygenase (COX) [68]. Treatment of Hep-2 cells with ANXA1_{2–26} reduced *MMP2* expression and increased *MMP9* expression. Unexpectedly, up-regulation of *EP4*, *MMP2* and *MMP9* and down-regulation of *EP3* expression were observed after treatment with ANXA1_{2–26} and Boc2. The latter findings show that ANXA1 may affect the expression of MMPs and EP receptors through its interaction with the receptor FPR2/ALX. Therefore, may interact with different receptors, generating signals that are interconnected with the prostaglandin pathway. Clearly, further investigation is needed.

Overall, our *in vivo* data indicated that mast cells and neutrophils, under activation by the tumor microenvironment, demonstrate up-regulation of ANXA1/FPR2 as an anti-inflammatory response mechanism to resolve inflammation and proliferation in laryngeal cancer. However, *in vitro* results have indicated that treatment with the peptide mimetic ANXA1_{2–26} promotes the reduction of Hep-2 cell proliferation, up-regulation of ANXA1/FPR2 expression, down-regulation of inflammatory cytokines (IL-6, IL-8 and MCP-1) and *MMP2*.

Conclusions

Integration of the *in vivo* and *in vitro* data showed that ANXA1 may be effective in the regulation of tumor growth and metastasis through paracrine mechanisms mediated by FPR2/ALX. Detailed studies of mast cell and neutrophil function in different types of cancer should aid the development of effective antitumor strategies. In light of the potential importance ascribed to ANXA1, our study not only has generated novel and important information about ANXA1 mechanisms but also may justify further investigation of the roles of the complex FPR receptor family. In addition, a combination of anti-inflammatory approaches that target

the tumor microenvironment with more sophisticated and selective tumoricidal drugs may lead to future therapeutic intervention in human laryngeal cancer.

Supporting Information

S1 Figure. Analysis of Hep-2 cell viability. Treatment with ANXA1₂₋₂₆, ANXA1₂₋₂₆+Boc2, Dexamethasone (Dexa), Dexa+Boc2 or Boc2 did not affect the percentage of cellular viability during growth curve analysis. The Hep-2 cells were seeded in MEM-Earle medium at a density of 2×10^6 cells in 75-cm² culture flasks, and then were incubated with serum-free medium, 24 hours prior to the addition of ANXA1₂₋₂₆ (1 μ M), ANXA1₂₋₂₆ (1 μ M)+Boc2 (10 μ M), Dexa (0.01 μ M), Dexa (0.01 μ M)+Boc2 (10 μ M) or Boc2 (10 μ M) alone. All experiments were performed in triplicate to confirm the results. Data are expressed as the mean $\pm$ SEM of the cell percentage number.

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S2 Figure. Effect of dexamethasone on the proliferation of Hep-2 cells. Treatment with dexamethasone (Dexa) reduced the cellular growth. The antagonist Boc2 had no effect on Dexa. Hep-2 cells were seeded in MEM-Earle medium at a density of 2×10^6 cells in 75-cm² culture flasks, and then were incubated with serum-free medium, 24 hours prior to the addition of Dexa (0.01 μ M) and Dexa (0.01 μ M)+Boc2 (10 μ M). All of the experiments were performed in triplicate to confirm the results. Data are expressed as the mean $\pm$ SEM of the cell number $\times 10^6$. ** $P < 0.01$, *** $P < 0.001$ vs. control.

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S3 Figure. Validation of mRNA and cDNA integrity. Agarose gels showing the quality of mRNA (A) and cDNA (B) from Hep-2 cells after treatment. Hep-2 cells were seeded in MEM-Earle medium at a density of 2×10^6 cells in 75-cm² culture flasks, and then were incubated with serum-free medium, 24 hours prior to the addition of ANXA1₂₋₂₆ (1 μ M) and ANXA1₂₋₂₆ (1 μ M)+Boc2 (10 μ M). All of the experiments were performed in triplicate to confirm the results.

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Author Contributions

Conceived and designed the experiments: SMO EHT. Performed the experiments: TSG BRC. Analyzed the data: TSG SMO EHT FCRL. Contributed reagents/materials/analysis tools: SMO EHT. Wrote the paper: TSG SMO EHT. Collected the surgical samples: LSR JVM. Performed the pathological analyses: PMC.

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Research paper

ANXA1_{Ac2-26} peptide reduces *ID1* expression in cervical carcinoma cultures Janesly Prates^a, Gabriela Bueno Franco-Salla^a, Anemari Ramos Dinarte dos Santos^b, Wilson Araújo da Silva Jr.^b, Bianca Rodrigues da Cunha^c, Eloiza Helena Tajara^c, Sonia Maria Oliani^a, Flávia Cristina Rodrigues-Lisoni^{d,*}^a Department of Biology, Institute of Biosciences, Letters and Science – IBILCE/UNESP, São José do Rio Preto, SP, Brazil^b Department of Clinical Medical, Foundation Blood Center of Ribeirão Preto, Faculty of Medicine of Ribeirão Preto, University of São Paulo – FCFRP/USP, Ribeirão Preto, SP, Brazil^c Department of Molecular, Biology Faculty of Medicine of São José do Rio Preto – FAMERP, São José do Rio Preto, SP, Brazil^d Department of Biology and Animal Science, Faculty of Engineering of Ilha Solteira – FEIS/UNESP, Ilha Solteira, SP, Brazil

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ABSTRACT

Cervical cancer is the second most frequent cancer in women worldwide and is associated with genetic alterations, infection with human papilloma virus (HPV), angiogenesis and inflammatory processes. The idea that inflammation is involved in tumorigenesis is supported by the frequent appearance of cancer in areas of chronic inflammation. On the other hand, the inflammatory response is controlled by the action of anti-inflammatory mediators, among these mediators, annexin A1 (ANXA1), a 37 kDa protein was detected as a modulator of inflammatory processes and is expressed by tumor cells.

The study was carried out on the epithelial cancer cell line (SiHa) treated with the peptide of annexin A1 (ANXA1_{Ac2-26}). We combined subtraction hybridization approach, Ingenuity Systems software and quantitative PCR, in order to evaluate gene expression influenced by ANXA1.

We observed that ANXA1_{Ac2-26} inhibited proliferation in SiHa cells after 72 h. In these cells, 55 genes exhibited changes in expression levels in response to peptide treatment. Six genes were selected and the expression results of 5 up-regulated genes (*TPT1*, *LDHA*, *NCOA3*, *HIF1A*, *RAB13*) and one down-regulated gene (*ID1*) were research by real time quantitative PCR. Four more genes (*BMP4*, *BMPR1B*, *SMAD1* and *SMAD4*) of the *ID1* pathway were investigated and only one (*BMPR1B*) shows the same down regulation.

The data indicate the involvement of ANXA1_{Ac2-26} in the altered expression of genes involved in tumorigenic processes, which could potentially be applied as a therapeutic indicator of cervical cancer.

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Abbreviations: [LaCl₂]₃, lanthanum citrate complex; µg, microgram; ACTB, beta-actin; ANX, annexin; ANXA1, annexin A1; BMP4, bone morphogenetic protein 4; BMPR1B, bone morphogenetic protein receptor, type 1B; cDNA, complementary DNA; CO₂, carbon dioxide; DNA, deoxyribonucleic acid; dNTP, deoxynucleotide-solution mix; DTT, DL-Dithiothreitol; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HCl, hydrochloric acid; HIF1A, hypoxia inducible factor 1, alpha subunit; HPV, human papilloma virus; ID1, DNA-binding protein inhibitor; kDa, kilodalton; LDHA, lactate dehydrogenase A; Mbol, enzyme gene from *Moraxella bovis*; MEM, minimum essential medium; MgCl₂, magnesium chloride; mL, milliliter; mRNA, messenger RNA; NCOA3, nuclear receptor coactivator 3; ng, nanogram; nM, nanomolar; p53, tumor suppressor p53; PCR, polymerase chain reaction; PLA2, phospholipase A2; pRb, retinoblastoma protein; RAB13, member RAS oncogene family; RaSH, Rapid Subtraction Hybridization; RNA, ribonucleic acid; SiHa, cells epidermoid carcinoma of the cervix; SMAD1, SMAD family member 1; SMAD4, SMAD family member 4; TPT1, tumor protein, translationally-controlled 1; XhoI, enzyme gene from *Xanthomonas holcicola*.

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1. Introduction

Carcinoma of the cervix, or cervical cancer, is the second most frequent neoplasm in women worldwide. Each year, approximately 530,000 new cases of cervical cancer arise. Cervical cancer is the fourth leading cause of death in developing countries, with approximately 275,000 deaths per year (Jemal et al., 2011).

A factor directly related to cervical cancer is infection with human papilloma virus (HPV), and approximately 90% of cervical cancer cases are associated with HPV as a causative agent (Jaafar et al., 2009; Walboomers et al., 1999). A common feature of this type of tumor is the high expression of two oncogenes, *E6* and *E7*, which encode multifunctional proteins known for their ability to inactivate the tumor suppressors p53 and pRb, respectively (Narisawa-Saito and Kiyono, 2007). This altered expression of oncogenes leads to instabilities in the genome by blocking the cell cycle and checkpoints deregulation in these cells, due to the loss of these tumor suppressors genes. These cells then can progress towards the tumorigenic process (Incassati et al., 2006).

Other factors that contribute to the risk of developing cervical cancer have been described. These factors include age; the number of sexual

partners, which has been especially associated with co-infection; prolonged use of hormonal contraceptives; a large number of full-term pregnancies; and cigarette smoking (Soto-De Leon et al., 2011).

The inflammatory response is controlled by the action of anti-inflammatory mediators, which act to maintain the homeostasis of the immune response to prevent tissue damage. These mediators include annexin-A1 (ANXA1), a 37 kDa protein expressed by tumor cells that act as a modulator of inflammation (Flower, 1988; Silistino-Souza et al., 2007; Alves et al., 2008).

The Annexins (ANX) are a well-conserved superfamily of structurally related proteins characterized by a C-terminal homologous domain containing four to eight repeats of 70–75 amino acids that are responsible for the calcium and phospholipid binding properties. The variable N-terminal region is unique in length and sequence and includes potential sites of phosphorylation, glycosylation and peptidase action (Gerke and Moss, 2002). Annexins are present in many organisms, including molds, plants, fungi and mammals. They are preferentially localized in the cytoplasm, where they associate with the plasma membrane or cytoskeleton, but some annexins, including ANXA1, have also been found to localize to the outside of the plasma membrane (Solito et al., 1994, 2003a). Though the function of the annexins is far from clear, they have been shown to be involved in signal transduction (Allridge et al., 1999; De Coupade et al., 2000), vesicle transport (Diakonova et al., 1997), cell transformation (Violette et al., 1990; Solito et al., 1998), inflammation (Perretti and Gavins, 2003), cell matrix interactions and apoptosis (Raynal and Pollard, 1994; Solito et al., 2003b).

Annexin A1 (ANXA1), originally known as lipocortin 1, was the first member of this family to be cloned (Wallner et al., 1986). ANXA1 mimics the anti-inflammatory activity of glucocorticoids by inhibiting the synthesis of eicosanoids and the activity of phospholipase A2 (PLA2), affecting the components of the inflammatory reaction and the release of arachidonic acid (Flower, 1988; Perretti and Gavins, 2003). This protein is especially found in many cell types related to defensive processes, which include neutrophils (Oliani et al., 2001), mast cells (Oliani et al., 2008), eosinophils (Oliani et al., 2002) and monocytes (Goulding et al., 1990).

The expression and localization of ANXA1 have been investigated in experimental models of acute inflammation induced by carrageenan in the rat mesentery through the analysis of extravasated leukocytes (Oliani et al., 2001; Gil et al., 2006). The vascular endothelium and chronic granulomatous inflammation have also been examined in the skin of mice (Oliani et al., 2008; Gibbs et al., 2002).

After it was discovered that the biological activity of ANXA1 could be reproduced by the first twenty-six amino acids in the N-terminus (peptide Ac2-26) as well as some smaller peptides (Cirino et al., 1993; Perretti et al., 2001), the use of these molecules in experimental models of acute (Damazo et al., 2006; Gavins et al., 2007), chronic (Gibbs et al., 2002) and systematic inflammation has become commonplace (Damazo et al., 2005). The management of these mimetic ANXA1 peptides causes the activation of the abundant ANXA1 receptors found in leukocytes, promoting cell activation and intracellular signaling, provoking detachment of adherent leukocytes (Gavins et al., 2003; Gastardelo et al., 2009).

In comparison to HPV negative squamous cell penile carcinoma samples, ANXA1 protein expression was significantly increased in high-risk HPV squamous cell penile carcinoma samples independently of the subtype of penile squamous cell carcinoma. ANXA1 is likely to play an oncogenic role in penile cancer associated with high-risk HPV's (Calmon et al., 2013). By contrast, the ANXA1 expression in cervical carcinogenesis has been scarcely investigated. The levels of this protein and its mRNA decrease as the cancer develops, suggesting that ANXA1 may be regarded as a biological marker in tumor progression (Lee et al., 2008). The down-regulation of ANXA1 expression in [LaCit2]3 (Lanthanum citrate complex)-treated SiHa cells suggests that ANXA1 might regulate growth factors

related to apoptosis to mediate programmed cell death (Shen et al., 2010).

Though the works related to ANXA1 have contributed to a greater understanding of the role of this protein in inflammatory and tumorigenic process, the molecular mechanisms by which ANXA1 modulates cellular responses in the inflammatory process have not yet been fully determined. Available data suggest that this protein may be significantly involved in cancer in addition to its important role in the inflammatory process, most likely through signaling cascades that include genes related to the cell cycle, differentiation and apoptosis.

To investigate the effects of ANXA1 in cervical cancer, we evaluated the influence of this peptide on the morphology, cell proliferation and gene expression in SiHa cells. We also identified the signaling pathways that genes modulated by ANXA1_{Ac2-26} participate which are important for tumorigenic and the inflammatory process.

2. Materials and methods

2.1. SiHa culture conditions

The SiHa cell line originally established from an epidermoid carcinoma of the cervix (ATCC, Rockville, Maryland, USA) was obtained and seeded at a density of 1×10^6 cells/mL per 75 cm² culture flask (Corning, NY, USA). MEM-Earle medium (Cultilab, Campinas, SP, Brazil) was used at pH 7.5 and supplemented with 10% fetal calf serum (Cultilab), 1% non-essential amino acids and 1% antibiotic/antimycotic (Invitrogen Corporation, Carlsbad, CA, USA) and cultured at 37 °C under an atmosphere of 5% CO₂.

2.2. Pharmacological treatment

The SiHa cells were subdivided into a control group that was not manipulated and an experimental group to which 3 μM of ANXA1_{Ac2-26} was added. The experiments were conducted for time periods of 2, 4, 24, 48 and 72 h to determine the statistically significant time for further use in the methodology.

2.3. Growth curve

SiHa cells were seeded in triplicate at a density of 5×10^4 cells in plastic 6-well plates and divided in two groups. The control group was grown on complete medium whereas peptide ANXA1_{Ac2-26} was added to the experimental group. 24 h later, when the cells had already adhered, SiHa cultures were incubated with serum free medium (MEM 0%). After an additional 24 h, peptide was added to the experimental group whereas the control group was maintained in complete medium without any pharmacological manipulation.

The cell morphology was observed on each day. The cytotoxic effects of peptide on the proliferation of SiHa cells were investigated in two experimental groups at 2, 4, 24, 48 and 72 h. The cells were harvested, stained with trypan blue and counted using a Neubauer hemocytometer. Significant differences between the groups were determined by one-way analysis of variance (one-way ANOVA) and if found to be significant, further analyzed using the Bonferroni test. The same experiment was repeated twice.

2.4. RNA extraction for Rapid Subtraction Hybridization (RaSH) and real time PCR experiments

SiHa cells were seeded at a density of 1×10^6 cells/mL in 75 cm² culture flasks in complete medium (control group) and incubated with 10 μg/mL of peptide Ac2-26 for 72 h (experimental group). The RNA from control and ANXA1_{Ac2-26} treated SiHa cells was extracted using Trizol (Sambrook and Russel, 2001) following treatment with DNase (Invitrogen). cDNA synthesis was performed using a High Capacity

cDNA Archive kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions.

2.5. Rapid Subtraction Hybridization (RaSH)

The RaSH technique (Jiang et al., 2000) was performed with two subtractions. The first was termed "Sub I", in which the SiHa control cells were referred to as *tester* and the cells treated with peptide ANXA1_{Ac2-26} were termed *driver*. In the second subtraction, "Sub II," the opposite test was performed.

The first cDNA strand was obtained by reverse transcriptase with 2 µL of oligo (dT) (50 mM) and 2 µL of 10 mM dNTPs at 65 °C for 5 min. The samples were immediately placed on ice for 1 min and 2.0 µL of 0.1 mM DTT, 1 µL of 40 U/µL RNAout and 4.0 µL of 5X First-Strand Buffer [250 mM Tris-HCl (pH 8.3), 375 mM KCl and 15 mM MgCl₂] were added. The samples were incubated at 42 °C for 2 min before 1.0 µL of Superscript™ II reverse transcriptase (RT) (200 U/µL) was added. The mixture was incubated again at 42 °C for 50 min and then at 70 °C for 15 min. Segments of β-actin cDNA were also amplified to verify the quality of these reactions.

The second strand of cDNA was synthesized in reactions containing 30 µL of 5X Second Strand Buffer, 3 µL of 10 mM dNTPs, 1 µL of DNA ligase (10 U/µL), 4 µL of DNA Pol I (10 U/µL) and 1.0 µL of RNase H (2 U/µL). The samples were incubated for 2 h at 16 °C before the addition of 2 µL of T4 DNA Pol (5 U/µL) and further incubation for an additional 5 min. The action of T4 DNA Pol was inhibited by the addition of 10 µL of 0.5 M EDTA. Phenol:chloroform extraction was used to purify the sample and the pellet was resuspended in 45 µL of water. The cDNA was used in amplification reactions for segments of GAPDH and was subsequently digested with 2 µL of MboI enzyme (10 U/µL) and incubated for 1 h at 37 °C. Finally, over 1 µL of this enzyme was added to the reaction, and the samples were incubated overnight at 37 °C.

To this cDNA was added 4.1 and 4.5 µL, respectively, of the adaptors XDPN-14 5'-CTGATCACTCGAGA and XDPN-12 5'-GATCTCTCGAGT (Sigma Chemical, final concentration 10 mM), 8 µL of 5X T4 DNA ligase buffer Invitrogen®, heated at 55 °C for 1 min, and cooled down to 14 °C within 1 h. The cDNA received 3 µL (9 U) of T4 DNA ligase (3 U/µL), ligation was carried out overnight at 14 °C. After phenol/chloroform extraction and ethanol/glycogen precipitation, the mixtures were diluted to 100 µL with TE buffer (10 mM Tris/1 mM EDTA); 40 µL of the mixtures were used for PCR amplification.

The PCR mixtures were set up using 10 µM XPDN-18 5'-CTGATCAC TCGAGAGATC, 0.4 mM dNTPs, 10 × PCR buffer, 1.5 mM MgCl₂ and 1 U Taq DNA polymerase (Invitrogen). Thermocycler conditions were 1 cycle at 72 °C for 5 min, followed by 25 cycles of 94 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min, ending in a final extension at 72 °C for 3 min. Ten microgram of purified PCR product (*tester*) was digested with 10 U/µL XhoI (Invitrogen) for 6 h at 37 °C and followed phenol/chloroform extraction and ethanol precipitation.

One-hundred nanograms of the *tester* cDNA were mixed with 5 µg of the *driver* cDNA in hybridization solution (0.5 M NaCl, 50 mM Tris/HCl, SDS2% and 40% formamide) and, after heating at 99.9 °C for 5 min, incubated at 42 °C for 1 h, and 42 °C for 48 h. After extraction and precipitation, the hybridization mixture (1 µg) was ligated with XhoI-digested pZero plasmid and transformed into competent bacteria. Bacterial colonies were picked and used as DNA template for PCR. Clones were sequenced using an automated DNA sequence and homologies sequences were searched using the BLAST program. Gene ontology (GO) annotation was used for the functional classification of up- and down-regulated genes using terms from Gene Ontology database.

We further analyzed the genes through IPA (Ingenuity Systems®) to relate the differentially expressed genes with biological functions and processes in which these genes are involved and evaluate what the main networks of interaction between them.

2.6. Quantitative PCR – RT-qPCR

The RNA from control and ANXA1_{Ac2-26} treated SiHa cells was extracted and cDNA synthesis was performed as described above.

Six differentially expressed genes were selected for validation by quantitative real time PCR experiments according to their direct or indirect involvement in tumorigenesis. Their expression was checked in treated samples relative to matched non-treated samples.

The primers were manually designed with: 19–23 bp length, 30–80% GC content and a short amplicon size (80–120 bp). Their sequences are available upon request (Table 1). Real time PCR was performed in triplicate using a 7500 Fast Real-Time PCR System (Applied Biosystems). Reaction mixture consisted of a 20 µL volume solution containing 100–500 ng of Power SYBR Green PCR Master Mix (Applied Biosystems), 100 nM of each primer and 100 ng cDNA (control and treated with peptide ANXA1_{Ac2-26} [3 µM]). The PCR conditions were 95 °C for 10 min followed by 40 cycles of 95° for 15 s and 60° for 1 min. Melting curve analysis was performed for each gene to check the specificity and identity of the RT-PCR products.

For each primer set, the efficiency of the PCR reaction (linear equation: $y = \text{slope} + \text{intercept}$) was measured in triplicate on serial dilutions of the same cDNA sample. The PCR efficiency (E) was calculated by the formula $E = [10^{(-1/\text{slope})}]$ and ranged from 1.96 to 2.02 in the different assays.

Two control genes (*GAPDH*, *ACTB*) were used as internal standards. The relative expression ratio (fold change) of the target genes was calculated according to Pfaffl (2001). Statistical analysis was performed by a two-tailed unpaired t test using GraphPad prism software.

To investigate the pathway of *ID1* gene, were analyzed four genes (*BMP4*, *BMPRII*, *SMAD1* and *SMAD4*) following the methodology described above. The *ID1* gene is connected with more four genes (*BMP4*, *BMPRII*, *SMAD1* and *SMAD4*) in TGF-Beta signaling pathway, from KEGG (<http://www.kegg.jp/pathway/ko04350>), according to Pathways from Biosystems (<http://www.ncbi.nlm.nih.gov/gene/3397>).

3. Results

3.1. Cellular morphology

SiHa cells were initially cultured and observed by phase contrast microscopy. These cells have a fusiform appearance and central core, with multiple nucleoli and a eucromatic position, which are characteristics of a cell with high metabolic activity (Fig. 1A). In cells treated with ANXA1_{Ac2-26} [3 µM], there was no morphological change, and cytoplasmic processes were more clearly observed due to reduced cell proliferation (Fig. 1B).

3.2. Proliferation assay

In the proliferation assay, one-way ANOVA using the GraphPad Prism 5 program was used to observe the growth inhibitory effect

Table 1
Primers for the interesting genes.

Primer	Sequence
HIF1A right	5' TCCTCACACGCAATAGCTG 3'
HIF1A left	5' TGCTCATCAGTGGCACTTC 3'
ID1 right	5' TAGTCGATGACGTGCTGGAG 3'
ID1 left	5' AAACGTGCTGCTCTACGACA 3'
LDHA right	5' GCCGTGATAATGACAGCTT 3'
LDHA left	5' TGGCAGCCTTTTCCTTAGAA 3'
NCOA3 right	5' AAGTCCCAACACCTTCACTG 3'
NCOA3 left	5' TTCTGGCAATACTGGGAACC 3'
RAB13 right	5' CTGCAAGCGAATGATCAGA 3'
RAB13 left	5' AGCTACGACCACTTCTTCA 3'
TPT1 right	5' TAGAGCAACCATGCCATCTG 3'
TPT1 left	5' GCTGCAACAAATCAAGCA 3'

Fig. 1. Morphological analysis and growth curve of ANXA1_{Ac2-26} peptide treatment in SiHa cell line. SiHa cells were seeded in complete MEM 10% at a concentration of 5×10^4 per well (6 well plate). The cell morphology observed in (A) the control group, characterized by a monolayer of nucleated cells was not altered in (B) the ANXA1_{Ac2-26} peptide [10 μ g/mL] treated group. 400 $\times$ magnification. (C) SiHa cells were cultured, counted after 2, 4, 24, 48 and 72 h and these results were plotted on a growth curve. Assays were performed in triplicate. Graph $\times$ = time (hours) and y = number of cells $\times 10^4$. P values < 0.05 were significant.

under ANXA1_{Ac2-26} treatment. The peptide was shown to decrease the proliferation in treated cells in comparison to the control group (Fig. 1C). Statistical analysis showed that these differences were most significant ($P \leq 0.05$) at a concentration of 10 μ g/mL of peptide and a culture time of 72 h.

3.3. Genes identified using the RaSH approach

Rapid subtractive hybridization (RaSH) compares two distinct experiments to find differential gene expression. RaSH was performed on SiHa cells from the control group and those treated with ANXA1_{Ac2-26} for 72 h, and 82 clones were isolated and sequenced. By screening these sequences through GenBank (Blast) and by rigorous classification and selection, 55 genes were found to be differentially expressed. Two libraries of genes (Sub I and Sub II) were acquired. Twenty-three genes of the "Sub I" library exhibited changes in expression levels in the control cells, whereas 32 genes of the "Sub II" library changed in expression under treatment with peptide. Therefore, it was expected that the gene expression of one library would be lower than that of the other one. The libraries of the genes obtained along with their different functions and processes described in the Gene Ontology (GO) databases.

IPA (Ingenuity Systems©) was used to further analyze the 55 differentially expressed genes, which were especially associated with tumorigenesis, apoptosis, necrosis and cell proliferation (Supplements 1 and 2). Moreover, some of these genes were chosen because they form a network of important interactions with other genes such as NF- κ B, Ubiquitin and HSP in the "Sub I" library or with genes associated with cancer,

inflammatory diseases, the cell cycle, senescence, replication and cell death in the "Sub II" library.

Six differentially expressed genes, *TPT1*, *LDHA*, *ID1*, *NCOA3*, *HIF1A* and *RAB13*, were selected for validation by quantitative real time PCR experiments (Table 1). These genes are associated with calcium ion transport, cellular response to extracellular stimulus, apoptotic process, regulation of transcription, response to hypoxia and regulating membrane trafficking, respectively and were chosen according to their functions related to their direct or indirect involvement in tumorigenic and inflammatory processes.

3.4. Real-time PCR validation of differentially expressed genes

The RaSH analysis indicated the down-regulation of four genes (*ID1*, *LDHA*, *RAB13* and *TPT1*) and up-regulation of two genes (*HIF1A* and *NCOA3*) in SiHa cells treated with ANXA1_{Ac2-26}. However, the quantitative PCR experiment showed that only the expression of *ID1* was significantly reduced in these cells after peptide treatment (Fig. 2). The expression of other genes oscillated without statistical significance, which was most likely because the peptide does not quantitatively modify the expression of the selected genes despite its interaction with the cell.

3.5. Real-time PCR gene-related pathway gene *ID1*

Four genes (*BMP4*, *BMPR1B*, *SMAD1* and *SMAD4*) of *ID1* pathway were analyzed, however, the quantitative PCR experiment showed that only the expression of *BMPR1B* was significantly reduced in SiHa

Fig. 2. Quantitative real time PCR gene expression in SiHa cells treated with ANXA1_{A2-26} peptide. Expression of *HIF1A*, *ID1*, *LDHA*, *NCOA3*, *RAB13* and *TPT1* genes in SiHa cells treated with ANXA1_{A2-26} peptide (10 µg/mL), compared with control cells. Assays were performed in triplicate to confirm the results and the data represented in base 2 logarithm.

cells after ANXA1_{A2-26} treatment (Fig. 3), like the *ID1* gene expression. The expression of other genes oscillated without statistical significance (*BMP4*, *SMAD1* and *SMAD4*).

4. Discussion

Although cervical cancer can be prevented, it still represents a major public health problem for women, especially in underdeveloped countries where there is little monitoring and prevention.

The anti-inflammatory protein Annexin A1 (ANXA1) could be used as an indicator of treatment. Though the literature on the in vitro (D'Acquisto et al., 2008) and in vivo (Gastardelo et al., 2009; Facio et al., 2011) pharmacological effects of this protein and its mimetic peptides in inflammatory processes is extensive, the expression of this protein in cervical carcinogenesis has been scarcely investigated. Some studies found the low expression of ANXA1 in cervical cancer, suggesting that this protein could be considered a biological marker in the progression of invasive cervical cancer or regulates apoptosis-related growth factors, point to a negative effect of ANXA1 in the cervical cancer, low expression worst prognostic (Lee et al., 2008; Shen et al., 2010). Thus, we explored the action of ANXA1_{A2-26} on SiHa cells derived from cervix squamous cell carcinoma, a cell line that provided the best conditions for the experiments. These cells contained HPV16, with an estimated 10 copies of HPV 16 DNA integrated into the cellular DNA (Yee et al., 1985).

In analyzing the SiHa cell line, we observed that the morphology did not change after treatment with peptide ANXA1_{A2-26}. However, cell proliferation decreased after 72 h of treatment. ANXA1 mediates the disruption of normal cell morphology and inhibits cyclin D1 expression, reducing cell proliferation through proximal modulation of the ERK signal transduction pathway (Alldridge and Bryant, 2003). ANXA1 has direct anti-proliferative effects on cells possibly through the constitutive

Fig. 3. Quantitative real time PCR gene expression in SiHa cells treated with ANXA1_{A2-26} peptide. Expression of *BMP4*, *BMPR1B*, *SMad1* and *SMad4* genes in SiHa cells treated with ANXA1_{A2-26} peptide (10 µg/mL), compared with control cells. Assays were performed in triplicate to confirm the results and the data represented in base 2 logarithm.

over-activation of ERK1/2. Thus, the anti-proliferative role of ANXA1 may be directly responsible, at least in part, for the growth arrest. These findings suggest that ANXA1 may act as a tumor suppressor (Ang et al., 2009).

But in the other hand, this anti-proliferative effect can be explained by apoptosis too, Parente and Solito (2004) comment that ANXA1 promotes inflammatory cell apoptosis associated with transient rise in intracellular calcium and caspase-3 activation. Then in these SiHa cells, how there are inflammatory cells in cancer process, we can suppose that this negative effect can be explained by apoptosis (Parente and Solito, 2004).

Apoptosis was also a criterion for selecting the differentially expressed genes for validation by real-time RT-PCR. Of the 55 genes identified by RaSH approach was very difficult choose only six genes to validation, because all of them have some interesting function, but IPA (Ingenuity Systems®) was helpful to choose which genes were especially associated with tumorigenesis, apoptosis, necrosis, regulation of transcriptional and in the same gene network.

After an analysis of the literature, six genes (*HIF1A*, *ID1*, *LDHA*, *NCOA3*, *RAB13* and *TPT1*) were selected for validation by real-time RT-PCR using treated and non-treated cell lines. These genes show potential involvement in signaling cascades related to tumorigenesis and/or the interaction between inflammation and tumor cells through angiogenesis, metastasis, lactate dehydrogenase activity, regulation of gene expression, cell adhesion and binding and calcium ion transport. The RT-PCR results for the *ID1* gene were consistent with the RaSH data. For the gene expression of *NCOA3* and *HIF1A*, the results were not significantly increased. Thus, these genes can be considered to be good molecular markers if their high expression levels are confirmed in other cells. *LDHA*, *RAB13* and *TPT1* genes still require further testing. These results are most likely due to the higher concentrations of the peptide that are required in these cells to observe the mimetic effects.

The different expression levels of the *ID1* gene are related to angiogenesis, differentiation and metastasis. The overexpression of *ID1* was detected in esophageal squamous cell carcinoma, which suggests that in our study, ANXA1 may regulate this gene by decreasing its expression and may be used as a possible therapeutic target. *ID1* belongs to a family of small molecules of short length (13 to 20 kDa) and acts as an inhibitor of differentiation or DNA binding (Benezra et al., 1990). The *ID1* gene is not a transcription factor, it is a transcriptional regulator (lacking a basic DNA binding domain) which negatively regulates the basic helix-loop-helix (bHLH) transcription factors by forming heterodimers and inhibiting their DNA binding and transcriptional activity (Zheng et al., 2004). Thus, *ID1* regulates the effects of transcription factors on target genes. Infection by high-risk human papilloma viruses (HPVs) is considered to be the central cause of invasive cervical cancer, suggesting that high-risk HPVs can enhance the progression of human cervical cancer through *ID1* regulation (Darnel et al., 2010).

The overexpression of *ID1* alone can induce angiogenic processes in endothelial progenitor cells (EPCs) in ovarian cancer. The expression of *ID1* and matrix metalloproteinase-2 (MMP-2) was increased in EPCs isolated from ovarian cancer patients compared to those obtained from healthy subjects. It can thus be concluded that *ID1* can enhance EPC angiogenesis in ovarian cancer, which is mainly mediated by the PI3K/Akt and NF-κB/MMP-2 signaling pathways (Su et al., 2013).

In most metastatic cancer patients, epithelial tumor cells from primary tumor samples express any grade *ID1* positivity. In addition, higher protein expression levels seem to correlate with poorer prognoses in prostate cancer (Ponz-Sarvisé et al., 2014). The overexpression of *ID1* protein is associated with more aggressive behavior of tumor cells in human cervical cancer and is an independent marker for the progression of this tumor type (Schindl et al., 2001).

The *ID1* is considered as a BMP (bone morphogenetic protein) specific target gene that binds either to a type 1 or a type 2 receptor to form the ternary signaling complex (Chang et al., 2002). BMPs belong to the TGF-β superfamily and act via their specific receptors, the

BMPRs. Among the receptors, BMPR1B has been previously analyzed and shown to be linked to the advancement of breast cancer and poor prognosis. Analysis revealed lower expression levels of *BMPR-1B* transcript in poor prognosis patient samples compared to good prognosis samples, as well as, down-regulation of *BMPR-1B* in MDA-MB-231 cells and leads to increased cell proliferation of breast cancer cells in vitro (Bokobza et al., 2009). Probably the down expression of the *BMPR1B* receptor is according to the *ID1* gene expression.

In the literature, the overexpression of *ID1* gene is observed in cervical cancer, but our data showed decreased expression of *ID1* and *BMPR-1B* genes after the peptide treatment, suggesting that this peptide can modify the expression of some genes in cervical carcinoma cells. As consequence of the decreased expression of *ID1* gene by the peptide, *ID1*-targeted transcription factors will be relieved to regulate their downstream signals.

The genes evaluated in this study showed differential expression in SiHa cells in a manner dependent on ANXA1_{Ac2-26} treatment. In summary, this study highlights the importance of the RaSH technique as a fast and efficient method for identifying genes modulated by ANXA1 in cells of epithelial origin. Further experiments are needed to determine whether other genes obtained by subtraction are regulated by ANXA1 and whether these are causally related to treatment-induced changes in this protein. The functions of the genes identified in this study are important in tumor progression and are amenable to changes through altering the metabolism of the cervical epithelial cells.

The cervix is difficult to access, and treatments for cervical carcinoma commonly involve surgery, chemotherapy or radiotherapy. However, new therapeutics such as the use of gene therapy associated with nanoparticles tested in cervical cancer derived HeLa cells are being developed, allowing the delivery of drugs into target cells (Zheng et al., 2013).

Finally, the ANXA1_{Ac2-26} peptide alters the proliferation and gene expression of SiHa cells. These changes are most likely related to inflammatory and tumorigenic processes. Thus, we suggest that the peptide has modulatory effects on the metabolism of this cell type. Few investigations have evaluated the effects of this peptide on the expression of selected genes. Our data suggests the mechanisms of action of this anti-inflammatory protein, and future molecular studies must be conducted to verify these processes. Our findings suggest the importance of future studies using SiHa cells for the development of new therapies. These studies should focus on gene activities that involve the action of ANXA1 and applications of this peptide as a possible therapeutic in the treatment cervical carcinoma.

5. Conclusions

These findings show that the ANXA1_{Ac2-26} alters the proliferation of SiHa cells and the expression of genes with roles in signaling cascades involved in the inflammatory and tumorigenic processes. Thus, our results point to possible applications of the ANXA1_{Ac2-26} peptide as an indicator of innovative therapies for the treatment of cervical carcinoma.

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Disclosures

The authors have declared that no competing interests exist.

Author contributions statement

JP carried out the cell culture experiments, RaSH assay, real-time PCR assay, performed the statistical analysis, and drafted the manuscript. GBF-S assisted with the cell culture experiments and RaSH assay. ARDS and WASJr assisted with the RaSH assay. BRC assisted with the real-time PCR assay. EHT and SMO were involved in drafting the manuscript or revising it critically for important intellectual content. FCR-L

conceived the study, and participated in its design and coordination and helped to draft the manuscript. All of the authors read and approved the final manuscript.

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Review Article

Involvement of Kallikrein-Related Peptidases in Normal and Pathologic Processes

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Human kallikrein-related peptidases (KLKs) are a subgroup of serine proteases that participate in proteolytic pathways and control protein levels in normal physiology as well as in several pathological conditions. Their complex network of stimulatory and inhibitory interactions may induce inflammatory and immune responses and contribute to the neoplastic phenotype through the regulation of several cellular processes, such as proliferation, survival, migration, and invasion. This family of proteases, which includes one of the most useful cancer biomarkers, kallikrein-related peptidase 3 or PSA, also has a protective effect against cancer promoting apoptosis or counteracting angiogenesis and cell proliferation. Therefore, they represent attractive therapeutic targets and may have important applications in clinical oncology. Despite being intensively studied, many gaps in our knowledge on several molecular aspects of KLK functions still exist. This review aims to summarize recent data on their involvement in different processes related to health and disease, in particular those directly or indirectly linked to the neoplastic process.

1. Introduction

Human kallikrein-related peptidases (KLKs) are a subgroup of serine proteases that have important roles in regulating normal physiological functions, such as immune response, skin desquamation, enamel formation, and semen liquefaction, and the corresponding pathological conditions. There is growing evidence in the literature supporting the view that KLKs are also implicated in tumorigenesis by activating proteolytic processes associated with the neoplastic phenotype. The potential mechanisms involved include the modulation of growth factor bioavailability and activation of hormone and protease-activated receptors (PARs) resulting in proliferative signaling pathways, the degradation of extracellular matrix, cleavage of junction proteins and induction of an epithelial-mesenchymal transition (EMT) phenotype leading to increased tumor cell migration and invasion, and the modulation of interactions between cancer cells and their

microenvironment promoting angiogenesis and other protumorigenic processes (reviewed by [1–3]).

The potential of KLKs as cancer markers has been suggested for several members of this protease family [2, 4–6], particularly for kallikrein-related peptidase 3 or prostate-specific antigen (PSA) [7]. PSA is well accepted for assessing recurrence risk in patients with prostate cancer, but its predictive power for diagnosis has been questioned, since several factors other than malignancy may be associated with its high levels in serum, such as preanalytical variables, benign diseases, and drugs [8, 9]. Biomarker panels combining PSA and other promising markers, including members of the KLK family, are expected to improve prostate cancer screening and reduce unnecessary treatments, a strategy that may also be used for detection and monitoring of other malignancies and nonmalignant diseases.

In this paper, we review the current knowledge about the evolution and functions of human kallikrein-related

peptidases, their substrates, and their role in health and disease, particularly in the context of cancer.

2. The Human Degradome

Protein synthesis is essential for living, metabolically active cells, but its counterpart, protein degradation, is no less important. Proteolytic mechanisms driven by proteases maintain appropriate protein levels and recognize and degrade the misfolded or mislocalized ones. In addition to acting in nonspecific catabolism, proteases are involved in selective cleavages and activations, modulating protein-protein interactions and contributing to cell signaling both as catalytic units and as multicatalytic complexes. Due to their broad-spectrum actions, proteases play critical roles in regulating normal biological processes, including DNA replication and transcription, cell proliferation, differentiation, and apoptosis. When altered, they may facilitate the development of pathological conditions such as inflammatory and degenerative disorders (reviewed by [10]). The importance of these hydrolytic enzymes is reflected by the number of genes already identified in several mammalian species, with more than 500 in human and primates and even more in rodents [11–14].

The complete set of human proteases—named the human degradome—is distributed in aspartic-, threonine-, cysteine-, serine-, and metalloprotease classes according to the chemical group involved in their catalytic activity [15, 16], and the latter three are the most populated classes [10]. Their substrate cleavage patterns may be specific for a single peptide, as in the case of proteases involved in signaling pathways, or common for a broad range of peptides, which is well exemplified by digestive enzymes [17]. Otherwise, inactive proteases or pseudoproteases bind to their cognate substrate without cleaving them, thus exerting a regulatory function [18].

Detailed information on proteases in prokaryotes and eukaryotes, protease families, pseudogenes, the sequences derived from endogenous retroviruses, 3D structures, substrates, and proteolytic events has been accumulated in different databases such as MEROPS [19] and Degradome [20].

3. The Serine Protease Group

Approximately one-third of proteolytic enzymes are serine proteases, usually endopeptidases. These enzymes use the serine residue present in their active site as a nucleophile to attack the peptide bond of the substrate [21]. In humans, many serine proteases are involved in extra- and intracellular processes mainly related to food digestion, blood coagulation, and immunity (reviewed by [1, 22]). Although these processes are essential for the purposes of catabolism or selective cleavages required for cell signaling, serine protease activity (as well as that of other proteases) is potentially devastating, and several cellular mechanisms were selected to modulate and keep them within limits. For example, they are stored as inactive zymogens or inside granules and can access the substrates only through controlled actions. In addition, serpins, a superfamily of serine protease inhibitors, antagonize their

activities in many metabolic pathways, arresting the proteases into an irreversible complex (reviewed by [1]).

Although tightly controlled, several serine proteases have been associated with human diseases. For example, high granzyme levels (granule-secreted enzymes found in cytotoxic T cells and natural killer cells) have been observed in chronic inflammatory diseases such as rheumatoid arthritis [23], asthma [24], diabetes [25], atherosclerosis [26], and chronic obstructive pulmonary [27] and cardiovascular diseases [28]. They have also been implicated in susceptibility to skin tearing and disorganized collagen as observed in chronic wounds and aged/sun-damaged skin (reviewed by [29]). The role of granzymes in these conditions resides in their ability to cleave many substrates, inducing apoptosis through caspase-dependent and caspase-independent pathways [30]. Their potential to create or destroy autoimmune epitopes [31] and be improperly regulated in chronic wounds or released non-specifically from immune cell into extracellular spaces also contributes to chronic inflammation or extracellular matrix disorganization [27, 32].

Increased levels of neutrophil proteases such as elastase, cathepsin G, and myeloblastin have also been correlated with the severity of cystic fibrosis and chronic obstructive pulmonary disease [33]. Similarly, tryptase and chymase, two serine proteases stored in mast cell granules, take part in the pathophysiology of asthma [34], psoriasis [35], atherothrombosis [36], and fibrotic [37] and inflammatory kidney diseases [38].

With respect to cancer, several serine proteases have been linked to tumor development and progression by activating proteolytic processes that are associated with the neoplastic phenotype (reviewed by [1]). Specifically, a family of serine proteases expressed and secreted in many tissues participates in complex networks of cell signaling pathways that are related to cancer [4–7]. One of the most useful cancer biomarkers in clinical medicine is kallikrein-related peptidase 3 or PSA, which is a member of this family (reviewed by [7]), and there is evidence that other KLKs are also deregulated in cancer and other diseases [4, 39–147] as summarized in Table 1.

4. The Human Kallikreins

Human kallikreins, initially detected at high levels in pancreas, *kallikreas* in Greek, include plasma and tissue serine proteases, which are two categories that differ in molecular weight, substrate specificity, and gene structure. The unique plasma kallikrein (PKK) is a glycoprotein encoded by the *KLKB1* gene on chromosome region 4q35 and is predominantly synthesized in the liver as an inactive precursor. After activation by the coagulation factor XII, PKK cleaves high molecular weight kininogen to release bradykinin, a mediator of blood coagulation, inflammation, blood pressure, and thrombosis risk [148].

4.1. Kallikrein-Related Peptidases at DNA Level: Genomic Organization and Evolutionary Aspects. The 15 tissue kallikreins or kallikrein-related peptidases (KLKs) are encoded by genes that are tightly clustered in an approximately

TABLE 1: Kallikrein-related peptidases. Gene expression pattern, SNPs, and promoter methylation related to cancer and other diseases. CSF = cerebrospinal fluid.

Disease	Kallikrein	Factor	Observation	Reference
Alzheimer's disease	<i>KLKs 6 (CSF), 10 KLKs 6 (brain, blood), 7</i>	Increased expression Decreased expression		[39–42]
Amelogenesis imperfecta	<i>KLK4</i>	Mutation	Disease-causing mutation	[43–45]
Aneurism	<i>KLK6</i> <i>KLK8</i>	Decreased expression SNP	Suggestion of unfavorable prognosis Suggestion of unfavorable prognosis	[46, 47]
Asthma	<i>KLK3</i>	SNP		[48]
Atopic dermatitis	<i>KLK5</i>	Decreased expression		[49, 50]
Bipolar disease	<i>KLK8</i>	SNP	Suggestion of unfavorable prognosis	[51]
Coronary artery disease	<i>KLK1</i> <i>KLK1</i>	SNP Increased expression	Controversial prognosis Predictor of disease	[52, 53]
Kidney disease				
Lupus nephritis	<i>KLK1</i>	SNP	Disease-associated SNP	
Acute kidney injury	<i>KLK1</i>	SNP	Suggestion of unfavorable prognosis	[54–56]
Diabetic nephropathy	<i>KLK1</i>	Increased expression	Tubular inflammation	
Multiple sclerosis	<i>KLK6</i>	Increased expression	Advanced disease	[57–59]
Dementia with Lewy bodies	<i>KLK6</i>	Decreased expression	Suggestion of diagnostic marker	[60]
Other neurodegenerative diseases	<i>KLKs 1, 5, 6, 7, and 9</i>	Increased expression	Suggestion of disease-associated marker	[61–63]
Other skin diseases	<i>KLKs 5–8, 10–13, and 15</i>	Increased expression	Suggestion of unfavorable prognosis	[64–73]
Netherton syndrome	<i>KLK5</i>	Increased expression	Suggestion of unfavorable prognosis	
Psoriasis	<i>KLK8</i> <i>KLKs 6, 8, 10, and 13</i>	Increased expression Increased expression	Suggestion of unfavorable prognosis Severity of skin lesions	[74, 75]
Parkinson's disease	<i>KLK6</i>	Increased expression	Disease-associated marker	[76]
Sjogren disease	<i>KLK11</i>	Increased expression	Suggestion of disease-associated marker	[77]
Breast cancer	<i>KLKs 2, 4</i> <i>KLK3</i> <i>KLKs 5, 10, and 14</i> <i>KLKs 6, 12 variant 3, and 15</i> <i>KLKs 3, 8, and 12</i> <i>KLKs 5, 7</i> <i>KLK10</i>	SNP SNP Increased expression Increased expression Decreased expression Increased expression Methylation	Breast cancer risk Association with less aggressiveness Potential diagnostic biomarkers Suggestion of favorable prognosis Suggestion of favorable prognosis Suggestion of unfavorable prognosis Suggestion of favorable prognosis	[4, 47, 78–83]
Cervix cancer	<i>KLK7</i>	Increased expression	Controversial prognosis	[84, 85]
Colorectal cancer	<i>KLKs 4, 6, 7, and 10</i>	Increased expression	Suggestion of unfavorable prognosis	[86–90]

TABLE 1: Continued.

Disease	Kallikrein	Factor	Observation	Reference
Gastric cancer	<i>KLKs 6, 7, and 10</i>	Increased expression	Suggestion of unfavorable prognosis	[90–95]
	<i>KLK13</i>	Increased expression	Suggestion of favorable prognosis	
	<i>KLK11</i>	Decreased expression	Suggestion of unfavorable prognosis	
Head and neck cancer	<i>KLK10</i>	Methylation	Suggestion of unfavorable prognosis	[96–99]
	<i>KLKs 4–8, 10</i>	Increased expression	Suggestion of unfavorable prognosis	
Intracranial tumor	<i>KLKs 6–8</i>	Increased expression	Controversial prognosis	[100, 101]
Lung cancer	<i>KLK10</i>	Methylation		[102–106]
	<i>KLKs 5–7</i>	Increased expression	Suggestion of unfavorable prognosis	
	<i>KLKs 11, 13, and 14</i>	Increased expression	Diagnostic marker	
	<i>KLKs 8, 12</i>	Decreased expression	Suggestion of unfavorable prognosis	
Melanoma	<i>KLKs 6, 8, and 13</i>	Increased expression		[107, 108]
	<i>KLK7</i>	Increased expression	Suggestion of favorable prognosis	
Ovarian cancer	<i>KLKs 4, 6</i>	Increased expression	Advanced stage	[47, 78, 109–125]
	<i>KLKs 8–10, 11, 13, and 14</i>	Increased expression	Suggestion of favorable prognosis	
	<i>KLKs 5, 7</i>	Increased expression	Suggestion of unfavorable prognosis	
	<i>KLK10, KLKP1</i>	SNP		
	<i>KLKs 3, 15</i>	SNP	Suggestion of unfavorable prognosis	
Pancreatic cancer	<i>KLK7</i>	Increased expression	Controversial prognosis	[126]
Prostate cancer	<i>KLK3</i>	Increased expression	Disease monitoring and recurrent prediction	[47, 78, 127–147]
	<i>KLKs 1, 2, 4, and 15</i>	Increased expression		
	<i>KLKP1</i>	Decreased expression		
	<i>KLK7</i>	Increased expression	Controversial prognosis	
	<i>KLK11</i>	Decreased expression	Suggestion of unfavorable prognosis	
	<i>KLKs 2, 3, 4, and 10</i>	SNP	Suggestion of unfavorable prognosis	
	<i>KLK12</i>	SNP	Cancer predisposition	
	<i>KLKs 4, 14, and 15</i>	SNP	Suggestion of unfavorable prognosis	

300 kb sequence of the 19q13.33–13.41 chromosome region, all containing 5 coding exons with comparable lengths and sequence homology [149, 150]. A pseudogene (*KLKP1*) has also been assigned to this region [151], as well as multiple repetitive elements such as ALU, Tigger2, MER8, and MSR1 [152]. The large contiguous human *KLK* gene cluster is limited by the *ACPT* (testicular acid phosphatase) gene and the *Siglec* (sialic acid-binding immunoglobulin-like lectin) family of genes at centromeric and telomeric positions, respectively,

and other less characterized genes (*SNORD88C*, *C19orf48*, *MGC45922*, and *CTUI*) (Figure 1).

The colocation and sequence conservation in a wide variety of species make this human tissue serine proteinase family a very interesting target for evolutionary studies [153]. The phylogenetic analysis of *KLKs* performed by the Maximum Likelihood method [154], using the transcript isoforms of 15 *KLK* genes, the pseudogene-1 (*KLKP1*) sequence, and the *PRSSI* (trypsin 1) transcript sequence as an external group,

FIGURE 1: *KLK* gene cluster and schematic representation of the human *KLK* gene and protein structure. (a) *KLK* gene cluster on the 19q13.33–13.41 chromosome region including the pseudogene *KLKP1* and the transcriptional direction from centromere to telomere, except for *KLK2* and *KLK3*, which have the opposite transcriptional direction. The classic *KLK* genes (*KLKs* 1–3) are turquoise, *KLK4*–*KLK15* are medium purple, and the Ψ *KLK1* processed pseudogene is silver; the arrowheads represent the neighboring genes: *ACPT* (testicular acid phosphatase) and the Siglec (sialic acid-binding immunoglobulin-like lectin) gene family as well as other less characterized genes (*SNORDs*, *CI9orf48*, and *CTUI*). (b) The human *KLK* gene consists of 5 coding exons (orange boxes represent coding exons; silver boxes represent noncoding exons) and their 4 intervening introns. The positions of the catalytic residues are highly conserved with the histidine (H), aspartic acid (D) 3, and serine (S) codons on coding exons 2, 3, and 5, respectively. Most *KLK* genes demonstrate alternative splicing, which generates several transcript variants. Alternative 3' splice sites or skipped exons (shown in green) result in short variants of *KLKs* 2, 3, 5, 8, and 12 genes. Alternative 5' splice sites or start sites (shown in blue) also generate short variants of *KLKs* 2, 3, 6, 7, 8, and 11 genes. Utilization of the alternative exon 6 generates a long transcript encoding a variant of *KLK12* gene (shown in blue). (c) *KLK* proteins are single-chain proteases that are synthesized as preproenzymes and are proteolytically processed to pro-*KLKs* and secreted after removal of the terminal signal peptide (Pre). The *KLK* sequence also includes a propeptide (Pro) that maintains the inactive state of the enzyme, as well as a serine protease domain.

reveals five major branches: (a) the classic *KLKs* (*KLKs* 1–3), (b) *KLKs* 4, 5, 7, and 14 and *KLKP1*, (c) *KLKs* 9 and 11, (d) *KLKs* 8, 10, and 15, and (e) *KLKs* 12 and 13, and a separate branch with *KLK6*. The tree (Figure 2) is similar in several aspects to other phylogenetic analyses of this cluster [150, 153, 155–157] but also includes the isoforms and reinforces the idea that all *KLK* genes evolved from a single gene by successive tandem duplications and genomic rearrangements facilitated by repetitive elements.

The high similarity between *KLK2* and *KLK3* sequences and the highest support value also suggest that they might have formed by duplication later in evolution. The data grouping *KLK4*/*KLK5* and *KLK9*/*KLK11* also corroborate

previous studies [153, 156]. The isolated position of *KLK6* in this phylogenetic tree, unlike the findings of other authors, may explain the apparent distance of the remaining family members in respect to normal and pathological functions.

4.2. Kallikrein-Related Peptidases at RNA Level: Transcriptional Regulation Mechanisms. Kallikrein-related peptidase expression is regulated at transcriptional, translational, and posttranslational levels. At the transcriptional level, several response elements (REs) have been identified in the *KLK* promoters such as an estrogen-related receptor γ (ERR γ) response element [158], a GATA binding motif in *KLK1* [159], and functional retinoic acid response elements (RAREs) in

FIGURE 2: Phylogenetic relationships within the human tissue *KLK* gene family in humans. Phylogenetic analysis was performed using the MEGA5 [205] and Maximum Likelihood methods based on the GTR model (*General Time Reversible*) [154] with Gamma distribution. The bootstrap method was used (with 1000 data set replicates) to investigate node robustness [206]. The phylogenetic tree includes 15 *KLK* transcripts, the pseudogene-1 (*KLKP1*) sequence, and the trypsin 1 gene sequence (*PRSSI*) [155, 156]. The sequences were obtained from the NCBI Reference Sequence (RefSeq) database (<http://www.ncbi.nlm.nih.gov/>). Numbers indicate the percentage of 1000 bootstrap replicates at each node in the consensus. Bootstrap value ≤ 95 .

KLK10 [160]. Due to the importance of *KLK3* expression in prostate cancer, a number of REs have already been described for its promoter, including Sp1/Sp3 [161] and WT1 transcription factor-binding sites [162], a putative p53 RE [163], an XBE (X-factor-binding element that binds specifically to the NF-kappaB p65 subunit) in the AREc (androgen response element enhancer core) [164], and androgen-responsive elements (AREs), the last of which were also present in the *KLK2* promoter (reviewed by [127, 165]).

KLK gene expression can also be regulated by epigenetic mechanisms, including histone modifications such as DNA methylation as well as microRNAs (reviewed by [166]), which can affect normal cell physiology and facilitate tumorigenesis if altered. In fact, aberrant promoter methylation leading to

KLK10 downregulation has been described in acute lymphoblastic leukemia [167] as well as in breast [168], gastric [91], and prostate cancer [169]. Similarly, abnormal histone acetylation at *KLK2* and *KLK3* sequences and deregulated expression of miRNAs targeting *KLK* genes have also been reported in kidney, prostate, and breast cancer cell lines (reviewed by [166]).

In addition to epigenetic events, polymorphisms in regulatory sequences can potentially alter RNA transcription rates and protein levels, as was observed for the homozygous G base substitution (rs266882) in the androgen response element (ARE-1) of the *KLK3* promoter [170] and for polymorphic alleles in the 5'-flanking region of the *KLK1* gene [171]. *KLK* gene activity is likewise affected by polymorphisms in

FIGURE 3: Schematic representation of KLK functions related to physiological and pathological conditions. KLKs are involved in several normal processes including blood pressure, coagulation, semen liquefaction, and skin desquamation and can also protect against cardiac injury and ischemia. These proteases may also participate in skin inflammation, neurodegeneration, and autoimmune diseases.

the coding region or in the 3'-UTR and downstream sequences of the *KLK1*, *KLK2*, *KLK3*, and *KLK7* genes (reviewed by [47]).

According to the NCBI Reference Sequence Database (accessed in November 20, 2014), with the exceptions of *KLK1*, *KLK4*, *KLK9*, *KLK13*, and *KLK14*, human *KLK* genes have multiple isoforms. The alternative transcripts apparently are species specific [155], and a number of them are cancer specific (reviewed by [172]), which supports the idea that they are constantly evolving. The diversity of these isoforms, especially those with no peptidase catalytic motifs, may indicate a type of activity control, for example, by competing for the same substrates or performing different tissue-specific functions [155].

4.3. Kallikrein-Related Peptidases at Protein Level. The KLKs are proteins of 230 amino acids and 28 to 33 kDa, although some small isoforms reach only 3 kDa. Their standard tertiary structure consists of two juxtaposed six-stranded antiparallel β -barrels and two α -helices with the active site between the barrels [173, 174]. They are synthesized as preproenzymes, which are proteolytically processed to pro-KLKs and secreted after removal of the terminal signal peptide. Their ability to release kinins was initially viewed as the definition of a true kallikrein. However, besides plasma kallikrein, only *KLK1* has the ability to cleave kininogen (in this case, low molecular weight kininogen) to release kinin. The tissue kallikrein-kinin system can protect against cardiac injury and ischemia/reperfusion-induced cardiomyocyte apoptosis as well as against oxidative stress-induced renal cell apoptosis via stimulation of kinin B2 receptor-Akt [175]. Otherwise, this system appears to be involved in the development of lupus nephritis by increasing local tissue damage triggered by autoimmune inflammation [176] (Figure 3).

As mentioned above, *KLK* promoters have several hormone response elements, and their expression can be regulated by steroid hormones [177]. Therefore, *KLK* levels in different tissues are dependent not only on the presence of specific transcriptional and translational regulators, but also on proteolytic mechanisms, as previously referred to in the degradome section. Shaw and Diamandis [178] detected distinct expression profiles for several kallikrein-related peptidases: *KLK1* was highly expressed in the pancreas and salivary gland, *KLKs* 2, 3 (also observed in seminal plasma), and 11 were highly expressed in the prostate, *KLK5* was expressed in the skin, *KLK6* was expressed in the brain, *KLK9* was expressed in the heart, and *KLK12* was expressed in several anatomical sites. *KLKs* 4, 8, 14, and 15 exhibited a more homogeneous profile or were not detected in various tissues. Komatsu et al. [179] analyzed the skin stratum corneum and identified the presence of many KLKs (*KLKs* 5–8, 10, 11, 13, and 14). Generally, expression patterns are compatible with their origins—duplicate genes have similar expression patterns in the same tissues, and coexpression patterns are compatible with their physiological functions [153].

5. Kallikrein-Related Peptidases and Their Relationship to Health and Disease

5.1. Normal Physiological Processes and Nonmalignant Diseases. Similar to what has been observed for other proteases, several regulatory mechanisms protect tissues from harmful proteolysis by KLKs. In addition to controlled proenzyme activation and endogenous inhibitors (such as α_2 -macroglobulin and serpins), there are also inactivating cleavages and allosteric regulation (reviewed by [165]). Regulatory steps may be performed by other proteases including members of the *KLK* family, which are supported by their

coexpression in the same tissue. For example, a KLK cascade including KLK2, KLK14, and probably other KLKs activates pro-KLK3 to generate the mature proteinase that directly cleaves the semenogelins SgI and SgII resulting in seminal clot liquefaction and spermatozoa release [180]. Recently, Yoon et al. [181] observed that MMP-20, which is usually expressed only in dental enamel, processes the prosequence of nine different KLKs and may be a nonspecific activator of the KLK family in pathological conditions.

Another proteolytic cascade has been described for the skin desquamation process in which KLK5 may be autoactivated or activated by KLK14 at neutral pH and then process KLK7, regulating skin desquamation. This cascade may start by KLK6 autoactivation following the cleavage of KLK11, which in turn activates KLK14. Although not completely understood, skin desquamation also depends on other proteases, including cathepsins, aspartic proteases, urokinase, plasmin, and the inflammatory metalloproteinases. Because KLK regulation is critical for proper desquamation, various endogenous inhibitors participate as attenuators of their activities, mainly LEKTI (serine protease inhibitor Kazal-type 5), a protein encoded by the *SPINK5* gene. Other factors such as an acidic environment and UV irradiation (and resulting inflammation) may inhibit LEKTI, also contributing to increased KLK expression and enhanced desquamation [64]. The lack of LEKTI expression in Netherton syndrome, a rare genetic skin disease characterized by congenital ichthyosis and severe allergic manifestations, indeed results in increased proteolytic activities of KLK5 and KLK7, which trigger an inflammatory process by activating protease-activated receptor-2 (PAR-2) and stimulating cytokine production [70] (Figure 3).

KLK deregulation is also observed in several other pathological conditions, of which neurodegenerative disorders are good examples (Figure 3). Alzheimer's disease (AD) and Parkinson's disease (PD) are the most prevalent human neurodegenerative disorders. Both are caused by the aggregation of proteins: AD is characterized by extracellular deposits of amyloid β (A β) and intraneuronal aggregates of tau protein in specific brain regions, and PD is characterized by intracellular neuronal deposits (Lewy bodies and neurites) formed by insoluble α -synuclein [182, 183].

There is convincing evidence from the literature on Alzheimer's disease that KLK6, the most abundant kallikrein-related peptidase in the central nervous system, cleaves the amyloid precursor protein (APP), a transmembrane glycoprotein from which A β derives. The proteolytic activity of KLK6 against APP and substrates in the extracellular matrix and perineuronal net places this peptidase as a potential component of AD pathogenesis. KLK6 expression is reduced in brain tissues, as well as in cerebrospinal fluid of AD patients [42, 184, 185], but the mechanisms behind these findings and their functional consequences are not yet known. Actually, other enzymes (α -, β -, and γ -secretases) cleave APP in different sites and generate several fragments; some of them are aggregation-prone [183]. KLKs may, for example, promote a bias toward synthesis of these toxic fragments by β - and γ -secretases.

Besides KLK6, the kallikrein-related peptidases 7 and 10 show decreased and increased levels, respectively, in cerebrospinal fluid of AD patients [39]. Recently, Shropshire and collaborators observed that KLK7 is able to cleave the core of A β in vitro, inhibiting A β aggregation and reducing neuronal toxicity [186]. This result may open new opportunities towards treatments for AD.

Several studies on Parkinson's disease have implicated KLK6 in the degradation of intracellular α -synuclein [187]. Recent data suggested that secreted α -synuclein is also involved in the development of PD by affecting neuronal cell viability [188] and activating inflammatory response [189]. Although still controversial with respect to the intracellular type, KLK6 inefficiency in α -synuclein degradation seems to contribute to PD pathogenesis, probably due to an altered trafficking of KLK6 [187, 190] or to the resistance of certain forms of α -synuclein to KLK6-proteolysis [76, 191].

Multiple sclerosis (MS) is another example of neurodegenerative disorder in which KLK6 levels are altered. In MS patients, KLK6 is abundantly expressed and cleaves myelin proteins, resulting in demyelination and oligodendrogliaopathy [192].

As may be noted from AD, PD, and MS data, KLK6 seems to be important for the neuronal homeostasis and survival. However, other kallikrein-related peptidases are probably involved in these processes, as can be deduced from the data on overexpression of KLK1 in epilepsy [193] and on the ability of a set of KLKs (KLK1, KLKs 5–7, and KLK9) to promote neural injury [62].

5.2. Malignant Diseases. As evidenced by the literature, particularly in prostate cancer, KLKs participate in proteolytic pathways that contribute to the neoplastic process (Figure 4). With respect to tumor growth, KLK1 facilitates EGFR and ERK1/2 cascade activation, which is involved in cell proliferation [194]. Similarly, KLK1, KLK2, and KLK3 can regulate tumor growth through IGF-binding protein (IGFBP) degradation, thereby allowing the release of the insulin-like growth factors (IGFs) and proliferative signals. However, a negative regulatory role for KLK3 in cancer has also been suggested because this protease can activate latent transforming growth factor- β (TGF β), a known suppressor of growth and promoter of apoptosis [2].

Recent data have demonstrated that kallikrein-related peptidase 4 and its substrate, promyelocytic leukemia zinc finger protein (PLZF), modulate androgen receptor (AR) and mTOR signaling in prostate cells to regulate cell survival. In fact, KLK4 negatively regulates PLZF, thus preventing its binding and inhibition by AR, which keeps mTORC1 signaling active and ensures cell survival [195].

During neoplastic progression, different KLKs can regulate new vessel formation, which are essential to provide oxygen and nutrients to proliferating cancerous cells. KLKs 1 and 4 stimulate angiogenesis by cleaving kininogen to kinin or activating prometalloproteinases 2 and 9 to their active forms, thereby potentiating extracellular matrix hydrolysis and enabling endothelial cell migration and neovascularization [196–198]. Other kallikrein-related peptidases (KLKs 2 and 4) can stimulate the urokinase plasminogen activator

Tumors have an increased acidic microenvironment resulting from accelerated glycolysis and lactate accumulation and thus low pH in the extracellular space [200]. Because an acidic environment may block the kallikrein inhibitor LEKTI, contributing to increased KLK expression and loss of cellular adhesion in skin desquamation, it is reasonable to consider a similar mechanism during neoplastic dissemination [201]. In fact, the metastatic process is associated with a transition from tightly connected cells to cells with increased motility, namely, the epithelial-mesenchymal transition, where KLKs play important roles. For example, KLKs activate latent TGF β , which induces EMT, and are associated with the loss of E-cadherin in tumor cells and thus with decreased cell-cell adhesion [202]. They also trigger extracellular matrix degradation via prometalloprotease activation and hence promote tissue invasion [2].

The biological processes in which they participate are related to diseases other than cancer but are directly connected with cancer pathways, including cell proliferation, adhesion, inflammation, and apoptosis.

6. Therapeutic Relevance of KLKs

As discussed in previous sections, KLKs have been associated with different pathologic processes, from skin diseases to neurodegenerative disorders and cancer. The progress in our knowledge on all members of this protein family, functions, 3D structures, substrates, and physiological roles, has provided opportunities to develop new therapeutic approaches for different disorders.

KLKs are targeted by several types of inhibitors, including small-molecule inhibitors, antibody-, protein-, and peptide-based inhibitors, KLK-activated prodrugs, interfering RNAs, and immunotherapeutic vaccines (reviewed by [3]). PROST-VAC, for example, is a prostate cancer vaccine consisting of a KLK3 recombinant vector that contains transgenes for three T-cell costimulatory molecules (TRICOM). This vaccine has demonstrated success in inhibiting, with few side effects, cell proliferation and tumor growth and in improving overall survival [203].

Prodrugs activated by KLKs are another strategy that has been investigated. For instance, KLK3-activated peptides have the ability to target the prostate since most KLK3 is expressed in the gland whereas circulating KLK3 is normally inactivated in plasma by endogenous inhibitors [204]. This

drug has overcome the challenge of specificity, although similar successful results are not always achieved. The reasons for that include the fact that the active sites of members of KLK family are conserved, which hampers drug design. The resolution of 3D structure of KLKs should help in this regard. However, KLKs also have overlapping and even opposing actions, which certainly depend on the physiologic, tissue, and disease context [203].

7. Conclusions

The KLK network is impressive. Its intricate signaling pathways and protein interactions strongly show that this group of proteases contributes to normal and pathological metabolisms. However, despite being intensively studied, there are many gaps in our knowledge on the molecular aspects of the KLK family. For example, there is no doubt that KLK expression deregulation participates in the development of neurodegenerative disorders. But what exactly is its role? Would it be a primary and direct one, promoting erroneously protein degradation, which results in pathogenic fragments? Or would it be one that implies cooperating with specific secretases and other enzymes to generate toxic deposits?

In cancer, it is not clear whether KLKs alterations are driver mutations or deleterious passenger mutations. The fact that similar sets of KLKs are associated with different tumor types and facilitate proliferation, migration, and other cancer hallmarks aligns with driver mutations. Differently, antiproliferative effects of KLKs and similar regulatory factors for different members of this family may argue in favor of random passenger mutations. However, both statements are not mutually exclusive and may occur simultaneously or sequentially. In fact, the idea of sequential occurrence is interesting: considering the complexity of human proteolytic system, it is reasonable to assume that the expression of specific KLKs may counteract the under- or overexpression of other KLKs or enzymes or even that those KLKs are activated, one after the other, to neutralize the expression of a driver mutation, but without success. The analysis of KLK panels in large sets of samples from diverse stages of the disease, including premalignant phases, will probably help to reveal how the expression profile evolves during the course of the disease.

Many questions are still unanswered and the scenario is therefore incomplete. Many more data are necessary to improve our understanding on the function, substrates, and role of KLKs in health and disease in order to distinguish in each case whether they are heroes, villains, or supporting actors.

Conflict of Interests

The authors declare that they have no conflict of interests.

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RESEARCH ARTICLE

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Euphorbia tirucalli modulates gene expression in larynx squamous cell carcinoma

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Abstract

Background: Some plants had been used in the treatment of cancer and one of these has attracted scientific interest, the *Euphorbia tirucalli* (*E. tirucalli*), used in the treatment of asthma, ulcers, warts has active components with activities scientifically proven as antimutagenic, anti-inflammatory and anticancer.

Methods: We evaluate the influence of the antitumoral fraction of the *E. tirucalli* latex in the larynx squamous cell carcinoma (Hep-2), on the morphology, cell proliferation and gene expression. The Hep-2 cells were cultivated in complete medium (MEM 10 %) and treated with *E. tirucalli* latex for 1, 3, 5 and 7 days. After statistically analyzing the proliferation of the tested cells, the cells were cultivated again for RNA extraction and the Rapid Subtractive Hybridization (*RaSH*) technique was used to identify genes with altered expression. The genes found using the *RaSH* technique were analyzed by Gene Ontology (GO) using Ingenuity Systems.

Results: The five genes found to have differential expression were validated by real-time quantitative PCR. Though treatment with *E. tirucalli* latex did not change the cell morphology in comparison to control samples, but the cell growth was significantly decreased. The *RaSH* showed change in the expression of some genes, including *ANXA1*, *TCEA1*, *NGFRAP1*, *ITPR1* and *CD55*, which are associated with inflammatory response, transcriptional regulation, apoptosis, calcium ion transport regulation and complement system, respectively. The *E. tirucalli* latex treatment down-regulated *ITPR1* and up-regulated *ANXA1* and *CD55* genes, and was validated by real-time quantitative PCR.

Conclusions: The data indicate the involvement of *E. tirucalli* latex in the altered expression of genes involved in tumorigenic processes, which could potentially be applied as a therapeutic indicator of larynx cancer.

Keywords: Differential gene expression, Cell culture, Subtractive hybridization, Quantitative PCR

Background

The head and neck squamous cell carcinoma (HNSCC) represents malignant tumors arising primarily in the oral cavity, tongue, floor of the mouth, tonsils, pharynx and larynx. Worldwide HNSCC is the fifth most common cancer and it accounts for about 6 % among all cancers [1]. This tumor originates from various anatomic structures

including the craniofacial bones, soft tissues, salivary glands, skin, and mucosal membranes [2].

The main risk factors for larynx squamous cell carcinoma (LSCC) are smoking and alcohol consumption [3]. Family history is another important risk factor for this cancer, suggesting that genetic factors may contribute to susceptibility of this disease [4]. Others aspects that can modify cancer risk factors of the head and neck are the polymorphisms in drug metabolizing enzymes (DMEs) and prevalence of variant genotypes of cytochrome P450 (CYP) 1A1, 1B1, 2E1 or glutathione-S-transferase M1 (null). Furthermore, strong associations of the polymorphic genotypes of DMEs with cases of pharyngeal

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and oral cavity cancer who were tobacco chewers or alcohol users demonstrate that gene-environment interactions may explain differences in genetic susceptibility for cancers of the oral cavity, pharynx and larynx [5].

Some authors have reported that DNA alterations are responsible for the onset of carcinogenesis that usually occur in genes involved with proteins regulating cell cycle, cell growth, and differentiation. These are classified as oncogenes and tumor suppressor genes [6]. In HNSCC, the oncogenes *H-ras*, *c-myc*, *EGFR* and *TGF- β* are often activated and the tumor suppressor genes such as *TP53*, *p16*, *FHIT*, *PTEN*, *Rb* and *APC* are inactivated [7, 8].

Other factors that may contribute to LSCC include diet, oral hygiene, body mass, environmental pollution, certain working conditions associated with industries such as metallurgy and petrochemical and infections by human papillomavirus - HPV, particularly types 16 and 18 and Epstein-Barr virus which appear to be associated with the development of more aggressive tumors [7, 9–11]. The treatment for this neoplasm is defined depending on the type of cell, degree of differentiation, location, extent, presence of lymph node metastases, and macroscopic characteristics with bone and muscle tumor involvement. Conventional methods used for treatment are surgery, chemotherapy, and radiation therapies, but all these methods are considered highly invasive. Surgery can cause irreversible aesthetic injuries with possible functional alterations [12]. However, some plants have been used in the treatment of cancer as alternative therapies in an attempt to soften the damage caused by conventional methods. The *Euphorbia tirucalli* (*E. tirucalli*), popularly known as aveloz, which is used in the herbal treatment of asthma, ulcers, warts and tumors in general has attracted scientific interest [13, 14].

Originating from Africa, *E. tirucalli* belonging to the family Euphorbiaceae, was subsequently introduced in other countries, it has some compounds that have been scientifically proven to have biological activities such as antiviral, anticancer for breast, antibacterial, anti-inflammatory, antimutagenic, antitumor and larvicidal [13, 15–18]. In addition, some authors have reported that a lectin isolated from the latex of *E. tirucalli*, called Eutirucallin showed biological activities such as neutrophils recruitment and cytokine production by macrophages [19]. An alternative that justifies its antitumorigenic potential is antimutagenicity. Substances with antimutagenic potentials increase the efficiency of repair mechanisms thereby acting as protective agents that decrease the frequency of DNA damage while controlling the disorderly proliferation of tumor cells [17].

The euphol (tetracyclic triterpene alcohol isolated from the latex of *E. tirucalli*) showed anticancer effects, inhibiting the growth of human gastric cancer cells and tumor development, demonstrating its great value and

potential for use in disease therapy [20]. Anti-inflammatory properties of euphol was observed in skin diseases, because it markedly inhibited TPA (12-O-tetradecanoylphorbol-13-acetate)-induced inflammatory response, demonstrating that euphol can be used as an alternative therapy in skin diseases [21].

To investigate the effects of *E. tirucalli* in LSCC, we evaluated the influence of this herbal medicine on the morphology, cell proliferation and gene expression in Hep-2 cells. We also identified the signaling pathways where genes modulated by *E. tirucalli* participate, which are important for tumorigenic and the inflammatory process.

Methods

Hep-2 culture conditions

The Hep-2 cell line originally established from an epidermoid carcinoma of the larynx (ATCC, Rockville, Maryland, USA), was obtained and seeded at a density of 1×10^6 cells/ml per 75 cm² culture flask (Corning, NY, USA). Minimum Essential Medium (MEM - Cultilab, Campinas, SP, Brazil) was used at pH 7.5 and supplemented with 10 % fetal calf serum (Cultilab), 1 % non-essential amino acids and 1 % antibiotic/antimycotic (Invitrogen Corporation, Carlsbad, CA, USA) and cultured at 37 °C under an atmosphere of 5 % CO₂.

Pharmacological treatments

The Hep-2 cells were subdivided into three groups: a) control that was not manipulated, b) the group that was submitted to the addition of vehicle (ethanol) and c) an experimental group where *E. tirucalli* latex was added at concentrations of 0.25 to 250 μ g/ml. The experiments were conducted for periods of 1, 3, 5, and 7 days to determine the statistically significant time for further use in the methodology, for all the three groups.

The sap from *E. tirucalli* was initially extracted with hexane through an incision in the trunk and branches of the adult plant, and the resulting precipitate was extracted with n-butanol. The active fraction of this latex, diluted in this polar solvent, was used to treat the cells. In this butanol fraction of the *E. tirucalli* latex, ingenol-3-hexanoate was used to obtain a useful composition for the treatment of disease. Synthesis and modifications were done by Kyolab Laboratories (Campinas, Brazil), by Dr. Luiz Francisco Pianowski.

Separation of the butanol fraction by HPLC (High Performance Liquid Chromatography) allowed the compounds present to be taken off in group scales, mainly by size. In a column chromatography separation with silica gel Sephadex G75, using a mixture of hexane:ethyl acetate (0 to 100 %), eight fractions were separated from the butanol fraction of the latex extract. But one of them show the best presence of components with higher polarity, with solubility and affinity characteristics with the

substrate, then this fraction was submitted to tests to verify its anticancer action, as disclosed in the “patent of the active fraction of a polar solvent extract from the latex of Euphorbiaceae plants” (Additional file 1) and results in decreased growth of seven cancer cell lines [22].

Growth curve

Hep-2 cells were seeded in triplicate at a density of 5×10^4 cells in plastic 6-well plates and divided in three groups. The control group was grown on complete medium, the other groups were submitted to the addition of vehicle (ethanol), whereas *E. tirucalli* latex was added to the experimental group. Twenty four (24) hours later, when the cells had already adhered, Hep-2 cultures were incubated with serum free medium (MEM 0 %). After an additional 24 h, the control group was maintained in complete medium without any pharmacological manipulation, whereas vehicle (ethanol) was added to the other groups and *E. tirucalli* latex was added to the experimental group.

The cell morphology was observed on each day. The cytotoxic effects of the *E. tirucalli* on the proliferation of Hep-2 cells were investigated in three experimental groups at 1, 3, 5 and 7 days. The cells were harvested, stained with trypan blue and counted using the Countess® Automated Cell Counter (Invitrogen®). Significant differences between the groups were determined by one-way analysis of variance (GraphPad Prism 5) and statistical significance was assumed when $P < 0.05$. The same experiment was repeated twice.

RNA extraction for Rapid Subtraction Hybridization (RaSH) and real time PCR experiments

Hep-2 cells were seeded at a density of 1×10^6 cells/ml in 75 cm² culture flasks in complete medium (control group), vehicle (ethanol) was added and they were incubated with *E. tirucalli* latex for 3 days (experimental group). The RNA from Hep-2 cells (three groups) was extracted using Trizol [23] following treatment with DNase (Invitrogen). cDNA synthesis was performed using a High Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions.

Rapid Subtraction Hybridization (RaSH)

The RaSH technique [24] was performed with two subtractions. The first was termed “Sub I”, in which the Hep-2 control cells were referred to as *tester* and the cells treated with *E. tirucalli* latex were termed *driver*. In the second subtraction, “Sub II,” the opposite test was performed.

The first cDNA strand was obtained by reverse transcriptase with 2 µl of oligo (dT) (50 mM) and 2 µl of 10 mM dNTPs at 65 °C for 5 min. The samples were immediately placed on ice for 1 min and 2.0 µl of 0.1 mM DTT, 1 µl of 40 U/µl RNA-OUT and 4.0 µl of 5X First-

Strand Buffer [250 mM Tris-HCl (pH 8.3), 375 mM KCl, 15 mM MgCl₂] were added. The samples were incubated at 42 °C for 2 min and 1.0 µl of Superscript™ II reverse transcriptase (RT) (200 U/µl) was added. The samples were incubated again at 42 °C for 50 min and then at 70 °C for 15 min. Segments of β-actin cDNA were also amplified to verify the quality of these reactions.

The second strand of cDNA was synthesized in reactions containing 30 µl of 5X Second Strand Buffer, 3 µl of 10 mM dNTPs, 1 µl of DNA ligase (10 U/µl), 4 µl of DNA Pol I (10 U/µl) and 1.0 µl of RNase H (2 U/µl). The samples were incubated for 2 h at 16 °C before the addition of 2 µl of T4 DNA Pol (5 U/µl) and further incubation for an additional 5 min. The action of the enzyme T4 DNA Pol was inhibited by the addition of 10 µl of 0.5 M EDTA. Phenol/chloroform extraction was used to purify the sample and the pellet was resuspended in 45 µl of water. The cDNA was used in amplification reactions for segments of GAPDH and was subsequently digested with 2 µl of MboI enzyme (10 U/µl) and incubated for 1 h at 37 °C. Finally, over 1 µl of this enzyme was added to the reaction, and the samples were incubated overnight at 37 °C.

A volume of 4.1 and 4.5 µl, of the adapters XDPN-14 5'-CTGATCACTCGAGA and XDPN-12 5'-GATCTCTC-GAGT (Sigma Chemical, final concentration 10 mM) was added to the cDNA respectively, 8 µl of 5X T4 DNA ligase buffer Invitrogen®, heated at 55 °C for 1 min, and cooled down to 14 °C within 1 h was also added. The cDNA received 3 µl (9 U) of T4 DNA ligase (3 U/µl), ligation was carried out overnight at 14 °C. After phenol/chloroform extraction and ethanol/glycogen precipitation, the mixtures were diluted to 100 µl with TE buffer (10 mM Tris/1 mM EDTA); 40 µl of the mixtures were used for PCR amplification.

The PCR mixtures were set up using 10 µM XDPN-18 5'-CTGATCACTCGAGAGATC, 0.4 mM dNTPs, 10 × PCR buffer, 1.5 mM MgCl₂ and 1U Taq DNA polymerase (Invitrogen). Thermocycler conditions were one cycle at 72 °C for 5 min, followed by 25 cycles of 94 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min, ending in a final extension at 72 °C for 3 min. Ten (10 µg) micrograms of purified PCR product (tester) was digested with 10 U/µl XhoI (Invitrogen) for 6 h at 37 °C and followed phenol/chloroform extraction and ethanol precipitation.

One-hundred nanograms of the tester cDNA were mixed with 5 µg of the driver cDNA in hybridization solution (0.5 M NaCl, 50 mM Tris/HCl, SDS 2 % and 40 % formamide) and, after heating at 99.9 °C for 5 min, they were incubated at 42 °C for 1 h, and 42 °C for 48 h. After extraction and precipitation, the hybridization mixture (1 µg) was ligated with XhoI-digested pZero plasmid and transformed into competent bacteria. Bacterial colonies were picked and used as DNA

template for PCR. Clones were sequenced using an automated DNA sequence and homologies sequences were searched using the BLAST program. Gene ontology (GO) annotation was used for the functional classification of up- and down-regulated genes using terms from Gene Ontology database.

The genes were further analyzed through Ingenuity Pathway (IPA - Ingenuity Systems®) to relate the differentially expressed genes with biological functions and processes in which these genes are involved and evaluate the main networks of interaction between them.

Quantitative PCR - RT-qPCR

The RNA from control, vehicle (ethanol) and *E. tirucalli* latex treated Hep-2 cells was extracted and cDNA synthesis was performed as described above.

Five differentially expressed genes were selected for validation by quantitative real time PCR experiments according to their direct or indirect involvement in tumorigenesis. Their expression was checked in treated samples relative to matched non-treated samples.

The primers were manually designed with: 19–23 bp length, 30–80 % GC content and a short amplicon size (80–120 bp). Their sequences are shown in Table 1. Real-time PCR was performed in triplicate using a 7500 Fast Real-Time PCR System (Applied Biosystems). Reaction mixture consisted of a 20 µl volume solution containing 10 µl of Power SYBR Green PCR Master Mix (Applied Biosystems).

Reaction mixture consisted of a 20 µl volume solution containing 100 and 500 ng of Power SYBR Green PCR Master Mix (Applied Biosystems), 100 nM of each primer and 100 ng cDNA (control and treated with *E. tirucalli* latex). The PCR conditions were 95 °C for 10 min followed by 40 cycles of 95° for 15 s and 60° for 1 min. Melting curve analysis was performed for each gene to check the specificity and identity of the RT-PCR products. For each primer set, the efficiency of the PCR

reaction (linear equation: $y = \text{slope} + \text{intercept}$) was measured in triplicate on serial dilutions of the same cDNA sample. The PCR efficiency (E) was calculated by the formula $E = [10^{(-1/\text{slope})}]$ and ranged from 1.96 to 2.02 in the different assays.

Two control genes (*GAPDH*, *ACTB*) were used as internal standards. The relative expression ratio (fold change) of the target genes was calculated according to Pfaffl [25]. Statistical analysis was performed by a two-tailed unpaired *t* test using GraphPad prism software.

Results

Cellular morphology

Hep-2 cells were initially cultured and observed by phase contrast microscopy. These cells have a fusiform appearance and central core, with multiple nucleoli and a eucromatic position, which are characteristics of a cell with high metabolic activity. In cells treated with *E. tirucalli* latex [25 µg/ml], there was no morphological change, and cytoplasmic processes were more clearly observed due to reduced cell proliferation (Fig. 1a and b).

Proliferation assay

In the proliferation assay (Fig. 1c), it was possible to observe the growth inhibitory effect under treatment with *E. tirucalli* latex, using the GraphPad Prism 5 (GraphPad Software). *E. tirucalli* was shown to decrease the proliferation in treated cells in comparison to the control group. Statistical analysis showed that these differences were most significant ($P < 0.05$) at a concentration of 25 µg/ml *E. tirucalli* and a culture time of 3 days.

We also observed, using the same statistical test, that the addition of ethanol reduced cell proliferation, however, they did not exhibit any statistically significant difference compared to control group (Fig. 1d), confirming that the reduced growth of Hep2 cells was due to *E. tirucalli* and not its diluent's (ethanol), (Additional files 2 and 3).

Genes identified using the RaSH approach

Rapid subtractive hybridization (*RaSH*) compares two distinct experiments aiming to find differential gene expression. *RaSH* was performed on Hep-2 cells from the control group and those treated with *E. tirucalli* latex for 3 days and 85 clones were isolated and sequenced. By screening these sequences through GenBank (*Blast*) and by rigorous classification and selection, 19 genes were found to be differentially expressed, and two libraries of genes (Sub I and Sub II) were acquired. Fifteen genes of the "Sub I" library exhibited changes in expression levels in the control cells, whereas four genes of the "Sub II" library changed in expression under treatment with *E. tirucalli* latex. Therefore, it was expected that the gene expression of one library would be lower than that of the other one. The libraries of the genes obtained

Table 1 Primers for the selected genes. *ANXA1*, *ITPR1*, *CD55*, *NGFRAP1* and *TCEA1* primers to quantitative PCR validation

Primer	Sequence
ANXA1 antisense	5' TAAGGGTGACCGATCTGAGG 3'
ANXA1 sense	5' ACGTCTGTCCCTTCTCTCT 3'
ITPR1 antisense	5' GCTCTATGAGCAGGGGTGAG 3'
ITPR1 sense	5' GGAACACTCGGTCACTGGAT 3'
CD55 antisense	5' CAGCACCACCACAAATTGAC 3'
CD55 sense	5' TGCTCTCCAATCATGGTGAA 3'
NGFRAP1 antisense	5' GGGGAGCTCTCTAATCACC 3'
NGFRAP1 sense	5' AAAGAAACAGCGGGAATCA 3'
TCEA1 antisense	5' AGCTGAAAGAGATGCGGAAA 3'
TCEA1 sense	5' TGCCACATGTGAACAAGTCA 3'

Fig. 1 Morphological analysis and growth curve of *E. tirucalli* latex treatment in Hep-2 cell line. Hep-2 cells were seeded in complete MEM 10 % at a concentration of 5×10^4 per well (6 well plate). The cell morphology observed in (a) the control group, characterized by a monolayer of nucleated cells was not altered in (b) the *E. tirucalli* latex [25 µg/mL] treated group for 3 days. 400 $\times$ magnification. The growth curve observed to Hep-2 cells treated with (c) *E. tirucalli* latex [0.25, 2.5, 25, 250 µg/mL] for 1, 3, 5 and 7 days and (d) ethanol (vehicle) and *E. tirucalli* latex [25 µg/mL] for 1, 3, 5 and 7 days. Assays were performed in triplicate. Graph x = time (hours) and y = number of cells $\times 10^4$. P values <0.05 were significant

along with their different functions and processes are described in the Gene Ontology (GO) databases.

IPA was used to further analyze the 19 differentially expressed genes, which were especially associated with tumorigenesis, apoptosis, cell proliferation, cell cycle, DNA repair, complement activation and inflammatory response (Table 2). Moreover, some of these genes were

chosen because they form a network of important interactions with other genes such as *NF-kB*.

Five differentially expressed genes, *ITPR1*, *ANXA1*, *TCEA1*, *CD55* and *NGFRAP1*, were selected for validation by quantitative real-time PCR experiments. These genes are associated with calcium ion transport, inflammatory response, DNA repair, complement

Table 2 Genes selected by *RaSH* in the subtractive library I expressed in Hep-2 cell line. NM is the access number in GenBank (www.ncbi.nlm.nih.gov)

NM	Name	Abbreviation	Chromosomal location	Functions and processes
NM_001099952.2	Homo sapiens inositol 1,4,5-trisphosphate receptor, type 1	ITPR1	3p26.1	Protein binding; calcium ion transmembrane transporter activity; intrinsic apoptotic signaling pathway and signal transduction.
NM_000700.1	Homo sapiens annexin A1	ANXA1	9q21.13	Calcium ion binding; regulation of cell proliferation; inflammatory response; cell surface receptor signaling pathway; negative regulation of apoptotic process.
NM_006756.2	Homo sapiens transcription elongation factor A (SII), 1	TCEA1	8q11.2	DNA repair; positive regulation of transcription from RNA polymerase II promoter and gene expression.
NM_000574.3	Homo sapiens <i>CD55</i> molecule, decay accelerating factor for complement	<i>CD55</i>	1q32	Enzyme inhibitor activity; elevation of cytosolic calcium ion concentration and regulation of complement activation.
NM_206917.1	Homo sapiens nerve growth factor receptor (TNFRSF1) associated protein 1	NGFRAP1	Xq22.2	Protein binding; induction of apoptosis.

activation and apoptotic process respectively. They were chosen because they are directly or indirectly involved in tumorigenic and inflammatory processes (Table 2 and Fig. 2).

Real-time PCR validation of differentially expressed genes

The RaSH analysis indicated the down-regulation of one gene (*ITPR1*) and up-regulation of four genes (*ANXA1*, *TCEA1*, *CD55* and *NGFRAP1*) in Hep-2 cells treated with *E. tirucalli* latex. The quantitative PCR experiment showed that only the expression of *ITPR1* was significantly reduced, however the expression of *CD55* and *ANXA1* genes was significantly increased in Hep-2 cells after treatment with *E. tirucalli* latex (Fig. 3). The expression of other genes oscillated without statistical significance, which was most likely because the *E. tirucalli* does not quantitatively modify the expression of the selected genes despite its interaction with the cell (Additional file 4).

Discussion

The active fraction present in the *E. tirucalli* latex that was used in our experiments inhibits proliferation of tumor-related enzymes and presents potential anti-inflammatory and analgesic effects [26]. Also, due to the importance of its antitumorigenic action, we analyzed the effect of herbal medicine in the cell line of laryngeal squamous cell carcinoma (Hep-2) and it was observed that the morphology did not change after treatment with *E. tirucalli* latex, however, cell proliferation decreased significantly. Other authors corroborate these results, they also observed that treatment with *E. tirucalli* in cell line of breast cancer did not alter the morphology of these cells independent on the vehicle [15]. Regarding the reduced proliferation, *E. tirucalli* must have acted in tumorigenic cells causing cell death and also causing the growth of these cells to be reduced. In vitro, it is known

that latex works in the cell colonies fighting breast cancer and gastric cancer [19, 27].

The *E. tirucalli* latex effect was also analyzed in the gene expression, for the first time, by RaSH that isolates differentially expressed genes including new genes and rare transcripts as can be seen in some researches [28] and others who use this technology [29]. It is possible to generate significant genomic libraries that can assist in the search for genes differentially expressed in treated Hep-2 cells with herbal cell lines which is faster and less costly than other alternative hybridization techniques [24].

The results showed changes in the expression of some genes in the cells of LSCC after treatment with *E. tirucalli* latex, including *ANXA1*, *TCEA1*, *NGFRAP1*, *ITPR1* and *CD55* genes. These genes belong to the same gene signaling network related by software IPA, correlating the tumorigenic and inflammatory processes. Other authors used the software IPA to identify the biological activities of the genes that distinguish between the thermal properties, hot (warm effects, disperse cold) or cold (cooling effect, removes heat) of Chinese medicinal herb. It was demonstrated that the main biological gene networks in the herbs with hot properties include inflammation and immunity regulation while the genes of the herbs with cold properties affect cell growth, proliferation and development [30].

The selection of the validated *ITPR1* gene (inositol 1,4,5-trisphosphate receptor, type 1) can contribute significantly to the understanding of processes including cancer of the larynx since it has functions related to apoptosis, tumorigenesis of cells squamous and cell proliferation. Inositol 1,4,5-trisphosphate receptor type1 (IP3R1) is known as an IP3-gated Ca^{2+} channel that is located on the endoplasmic reticulum (ER). The ER stores intracellular Ca^{2+} and it has been reported to play a critical role in a variety of cell functions including cell

proliferation, contraction of smooth muscle and apoptosis by pumping out Ca^{2+} from ER to cytosol [31–33].

We observed reduced *ITPR1* gene expression in Hep-2 cells after treatment with *E. tirucalli* latex. Other project showed that IP3 receptors act as in vivo substrates for Akt kinase, which plays a key role in suppressing apoptosis [34]. According to our tests, it can be inferred that the *E. tirucalli* latex decreases the expression of *ITPR1* gene by restricting the action of the enzyme Akt kinase and activating apoptosis, decreasing cell growth of LSCC since these receptors are substrates for the action of this enzyme.

Another gene studied was *ANXA1* (Annexin 1), there was increased expression in Hep-2 cells after treatment with *E. tirucalli* latex. This gene is important for the carcinogenic process such as cell cycle control, regulation of apoptosis, cell proliferation and growth, inflammatory response and calcium ion transport regulation [35]. The annexin family consists of ubiquitous proteins that interact with phospholipids in a Ca^{2+} -dependent manner. Each annexin consists of an N-terminal domain and a typical core domain. The annexin core is conserved among all members of the annexin family. In contrast, N-terminal domains are variable in length and are believed to regulate annexin functions, forming a truncated molecule with altered sensitivity to calcium [36].

Changes in the expression of *ANXA1* have been reported in various cancers and, furthermore, its expression has been compared to that of other proteins. This scenario prompted Jorge et al. [37] to investigate the relationship between the gene and protein expression of annexin-A1 (*ANXA1/AnxA1*) and galectin-1 (*LGALS1/Gal-1*) in an inflammatory gastric lesion as chronic gastritis (CG) and gastric adenocarcinoma (GA) and its association with *H. pylori* infection. High *ANXA1* mRNA expression levels were observed in 90 % of CG cases and in 80 % of GA cases. However, *LGALS1* mRNA levels were high in 60 % of the GA cases, while low expression was found in CG. These results have provided evidence that galectin-1 and mainly annexin-A1 are overexpressed in both gastritis and gastric cancer, suggesting a strong association of these proteins with chronic gastric inflammation and carcinogenesis [37].

The expression of *ANXA1* was evaluated in larynx cancer and it was observed that this protein plays a regulatory role in Hep-2 cells, decreasing the growth of these cells [38]. We infer that the *E. tirucalli* latex contributes to increased expression of the gene *ANXA1* in the larynx squamous cell carcinoma, confirming the role of Annexin A1 in the regulation of cell proliferation, besides the anti-inflammatory function.

The gene *CD55* (decay accelerating factor) presents functions related with squamous tumor cells, apoptosis, cell growth and proliferation, inflammatory response,

calcium ion transport regulation and innate immune response, according to the reported data Gene Ontology (GO).

This gene is a key regulator affecting all three complement activation pathways and encoding a globular glycoprotein known as DAF - decay accelerating factor. DAF inhibits the activation of C3 and C5 by preventing the formation of new and accelerating the decay of pre-formed C3 and C5 convertases which can mediate the downstream effect of all 3 complement-activation pathways, therefore, *CD55* functions to protect host cells from complement attack and inhibits amplification of the complement cascade [39].

CD55 is an anti-inflammatory and immunological anti-adhesive molecule implicated in the resolution of ongoing inflammation of mucosal epithelia through clearance of transmigrating neutrophils [40]. In our tests, it was demonstrated that the expression of *CD55* was elevated in cell lines of laryngeal squamous cell carcinoma after treatment with *E. tirucalli* latex, confirming its anti-inflammatory role.

The genes evaluated in this study showed differential expression in Hep-2 cells in a manner dependent on *E. tirucalli* latex treatment. This study highlights the importance of the *RaSH* technique as a fast and efficient method for identifying genes modulated by *E. tirucalli* latex in cells of epithelial origin. The functions of the genes identified in this study are important in tumor progression and are amenable to changes through interactions with the metabolism of the Hep-2 cells. The treatment for larynx carcinoma commonly involves surgery, chemotherapy or radiotherapy. However, new therapeutics such as the use of gene therapy associated with nanoparticles tested in larynx cancer derived Hep-2 cells are being developed, allowing the delivery of drugs into target cells.

Furthermore, the *E. tirucalli* latex alters the proliferation and gene expression of Hep-2 cells. These changes are most likely related to inflammatory and tumorigenic processes. Thus, we suggest that the this herb has modulatory effects on the metabolism of this cell type. Our findings suggest the importance of future studies using Hep-2 cells for the development of new therapies. These studies should focus on gene activities that involve the action of *E. tirucalli* and applications of this herb as a possible therapeutic in the treatment larynx carcinoma.

Conclusions

These findings show that the *E. tirucalli* latex alters the expression of genes with roles in signaling cascades involved in the inflammatory and tumorigenic processes. *E. tirucalli* latex was also shown to alter the proliferation of Hep-2 cells. Thus, our results point to possible applications of this herb as an indicator of innovative therapies for the treatment of LSCC.

Ethics approval and consent to participate

Not applicable in this section.

Consent for publication

Not applicable in this section.

Additional files

Additional file 1: Patente *Euphorbia tirucalli*. (PDF 96 kb)

Additional file 2: Excel spreadsheet about Figure 1C – raw data on cell count. (XLS 41 kb)

Additional file 3: Excel spreadsheet about Figure 1D – raw data on cell count. (XLS 23 kb)

Additional file 4: Excel spreadsheet about Figure 3 – raw data on quantitative PCR. (XLS 74 kb)

Abbreviations

ACTB: beta-actin; AKT: protein kinase; ANXA1: annexin A1; APC: adenomatous polyposis coli; BLAST: basic local alignment search tool; C3: C3 convertase enzyme; C5: C5 convertase enzyme; Ca²⁺: calcium; CD55: decay accelerating factor for complement; cDNA: complementary DNA; CG: chronic gastritis; c-myc: myelocytomatosis oncogene; CO₂: carbon dioxide; CYP1A1: cytochrome P450, family 1, subfamily A, member 1; CYP1B1: cytochrome P450, family 1, subfamily B, polypeptide 1; CYP2E1: cytochrome P450, family 2, subfamily E, member 1; DAF: decay accelerating factor; DMEs: polymorphisms in drug-metabolizing enzymes; DNA: deoxyribonucleic acid; DNA Pol I: DNA Polymerase I; DNase: deoxyribonuclease; dNTP: deoxynucleotide - solution mix; DTT: DL-Dithiothreitol; EDTA: ethylenediaminetetraacetic acid; EGFR: epidermal growth factor receptor; ER: endoplasmic reticulum; FHIT: fragile histidine triad; GA: gastric adenocarcinoma; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; GC: guanine and cytosine; GO: gene ontology; Hep-2: cells epidermoid carcinoma of the larynx; HNSCC: head and neck squamous cell carcinoma; HPLC: high performance liquid chromatography; HPV: human papilloma virus; H-ras: harvey rat sarcoma virus oncogene homolog; IP3: inositol 1,4,5-triphosphate; IP3R1: inositol 1,4,5-trisphosphate receptor type1; IPA: ingenuity pathway - ingenuity systems®; ITPR1: inositol 1,4,5-trisphosphate receptor, type 1; KCl: potassium chloride; LGALS1/Gal-1: galectin-1; LSCC: larynx squamous cell carcinoma; Mbol: enzyme gene from *Moraxella bovis*; MEM: minimum essential medium; MgCl₂: magnesium chloride; min: minutes; mL: milliliter; mM: millimolar; mRNA: messenger RNA; NaCl: sodium chloride; NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells; ng: nanogram; NGFRAP1: nerve growth factor receptor (TNFRSF16) associated protein 1; nM: nanomolar; p16: cyclin-dependent kinase inhibitor 2A; P450: cytochrome P450; pb: base pairs; PCR: polymerase chain reaction; PTEN: phosphatase and tensin homolog; RaSH: rapid subtraction hybridization; Rb: retinoblastoma; RNA: ribonucleic acid; RNase H: ribonuclease H; RT: reverse transcriptase; RT-qPCR: quantitative PCR; SDS: sodium dodecyl sulfate; Sub I: subtraction I - tester = Hep-2 control cells (no treatment) versus driver = Hep-2 cells treated with aveloz; Sub II: subtraction II - tester = Hep-2 cells treated with aveloz versus driver = Hep-2 control cells (no treatment); T4 DNA Pol: T4 DNA Polymerase; TCEA1: transcription elongation factor A protein 1; TE: buffer Tris-EDTA; TGF-β: transforming growth factor beta; TP53: tumor protein p53; TPA: 12-O-tetradecanoylphorbol-13-acetate; Tris-HCl: hydroxymethyl aminomethane hydrochloride; Trizol: monophasic solution of phenol and guanidine thiocyanate; U: units; Xhol: enzyme gene from *Xanthomonas holcicola*; µg: microgram; µL: microliters; µM: micromolar.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

GBF-S carried out the cell culture experiments, RaSH assay, real-time PCR assay, performed the statistical analysis, and drafted the manuscript. JP assisted with the cell culture experiments and RaSH assay. ARDS and WAS Jr assisted with the RaSH assay. BRC assisted with the real-time PCR assay. EHT and SMO were

involved in drafting the manuscript or revising it critically for important intellectual content. FCR-L conceived of the study, and participated in its design and coordination and helped to draft the manuscript. All of the authors read and approved the final manuscript.

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