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CROSSTALK CELULAR: INFLUÊNCIA DO CARCINOMA ESPINOCELULAR ORAL SOBRE FIBROBLASTOS

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Linha de pesquisa: Câncer Bucal

Dissertação de Mestrado apresentada ao Programa de Pós-Graduação em Odontologia da Universidade Federal do Rio Grande do Sul como requisito a obtenção do título de Mestre em Odontologia.

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Orientador: Prof. Dr. Marcelo Lazzaron Lamers

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“Becoming is never giving up on the idea that there’s more growing to be done.”

Michelle Obama

RESUMO

O carcinoma espinocelular oral (CEC) é o tipo mais frequente de câncer na cavidade oral. É caracterizado histopatologicamente por células epiteliais que invadem o tecido conjuntivo adjacente e por apresentar um microambiente extremamente complexo e diversificado, contendo diversos fatores químicos, físicos e biológicos que influenciam na progressão do tumor. Um dos diversos fatores que caracteriza a alteração do microambiente tumoral é a composição da matriz extracelular (MEC), a qual apresenta maior quantidade de colágeno nos tumores e, assim, pode influenciar em sua progressão. Dentre os componentes do microambiente tumoral, tem sido relatado na literatura que fibroblastos normais (NOFs) adquirem um fenótipo alterado, demonstrando ter maior proliferação e maior migração promovendo mudanças composicionais na MEC, assim sendo caracterizados de fibroblastos associados ao câncer (CAFs). Os CAFs influenciam na progressão e invasão tumoral, porém a sua origem ainda não está bem estabelecida. Neste trabalho, avaliamos o comportamento de NOFs quando associados aos fatores liberados por queratinócitos e células de diferentes graus de agressividade de CEC. Para tal finalidade foram realizados ensaios *in vitro* utilizando cinco diferentes linhagens celulares: fibroblastos primários de mucosa oral normal, queratinóticos (HaCat), CEC pouco (Cal27) ou muito (SCC9 e SCC25) invasivos. A partir de meio condicionado e/ou co-cultura destas células em ambiente bi ou tri-dimensional, foi analisada a proliferação, atividade contrátil e remodelamento da MEC por NOFs. Apenas os fatores liberados pela linhagem tumoral agressiva de CEC (SCC9) induziram a um aumento de proliferação de NOFs ($p<0.01$). Através de cultura tridimensional, foi observado que o meio condicionado das células de CEC aumentaram a contratilidade dos NOFs ($p<0.001$). Em um modelo de cultura organotípica, foi possível observar o *crosstalk* entre os diferentes tipos celulares reproduzindo as características histológicas observadas *in vivo*. Além disso, a linhagem tumoral de maior agressividade influenciou os NOFs a aumentarem a produção de fibras de colágeno ($P<0.001$) e a realizarem remodelamento da MEC. Portanto, estes dados reforçam o papel dos fatores liberados pelas células do tumor sobre as células do microambiente tumoral, como os fibroblastos, e sugerem um caminho alternativo de alvos terapêuticos pela modulação do perfil de fibroblastos.

Palavras-chave: microambiente tumoral; fibroblastos associados ao câncer; colágeno; cultura organotípica, carcinoma espinocelular oral.

ABSTRACT

Oral squamous cell carcinoma (OSCC) is the most common cancer in the oral cavity. It is histopathologically characterized by invading epithelial cells into the adjacent connective tissue and has an extremely complex and diverse microenvironment, including, chemical, physical and biological factors that influence tumor progress. One of several factors that characterize the alteration of the tumor microenvironment is the extracellular matrix composition (ECM), which presents a higher quantity of collagen in the tumors and, therefore, can influence its progress. Among the components of the tumor microenvironment, it has been reported in the literature that normal fibroblasts (NOFs) acquire an altered phenotype, demonstrating higher proliferation and higher migration, promotion changing compositional in ECM, as well as characterized by cancerassociated fibroblasts (CAFs). CAFs influence tumor progression and invasion however, its origin is not yet well defined. In this paper, we evaluated the behavior of NOFs when associated with factors released by keratinocytes and OSCC cell lines of different invasive profile. For this purpose, *in vitro* assays were performed using five different cell lines: primary normal oral mucosa fibroblasts, keratinocyte (HaCat), low (Cal27), or higher (SCC9 and SCC25) invasive OSCC cell. From conditioned medium and/ or co-culture of these cells in two or threedimensional environment, proliferation, contractile activity and remodeling of ECM by NOFs were analyzed. Only factors released by aggressive OSCC tumor cell line (SCC9) induced an increase in NOF proliferation ($p < 0.01$). Through threedimensional culture, it was observed that the conditioned media of the OSCC cells increased NOF contractility ($p < 0.001$). In an organotypic culture model, it was possible to observe a crosstalk between different cell types reproducing the histological characteristics observed *in vivo*. In addition, the high aggressive OSCC cell line influenced NOFs to increase production of collagen fiber ($P < 0.001$) and to perform the ECM remodeling. Therefore, these data reinforce the role of factors released by tumor cells on tumor microenvironment, as in fibroblasts, and suggest an alternative pathway for therapeutic targets by modulating the fibroblast profile.

Keywords: tumor microenvironment; cancer associated fibroblasts; collagen; organotypic culture, oral squamous cell carcinoma.

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LISTA DE ABREVIATURAS E SIGLAS

Antecedentes e justificativa

CEC -carcinoma espinocelular oral

% - por cento

INCA –insituto nacional de câncer

HPV - papilomavírus humano

UV – ultravioleta

DNA – ácido desoxirribonucleico

OMS - organização mundial de saúde

EMT – transição epitélio-mesênquima

miRNA – microácido ribonucleico

MEC - matriz extracelular

CAF – fibroblasto associado ao tumor

α-SMA- alfa-actina do músculo liso

SDF1 – secreção e fator -1

FAP – proteína ativadora e fibroblastos

NOF – fibroblastos normais

TGF-β - fator de crescimento beta

MMP- metaloproteinase

3D - tridimensional

2D – bidimensional

Artigo científico 1

OSCC – *oral squamous cell carcinoma*

HPV – *human papillomavius*

TME – *tumor microenvironment*

ECM - *extracellular matrix*

NOF- *normal fibroblast*

CAF – *cancer-associated fibroblast*

DMEM - *dulbecco's modified eagle media*

FBS - *fetal bovine serum*

α-SMA- *alpha-smooth muscle actin*

TGF-β – *growth factor beta*

SUMÁRIO

1. ANTECEDENTES E JUSTIFICATIVA

1.1 Carcinoma espinocelular oral

O câncer de cabeça e pescoço é responsável por 600 mil novos casos de câncer a cada ano no mundo (GLOBAL CÂNCER STATISTICS IN THE YEAR, 2018), sendo o carcinoma espinocelular oral (CEC) o tipo mais comum nesta região. Estima-se que 50% dos casos de CEC e faringe são diagnosticados com metástase regional e a distância, o que está relacionado com a taxa de sobrevida de 65% após 5 anos (Siegel *et al*., 2019). Para o ano de 2018, no Brasil, estimase que foram diagnosticados 14.700 novos casos de CEC, sendo 11.200 em homens e 3.500 em mulheres. Destes, estima-se que 1.100 casos foram diagnosticados somente no estado do Rio Grande do Sul. Assim, é a 5° neoplasia mais frequente em homens e a 12ª em mulheres no Brasil (INCA, 2018). Normalmente o tratamento do CEC é agressivo, baseado em cirurgia, radioterapia, quimioterapia ou associação entre os tipos o que resulta em redução da qualidade de vida do paciente (Van Der Waal, 2013). Clinicamente, o CEC é caracterizado como uma lesão ulcerada que não cicatriza com tempo de evolução de mais de duas semanas, contendo bordas irregulares e endurecidas (Neville *et al*., 2015).

A etiologia do CEC é multifatorial, podendo ser associada a fatores intrínsecos como condições sistêmicas e hereditariedade, ou extrínsecos associada a exposição ao tabaco, álcool, ao papilomavírus humano (HPV) e à radiação ultravioleta (UV). Ao fumo atribui-se papel principal, sendo considerado um agente iniciador, provocando mutações nos genes que regulam a proliferação e a morte celular (Ogden *et al*., 1999). Ao álcool também se atribui o aumento da proliferação epitelial e modificação no seu processo de maturação (Carrard *et al*., 2004). A infecção pelo HPV é um importante fator de risco para o câncer de cabeça e pescoço, especialmente na região da orofaringe, atingindo a base da língua, as tonsilas palatinas e a parte lateral e posterior da garganta. Existem diversos subtipos do vírus, sendo que o subtipo 16 é o que está mais relacionado ao desenvolvimento de CEC (Wichmann, 2017). A exposição solar representa um fator de risco importante para o CEC da região do lábio inferior. Seu risco depende da intensidade e o tempo de exposição, sendo comum em agricultores.

Diversos estudos mostram que o CEC decorre de acúmulo de eventos mutagênicos e alterações no DNA (como mutações no p53 e metilação do DNA), consequência principal do efeito do tabaco e do álcool (Jetwa e Khariwala, 2017). De acordo com o tempo de exposição a estes fatores de risco e outros fatores envolvidos, o processo da carcinogênese é decorrente de acúmulo de mutações.

1.2 Processo de carcinogênese

O processo em que a célula epitelial atravessa a lâmina basal e invade o tecido conjuntivo é necessário para a definição de CEC. A invasão das células tumorais ao tecido subjacente ocorre através de um estado mesenquimal transitório, que faz com que as células tenham a capacidade de migração - esse processo é definido como transição epitélio-mesênquima (EMT) (Kalluri e Weinberg, 2009; Markwell e Weed, 2015). A EMT é marcada pela perda do contato celular para assim ter aumento da motilidade, invasão e resistência a apoptose (Zhou *et al.,* 2014). A redução da expressão de proteínas relacionadas a origem epitelial da célula, como, por exemplo, E-caderina, e aumento da expressão de fatores de transcrição e proteínas relacionadas a células mesenquimais, como a N-caderina também são marcadores principais para a definição dessa transição (Scanlon *et al*., 2013). Vered *et al*., 2010 relataram que a expressão reduzida de E-caderina está localizada na periferia do tumor em contato com fibroblastos associados ao câncer (CAFs), sugerindo que a EMT pode ser modulada pelos CAFs (Vered *et al.,* 2010). Thiery em 2002, relacionou o processo de EMT com a expressão de fatores específicos de transcrição, como miRNAS e outros reguladores epigenéticos, os quais estão envolvidos no desenvolvimento embrionário, fibrose e metástase. Os principais eventos relacionados ao EMT são: ativação de fatores de transcrição, expressão de proteínas específicas da superfície celular, reorganização das proteínas do citoesqueleto e produção de enzimas que degradam a matriz extracelular (MEC) (Gao *et al.,* 2015).

É observado, em lâminas histológicas, que as células neoplásicas epiteliais invadem o tecido conjuntivo, podendo apresentar pérolas de ceratina, ilhas de invasão, apresentam mitoses atípicas, e apresentam diferentes graus de pleomorfismo celular. Estas alterações são consequências de mutações genéticas e alterações epigenéticas que resultam em atividades descontroladas como

proliferação celular e invasão de tecidos adjacentes, caracterizando uma patologia complexa e heterogênea (Thiery, 2002; Neville *et al.,* 2015). Para que a formação do tumor aconteça, Hanahan e Weinberg em 2011, propuseram características que são similares a diversos tipos de tumores e que são processos importantes para a progressão tumoral. Estas características são: fuga da destruição imune, imortalidade replicativa, metástase, angiogênese, mutação e instabilidade genômica, resistência a morte celular, inflamação promotora de tumor, desregulação do metabolismo energético, ativação de invasão e sinalização proliferativa contínua (Hanahan e Weinberg 2011; Hanahan e Coussens, 2012).

Apesar de muitos estudos terem sido realizados para compreender estas características, também se reconhece a importância das interações dinâmicas e recíprocas entre células tumorais e o estroma adjacente ao tumor, isto é, o microambiente tumoral.

1.3 Microambiente tumoral

O microambiente tumoral é definido como um conjunto de células e moléculas que são liberadas pelas células tumorais, composto por uma matriz extracelular remodelada, células tumorais, inflamatórias, endoteliais, fibroblastos e células recrutadas da medula óssea, que se comunicam entre si por meio de fatores liberados e citocinas (Becker *et al.,* 2013; Adjei e Blanka, 2015). Através da interação com as células tumorais, o componente estromal ajuda a promover o crescimento e a progressão do tumor.

Alterações são frequentes no microambiente tumoral e podem afetar de forma importante a resposta imune no interior do tumor. Muitas das células neste nicho podem inicialmente trabalhar para frear o desenvolvimento do câncer, no entanto, durante a progressão da doença, acabam sendo moduladas pelas células do tumor e pelo próprio microambiente a sofrerem essa mudança de função, tornando-se pró-tumorais (Quail e Joyce, 2013). Por exemplo os fibroblastos que podem inibir inicialmente o crescimento de tumores (Kalluri e Zeisberg, 2006). Porém, processos como a inflamação crônica gerada no microambiente acabam reprogramando-os para um estado promotor do crescimento desses tumores. Os

fibroblastos de ação pró-tumoral são chamados de fibroblastos associados ao tumor (Zhou *et al.,* 2014).

1.4 Fibroblastos associados ao tumor (CAFs).

A origem dos CAFs é um evento importante no processo da tumorigênese em que são mediados por fatores de crescimento e citocinas expressas pelas células tumorais (Parsonage *et al.,* 2005). São uma subpopulação de células que residem no microambiente tumoral estimulando o crescimento do tumor, angiogênese, inflamação e metástase. Secretam numerosos mediadores inflamatórios estimulando a proliferação celular, capazes de expressar α-actina do músculo liso (α-SMA), e caracterizados por síntese intensa de proteínas da matriz extracelular (Desmouliere *et al.,* 2004; Kellermann *et al.,* 2007; Tisty e Coussens, 2006). Os CAFs apresentam também maior aumento da secreção de fator-1 derivado de células estromais (SDF1), níveis aumentados de miRNA e proteína ativadora de fibroblastos (FAP), comparados aos fibroblastos normais (NOFs) (Zhou *et al.,* 2014). São definidos como NOFs que foram alterados como resultado da exposição contínua de fatores secretados pelas células tumorais, como por exemplo interleucina-1 (Kalluri e Zeisberg, 2006; Rollins *et al.,* 1989). Essa alteração é induzida por vários fatores de crescimento, um dos mais conhecidos é o fator de crescimento-β (TGF-β) que são liberados pelas células epiteliais danificadas ou pelas células inflamatórias mononucleares como macrófagos, após ocorrer o processo de injuria tecidual. Muitos estudos demonstram que os CAFs no microambiente tumoral podem se comunicar com as células tumorais através de exossomos, modificando diretamente a capacidade invasiva das células tumorais, servindo como caminho para que iniciem o processo de EMT (Yang *et al.,* 2017; Chen *et al.,* 2018). Também podem ser ativados pela comunicação direta célula-célula e por contatos com leucócitos mediados por moléculas de adesão e por componentes alterados da MEC (Kalluri e Zeisberg, 2006).

Os CAFs normalmente representam a maioria dos fibroblastos no estroma tumoral de vários tipos de carcinoma humano. Por exemplo em carcinomas de mama, em torno de 80% podem adquirir esse fenótipo alterado (Franco *et al.,* 2009). Também demonstram ter aumentada capacidade de mitose, proliferação mais rápida e maior migração que os NOFs (Commandeur *et al.,* 2011). Os CAFs

surgiram como um dos componentes mais importantes do microambiente tumoral, que desempenha um papel ativo na carcinogênese, promovendo a progressão tumoral através de comunicações específicas com as células tumorais, podendo transmitir sinais entre a MEC e as células epiteliais, promovendo mudanças dinâmicas e composicionais no estroma (Schauer *et al.,* 2011; Franco *et al.,* 2009).

Os CAFs também são importantes no processo de metástase tumoral. Por exemplo, estudos demonstram que os CAFS abrem “caminhos” para as células tumorais migrarem, remodelando o colágeno e facilitando a migração (Glentis *et al.,* 2017; Pankova *et al.,* 2016). Estudos evidenciam que os CAFs aumentam a invasão e as metástases de células tumorais através de metaloproteinases da matriz (MMPs), destruindo barreiras do tecido e aumentando a invasão (Cai *et al.,* 2011). Ainda que já é reconhecido o papel fundamental dos CAFs para a progressão tumoral, a origem exata destas células ainda não é compreendida totalmente.

1.5 Matriz extracelular

Outro componente importante para o microambiente tumoral, é a composição da matriz extracelular (MEC), o que também contribui para a heterogeneidade e complexidade dos tumores (Franco *et al.,* 2009; Liotta e Kohn, 2001). A MEC é uma rede estrutural complexa que rodeia e suporta as células dos tecidos e são essas células que secretam as diferentes moléculas que constituem a matriz extracelular (Ross e Pawlina, 2016). Sua composição pode ser didaticamente classificada como substância fundamental e componente fibrilar. A substância fundamental é constituída por três grupos de moléculas, proteoglicanos, glicosaminoglicanos e as glicoproteínas multiadesivas (Taylor e Gallo, 2006). Já o componente fibrilar é composto por fibras colágenas, fibras elásticas e reticulares (Souza e Pinhal, 2011).

A MEC possui capacidade de ligar e reter fatores de crescimento, que por sua vez modula o crescimento celular e transmite informações através da membrana plasmática das células do tecido conjuntivo. Além disso, a rigidez e elasticidade da MEC influenciam na diferenciação e migração celular e na expressão de genes (Orimo e Weinberg, 2006).

No tumor, a MEC apresenta quantidades aumentadas de colágeno, fibronectina, proteoglicanos e glicosaminoglicanos, presença de infiltrado inflamatório e CAFs (Wever e Mareel, 2003). A fibronectina, uma glicoproteína adesiva da MEC, durante a carcinogênese pode permitir a livre migração das células neoplásicas e sua expressão vem sendo relacionada a um pior prognóstico em tumores de cabeça e pescoço (Ramos *et al.,* 2016). Os mecanismos pelos quais as interações célula-MEC influenciam o comportamento metastático das células tumorais incluem: alteração da adesão celular e sua motilidade, aumento de proteases celular que degradam a MEC facilitando o movimento para degradação da membrana basal e barreiras estromais, liberação de fragmentos da MEC e fatores de crescimento que promovem a progressão da célula tumoral (Werb, 1997). Durante a progressão do câncer, as propriedades biomecânicas e bioquímicas da MEC são desreguladas (Lu *et al.,* 2011), algumas proteínas como colágeno e fibronectina são enriquecidas e outras que não estão presentes aparecem, como a laminina. As fibras colágenas se tornam mais reticuladas, enrijecidas e alinhadas, os fibroblastos geram e modificam o ambiente durante a progressão do tumor, deixando-o mais passível para invasão do câncer (Goetz *et al.,* 2011; Matte *et al.,* 2018).

A composição da MEC é capaz de influenciar o processo de invasão e metástase tumoral conforme o nível de diferenciação celular, através do desequilíbrio das interações célula-célula e adesões célula-MEC (Ramos *et al.,* 2016). Em estudo realizado por Matte et al. 2018 dados sugerem que as células menos invasivas do CEC podem ser induzidas pela rigidez da MEC, apresentando um caráter mais invasivo quando expostas a longos períodos em ambiente mais rígido.

1.6 Modelos de estudo

Um modelo ideal de câncer deve replicar muitos dos recursos que ocorrem no organismo humano, incluindo como ele se desenvolve e progride quando enfrenta um sistema imunológico humano, como se espalha de sua fonte primária para outras partes do corpo (metástase) e como ele reage à terapia (Taubenberger *et al.,* 2016). Devido a grande heterogeneidade de fenótipos existentes entre os tumores, busca-se novas formas de entender a complexidade do microambiente

tumoral e suas características. Entretanto, todos os modelos (*in vivo, in vitro, ex vivo*) apresentam vantagens, desvantagens e aspectos éticos a serem avaliados.

O uso de tecnologias *in vitro* são estratégias que visam a minimizar os custos, questões éticas e o estudo em animais. Contudo, ainda é necessário desenvolver e aperfeiçoar os modelos de estudo *in vitro* para que estes possam mimetizar os tumores observados em organismos vivos.

1.6.1 Modelo de estudo 2D

O cultivo de células teve início em torno da década de 1950 e trouxe várias vantagens, permitindo o estudo de respostas celulares, sinais bioquímicos e estudos da biologia celular mais aprofundada assim como compreensão da carcinogênese (Duval *et al.,* 2017).

O cultivo celular é realizado a partir de isolamento de células de tecidos vivos, sendo estas células cultivadas em placas de poliestireno em duas dimensões (2D) e nutridas com meios de cultura com formulações especí ficas (Edmondson *et al.,* 2014). A partir destas culturas, é possível analisar expressão proteica, novas terapias, dentre outros. Como vantagem, este sistema apresenta bom custo-benefício e permite a análise de alto rendimento (*high-throughput screening)* de novas terapias, por exemplo. Contudo, o ambiente presente na placa de poliestireno não reproduz os tecidos presentes nos seres vivos, devido a falta de diversas proteínas, como MEC, além de uma rigidez incompatível com os tecidos dos organismos vivos (Choi *et al.,* 2010).

1.6.2 Modelos de estudo 3D

A partir do entendimento da influência da MEC no comportamento celular, diversas metodologias foram desenvolvidas para aprimorar o estudo *in vitro* utilizando proteínas da MEC. Por exemplo, Bissel em 2011 utilizaram matrigel para demonstrar a importância da MEC para a morfologia das células. Outra proteína amplamente utilizada é o colágeno tipo I devido a sua abundância nos tecidos dos organismos vivos (Bissel e Hines, 2011; Nelson e Bissel, 2006).

Além disso, a utilização de proteínas da MEC em cultivo celular 3D também permite a co-cultura de diferentes tipos celulares. Desta maneira, é possível

realizar estudos sobre como as células interagem entre si. Um dos modelos desenvolvidos para mimetizar o CEC é a cultura organotípica. A partir de uma matriz de colágeno embebida com NOFs e presença de células epiteliais na superfície, é possível desenvolver modelos de estudos semelhantes morfologicamente ao que observamos nos organismos vivos. Por exemplo, Colley et al. (2011) demonstraram que culturas organotípicas realizadas a partir de linhagens celulares de CEC apresentou marcação de proteínas com distribuição semelhante ao observado em espécimes humanas de CEC e displasia (Colley *et al.,* 2011). Portanto, demonstra-se que estes modelos possuem vantagens frente a modelos 2D. Estes modelos 3D apresentam maior custo envolvido, além de ainda não apresentarem o mesmo número de metodologias para avaliação como nos modelos 2D. No entanto, com os modelos de cultura 3D é possível avaliar a interação celular que ocorre dentro do microambiente tumoral, bem como a progressão dos CAFs.

2. OBJETIVOS

2.1 Objetivo geral

Avaliar se fatores liberados por queratinócitos e por células do carcinoma espinocelular oral de diferentes graus de agressividade influenciam no comportamento de fibroblastos normais.

2.2 Objetivos específicos

- Avaliar a influência de meios condicionados de queratinócitos e linhagens celulares do carcinoma espinocelular oral sobre a proliferação de fibroblastos;
- Analisar a influência de meios condicionados de queratinócitos e linhagens celulares do carcinoma espinocelular oral sobre a contratilidade de fibroblastos em gel de colágeno;
- Investigar se os fibroblastos mudam seu comportamento em relação a produção de colágeno através de cultura organotípica com queratinócitos e diferentes linhagens do carcinoma espinocelular oral.

3. ARTIGO CIENTÍFICO

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HIGHLY INVASIVE ORAL SQUAMOUS CELL CARCINOMA MODIFIES FIBROBLAST BEHAVIOR AND EXTRACELLULAR MATRIX ORGANIZATION

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Abstract

Oral squamous cell carcinoma (OSCC) is the most common cancer in the oral cavity. It is characterized as a complex disease with a diverse tumor microenvironment (TME) that influences tumor progression. Among the components of the TME, it has been reported that normal fibroblasts (NOFs) acquire an altered phenotype, demonstrating higher proliferation and extracellular matrix (ECM) alteration, as characterized as cancer-associated fibroblasts (CAFs). In this paper, we evaluated the behavior of NOFs when associated with factors released by keratinocytes and OSCC cell lines of different invasive profile. For this purpose, *in vitro* assays were performed using five different cell lines: primary normal oral mucosa fibroblasts, keratinocyte (HaCat), low (Cal27), or higher (SCC9 and SCC25) invasive OSCC cell. From conditioned medium and/ or co-culture of these cells in two or three-dimensional environment, proliferation, contractile activity and remodeling of ECM by NOFs were analyzed. Only factors released by aggressive OSCC tumor cell lines induced an increase in NOF proliferation ($p < 0.01$). Through three-dimensional culture, it was observed that the conditioned media of the OSCC cells increased NOF contractility ($p < 0.001$). In an organotypic culture model, it was possible to observe a crosstalk between different cell types reproducing the histological characteristics observed *in vivo*. In addition, the highly aggressive OSCC cell line influenced NOFs to increase production of collagen fiber ($P < 0.001$). Therefore, these data reinforce the role of factors released by tumor cells on tumor microenvironment, as in fibroblasts, and suggest an alternative pathway for therapeutic targets by modulating the fibroblast profile.

Keywords: Oral Squamous cell carcinoma; cancer-associated fibroblasts; extracellular matrix.

Introduction

Oral Squamous cell carcinoma (OSCC) is the most common malignant epithelial neoplasm found in 80-90% of head and neck cancer (Leemans et al., 2018). It is originated from genetically altered keratinocytes arising from sun exposure, tobacco and alcohol users, genetic profiles (Ng et al., 2016). Clinically, OSCC is usually presented as an ulcer with irregular and indurated margins (Neville et al., 2015). In the histopathological specimen, it is observed epithelial neoplastic cells invade the adjacent connective tissue. The prognosis of the disease is correlated with the stage of diagnosis (Rivera and Venegas, 2014).

During the carcinogenesis process and once the tumor is formed, it is highlighted the fundamental role of other non-cancer cells components that is classified as the tumor microenvironment (TME). The TME is constituted by complex and diversified biological, chemical and physical factors that influence OSCC progression (Nieto et. al., 2016). The extracellular matrix (ECM) is an important physical component of the TME and it has been observed that its composition and stiffness can alter OSCC behavior and prognosis (Ramos et al., 2016; Matte et al., 2018). For example, an increased stiffness is correlated with increased migration of OSCC and a poor prognosis for OSCC patients (Matte, 2018). ECM alteration is a consequence of the different biological and chemical components of the TME.

As a biological component, fibroblasts, endothelial and inflammatory cells also have a fundamental role in the TME. Among them, it is evidenced the importance of cancer-associated fibroblasts (CAFs). CAFs are a cell population in the TME contribute to tumor growth, invasion and metastasis (Bissel and Hines, 2011). It has been demonstrated, for example, that CAFs can alter the ECM and facilitate cancer cell invasion (Glentis et al., 2017). Also, it has been reported that there is a cross talk between CAFs and OSCC cells that promote tumor progression (Bae et al., 2014; Li et al., 2018). However, the origin of CAFs is not fully understood (Attieh et al., 2017).

Therefore, the aim of this study is to evaluate if normal human oral fibroblasts (NOFs) are influenced by growth factors release by keratinocytes and OSCC cell lines of different aggressiveness profiles.

Materials and methods

Cell culture

HaCat (human keratinocyte cell line), SCC-9 and SCC-25 (OSCC lineages) cell lines were obtained from Rio de Janeiro Cell Bank (BCRJ, Rio de Janeiro, RJ). Cal27 (OSCC lineage) was obtained from the Tissue Culture Facility at School of Medicine of University of Virginia. HaCat, Cal27 and SCC-9 were cultivated in Dulbecco's modified Eagle's media (DMEM) with high glucose (Gibco) supplemented with 10% Fetal Bovine Serum (FBS) (Gibco) and 1% penicillin/streptomycin (Gibco). SCC-25 cells were cultivated in DMEM/F12 with 15mM HEPES and 0.5mM sodium pyruvate (Gibco) supplemented with 10%FBS, 1% penicillin/streptomycin (Gibco) and hydrocortisone (400ng/ml, Sigma). The primary normal fibroblasts (NOFs) were obtained from donated oral mucosa fragment, as approved by the Ethical Committee of UFRGS (CAE#59124916.6.0000.5327). NOFs were cultivated in Dulbecco's modified Eagle's media (DMEM) with low glucose (Gibco) supplemented with 10% Fetal Bovine Serum (FBS) (Gibco) and 1% penicillin/streptomycin (Gibco). All cells were maintained in incubator at 37°C with 5% CO_2.

Conditioned media preparation

Conditioned media were prepared with NOFs, a normal keratinocyte cell line (HaCat) and low (Cal27) or highly (SCC9 and SCC25) invasive OSCC cell lines. Cells were seeded in $5x10^5$ on a 6mm plate with regular media. After 24 hours, the media was changed for DMEM formulation without FBS and collected after 48 hours. The media was centrifuged (2000 rpm) for 5 mins and the supernatant was collected and used in the future experiments.

Proliferation assay

Cell proliferation was evaluated by Neutral Red (3-amino-7-dimethylamino-2methyl-phenazine hydrochloride) assay. A total of $1x10^3$ NOFs/well was seeded at a 96-well plate and incubated for 24 hours. Next, the media was removed, and

cells were treated with DMEM 0% FBS (control group) and with HaCat, Cal27, SCC9, SCC25 conditioned media (experimental groups) for 24 hours. For the proliferation analysis, it was used the Neutral Red assay according to previous studies (Repetto et al., 2008). Briefly, the media was removed, and it was added media containing neutral red and incubated for 3 hours. Next, the Neutral Red media was removed and washed with 0.15 µl of desorb solution in a shaker for 10 minutes. The results were analyzed at 540nm in the spectrophotometer multiskan Thermo Fisher®.

Extraction of collagen I from rat tails

Collagen type I was extracted from rat tails according to previously established protocols (Timpson et al. 2011). Briefly, we removed rat tails tendons slicing in the middle of the tail from top to bottom with a scalpel and pealing along the length of the tail. From 1 g of tendon extracted, it was mixed with 250 ml of 0.5 M acetic acid by stirring at 4°C for 48 h. The extract was centrifuged (7,500 x g) for 30 mins and the pellet was discarded. It was added an equal volume of 10% (w/v) NaCl to the supernatant, and stirred for 30-60 min. Next, it was centrifuged (10,000 x g) for 30 mins, the supernatant was discarded, and re-dissolved the precipitate in 0.25 M acetic acid at 1:1 ratio by stirring for 24 h at 4°C. Finally, the collagen was dialyzed against 6-8 changes of 2L of 2.0 mM acetic acid and was lyophilized. It was then diluted at a final concentration of 3 mg/ml using 0.02M of acetic acid to perform the experiments.

Contraction assay

It was performed a fibroblast embedded collagen gel on a 24-well plate in order to analyze cell contraction. Briefly, collagen gel was prepared at a final concentration of 1.8 mg/ml embedded with NOFs at $5x10^5$ cells/gel. After polymerization, it was added NOFs media with 10% FBS as the control group. The conditioned media from HaCat, Cal27, SCC9, SCC25 was added as the experimental groups and the same media formulations used for the conditioned media were added as internal control. As a negative control, it was performed a collagen gel without fibroblasts.

After 5 days, the diameter and height of collagen gels were measured with a ruler. The final volume was calculated as $v = h\pi r^2$.

OSCC organotypic culture

Rat-tail-derived collagen type I was used to produce a 3D matrix with a final concentration of 1.8 mg/ml. Briefly, rat-tail collagen type I was added on ice to 10× DMEM and the pH adjusted with 0.1 M NaOH to 7. 5×10^5 of NOFs were embedded in the matrix. On top of the matrix, 5×10^5 of HaCat, Cal27 or SCC9 cells were added. Once cells had reached confluence, the matrix was lifted to create an air–liquid interface. The system was cultivated for 14 days, formalin fixed, and paraffin embedded, and then sectioned. Sections were deparaffinized and re-hydrated, followed by staining with hematoxylin and eosin and Picrosirius Red. Pictures were taken with an Olympus BX51 microscope coupled to an Olympus Q-color 5 RTV at 10x and 20× magnification. For collagen quantification, slides stained with Picrosirius Red was analyzed for the sum of total fiber area with ImageJ software.

Statistical analysis

All experiments were performed with at least three biological replicates. The relationship between independent variables was analyzed with one-way analysis of variance ANOVA followed by Tukey test. Statistical analyzes were performed using Prism GraphPad software (San Diego, CA, U.S.A). A P-value < 0.05 was considered statistically significant.

Results

Highly invasive OSCC cell line induced increase on NOFs proliferation

In order to evaluate the possible effects of crosstalk between NOFs and keratinocytes or different OSCC cell types, we performed a conditioned media from different cell lines. We used a normal keratinocyte line (HaCat) and three other lines with different aggressiveness profiles. Cal27 is considered in the literature as a low aggressive OSCC cell line and SCC9 and SCC25 are considered highly

invasive cell lines (Ramos et al. 2016; Matte et al. 2018). Once this conditioned media was collected and added to the NOFs cell culture, we evaluated cell proliferation after 24 hours. All conditioned media evaluated showed an increased in NOFs proliferation (Fig. 1A). However, only SCC9 conditioned media showed biological relevance (0.792±0.078, n=3) and statistics significance ($p < 0.01$) in NOFs proliferation. Therefore, NOFs can have their behavior modulated by conditioned media of different cell lines and, more specifically, a highly invasive cell line demonstrated to induce NOFs proliferation.

Tumor cell conditioned media increased fibroblast contractility degree

It is attributed that CAFs have a high level of alfa-smooth muscle actin (α-SMA) and are responsible for high contractility of the tumor microenvironment, leading to increased stiffness and an interlaced arrangement manner of collagen fibers (Ding et al. 2014). In order to understand the influence of tumor cell-related factors on the contractility level of NOFs, we performed a contractility assay using a three-dimensional (3D) collagen matrix. We embedded NOFs in a 3D collagen gel and after polymerized, conditioned media from 4 different cell lines was added on the gels and observed for 5 days. At the end, the final volume of the gels was evaluated (Fig. 2A). As a negative control, it was performed a collagen gel without fibroblasts, and in control group, it was evaluated the own action of fibroblasts exposed to their media with FBS. For an inner control of the different media formulations that was used to prepare the conditioned media, it was analyzed three DMEM formulations without FBS in order to observe the isolated effect of the cell-released factors. It was observed that NOFs are contractile cells since there was a reduction on the final gel size compared to the negative control. Moreover, the conditioned media from the OSCC cell lines induced a higher contractility in the NOFs (Fig. 2B; $p<0.001$) which was more evident in highly aggressive OSCC (Cal27= 95.03±36.88; SCC9= 49.04±21.08; SCC25= 24.54±11.79; n=3). Interestingly, HaCat conditioned media and regular unconditioned media did not have the same influence on NOFs contractility. On the basis of these differences, we observed that the factors released by OSCC cells influence NOFs contractility activity.

Increased collagen fibers in organotypic culture is influenced by highly invasive OSCC cells

In order to evaluate the behavior of the NOFs associated with normal keratinocytes or OSCC cell lines, we performed an organotypic culture since it resembles the morphological structure of *in vivo* oral mucosa and it allows to perform a co-culture analysis. In this technique, a collagen gel embedded with fibroblasts and keratinocytes or OSCC cell lines were seeded on top of the gel (Fig. 3A), resulting in fibroblasts embedded in a three-dimensional way (Fig. 3B). Once top-layer cells had reached confluence, the matrix was lifted to create an air–liquid interface and cultured for 14 days. Histological sections were made to observe the behavior of co-cultured cell types. It was observed that, as a result of the air-liquid interface, keratinocytes (HaCat) produce layers (stratification) similar to what is observed in a normal oral mucosa. The organotypic culture with OSCC cell lines demonstrated invasion into the collagen gel what is also observed in OSCC specimens *in vivo*. Also, a highly invasive OSCC cell line (SCC9) invaded the collagen gel and even formed tumor islands in it. Meanwhile, a low invasive OSCC cell line (Cal27) did not show this same behavior (Fig. 3C).

Next, it was analyzed the collagen organization in the organotypic cultures, using Picrosirius Red staining and the images were acquired by polarized light microscope (Fig. 4A). The quantification of the images was analyzed by summing the fiber area per field, and it was observed that a highly invasive OSCC cell led to an increase in collagen fibers compared to the other groups (Fig. 4B; $p<0.01$), which may have influenced the fibroblasts to modify the surrounding environment. Therefore, we observed in an organotypic culture, that there is a crosstalk between highly invasive OSCC cell line, inducing increase on proliferation, production of collagen fibers and contractility in NOFs.

Discussion

The TME is defined as the sum of all elements residing within or in the vicinity of a tumor and is widely acknowledged as a significant contributor to cancer initiation and progression (Madar et al. 2013; Friedl and Alexander, 2011). Cancer

cells are influenced by this microenvironment, expressing different oncogenes and inducing a migration phenotype (Alexander et al. 2013). These cells recruit other TME cells to facilitate tumor growth, survival, invasion and dissemination (Joyce and Pollard, 2008). As the most abundant component of TME, CAFs are widely known to be co-conspirators in tumor initiation and progression (Zhou et al. 2014). They are involved in carcinogenesis process and progress by remodeling the ECM, secreting growth factors, affecting tumor cell growth and cellular invasion (Marsh et al. 2013). Thus, there is a crosstalk between cancer cells and CAFs, and this has earned recognition as an interaction that plays a pivotal role in carcinogenesis (Bae et al. 2014). However, the exact origin of CAFs is still not fully understood.

In our study, we evaluated the influence of keratinocytes and tumor cellreleased factors on NOFs behavior. First, we observed that a highly invasive OSCC cell line induced proliferation of NOFs. Other studies also reported the same influence of cancer cells on NOFs proliferation and it has been shown that this pathway is regulated by transforming growth factor beta (TGFb) increase (Kellerman et al., 2007; Mahjour et al., 2019). Interestingly, it is also observed that CAFs conditioned media can induce proliferation and invasion of OSCC cells (Zhou et al., 2014). Therefore, it is shown that there is a crosstalk between OSCC cells and cells present in the TME.

Tumor stiffness is characterized by alignment of collagen fibers that results in a worse prognosis (Matte et al., 2018). Since it has been demonstrated that CAFs have a high α-SMA expression and this protein is associated with an increase contractility effect, we evaluated the influence of conditioned media in the contraction of NOF-embedded collagen gels. It was observed that OSCC cells released factors induced NOFs into a more contractile phenotype. The same effect was observed previously by hepatocellular carcinoma cells and breast cancer cells (Zhou et al., 2018; Calvo et al., 2013). Also, the identification of CAFs by α-SMA staining in immunohistochemistry has been correlated with patient survival. In a recent systematic review and meta-analysis, it was observed that the presence of high levels of CAFs in OSCC is correlated with overall decreased survival (Dourado et al., 2017). Thus, highly aggressive oral OSCC cells might modulate CAFs activity during OSCC progression.

One of the characteristics that CAFs exert in the TME is to remodel the

ECM, by pulling and stretching the fibers, facilitating cancer cells to migrate (Glentis et al., 2018). In order to analyze if there is a crosstalk between OSCC cells and NOFs, we employed an organotypic culture model (Colley et al., 2011). In this model, we co-cultured keratinocytes or OSCC cells on top of NOFs embedded collagen gel in an air-liquid interface for 14 days. It was observed the keratinocytes stratified while the OSCC cells invaded the adjacent collagen. Also, it was observed a higher amount of collagen with the presence of a highly invasive OSCC cell line. Since both invasion and collagen organization resembled to what we observe in *in vivo* histological specimens, our data indicate that the crosstalk among tumor cells and fibroblast is essential to ECM remodeling. This is also observed in other tumors, like mammary and ovarian cancers cells, that have a more invasive phenotype in increased ECM stiffness (Wei et al., 2015). Also, it is important to highlight that organotypic culture is a relevant model in order to evaluate the crosstalk between different cell types and can be useful to analyze new therapeutic options targeting cancer cells and TME cells.

Altogether, we have shown that OSCC cells-released factors influence NOFs behavior by inducing proliferation, contraction and ECM remodeling. Even though we did not evaluate if NOFs have become CAFs after exposure to OSCC conditioned media, our results suggest that OSCC cells promote NOFs activation. Therefore, it is concluded that there is a crosstalk among TME cells and that this can impact on tumor progression.

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Conflict of interest statement

All authors declare no conflicts of interest.

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Figures

Figure 1

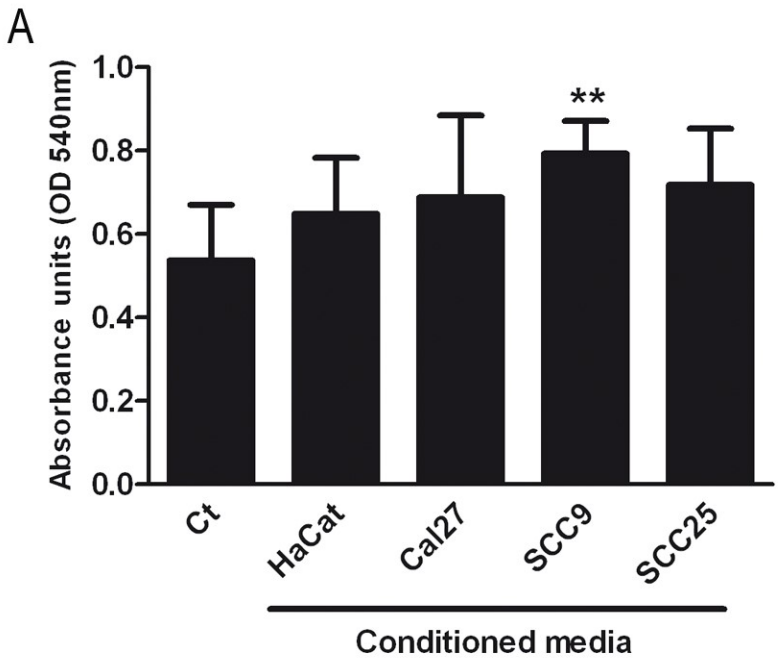

Figure 1. Conditioned media of OSCC cell lines increase NOFs proliferation. **(A)** Regular NOF media without FBS and conditioned media of HaCat (keratinocytes), Cal27, SCC9 and SCC25 (OSCC cell lines) were exposed to NOFs for 24 hours and proliferation was analyzed by Neutral Red assay. ** $p<0.01$ for one-way ANOVA and Tukey's multiple comparison test (n=3).

Figure 2

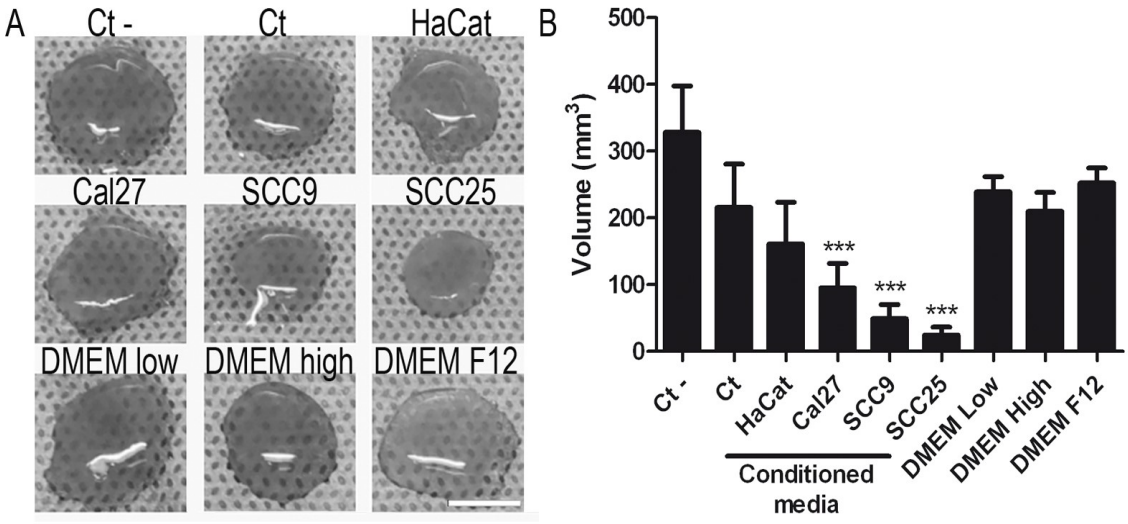

Figure 2. OSCC cell lines conditioned media increased NOFs contractility (A) Images of representative gels after 5 days of the following groups: Ct- represents collagen gel without NOFs; Ct represents NOFs embedded collagen gel with regular 10% FBS media; conditioned media of Hacat, Cal27, SCC9, SCC25 and inner control of 0% FBS DMEM media (Low, High, F12); Scale bar is 1cm. **(B)** Final volume quantification of collagen gels; *** $p<0.001$ for one-way ANOVA and Tukey's multiple comparison test compared to Ct (n=3).

Figure 3

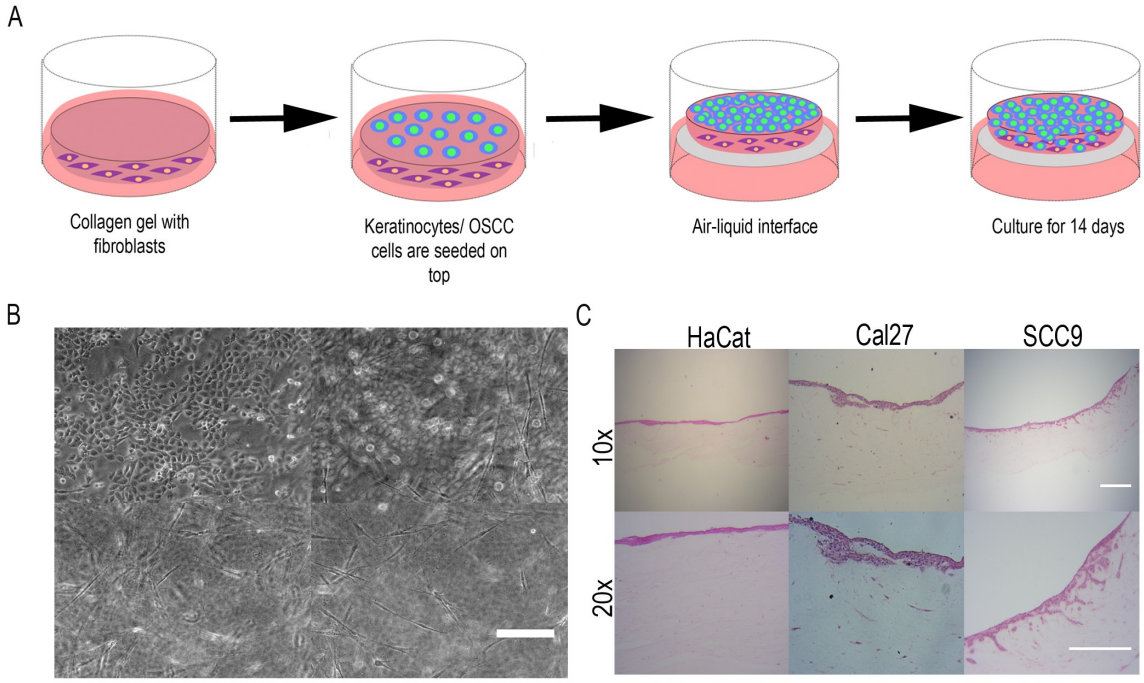

Figure 3. Organotypic culture of keratinocytes and OSCC cells with NOFs. **(A)** Schematic illustration showing the methodology of organotypic culture. **(B)** Phase-contrast images of organotypic culture. First image represents the OSCC cell on top of the gel and following images demonstrate fibroblasts embedded in a three-dimensional gel. Scale bar is 50µm. **(C)** Histological sections of organotypic culture stained with H&E with HaCat, Cal27 and SCC9 on the surface in10x and 20x magnifiation. Scale bar in 10x and 20x are 0.2mm.

Figure 4

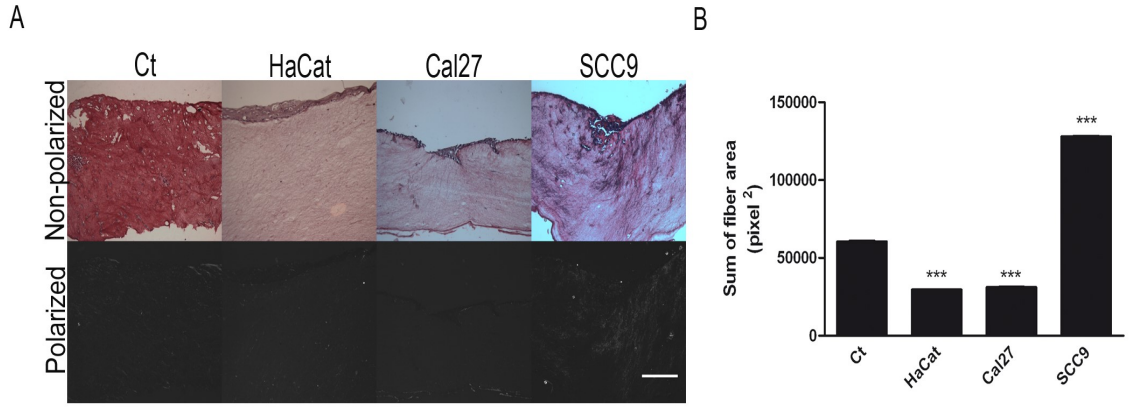

Figure 4. Highly invasive OSCC cell line led to an increase in collagen fibers. **(A)** Histological sections of organotypic culture stained with Picrosirius Red of Ct (NOFs embedded-collagen gel), HaCat, Cal27 and SCC9 on the surface in 20x magnifiation. Scale bar is 0.8mm. **(B)** Quantification of collagen fiber area stained by Picrosirius Red; *** $p<0.001$ for one-way ANOVA and Tukey's multiple comparison test

4. CONSIDERAÇÕES FINAIS

A relação de *crosstalk* entre os fatores liberados pelas células do CEC e pelas células do microambiente tumoral impacta a progressão do tumor. Foi observado que as células relacionadas a um perfil tumoral mais agressivo modificam o comportamento dos NOFs em relação a sua proliferação, contratilidade e produção de fibras colágenas, modificando a matriz extracelular. Essa modificação pode modular o caráter invasivo e a proliferação das células tumorais tornando essa relação uma via de mão dupla. Assim, através de um modelo tridimensional foi possível observar o *crosstalk* das células do microambiente tumoral, reproduzindo as características histológicas que observamos *in vivo*. Portanto, este modelo é útil para as futuras análises do microambiente tumoral e inclusive como base para análise de novas opções terapêuticas.

A presente dissertação demonstrou que:

- Os fatores liberados por células tumorais mais agressivas do CEC aumentam a proliferação dos NOFs;
- O perfil contrátil de NOFs foi aumentado quando expostos aos fatores liberados pelas células de CEC;
- A presença de células mais agressivas de CEC em um modelo de cultura organotípica influenciou os NOFs a remodelarem a matriz extracelular no seu entorno;
- A utilização de um modelo tridimensional possibilita melhor avaliação da interação entre as células do microambiente tumoral, sendo um potencial para novos campos de estudo.

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