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Participação de Smad7 e CTGF na transdiferenciação de miofibroblastos gengivais e análise da influência dos miofibroblastos na proliferação e invasão de carcinomas espinocelulares orais

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Orientador: Prof. Dr. Ricardo Della Coletta

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Resumo

Miofibroblastos são células mesenquimais caracterizadas pela expressão da isoforma α da actina de músculo liso (α-SMA) e pela secreção de proteínas da matriz extracelular, fatores de crescimento e proteases. Estas células desempenham um papel importante na reparação de feridas e em processos patológicos, incluindo fibroses e cânceres. Os objetivos deste estudo foram 1) analisar o papel do fator de crescimento de tecido conjuntivo bem como o efeito da superexpressão de Smad7 na transdiferenciação de miofibroblastos gengivais induzida pelo fator de crescimento transformante-β1 (TGF-β1), 2) isolar e caracterizar linhagens celulares de miofibroblastos do estroma de carcinomas espinocelulares (CEC) orais e comparar o potencial proliferativo e produção de metaloproteinases de matriz (MMP) com linhagens celulares de fibroblastos do estroma de CEC orais, e 3) analisar a influência de miofibroblastos na modulação da proliferação e invasão de linhagens celulares de CEC oral. Nossos resultados demonstraram que o tratamento com TGF-β1 induziu simultaneamente a expressão de α-SMA e CTGF e a neutralização de CTGF com RNA de interferência (siRNA) bloqueou o efeito de TGF-β1 na indução da transdiferenciação de células de gengiva normal em miofibroblastos. A superexpressão de Smad7 em células de GN inibiu a cascata de ativação de TGF-β1, caracterizada pela fosforilação de Smad2 e expressão de α-SMA, CTGF e colágeno tipo I. Similarmente, miofibroblastos isolados do tecido gengival de fibromatose gengival hereditária (FGH) superexpressando Smad7 demonstraram níveis reduzidos de α-SMA e pSmad2, além de baixos níveis de expressão de CTGF e colágeno tipo I. Três linhagens celulares de miofibroblastos foram isoladas do estroma de CEC de língua e caracterizadas pela expressão de α-SMA e por níveis elevados de produção de colágeno tipo I. Embora o potencial proliferativo dos clones de fibroblastos e miofibroblastos tenham sido semelhantes, as produções de MMP-1, -2, -9 e -13 foram significantemente maiores em miofibroblastos. Finalmente, nós demonstramos que miofibroblastos do estroma

tumoral produzem níveis elevados de alguns fatores de crescimento comparado com fibroblastos, incluindo ativina A. Meios de cultura condicionados por miofibroblastos contendo ativina A significantemente induziu a proliferação de linhagens celulares de CEC oral e uma maior progressão tumoral *in vivo*, enquanto que o bloqueio de ativina A por siRNA diminuiu significantemente a proliferação das células de CEC oral. In vitro, miofibroblastos induziram a invasão de linhagens celulares de CEC oral, o qual foi acompanhado por uma indução na produção de MMPs, e *in vivo* uma significante correlação entre presença de miofibroblastos e atividades de MMP-2 e MMP-9 foi observada. O bloqueio da síntese de ativina A por siRNA em miofibroblastos não alterou a capacidade de indução da invasão e síntese de MMPs. Os resultados deste estudo demonstram 1) que Smad7 bloqueia a transdiferenciação de miofibroblastos gengivais por meio da inibição da fosforilação de Smad2 e da transcrição de CTGF, 2) que miofibroblastos no estroma de CEC orais podem contribuir para um fenótipo mais invasivo via secreção de elevados níveis de MMPs e 3) que produtos de síntese dos miofibroblastos induzem a proliferação e invasão das células de CEC oral e os estímulos proliferativos são controlados pela produção de ativina A.

Abstract

Myofibroblasts are mesenchymal cells, characterized by the specific isoform α of the smooth muscle actin (α-SMA) expression and the extracellular matrix proteins, growth factors and proteases secretion. These cells play a central role on wound healings and in pathologic process, including fibrosis and cancers. The aims of this study were 1) analyze the connective tissue growth factor (CTGF) role and the superexpression of Smad7 effect on TGF-β1-induced gingival myofibroblasts transdifferentiation, 2) isolate and characterize myofibroblast cell lines from oral squamous cell carcinomas stroma (OSCC) and compare the proliferative potential and matrix metalloproteinases (MMP) production with fibroblast cell lines from OSCCs stroma, and 3) analyze the myofibroblasts influence on the modulation of OSCC cell lines proliferation and invasion. Our results demonstrated that the TGF-β1 treatment induced simultaneously the α-SMA and CTGF expression and the CTGF neutralization using the small interference RNA (siRNA) blocked the TGF-β1-induced gingival myofibroblasts transdifferentiation. Smad 7 superexpression in normal gingival cells (NG) inhibit the TGF-β1 cascade activation, characterized by the Smad2 phosphorilation and α-SMA, CTGF and type I collagen expression. Similarly, hereditary gingival fibromatosis (HGF) myofibroblasts superexpressing Smad 7, demonstrated reduced levels of α-SMA and phospho-Smad2, and low expression levels of CTGF and type I collagen. Three myofibroblast cell lines were isolated from tongue OSCC stroma and characterized by the α-SMA expression and high levels of type I collagen. Although the proliferative potential of fibroblast and myofibroblast clones has been similar, the MMP-1, -2, -9 and -13 were significantly higher in myofibroblasts. Finally, we demonstrated that tumor stroma myofibroblasts produce high levels of some growth factors compared with fibroblasts, including activin A. Myofibroblasts conditioned medium containing activin A induce significantly the OSCC cell lines proliferation and a tumor progression *in vivo*, while the activin A dowregulation by siRNA significantly decreased the OSCC cells proliferation. In vitro, myofibroblasts induced OSCC

cells invasion, accompanied by an induction of MMPs production, and *in vivo* was observed a significant correlation between the myofibroblasts presence and the MMP-2 and MMP-9 activity. The myofibroblasts dowregulation of activin A by siRNA did not affect the induction of invasion and MMPs synthesis. The results of this study demonstrate that 1) Smad 7 blockage the gingival myofibroblasts transdifferantiation through the inhibition of Smad 2 phosphorilation and CTGF transcription, 2) myofibroblasts on the OSCCs stroma can contribute to a more invasive phenotype via elevated levels of MMPs secretion, 3) myofibroblasts released products induce an OSCC cells proliferation and invasion and the proliferative stimulus are controlled by the activin A production.

Sumário

1. Introdução

Miofibroblastos são células mesenquimais altamente especializadas que adquirem a capacidade de expressar a isoforma α da actina de musculatura lisa (α-SMA) e de sintetizar níveis elevados de colágeno e outros componentes da matriz extracelular (MEC) (Gabbiani, 1992). Estas células apresentam características intermediárias entre fibroblastos e células da musculatura lisa (Badid *et al.,* 2000) e são caracterizadas morfologicamente como células alongadas, fusiformes ou estreladas com núcleo regular e central (Micke & Ostman, 2004). Miofibroblastos apresentam um citoplasma proeminente, o qual é rico em microfilamentos de actina (fibras de estresse) e retículo endoplasmático, e podem estar conectados uns aos outros através de aderências e junções do tipo gap (Darby *et al*., 1990; Tang *et al*., 1996; Micke & Ostman, 2004). Estas células também estabelecem contatos com os componentes da MEC através de fibronexus, um complexo transmembrânico formado por actina, integrina e fibronectina (Eyden, 2001; Powell *et al*., 2005). Miofibroblastos são identificados através da expressão de α-SMA (Desmouliere *et al*., 2004), porém este marcador citoplasmático é encontrado adicionalmente em outros dois tipos celulares: células musculares lisas e células mioepiteliais. A presença de outros marcadores como laminina, desmina, calponina, miosina de músculo liso, caldesmonina e proteína de ativação dos fibroblastos tem sido utilizada para caracterizar os miofibroblastos, mas o padrão de expressão é variável e dependente principalmente da origem, localização e condição patológica (Micke & Ostman, 2004). Recentemente, De Wever e colaboradores (2008) sugeriram alguns critérios mínimos para a caracterização dos miofibroblastos, que incluem a positividade para α-SMA, vimentina e a enzima de maturação do colágeno tipo I prolil-4-hidroxilase e negatividade para citoqueratinas.

Miofibroblastos estão presentes fisiologicamente em pequenas populações no tecido conjuntivo de quase todos os órgãos, particularmente em locais onde a força mecânica é necessária (Desmouliere *et al*., 2004), e patologicamente em

condições como cicatrizes hipertróficas, fibromatoses, doenças fibro-contráteis e neoplasias (Gailit *et al*., 2001; Desmouliere *et al*., 2004; Powell *et al*., 2005). Embora os mecanismos que induzam o aparecimento dos miofibroblastos sejam parcialmente desconhecidos, estudos demonstraram uma origem a partir da diferenciação de células mesenquimais indiferenciadas ou por meio da transdiferenciação de fibroblastos e outras células mesenquimais (Tomasek *et al*., 2002; Smith *et al*., 2006; Bitu *et al*., 2006). É importante destacar que o termo transdiferenciação é aplicado quando uma célula diferenciada sofre transformação (conversão) em outro tipo celular também diferenciado. Em ambas as situações, o fator de crescimento transformante-beta1 (TGF-β1) parece ter um papel importante via ativação do fator de crescimento de tecido conjuntivo (CTGF) (Garrett *et al*., 2004; Grotendorst *et al*., 2004; Zhang *et al*., 2004; Sobral *et al*., 2007).

TGF-β1 é um peptídeo multifuncional que regula várias atividades celulares, incluindo crescimento e diferenciação celular e expressão e metabolismo de macromoléculas da MEC (Huang & Lee, 2003). Os efeitos de TGF-β1 na homeostasia do tecido conjuntivo parecem ser mediados pela ativação dos receptores citoplasmáticos Smads e de CTGF (Tabibzadeh, 2002). TGF-β1 é secretado na em uma forma latente e sua ativação é regulada por proteases, incluindo plasmina, catepsina, metaloproteinases de matriz-2 (MMP-2), MMP-9, entre outras (Annes *et al*., 2003). Este peptídeo desempenha um papel fundamental na transdiferenciação dos fibroblastos para miofibroblastos, como revelado pela sua capacidade de super-regular a expressão de colágeno e α-SMA em modelos *in vivo* e in vitro (Desmouliere *et al*., 2004; Orimo & Weinberg, 2006; Powell *et al*., 2005). No tecido tumoral, TGF-β1 pode ser derivado de células epiteliais, células inflamatórias ou dos próprios miofibroblastos (Galliher *et al*., 2006). Tuxhorn *et al*. (2002b) demonstraram *in vivo* e in vitro a participação de TGF-β1 na conversão dos fibroblastos em miofibroblastos. No modelo in vitro de cobaias com câncer de cólon, os autores demonstraram através de imuno-histoquímica que as áreas que continham a presença de miofibroblastos apresentavam uma forte expressão de TGF-β1. Adicionalmente, em ensaios in vitro

com anticorpos neutralizantes anti-TGF-β1 revelaram que a inibição de TGF-β1 foi acompanha por uma inibição na proliferação dos fibroblastos intestinais e uma significante indução na transdiferenciação em miofibroblastos (Tuxhorn *et al*., 2002a). Interessantemente, Untergasser *et al*. (2005) demonstraram que fibroblastos de próstata tratados com TGF-β1 transdiferenciaram em miofibroblastos, como revelado pela aquisição de densos feixes de fibras no citoesqueleto e um aumento na expressão de α-SMA, calponina e tenascina. A via de sinalização de TGF-β1 é iniciada a partir da ligação com seus receptores transmembrânicos que ativam os mensageiros citoplasmáticos de 2 principais cascatas: cascata Smad (mais importante e que será detalhada neste estudo) e a cascata MAPK (Derynck & Zhang, 2003; Colwell, 2006). Seguindo a ligação de TGF-β1 aos seus receptores de superfície, Smad2 e/ou Smad3 são fosforilados e formam um complexo com Smad4, sendo então translocados para o núcleo onde irão agir como fatores de transcrição ou associarem a outras proteínas nucleares (Massague & Wotton, 2000; Massague & Gomis, 2006). Smad6 e Smad7 possuem uma atividade inibitória nesta cascata, impedindo a fosforilação de Smad2 e Smad3 (Derynck & Zhang, 2003). Recentemente, nós demonstramos que TGF-β1 induz concomitantemente a expressão de CTGF e a transdiferenciação de fibroblastos gengivais normais em uma maneira dose- e tempo-dependentes (Sobral *et al*., 2007). Adicionalmente, observamos que interferon gama (IFN-γ) bloqueia os efeitos de TGF-β1 na transdiferenciação dos miofibroblastos, estimulando a expressão de Smad7 e inibindo CTGF. Estes achados suportam a hipótese de que a cascata de TGF-β1 resultando na ativação transcricional de CTGF desempenha um papel importante na transdiferenciação de miofibroblastos gengivais (Sobral *et al*., 2007).

A presença dos miofibroblastos já foi identificada no estroma de inúmeros tumores onde estão relacionadas a um fenótipo tumoral mais agressivo (Sieuwerts *et al*., 1998; Olumi *et al*.,1999; Ronnov-Jessen *et al*., 2002; Barth *et al*., 2004; Lewis *et al*., 2004; Kojc *et al*., 2005; Untergasser *et al*., 2005; Cekanova *et al*., 2006; Kuroda *et al*., 2006; Mikula *et al*., 2006; Kellermann *et al*., 2008). Por exemplo, em cânceres de mama a presença de miofibroblastos no estroma tumoral

correlacionou com um prognóstico desfavorável para os pacientes (Offersen *et al*., 2003). Adicionalmente, miofibroblastos são considerados os principais componentes do estroma dos carcinomas hepáticos, onde podem influenciar a invasão das células tumorais (Desmouliere, 2004). Estudos *in vitro* demonstraram que os produtos derivados da síntese dos miofibroblastos podem modular inúmeros eventos biológicos associados ao fenótipo maligno, incluindo crescimento, diferenciação, adesão, migração e invasão das células tumorais (Kunz-Schughart & Knuechel, 2002; Pourreyron *et al*., 2003; Lewis *et al*., 2004; De Wever *et al*., 2004; Mukaratirwa *et al*., 2005; Orimo *et al*., 2005; Powell *et al*., 2005; Baglole *et al*., 2006; Vered *et al*., 2010).

A presença dos miofibroblastos também foi demonstrada em carcinomas espinocelulares (CECs) da região de cabeça e pescoço (Barth *et al*., 2004; Kojc *et al*., 2005; Kellermann *et al*., 2007). Em estudos prévios, nós demonstramos que miofibroblastos são encontrados em aproximadamente 60% dos CECs orais, mas não estão presentes no estroma das amostras de mucosa oral normal e leucoplasias com o diagnóstico histológico de displasia (Kellermann *et al*., 2007). Adicionalmente, a presença de grandes quantidades de miofibroblastos no estroma ou fronte invasivo do tumor correlacionou significantemente com o estádio clínico avançado, estádio N, invasão linfática e vascular, presença de metástases histologicamente confirmadas em linfonodos e infiltração extra-capsular de metástases linfonodais. A presença abundante de miofibroblastos foi também correlacionada com uma menor sobrevida global dos pacientes e com um maior potencial proliferativo das células tumorais. In vitro, nossos resultados demonstraram que os produtos de síntese dos miofibroblastos induzem significantemente a proliferação das linhagens celulares de CEC oral, como revelado pelos ensaios de incorporação de bromodeoxiuridina (BrdU) e expressão imunocitoquímica de Ki-67 *(*Kellermann *et al*., 2008). Etemad-Moghadam e colaboradores (2009), analisando amostras de mucosa oral normal, displasia epitelial e CEC oral, detectaram miofibroblastos em todos os casos de CEC, enquanto que as amostras de mucosa normal e displasia foram negativas. Em

CECs de língua, Vered *et al*. (2009) detectaram miofibroblastos em 54% das amostras, sendo que esta presença foi correlacionada com uma incidência maior de recorrência local e uma redução na sobrevida global dos pacientes. Kawashiri *et al*. (2009) relataram em um estudo contendo 80 amostras de CEC oral e uma análise de sobrevida de 5 anos, que a abundante presença de miofibroblastos no estroma tumoral significantemente correlacionou com metástase linfonodal e diminuição da sobrevida global. Recentemente, nós observamos uma significante correlação entre miofibroblastos, produção de MMP-2 e ruptura da cortical óssea em ameloblastomas, sugerindo que a detecção de miofibroblastos pode ser um importante marcador prognóstico de agressividade para pacientes com este tumor odontogênico (Fregnani *et al*., 2009).

As MMPs constituem uma família com mais de 25 endopeptidases dependentes de cálcio e zinco, que exercem atividades proteolíticas na MEC em condições fisiológicas e patológicas (Konstantinopoulos *et al*., 2008). Estudos demonstram que a expressão e atividade das MMPs estão aumentadas em quase todos os tipos de cânceres humanos, sendo que esta presença está frequentemente correlacionada com estágio tumoral avançado, aumento da invasão e metástase e diminuição do tempo de sobrevida do paciente (Ohashi *et al*. 2000; Egeblad & Werb, 2002; Samantaray *et al.* 2004; Gu *et al*., 2005; Kessenbrock *et al*. 2010; Yamada *et. al*. 2010; Zhou *et al*. 2010; Kim *et al*. 2010; Garavello *et al*. 2010). O processo de metástase envolve a degradação proteolítica da MEC e a invasão das células tumorais no estroma adjacente, sendo que as MMPs são consideradas as principais responsáveis por este processo (Jodele *et al.,* 2006; Roy *et al.,* 2009).

Estudos in vitro demonstram que linhagens celulares de CEC oral capazes de secretar MMP-1, MMP-2 e MMP-9 possuem uma característica mais invasiva, sugerindo que a habilidade das células tumorais em secretar MMPs exerce uma função importante no comportamento maligno destes tumores (Kusukawa *et al* 1992; Kawahara et al. 1993; Juarez *et al*. 1993; Shindoh *et al*. 1996; Kawamata *et al*. 1997). Tsai e colaboradores (2003) demonstraram que linhagens celulares de

CEC oral apresentavam uma produção significativamente maior de MMP-2 e MMP-9 comparadas com linhagens celulares de queratinócitos orais normais. A presença de MMPs também foi identificada *in vivo* em amostras de CEC oral. Kuzukawa e colaboradores (1993) observaram que 76% dos pacientes com CEC oral com presença de metástase linfonodal apresentavam uma elevada expressão de MMP-2 nas células tumorais, enquanto que nos casos de ausência de metástase, esta marcação foi encontrada em apenas 25% dos casos. A expressão de MMP-3 também já foi relatada em amostras de CEC oral, sendo significativamente relacionada ao tamanho do tumor, invasão tumoral e alta incidência de metástase linfonodal (Kuzukawa *et al*., 1995; Kuzukawa *et al*., 1996). Estudos mais recentes correlacionam a expressão de MMP-2 e MMP-9 pelas células de CEC oral com um comportamento tumoral mais agressivo, caracterizado por uma maior capacidade de invasão tumoral, presença de metástases linfonodais, recorrência e pior prognóstico (Ikebe *et al*., 1999; Hong *et al*., 2000; Yoshizaki *et al*., 2001; Katayama *et al*., 2004; Patel *et al*., 2007; Sun *et al*., 2008). Um estudo realizado em nosso laboratório correlacionou o aumento da atividade de MMP-2 e MMP-9 por amostras de CEC oral com a diminuição da sobrevida global destes pacientes (Yorioka *et al*., 2005).

Sabe-se hoje, que a interação epitélio-mesênquima, através da produção de diversos fatores pelas células mesenquimais exerce uma importante função no comportamento tumoral. Em relação as MMPs, evidências demonstram que a produção destas enzimas pelas células mesenquimais, como células endoteliais, inflamatórias, pericitos, fibroblastos e miofibroblastos, contribuem para a modulação e degradação da MEC e consequente invasão e metástase tumoral (Jodele *et al*. 2006). Além de produzir estas enzimas, as células do estroma, também podem induzir as células tumorais a produzi-las, contribuindo para um fenótipo mais agressivo. Hayashido *et al.* (2003) demonstraram que linhagens celulares de CEC oral quando tratadas com meio condicionado por fibroblastos, apresentavam um aumento da atividade MMP-2, sugerindo que os fibroblastos estariam facilitando o processo de invasão tumoral. Uma análise, por meio de

hibridização in situ em 30 casos de CEC oral, demonstrou que tanto as células tumorais quanto as células do estroma apresentavam positividade para MMP-2 e MMP-9, sendo que pacientes com metástase linfonodal possuíam uma maior expressão destas enzimas (Liu *et al*.; 2001). Em outro estudo, utilizando análises de microarranjo de expressão, os autores demonstraram que miofibroblastos no estroma de CECs orais apresentaram uma superexpressão da MMP de membrana do tipo I (MT1-MMP) (Rosenthal *et al*.; 2004). Zhang e colaboradores (2006) inibiram a expressão de MMP-2, MMP-9 e MT1-MMP em linhagens celulares de fibroblastos e avaliaram a capacidade destas células em promover invasão tumoral e induziram a formar, por meio da injeção conjunta com células de CEC de cabeça e pescoço, tumores em camundongos. Estes autores observaram uma significante redução no tamanho tumoral *in vivo* e na capacidade invasiva in vitro, sugerindo que a síntese de MMPs pelas células do estroma tumoral ser um fator determinante para o fenótipo agressivo dos tumores. Em amostras de melanoma, a expressão de MMP-13 foi encontrada predominantemente em células do estroma, sendo que esta expressão foi necessária para o processo de invasão e o desenvolvimento de mestástase em modelo xenográfico. (Zigrino *et al*., 2009).

Ativina A é um membro da superfamília de TGF-β que participa da mediação de alguns eventos celulares, como crescimento e diferenciação celular (Kingsley, 1994). Esta proteína foi originalmente purificada do fluido ovariano e identificada como um fator pituitário de secreção do hormônio folículo estimulante (FSH), porém, posteriormente, foi identificada como tendo funções regulatórias em diversos órgãos e tecidos (Ling *et al*., 1986; Vale *et al*., 1986). Ativina A é um homodímero composto por 2 subunidades βA ligadas por um ligação bissulfídica que é codificada pelo gene inibina βA (*INHBA)* e tem sido considerada um importante regulador no desenvolvimento de órgãos e no processo de cicatrização de feridas (Welt *et al*., 2002; Vale *et al*., 2004). A superexpressão de ativina A já foi identificada em alguns tipos de câncer, sendo principalmente correlacionada com uma maior progressão tumoral e pior prognóstico (Risbridger *et al*., 2001; Devouassoux-Shisheboran *et al*., 2003; Tanaka *et al*., 2004; Yoshinaga *et al*., 2008;

Seder *et al*., 2009a e b; Chang *et al*., 2010). Yosinaga e colaboradores (2008) avaliaram o efeito da superexpressão de ativina A em linhagens celulares de câncer esofágico humano e observaram um aumento da proliferação, invasão e tolerância a apoptose celular in vitro e uma maior progressão tumoral em modelo xenográfico. Adicionalmente, o bloqueio da atividade protéica com anticorpos neutralizantes inibiu a migração celular das células tumorais. A participação de ativina A também foi descrita em adenocarcinomas esofágicos (Seder *et. al.,* 2009b). Por meio de ensaios de microarranjo de expressão e confirmação por PCR em tempo real, os autores demonstraram que a expressão de *INHBA* é ~5,7 vezes maior nestes tumores comparado com amostras de metaplasia de Barret. Análise imuno-histoquímica destes tumores revelou a expressão de ativina A em 69% dos espécimes tumorais, enquanto em amostras de displasia e metaplasia de Barret foram encontrados em apenas 37% e 33% das amostras, respectivamente. In vitro, este estudo demonstrou que o tratamento de linhagens celulares de adenocarcinoma esofágico com ativina A exógena induz proliferação celular, enquanto o silenciamento deste gene utilizando técnicas de RNA de interferência reduz este efeito (Seder *et. al*., 2009b). Em outro estudo, estes mesmos autores encontraram uma expressão aumentada de *INHBA* em adenocarcinomas pulmonares comparada com amostras de pulmão normal, e pacientes com estágio clínico do tumor nível I, os altos níves de expressão de *INHBA* foram correlacionados com um pior prognóstico (Seder *et al*., 2009a). Adicionalmente, a presença de níveis elevados de ativina A no soro, tem sido proposta como sendo um marcador para câncer de mama, carcinoma hepatocelular, endometrial e cervical (Petraglia *et al.*, 1998; Pirisi *et al*., 2000; Reis *et al.*, 2002).

A superexpressão de ativina A também já foi encontrada em cânceres da região de cabeça e pescoço, incluindo carcinomas espinocelulares orais. Shimizu e colaboradores (2007) detectaram, por meio da técnica de microarranjos de expressão, a superexpressão de *INHBA* em linhagens celulares de câncer de cabeça e pescoço e boca. Clinicamente, estes autores detectaram um aumento na expressão de *INHBA* em amostras tumorais comparadas com amostras de tecido

normal, sendo que esta presença foi significantemente relacionada com diminuição da sobrevida global dos pacientes. Outro estudo, utilizando amostras de carcinoma espinocelular de língua e análise de transcriptoma, revelou que *INHBA* está entre os genes superexpressos no câncer de boca (Ye *et al*., 2008). Recentemente, Chang e colaboradores (2010) demonstraram que a superexpressão de ativina A em casos de CEC oral correlacionou significativamente com estadiamento N, presença de invasão perineural, tumores histologicamente classificados como pouco diferenciados e pior prognóstico. É importante salientar que as micrografias demonstradas neste estudo claramente apontaram para a expressão de ativina A pelas células tumorais e pelas células do estroma do tumor. In vitro, este estudo demonstrou que a inibição da expressão de ativina A em linhagens celulares de carcinoma espinocelular oral, ocasionou uma diminuição na proliferação, migração e invasão celular. (Chang *et al*., 2010) Estas evidências sugerem que ativina A possa estar envolvida na carcinogênese, progressão e metástase de alguns tipos de cânceres, contudo o exato mecanismo de ação de ativina A nestes processos ainda não está totalmente esclarecido.

Diante dos argumentos dispostos, os objetivos deste estudo foram 1) avaliar a participação de CTGF na transdiferenciação de miofibroblastos gengivais induzida por TGF-β1 e determinar o efeito da superexpressão de Smad7 neste processo, 2) estabelecer linhagens celulares de fibroblastos e miofibroblastos do estroma de CECs orais e comparar seus potenciais proliferativos e de produção de MMPs, e 3) analisar o efeito dos fatores de síntese dos miofibroblastos na modulação da proliferação e invasão de células tumorais, bem como avaliar a importância de ativina A neste processo.

2. Capítulo 1

Smad7 blocks transforming growth factor-β1-induced gingival fibroblast-myofibroblast transition via inhibitory regulation of Smad2 and connective tissue growth factor

Short title: Inhibitory effect of Smad7 on myofibroblast.

Key words: Myofibroblast; TGF-β1; Smad7; Smad2; CTGF; gingival fibromatosis.

One-sentence summary

We demonstrate the participation of TGF-β1, Smad2 and CTGF on transition of gingival fibroblast into myofibroblast and further showed that Smad7 overexpression significantly blocks this process.

Abstract

Background: Transforming growth factor-β1 (TGF-β1), its downstream signaling mediators (Smad proteins) and specific targets, including connective tissue growth factor (CTGF), play important roles in tissue remodeling and fibrosis via myofibroblast activation. We investigated the effect of overexpression of Smad7, a TGF-β1 signaling inhibitor, on transition of gingival fibroblast to myofibroblast. Moreover, we analyzed the participation of CTGF on TGF-β1-mediated myofibroblast transformation.

Methods: To study the inhibitory effect of Smad7 on TGF-β1/CTGF-mediating gingival fibroblast transition into myofibroblasts, we stably overexpressed Smad7 in normal gingival fibroblasts and in myofibroblasts from hereditary gingival fibromatosis (HGF). Myofibroblasts were characterized by the expression of its specific marker isoform α of the smooth muscle isoform actin (α-SMA) by western

blot, flow cytometry and immunofluorescence. Enzyme-linked immunosorbent assay (ELISA) for type I collagen was performed to measure myofibroblast activity. CTGF role on myofibroblast transformation was examined by ELISA and small interference RNA (siRNA).

Results: TGF-β1 induced the expression of α-SMA and CTGF, and siRNA-mediating CTGF silencing prevented fibroblast-myofibroblast switch induced by TGF-β1. In Smad7-overexpressing fibroblasts, ablation of TGF-β1-induced Smad2 phosphorylation marked decreased α-SMA, CTGF and type I collagen expression. Similarly, HGF transfectants overexpressing Smad7 demonstrated low levels of α-SMA and phospho-Smad2 and significant reduction on CTGF and type I collagen production.

Conclusions: CTGF is critical for TGF-β1-induced gingival fibroblast-myofibroblast transition, and Smad7 overexpression is effective in the blockage of myofibroblast transformation and activation, suggesting that treatments targeting myofibroblasts by Smad7 overexpression may be clinically effective in gingival fibrotic diseases, such as HGF.

Introduction

Myofibroblasts are mesenchymal cells that exhibit a phenotype between fibroblasts and smooth muscle cells, and are characterized by the expression of the specific isoform α of the smooth muscle actin (α-SMA).[1,2] Those cells were first identified in the granulation tissue, where contribute for the wound healing, but they are present as a minor cell subpopulation in almost all organs.[3] Myofibroblasts were late described as the main cell type associate with fibrotic process, including hypertrophic scarring and pulmonary, renal and hepatic fibrosis.[4-6] Myofibroblasts are able to express and secrete an extensive repertoire of cytokines, growth factors, chemokines, hormones, neurotransmitters, inflammatory mediators, adhesion proteins, and extracellular matrix molecules.[7] There is little understanding about the underlying mechanisms that regulate myofibroblast emergence, however the regulatory cytokine transforming growth factor-β1 (TGF-β1) has been traditionally considered as an inducer of the myofibroblastic phenotype via activation of the connective tissue growth factor (CTGF)-dependent pathway.[8-10]

Our previous study demonstrated that the presence of myofibroblasts in hereditary gingival fibromatosis (HGF), a fibrotic gingival disease characterized by connective tissue accumulation, is heterogeneous and associated with CTGF expression levels.[11] Recently we demonstrated that TGF-β1 concomitantly induces CTGF expression and leads to a dose- and time-dependent induction of normal gingiva (NG) fibroblast transformation into myofibroblast[12]. In addition, it was revealed that interferon-γ blocks the effects of TGF-β1 on myofibroblast transformation, stimulating Smad7 expression and inhibiting CTGF.[12] These findings support the hypothesis that CTGF plays a crucial role in mediating TGF-β1-induced NG myofibroblast transformation, and that the blockage of TGF-β1 signaling may be clinically effective in the attenuation of myofibroblast emergence and, consequently, excessive accumulation of extracellular matrix. In the present study we have explored the biological role of CTGF on gingival fibroblast to myofibroblast transition induced by TGF-β1, and analyzed whether the

overexpression of Smad7 could block TGF-β1-induced fibroblast-myofibroblast switch and could inhibit the activity of HGF myofibroblasts.

Materials and Methods

Cell cultures, plasmids and treatments

NG fibroblasts (NG1, NG2, NG3, NG4 and NG5 cell lines) and HGF myofibroblasts (HGF cell line) were described previously.[12] Cells were maintained in Dulbecco's modified Eagle's medium* (DMEM) containing 10% fetal bovine serum (FBS), 100 μg/ml penicillin and 100 μg/ml streptomycin at 37°C in a 5% CO_2 air atmosphere. NG-Smad7 (from transfection of NG1 cell line) and HGF-Smad7 stable cells were generated as previously described[13] using the Smad7-pcDNA3 plasmid.[14] Control cells for Smad7 transfectants constitute the cell lines transfected with vector alone. Lyophilized TGF-β1† was dissolved in culture medium, aliquoted and stored at -80°C. To assess the effect of this cytokine on myofibroblast transformation and CTGF production, cells were serum starved for 24 h before treatment with 10 ng/ml of TGF-β1 for 3 days. To determine the effect of TGF-β1 on phosphorylation of Smad2, clones were treated with TGF-β1 for 1 h. The study protocol was approved by the Ethical Committee in Research at the School of Dentistry, State University of Campinas.

Western blot analysis

Cells were washed with cold PBS and lysed in RIPA buffer (50 mM Tris HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40%, 1% deoxycholic acid, 0.5% sodium dodecyl sulphate, 1mM phenymethylsulphony fluoride, 1mM N-ethylmaleimide, 1mM dithiothreitol, 10 mg/ml soybean trypsin inhibitor, 1 mg/ml leupeptin, and 1 mg/ml aprotinin) for detection of α-SMA, or in detergent-free buffer (10 mM Tris-HCl pH 7.4, 5 mM NaCl, 1 mM EDTA and protein inhibitors) associated with mechanical disruption for detection of Smad proteins. After centrifugation, protein

* Invitrogen, Carlsbad, CA, USA.
† R&D Systems, Minneapolis, MN, USA.

concentrations were measured using a protein assay[‡] according to the manufacturer's instructions. Fifty micrograms of total protein per sample was resolved in 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions, and transferred onto nitrocellulose membranes. The membranes were blocked overnight with 10% non-fat dry milk in PBS containing 0.1% Tween 20, rinsed in the same buffer, and incubated for 2 h with the following antibodies: anti-α-SMA[§], anti-Smad7[**], anti-pSmad2 and anti-β-actin[††]. Reactions were developed using a chemiluminescent western blot system[‡‡].

Flow cytometry

After incubation with TGF-β1 for 3 days, NG fibroblasts were released from the cell culture flasks, and single-cell suspensions were fixed with 70% ethanol and stained with anti-α-SMA antibody followed by goat anti-mouse IgG conjugated with fluorescein[§§]. Cells were washed, resuspended in PBS, and analyzed on a flow cytometer equipped with an argon laser[***]. A minimum of 10,000 events was collected on each sample, and only cells with forward and orthogonal light scatter characteristics similar to whole and intact cells were included in the analysis. Quantitative flow cytometric analysis was performed with the aid of software[†††], measuring the percentage of α-SMA-positive cells.

Immunofluorescence

Ten thousand cells were plated in each well of a 8-well culture chamber slides and incubated at 37ºC in humidified air containing 5% CO_2 for 24 h. Following incubation, cells were washed in PBS and fixed in 70% ethanol for 15 min. To prevent non-specific binding, the cells were blocked with 3% BSA in PBS for 1 h.

[‡] Bradford protein assay, Bio Rad, Hercules, CA, USA.
[§] Dako Corp., Carpenteria, CA, USA.
[**] Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA.
[††] Sigma-Aldrich, St. Louis, MO, USA.
[‡‡] Enhanced chemiluminescent western blot kit, GE Healthcare, Vienna, Austria.
[§§] Vector Labs, Burlingame, CA, USA.
[***] FACScalibur, Becton Dickinson, San Jose, CA, USA.
[†††] CellQuest, Becton Dickinson, San Jose, CA, USA.

Cells were then incubated for 1 h with anti-α-SMA or anti-pSmad2 antibodies diluted 1:100, followed by incubation with secondary anti-IgG conjugated with fluorescein at 1:250. Cells were mounted with a fluorescent mounting media containing DAPI[‡‡‡] and examined under a photomicroscope equipped with epifluorescence[§§§]. To generate fluorescent labeled images, cells were excited at 480/40 nm with a 527/30 band pass filter. Cells untreated with primary antibodies were used as negative controls.

Small interference RNA (siRNA)-mediated silencing of CTGF

To determine the role of CTGF in TGF-β1-induced fibroblast to myofibroblast transition, we examined the effect of CTGF siRNA transfection on α-SMA expression induced by TGF-β1 on NG1 cell line. The 25-mer RNA molecules were chemically synthesized, annealed and purified by the manufacturer[*]. Three sequenced targeting CTGF (NM_001901) were used, corresponding to nucleotides 746-770 (5'AAA CGT GTC TTC CAG TCG GTA AGC C3'), 1027-1051 (5'TTA GCT CGG TAT GTC TTC ATG CTG G3'), and 1135-1159 (5'ATC ATG TTC TTC TTC ATG ACC TCG C3'). In essence, fibroblasts grown to 50%-confluence were transfected with 100 nM of a mixture containing equal parts of the 3 CTGF siRNAs using a liposome based reagent[****] according to manufacturer's instructions. In parallel, to act as negative controls, cell were transfected with a nonspecific siRNA. Thirty hours after transfection, cells were washed with PBS and exposed to TGF-β1 for 48 h. Thus, the experimental conditions were: nonspecific siRNA, nonspecific siRNA plus 10 ng/ml TGF-β1, and CTGF siRNA plus 10 ng/ml TGF-β1. CTGF mRNA and protein levels were determined by semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) and enzyme-linked immunosorbent assay (ELISA) respectively, and α-SMA protein levels were determined by western blot.

[‡‡‡] Vectashield, Vector Labs, Burlingame, CA, USA.
[§§§] Leica Microsystems, Wetzlar, Germany.
[****] Lipofectamine 2000, Invitrogen, Carlsbad, CA, USA.

RT-PCR assay

After total RNA isolation and DNase I treatment in order to eliminate genomic DNA contamination, 2 μg of total RNA per sample were used to generate cDNA using a superscript enzyme[††††]. The resulting cDNAs were subsequently amplified, analyzed and quantified as described previously.[15] Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as a reference gene. Primer sequences, PCR conditions and the amplified lengths have been described elsewhere.[11]

ELISA

Production of CTGF and type I collagen was determined by ELISA. Cells were plated in 24-well culture plates at a density of 80,000 cells/well in DMEM containing 10% FBS. After 16 h, the cells were rinsed with PBS and the medium replaced by 0.1% FBS-DMEM with or without TGF-β1. ELISA for type I collagen was performed after the methods of Sobral et al.[12] For CTGF quantification, culture medium was collected and remaining cells fixed and used for cell counting by toluidine blue stain technique.[16] In essence, microtite plate wells were coated with 200 μl of the culture medium for 2 h at room temperature. The wells were then washed 3 times with 400 μl of 1% Tween 20 in PBS and non-specific binding sites were blocked with 3% BSA in PBS for 2 h. After washing, anti-CTGF antibodies[§] diluted 1:200 in PBS were added to the wells and incubated for 2 h. After another washing step, peroxidase-conjugated streptavidin diluted 1:500 in PBS was added and maintained for 1 h. The reaction was developed with 0.5 mg/ml of o-phenylenediamine in 0.5 M citric buffer pH 5.5 containing 0.01% H_2O_2 for 20 min. After terminating the reaction with 50 μl of 2 N H_2SO_4, absorbance was read at 450 nm. The values were expressed as CTGF/cell.

[††††] Superscript II RT enzyme, Invitrogen, Carlsbad, CA, USA.

Statistical analysis

When appropriate, one-way analysis of variance (ANOVA) with post hoc comparisons base on the Tukey test was performed. In the current comparisons, $p<0.05$ was considered to indicate statistical significance.

Results

Myofibroblast transformation induced by TGF-β1 is dependent of CTGF activation

The effect of TGF-β1 on transformation of human NG fibroblasts into myofibroblasts was assessed by western blot and flow cytometric analysis. As depicted in Fig. 1A, TGF-β1 clearly induces the transformation of myofibroblasts, as revealed by the increased expression of the myofibroblast marker α-SMA in the cells treated with 10 ng/ml of TGF-β1 in comparison with unstimulated cells. Three NG fibroblast cell lines were selected and subjected to flow cytometric analysis to determine the proportion of α-SMA-positive cells after TGF-β1 treatment. TGF-β1-treated cells exhibited an upward and unimodal shift of staining for α-SMA, indicating that most of the cells were responsive to TGF-β1 (more than 90% of the treated-cells were α-SMA-positive) (Fig. 1B). NG cell lines treated with TGF-β1 also demonstrated an increase in the CTGF production (Fig. 1C, $p<0.01$ between stimulated and unstimulated cells). All the cell lines showed significantly higher levels of production of CTGF than unstimulated controls, with exception of NG2 that showed elevation on CTGF production but not significantly different than control.

To determine the role of CTGF on TGF-β1-induced fibroblast to myofibroblast transition, we assessed the effect of CTGF siRNA on expression of α-SMA induced by TGF-β1. Specific-stranded RNA oligonucleotides against CTGF (CTGF siRNA) or nonspecific siRNA (control siRNA) were transfected into NG1 cells. When CTGF-specific oligonucleotides were used, a rapid down-regulation of CTGF mRNA and protein even after TGF-β1 treatment was observed (Fig. 1D and 1E). In the absence of CTGF induction, transformation of myofibroblasts was markedly inhibited (Fig.

1F). Together, these data indicate that CTGF stimulation is a crucial event in TGF-β1-induced myofibroblast transformation.

Overexpression of Smad7 inhibits TGF-β1-induced myofibroblast transformation

Since interferon-γ blocks TGF-β1-induced myofibroblast transformation via stimulation of Smad7, we determined whether Smad7 overexpression prevents the effect of TGF-β1 on transition of NG fibroblasts in myofibroblasts. Stable Smad7 and control transfectants were generated and examined for Smad7 protein levels (Fig.2A). As anticipated, exposure of NG fibroblasts and NG-Control fibroblasts to 10 ng/ml of TGF-β1 for 3 days was associated with marked increase of α-SMA production compared with untreated cells (Fig.2B); however, TGF-β1 treatment of Smad7-overexpressing cells resulted in an attenuation of α-SMA production. To confirm these findings, immunofluorescence reactions showed that NG and NG-Control cells treated with 10 ng/ml of TGF-β1 exhibited abundant bundles of α-SMA and had typical flattened myofibroblast morphology, which was not observed in TGF-β1-treated Smad7-overexpressing cells (Fig. 2C).

Since TGF-β1 signaling starts with activation of Smad2 in many cells lines,[17] we examined the activation pattern of phosphorylated Smad2 (pSmad2) in the control and Smad7-overexpressing cells. Treatment with TGF-β1 induced phosphorylation of Smad2 in both NG and NG-Control cells, whereas pSmad2 was fairly low in NG-Smad7 cells in the absence or presence of TGF-β1 (Fig. 3A). To examine the localization of pSmad2 after TGF-β1 treatment we performed immunofluorescence analysis. TGF-β1 treatment of NG and NG-Control fibroblasts caused a marked transnuclear location of pSmad2, which was in contrast to observed in NG-Smad7 cells (Fig. 3B). Few nuclear-positive cells were found in NG-Smad7 cells after treatment with 10 ng/ml of TGF-β1. Interestingly, the attenuation in the transformation of Smad7-overexpressing cells in myofibroblasts by TGF-β1 was accompanied by a significant reduction on CTGF levels (Fig. 4A).

To demonstrate that the increased type I collagen activity of the myofibroblasts induced by TGF-β1 is also inhibited by Smad7 overexpression, we performed ELISA. Overexpression of Smad7 significantly decreased type I collagen production induced by TGF-β1 in NG cultures (Fig. 4B).

Overexpression of Smad7 inhibits α-SMA and type I collagen expression in human HGF myofibroblastic cells

We previously demonstrated that interferon-γ reverse the myofibroblastic phenotype of HGF cells due to an increase in Smad7 expression.[12] Thus, we set out to determine whether Smad7 overexpression could affect HGF myofibroblastic cells. To this end, we established HGF myofibroblastic cells expressing high levels of Smad7. In opposite to high Smad7 levels, stable Smad7-overexpressing HGF cells demonstrated reduced levels of pSmad2 and α-SMA (Fig. 5A). Consistent with these observations, immunofluorescence analyses showed a marked reduction of the characteristic brilliant striated α-SMA-staining throughout the cytoplasm and of the pSmad2 nuclear staining pattern (Fig. 5B and 5C). To determine whether this phenotype was accompanied by the loss of myofibroblast activity, we analyzed CTGF and type I collagen production by ELISA. Both CTGF and type I collagen production were significantly lower in Smad7-overexpressing HGF cells compared with control cells (Fig. 6).

Discussion

The development of gingival overgrowth can be inherited (recognized as HGF), induced as a side-effect of systemic drugs, such as phenytoin, cyclosporine A or nifedipine, or idiopathic (idiopathic gingival overgrowth). Independent of the etiology, the gingival overgrowth is resulted of a connective tissue fibrosis histologically characterized by an excessive accumulation of extracellular matrix proteins, particularly type I collagen.[18] In HGF, gingival fibrosis is associated with increased production of TGF-β1 and collagen, and elevated proportion of myofibroblast

cells.[11,12,15] In this study, we have found that TGF-β1 activates Smad2 and CTGF in gingival fibroblasts promoting myofibroblast transformation with increased type I collagen production, and that overexpression of Smad7 blocks this process.

TGF-β1 is largely recognized to play a pathogenic role in fibrotic disorders, and most of its effects have been associated with myofibroblast stimulation.[19] Thus, understanding TGF-β1 intracellular signal transduction pathways involved in myofibroblast activation is crucial for the development of therapeutic approaches to fibrosis. In the past few years, the receptors and signal transduction pathways mediating TGF-β1 effects have been identified, enabling the delineating of the specific pathways involved in pathogenic events dependent on this cytokine. TGF-β type I and type II transmembrane receptor serine/threonine kinases transduce downstream signals via cytoplasmic latent factors called Smad proteins. Smad2 and Smad3 are phosphorylated directly by the receptor kinases, after which they partner with Smad4 and translocate to the nucleus, where they act as transcriptional regulators of target genes, including CTGF.[8,10,20] In addition, activation of TGF-β1 signaling also results in the expression of inhibitory Smads, which include Smad7. Smad7 is not constitutively expressed but is rapidly induced by TGF-β1 in several cell types, including fibroblasts.[21,22] The inhibitory Smads decrease Smad2 and Smad3 phosphorylation by blocking their access to TGF-β receptors or causing degradation of TGF-β receptors via a negative feedback mechanism.[20,23,24] Previous studies of our laboratory showed that in parallel with the myofibroblast transformation induced by TGF-β1, CTGF expression is also stimulated. Indeed, TGF-β1-induced CTGF expression occurred earlier than the α-SMA expression, leading to the hypothesis that CTGF may serve as a mediator of TGF-β1 stimulation of gingival myofibroblast transformation.[12] In support, we demonstrated that the presence of myofibroblasts in HGF is associated with the CTGF expression levels.[11]

In the first assays of the present study we explored the dependency of CTGF on TGF-β1-induced NG fibroblast transition in myofibroblast. TGF-β1 clearly induced myofibroblast transformation and CTGF production. When siRNAs specifics

for CTGF were used, a rapid down-regulation of CTGF expression and production compared with NG cells treated with nonspecific siRNA sequences was observed (data not show). Interestingly, TGF-β1-induced transition of NG fibroblasts in myofibroblasts was significantly blocked in cells with down-regulation of CTGF, revealing that CTGF expression is needed for TGF-β1 induction of NG myofibroblast transformation. Some lines of evidence have suggested that CTGF is required for myofibroblast transformation in in vitro and in vivo studies. For example, CTGF was found in elevate levels in renal, pulmonary and hepatic fibrotic disorders,[25-27] and its expression was strongly induced by TGF-β in vitro.[28] Zhang *et al.*[10] demonstrated that addition of CTGF induces myofibroblast transformation in human renal cells, and that antisense oligonucleotides against CTGF abolished myofibroblast induction by TGF-β1. Similar findings were also observed in human corneal fibroblasts.[29] Recently Mori *et al.*[30] showed that myofibroblast transformation induced by TGF-β1 is impaired in mouse embryonic fibroblasts isolated from CCN2-null mice (CTGF is encoded by CCN2 gene). Together, these findings indicate that CTGF is functionally involved in mediating TGF-β1 induction of myofibroblast transformation.

The present study also revealed that the fibrotic effects of TGF-β1 on NG and HGF, in terms of myofibroblast transformation and activity (production of type I collagen), signals though activation of Smad2. Indeed, blockage of Smad2 phosphorylation and translocation to nucleus by inducing overexpression of Smad7 resulted in inhibition of TGF-β1-induced myofibroblast transformation. This is further demonstrated by the finding that Smad7 overexpression in HGF myofibroblastic cells reduced Smad2 phosphorylation and nuclear localization, and inhibited CTGF, α-SMA and type I collagen expression. Interestingly, Smad7 overexpression did alter the high levels of TGF-β1 expression and production of HGF cells and neither modulated Smad3 phosphorylation (data not shown). In both TGF-β1-induced transformation and HGF myofibroblastic cells, overexpression of Smad7 was not able to completely block α-SMA expression, suggesting that pathways independent

of Smad7 may exist. Indeed, TGF-β1 signaling involves both Smad-dependent and Smad-independent pathways, such as activation of MAPKs (mitogen-activated protein kinases), including extracellular signal-regulated kinase (ERK)1/2, c-Jun N-terminal kinase and p38, and effectors of Rho GTPases.[31-34] Interestingly, it has been demonstrated that both Smad3 and ERK1/2, but not Smad2, cooperatively mediates TGF-β stimulatory effect on CTGF production in gingival fibroblasts.[35] However, participation of non-Smad proteins on myofibroblast transformation was not reported yet.

Smad proteins have been implicated in other models of fibrosis. It has been shown that Smad7, but not Smad6, is a TGF-β-induced attenuator of Smad2-mediated inhibition of embryonic morphogenesis,[36] and overexpression of Smad7, but not Smad6, inhibits TGF-β-induced hemeoxygenase-1 by human tubular epithelial cells.[37] Impaired of Smad2 activation and downregulation of collagen was observed in Smad7 overexpressing cells from renal tubular epithelia and smooth muscle.[38,39] In stellate cells (hepatic myofibroblasts) ectopic expression of Smad7 also leads to abrogation of Smad2 activation.[40] Furthermore, expression of Smad7 transgene blocked Smad2 phosphorylation induced by bleomycin in mouse lung, and gene transfer of Smad7 prevented bleomycin-induced lung fibrosis.[41] Additionally, overexpression of Smad7 resulted in marked inhibition of TGF-β-induced Smad2 activation with the prevention of collagen synthesis and myofibroblast transformation[42] and, in contrast, decreased Smad7 expression contributed to cardiac fibrosis in the infarcted rat heart.[43] More recently, Wang *et al.*[17] demonstrated that ectopic expression of Smad7 inhibit TGF-β-induced collagen production by cardiac myofibroblasts via blockage of Smad2, but not Smad3, phosphorylation. Together with our previous results showing that interferon-γ blocks TGF-β1 signaling on NG myofibroblast transformation by stimulating Smad7, but not Smad6, expression,[12] the current findings suggest that activation of Smad2 and CTGF might be the intracellular mechanism by which TGF-β1 mediates gingival

fibrosis in HGF, and that forced expression of Smad7 is capable of attenuate this process.

It is well known that treatments that block TGF-β1 expression or its biological activity have therapeutic potential by reduce pathological fibrosis.[44] However, most anti-fibrotic therapies currently in use are not specific and frequently cause severe side effects. Furthermore, whether TGF-β1 blockade interferes with myofibroblast generation and/or activity is still unclear. Thus, myofibroblast transformation via Smad2 phosphorylation and CTGF activation, and its blockage by the overexpression of Smad7 may lead to the development of a novel and more specific therapy for HGF as well as other interstitial fibrosis. Importantly, overexpression of Smad7 suppressed an injury induced fibrogenic reaction of the corneal endothelium in vivo in rats, which was associated with almost completely abolishment of Smad2 phosphorylation, expression of α-SMA and the accumulation of type I collagen in the mesenchymal cells.[45]

In conclusion, the present findings provide evidence that gingival fibroblast transition in myofibroblast induced by TGF-β1 is dependent of phosphorylation of Smad2, and CTFG induction and activity. This study also demonstrated that overexpression of Smad7 inhibits TGF-β1-induced myofibroblast transformation and reverts the myofibroblastic phenotype of HGF cells with reduction on type I collagen production. Thus, interference of TGF-β1 signaling by overexpression of Smad7 might have potential to be clinically effective in attenuating excessive accumulation of extracellular matrix produced by myofibroblasts in HGF.

Conflict of interest and source of funding statement

There are no conflicts of interest associated with this work.

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References

1. Badid C, Mounier N, Costa AM, Desmouliere A. Role of myofibroblasts during normal tissue repair and excessive scarring: interest of their assessment in nephropathies. *Histol Histopathol* 2000;15:269-280.
2. Gabbiani G. The biology of the myofibroblast. *Kidney Int* 1992;41:530-532.
3. Desmouliere A., Guyot, C. & Gabbiani, G. The stroma reaction myofibroblast: a key player in the control of tumor cell behavior. *Int J Dev Biol* 2004;48:509-517.
4. Desmouliere A, Chaponnier C, Gabbiani G. Tissue repair, contraction, and the myofibroblast. *Wound Repair Regen* 2005;13:7-12.
5. Hinz B, Gabbiani G. Cell-matrix and cell-cell contacts of myofibroblasts: role in connective tissue remodeling. *Thromb Haemost* 2003;90:993-1002.
6. Willis BC, duBois RM, Borok Z. Epithelial origin of myofibroblasts during fibrosis in the lung. *Proc Am Thorac Soc* 2006;3:377-382.
7. Powell DW, Adegboyega PA, Di Mari JF, Mifflin RC. Epithelial cells and their neighbors I. Role of intestinal myofibroblasts in development, repair, and cancer. *Am J Physiol Gastrointest Liver Physiol* 2005;289:G2-7.
8. Grotendorst GR, Duncan MR. Individual domains of connective tissue growth factor regulate fibroblast proliferation and myofibroblast differentiation. *FASEB J* 2005;19:729-738.
9. Grotendorst GR, Rahmanie H, Duncan MR. Combinatorial signaling pathways determine fibroblast proliferation and myofibroblast differentiation. *FASEB J* 2004;18:469-479.
10. Zhang C, Meng X, Zhu Z, Liu J, Deng A. Connective tissue growth factor regulates the key events in tubular epithelial to myofibroblast transition in vitro. *Cell Biol Int* 2004;28:863-873.
11. Bitu CC, Sobral LM, Kellermann MG, et al. Heterogeneous presence of myofibroblasts in hereditary gingival fibromatosis. *J Clin Periodontol* 2006; 33,393-400.
12. Sobral LM, Montan PF, Martelli-Junior H, Graner E, Coletta RD. Opposite effects of TGF-beta1 and IFN-gamma on transdifferentiation of myofibroblast in human gingival cell cultures. *J Clin Periodontol* 2007;34: 397-406.
13. Coletta RD, Christensen K, Reichenberger KJ, et al. The Six1 homeoprotein stimulates tumorigenesis by reactivation of cyclin A1. *Proc Natl Acad Sci USA* 2004;101:6478-6483.

14. Mazars A, Lallemand F, Prunier C, et al. Evidence for a role of the JNK cascade in Smad7-mediated apoptosis. *J Biol Chem* 2001;276:36797-36803.
15. Martelli-Junior H, Cotrim P, Graner E, Sauk JJ, Coletta RD. Effect of transforming growth factor-beta1, interleukin-6, and interferon-gamma on the expression of type I collagen, heat shock protein 47, matrix metalloproteinase (MMP)-1 and MMP-2 by fibroblasts from normal gingiva and hereditary gingival fibromatosis. *J Periodontol* 2003;74:296-306.
16. Scragg MA, Ferreira LR. Evaluation of different staining procedures for the quantification of fibroblasts cultured in 96-well plates. *Anal Biochem* 1991;198:80-85.
17. Wang B, Omar A, Angelovska T, et al. Regulation of collagen synthesis by inhibitory Smad7 in cardiac myofibroblasts. *Am J Physiol Heart Circ Physiol* 2007;293:H1282-1290.
18. Coletta RD, Graner E. Hereditary gingival fibromatosis: a systematic review. *J Periodontol* 2006;77:753-764.
19. Scotton CJ, Chambers RC. Molecular targets in pulmonary fibrosis: the myofibroblast in focus. *Chest* 2007;132:1311-1321.
20. Massague J, Gomis RR. The logic of TGFbeta signaling. *FEBS Lett* 2006;580:2811-2820.
21. Chen SJ, Yuan W, Mori Y, et al. Stimulation of type I collagen transcription in human skin fibroblasts by TGF-beta: involvement of Smad 3. *J Invest Dermatol* 1999;112:49-57.
22. Landstrom M, Heldin NE, Bu S, et al. Smad7 mediates apoptosis induced by transforming growth factor beta in prostatic carcinoma cells. *Curr Biol* 2000;10:535-538.
23. Mehra A, Wrana JL. TGF-beta and the Smad signal transduction pathway. *Biochem Cell Biol* 2002;80:605-622.
24. Moustakas A, Souchelnytskyi S, Heldin CH. Smad regulation in TGF-beta signal transduction. *J Cell Sci* 2001;114:4359-4369.
25. Burns WC, Twigg SM, Forbes JM, et al. Connective tissue growth factor plays an important role in advanced glycation end product-induced tubular epithelial-to-mesenchymal transition: implications for diabetic renal disease. *J Am Soc Nephrol* 2006;17:2484-2494.
26. Gressner OA, Gressner AM. Connective tissue growth factor: a fibrogenic master switch in fibrotic liver diseases. *Liver Int* 2008;28:1065-1079.
27. Ponticos M, Holmes AM, Shi-Wen X, et al. Pivotal role of connective tissue growth factor in lung fibrosis: MAPK-dependent transcriptional activation of type I collagen. *Arthritis Rheum* 2009;60:2142-2155.

28. Qi W, Chen X, Polhill TS, Sumual S, et al. TGF-beta1 induces IL-8 and MCP-1 through a connective tissue growth factor-independent pathway. *Am J Physiol Renal Physiol* 2006;290:F703-709.
29. Garrett Q, Khaw PT, Blalock TD, et al. Involvement of CTGF in TGF-beta1-stimulation of myofibroblast differentiation and collagen matrix contraction in the presence of mechanical stress. *Invest Ophthalmol Vis Sci* 2004;45:1109-1116.
30. Mori Y, Hinchcliff M, Wu M, Warner-Blankenship MKML, Varga J. Connective tissue growth factor/CCN2-null mouse embryonic fibroblasts retain intact transforming growth factor-beta responsiveness. *Exp Cell Res* 2008;314:1094-1104.
31. Atfi A, Djelloul S, Chastre E, Davis R, Gespach C. Evidence for a role of Rho-like GTPases and stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) in transforming growth factor beta-mediated signaling. *J Biol Chem* 1997;272:1429-1432.
32. Engel ME, McDonnell MA, Law BK, Moses HL. Interdependent SMAD and JNK signaling in transforming growth factor-beta-mediated transcription. *J Biol Chem* 1999;274:37413-37420.
33. Bhowmick NA, Ghiassi M, Bakin A, et al. Transforming growth factor-beta1 mediates epithelial to mesenchymal transdifferentiation through a RhoA-dependent mechanism. *Mol Biol Cell* 2001;12:27-36.
34. Edlund S, Landstrom M, Heldin CH, Aspenstrom P. Transforming growth factor-beta-induced mobilization of actin cytoskeleton requires signaling by small GTPases Cdc42 and RhoA. *Mol Biol Cell* 2002;13:902-914.
35. Leivonen SK, Häkkinen L, Liu D, Kähäri VM. Smad3 and extracellular signal-regulated kinase 1/2 coordinately mediate transforming growth factor-beta-induced expression of connective tissue growth factor in human fibroblasts. *J Invest Dermatol* 2005;124:1162-1169.
36. Zhao J, Shi W, Chen H, Warburton D. Smad7 and Smad6 differentially modulate transforming growth factor beta -induced inhibition of embryonic lung morphogenesis. *J Biol Chem* 2000;275:23992-23997.
37. Hill-Kapturczak N, Truong L, Thamilselvan V, et al. Smad7-dependent regulation of heme oxygenase-1 by transforming growth factor-beta in human renal epithelial cells. *J Biol Chem* 2000;275:40904-40909.
38. Kato S, Ueda S, Tamaki K, et al. Ectopic expression of Smad7 inhibits transforming growth factor-beta responses in vascular smooth muscle cells. *Life Sci* 2001;69:2641-2652.
39. Talwar S, Squire IB, O'Brien RJ, et al. Plasma cardiotrophin-1 following acute myocardial infarction: relationship with left ventricular systolic dysfunction. *Clin Sci (Lond)* 2002;102:9-14.

40. Dooley S, Delvoux B, Streckert M, et al. Transforming growth factor beta signal transduction in hepatic stellate cells via Smad2/3 phosphorylation, a pathway that is abrogated during in vitro progression to myofibroblasts. TGFbeta signal transduction during transdifferentiation of hepatic stellate cells. *FEBS Lett* 2001;502:4-10.
41. Nakao A, Fujii M, Matsumura R, et al. Transient gene transfer and expression of Smad7 prevents bleomycin-induced lung fibrosis in mice. *J Clin Invest* 1999;104:5-11.
42. Li JH, Zhu HJ, Huang XR, et al. Smad7 inhibits fibrotic effect of TGF-Beta on renal tubular epithelial cells by blocking Smad2 activation. *J Am Soc Nephrol* 2002;13:1464-1472.
43. Wang B, Hao J, Jones SC, et al. Decreased Smad 7 expression contributes to cardiac fibrosis in the infarcted rat heart. *Am J Physiol Heart Circ Physiol* 2002;282:H1685-1696.
44. Gressner AM, Weiskirchen R, Breitkopf K, Dooley S. Roles of TGF-beta in hepatic fibrosis. *Front Biosci* 2002;7:d793-807.
45. Sumioka T, Ikeda K, Okada Y, et al. Inhibitory effect of blocking TGF-beta/Smad signal on injury-induced fibrosis of corneal endothelium. *Mol Vis* 2008;14:2272-2281.

Figures

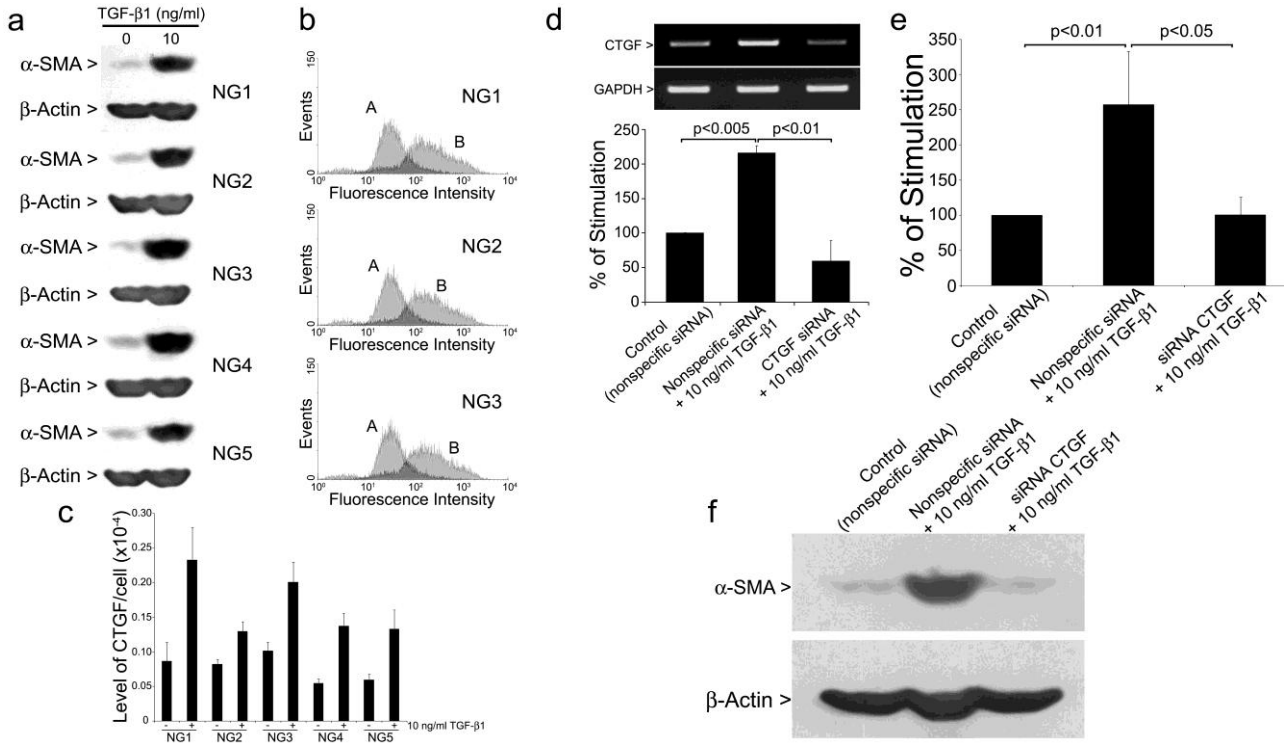

Figure 1. TGF-β1 induces transformation of gingival fibroblasts in myofibroblasts. (a) NG fibroblasts were cultured with 10 ng/ml of TGF-β1 for 3 days, and subjected to total protein isolation and western blot. (b) Flow cytometric analysis of NG fibroblasts with FITC-anti-α-SMA. Addition of 10 ng/ml of TGF-β1 to NG fibroblasts induced the expression of the myofibroblast marker α-SMA in more than 90% of the cells. (A) untreated control cells and (B) TGF-β1-treated cells. (c) Quantification of CTGF in supernant of TGF-β1-treated cells. TGF-β1 significantly induced the production of CTGF by NG fibroblasts. (d) Effectiveness of CTGF siRNA-mediated gene silencing. CTGF siRNA significantly inhibited CTGF expression by NG cells after TGF-β1 treatment. Graphic represent the average ± SD of 3 independent experiments. (e) ELISA analysis of CTGF in NG cells treated with CTGF siRNA or nonspecific siRNA. Production of CTGF was significantly knocked down by CTGF siRNA, even after treatment with TGF-β1. (f) Representative western blot analysis of NG1 cells treated simultaneously with CTGF siRNA and TGF-β1. TGF-β1 did not induce myofibroblast transformation in cells in which CTGF expression was significantly knocked down by the specific CTGF siRNA.

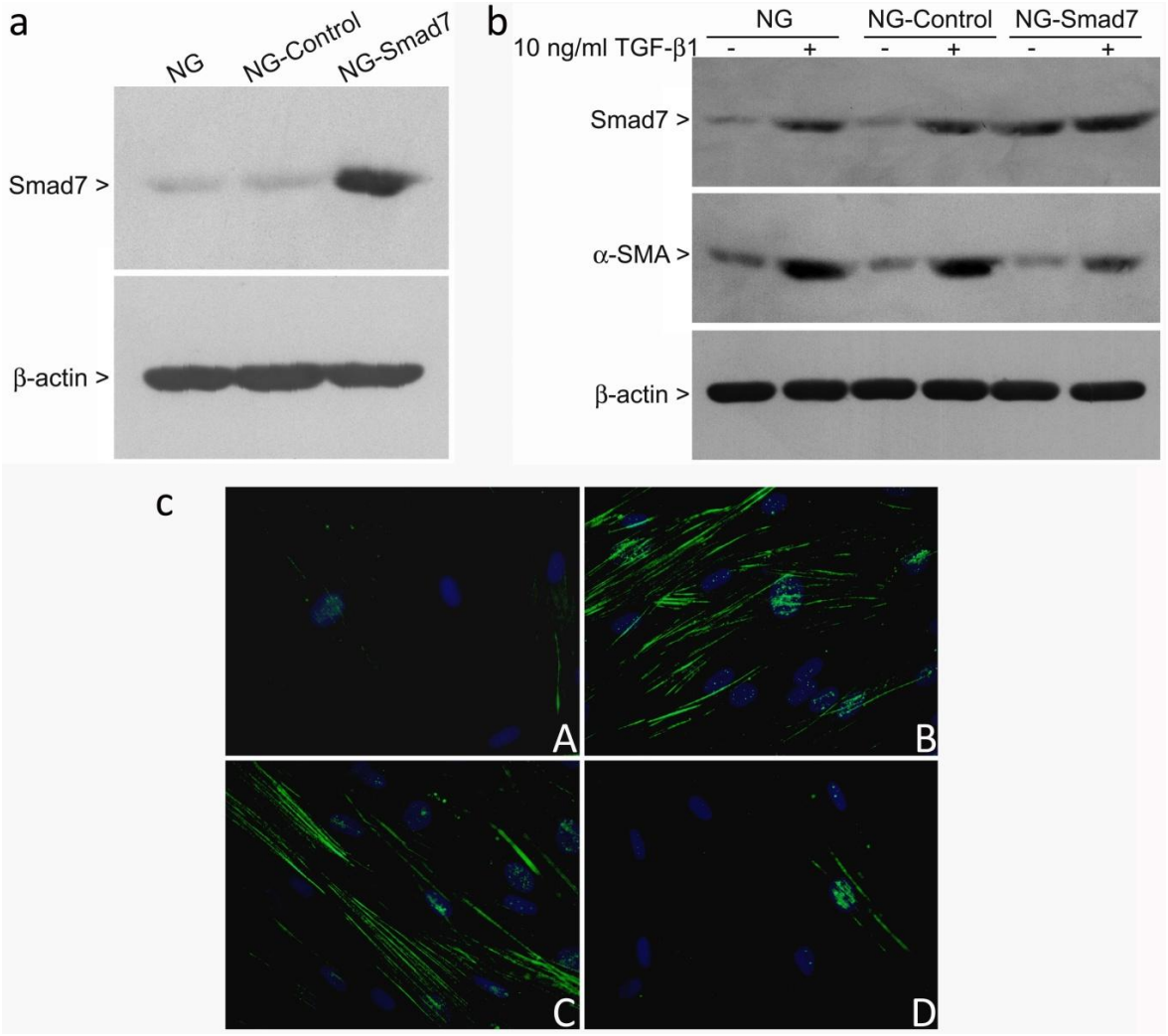

Figure 2. Overexpression of Smad7 blocks fibroblast-myofibroblast transition induced by TGF-β1. (a) Representative western blot analysis demonstrating the overexpression of Smad7 protein in stable NG-Smad7 cells. (b) Cells overexpressing Smad7 showed a marked inhibition on TGF-β1-induced myofibroblast transformation, as manifested by the downregulation of α-SMA. (c) Smad7 overexpression prevents the TGF-β1 switch of gingival fibroblasts into myofibroblasts. (A) NG untreated cells, (B) NG cells treated with 10 ng/ml TGF-β1, (C) NG-Control cells treated with 10 ng/ml TGF-β1, and (D) NG-Smad7 cells treated with 10 ng/ml TGF-β1. (original magnification x200)

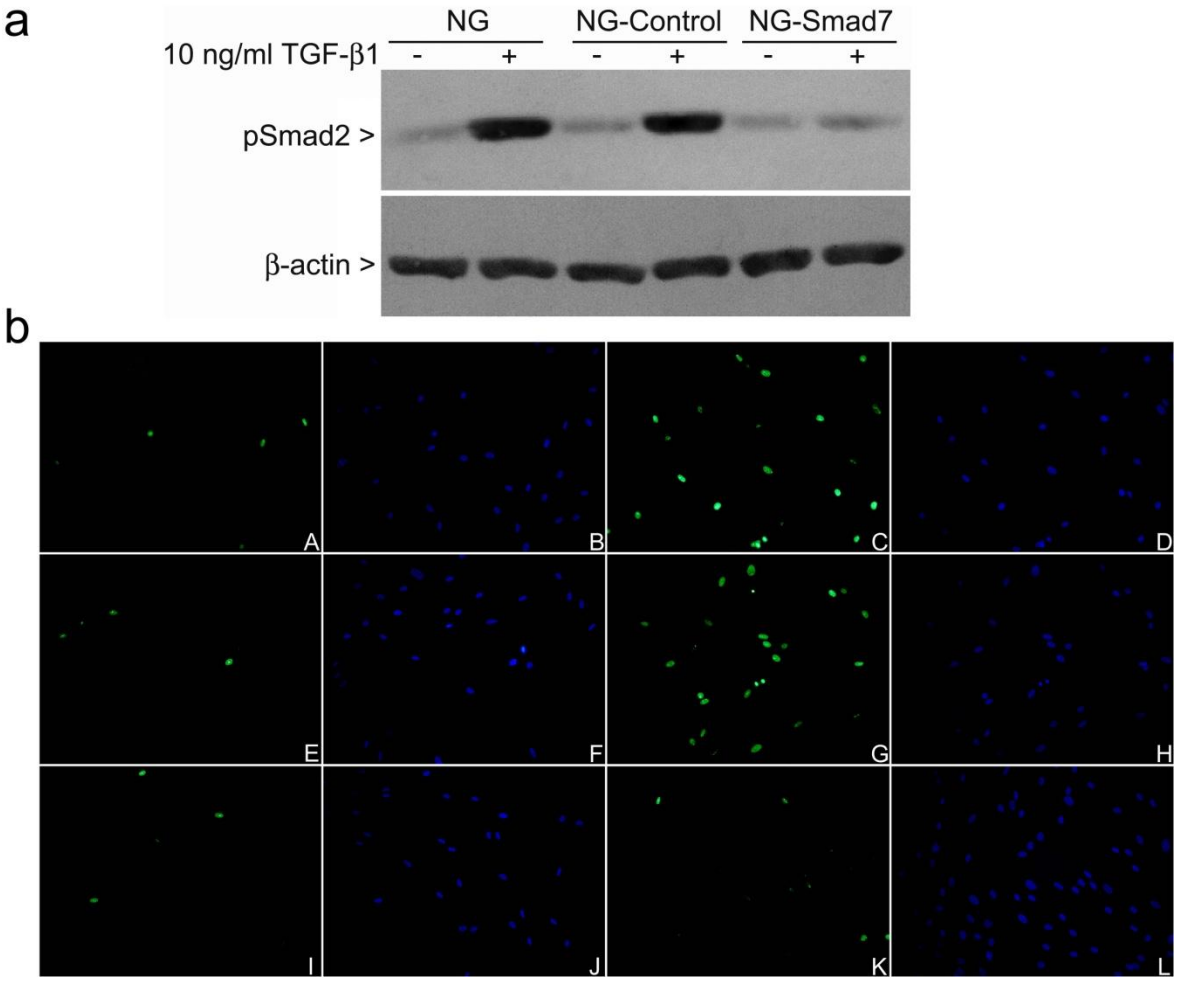

Figure 3. Phosphorylation and nuclear localization of Smad2 is attenuated in NG-Smad7 cells after treatment with TGF-β1. (a) Representative western blot analysis of phosphorylated Smad2 (pSmad2) in NG, NG-Control and NG-Smad7 cells treated with 10 ng/ml of TGF-β1. (b) Immunofluorescence analysis demonstrated that TGF-β1induced pSmad2 nuclear localization in both NG and NG-Control cells, but overexpression of Smad7 markedly blocked it. A-D: NG cells; E-H: NG-Control cells; I-L: NG-Smad7 cells. Unstimulated cells are in A, E and I, and TGF-β1 treated cells are in C, G and K. (original magnification x100)

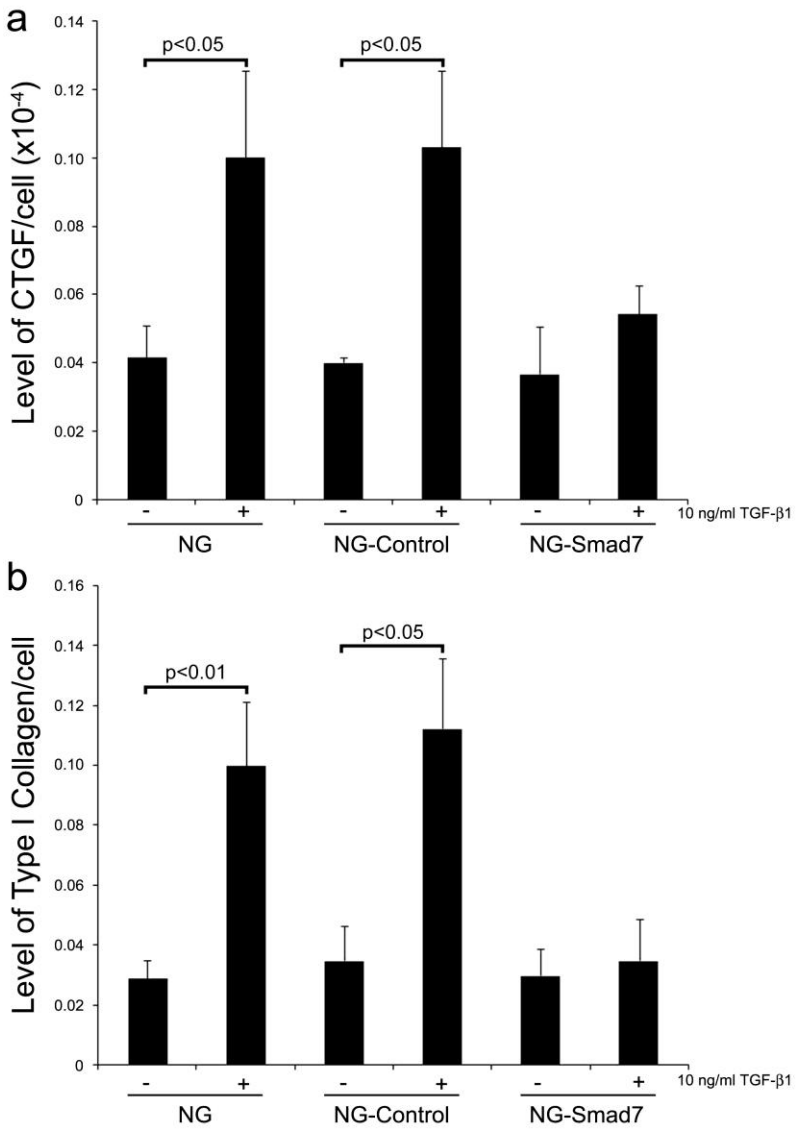

Figure 4. Overexpression of Smad7 blocks significantly the production of CTGF and type I collagen induced by TGF-β1. ELISA analysis revealed that overexpression of Smad7 significantly blocked TGF-β1 stimulatory effects on CTGF (a) and type I collagen production (b).

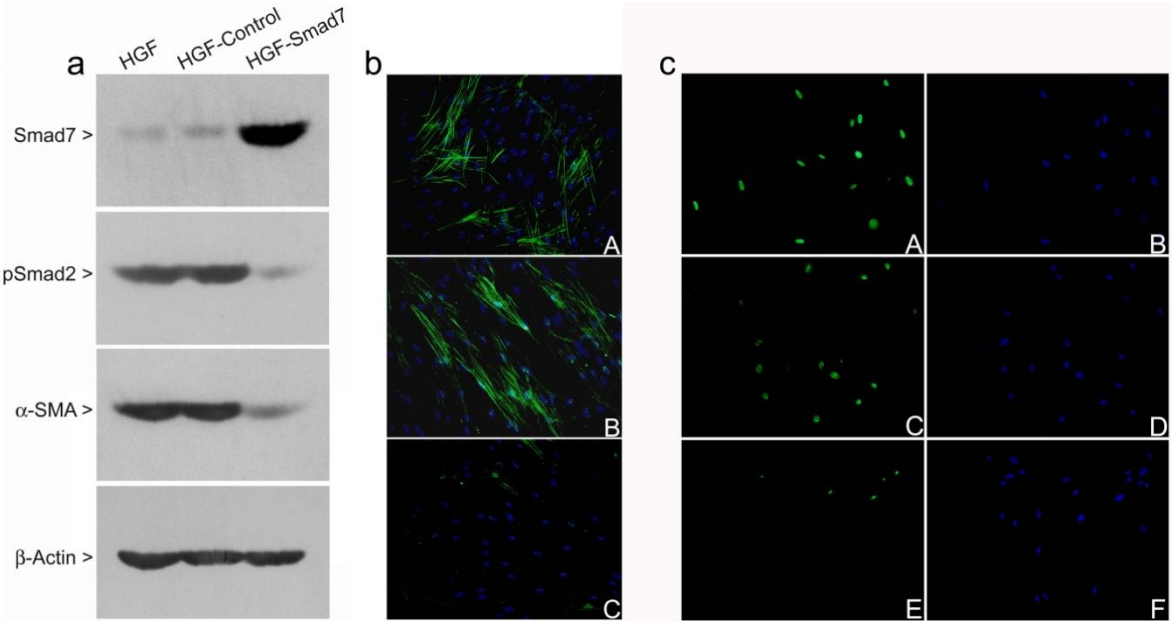

Figure 5. Reversion of the HGF myofibroblast phenotype with overexpression of Smad7. (a) Stable HGF clones overexpressing Smad7 demonstrated reduced levels of pSmad2 and α-SMA production. Western blot reaction against β-actin indicated relatively even protein loading among lanes. (b) Immunofluorescence analysis showed that HGF-Smad7 cells lost their α-SMA brilliant striated staining throughout the cytoplasm. (A) HGF myofibroblasts, (B) HGF-Control cells and, (C) HGF-Smad7 cells. (original magnification x200). (c) HGF cells overexpressing Smad7 demonstrated decreased nuclear positivity for phosphorylated Smad2. A marked reduction on nuclear staining for pSmad2 was observed in HGF-Smad7 cells (E and F) compared with HGF cells (A and B) or HGF-Control cells (C and D). (original magnification x100)

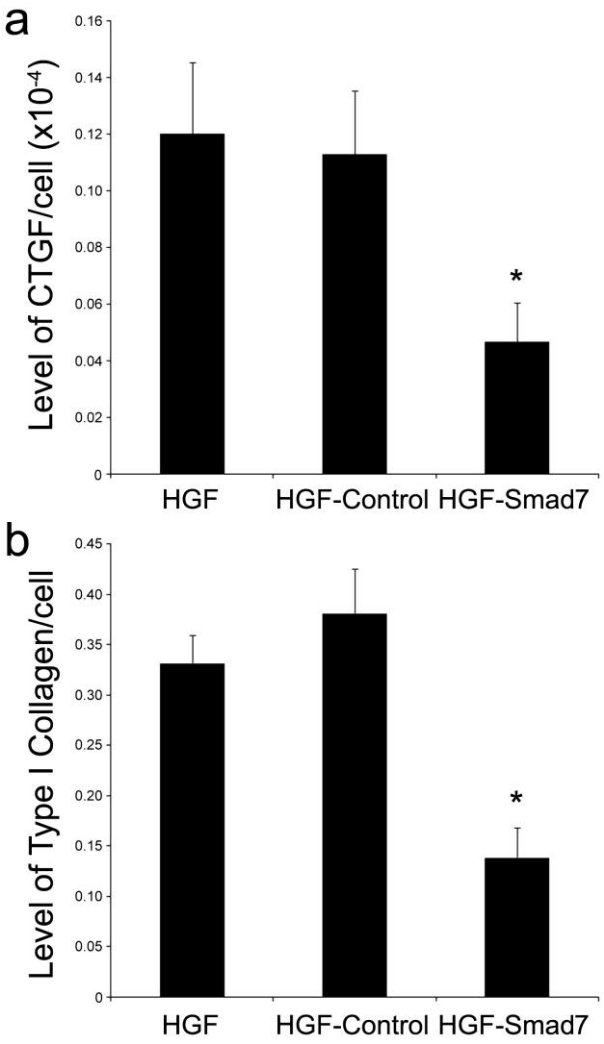

Figure 6. Overexpression of Smad7 inhibits HGF myofibroblasts activity as revealed by decreased CTGF and type I collagen production. (a) ELISA for CTGF and (b) ELISA for type I collagen.

3. Capítulo 2

Isolation and characterization of myofibroblast cell lines from oral squamous cell carcinoma

***Key words*:** Oral cancer, myofibroblast, cell culture, proliferation, matrix metalloproteinase.

Running title: Myofibroblast cell lines from OSCC.

Abstract. Oral squamous cell carcinoma (OSCC) invasion is followed by several stromal events such as inflammatory and immune cell infiltration, neo-vascularization, fibroblast activation and occasionally myofibroblast emergence. Our previous study demonstrated that myofibroblast in the stroma of OSCC is associated with a more aggressive behavior, leading to shorter patient's overall survival. Therefore, we evaluated whether OSCC-associated myofibroblasts have different characteristics as compared to OSCC-associated fibroblasts. OSCC myofibroblast cell lines were isolated, cultured and characterized on the basis of the specific isoform α of the smooth muscle actin (α-SMA) expression and of the excessive production of type I collagen. To assess the proliferative potential of the cell lines, growth curves were constructed, whereas the production and activity of matrix metalloproteinases (MMP) were analyzed by ELISA and enzymography, respectively. Myofibroblast clones were positive for α-SMA and vimentin, and negative for pan-cytokeratin and CD34. In long time culture, western blot, flow cytometric analysis and ELISA revealed constant α-SMA expression and elevated production of type I collagen. There were no differences on the proliferative potential between fibroblast and myofibroblast clones, but myofibroblast cells secreted significantly higher levels of MMP-1, -2, -9 and -13. MMP-2 gelatinolytic activity was significantly higher in myofibroblast clones. The results of this study suggest that myofibroblasts may contribute to OSCC invasion throughout elevate synthesis of MMPs.

Introduction

Oral squamous cell carcinomas (OSCC) are tumors formed by neoplastic epithelial cells surrounded by a biologically complex stroma composed of various types of host cells and extracellular matrix (ECM) molecules, both of which create a unique tumor microenvironment (1). For many years the research focus have been in the epithelial cells or more specifically, on genetic changes that occur in the epithelial cells as they progress from normal to malignant. However, the stroma has recently received increasing attention because of its recognized participation on tumor development, including invasion and metastasis, and of its influence on therapeutic response (2-5). Tumor-associated cells comprise immunocompetent and inflammatory cells, blood and lymph endothelial cells, fibroblasts, and eventually myofibroblasts. Evidences demonstrated that all of them may critically influence the processes of tumorigenesis (6-8), although the role of the myofibroblasts in oral cancer has not been fully elucidated.

Myofibroblasts were first described in skin wounds where they are co-opted by the remodeling tissues, facilitating wound healings (9,10). Morphologically they are large spindle-shaped mesenchymal cells that share some characteristics with smooth muscle cells and fibroblasts (11). Myofibroblasts are characterized by expression of the specific isoform α of the smooth muscle actin (α-SMA) and by the excessive synthesis of collagen (12). Through cell-cell contacts and through secretion of an extensive repertoire of molecules, including cytokines, growth factors, chemokines, hormones, neurotransmitters, inflammatory mediators, adhesion proteins, and ECM proteins, myofibroblasts promote effects in both physiological and pathological conditions (13-15). Those cells also secrete enzymes responsible for matrix remodeling, including matrix metalloproteinases (MMP) (16,17). Presence of myofibroblasts in the stroma of several cancers, including those of the oral cavity, is correlated with a worse prognosis (18,19). In oral squamous cell carcinomas, increased amount of myofibroblasts was significantly correlated with lymph node metastasis, vascular, lymphatic and perineural invasion of the tumor cells, and patient's shorter overall survival (18). Moreover, mutual

interactions between OSCC cells and myofibroblasts exist and are dependents on multiple invasive growth-promoting factors via paracrine signals (20,21).

The aim of this study was to establish myofibroblast cell lines from OSCC stroma and to compare their proliferative potential and production of MMPs with OSCC-stromal fibroblast cell lines.

Material and Methods

Tissue samples. Tissue fragments from 3 patients with tongue SCC were removed during tumor biopsy and divided into two parts: one was fixed in formalin and embedded in paraffin for hematoxylin and eosin staining and immunohistochemistry with α-SMA antibodies (22), while the other was washed with phosphate buffered salina (PBS) and incubated in 500 μl of Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA, USA) supplemented with 10% calf serum (CS) and antibiotics for 16 h at 4ºC.

Cell Culture. Cells were established using tissue explants as described previously (23). Briefly, specimens were washed 3 times with PBS, minced, placed in 25 cm^2 cell culture flasks containing 1 ml of DMEM plus 10% CS and antibiotics, and incubated at 37ºC in a humidified atmosphere of 5% CO_2. The cell growth was monitored every day and the medium was replaced every 2-3 days. When the cells growing out from the explant reached confluence, they were trypsinized and plated in very low density to select individual clones with cloning rings. Individual clones were propagated, and characterized as fibroblast or myofibroblast cell lines by immunocytochemistry, flow cytometry, and western blot. As marker of myofibroblast activity, type I collagen production was evaluated by enzyme-linked immunosorbent assay (ELISA).

Immunocytochemistry. Ten thousand cells were plated in each well of a 8-well culture chamber slides (Lab Tek, Nunc, Naperville, IL, USA) and incubated at 37ºC in humidified air containing 5% CO_2 for 24 h. Following incubation, cells were fixed

in 10% formalin, permeabilized with 0.5% Triton X100 in PBS, treated with 3% H_2O_2 for endogenous peroxidase inactivation, and thus incubated with primary antibodies for 16 h at 4ºC. The primary antibodies used were as follows: anti-α-SMA diluted 1:100, anti-vimentin diluted 1:200; anti-pan-cytokeratin diluted 1:200 and anti-CD34 1:50. All antibodies were purchased from Dako Co. (Carpenteria, CA, USA). Subsequent incubations were with biotinylated IgG followed by streptavidin-biotin peroxidase complex (LSAB+ System-HRP, Dako Co.). Reactions were developed with 0.6 mg/ml 3,3'-diaminobenzidine tetrahydrochloride (DAB, Sigma-Aldrich, St. Louis, MO, USA) containing 0.01% H_2O_2 and counterstained with Mayer's haematoxylin. Omission of the primary antibodies was used as negative controls.

Flow cytometry. After released from culture flasks, single-cell suspensions were fixed with 70% ethanol and stained with anti-α-SMA antibody followed by goat anti-mouse IgG conjugated with fluorescein (Vector Labs, Burlingame, CA, USA). Cells were washed, resuspended in PBS, and analyzed on a FACScalibur flow cytometer equipped with an argon laser (Becton Dickinson, San Jose, CA, USA). A minimum of 10,000 events was collected on each sample, and only cells with forward and orthogonal light scatter characteristics similar to whole and intact cells were included in the analysis. Quantitative flow cytometric analysis was performed with the aid of CellQuest software (Becton Dickinson), measuring the percentage of α-SMA-positive cells.

Western blot analysis. Cells were washed with cold PBS and lysed in RIPA buffer (50 mM Tris HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40%, 1% deoxycholic acid, 0.5% sodium dodecyl sulphate, 1 mM phenymethylsulphony fluoride, 1 mM N-ethylmaleimide, 1 mM dithiothreitol, 10 mg/ml soybean trypsin inhibitor, 1 mg/ml leupeptin, and 1 mg/ml aprotinin) for detection of α-SMA. After centrifugation, protein concentrations were measured using a protein assay according to the manufacturer's instructions (Bio Rad protein assay, Bio Rad, Hercules, CA, USA). Thirty micrograms of total protein per sample was resolved in a 10% sodium

dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions, and transferred onto nitrocellulose membranes (GE Healthcare, Vienna, Austria). The membranes were blocked overnight with 10% non-fat dry milk in PBS containing 0.1% Tween 20, rinsed in the same buffer, and incubated for 2 h with a of the follow antibodies: anti-α-SMA (Dako Corp.) and anti-β-actin (Sigma-Aldrich). Reactions were developed using a chemiluminescent western blot system (Enhanced chemiluminescent western blot kit, GE Healthcare).

Cell growth assay. Cells in 2 ml of DMEM containing 10% CS were allowed to settle in triplicate wells of 24-well culture plate (Corning Corp. Cambridge, MA, USA) for 24 h. After washing with PBS, the medium was replaced by serum-free DMEM for 24 h to reach cellular synchronism. Cells were then cultured in DMEM supplemented by 10% CS and the number of cells was determined at days 3, 5, 7, 9 and 11 with the toluidine-blue stain method. Briefly, cells were fixed with 10% formalin for 15 min, washed in PBS and stain with a 1% toluidine blue in 1% borax solution for 5 min. After washing and solubilization of the toluidine blue with 1% SDS, absorbance was determined using an automated microplate photometer at 650 nm. In parallel, a standard curve with crescent number of cells (4,000 to 128,000 cells/well) was constructed.

ELISA. Type I collagen production was determined according the method described for Sobral et. al. (12). For MMP quantification, culture mediums were collected, whereas the cells were fixed and used for cell count by toluidine blue stain method. In essence, microtite plate wells were coated with 100 µl of the culture mediums for 2 h at room temperature. The wells were then washed 3 times with 400 µl of 1% Tween 20 in PBS and non-specific binding sites were blocked with 3% BSA in PBS for 2 h. After washing, anti-MMP-1 (diluted 1:10,000, Calbiochem-Merck KGaA, Darmstadt, Germany), anti-MMP-2 (diluted 1:3,500, Calbiochem), anti-MMP-9 (diluted 1:2,000, Calbiochem) and anti-MMP-13 (1:2,000, Calbiochem) antibodies in PBS were added to the wells and incubated for 2 h. After another washing step,

goat anti-mouse peroxidase-conjugated IgG (Vector Labs) diluted 1:1,000 in PBS was added and maintained for 1 h. The reaction was developed with 0.5 mg/ml of o-phenylenediamine (Sigma-Aldrich) in 0.5 M citric buffer pH 5.5 containing 0.01% H_2O_2 for 20 min. After terminating the reaction with 50 μl of 2 N H_2SO_4, absorbance was read at 450 nm. The values were expressed as MMPs/cell.

Enzymography and densitometry. Enzymographic analysis was performed as previously described (23). Same amounts of protein per sample were mixed with non-reducing sample buffer and resolved in 10% SDS-PAGE copolymerized with 1.6 mg/ml of gelatin (Bio Rad Lab, Hercules, CA, USA) as substrate. After electrophoresis, the gels were washed for 1 h in 2% Triton X-100 and incubated for 16 h in activation buffer (10 mM Tris-HCl pH 8.0, 5 mM $CaCl_2$) at 37ºC. Gelatinolytic activity was visualized after staining with Coomassie blue R-250 (Bio Rad). The intensities of the negative bands were determined using a GS-700 imaging densitometer (Bio Rad). To confirm the identity of the enzymes, 1 mM of 1,10-phenanthroline (Sigma), a specific inhibitor of MMP activity by chelating Zn^{++} ions of their catalytic domain, was utilized.

Statistical analysis. The Kruskal-Wallis multiple comparison test was used to test group effects at 5% significance ($p<0.05$). All assays were performed at least three times in triplicates or quadruplicates.

Results

Characterization of fibroblast and myofibroblast clones isolated from OSCC. Immunohistochemical analysis of the tumor tissues used for explants cultures demonstrated a significant proportion of α-SMA-positive cells (myofibroblasts) in close contact to tumor epithelial cells, but fibroblasts (α-SMA-negative fusiform cells) were also observed in all samples (Fig. 1A). After 2 weeks of culture, fusiform cells extended from almost all explant fragments, and by day 30, cells were confluent and ready to be trypsinized and sub-cultured (Fig. 1B). Since flow

cytometric analysis confirmed that all cultures were formed by both α-SMA-negative and α-SMA-positive cells (Fig. 1C), individual clones were selected and screened for α-SMA expression and type I collagen production.

To assess α-SMA a number of modalities were employed that included immunocytochemistry, flow cytometry and western blot analysis. Immunocytochemical analysis demonstrated a strong cytoplasmatic staining for α-SMA in the myofibroblast clones, which was not observed in the fibroblast clones (Fig. 2). Immunoreactivity for vimentin was detected in almost 100% of both fibroblast and myofibroblast clones, whereas cells were negative for pan-cytokeratin and CD34 marker (Fig. 2). Western blot analyses of cell homogenates revealed a significant difference in the production of α-SMA between clones, without detection in the fibroblast ones (Fig. 3). All 3 myofibroblast clones expressed high levels of α-SMA. The expression of α-SMA in fibroblast and myofibroblast clones was also confirmed by flow cytometric assay (Fig. 4). Since one of the major features of myofibroblasts is the elevate production of type I collagen (24), we further characterized myofibroblast phenotype by quantification of type I collagen. As expected, myofibroblast clones shown a significantly higher production of type I collagen production compared with fibroblast cells (Fig. 5). Most importantly, myofibroblast clones retained their morphology, α-SMA expression and higher type I collagen production for at least 20 passages.

Proliferation and production of MMPs of fibroblast and myofibroblast clones. Growth curves of fibroblast and myofibroblast clones are depicted in Fig. 6. No differences on proliferative potential were observed between groups, although myofibroblast clone 3 showed a slight higher proliferation. Our results also showed that myofibroblast cell lines can be maintained in a serum-free medium without cellular growth, and all clones demonstrated a contact inhibition of growth (data not shown), arguing for the nontransformed nature of the clones. Next, we compared the production of MMPs between fibroblast and myofibroblast clones by ELISA (Fig. 7). The productions of MMP-1, -2, -9 and -13 were significantly higher in myofibroblasts

compared with fibroblasts. Comparison of production of MMP-1, -2, -9 and -13 in fibroblast and myofibroblast clones indicated that in myofibroblasts, MMP-1 was increased approximately by 3.3-fold, MMP-2 by 4.7-fold, MMP-9 by 2.7-fold and MMP-13 by 3.1-fold of the value observed in corresponding normal control fibroblasts. One gelatinolytic band with approximately 70 kDa was detected in the supernatants of all studied clones (Fig. 8A). This activity was attributed to the activated form of MMP-2, since it was completely inhibited by 1,10-phenanthroline. Densitometric analysis of 3 independent experiments demonstrated that MMP-2 production by myofibroblast cells was dramatically higher than that observed in fibroblast cell lines (Fig. 8B).

Discussion

Nowadays is well recognized that tumor progression is characterized not just for the acquisition of genetic alterations of the tumor cells, but also for a crosstalk between malignant cells and the surrounded stroma (1,25,26). One of the characteristics of the tumor stroma is the emergence of a mesenchymal specific cell called myofibroblast (27). Both clinical and experimental data support the hypothesis that myofibroblasts may modulate growth, invasion and metastasis (28-33). The first evidence in favor of a role for myofibroblasts in tumor progression was obtained by Dimanche-Boitrel and collaborators (34). Those authors demonstrated that tumor cells isolated from rat colon cancers failed to invade in vitro, whereas suspensions containing tumor cells and tumor-associated stromal cells were invasive in vitro and in vivo. Similarly, it was demonstrated that myofibroblasts secrete high levels of HGF/SDF, which promotes OSCC invasion (20). Our previous data demonstrated that elevated amount of myofibroblasts is correlated with an infiltrative phenotype of the OSCC, as demonstrated by the presence of vascular, lymphatic and perineural invasion of the tumor cells and lymph node metastasis (18). We further demonstrated that myofibroblasts secrete tumor growth factors that stimulate OSCC cell proliferation (21), and its elevated production of MMP-2 contribute to the rupture of the cortical bone in ameloblastomas, one of the major prognostic markers of

aggressiveness of this odontogenic tumor (35). The precise role of myofibroblasts in tumor-stroma interactions in OSCC has not been completely defined, in part because of the difficulty in obtaining primary tumor-associated myofibroblasts for analysis.

In this study we have established 3 myofibroblast cell lines derived from the stroma of tongue SCCs. There is no myofibroblast-specific immunocytochemical marker, but α-SMA is the most reliable one (36). In one recent review, De Wever and collaborators (16) suggested a minimum criterium for myofibroblast characterization, which included positivity for α-SMA, vimentin and type I collagen maturation enzyme prolyl-4 hydroxylase (P4H), and negativity for cytokeratin. Here we demonstrated that myofibroblast cells expressed α-SMA and vimentin, but did not express pan-cytokeratin, the marker of epithelial cells, and CD34, the specific-endothelial cell marker. In addition, those cells produced great amounts of type I collagen. Consistently, myofibroblast cell lines demonstrated those features in a long culture period, without losing their untransformed characteristics such as growth factor dependency to proliferation and contact inhibition. Together, those features led us to conclude that those cells are myofibroblasts.

Myofibroblasts secrete several functional molecules, including growth factors, and express receptors for many of these ligands, allowing in both paracrine and autocrine manner the control of the growth, migration and invasion (16). Previous study demonstrated that OSCC-associated fibroblasts, which were α-SMA-positive, showed an elevated proliferation rate compared to normal oral mucosal fibroblasts (37). The current study did not find differences on the proliferative potential between OSCC stromal clones of fibroblasts and myofibroblasts. This lack of difference may be explained because during invasion tumor cell-released factors active receptors in both cell types allowing similar responsiveness to growth stimuli. However, myofibroblast cells produced significantly higher amounts of all analyzed MMPs (MMP-1, -2, -9 and -13) compared with fibroblast clones. MMP-2 activity was also significantly higher in myofibroblast cells, as revealed by gelatine enzymography. In

accordance, Webber et al. (38), by enzymographic analysis of secreted MMPs, showed a higher MMP-2 activity in WPMY-1 cells, an immortalized prostatic stromal myofibroblast cell line, compared to WPE1-10 epithelial cells.

MMPs are a large family of zinc-dependent extracellular matrix-degrading endoproteinases with pathogenetic significance in a broad range of disorders (39). In cancer, they are considered major end-stage effectors for ECM degradation and tumor invasion (17,40). MMP production is dramatically increased in OSCC, and study in our laboratory have previously shown that patients with tumors showing elevated activity of MMP-2 and MMP-9 had shorter disease-free survival period after treatment than patients with tumors exhibiting low MMP activities (41). Interestingly, previous reports demonstrated myofibroblasts as the main source of MMPs in fibrotic diseases (42, 43) and some types of cancers. (44-46) Furthermore, the observation that myofibroblasts may enhance the ability of cancer cells to invade is supported by the fact that in in vitro presence of MMPs secreted by myofibroblasts increased the invasion of cancer cells (47,48).

In conclusion, differences between fibroblasts and myofibroblasts derived from OSCC stroma exist, in favor of a higher production and secretion of MMPs by myofibroblast cells. The myofibroblast cell lines may be useful tool to study myofibroblast-epithelial cell interactions on tumorigenesis, mainly because it can provide new insights into the mechanism involved in the tumor growth, invasion and metastasis.

Acknowledgment

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References

1 Mueller MM, Fusenig NE: Friends or foes - bipolar effects of the tumour stroma in cancer. Nat Rev Cancer 4: 839-849, 2004.

2 Micke P, Ostman A: Tumour-stroma interaction: Cancer-associated fibroblasts as novel targets in anti-cancer therapy? Lung Cancer 45: Suppl 2: S163-175, 2004.

3 Micke P, Ostman A: Exploring the tumour environment: Cancer-associated fibroblasts as targets in cancer therapy. Expert Opin Ther Targets 9: 1217-1233, 2005.

4 Albini A, Sporn MB: The tumour microenvironment as a target for chemoprevention. Nat Rev Cancer 7: 139-147, 2007.

5 Madani I, De Neve W, Mareel M: Does ionizing radiation stimulate cancer invasion and metastasis? Bull Cancer 95: 292-300, 2008.

6 Ziober AF, Falls EM, Ziober BL: The extracellular matrix in oral squamous cell carcinoma: Friend or foe? Head Neck 28: 740-749, 2006.

7 Kim R, Emi M, Tanabe K, Arihiro K: Tumor-driven evolution of immunosuppressive networks during malignant progression. Cancer Res 66: 5527-5536, 2006.

8 Witz IP: Tumor-microenvironment interactions: The selectin-selectin ligand axis in tumor-endothelium cross talk. Cancer Treat Res 130: 125-140, 2006.

9 Desmouliere A, Guyot C, Gabbiani G: The stroma reaction myofibroblast: A key player in the control of tumor cell behavior. Int J Dev Biol 48: 509-517, 2004.

10 Desmouliere A, Chaponnier C, Gabbiani G: Tissue repair, contraction, and the myofibroblast. Wound Repair Regen 13: 7-12, 2005.

11 Gabbiani G: The myofibroblast in wound healing and fibrocontractive diseases. J Pathol 200: 500-503, 2003.

12 Sobral LM, Montan PF, Martelli-Junior H, Graner E, Coletta RD: Opposite effects of tgf-beta1 and ifn-gamma on transdifferentiation of myofibroblast in human gingival cell cultures. J Clin Periodontol 34: 397-406, 2007.

13 Powell DW, Adegboyega PA, Di Mari JF, Mifflin RC: Epithelial cells and their neighbors i. Role of intestinal myofibroblasts in development, repair, and cancer. Am J Physiol Gastrointest Liver Physiol 289: G2-7, 2005.

14 Hinz B, Gabbiani G: Cell-matrix and cell-cell contacts of myofibroblasts: Role in connective tissue remodeling. Thromb Haemost 90: 993-1002, 2003.

15 Sato T, Sakai T, Noguchi Y, Takita M, Hirakawa S, Ito A: Tumor-stromal cell contact promotes invasion of human uterine cervical carcinoma cells by augmenting the expression and activation of stromal matrix metalloproteinases. Gynecol Oncol 92: 47-56, 2004.

16 De Wever O, Demetter P, Mareel M, Bracke M: Stromal myofibroblasts are drivers of invasive cancer growth. Int J Cancer 123: 2229-2238, 2008.
17 Jodele S, Blavier L, Yoon JM, DeClerck YA: Modifying the soil to affect the seed: Role of stromal-derived matrix metalloproteinases in cancer progression. Cancer Metastasis Rev 25: 35-43, 2006.
18 Kellermann MG, Sobral LM, da Silva SD, Zecchin KG, Graner E, Lopes MA, Nishimoto I, Kowalski LP, Coletta RD: Myofibroblasts in the stroma of oral squamous cell carcinoma are associated with poor prognosis. Histopathology 51: 849-853, 2007.
19 Kawashiri S, Tanaka A, Noguchi N, Hase T, Nakaya H, Ohara T, Kato K, Yamamoto E: Significance of stromal desmoplasia and myofibroblast appearance at the invasive front in squamous cell carcinoma of the oral cavity. Head Neck 31: 1346-1353, 2009.
20 Lewis MP, Lygoe KA, Nystrom ML, Anderson WP, Speight PM, Marshall JF, Thomas GJ: Tumour-derived tgf-beta1 modulates myofibroblast differentiation and promotes hgf/sf-dependent invasion of squamous carcinoma cells. Br J Cancer 90: 822-832, 2004.
21 Kellermann MG, Sobral LM, da Silva SD, Zecchin KG, Graner E, Lopes MA, Kowalski LP, Coletta RD: Mutual paracrine effects of oral squamous cell carcinoma cells and normal oral fibroblasts: Induction of fibroblast to myofibroblast transdifferentiation and modulation of tumor cell proliferation. Oral Oncol 44: 509-517, 2008.
22 Bitu CC, Sobral LM, Kellermann MG, Martelli-Junior H, Zecchin KG, Graner E, Coletta RD: Heterogeneous presence of myofibroblasts in hereditary gingival fibromatosis. J Clin Periodontol 33: 393-400, 2006.
23 Coletta RD, Almeida OP, Reynolds MA, Sauk JJ: Alteration in expression of mmp-1 and mmp-2 but not timp-1 and timp-2 in hereditary gingival fibromatosis is mediated by tgf-beta 1 autocrine stimulation. J Periodontal Res 34: 457-463, 1999.
24 Petrov VV, Fagard RH, Lijnen PJ: Stimulation of collagen production by transforming growth factor-beta1 during differentiation of cardiac fibroblasts to myofibroblasts. Hypertension 39: 258-263, 2002.
25 Liotta LA, Kohn EC: The microenvironment of the tumour-host interface. Nature 411: 375-379, 2001.
26 Pietras K, Ostman A: Hallmarks of cancer: Interactions with the tumor stroma. Exp Cell Res 316: 1324-1331, 2010.
27 Franco OE, Shaw AK, Strand DW, Hayward SW: Cancer associated fibroblasts in cancer pathogenesis. Semin Cell Dev Biol 21: 33-39, 2010.

28 Olumi AF, Grossfeld GD, Hayward SW, Carroll PR, Tlsty TD, Cunha GR: Carcinoma-associated fibroblasts direct tumor progression of initiated human prostatic epithelium. Cancer Res 59: 5002-5011, 1999.
29 Bhowmick NA, Neilson EG, Moses HL: Stromal fibroblasts in cancer initiation and progression. Nature 432: 332-337, 2004.
30 Casey TM, Eneman J, Crocker A, White J, Tessitore J, Stanley M, Harlow S, Bunn JY, Weaver D, Muss H, Plaut K: Cancer associated fibroblasts stimulated by transforming growth factor beta1 (tgf-beta 1) increase invasion rate of tumor cells: A population study. Breast Cancer Res Treat 110: 39-49, 2008.
31 Hwang RF, Moore T, Arumugam T, Ramachandran V, Amos KD, Rivera A, Ji B, Evans DB, Logsdon CD: Cancer-associated stromal fibroblasts promote pancreatic tumor progression. Cancer Res 68: 918-926, 2008.
32 Liao D, Luo Y, Markowitz D, Xiang R, Reisfeld RA: Cancer associated fibroblasts promote tumor growth and metastasis by modulating the tumor immune microenvironment in a 4t1 murine breast cancer model. PLoS One 4: e7965, 2009.
33 Vered M, Dayan D, Dobriyan A, Yahalom R, Shalmon B, Barshack I, Bedrin L, Talmi YP, Taicher S: Oral tongue squamous cell carcinoma: Recurrent disease is associated with histopathologic risk score and young age. J Cancer Res Clin Oncol 136: 1039-1048, 2010.
34 Dimanche-Boitrel MT, Vakaet L, Jr., Pujuguet P, Chauffert B, Martin MS, Hammann A, Van Roy F, Mareel M, Martin F: In vivo and in vitro invasiveness of a rat colon-cancer cell line maintaining e-cadherin expression: An enhancing role of tumor-associated myofibroblasts. Int J Cancer 56: 512-521, 1994.
35 Fregnani ER, Sobral LM, Alves FA, Soares FA, Kowalski LP, Coletta RD: Presence of myofibroblasts and expression of matrix metalloproteinase-2 (mmp-2) in ameloblastomas correlate with rupture of the osseous cortical. Pathol Oncol Res 15: 231-240, 2009.
36 Eyden B: The myofibroblast: A study of normal, reactive and neoplastic tissues, with an emphasis on ultrastructure. Part 2 - tumours and tumour-like lesions. J Submicrosc Cytol Pathol 37: 231-296, 2005.
37 Liu Y, Hu T, Shen J, Li SF, Lin JW, Zheng XH, Gao QH, Zhou HM: Separation, cultivation and biological characteristics of oral carcinoma-associated fibroblasts. Oral Dis 12: 375-380, 2006.
38 Webber MM, Trakul N, Thraves PS, Bello-DeOcampo D, Chu WW, Storto PD, Huard TK, Rhim JS, Williams DE: A human prostatic stromal myofibroblast cell line wpmy-1: A model for stromal-epithelial interactions in prostatic neoplasia. Carcinogenesis 20: 1185-1192, 1999.

39 Visse R, Nagase H: Matrix metalloproteinases and tissue inhibitors of metalloproteinases: Structure, function, and biochemistry. Circ Res 92: 827-839, 2003.
40 Roy R, Yang J, Moses MA: Matrix metalloproteinases as novel biomarkers and potential therapeutic targets in human cancer. J Clin Oncol 27: 5287-5297, 2009.
41 Yorioka CW, Coletta RD, Alves F, Nishimoto IN, Kowalski LP, Graner E: Matrix metalloproteinase-2 and -9 activities correlate with the disease-free survival of oral squamous cell carcinoma patients. Int J Oncol 20: 189-194, 2002.
42 McKaig BC, McWilliams D, Watson SA, Mahida YR: Expression and regulation of tissue inhibitor of metalloproteinase-1 and matrix metalloproteinases by intestinal myofibroblasts in inflammatory bowel disease. Am J Pathol 162: 1355-1360, 2003.
43 Czochra P, Klopcic B, Meyer E, Herkel J, Garcia-Lazaro JF, Thieringer F, Schirmacher P, Biesterfeld S, Galle PR, Lohse AW, Kanzler S: Liver fibrosis induced by hepatic overexpression of pdgf-b in transgenic mice. J Hepatol 45: 419-428, 2006.
44 Okada A, Bellocq JP, Rouyer N, Chenard MP, Rio MC, Chambon P, Basset P: Membrane-type matrix metalloproteinase (mt-mmp) gene is expressed in stromal cells of human colon, breast, and head and neck carcinomas. Proc Natl Acad Sci USA 92: 2730-2734, 1995.
45 Seomun Y, Kim J, Lee EH, Joo CK: Overexpression of matrix metalloproteinase-2 mediates phenotypic transformation of lens epithelial cells. Biochem J 358: 41-48, 2001.
46 Bisson C, Blacher S, Polette M, Blanc JF, Kebers F, Desreux J, Tetu B, Rosenbaum J, Foidart JM, Birembaut P, Noel A: Restricted expression of membrane type 1-matrix metalloproteinase by myofibroblasts adjacent to human breast cancer cells. Int J Cancer 105: 7-13, 2003.
47 Stamenkovic I: Matrix metalloproteinases in tumor invasion and metastasis: Semin Cancer Biol 10: 415-433, 2000.
48 Nielsen BS, Rank F, Lopez JM, Balbin M, Vizoso F, Lund LR, Dano K, Lopez-Otin C: Collagenase-3 expression in breast myofibroblasts as a molecular marker of transition of ductal carcinoma in situ lesions to invasive ductal carcinomas. Cancer Res 61: 7091-7100, 2001.

Figures

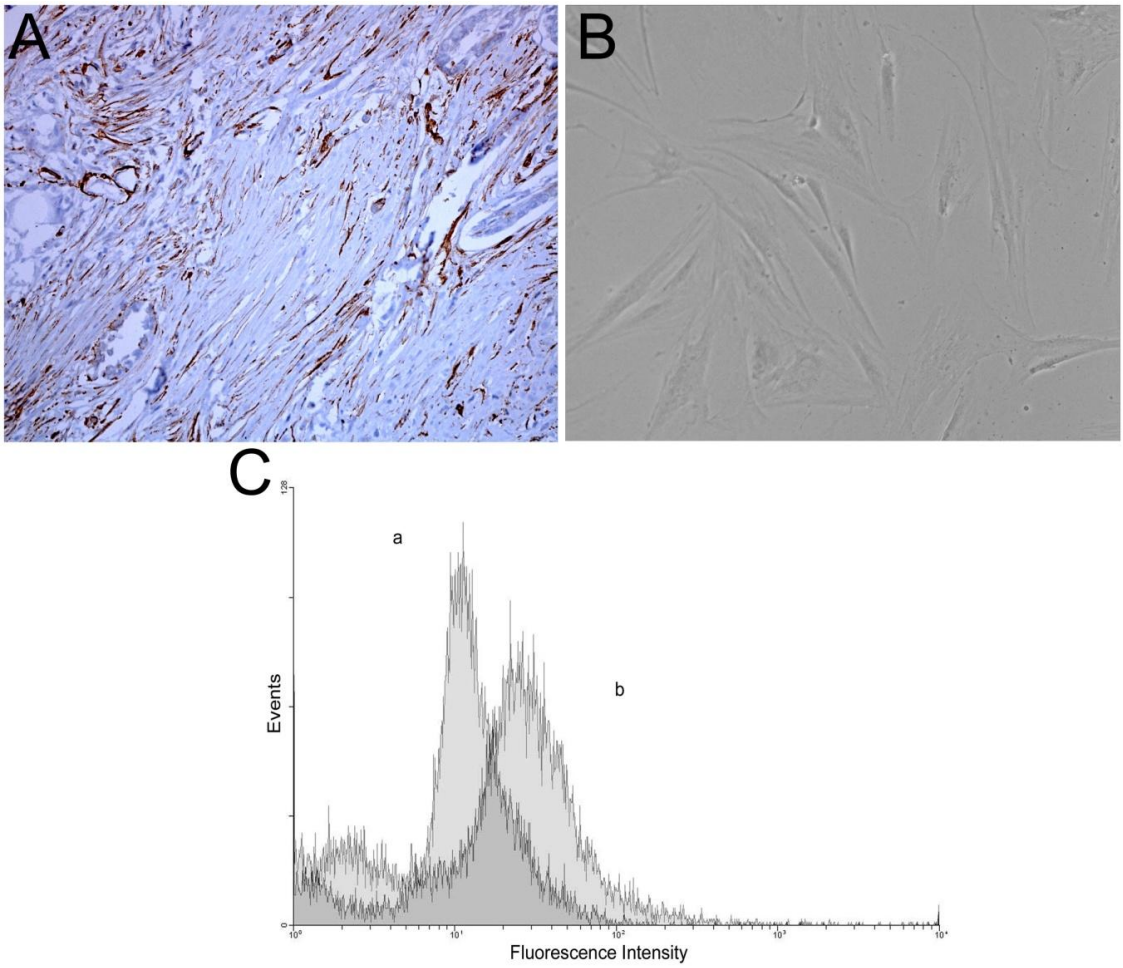

Figure 1. (A) Immunohistochemical expression of α-SMA in one of the tumor samples of this study. Myofibroblasts were found immediately adjacent to the islands of tumor cells. (Original magnification x100) (B) Phase-contrast microscopic features of cells cultured from one of the tumors. Note the typical spindle characteristics with a central spherical nucleus and typical cytoplasmic prolongations. (C) Flow cytometric analysis revealed a mix population of mesenchymal cells formed by both α-SMA-negative (a) and α-SMA-positive cells (b)

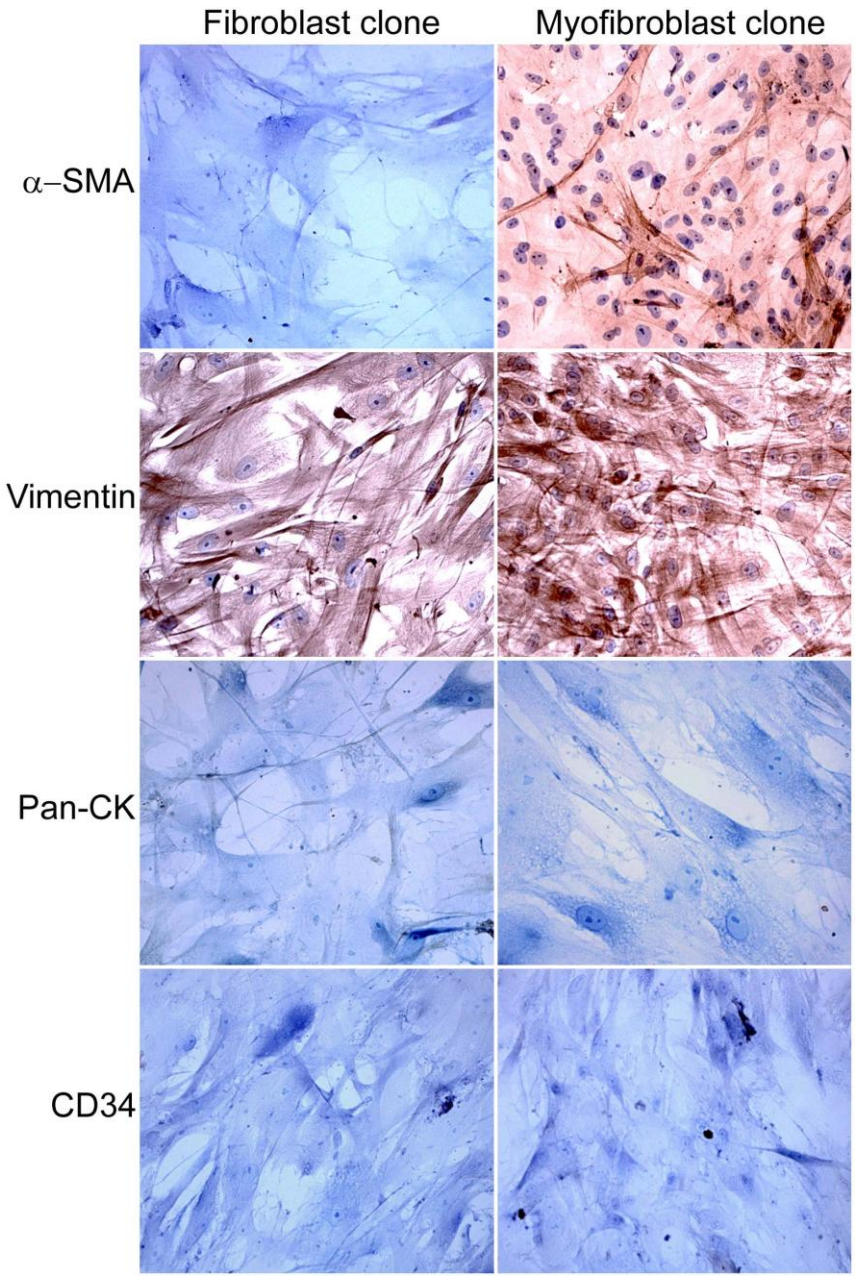

Figure 2. Immunocytochemistry analysis of α-SMA and vimentin. Myofibroblast clones demonstrated a vivid cytoplasmatic staining for α-SMA, which was not observed in the fibroblast clones. Immunereactivity for vimentin was detected in almost 100% of both fibroblast and myofibroblast clones. Both fibroblast and myofibroblast clones were negative to pan-cytokeratin (pan-CK) and CD34.

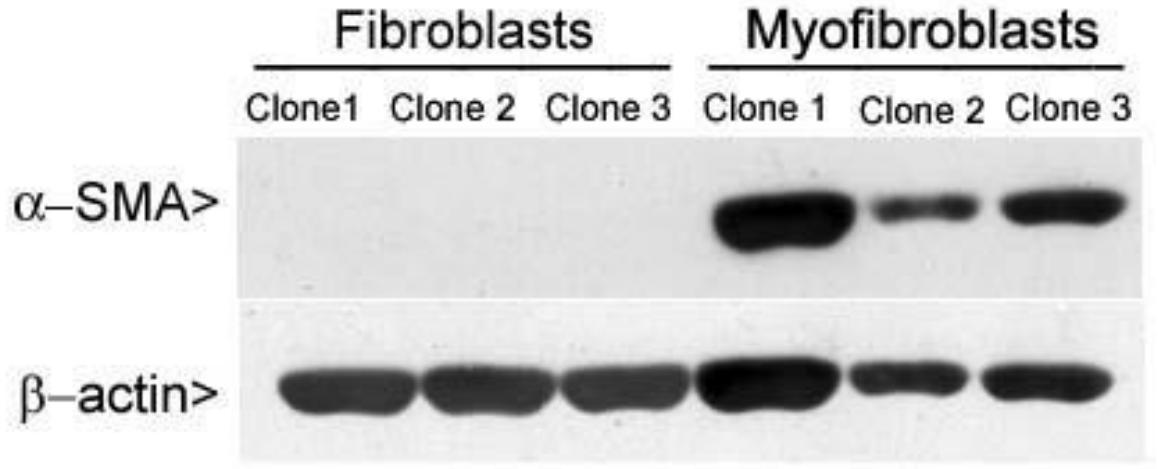

Figure 3. Western blot analysis performed with total cellular proteins from fibroblast and myofibroblast clones. Similar amount of protein of each sample was separated on SDS-PAGE, transferred to nitrocellulose membranes, and α-SMA production was analyzed utilizing anti-α-SMA antibodies. Blots were stripped and reprobed with anti-β-actin antibodies. Note that myofibroblast clones expressed high levels of α-SMA, whereas fibroblast clones lacked expression.

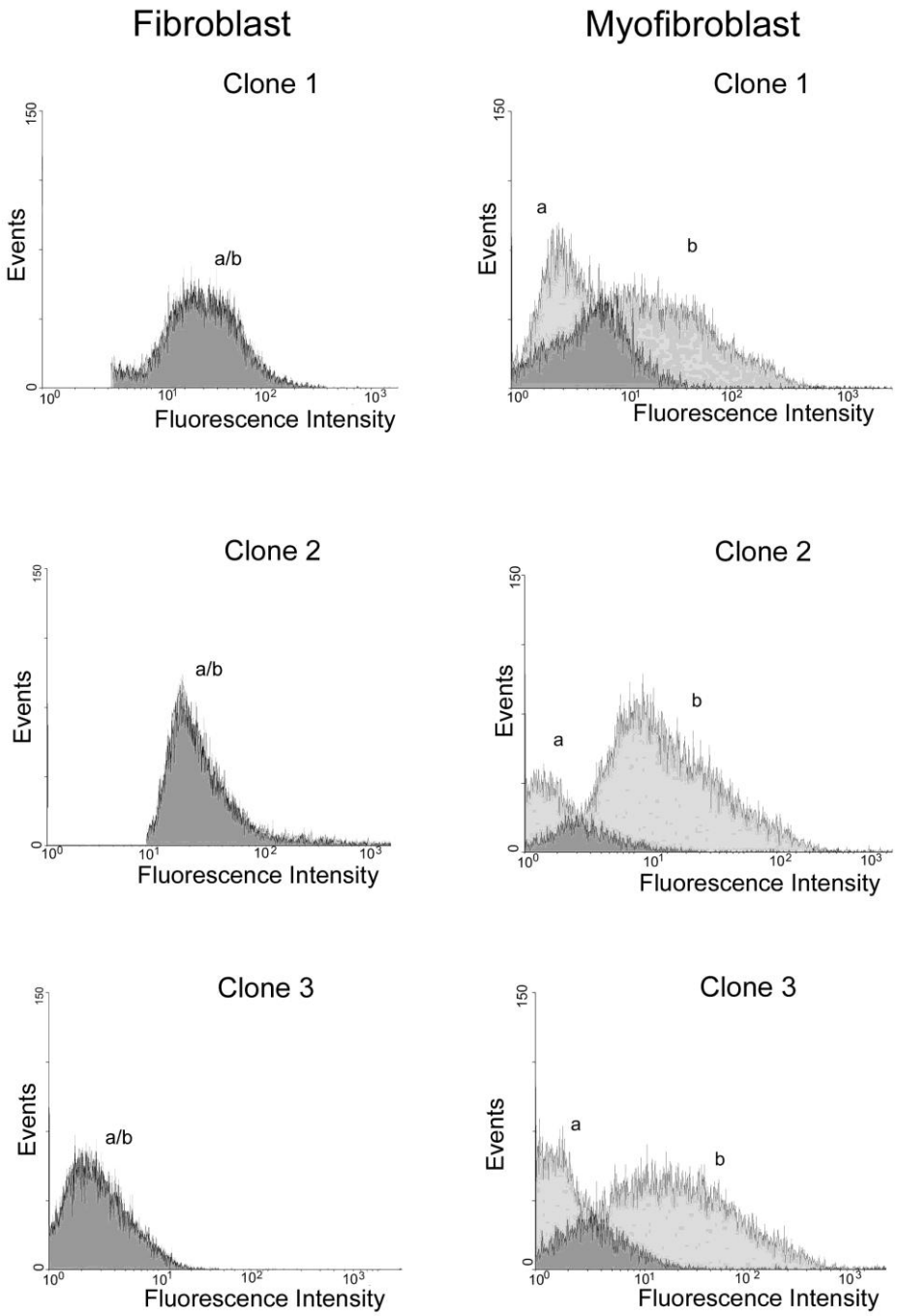

Figure 4. Flow cytometric analysis of fibroblast and myofibroblast clones with FITC-anti-α-SMA antibodies. Representative histograms of each clone are depicted. On left are fibroblast clones and on right are myofibroblast ones. (a) control negative cells (FITC antibodies alone) and (b) FITC-anti-α-SMA stain. Myofibroblast clones were formed by almost 100% of α-SMA-positive cells, confirming the purity of the cell lines.

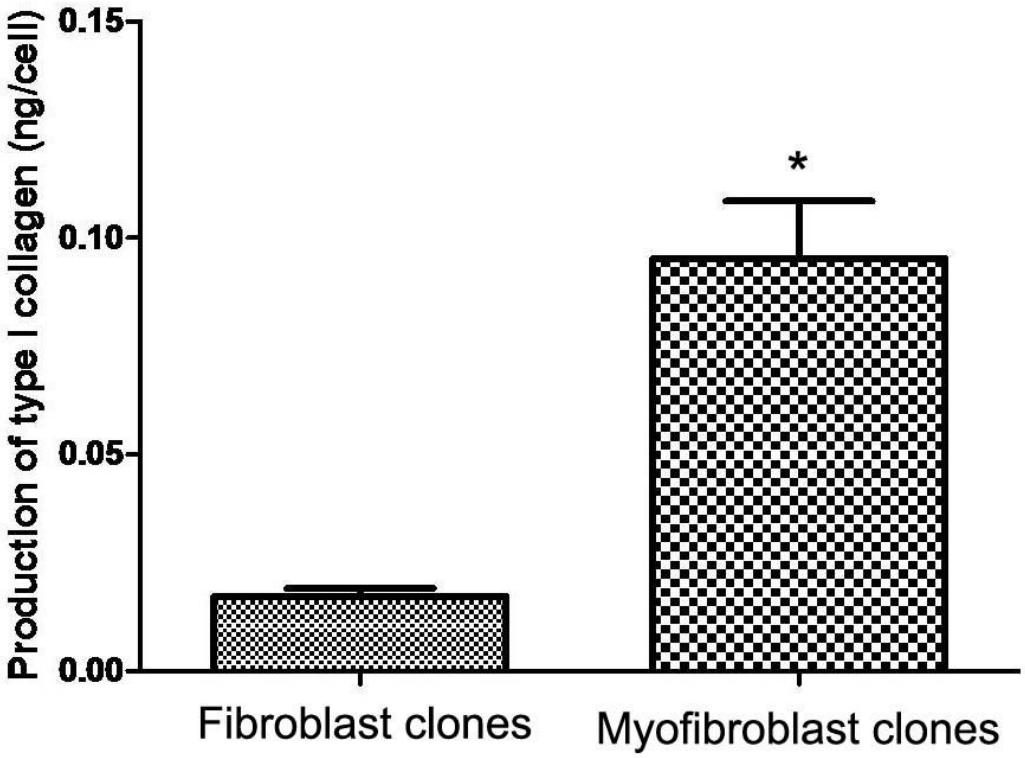

Figure 5. Myofibroblasts produce significantly higher levels of type I collagen than fibroblasts. Production of type I collagen by monolayers of cells as determined by ELISA. The values represent the mean of ng/cell of type I collagen production for triplicate wells ± SD. *p<0.005

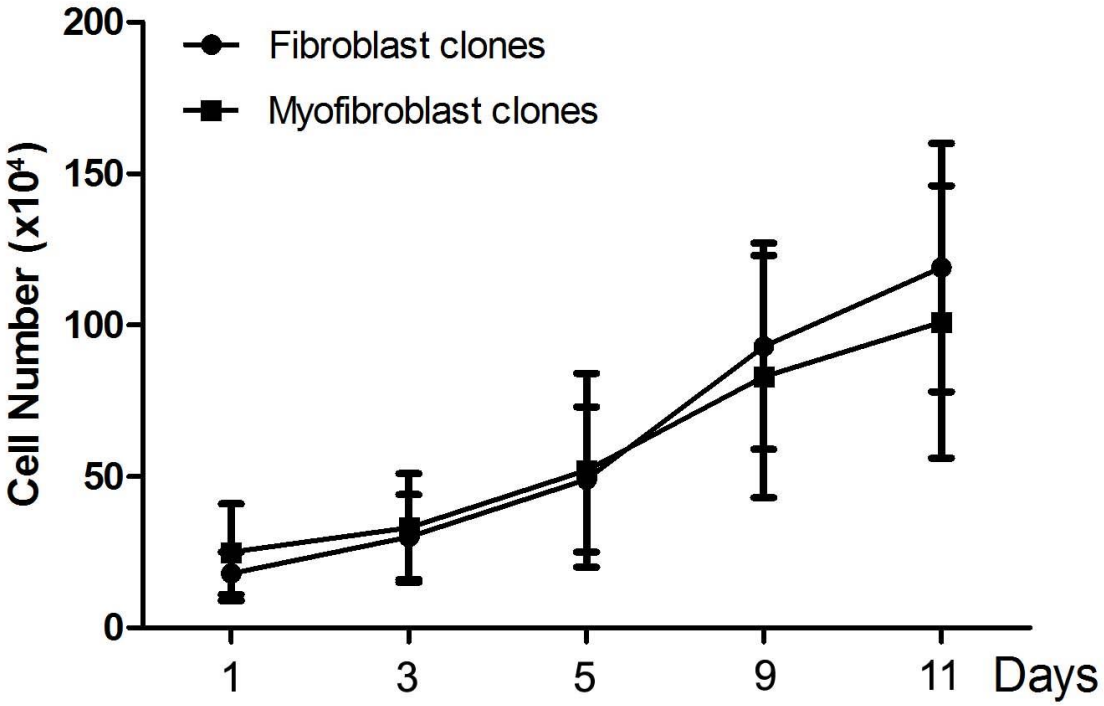

Figure 6. Growth rate of fibroblast and myofibroblast clones. Each timepoint represent the mean of 3 counts in quadruplicate for each strain. From day 3 to 11, the growth rates of fibroblast and myofibroblast cell lines were quite similar.

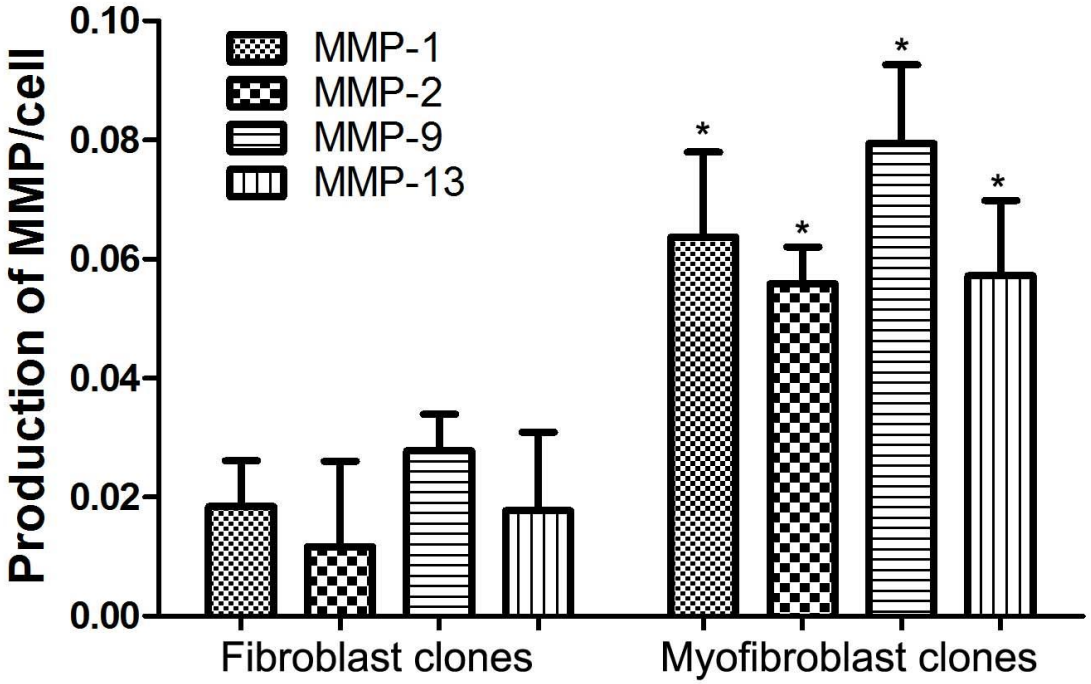

Figure 7. Production of MMPs by fibroblast and myofibroblast cell lines. Data are expressed as production of protein/cell. The amount of MMP-1, MMP-2, MMP-9 and MMP-13 synthesized by myofibroblast clones was significantly higher than fibroblast cell lines ($p<0.01$).

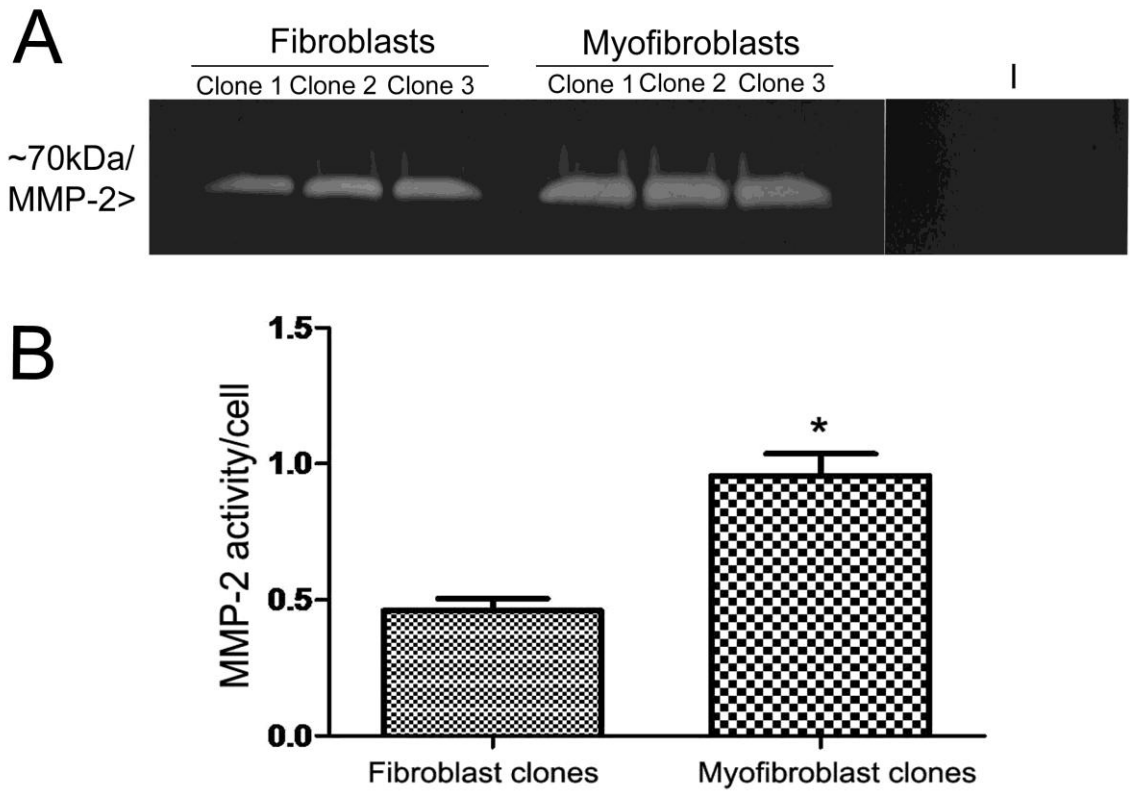

Figure 8. Activity of MMP-2 is significantly higher in myofibroblast clones. (A) Enzymographic analysis of the culture supernates of fibroblast and myofibroblast clones. Gelatinolytic activities were detected at ~70 kDa consistent with the presence of the active form of MMP-2. Lanes I show the complete inhibition of gelatinolytic activity of MMP-2 with 0.5 mM of 1,10-phenanthroline. (B) Densitometric analysis of the MMP-2 band demonstrated a significant higher MMP-2 activity in the myofibroblast clones. *$p<0.005$

4. Capítulo 3

Myofibroblasts in the stroma of oral cancer promote tumorigenesis via secretion of activin A

Short title: Myofibroblast in oral cancer.

Summary

Myofibroblasts are essential during wound healing and are often found in the stroma of oral squamous cell carcinomas (OSCC). Although the molecular mechanisms by which myofibroblasts influence OSCC remain largely unknown, previous studies demonstrated that presence of myofibroblast in OSCC stroma is an important risk factor of patient's shortened survival. Here we showed that some growth factors are produced in higher levels by tumor-associated myofibroblasts compared to tumor-associated fibroblasts, including activin A. Myofibroblast-conditioned medium containing activin A significantly increased OSCC cell proliferation and tumor volume, whereas down-regulation of activin A in the conditioned medium decreased proliferation. In addition, myofibroblasts induced in vitro invasion of OSCC cells, which was accompanied by an increased production of matrix metalloproteinases (MMP). *In vivo*, a significant correlation between presence of myofibroblasts and activities of MMP-2 and MMP-9 was observed in OSCC samples. However, blockage of activin A synthesis by myofibroblasts did not affect invasion and MMP production by OSCC cells. Together, our data demonstrate that activin A is required for the proliferative effects of myofibroblasts on OSCC cells. We conclude that myofibroblasts in the stroma of OSCC may influence proliferation and invasion, resulting in more aggressive tumor.

Keywords: oral cancer; myofibroblast; proliferation; invasion; matrix metalloproteinase; activin A.

Introduction

Myofibroblasts secrete extracellular matrix molecules and degrading enzymes, angiogenic and pro- and anti-inflammatory factors, and stimulate epithelial cell proliferation and invasion.[1-3] Myofibroblasts are involved transiently in wound healing but as they are also found in the reactive tumor stroma,[4, 5] the processes involved in their activation and function are an area of active investigation. Although the knowledge of myofibroblast participation in tumors is evolving, there has been little work investigating the role of myofibroblasts in oral squamous cell carcinomas (OSCC).

The first evidence in favor of a role for myofibroblasts in OSCC was obtained by Barth and collaborators in 2004,[6] and later of this year Lewis et al.[7] demonstrated that myofibroblasts induced by TGF-β1 secrete hepatocyte growth factor, which promotes OSCC invasion in vitro. Clinicopathologic studies demonstrated that myofibroblasts in the stroma of OSCC significantly correlate with lymph node metastasis, vascular, lymphatic and perineural invasion of the tumor cells, and patient's shorter survival, suggesting that myofibroblasts may assist tumor invasion and metastasis.[8, 9] Moreover, it was demonstrated that mutual interactions between OSCC cells and myofibroblasts exist, and that conditioned medium from myofibroblasts enhances cell growth of OSCC cells.[10] In this study we examine the role of myofibroblasts in tumor proliferation and invasion and identify the molecular mechanism by which myofibroblasts influence cellular proliferation. By secretion of activin A, myofibroblasts set in motion a pathway for proliferation of OSCC cells. We further show that myofibroblast-released factors, others than activin A, induce invasion and secretion of matrix metalloproteinases (MMP) by OSCC cells, and that presence of myofibroblasts in OSCCs correlates with increased tumor production of MMP-2 and MMP-9.

Material and methods

Cell cultures

Tumor-associated fibroblast and myofibroblast cell lines were established from fragments of 3 tongue SCCs using tissue explants as described previously.[11] Cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA, USA) containing 10% donor calf serum (DCS) and antibiotics at 37ºC in a 5% CO_2 air atmosphere. Individual clones were selected by cloning ring strategy and screened for α-SMA expression by western blot and flow cytometric analysis[12] and for type I collagen production by ELISA.[13]

SCC9 and SCC25 oral cancer cell lines were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured as recommended.

Preparation of the medium conditioned by fibroblast and myofibroblast clones

Subconfluent monolayers (2,500 cells/cm^2) of 3 fibroblast clones and 2 myofibroblast clones were washed twice with PBS and once with serum-free DMEM, and subsequently cultured in serum-free DMEM for 24 h. The conditioned medium was clarified by centrifugation, aliquoted and stored at -70°C until used to determine the effect on tumor cell proliferation or MMP production.

Proliferation assay

The effect of fibroblast and myofibroblast clones on OSCC cell proliferation was determined by bromodeoxyuridine (BrdU) incorporation index,[10] 24 h after release from a serum-starved synchrony. BrdU-labeling index, expressed as the percentage of cells labeled with BrdU, was determined by counting 500 cells in 2 independent reactions using the Kontron 400 image analysis system (Zeiss).

Invasion assay

The in vitro cell invasion assay was performed by using a modified Boyden chamber insert with polycarbonate filter membrane containing 8-μm pores in 24-well plates (Corning Inc, Corning, NY, USA). Matrigel (BD Biosciences, San Jose, CA, USA)

was diluted to 1:1 with serum-free medium and used to coat the filter membranes. In the lower chamber, monolayers of fibroblast and myofibroblast clones were plated in triplicate and cultured in serum-free DMEM. Tumor cells ($1x10^5$) suspended in 200 µl of serum-free DMEM/F12 were seeded onto the upper compartment of the transwell chamber. After incubation for 72 h, the medium in the upper chamber was removed and the filters were fixed with 10% formalin for 15 min. The cells remaining on the upper surface of the filter membrane were then completely removed by wiping with a cotton swab, and the cells on the opposite surface of the filter membrane were stained with 1% toluidine blue in 1% borax for 5 min and then lysed with 1% SDS. Absorbance was read at 650 nm to determine relative cell number.

ELISA

For MMP quantification, OSCC cells were cultured with fibroblast or myofibroblast-conditioned mediums for 24 h. The medium were collected, whereas the cells were fixed and used for cell count by toluidine blue stain method. In essence, microtite plate wells were coated with 100 µl of the culture mediums for 2 h at room temperature. The wells were then washed and non-specific binding sites were blocked with 3% BSA in PBS for 2 h. After washing, anti-MMP-1 (diluted 1:10,000), MMP-2 (diluted 1:3,500), MMP-9 (diluted 1:2,000) and MMP-13 (1:2,000) antibodies in PBS were added to the wells and incubated for 2 h. All antibodies were purchased from Calbiochem (Merck KGaA, Darmstadt, Germany). After another washing step, goat anti-mouse peroxidase-conjugated IgG (Vector Labs, Burlingame, CA, USA) diluted 1:1,000 in PBS was added and maintained for 1 h. The reaction was developed with 0.5 mg/ml of o-phenylenediamine (Sigma) in 0.5 M citric buffer pH 5.5 containing 0.01% H_2O_2 for 20 min. After terminating the reaction with 50 µl of 2 N H_2SO_4, absorbance was read at 450 nm. The values were expressed as MMPs/cell.

Real-time PCR array and quantitative real-time PCR

RNA isolation and cDNA synthesis were performed as previously described.[13] cDNAs were used to quantify human growth factor expression in a 96-well plate array (RT^2 Profiler™ PCR Array System, SABiosciences, Qiagen, Frederick, MD, USA), according to the manufacturer's instructions. Up-regulated genes were defined as those whose expression level in myofibroblast clones was greater than 2-fold above that in the fibroblast clones, in an average of 3 independent experiments with 2 individual clones for each group.

Quantitative reverse transcription-PCR (qRT-PCR) was performed with the StepOne Plus instrument (Applied Biosystems), and amplicons were detected by using SYBR green fluorescence method. Target genes were analyzed by using standard curves to determine relative levels of gene expression, and individual RNA samples were normalized according to the levels of GAPDH. All primers used in this study are described in Supplementary Table 1.

Small interference RNA (siRNA)

siRNA molecules to target INHBA and nontargeting negative control were purchased from Invitrogen and transfected as previously described.[14] After transfection, the efficacy of the INHBA knock down was determined by qRT-PCR and ELISA.

Tumorigenicity assay

To assess the growth of xenographic tumors in nude mice, eight 12 week-old nude mice per cell line were injected s.c. in the flank with SCC9 cells mixture with either fibroblast clones or myofibroblast clones. Tumor size was measured weekly with a calipers, and volumes are reported as mm^3, calculated by using the formula volume=0.5 x length x $width^2$. At 12 week postinjection, all mice were euthanized, and tumors and multiple organs were examined grossly, collected, fixed in 10% formalin and then embedded in paraffin. Five-micrometer sections were stained with H&E for histopathologic analysis.

Immunohistochemistry and zymography

Thirty three consecutive OSCC samples removed during tumor resection were divided into 2 parts: one was fixed in formalin and embedded in paraffin for H&E staining or immunohistochemistry against α-SMA,[12] while the other was used to zymographic analysis as previously described.[15]

Statistical analysis

The Kruskal-Wallis multiple comparison test was used to test group effects at 5% significance ($p<0.05$).

Results

Characterization of the cancer-associated myofibroblast clones

From 3 tongue SCCs we established several spindle-shaped cells with indented nuclei that were plated in low density, isolated with aid the cloning rings and characterized as myofibroblasts by the expression of α-SMA and elevated type I collagen synthesis. Western blot analysis revealed an excessive production of α-SMA by myofibroblast clones, whereas fibroblasts lacked its production (Fig. 1A). Flow cytometric analysis showed that more than 90% of the cells express α-SMA in the myofibroblast clones (Fig. 1B). As a marker of myofibroblast activity, type I collagen production was evaluated. Myofibroblast clones produced significantly higher amounts of type I collagen than fibroblasts (Fig. 1C). Thus, those clones were used to further characterization of myofibroblast-effects on tumor proliferation and invasion.

Myofibroblasts promote proliferation and enhance tumor volume in nude mice

The comparative effect of myofibroblast-conditioned medium and fibroblast-conditioned medium on OSCC proliferation was assessed by measuring BrdU incorporation into DNA. Figure 2A shows that myofibroblast-conditioned medium significantly stimulated BrdU-labeling index of both SCC9 and SCC25 oral cancer cell lines compared to fibroblast-conditioned medium ($p<0.001$ for SCC9 and

$p<0.05$ for SCC25). Interestingly, BrdU-labeling index of both oral cancer cell lines induced by myofibroblast-conditioned medium was similar to those of the positive control (fresh medium containing 10% of DCS). When myofibroblast cell lines were injected with SCC9 cells into nude mice, tumors formed were significantly larger than those formed by the fibroblast clones and SCC9 (Fig. 2B). Tumors formed in the presence of the myofibroblast clone 1 were significantly larger than controls from week 2 to 12 ($p<0.001$), whereas differences for myofibroblast clone 2 was observed later, from week 6 to 12 ($p<0.01$). No metastases were observed. As an uninspected result, SCC25 cells were not capable to induce tumors either when injected alone or when injected in association with fibroblasts or myofibroblasts.

Myofibroblasts induce invasion and synthesis of MMPs, and MMP activities are correlated with myofibroblasts in vivo

We compared the effect of factors released from fibroblasts and myofibroblasts on invasion of SCC9 and SCC25 cells. We found that myofibroblasts significantly promoted invasion of SCC9 cells compared with fibroblasts ($p<0.0001$, Fig. 3A). Since myofibroblast clones stimulated invasion of SCC9 cells, we further investigated whether this event is accompanied by increased MMP production. Myofibroblast clones significantly induced production of MMP-1, MMP-2, MMP-9 and MMP-13 by SCC9 cells compared with fibroblast clones (Fig. 3B). Although myofibroblast clones induced MMP production by SCC25 cells, those cells were not capable to invade towards any of the stimuli (data not shown).

To investigate whether myofibroblasts are associated with highly invasive oral cancers, we used 33 samples of OSCC to examine myofibroblast presence concurrent with MMP activities. As previously reported,[8] myofibroblasts was detected by immunohistochemistry and scored as undetectable (0), scanty (1) or abundant (2), whereas MMP activities were characterized by gelatin zymography. Four major gelatinolytic bands were produced by the tumor samples, a doublet with approximately 66 and 72 kDa, which correspond to the active and latent forms of MMP-2, and two more diffuse bands with approximately 85 and 92 kDa

corresponding to active and latent forms of MMP-9, respectively. These activities were attributed to MMPs since they were completely inhibited by 1,10-phenanthroline (Fig. 3C). We found that tumors with abundant presence of myofibroblasts showed significantly higher activities of MMP-2 and MMP-9 compared with tumors classified as negative or scanty for myofibroblasts (Fig. 3D). These data are consistent with the notion that abundant presence of myofibroblasts results in highly invasive oral tumors.

Myofibroblasts are dependent on activin A secretion to stimulate proliferation but not invasion of OSCC cells

To gain insight into the molecular mechanism by which myofibroblasts affect proliferation and invasion, we examined the expression profile of growth factors by using a quantitative real-time PCR array. When the criteria outlined in Material and Methods were used, 5 significantly up-regulated genes were identified (Table 1). All 5 genes were reproducibly and significantly up-regulated in myofibroblasts as revealed by qRT-PCR. In 3 independent experiments we found up-regulation of *INHBA* in myofibroblast clones compared to fibroblast clones, whereas the levels of *INHBB and INHA expression were quite similar.* As the product of *INHBA* (activin A) has important role in both cell cycle and invasion control,[16] we focused our attention on activin A. The amount of activin A was significantly higher in the supernatants of myofibroblast clones compared with fibroblast clones (data not shown).

To determine whether myofibroblast-released activin A promotes proliferation and invasion, activin A was knocked down by using siRNA. When activin A-specific oligonucleotides were used, a rapid and significant downregulation of activin A mRNA and protein was observed and maintained for 3 days (Fig. 4A). The decrease in activin A levels was concomitant with a significant decrease in the proliferation of both SCC9 and SCC25 cells cultured with myofibroblast-conditioned medium, demonstrating that activin A released by myofibroblasts regulates OSCC cell proliferation (Fig. 4B). On the other hand, downregulation of activin A on myofibroblast conditioned medium did not alter SCC9 invasion (Fig. 4C). Since

SCC9 invasion induced by myofibroblast clones was associated with increased MMP production, we performed ELISA for all MMPs in the conditioned mediums. MMP levels remained constant even in activin A downregulated cells (Fig. 4D). Together, these data suggest that myofibroblast-induced OSCC invasion and MMP synthesis are independent of activin A.

Discussion

Emergence of myofibroblasts is commonly observed in human cancers. Although through secretion of an extensive repertoire of molecules these cells are known to affect numerous processes important in both would healing and fibrosis,[17] a causal role for myofibroblasts in tumor progression remains controversial. The evidence presented in this article that myofibroblasts secrete several growth factors which induce proliferation, invasion and production of MMPs in OSCC cells in culture, and that their presence correlates with indices of MMP-2 and MMP-9 activity in OSCCs, strongly support a role for these cells on tumor progression and invasion. Furthermore, our current data, coupled with previously published work,[10] show that myofibroblasts promote proliferative activity of OSCC by upregulating activin A, a member of the transforming growth factor-β superfamily of proteins.[18] Activins are homo or heterodimeric proteins consisting of two β subunits (βA and βB), and INHBA is one of the β subunits (βA) that comprise activin A (βAβA), activin AB (βAβB) and inhibin A (αβA).[19] Activin A regulates normal embryogenesis,[18] and its dysregulation is observed in endometrial carcinoma,[20] testicular cancer,[21, 22] esophageal squamous cell carcinoma,[23] and lung adenocarcinoma.[24] In the later, activin A overexpression promotes tumor proliferation and is associated with worse survival. Thus, elevated expression of *INHBA*, β subunits of activin A, by stromal myofibroblasts induces tumor cell growth. It would be interesting to examine whether activin A is related to OSCC growth *in vivo*.

Myofibroblasts are known to induce migration and invasion in a number of contexts both in normal development[25] and tumorigenesis.[3] Such a role for myofibroblasts in tumor progression is supported by observations that they are

critical for invasion and metastasis of colon and prostate cancer and basal cell carcinoma,[26-28] and are associated with increased lymph node metastasis as well as poor survival in OSCC,[9, 10] supporting a role for these cells in invasion and metastasis. Indeed, our in vitro system demonstrated that myofibroblasts induce secretion of MMPs with subsequent increment in OSCC cell invasion. Although we did not observe organ metastases in the xenographic model, the study was not designed to examine metastasis, and thus, it remains a possibility that myofibroblasts not only promote proliferation of cancer cells but facility tumor invasion and metastasis. This is in line with previous reports that demonstrated myofibroblasts as the main source of MMPs in some types of cancers.[29-31] In ameloblastomas, presence of myofibroblasts as a releasing font of MMP-2 was associated with rupture of the osseous cortical, which has been considered an important prognostic marker of ameloblastoma aggressiveness.[32] Thus, myofibroblasts not only promotes OSCC cell proliferation, but also is likely to contribute to subsequent stages of tumor progression.

In summary, myofibroblasts are powerful regulators affecting many cellular processes involved in tumorigenesis including proliferation, migration, invasion, and neovascularization.[33] Here, we present strong evidences that myofibroblasts in OSCC induce proliferation via secretion of activin A, and promote invasion throughout secretion of MMPs. Together with previous clinicopathological studies, our data suggest that myofibroblast promotes tumorigenesis in OSCC, supporting its verification and monitoring as a marker of OSCC behaviour.

Conflict of interest statement

None declared.

Acknowledgments

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References

1. Olumi AF, Grossfeld GD, Hayward SW, Carroll PR, Tlsty TD, Cunha GR. Carcinoma-associated fibroblasts direct tumor progression of initiated human prostatic epithelium. *Cancer Res* 1999;**59**(19):5002-11.
2. Zhang C, Fu L, Fu J, Hu L, Yang H, Rong TH, et al. Fibroblast growth factor receptor 2-positive fibroblasts provide a suitable microenvironment for tumor development and progression in esophageal carcinoma. Clin *Cancer Res* 2009;**15**(12):4017-27.
3. Franco OE, Shaw AK, Strand DW, Hayward SW. Cancer associated fibroblasts in cancer pathogenesis. *Semin Cell Dev Biol*;**21**(1):33-9.
4. Tomasek JJ, Gabbiani G, Hinz B, Chaponnier C, Brown RA. Myofibroblasts and mechano-regulation of connective tissue remodelling. *Nat Rev Mol Cell Biol*. 2002/05/04 ed; 2002. p. 349-63.
5. Kalluri R, Zeisberg M. Fibroblasts in cancer. *Nat Rev Cancer* 2006;**6**(5):392-401.
6. Barth PJ, Schenck zu Schweinsberg T, Ramaswamy A, Moll R. CD34+ fibrocytes, alpha-smooth muscle antigen-positive myofibroblasts, and CD117 expression in the stroma of invasive squamous cell carcinomas of the oral cavity, pharynx, and larynx. *Virchows Arch* 2004;**444**(3):231-4.
7. Lewis MP, Lygoe KA, Nystrom ML, Anderson WP, Speight PM, Marshall JF, et al. Tumour-derived TGF-beta1 modulates myofibroblast differentiation and promotes HGF/SF-dependent invasion of squamous carcinoma cells. *Br J Cancer* 2004;**90**(4):822-32.
8. Kellermann MG, Sobral LM, da Silva SD, Zecchin KG, Graner E, Lopes MA, et al. Myofibroblasts in the stroma of oral squamous cell carcinoma are associated with poor prognosis. *Histopathology* 2007;**51**(6):849-53.
9. Kawashiri S, Tanaka A, Noguchi N, Hase T, Nakaya H, Ohara T, et al. Significance of stromal desmoplasia and myofibroblast appearance at the invasive front in squamous cell carcinoma of the oral cavity. *Head Neck* 2009;**31**(10):1346-53.
10. Kellermann MG, Sobral LM, da Silva SD, Zecchin KG, Graner E, Lopes MA, et al. Mutual paracrine effects of oral squamous cell carcinoma cells and normal oral fibroblasts: induction of fibroblast to myofibroblast transdifferentiation and modulation of tumor cell proliferation. *Oral Oncol* 2008;**44**(5):509-17.

11. Coletta RD, Almeida OP, Graner E, Page RC, Bozzo L. Differential proliferation of fibroblasts cultured from hereditary gingival fibromatosis and normal gingiva. *J Periodontal Res* 1998;**33**(8):469-75.
12. Bitu CC, Sobral LM, Kellermann MG, Martelli-Junior H, Zecchin KG, Graner E, et al. Heterogeneous presence of myofibroblasts in hereditary gingival fibromatosis. *J Clin Periodontol* 2006;**33**(6):393-400.
13. Sobral LM, Montan PF, Martelli-Junior H, Graner E, Coletta RD. Opposite effects of TGF-beta1 and IFN-gamma on transdifferentiation of myofibroblast in human gingival cell cultures. *J Clin Periodontol* 2007;**34**(5):397-406.
14. De Souza Setubal Destro MF, Bitu CC, Zecchin KG, Graner E, Lopes MA, Kowalski LP, et al. Overexpression of HOXB7 homeobox gene in oral cancer induces cellular proliferation and is associated with poor prognosis. *Int J Oncol*;**36**(1):141-9.
15. Coletta RD, Almeida OP, Reynolds MA, Sauk JJ. Alteration in expression of MMP-1 and MMP-2 but not TIMP-1 and TIMP-2 in hereditary gingival fibromatosis is mediated by TGF-beta 1 autocrine stimulation. *J Periodontal Res* 1999;**34**(8):457-63.
16. Chen YG, Wang Q, Lin SL, Chang CD, Chuang J, Ying SY. Activin signaling and its role in regulation of cell proliferation, apoptosis, and carcinogenesis. *Exp Biol Med (Maywood)* 2006;**231**(5):534-44.
17. Desmouliere A, Chaponnier C, Gabbiani G. Tissue repair, contraction, and the myofibroblast. *Wound Repair Regen* 2005;**13**(1):7-12.
18. Welt C, Sidis Y, Keutmann H, Schneyer A. Activins, inhibins, and follistatins: from endocrinology to signaling. A paradigm for the new millennium. *Exp Biol Med (Maywood)* 2002;**227**(9):724-52.
19. Vale W, Wiater E, Gray P, Harrison C, Bilezikjian L, Choe S. Activins and inhibins and their signaling. *Ann N Y Acad Sci* 2004;**1038**:142-7.
20. Tanaka T, Toujima S, Umesaki N. Activin A inhibits growth-inhibitory signals by TGF-beta1 in differentiated human endometrial adenocarcinoma cells. *Oncol Rep* 2004;**11**(4):875-9.
21. Devouassoux-Shisheboran M, Mauduit C, Tabone E, Droz JP, Benahmed M. Growth regulatory factors and signalling proteins in testicular germ cell tumours. *APMIS 2003*;**111**(1):212-24; discussion 24.
22. Risbridger GP, Schmitt JF, Robertson DM. Activins and inhibins in endocrine and other tumors. *Endocr Rev* 2001;**22**(6):836-58.
23. Yoshinaga K, Yamashita K, Mimori K, Tanaka F, Inoue H, Mori M. Activin a causes cancer cell aggressiveness in esophageal squamous cell carcinoma cells. *Ann Surg Oncol* 2008;**15**(1):96-103.

24. Seder CW, Hartojo W, Lin L, Silvers AL, Wang Z, Thomas DG, et al. Upregulated INHBA expression may promote cell proliferation and is associated with poor survival in lung adenocarcinoma. *Neoplasia* 2009;**11**(4):388-96.
25. Serini G, Gabbiani G. Mechanisms of myofibroblast activity and phenotypic modulation. *Exp Cell Res* 1999;**250(**2):273-83.
26. De Wever O, Demetter P, Mareel M, Bracke M. Stromal myofibroblasts are drivers of invasive cancer growth. *Int J Cancer* 2008;**123**(10):2229-38.
27. Hill R, Song Y, Cardiff RD, Van Dyke T. Selective evolution of stromal mesenchyme with p53 loss in response to epithelial tumorigenesis. *Cell* 2005;**123**(6):1001-11.
28. Marsh D, Dickinson S, Neill GW, Marshall JF, Hart IR, Thomas GJ. alpha vbeta 6 Integrin promotes the invasion of morphoeic basal cell carcinoma through stromal modulation. *Cancer Res* 2008;**68**(9):3295-303.
29. Okada A, Bellocq JP, Rouyer N, Chenard MP, Rio MC, Chambon P, et al. Membrane-type matrix metalloproteinase (MT-MMP) gene is expressed in stromal cells of human colon, breast, and head and neck carcinomas. *Proc Natl Acad Sci U S A* 1995;**92**(7):2730-4.
30. Seomun Y, Kim J, Lee EH, Joo CK. Overexpression of matrix metalloproteinase-2 mediates phenotypic transformation of lens epithelial cells. *Biochem J* 2001;**358**(Pt 1):41-8.
31. Bisson C, Blacher S, Polette M, Blanc JF, Kebers F, Desreux J, et al. Restricted expression of membrane type 1-matrix metalloproteinase by myofibroblasts adjacent to human breast cancer cells. *Int J Cancer* 2003;**105**(1):7-13.
32. Fregnani ER, Sobral LM, Alves FA, Soares FA, Kowalski LP, Coletta RD. Presence of myofibroblasts and expression of matrix metalloproteinase-2 (MMP-2) in ameloblastomas correlate with rupture of the osseous cortical. *Pathol Oncol Res* 2009;**15**(2):231-40.
33. Kunz-Schughart LA, Knuechel R. Tumor-associated fibroblasts (part II): Functional impact on tumor tissue. *Histol Histopathol* 2002;**17**(2):623-37.

Figures

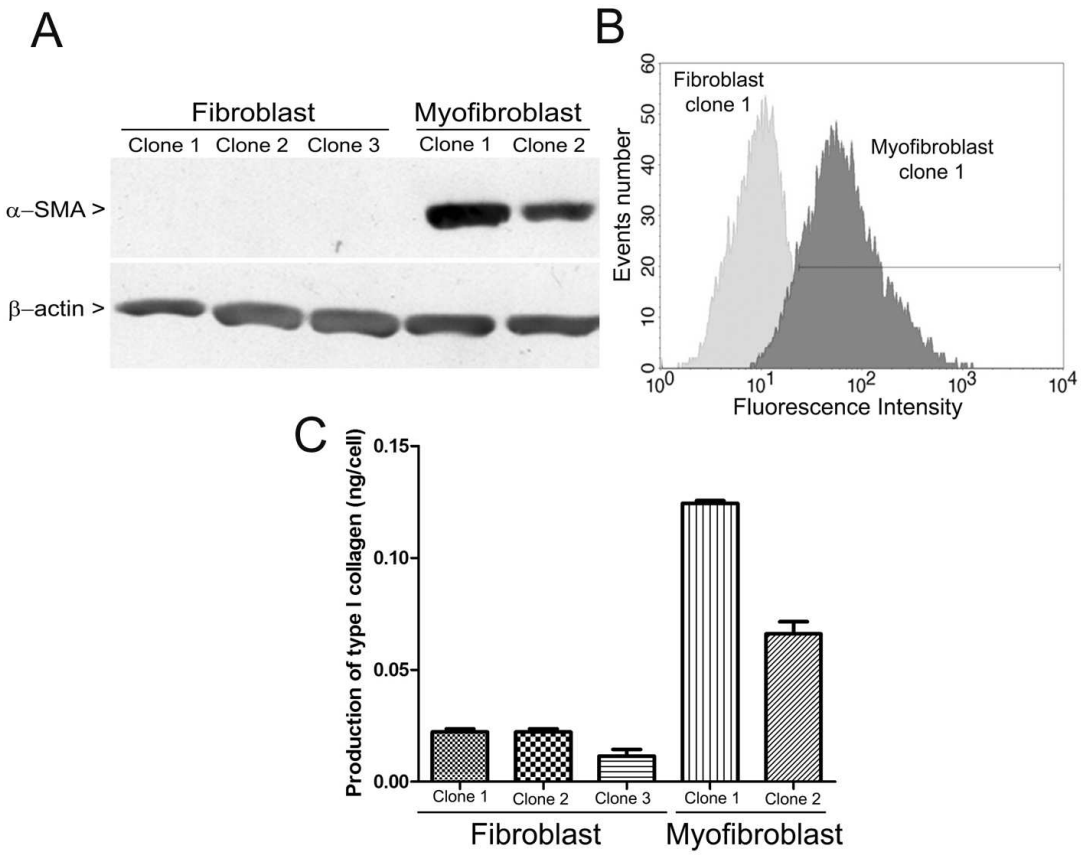

Figure 1. Characterization of the myofibroblast clones. Individual clones of OSCC-associated mesenchymal fusiform cells were cloned, screened for α-SMA expression, and characterized as fibroblast or myofibroblast cell lines. (A) Western blot analysis showed a lack of α-SMA expression in the fibroblast cell lines, and a high expression in the myofibroblasts. (B) Representative flow cytometric analysis showed that more than 90% of the cells express α-SMA in the myofibroblast clones. (C) ELISA for type I collagen showed that myofibroblast clones produced significantly higher amounts of type I collagen than fibroblasts ($p<0.01$).

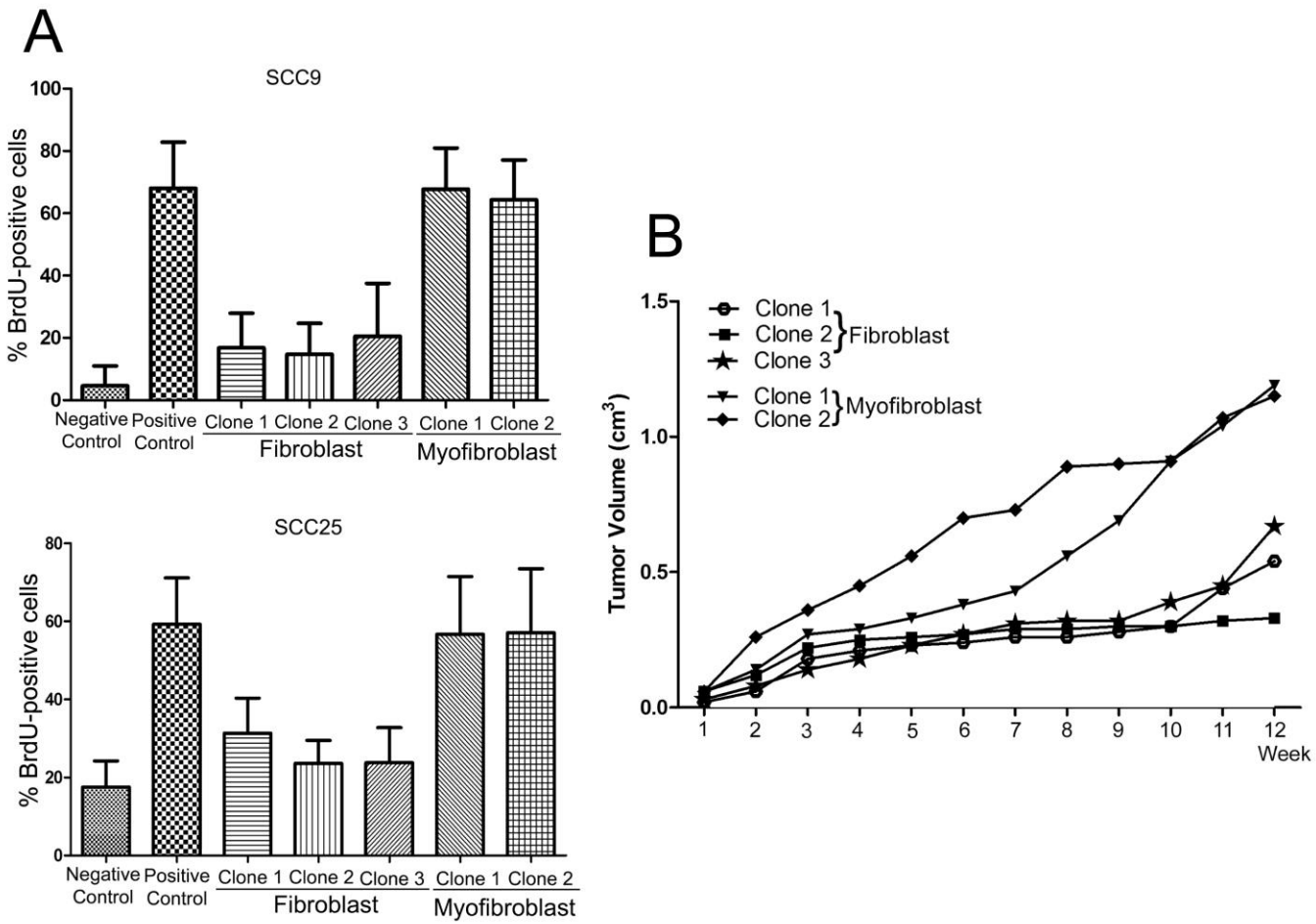

Figure 2. Myofibroblast stimulates OSCC proliferation. (A) Myofibroblast-conditioned medium significantly stimulated BrdU-labeling index of both SCC9 and SCC25 oral cancer cell lines compared to fibroblast-conditioned medium ($p<0.001$ for SCC9 and $p<0.05$ for SCC25). (B) Myofibroblasts significantly increases tumor burden in nude mice. SCC9 cells with either fibroblast or myofibroblast clones were injected into the flank of 12-week-old nude mice, and tumor size was measured over a 12-week time period. Data are shown as mean ± SD. In the presence of the myofibroblast clone 1, tumors were significantly larger than controls from week 2 to 12 ($p<0.001$), whereas differences for myofibroblast clone 2 was observed later, from week 6 to 12 ($p<0.01$).

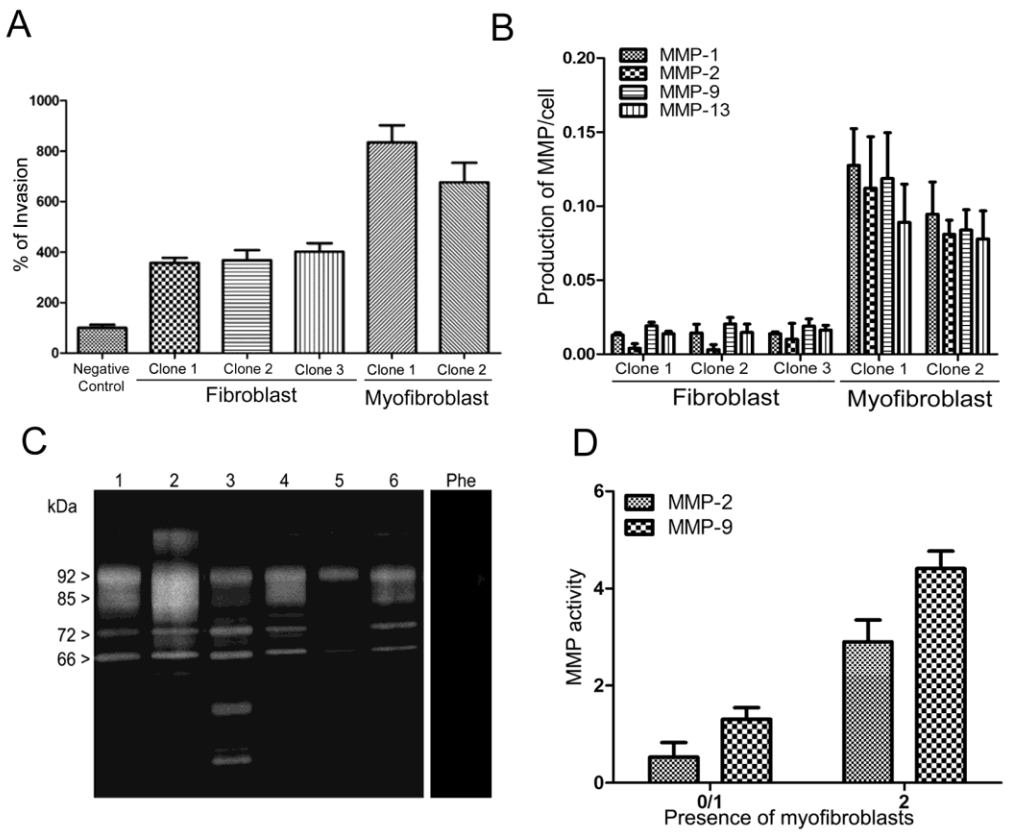

Figure 3. Myofibroblast promotes invasion and MMP synthesis of OSCC cells. (A) SCC9 cells were subjected to transwell chamber invasion assay with factors released by fibroblast or myofibroblast clones as chemotatics. Myofibroblasts significantly promoted invasion of SCC9 cells compared with fibroblasts ($p<0.0001$). (B) Myofibroblast-induced SCC9 invasion was associated with increased production of MMP-1, MMP-2, MMP-9 and MMP-13, as revealed by ELISA ($p<0.001$). (C) Zymographic analysis of MMPs secreted by 6 representative OSCC samples. Gelatinolytic activities were detected at ~66-72 kDa and ~85-92 kDa, consistent with MMP-2 and MMP-9, respectively. Lanes 1, 2 and 6 depict conditioned cell culture media from OSCCs of the tongue, lane 3 of the retromolar region, and lanes 4 and 5 of the floor of mouth. Last lane represents sample 6 incubated with 1 mM 1,10-phenanthroline, a specific MMP inhibitor. (D) Presence of myofibroblasts [from undetectable (0), scanty (1) and abundant (2)] was significantly associated with an increasing in the activity of the MMP-2 and MMP-9 of the OSCCs ($p<0.05$ for MMP-2 and $p<0.008$ for MMP-9).

p

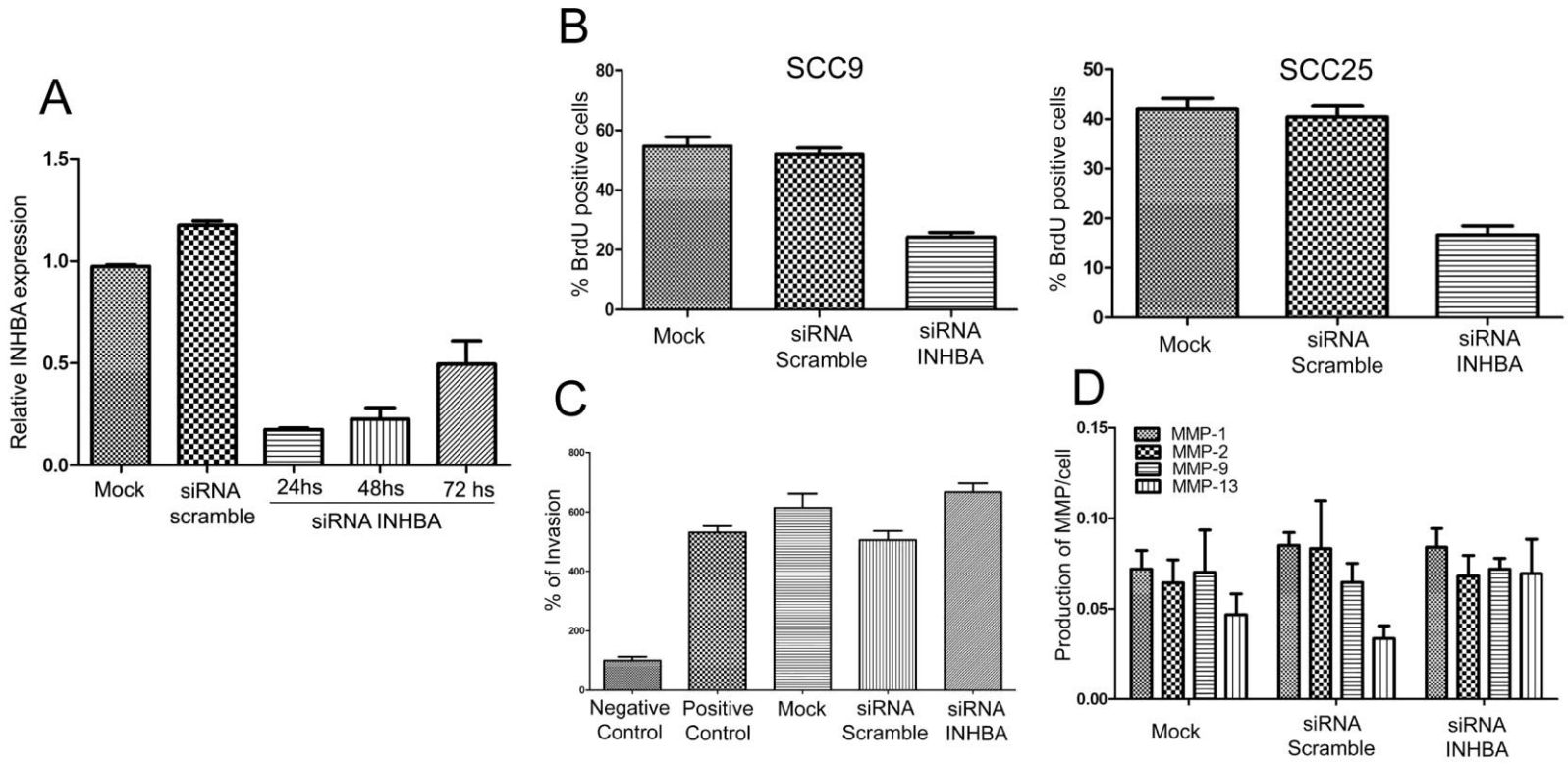

Figure 4. Inhibition of activin A production by myofibroblasts decreases effect on OSCC cell proliferation. (A) Effect of siRNA against *INHBA* in myofibroblast clone 2 over a 3-day time course. Cells were exposed to transfectant agent only (mock), siRNA control or siRNA INHBA. qRT-PCR analysis demonstrate that *INHBA* levels are significantly decreased after introduction of the specific siRNA oligonucleotides. (B) BrdU incorporation assay demonstrated a statistically significant decrease in both SCC9 and SCC25 proliferation when *INHBA* was downregulated by siRNA in myofibroblast clone 2 ($p<0.0001$ for SCC9 and $p<0.0001$ for SCC25). Downregulation of activin A on myofibroblast clone 2 conditioned medium did not alter invasion (C) and MMP production of SCC9 cells.

Supplementary Table 1 Description of the primers used in the qRT-PCR.

Primers	Sequence (5’ → 3’)
BMP4	Forward: GCCCGCAGCCTAGCA
	Reverse: CGGTAAAGATCCCGCATGTAG
FIGF	Forward: GCAGGCTGAGGCTCAAAAGT
	Reverse: AGTGGACCGATGGGATGCT
INHBA	Forward: CCCCTTTGCCAACCTCAAA
	Reverse: CATGGACATGGGTCTCAGCTT
MDK	Forward: AGTTTGGAGCCGACTGCAA
	Reverse: CCTGTGCCCCCATCACA
NTF3	Forward: GCAGGCTGAGGCTCAAAAGT
	Reverse: AGTGGACCGATGGGATGCT
GAPDH	Forward: GAAGGTGAAGGTCGGA
	Reverse: GGGTCATTGATGGCAAC

Table 1 Genes upregulated in myofibroblasts. Values represent the fold of expression in myofibroblast clones in relation to fibroblast clones. After selection using the method outlined in Material and Methods, upregulated genes were validated by qRT-PCR in 3 independent experiments

Genes	Real-time PCR Array	qRT-PCR	p value
BMP4	7.63	4.45	0.01
FIGF	6.24	3.54	0.013
INHBA	2.09	3.06	0.003
MDK	3.44	1.80	0.007
NTF3	16.53	2.75	0.02

Conclusões

1. A expressão de CTGF é fundamental para a transdiferenciação de miofibroblastos gengivais via TGF-β1.
2. A superexpressão de Smad 7 é efetiva no bloqueio da transdiferenciação e ativação dos miofibroblastos.
3. Miofibroblastos do estroma de CECs orais apresentam uma maior produção e secreção de MMPs comparados com fibroblastos do estroma de CECs orais.
4. A presença de miofibroblastos no estroma de CECs orais induz um aumento da proliferação das células tumorais via secreção de ativina A.
5. A presença de miofibroblastos induz uma maior progressão tumoral em modelo xenográfico
6. Miofibroblastos induzem um aumento da invasão das células tumorais através da indução da secreção de MMPs.

Referências*

Annes JP, Munger JS, Rifkin D. Making sense of latent TGFbeta activation. J Cell Sci. 2003; 116(Pt 2):217-24.

Baglole CJ, Ray DM, Bernstein SH, Feldon SE, Smith TJ, Sime PJ, *et al.* More than structural cells, fibroblasts create and orchestrate the tumor microenvironment. Immunol Invest. 2006; 35(3-4):297-325.

Cekanova M, Masi T, Plummer HK, 3rd, Majidi M, Fedorocko P, Schuller HM. Pulmonary fibroblasts stimulate the proliferation of cell lines from human lung adenocarcinomas. Anticancer Drugs. 2006; 17(7):771-81.

Chang KP, Kao HK, Liang Y, Cheng MH, Chang YL, Liu SC, *et al.* Overexpression of activin A in oral squamous cell carcinoma: association with poor prognosis and tumor progression. Ann Surg Oncol. 2010; 17(7):1945-56.

Colwell AS, Krummel TM, Longaker MT, Lorenz HP. Fetal and adult fibroblasts have similar TGF-beta-mediated, Smad-dependent signaling pathways. Plast Reconstr Surg. 2006; 117(7):2277-83.

Darby I, Skalli O, Gabbiani G. Alpha-smooth muscle actin is transiently expressed by myofibroblasts during experimental wound healing. Lab Invest. 1990; 63(1):21-9.

De Wever O, Westbroek W, Verloes A, Bloemen N, Bracke M, Gespach C, *et al.* Critical role of N-cadherin in myofibroblast invasion and migration in vitro stimulated by colon-cancer-cell-derived TGF-beta or wounding. J Cell Sci. 2004; 117(Pt 20):4691-703.

* De acordo com a norma UNICAMP/FOP, baseadas na norma do International Committee of Medical Journal Editors – Grupo Vancouver. Abreviatura dos periódicos em conformidade com o Medline.

Derynck R, Zhang YE. Smad-dependent and Smad-independent pathways in TGF-beta family signalling. Nature. 2003; 425(6958):577-84.

Egeblad M, Werb Z. New functions for the matrix metalloproteinases in cancer progression. Nat Rev Cancer. 2002; 2(3):161-74.

Etemad-Moghadam S, Khalili M, Tirgary F, Alaeddini M. Evaluation of myofibroblasts in oral epithelial dysplasia and squamous cell carcinoma. J Oral Pathol Med. 2009; 38(8):639-43.

Eyden B. The fibronexus in reactive and tumoral myofibroblasts: further characterisation by electron microscopy. Histol Histopathol. 200; 1 16(1):57-70.

Gailit J, Marchese MJ, Kew RR, Gruber BL. The differentiation and function of myofibroblasts is regulated by mast cell mediators. J Invest Dermatol. 2001; 117(5):1113-9.

Galliher AJ, Neil JR, Schiemann WP. Role of transforming growth factor-beta in cancer progression. Future Oncol. 2006; 2(6):743-63.

Hayashido Y, Urabe K, Yoshioka Y, Kitano H, Okamoto T, Matsuya T. Participation of fibroblasts in MMP-2 binding and activation on the surface of oral squamous cell carcinoma cells. Int J Oncol. 2003; 22(3):657-62.

Hong SD, Hong SP, Lee JI, Lim CY. Expression of matrix metalloproteinase-2 and -9 in oral squamous cell carcinomas with regard to the metastatic potential. Oral Oncol. 2000; 36(2):207-13.

Huang X, Lee C. Regulation of stromal proliferation, growth arrest, differentiation and apoptosis in benign prostatic hyperplasia by TGF-beta. Front Biosci. 2003; 8(s740-9.

Ikebe T, Shinohara M, Takeuchi H, Beppu M, Kurahara S, Nakamura S, *et al.* Gelatinolytic activity of matrix metalloproteinase in tumor tissues correlates with the invasiveness of oral cancer. Clin Exp Metastasis. 1999; 17(4):315-23.

Juarez J, Clayman G, Nakajima M, Tanabe KK, Saya H, Nicolson GL, *et al.* Role and regulation of expression of 92-kDa type-IV collagenase (MMP-9) in 2 invasive squamous-cell-carcinoma cell lines of the oral cavity. Int J Cancer. 1993; 55(1):10-8.

Katayama A, Bandoh N, Kishibe K, Takahara M, Ogino T, Nonaka S, *et al.* Expressions of matrix metalloproteinases in early-stage oral squamous cell carcinoma as predictive indicators for tumor metastases and prognosis. Clin Cancer Res. 2004; 10(2):634-40.

Kawahara E, Okada Y, Nakanishi I, Iwata K, Kojima S, Kumagai S, *et al.* The expression of invasive behavior of differentiated squamous carcinoma cell line evaluated by an in vitro invasion model. Jpn J Cancer Res. 1993; 84(4):409-18.

Kawamata H, Nakashiro K, Uchida D, Harada K, Yoshida H, Sato M. Possible contribution of active MMP2 to lymph-node metastasis and secreted cathepsin L to bone invasion of newly established human oral-squamous-cancer cell lines. Int J Cancer. 1997; 70(1):120-7.

Kessenbrock K, Plaks V, Werb Z Matrix metalloproteinases: regulators of the tumor microenvironment. Cell. 2010; 141(1):52-67.

Kingsley DM. The TGF-beta superfamily: new members, new receptors, and new genetic tests of function in different organisms. Genes Dev. 1994; 8(2):133-46.

Kojc N, Zidar N, Vodopivec B, Gale N. Expression of CD34, alpha-smooth muscle actin, and transforming growth factor beta1 in squamous intraepithelial lesions and squamous cell carcinoma of the larynx and hypopharynx. Hum Pathol. 2005; 36(1):16-21.

Konstantinopoulos PA, Karamouzis MV, Papatsoris AG, Papavassiliou AG. Matrix metalloproteinase inhibitors as anticancer agents. Int J Biochem Cell Biol. 2008; 40(6-7):1156-68.

Kunz-Schughart LA, Knuechel R. Tumor-associated fibroblasts (part I): Active stromal participants in tumor development and progression? Histol Histopathol. 2002; 17(2):599-621.

Kuroda N, Shimasaki N, Miyazaki E, Hamauzu T, Toi M, Hiroi M, *et al.* The distribution of myofibroblasts and CD34-positive stromal cells in normal renal pelvis and ureter and their cancers. Histol Histopathol. 2006; 21(12):1303-7.

Kusukawa J, Sasaguri Y, Shima I, Kameyama T, Morimatsu M. Production of matrix metalloproteinase 2 (gelatinase/type IV collagenase) and 3 (stromelysin) by cultured oral squamous cell carcinoma. J Oral Pathol Med. 1992; 21(5):221-4.

Kusukawa J, Sasaguri Y, Shima I, Kameyama T, Morimatsu M. Expression of matrix metalloproteinase-2 related to lymph node metastasis of oral squamous cell carcinoma. A clinicopathologic study. Am J Clin Pathol. 1993; 99(1):18-23.

Kusukawa J, Sasaguri Y, Morimatsu M, Kameyama T. Expression of matrix metalloproteinase-3 in stage I and II squamous cell carcinoma of the oral cavity. J Oral Maxillofac Surg. 1995; 53(5):530-4.

Kusukawa J, Harada H, Shima I, Sasaguri Y, Kameyama T, Morimatsu M. The significance of epidermal growth factor receptor and matrix metalloproteinase-3 in squamous cell carcinoma of the oral cavity. Eur J Cancer B Oral Oncol. 1996; 32B(4):217-21.

Ling N, Ying SY, Ueno N, Shimasaki S, Esch F, Hotta M, *et al.* A homodimer of the beta-subunits of inhibin A stimulates the secretion of pituitary follicle stimulating hormone. Biochem Biophys Res Commun. 1986; 138(3):1129-37.

Liu G, Li J, Li Z, Chen X. [The effects of MMPs and TIMPs on the metastasis of oral squamous cell carcinoma to neck lymph nodes]. Hua Xi Kou Qiang Yi Xue Za Zhi. 2001; 19(4):216-8, 224.

Massague J, Wotton D. Transcriptional control by the TGF-beta/Smad signaling system. EMBO J. 2000; 19(8):1745-54.

Mikula M, Proell V, Fischer AN, Mikulits W. Activated hepatic stellate cells induce tumor progression of neoplastic hepatocytes in a TGF-beta dependent fashion. J Cell Physiol. 2006; 209(2):560-7.

Mukaratirwa S, Koninkx JF, Gruys E, Nederbragt H. Mutual paracrine effects of colorectal tumour cells and stromal cells: modulation of tumour and stromal cell differentiation and extracellular matrix component production in culture. Int J Exp Pathol. 2005; 86(4):219-29.

Offersen BV, Nielsen BS, Hoyer-Hansen G, Rank F, Hamilton-Dutoit S, Overgaard J, *et al.*. The myofibroblast is the predominant plasminogen activator inhibitor-1-expressing cell type in human breast carcinomas. Am J Pathol. 2003; 163(5):1887-99.

Orimo A, Gupta PB, Sgroi DC, Arenzana-Seisdedos F, Delaunay T, Naeem R, *et al.* Stromal fibroblasts present in invasive human breast carcinomas promote tumor growth and angiogenesis through elevated SDF-1/CXCL12 secretion. Cell. 2005; 121(3):335-48.

Orimo A, Weinberg RA. Stromal fibroblasts in cancer: a novel tumor-promoting cell type. Cell Cycle. 2006; 5(15):1597-601.

Patel BP, Shah SV, Shukla SN, Shah PM, Patel PS. Clinical significance of MMP-2 and MMP-9 in patients with oral cancer. Head Neck. 2007; 29(6):564-72.

Petraglia F, Florio P, Luisi S, Gallo R, Gadducci A, Vigano P, *et al.* Expression and secretion of inhibin and activin in normal and neoplastic uterine tissues. High levels of serum activin A in women with endometrial and cervical carcinoma. J Clin Endocrinol Metab. 1998; 83(4):1194-200.

Pirisi M, Fabris C, Luisi S, Santuz M, Toniutto P, Vitulli D, *et al.* Evaluation of circulating activin-A as a serum marker of hepatocellular carcinoma. Cancer Detect Prev. 2000; 24(2):150-5.

Pourreyron C, Dumortier J, Ratineau C, Nejjari M, Beatrix O, Jacquier MF, *et al.* Age-dependent variations of human and rat colon myofibroblasts in culture: Influence on their functional interactions with colon cancer cells. Int J Cancer. 2003; 104(1):28-35.

Reis FM, Cobellis L, Tameirao LC, Anania G, Luisi S, Silva IS, *et al.* Serum and tissue expression of activin a in postmenopausal women with breast cancer. J Clin Endocrinol Metab. 2002; 87(5):2277-82.

Risbridger GP, Mellor SL, McPherson SJ, Schmitt JF. The contribution of inhibins and activins to malignant prostate disease. Mol Cell Endocrinol. 2001; 180(1-2):149-53.

Ronnov-Jessen L, Villadsen R, Edwards JC, Petersen OW. Differential expression of a chloride intracellular channel gene, CLIC4, in transforming growth factor-beta1-mediated conversion of fibroblasts to myofibroblasts. Am J Pathol. 2002; 161(2):471-80.

Rosenthal EL, McCrory A, Talbert M, Carroll W, Magnuson JS, Peters GE. Expression of proteolytic enzymes in head and neck cancer-associated fibroblasts. Arch Otolaryngol Head Neck Surg. 2004; 130(8):943-7.

Seder CW, Hartojo W, Lin L, Silvers AL, Wang Z, Thomas DG, *et al.* INHBA overexpression promotes cell proliferation and may be epigenetically regulated in esophageal adenocarcinoma. J Thorac Oncol. 2009b; 4(4):455-62.

Shimizu S, Seki N, Sugimoto T, Horiguchi S, Tanzawa H, Hanazawa T, *et al.* Identification of molecular targets in head and neck squamous cell carcinomas based on genome-wide gene expression profiling. Oncol Rep. 2007; 18(6):1489-97.

Shindoh M, Higashino F, Kaya M, Yasuda M, Funaoka K, Hanzawa M, *et al.* Correlated expression of matrix metalloproteinases and ets family transcription factor E1A-F in invasive oral squamous-cell-carcinoma-derived cell lines. Am J Pathol. 1996; 148(3):693-700.

Sieuwerts AM, Klijn JG, Henzen-Logmand SC, Bouwman I, Van Roozendaal KE, Peters HA, *et al.* Urokinase-type-plasminogen-activator (uPA) production by human breast (myo) fibroblasts in vitro: influence of transforming growth factor-beta(1) (TGF beta(1)) compared with factor(s) released by human epithelial-carcinoma cells. Int J Cancer. 1998; 76(6):829-35.

Smith PC, Caceres M, Martinez J. Induction of the myofibroblastic phenotype in human gingival fibroblasts by transforming growth factor-beta1: role of RhoA-ROCK and c-Jun N-terminal kinase signaling pathways. J Periodontal Res. 2006; 41(5):418-25.

Sun L, Diamond ME, Ottaviano AJ, Joseph MJ, Ananthanarayan V, Munshi HG. Transforming growth factor-beta 1 promotes matrix metalloproteinase-9-mediated oral cancer invasion through snail expression. Mol Cancer Res. 2008; 6(1):10-20.

Tabibzadeh S. Homeostasis of extracellular matrix by TGF-beta and lefty. Front Biosci. 2002; 7(d1231-46.

Tang WW, Ulich TR, Lacey DL, Hill DC, Qi M, Kaufman SA, *et al.* Platelet-derived growth factor-BB induces renal tubulointerstitial myofibroblast formation and tubulointerstitial fibrosis. Am J Pathol. 1996; 148(4):1169-80.

Tsai CH, Hsieh YS, Yang SF, Chou MY, Chang YC. Matrix metalloproteinase 2 and matrix metalloproteinase 9 expression in human oral squamous cell carcinoma and the effect of protein kinase C inhibitors: preliminary observations. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2003; 95(6):710-6.

Tuxhorn JA, Ayala GE, Smith MJ, Smith VC, Dang TD, Rowley DR. Reactive stroma in human prostate cancer: induction of myofibroblast phenotype and extracellular matrix remodeling. Clin Cancer Res. 2002a 8(9):2912-23.

Tuxhorn JA, McAlhany SJ, Yang F, Dang TD, Rowley DR. Inhibition of transforming growth factor-beta activity decreases angiogenesis in a human prostate cancer-reactive stroma xenograft model. Cancer Res. 2002b 62(21):6021-5.

Untergasser G, Gander R, Lilg C, Lepperdinger G, Plas E, Berger P. Profiling molecular targets of TGF-beta1 in prostate fibroblast-to-myofibroblast transdifferentiation. Mech Ageing Dev. 2005; 126(1):59-69.

Vale W, Rivier J, Vaughan J, McClintock R, Corrigan A, Woo W, *et al.* Purification and characterization of an FSH releasing protein from porcine ovarian follicular fluid. Nature. 1986; 321(6072):776-9.

Ye H, Yu T, Temam S, Ziober BL, Wang J, Schwartz JL, *et al.* Transcriptomic dissection of tongue squamous cell carcinoma. BMC Genomics. 2008; 9:69.

Yoshinaga K, Mimori K, Inoue H, Kamohara Y, Yamashita K, Tanaka F, *et al.* Activin A enhances MMP-7 activity via the transcription factor AP-1 in an esophageal squamous cell carcinoma cell line. Int J Oncol. 2008; 33(3):453-9.

Yoshizaki T, Maruyama Y, Sato H, Furukawa M. Expression of tissue inhibitor of matrix metalloproteinase-2 correlates with activation of matrix metalloproteinase-2 and predicts poor prognosis in tongue squamous cell carcinoma. Int J Cancer. 2001; 95(1):44-50.

Zhang W, Matrisian LM, Holmbeck K, Vick CC, Rosenthal EL. Fibroblast-derived MT1-MMP promotes tumor progression in vitro and *in vivo*. BMC Cancer. 2006; 6:52.

Zigrino P, Kuhn I, Bauerle T, Zamek J, Fox JW, Neumann S, *et al.* Stromal expression of MMP-13 is required for melanoma invasion and metastasis. J Invest Dermatol. 2009; 129(11):2686-93.

Anexo 1.

UNICAMP

CEEA/Unicamp

Comissão de Ética na Experimentação Animal
CEEA/Unicamp

CERTIFICADO

Certificamos que o Protocolo nº **1353-1**, sobre **"Análise comparativa entre as características dos fibroblastos e miofibroblastos do estroma de CECs orais e suas influências na invasão e formação de tumores em modelo xenográfico"**, sob a responsabilidade de **Prof. Dr. Ricardo Della Coletta / Lays Martin Sobral**, está de acordo com os Princípios Éticos na Experimentação Animal adotados pelo Colégio Brasileiro de Experimentação Animal (COBEA), tendo sido aprovado pela Comissão de Ética na Experimentação Animal – CEEA/Unicamp em **26 de setembro de 2007**.

CERTIFICATE

We certify that the protocol nº **1353-1**, entitled **"Comparative analysis between fibroblasts and myofobroblasts from stroma of oral squamous cell carcinomas and influence of myofibroblast on tumor development and progression in xenographic model"**, is in agreement with the Ethical Principles for Animal Research established by the Brazilian College for Animal Experimentation (COBEA). This project was approved by the institutional Committee for Ethics in Animal Research (State University of Campinas - Unicamp) on **september 26, 2007**.

Campinas, 26 de setembro de 2007.

Profa. Dra. Ana Maria A. Guaraldo
Presidente

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Anexo 2

COMITÊ DE ÉTICA EM PESQUISA
FACULDADE DE ODONTOLOGIA DE PIRACICABA
UNIVERSIDADE ESTADUAL DE CAMPINAS

CERTIFICADO

O Comitê de Ética em Pesquisa da FOP-UNICAMP certifica que o projeto de pesquisa **"Análise comparativa entre as características dos fibroblastos e miofibroblastos do estroma de CECs orais e suas influências na invasão e formação de tumores em modelo xenográfico"**, protocolo nº 070/2007, dos pesquisadores Ricardo Della Coletta e Lays Martin Sobral, satisfaz as exigências do Conselho Nacional de Saúde – Ministério da Saúde para as pesquisas em seres humanos e foi aprovado por este comitê em 10/10/2007.

The Ethics Committee in Research of the School of Dentistry of Piracicaba - State University of Campinas, certify that the project **"Comparative analysis between fibroblasts and myofibroblasts from stroma of oral squamous cell carcinomas and influence of myofibroblast on tumor development and progression in xenographic model"**, register number 070/2007, of and Lays Martin Sobral, comply with the recommendations of the National Health Council – Ministry of Health of Brazil for research in human subjects and therefore was approved by this committee at 10/10/2007.

Profa. Dra. Cinthia Pereira Machado Tabchoury
Secretária
CEP/FOP/UNICAMP

Prof. Dr. Jacks Jorge Junior
Coordenador
CEP/FOP/UNICAMP

Nota: O título do protocolo aparece como fornecido pelos pesquisadores, sem qualquer edição.
Notice: The title of the project appears as provided by the authors, without editing.