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Camila Leonel da Silva

INIBIÇÃO DA METÁSTASE VIA TRANSIÇÃO EPITÉLIO-MESENQUIMAL POR shRNA, METFORMINA E Y27632 EM NEOPLASIA MAMÁRIA

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Orientador: Profª. Drª. Debora Ap. Pires de Campos Zuccari

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“Quando uma criatura humana desperta para um grande sonho e sobre ele lança toda a força de sua alma, todo o universo conspira a seu favor”.

Johann Wolfgang von Goethe

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Sumário

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Lista de abreviaturas e símbolos

Lista de abreviaturas e símbolos

µL	Microlitros
µM	Micromolar
AMPK	do inglês *AMP-activated protein kinase*
ANOVA	análise de variância
ATCC	do inglês *American type culture collection*
ATP	do inglês *Adenosine triphosphate*
a.u.	do inglês *arbitrary unit*
BCSC	do inglês *Breast Cancer Stem Cells*
CAM	do inglês *Cell adhesion molecules*
cDNA	DNA complementar
CEUA	Comissão de Ética no Uso de Animais
CO_2	fórmula química do gás carbônico
CSC	do inglês *Cancer Stem Cells*
DAB	Diaminobenzidina 3,3'
DAPI	*4',6-diamidino-2-phenylindole*
dL	Decilitro
DMEM	do inglês *Dulbecco's modified Eagle's medium*
D.O.M.	Densidade Óptica Média
FAMERP	Faculdade de Medicina de São José do Rio Preto
FAPESP	Fundação de Amparo à Pesquisa do Estado de São Paulo
FBS	soro fetal bovino, do inglês *fetal boine serum*
FGF	do inglês *Fibroblast growth factor*
GFP	do inglês *green fluorescent protein*
GFPsh	*green fluorescent protein reporter into CF41 cells*
H&E	Hematoxilina e eosina
HER2	do inglês *Human Epidermal Growth Factor Receptor 2*
IARC	do inglês *International Agency for Research on Cancer*
IGF	do inglês *Insulin-like Growth Factor*
INCA	Instituto Nacional do Câncer
JAM	do inglês *Junctional Adhesion Molecules*
kDa	kilodaltons
MET	do inglês *Mesenquimal-Epithelial Transition*
mg	Miligrama
mL	Mililitro
mM	Milimolar
MTT	*3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide*
mTOR	do inglês *Mammalian Target of Rapamycin*
PCR	do inglês *Polymerase Chain Reaction*
qRT-PCR	*Real-Time Polymerase Chain Reaction*
RE	Receptor de estrógeno
RNA	Ácido ribonucléico
RPS5	gene *Ribosomal Protein-5*
RPS19	gene *Ribosomal Protein-19*
ROCK-1	do inglês *Rho-associated Protein Kinase*

RP	Receptor de Progesterona
shRNA	do inglês *Small Hairpin RNA*
TGF-β1	do inglês *Transforming Growth Factor Beta*
TGF-β1sh	*Small hairpin RNA Constructs Targeting TGF-β1 in CF41 cells*
TNM	Sistema de estadiamento clínico, T= tumor, N=linfonodo (do inglês node), M= metástase, utilizado pela UICC (*Union for International Cancer Control*) e pela AJCC (*American Joint Committee on Cancer*).

Resumo

RESUMO

A transição epitélio-mesenquimal (EMT) é o processo pelo qual as células cancerosas a partir de tumores primários passam por uma conversão fenotípica para invadir e migrar, gerar metástases em tecidos ou órgãos distantes. Este processo pode ser induzido por fatores de crescimento, tais como Fator de Crescimento Transformante beta (TGF-β) e sua alta expressão tem sido implicada na angiogênese tumoral, na migração e invasão celular em muitos tipos de tumores. A expressão de ROCK-1 está associada com a malignidade dos tumores, enquanto a inibição desta molécula resulta em uma supressão significativa de metástases tumorais. A metformina, um fármaco utilizado no tratamento da diabetes, demonstrou inicialmente inibir a EMT e impedir o fenótipo mesenquimal pela repressão transcricional de pontos chave da regulação da EMT (ZEB1, TWIST1, SNAIL2, TGF-β) em células de câncer de mama. Os objetivos foram avaliar a expressão gênica e proteica de marcadores relacionados a metástase, em um estudo *in vitro* e *in vivo*, em linhagens de câncer de mama, após o tratamento com metformina, além do silenciamento gênico do TGF-β1 para inibição da transição epitélio-mesenquimal. Foi realizado a transfecção da linhagem celular metastática de tumor mamário canino CF41 de forma estável após a construção de um pequeno RNA de interferência para desenvolver derivados clonais que expressam níveis reduzidos de TGF-β1 (células TGF-β1sh). Este foi subsequentemente combinado com o tratamento com metformina, para analisar os efeitos sobre a migração de células, assim como a expressão dos marcadores de EMT E-caderina e N-caderina, quantificados através de imunofluorescência e do qRT-PCR. As linhagens mamárias humanas MCF-7 (não-metastática) e MDA-MB-231 (metastática) foram tratadas com metformina e inibidor Y27632, após a indução da EMT por TGF-β1 para examinar os efeitos sobre a migração destas células, bem como a expressão proteica dos marcadores ROCK-1, vimentina, E-caderina, CD44 e CD24 por imunocitoquímica. Em um estudo *in vivo*, as células não modificadas CF41 ou que expressam TGF-β1 shRNA foram injetadas na região inguinal de camundongos fêmea nude atímicos tratados com metformina. Os camundongos foram eutanasiados após o tratamento e os pulmões foram recolhidos para avaliação do

número de metástases. As regiões metastáticas foram subsequentemente avaliadas pela expressão de N-caderina, E-caderina, vimentina e claudina-7 através da imuno-histoquímica. Foi possível avaliar que a taxa de migração e invasão foi menor em células TGF-β1sh, em comparação com as células parentais CF41 e esta inibição foi significativa quando combinado com o tratamento com metformina. As análises *in vitro* demonstraram que o tratamento com metformina reduziu a expressão de N-caderina e aumentou a expressão de E-caderina nas células CF41 e TGF-β1sh. Os resultados demonstram também que após a indução do TGF-β1 nas linhagens MCF-7 e MDA-MB-231 houve menor expressão das proteínas ROCK-1, vimentina, CD44 e CD24 em ambas as linhagens após tratamento com metformina e Y27632. Nas células MDA-MB-231 a expressão de E-caderina foi maior em todos os grupos de tratamento. O tratamento da linhagems MDA-MB-231 com metformina e Y27632 reduziu significativamente a invasão destas células. O estudo *in vivo* demonstrou que o tratamento com metformina reduziu o número de metástases pulmonares em animais portadores de tumores induzidos com as células TGF-β1sh. Houve diminuição da expressão de marcadores mesenquimais N-caderina e vimentina, e aumento da expressão de marcadores epiteliais E-caderina e claudina-7 nas metástases pulmonares. Assim, concluimos que este estudo confirma os benefícios do silenciamento do TGF-β1, além do tratamento com metformina e Y27632 como potenciais agentes terapêuticos em tumores de mama, bloqueando o processo de EMT e seu potencial metastático.

Palavras-chave: Transição epitélio-mesenquimal, metformina, RNA de interferência, câncer de mama, metástase, TGF-β, agentes anticarcinogênicos

Abstract

ABSTRACT

Epithelial mesenchymal transition (EMT) is the process by which cancer cells from primary tumors pass through a phenotypic conversion to invade and migrate, generating metastases in organs or tissues distant. This process can be induced by growth factors such as transforming growth factor beta (TGF-β) and its overexpression has been implicated in tumor angiogenesis, cell migration and invasion in many cancers. ROCK-1 expression is associated with the malignant character of tumors, while inhibiting this molecule results in a significant suppression of tumor metastasis. Metformin, a drug use for the treatment of diabetes, was previously shown to inhibit EMT by suppressing expression of key transcription factors in breast cancer cells. The aims were to evaluate the gene expression and protein expression of related markers metastasis, in a study *in vitro* and *in vivo* in breast cancer cell lines after treatment with metformin in addition to the gene silencing of TGF-β1 for inhibiting epithelial-mesenquimal transition. These aims were contemplated performing transfected of canine metastatic mammary tumor cell line CF41 with small interfering RNA constructs to develop clonal derivatives expressing reduced levels of TGF-β1 (TGF-β1sh cells). This was subsequently combined with metformin treatment, to look at effects on cell migration, as well as the expression of the EMT markers E-cadherin and N-cadherin, which were quantified by immunofluorescence and qRT-PCR. MCF-7 and MDA-MB-231 cell lines were treated with metformin and Y27632, after induction of EMT by TGF-β1, to examine the effects on cell migration as well as the protein expression of the ROCK-1 markers, vimentin, E-cadherin, CD44 and CD24 by immunocitochemistry. In an *in vivo* study, unmodified or TGF-β1 shRNA-expressing CF41 cells were injected in the inguinal region of nude athymic female mice that were treated with metformin. Mice were sacrificed after treatment and the lungs were collected to assess the number of metastases. Metastatic nodules were subsequently assessed for, N-cadherin, E-cadherin, vimentin and claudin-7 expression via immunohistochemistry. With the obtained results it was possible to assess the migration and invasion rate was lower in TGF-β1sh cells as compared to parental CF41 cells and this inhibition was significant when combined with metformin treatment. *In vitro* analyses demonstrated that metformin treatment reduced n-cadherin

expression and increased E-cadherin expression in both CF41 and TGF-β1sh cells. After TGF-β1 induction in MDA-MB231 and MCF-7 cell lines, there was a lower protein expression of ROCK-1, vimentin, CD44 and CD24 in both cell lines after treatment with metformin and Y27632. In MDA-MB-231 cells, E-cadherin expression was increased in all treatment groups. Treatment of MDA-MB-231 cell line with metformin and Y27632 significantly reduced the invasion of these cells. *In vivo* studies demonstrated that metformin treatment reduced the number of lung metastases in animals bearing TGF-β1sh tumors. This paralleled a decreased expression of mesenchymal markers N-cadherin and vimentin, and increased expression of epithelial markers E-cadherin and claudin-7 in lung metastases. This study confirms the benefits of TGF-β1 silencing in addition to metformin and Y27632 as potential therapeutic agents in mammary tumors, by blocking EMT process and metastatic potential.

Keywords: Epithelial-mesenchymal transition, metformin, interference RNA, breast cancer, metastasis, TGF-β, anticarcinogenic agents

Introdução

I. INTRODUÇÃO

1. Câncer de mama

1.1 Aspectos gerais

O câncer de mama representa a maior causa de morte por câncer em mulheres Dados do IARC (Agência Internacional de Pesquisa no Câncer; Julho, 2015) estimam 14 milhões de novos casos de câncer no mundo para este ano, com previsão de 8.2 milhões de óbito. No Brasil, estima-se a ocorrência de 57.960 novos casos para o ano de 2016 (INCA, 2015).

Na população mundial, a sobrevida média das pacientes após cinco anos de acompanhamento é de 61% e, apesar do progresso no diagnóstico e tratamento nos últimos 30 anos, essa doença é ainda responsável por quase meio milhão de mortes por ano no mundo (Snoussi et al., 2010). Apesar de todas as ações para controle, a taxa de mortalidade por câncer de mama continua elevada, sendo a capacidade de invasão das células tumorais e consequente desenvolvimento de metástases as principais causas de mortalidade em mulheres com neoplasias mamárias (Santos et al., 2011). (**Figura 1**).

Figura 1. Representação espacial das taxas brutas de incidência de câncer de mama no Brasil por 100 mil mulheres, estimadas para o ano de 2016 (INCA, 2016).

Dentre todos os mamíferos, a espécie canina apresenta a maior incidência de neoplasias mamárias, representando aproximadamente 52% do total (Brodey, Goldschmidt e Roszel, 1983; Sleeckx et al., 2013), e, quando comparada à mulher, a fêmea canina possui três vezes mais chances de desenvolver essa neoplasia (Krol et al., 2009; Gelaleti et al., 2012; Michel et al., 2012; Pawłowski et al., 2013). A prevalência de câncer de mama nessa espécie tem aumentado ao longo dos anos, sendo que a incidência de lesões malignas varia de 26 a 73% (MacEwen, 1990; Gelaleti et al., 2012; Zuccari et al., 2012).

Tumores da glândula mamária compartilham características comuns entre cães e seres humanos. Por esta razão, são considerados excelentes modelos para o estudo da biologia do câncer assim como para testes de agentes terapêuticos, já que animais de estimação têm tumores com apresentação histopatológica similares àqueles que acometem os seres humanos e compartilham uma resposta semelhante à cirurgia e à quimioterapia (Andrade et *al.*, 2010; Phillips, Lembcke e Chamberlin, 2010; Rasotto et al., 2014; Rismanchi et al., 2014).

As condutas preventivas, apesar de válidas tem valor questionável na mulher devido à variabilidade dos fatores de risco e às características genéticas relacionadas à sua etiologia (SNOUSSI et al., 2006). Na cadela, alguns aspectos já estão bem estabelecidos no desenvolvimento da neoplasia mamária: cães de raça têm duas vezes mais chances de desenvolverem tumores mamários, a ausência de receptores de estrógeno e progesterona e o uso de contraceptivos, além de dieta imprópria e idade avançada aumentam as chances de desenvolver a neoplasia. Por outro lado, sabe-se que a castração antes do primeiro cio reduz o risco em até 90%; no entanto, o problema maior é a negligência dos proprietários, que só levam o animal ao especialista quando o nódulo já tem grande diâmetro, diminuindo as chances de tratamento e cura (QUEIROGA; LOPES, 2002). Além disso, os tumores de mama em cadelas constituem um desafio para clínicos e, principalmente, para patologistas, uma vez que a nomenclatura e classificação desses tumores têm se revelado muito difíceis e controversas, impossibilitando há muito tempo, estudos comparativos entre os resultados de investigação reportados por diferentes pesquisadores (BRODEY et al., 1983; QUEIROGA; LOPES, 2002).

O estabelecimento do prognóstico e do planejamento terapêutico do câncer de mama baseia-se no tipo, grau histológico do tumor e classificação pelo sistema de estadiamento TNM, que abrange a avaliação do tamanho tumoral, presença de metástase em linfonodos regionais e de metástase à distância (Pedersen et al., 2004). Além dos fatores clínico-patológicos, o conhecimento das características moleculares dos tumores tem contribuído com a avaliação mais precisa do prognóstico e com o desenvolvimento e incorporação de novos agentes e estratégias terapêuticas (Gonzalez-Angulo, Morales-Vasquez e Hortobagyi, 2007; Gralow et al., 2008; Hicks e Kulkarni, 2008; Duffy, O'Donovan e Crown, 2011).

A classificação prognóstica atual considera além dos subtipos histológicos os subtipos moleculares (Perou, 2011), caracterizados nos seguintes fenótipos: Luminal A (receptor de estrogênio (RE) positivo e/ou receptor de progesterona (RP) positivo e receptor do fator de crescimento epidermal 2 negativo ($HER2^-$); Luminal B (RE^+ e/ou RP^+, $HER2^+$); HER2 superexpresso **(**RE^-, RP^-, $HER2^+$); Basal like (RE^-, RP^-, $HER2^-$, citoqueratina $5/6^+$ e/ou $HER1^+$); Triplo-negativo (RE^-, RP^-, $HER2^-$, citoqueratina $5/6^-$ e/ou $HER1^-$) . Na classificação molecular cerca de 80% dos tumores triplo negativos são basal-*like*. Devido à ausência de imunomarcação de RE, RP e HER2, tumores basal-*like* são ainda chamados por alguns autores de “tumores triplo-negativos” (TTN), no entanto, sabe-se que parte dos tumores triplo-negativos não se equivale aos basaloides, podendo ser mais bem descritos como “tumores triplo-negativos não basaloides” (Kennecke et al., 2010; Voduc et al., 2010).

Esses subtipos moleculares apresentam comportamentos distintos relacionados com a sobrevivência, prognóstico e resposta à terapêutica específica. Os subtipos luminais A tem baixa taxa de proliferação e são acompanhados de um bom prognóstico. Os luminais B tem alta taxa de proliferação e um prognóstico mais pobre que o luminal A. Portanto, o status do HER2 e dos receptores hormonais, RE e RP, são fatores prognósticos e preditivos que foram incorporados à rotina clínica e permitem que se estabeleça um tratamento individualizado (Hsiao et al., 2010). A imunoexpressão do RE e RP, por exemplo, está associada ao melhor prognóstico da doença (Duffy, O'Donovan e Crown, 2011). O HER2 tem expressão alterada em aproximadamente 10-15% dos casos. Essa alteração está diretamente associada com o pior prognóstico,

resistência à quimioterapia e à terapia hormonal e aumento da proliferação celular (Jukkola *et al.*, 2001).

Quanto ao tratamento personalizado, o uso de trastuzumabe (Herceptin) em pacientes com câncer de mama que apresentam superexpressão da proteína HER2 é um exemplo de sucesso da terapia-alvo com anticorpos monoclonais. Já a positividade para o RE é um fator preditivo de resposta ao quimioterápico Tamoxifeno (Hsiao et al., 2010). Porém, os tumores triplo-negativos, são caracterizados por um pior prognóstico, uma vez que não respondem a tratamentos específicos, relacionando-os à ocorrência de metástases e menor sobrevida das pacientes. Esses dados mostram a importância da expressão de um ou mais imunomarcadores como uma informação útil, e muitas vezes conclusiva, na prática clínica (Thomas e Berner, 2000), permitindo que estratégias de tratamento sejam definidas de maneira mais eficaz e com menor toxicidade (Gonzalez-Ângulo et al., 2007; Hicks e Kulkarni, 2008; Duffy et al., 2011).

1.2. Metástase

A alta taxa de mortalidade no câncer de mama é devida principalmente à invasão tumoral e ocorrência de metástases. A maioria das mortes por câncer é causada pelas consequências da lesão metastática e não pelo tumor primário, sendo a principal causa de morbidade e mortalidade, responsável por cerca de 90% de óbitos (Seyfried e Huysentruyt, 2013; Guan, 2015)

A metástase é um processo de disseminação de células tumorais do tumor primário para um sítio diferente através dos vasos sanguíneos e linfáticos. É uma sucessão complexa de uma série de eventos biológicos (Hu et al., 2011; Daenen et al., 2012). Estes eventos envolvem o crescimento de novos vasos sanguíneos (angiogênese), a saída de células tumorais do tumor primário (descolamento e migração), invasão através da membrana basal e da matriz extracelular que envolvem o tumor, invasão da membrana basal que apoia o endotélio dos vasos sanguíneos e linfáticos locais, penetração das células tumorais no sangue e/ou de vasos linfáticos, adesão destas células metastáticas circulantes ao endotélio de capilares do órgão-alvo, invasão através da camada de células endoteliais e da membrana basal circundante, e, finalmente, a

fixação e o crescimento de tumores secundários no órgão-alvo (**Figura 2**) (Hu et al., 2011; Daenen et al., 2012; Guan, 2015).

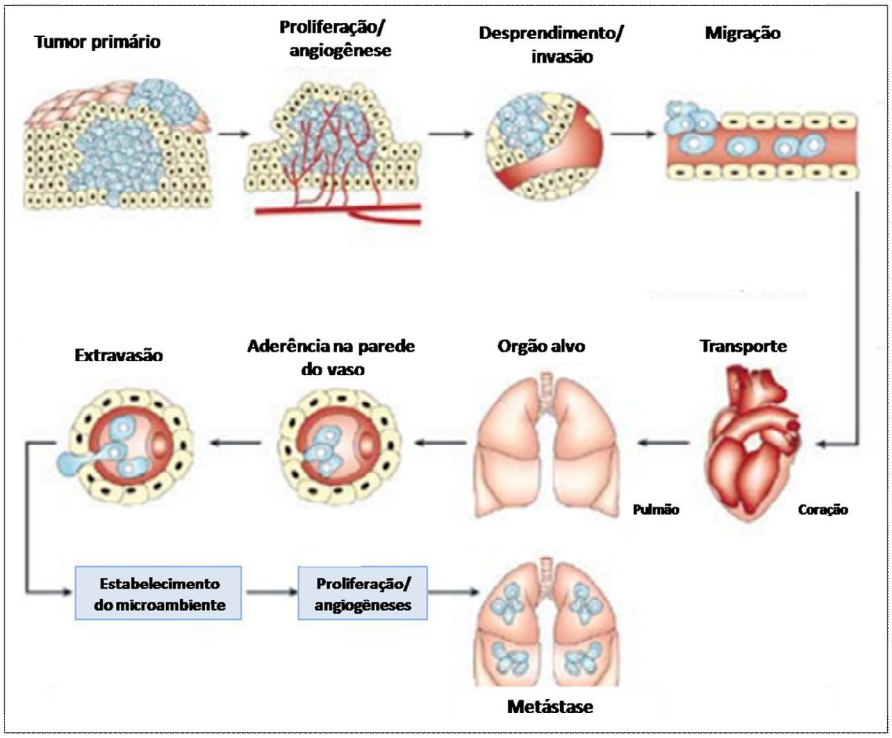

Figura 2. Etapas da metástase. Processo de disseminação de células tumorais do tumor primário para um sítio diferente através dos vasos sanguíneos e fixação e crescimento de tumores secundários no órgão-alvo (adaptado de Fidler**,** 2003).

1.2.1. Transição epitélio-mesenquimal

Ao mesmo tempo que as células tumorais se soltam do tumor primário por diminuição da interação célula-célula, elas devem ter a capacidade de migrar e invadir o estroma adjacente. As células epiteliais malignas passam por um processo denominado de transição epitélio-mesenquimal (EMT, do inglês *epithelial-mesenchymal transition*), caracterizado pela mudança no fenótipo epitelial da célula para o mesenquimal, que leva a perda ou expressão reduzida dos marcadores específicos das células epiteliais e o aumento da expressão de marcadores específicos de células mesenquimais (Foroni et al., 2011; Sigurdsson et al., 2011).

O processo de EMT pode ser delineado em várias etapas, algumas das quais podem ocorrer simultaneamente (**Figura 3**).

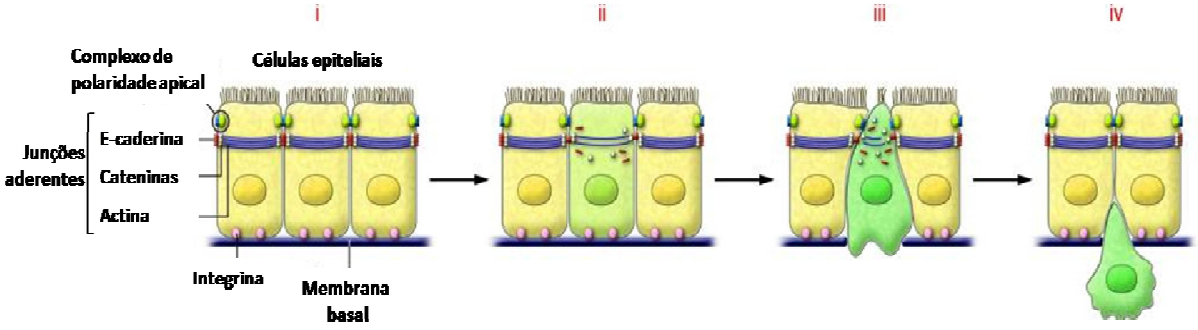

Figura 3. Aspectos celulares da EMT. (i) as células epiteliais normais contêm junções aderentes compostas por E-caderina em conjunto com anéis de catenina e actina. As junções de oclusão estão associadas com os complexos de polaridade apical, ao passo que as integrinas interagem com componentes da membrana basal. (ii) perda de adesão célula-célula. Indutores de EMT reprimem a transcrição dos genes que codificam os componentes de aderência e das junções de oclusão, induzindo a perda da polaridade celular. E-caderina é alvo da degradação. (iii) Decomposição da membrana basal e constrição apical. Profunda remodelação do citoesqueleto favorecerá a delaminação das células através da indução da constrição apical e desorganização da membrana basal. (iv) delaminação celular e invasão. A expressão de receptores de integrina e ativação contínua de metaloproteases favorece a migração através da matriz extracelular e a invasão dos tecidos adjacentes (Adaptado de Acloque, 2009).

Uma vez que as células epiteliais se tornar competentes para responder aos sinais induzidos pela EMT, estes sinais podem promover a ruptura dos complexos de adesão intercelular e a perda das características de polaridade apico-basal das células epiteliais (Barrallo-Gimeno e Nieto 2005, Moreno-Bueno et al., 2008; Acloque et al., 2009). Alterações do citoesqueleto são cruciais para as células deixarem o epitélio e começarem a migrar individualmente. Estas modificações ocorrem inicialmente por formação de constrições apicais e desorganização do citosqueleto basal (Nakaya et al., 2008, Martin et al., 2009). Simultaneamente, a atividade de proteases conduz à ruptura da membrana basal e ingresso celular. Assim, as células adquirem propriedades migratórias e invasivas que lhes permitem migrarem através da matriz extracelular (**Figura 4**) (Haraguchi et al., 2008).

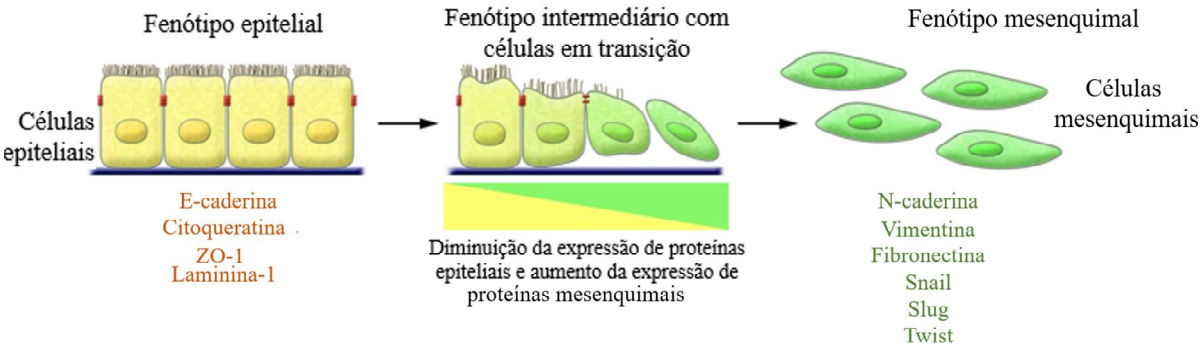

Figura 4. EMT no câncer. A transição epitélio-mesenquimal faz com que a célula converta seu fenótipo epitelial para o fenótipo mesenquimal, diminuindo a expressão de proteínas epiteliais e aumentando a expressão de proteínas mesenquimais, responsáveis pela motilidade da célula (Adaptado de Kalluri e Weinberg, 2009).

O conceito de EMT foi desenvolvido no campo da embriologia, mas foi estendido para o estudo da progressão tumoral e metástase. EMT é classificada em três tipos com base no contexto biológico em que ocorre (**Figura 5**) (Kalluri e Weinberg, 2009). A EMT tipo 1 (descreve os eventos de transição que permitem que as células epiteliais se tornem células mesenquimais móveis durante a implantação, formação do embrião, gastrulação e migração da crista neural. Estas células primárias mesenquimais atuam como progenitoras e geram epitélios secundários em órgãos de origem mesodérmica e endodérmica através da transição mesenquimal-epitelial (MET). A EMT tipo 2 está associada com a cura de feridas, a regeneração dos tecidos e fibrose de órgãos. Neste processo, os fibroblastos do tecido são gerados a partir de células epiteliais ou endoteliais durante a lesão e inflamação crônica. O tipo 3, que ocorre em as células epiteliais neoplásicas, é o processo pelo qual células cancerosas na frente invasiva dos tumores primários, passam por uma conversão fenotípica para invadir e migrar via circulação sanguínea e linfática, gerando metástase em tecidos ou órgãos distantes (Acloque et al., 2009, Wang e Zhou, 2011).

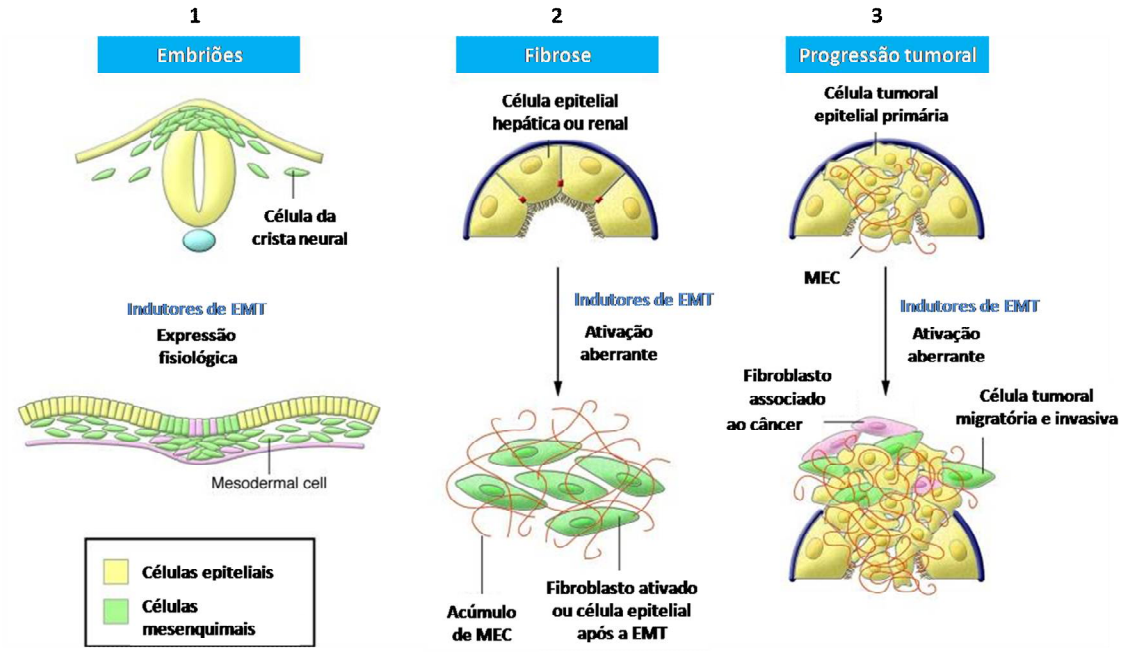

Figura 5. Os três estágios da EMT. EMTs (células em verde) ocorrem durante o desenvolvimento embrionário normal, como durante a delaminação de células da crista neural do tubo neural dorsal e ingresso da mesentoderme da linha primitiva. Enquanto indutores de EMT são geralmente mantidos num estado silencioso no adulto, eles são reativados durante a fibrose do órgão e na frente invasiva de carcinomas humanos durante a progressão tumoral (Adaptado de Kalluri e Weinberg, 2009).

Dessa forma, a EMT pode ser induzida por componentes de matriz extracelular e fatores de crescimento, tais como o fator de transformação de crescimento beta (TGF-β) (Massaguè, 2008; Yang e Weinberg, 2008), Fator de Dispersão/Fator de Crescimento de Hepatócito (SF/HGF), Fator de Crescimento de Fibroblasto (FGF), membros da família do fator de crescimento epitelial (EGFs) e Fator de crescimento Insulina-like 1 e 2 (IGF-1 e -2) (Roussos et al., 2010). TGF-β1 e EGF, que estão envolvidos na EMT, assim como suas vias de transdução intracelulares correspondentes foram descritos por formar redes altamente interligadas, no entanto, os mecanismos envolvidos ainda não foram determinados (Li et al., 2015).

1.2.2. TGF-β1

O TGF-β está envolvido em diversos processos fisiológicos sendo responsável por regular a diferenciação e proliferação celular, migração e apoptose. Atualmente, acredita-se que o TGF-β exerça duplo papel na progressão e metástase do câncer, sendo considerado um importante supressor de proliferação em células epiteliais cancerosas nos primeiros estágios da carcinogênese, mas induzindo metástases em estágios avançados, através da permissão da motilidade e invasão celular, papel este ainda não totalmente esclarecido (Bierie et al., 2006; Ikushima et al., 2010).

Esta citocina atua como um potente inibidor de proliferação. Tem se mostrado inibidor da progressão do ciclo celular epitelial e promotor da apoptose que em conjunto contribuem de forma significativa para a função supressora de carcinomas durante a iniciação e progressão (Bierie e Moses, 2010; Parvani et al., 2013). No entanto, a capacidade de TGF-β1 para induzir e promover a EMT está associada com o aumento da motilidade celular e invasão tumoral (Massagué, 2012, Zarzynska et al., 2014). Assim, o TGF-β1 também é considerado um indutor de metástases, que participa na progressão maligna e angiogênese (Gomes et al., 2012, Parvani et al., 2013). Estes comportamentos contrastantes do TGF-β1 no desenvolvimento e progressão do câncer são conhecidos como "paradoxo do TGF-β1" (Taylor et al., 2010, Parvani et al., 2011, Zarzynska et al., 2014).

TGF-β é um membro da superfamília de TGF-β e há três isoformas descritas, o TGF-β1, TGF-β2 e TGF-β3. A sinalização destas isoformas é comparável, mas os níveis de expressão diferem entre os tecidos (Wu e Hill, 2009). O TGF-β1 em particular, desempenha um papel importante durante a progressão do câncer de mama, ocasionando metástase em vários ensaios experimentais com ratos e sendo considerado o principal componente encontrado no microambiente do tumor em metástase pulmonar (Siegel et al., 2003; Bandyopadhyay et al., 2006) e óssea provocadas por células de câncer de mama (Bandyopadhyay et al., 2006; Padua et al., 2008). Nos estudos realizados por Moore et al. (2008) em metástases tumorais, observou-se que houve diminuição da expressão do receptor do TGF-β1 (TGF-βR) e também limitou a sinalização das outras isoformas da família do TGF-β.

Os níveis de TGF-β estão associados positivamente com resistência tumoral à radioterapia ou quimioterapia. Zhao et al. (2010) observaram que o aumento dos níveis de TGF-β durante a terapia de radiação se relacionam fortemente com um mau prognóstico em pacientes com câncer de pulmão.

TGF-β desempenha um papel importante no desenvolvimento e progressão do câncer. A expressão de TGF-β pode predizer o prognóstico de pacientes com tumores malignos. Estudos têm investigado o papel prognóstico da expressão proteica e gênica do TGF-β no câncer. Estes estudos indicaram que uma elevada expressão de TGF-β pode prever mau prognóstico, TNM em fase avançada, a pequena sobrevida global, e metástase localmente avançada ou distante (Lin e Zhao, 2015, Zhu et al., 2016)

Na maioria dos cânceres de mama primários, mRNAs específicos de TGF-β1, -2, e -3 são detectados (Tan et al., 2009, Zarzynska et al., 2014). Os níveis plasmáticos de TGF-β também têm sido relatados como sendo elevados em pacientes com câncer de mama. Estes níveis se correlacionam com o estágio da doença e diminuição após a resseção do tumor primário (Tan et *al*., 2009, Zarzynska et al., 2014). Os membros da família TGF-β são preditores de resposta ruim à quimioterapia em mulheres com câncer de mama (Panis et al., 2013).

TGF-β1 mostrou desempenhar um papel regulador importante na EMT (Shi et al., 2013, Katz et al., 2013). Identificação do TGF-β como um indutor principal de EMT veio de estudos *in vitro* em culturas de células. O tratamento de células epiteliais normais da mama de ratas com TGF-β muda a forma cubóide para um eixo alongado, acompanhado por uma diminuição nos marcadores epiteliais e aumento da expressão de marcadores mesenquimais (Meulmeester e Dijke, 2011). Ela induz o aumento do tamanho da célula e o teor de proteínas durante EMT (como resultado da ativação de mTOR) (Mandel et al., 2013). Regula negativamente a sinalização das claudinas, ocludinas, e do ZO1, seguida por degradação das junções de oclusão (Lamouille et al., 2012).

2. Metformina e câncer

Muitas terapias têm sido estudadas no câncer, com destaque para a metformina (**Figura 6**), um derivado de biguanidas (N', N'dimetilbiguanida), medicamento utilizado por via oral para reduzir a concentração de glicose no sangue de pacientes com diabetes tipo 2 e síndrome metabólica (Ben Sahra, et al., 2010; Decensi et al., 2010)

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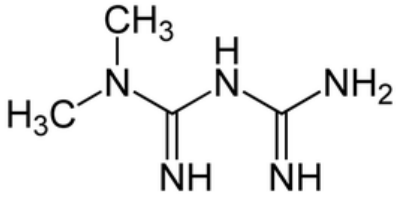

Figura 6. Fórmula estrutural da metformina (N', N'dimetilbiguanida).

Curiosamente, Hirsch et al. (2009) demonstraram que as células tronco tumorais (CSCs) parecem ter uma maior sensibilidade à metformina. Ainda segundo Cufí et al. (2010) o tratamento com metformina pode impedir o fenótipo mesenquimal pela repressão transcricional de pontos chave da regulação da EMT (ex. ZEB1, TWIST1, SNAIL2, TGF-β).

A metformina age por diversos mecanismos extra-hepáticos, aumentando a sensibilidade do organismo a ação da insulina endógena e controlando a resistência à insulina por mecanismos dependentes e independentes de insulina. A metformina ainda não tem seu mecanismo de ação totalmente elucidado, mas sabe-se que sua principal ação em pacientes com diabetes é inibir a produção de glicose hepática, reduzindo a gliconeogênse hepática, em parte atravéz da potencialização da ação da insulina e mediante a ativação da proteína quinase ativada por AMP (AMPK) (Seufert et al., 2004, Shaw et al., 2005, Sen et al., 2014).

AMPK é um sensor de energia celular localizado dentro do citoplasma, que está envolvido na regulação do metabolismo no interior das células e consiste de uma subunidade catalítica (α) e duas subunidades reguladoras (β e γ) sendo expresso em vários tecidos, incluindo fígado e no músculo esquelético (Fogarty e Hardie, 2010,

Pulito et al., 2013, Li et al., 2015). Ele é ativado quando os níveis de ATP (adenosina trifosfato) são diminuidos e os níveis de AMP (adenosina monofosfato) são elevados (Hardie, 2003).

Em mamíferos, a AMP ativa a AMPK por estimular a fosforilação do resíduo de treonina 172, localizado na subunidade α da AMPK, por ação de quinase regulatória, a AMPK quinase (AMPKK). Dentre as AMPKK há a LKB1, uma proteína supressora tumoral, e a proteína quinase quinase dependente de Ca^{+2}/calmodulina (CaMKK). Quando a AMPK está ativada, ela inativa as enzimas 3-hidroxi-3metilglutaril CoA redutase (HMG-CoA redutase), acetil CoA carboxilase (ACC) e mTOR (do inglês, *mammalian target of rapamycin*), exercendo efeitos sobre o metabolismo da glicose e dos lipídios, sobre a expressão gênica e síntese proteica (Hadad et al., 2008; Santomauro Jún et al., 2008).

A AMPK também pode ser ativada por drogas antidiabéticas orais, como a metformina (Hadad et al., 2008). Assim, a metformina parece ativar a AMPK pela LKB1 e esta ativação envolve a regulação de vias relevantes para o controle da proliferação celular. Estudos experimentais mostram que a metformina, ativando a AMPK, reduz a ativação da mTOR e consequentemente, diminui a proliferação de células epiteliais (Algire et al., 2008; Berstein et al., 2010, Sen et al., 2014).

A molécula mTOR é uma proteína serina/treonina quinase, pertencente à família das quinases relacionadas com a PI3K e está integrada a dois complexos multiproteicos: mTORC1 e mTORC2, sendo regulada por fatores de crescimento. Estes fatores aumentam a função da mTORC1, aumentando a fosforilação da proteína ribossomal S6 quinase (S6K) e do *eukaryote initiation factor 4E binding protein 1* (4EBP1), reguladores chave da tradução proteica. A mTOR está super ativada em muitas células de câncer, como resultado de alterações genéticas ou ativação aberratnte dos componentes da via PI3K/Akt. Isso contribui para a perda de controle da proliferação, crescimento, diferenciação e sobrevivência das células. Portanto, a metformina por inibição da mTOR, via AMPK, pode exercer um efeito anti-neoplásico importante nas células epiteliais **(Figura 7)** (Rattan et al., 2012; Zhang et al., 2013).

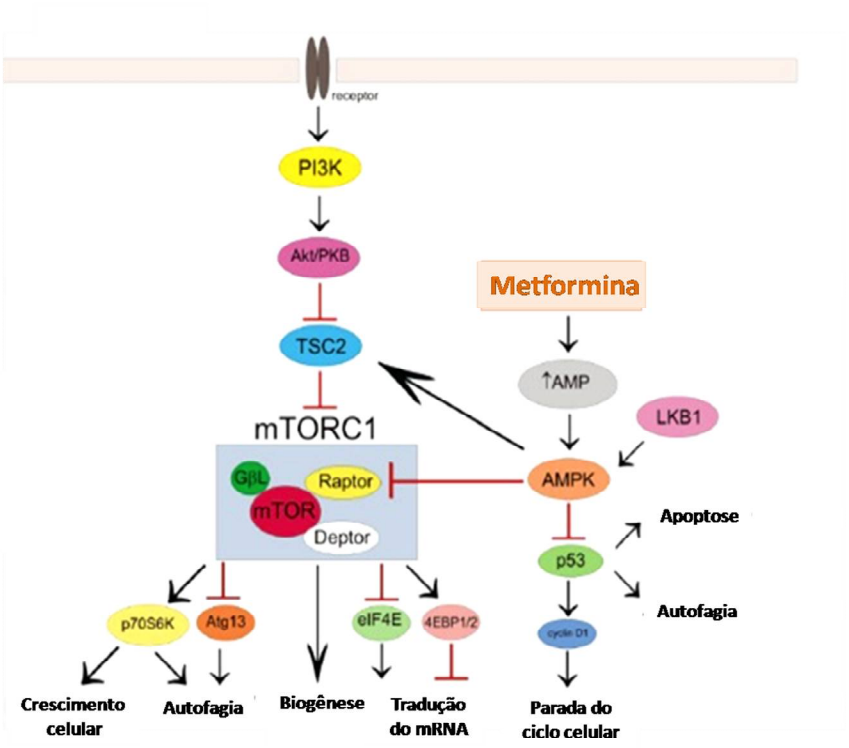

Figura 7. Ação molecular proposta de metformina em células cancerosas (adaptado de Kasznicki et al., 2014).

3. Adesão celular

A adesão celular refere-se à fixação entre as células (adesão célula-célula) e com o ambiente celular, principalmente com a matriz extracelular (MEC) (adesão célula-matriz). A adesão celular ajuda a estabelecer fortes conexões tanto entre as células como entre as células e a matriz. A adesão também está envolvida no estabelecimento de células metastáticas no sítio distante, quando ela está diminuida. Além disso, a adesão celular não é apenas uma maneira de vincular célula-a-célula ou célula à membrana basal, mas também se apresenta como um mecanismo para ativar a proliferação e a sobrevivência celular através das interações das integrinas com moléculas que são essenciais para a motilidade e sobrevivência celular (Alizadeh et al., 2014).

A adesão é obtida principalmente através da ligação intracelular entre o citoesqueleto das células (adesão célula-célula) ou ligando o citoesqueleto celular com componentes da matriz extracelular tais como colagéno, fibronectina e laminina (adesão célula-MEC) através de um grupo de moléculas de adesão celular (CAMs). As CAMs são glicoproteínas de superfície que são receptores transmembranares tipicamente composta por três domínios: o domínio intracelular, domio transmembranar e o domínio extracelular. CAMs incluem principalmente CAMs dependentes de cálcio, que compreendem as caderinas, integrinas e selectinas (Li et al., 2011, Guan et al., 2015).

3.1. Caderinas

As caderinas são moléculas de adesão dependentes de cálcio que permitem a ligação entre células vizinhas. Todas as células que são ligadas possuem o mesmo tipo de caderina, assim sendo, as interações entre elas são homofílicas (Alizadeh et al., 2014). Mais de 20 membros da família das caderinas foram relatados, sendo que a E-caderina está presente em células epiteliais e a N-caderina, em células mesenquimais (Li et *al*., 2011, Alizadeh et al., 2014).

A expressão baixa ou ausente de E-caderina é fundamental para a ocorrência da EMT e este fenômeno já foi descrito no câncer metastático. A baixa expressão de E-caderina leva a perda da ligação célula-célula, que permite que as células tumorais se disseminem e, eventualmente, ocorra a metástase, sendo também correlacionada com a perda da morfologia epitelial e a aquisição do potencial metastático das células do carcinoma de próstata, mama e fígado (Guan et al., 2015). Interessantemente, a E-caderina não foi encontrada transitoriamente em células migratórias, mas se re-expressa com o início da diferenciação celular nos tecidos epiteliais dos órgãos colonizados (Alizadeh et al., 2014).

Em contraste com a E-caderina, a N-caderina não é expressa em células epiteliais mamárias normais, mas está expressa em células do estroma, como por exemplo os fibroblastos, e verificou-se ser altamente expressa no câncer de próstata, mama e fígado. N-caderina, encontrada primeiramente em tecido neural e fibroblastos (Hatta et al., 1987), é uma das caderinas presentes nas células mesenquimais e está envolvida na adesão das células do estroma. A baixa expressão de E-caderina com

concomitante aumento da expressão de N-caderina reduz a capacidade de adesão das células epiteliais, aumenta a adesão de células estromais, e leva a subsequente invasão de células tumorais no estroma. A N-caderina promove a migração de células epiteliais e a metástase, independentemente da expressão e função da E-caderina. Esta importante função da N-caderina na adesão e migração de células tumorais faz com que esta proteína seja um alvo para a terapia do câncer (Li et et al., 2011, Guan et et al., 2015).

3.2. Claudinas

Os membros da família das claudinas representam a maior parte das proteínas de membrana, localizadas exclusivamente nas junções celulares e estas moléculas regulam as propriedades fisiológicas de dessas junções entre os tecidos (Lu et al., 2013, Rossetti et al., 2015). Existem atualmente pelo menos 24 claudinas diferentes já descritas nos seres humanos, e a expressão de cada uma parece ser específica do tecido e sua expressão é frequentemente alterada em diversos tipos de câncer (Hewitt et al., 2006, Lu et al., 2013).

As claudinas são componentes essenciais das junções apertadas na região apical de células epiteliais. As junções de oclusão, que incluem a JAM (do inglês, *Junctional Adhesion Molecule*) e as ocludinas, regulam a permeabilidade paracelular e polaridade de células epiteliais (Schulzke et al., 2012, Heiler et al., 2015). Como a disseminação de células tumorais requer perda de adesão célula-célula, espera-se que as claudinas estejam pouco expressas no câncer. As claudinas, mais frequentemente a claudina 7, também são expressas na superfície lateral ou basal das células epiteliais (Ding et al., 2013). No entanto, as atividades funcionais das claudinas nas junções de oclusão são pouco definidas (Kwon, 2013, Heiler et al., 2015).

Em concordância com o papel da EMT no câncer, a claudina-7 está associada com mau prognóstico de pacientes com carcinomas ductal e ductal invasivo da mama e nos subtipos moleculares ER negativos e basal-like, sugerindo que a perda de claudina-7 pode favorecer a disseminação de células tumorais e aumentar seu potencial metastático (Bernardi et al., 2012; Lu et al., 2013, Zhu et al., 2015).

Estes relatos de diminuição da expressão das claudinas no câncer é devido a ruptura das junções de oclusão na tumorigênese, um processo fundamental na perda de

coesão, capacidade de invasão, além da falta de diferenciação observada nas células do câncer (Zhu et al., 2015).

Por causa da elevada especificidade do padrão de expressão no câncer, tem sido proposto que as claudinas podem representar úteis marcadores de diagnóstico e prognóstico em neoplasias gastrointestinais, gênito-urinárias e pulmonares (Jung et al., 2009). Estas observações indicam que as claudinas podem contribuir para a resistência aos medicamentos e recorrência do tumor através do mecanismo da EMT (Kwon, 2013).

4. Vimentina

O citoesqueleto é uma estrutura celular, semelhante a uma rede, composta por um conjunto de três tipos diferentes de filamentos proteicos. São eles: filamentos de actina ou microfilamentos, filamentos intermediários e microtúbulos (Chung et al., 2013) (**Figura 8**).

Os filamentos de actina são constituídos por monómeros de actina. Para além da actina existem outras proteínas que interagem com estes filamentos e que contribuem para a maioria das suas funções celulares, como é o caso dos movimentos de superfície, da manutenção da forma, fagocitose, comunicação intracelular e distribuição de organelos. As proteínas que interagem com os filamentos de actina são proteínas que fazem a ligação de uns filamentos de actina aos outros e que permitem a formação de uma "rede" estrutural dentro da célula, o córtex celular (Standring, 2010).

Filamentos intermediários possuem uma cabeça aminoterminal, uma cauda carboxiterminal e um domínio central em forma de bastão. São estruturas estáveis formadas por proteínas específicas que variam de acordo com o tipo celular. A vimentina é uma proteína estrutural do citoesqueleto celular, constituído pelos filamentos intermediários das células mesenquimais (Liu et al., 2015) e está presente nos fibroblastos, células endoteliais, macrófagos, leucócitos e linfócitos, dentre outros. Nos adultos, a expressão de vimentina é vista nos tecidos conjuntivos, constituídos por células mesenquimais, e também nos músculos e sistema nervoso central (Kokkinos et al., 2007).

A alternância na expressão de proteínas de filamentos intermediários está associada à malignidade. Por exemplo, a baixa expressão de citoqueratina faz com que o marcador mesenquimal vimentina substitua a citoqueratina em células malignas de câncer de mama (Chung et al., 2013).

A vimentina é altamente expressa no carcinoma ductal da mama de alto grau ou em tumores com níveis baixos de ER, mas não nos carcinomas lobulares. Embora a expressão de vimentina seja encontrada em fases avançadas do câncer e se correlacionar com a malignidade, o papel da vimentina no controle da malignidade não está claro (Chung et al., 2013).

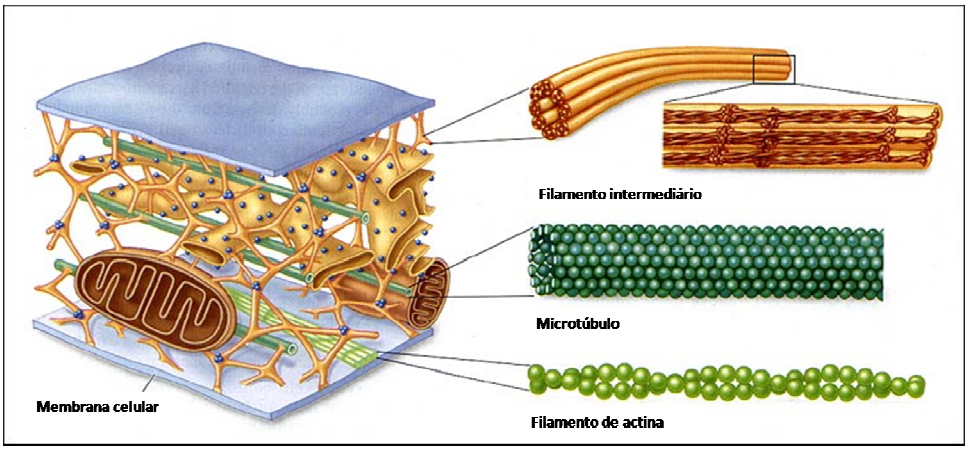

Figura 8. Componentes do citoesqueleto. Esquema representativo de uma célula mostrando os componentes do citoesqueleto: Filamento intermediário, microtúbulo e filamento de actina. Johnson, George B. *The Living World*. © 2006 The McGraw-Hill Companies, Inc.

5. ROCK-1

A proteína quinase associada à Rho (ROCK) tem um papel importante no processo de invasão e migração celular através da regulação dos rearranjos de actina do citoesqueleto da célula (Ortiz-Lopez et al., 2009). Esta quinase associada à Rho possui duas isoformas, ROCK-1 e ROCK-2, e sua ativação aumenta a ancoragem celular e está implicada na regulação da metástase no câncer de mama (Liao et al., 2007). Efetores da sinalização por Rho/ROCK também foram envolvidos com estágios tardios da

progressão tumoral e metástases e podem explicar sua associação com mau prognóstico em vários cânceres (Joshi et al., 2008).

Liu et al. (2009) verificaram alta expressão gênica de ROCK, principalmente ROCK-1, em tumores metastáticos de linhagens celulares e modelos animais de câncer mama, e demonstrou que um inibidor específico de ROCK-1, o Y27632, foi capaz de afetar a motilidade celular *in vitro* e a metástase *in vivo* (Liu et al., 2009). Em um estudo recente, o Y27632 mostrou ser eficaz no controle do câncer de mama metastático *in vitro* e *in vivo*, não só através da inibição da proliferação de células tumorais, mas também através do antagonismo direto do mecanismo metastático das células pela inibição do ROCK-1 (Borin et al., 2015).

6. CD44/CD24

A manutenção da heterogeneidade das células dentro do tumor não está completamente esclarecida e uma das hipóteses para explicar esta dinâmica e extrema heterogeneidade do câncer é a presença de células tronco tumorais (CSCs), também conhecidas como células iniciadoras de tumor. A hipótese de CSCs sugere que uma pequena população de células tumorais, tem a capacidade de auto regeneração e de diferenciação, podendo iniciar e manter o desenvolvimento do câncer e até mesmo a resistência ao tratamento quimioterápico e radioterápico (Spradling et al., 2001).

Evidências ligando a EMT a células-tronco do câncer de mama (BCSCs) foram apresentadas pela primeira vez por Mani et al. em 2008, que mostrou que a indução desse processo em células epiteliais mamárias transformadas in *vitro*, geram células com propriedades BCSCs.

A molécula de adesão, CD44, é uma glicoproteína transmembrana da superfície celular envolvida na ativação e recirculação de linfócitos, adesão da matriz extracelular, angiogênese, proliferação, diferenciação e migração celular (Phuc et al., 2011). CD24 é uma molécula de adesão *mucina-like* identificada como um ligante alternativo da P-selectina, presente em receptores de plaquetas ativados e de células endoteliais cuja expressão pode aumentar a capacidade de metastatizar de células tumorais (Simonetti et *al.*, 2012).

Em tumores de mama humanos, uma pequena subpopulação de células tumorais com o fenótipo CD44+/CD24- está presente e foram associadas com células-tronco tumorais (CSCs) (Honeth et al., 2008). CSCs de câncer da mama superexpressam a molécula de adesão CD44 em conjunto com baixa ou nenhuma expressão da molécula de adesão CD24 (Wang et al., 2011).

As células que foram fortemente positivas para CD44 e negativas para CD24 ($CD44^{+}/CD24^{-/low}$) tiveram maior capacidade tumorigênica e metastática em câncer de mama (Phu et al., 2011). Sugere-se que as células tumorais com uma subpopulação $CD44^{+}/CD24^{-}$, podem ter um pior comportamento clínico (Honeth et al., 2008). Em contraste, outros estudos não revelaram uma associação significativa de $CD44^{+}/CD24^{-}$ com um potencial de progresso ou de reincidência (Bernardi et al., 2012).

Objetivos

II. OBJETIVOS

O câncer é um processo complexo que envolve modificações na expressão de proteínas relacionadas a adesão celular, promovendo a metástase e o estabelecimento de um pior prognóstico. A identificação de novos agentes terapêuticos é fundamental para o controle da tumorigênese e direcionamento de estratégias terapêuticas no câncer de mama. Assim, o objetivo geral desse estudo foi, a partir da ação do TGF-β1, avaliar a eficácia da metformina e do inibidor de ROCK1, o Y27632 na transição epitélio-mesenquimal no câncer de mama.

- Verificar a viabilidade celular, expressão gênica e proteica de N-caderina, E-caderina, vimentina e claudina-7, em um estudo *in vitro* e *in vivo*, utilizando a linhagem mamária metastática canina CF41 após o tratamento com metformina e silenciamento gênico do TGF-β1 para inibição da transição epitélio-mesenquimal.

- Inibir a transição epitélio-mesenquimal utilizando metformina e Y27632, após sua indução com TGF-β1 nas linhagens MDA-MB-231 (metastática) e MCF-7 (não metastática). Verificar a ação dos tratamentos estabelecidos na viabilidade celular e na expressão proteica de ROCK1, E-caderina, vimentina, CD44 e CD24 em cultivo celular.

Capítulos

III. CAPÍTULOS

Os resultados referentes aos objetivos desta Tese serão apresentados a seguir na forma de dois artigos científicos, conforme as normas de publicações específicas de cada periódico.

Artigo I

Título: Inhibition of epithelial-mesenchymal transition and metastasis by combined TGFbeta knockdown and metformin treatment in a canine mammary cancer xenograft model

Autores: Camila Leonel da Silva, Gabriela Bottaro Gelaleti, Thaiz Ferraz Borin, Larissa Bazela Maschio, Marina Gobbe Moschetta, Bruna Victorasso Jardim-Perassi, Alicia M. Viloria-Petit, Debora Aparecida Pires de Campos Zuccari

Periódico: Journal of Mammary Gland Biology and Neoplasia, submetido.

Artigo II

Título: Inhibition of epithelial-mesenchymal transition in response to treatment with metformin and Y27632 in breast cancer cell lines

Autores: Camila Leonel da Silva, Lívia de Carvalho Ferreira, Thaiz Ferraz Borin, Marina Gobbe Moschetta, Gabriela Scavacini de Freitas, Michel Raineri Haddad, João Antônio de Camargos Pinto Robles, Debora Aparecida Pires de Campos Zuccari

Periódico: The Journal of Histochemistry and Citochemistry, a ser submetido.

Artigo I

Inhibition of epithelial-mesenchymal transition and metastasis by combined TGFbeta knockdown and metformin treatment in a canine mammary cancer xenograft model

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Abstract

Introduction

Epithelial mesenchymal transition (EMT) is the process by which cancer cells from primary tumors pass through a phenotypic conversion to invade and migrate, generating metastases in distant organs or tissues. This process can be induced by growth factors such as transforming growth factor beta (TGF-β) and its overexpression has been implicated in tumor angiogenesis, cell migration and invasion in many cancers. Metformin, a drug use for the treatment of diabetes, was previously shown to inhibit EMT by suppressing expression of key transcription factors in breast cancer cells. The aim of this study is to inhibit EMT in response to TGF-β1 silencing by RNA interference and metformin in metastatic canine mammary tumor cell line CF41.

Methods

The canine metastatic mammary tumor cell line CF41 was stably transfected with small interfering RNA constructs to develop clonal derivatives expressing reduced levels of TGF-β1 (TGF-β1sh cells). This was subsequently combined with metformin treatment, to look at effects on cell migration (scratch assay), as well as the expression of the EMT markers E-cadherin and N-cadherin, which were quantified by immunofluorescence and qRT-PCR. In an *in vivo* study, unmodified or TGF-β1 shRNA-expressing CF41 cells were injected in the inguinal region of nude athymic female mice that were treated with metformin. Mice were sacrificed after treatment and the lungs were collected to assess the number of metastases. Metastatic nodules were subsequently assessed for, N-cadherin, E-cadherin, vimentin and claudin-7 expression via immunohistochemistry.

Results

The migration and invasion rate was lower in TGF-β1sh cells as compared to parental CF41 cells and this inhibition was significant when combined with metformin treatment. *In vitro* analyses demonstrated that metformin treatment reduced n-cadherin expression and increased E-cadherin expression in both CF41 and TGF-β1sh cells. *In vivo* studies demonstrated that metformin treatment reduced the number of lung metastases in animals bearing TGF-β1sh tumors. This paralleled a decreased expression of mesenchymal markers N-cadherin and vimentin, and increased expression of epithelial markers E-cadherin and claudin-7 in lung metastases.

Conclusions

This study confirms the benefits of TGF-β1 silencing in addition to metformin as potential therapeutic agents for breast cancer patients, by blocking EMT process and metastatic potential. To the best of our knowledge, we are the first to report treatment of metformin in cells with TGF-β1 silencing and their effect on EMT.

Keywords: breast cancer, metastasis, anticarcinogenic agents, shRNA, TGF-β, EMT, metformin

INTRODUCTION

The high mortality rate in breast cancer is mainly due to metastatic disease. The mechanisms for the occurrence of metastases are variable and require several stages starting with the loss of contact with neighboring cells, following the penetration of the vessel walls, joining the new location and angiogenesis [1].

The migration and invasiveness of epithelial tumor cells has been linked to the re-activation of a developmental process called epithelial-mesenchymal transition (EMT) [2], characterized by the down-regulation of epithelial markers such as claudin, E-cadherin and keratins, loss of apical-basal cell polarity and cell–cell adhesion, and the up-regulation of mesenchymal markers such as N-cadherin and vimentin, as well as the production matrix-degrading enzymes. Altogether, this facilitates migration and invasiveness capacity during the process of malignant transformation, and tumor cell dissemination to surrounding tissues and distant sites [2, 3, 4]. EMT can be induced by extracellular matrix components and growth factors, such as transforming growth factor beta (TGF-β) and epidermal growth factor (EGF) [5, 6], which might cooperate with intrinsic transforming events (e.g., oncogenes) to promote mesenchymal phenotypes [7].

TGF-β is involved in many physiological processes responsible for regulating cellular differentiation and proliferation, migration and apoptosis. In breast and other epithelial cancers, TGF-β is well-documented to have a stage-dependent dual role, by acting as a suppressor of proliferation and/or inducer of cell death in early stages of carcinogenesis, but promoting survival, proliferation and invasiveness/metastasis in advanced stages [8, 9]. TGF-β is a member of the TGF-β superfamily comprised of 33 ligands, including bone morphogenic proteins (BMP) and activins. TGF- β itself exists in three isoforms, TGF-β1, TGF-β2 and TGF-β3. The signaling of these isoforms is comparable in some settings, and the expression levels varies among different tissues [10]. TGF-β1 in particular, is the best documented to play a role in the progression of breast cancer, promoting metastasis in many experimental rodent models. In some of these models, TGF- is supplied by the a tumor cell-dependent tumor microenvironment in the lung [11, 12] and bone [11, 13] .

Stem cells have been investigated for their central role in the development of tissues and organs. Research within the last two decades suggest that cells with stem/progenitor characteristics also play a critical role in tumor initiation and progression [14, 15]. Evidence linking EMT to breast cancer stem cells (BCSCs) was first reported by Mani et al. [16], who showed that the induction of this process in mammary epithelial cells transformed *in vitro*, generate cells with BCSCs properties.

The definition of the cellular and microenvironmental signals that induce the EMT during the progression of breast cancer is crucial to provide new therapeutic targets in an attempt to avoid metastasis and/or properly manage metastatic disease ro reduce cancer mortality. Currently, intervention tools have been employed to block cellular signaling pathways that induce EMT and subsequent metastasis. These include oncolytic adenoviruses [17], RNA interference [18, 19], and molecular-targeted drugs, such as pharmacological signalling inhibitors [4].

Several other anti-tumor and possibly EMT-targeting approaches have been more recently identified, based on the use of drugs initially intended to treat other conditions. One example of these is metformin, which is used orally to reduce the glucose concentration in the blood of patients with type 2 diabetes and metabolic syndrome, with well-established and manageable side effects and safety profiles. Following epidemiological findings linking the use of metforming to reduced incidence of certain cancers, particularly of the breast [20], this drug has been widely studied for its anti-tumor activities [21, 22]. Among the mechanims of metformin anti-tumor activity, the activation of AMPK (AMP-activated protein kinase) signaling has been shown to inhibit the mTOR pathway, and several studies showed metformin’s capacity to inhibit epithelial-mesenchymal transition (EMT) in human breast

cancer cells [23, 24, 25, 26]. In support of previous finding linking stemness to EMT [16], Hirsch et al. [27] recently demonstrated that tumor stem cells have a greater sensitivity to metformin, and according to a study by Cufí et al. [28], treatment with metformin suppresses the mesenchymal phenotype by transcriptional repression of key EMT-promoting genes, including (Zinc Finger E-Box Binding Homeobox 1 (*ZEB1*), *TWIST1*, *SNAIL2/SLUG*, and *TGF-β*).

Based on the aforementioned findings, this study addressed the possibility that blocking TGF-β signalling input in a mesenchymal metastatic breast cancer cell line, will enhance the metastasis-targeting effects of metformin. We show that TGF-β1 knockdown by RNA interference in mesenchymal CF41 canine mammary cells potentiates the EMT- and invasiveness-targeting effects of metformin in CF41 in vitro. A pilot animal study suggests enhanced EMT- and metastasis-suppressing effects by this combined approach but suggest complex interactions between the two strategies that warrant further invesitigation.

MATERIAL AND METHODS

This study was approved by the ethics committee on animal use (CEUA) of the Faculty of Medicine of Sao Jose do Rio Preto (FAMERP) # 3244/2012, which is developed following national and international standards of ethics in animal experimentation.

Antibodies

Antibodies used in this study were as follows: vimentin (#M0725) from DAKO (Carpinteria, CA, USA); N-cadherin (#sc7939) from Santa Cruz Biotechnology (Dallas, Texas, USA), E-cadherin (#3195S) from Cell Signaling Technology (Danvers, Massachusetts, USA),

claudin-7 (ab27487) from Abcam (Cambridge, UK) and Alexa-Fluor®-conjugated secondary antibodies from Life Technologies (Eugene, OR, USA).

Cell culture

The metastatic canine mammary tumor cell line CF41 (ATCC, Manassas, VA, USA) was cultured in 75 cm^2 culture flasks (Sarstedt, Nümbrecht, Germany) with Dulbecco's modified Eagle's medium – high glucose (DMEM) (Cultilab, Campinas, SP, Brazil) supplemented with 10% fetal bovine serum (FBS) (Cultilab, Campinas, SP, Brazil), penicillin (100 IU/mL) and streptomycin (10 mg/mL) (Sigma-Aldrich, St. Louis, MO, USA) in a humidified incubator at 5% CO_2 and 37 ºC.

Cell viability by MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay.

For MTT assay, 5 x 10^4 cells/well were plated in 96-well plates in 100 µL DMEM with 2% FBS. Then cells were treated for 24 hours with different concentrations (1 mM, 2 mM, 3 mM, 5 mM, 6 mM, 8 mM and 10 mM) of metformin (Sigma-Aldrich, St. Louis, MO, USA) diluted in ultrapure water. Thereafter, 10 µL of MTT solution (Vibrant MTT Cell Proliferation Assay Kit, Invitrogen - Life Technologies, Eugene, OR, USA) were added to each well and the plates were incubated at 37 ºC for an additional 4 hours. Absorbance was measured at 540 nm by ELISA plate reader (Thermo Fisher Scientific - Waltham, MA USA). Medium with 2% FBS was used as background and subtracted from the samples. Cell viability (%) was calculated for all groups compared to control sample (untreated), and all treatment were performed in triplicate.

Regression analysis was performed on the results of viability resulting in an equation

used to calculate the required substance concentration to produce 50% reduction in cell viability (IC50).

Construction of TGF-β1 shRNA and CF41 cells silencing

Four small interfering RNA (shRNA) sequences targeting TGF-β1 gene (Exxtend, Campinas, SP, Brazil) were designed, and inserted into the lentivirus vector pLKO 1.puro (Weinberg Laboratory of Cancer Biology – Massachusets Institute of Technology, Cambridge, MA, USA). Recombinant lentivirus and control were extracted after transfection of 293 T cells (ATCC, Manassas, VA, USA) with the recombinant vector and helper vectors. CF41 cells were infected with lentivirus and selected with 2 μg/mL of puromycin (Gibco, Grand Island, NY, USA). The best interfering effect was determined by real-time PCR, and cells with the most prominent TGF- β1 knockdown (TGF-β1sh cells) were selected to implant into the animals. The target sequences selected for TGF-β1 shRNA were: forward, 5'-CGG CAC ACT GCA AGT AGA CAT TAA CCT GAC CCA TTA ATG TCT ACT TGC AGT GTG TTT TT -3'; and reverse, 5'-AAT TAA AAA CAC ACT GCA AGT AGA CAT TAA TGG GTC AGG TTA ATG TCT ACT TGC AGT GTG -3'. The same method was used to transduce the negative control virus containing a shRNA against green fluorescent protein (GFP) reporter into cells (GFPsh) to control for the impact of the lentivirus vector into cells.

The annealed oligonucleotides (insert) were ligated to the vector pLKO 1.puro between AgeI (New England Biolabs, Ipswich, MA, USA) and EcoRI (New England Biolabs, Ipswich, MA, USA) restriction sites. After transformation into DH5-α bacteria and selection by ampicillin (Sigma-Aldrich, St. Louis, MO, USA), clones from each insert were selected and checked by sequencing to verify the sequences of interest. QIAquick Gel Extraction Kit

(Qiagen, Valencia, CA, USA) and Plasmid Midi Kit (Qiagen, Valencia, CA, USA) was used to extract the plasmidial DNA.

Quantitative RT-PCR

TGF-β1, *N-cadherin* and *E-cadherin* gene expression was determined by real-time PCR (qRT-PCR), according to Bustin et al. [29]. Total RNA was extracted from the cultured cells with TRIZOL reagent (Invitrogen - Life Technologies, Eugene, OR, USA). The concentration of RNA from each sample was determined using a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific - Waltham, MA, USA). Each sample of total RNA was subjected to reverse transcription using a High Capacity cDNA kit (Applied Biosystems, Foster City, CA, USA).

The qRT-PCR reaction was performed by StepOne Plus Real Time PCR System (Applied Biosystems, Foster City, CA, USA) using inventoried TaqMan assays (Life Technologies, Eugene, OR, USA): *TGF-β1* (Cf02623325_m1), *N-cadherin* (Cf02696084_m1) and *E-cadherin* (Cf02624269_m1). 40S ribosomal protein S5 (*RPS5*) and 40S ribosomal protein S19 (*RPS19*) were used as endogenos control genes to normalized the expression of genes of interest. Primers used for amplification were: *RPS5* forward: 5'- TCA CTG GTG AGA ACC CCC TG -3', reverse 5'- GCC TGA TTC ACA CGG CGT A -3', and *RPS19* forward 5'- GCC TTC CTC AAA AAG TCT GGG - 3', reverse 5'- GCT TGC TCC CTA CGA TGA GAA C – 3'.

The expression level of genes of interest was determined by relative quantification (RQ) value using the average of normalizing genes (ΔΔCt). The samples were tested in triplicate and all experiments included a negative control, lacking cDNA.

Migration and invasion assay

The migration and invasiveness of CF41 and TGF-β1sh cells were tested with 8 μm inserts matrigel-coated membranes in 24 well plates (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). In the upper compartment of the chamber, approximately 2.5 x 10^4 cells/insert were added into culture medium without serum, while 750 μL of culture medium (with 10% FBS) was added to the lower compartment with 5 mM of metformin treatment. For negative and positive controls, 0.5% and 10% FBS respectively, were used.

After 24 hours, the membranes were washed, fixed in paraformaldehyde, permeabilized in methanol, and stained with hematoxylin to detect the migrated cells. The counting was made with an inverted optic microscope (Nikon Eclipse E200, Melville, NY, USA) and photographed by putting the insert over a plate containing glycerol at 50%. The migration and invasion rate was calculated by dividing the average number of treated cells that migrated and invaded the matrigel membrane by the average of the positive control cells that did so.

N-cadherin and E-cadherin immunofluorescence staining

Cells were fixed in 4% paraformaldehyde solution and permeabilized with 0.5% triton X-100 (Sigma-Aldrich). The primary antibodies N-cadherin (Santa Cruz Biotechnology) and E-cadherin (Cell Signaling Technology) were used to assess the expression of respective antigens, and the secondary antibody was Alexa Fluor 488 anti-rabbit IgG (Life Technologies). Following secondary antibody, the structures were incubated with 4',6-diamidino-2-phenylindole (DAPI) solution (Life Technologies) and mounted with Prolong Gold (Life

Technologies). Nuclei and antigens were visualized with a confocal microscope (ZEISS LSM 710, software ZEN 2010, Thornwood, NY, USA) at 40X objective magnification.

Animal model

Athymic nude female mice (n = 30) weighing 20–25 grams were randomly separated into groups of treatment, and kept under pathogen-free conditions at room temperature (21 to 25 °C) on exposure to light for 12 hours and 12 hours in the dark. Food and water were offered *ad libitum*. The mice were purchased from central animal laboratory of medical school of Sao Paulo University (FM-USP).

For primary breast tumor and lung metastasis induction, 3×10^6 viable cells were washed with PBS, resuspended in 0.1 mL of serum free RPMI 1640 medium (Gibco, Grand Island, NY, USA), and injected into the fourth right inguinal mammary fat pad. Mice were divided according to the following six treatment groups (n=5 animals/group): Group I: animals that were not implanted with tumor cells and were left untreated (negative control), Group II: animals implanted with parental CF41 cells and untreated (positive control), Group III: animals implanted with CF41 cells treated with metformin alone, Group IV: animals implanted with GFPsh cells and untreated (shRNA control), Group V: animals implanted with TGF-β1sh cells and untreated, Group VI: animals implanted with TGF-β1sh cells and treated with metformin.

After one week of tumor cell implantation, the animals received metformin treatment for 4 weeks. The drug was administered intraperitoenaly (i.p.) at a dose of 200 mg/kg per day as previously reported [30]. Tumor volume and body weight were measured once a week . Tumor volume was calculated using the formula $V=\text{length} \times \text{width}^2/2$.

Measurement of blood glucose of the experimental group

The measurement of blood glucose was performed to verify the glycemic variation and the potential cytotoxicity of metformin in treated animals, as previously reported [30]. A drop of blood was collected using a 26 gauge needle to venipuncture of tail vein of animals, once a week throughout the treatment period. Glucose levels were detected using a standard blood glucose portable apparatus (Abbott Optium Xceed®), with individual reagent strips for rapid determination indicating amounts in milligrams per deciliter (mg/dL).

Histopathology and immunohistochemistry

After 4 weeks of treatment the animals were euthanized, and perfused with PBS and 4% paraformaldehyde (Acros Organics, New Jersey, USA). The lungs were collected, fixed in 4% paraformaldehyde, prepared for paraffin blocks and sectioned. Standard haematoxylin and eosin (H&E) staining was performed to evaluate the metastases presence and immunohistochemical (IHC) staining procedures were performed as recommended by the suppliers of primary antibodies. The following antibodies were used to delineate the expression of corresponding antigens: N-cadherin (Santa Cruz Biotechnology), E-cadherin (Cell Signaling Technology), claudin-7 (Abcam) and vimentin (Dako).

Briefly, the slides containing lung tissues were deparaffinized, rehydrated, incubated with citrate buffer at 96 ℃ for 30 minutes and blocked with 0.1% of hydrogen peroxide and 1% of BSA for 20 minutes each. Following this process, sections were incubated with the primary antibody (anti-N-cadherin, anti-E-cadherin, claudin-7 and vimentin) at 4 ºC overnight. Of note, The vimentin antibody did not detect mouse antigen, and was therefore used to identify metastatic colonies in serial sections that were parallely stained for the other antigens.

This was restricted to the areas of metastatic colonies. Then, incubated with Starr Trek Universal HRP Detection System kit (Biocare Medical, Concord, CA, USA) containing the secondary antibody (biotinylated anti-mouse, -rabbit, -goat immunoglobulins), peroxidase-streptavidin conjugates, and diaminobenzidine tetrachloride (DAB) chromogenic substrate. At last, all sections were counter stained with haematoxylin, dehydrated and cover slipped with Erv Mount (Easypath, Sao Paulo, SP, Brazil).

Data analysis and statistics

All results were submitted to descriptive analysis to determine statistical normality. An analysis of variance (ANOVA) was performed, followed by the Bonferroni´s test. Values of $p \leq 0.05$ were considered statistically significant. The GraphPadPrism 5 software (GraphPad Software, Inc., San Diego, CA, USA) was used.

RESULTS

MTT assay of cell viability

CF41 cell line was subjected to MTT cell viability testing, after 24 hours treatment with increasing doses of metformin. At 5 mM metformin, there was a significant 37% reduction in cell viability (63,17 ± 2,104%) compared to control group (100,0 ± 7,155%; $p < 0.05$; **Fig. 1**). After the calculation of metformin, the IC50 was 67.68, and this substance showed the best antitumor potential in concentration of 5 mM (63,17). Based on these results, the 5 mM concentration was used for all the following studies.

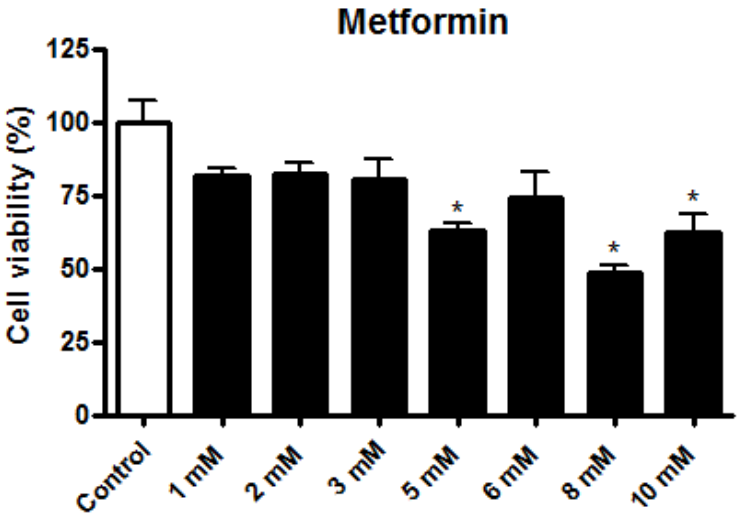

Figure 1. **Effect of metformin on viability of canine mammary tumor cell line.** CF41 cells were treated with 1 mM to 5 mM of metformin for 24 hours and cell viability was measured by MTT assay. Significance calculated by ANOVA followed by Bonferroni´s test (± S.E.M. *p < 0.05).

Metformin and TGF-β1 shRNA effect on cell migration and invasion

Expression of TGF-β1 was first assessed in CF41 cells by qRT-PCR (data not shown). This was important to infere the feasibility of using this cell line for our knockout experiments. Next, CF41 were transfected with the shRNA plasmid and selected for stable clones with demonstrated silencing of TGF-β1 expression. This was confirmed by qRT-PCR. TGF-β1 mRNA levels were decreased by TGF-β1sh #2, #3 and #4, but sequence #3 was more effective as compared to the other 2 (-1,149 ± 0,05634 a.u.; p = 0.0001) [for more details see additional file 1].

To verify whether metformin or TGF-β1sh alone or in combination would decrease cell invasive potential, we performed a transwell assay using matrigel-covered membranes (**Fig. 2**). After 24 hours of metformin treatment, there was a significant decrease (49,03 ±

5,227%) in invasion and migration of CF41 cells as compared to positive control (100,0 ± 5,853%; $p = 0.0001$). When compared to GFPsh cells untreated (96,74 ± 2,542 a.u.), there was a significant decrease (57,85 ± 5,394%; $p = 0.0001$) in invasion and migration of TGF-β1sh cells and of TGF-β1sh cells treated with metformin (10,48 ± 1,876%; $p = 0.0001$). For this assay, the positive control (10% serum at the bottom well) was used as the basal level to compare to all treatments. The negative control (2% serum) in this assay showed a 86% reduction (13,74 ± 1,195%) in the migration/invasion of the cells compared to that of positive control (100,0 ± 5,853%; $p = 0.0001$) (data not shown).

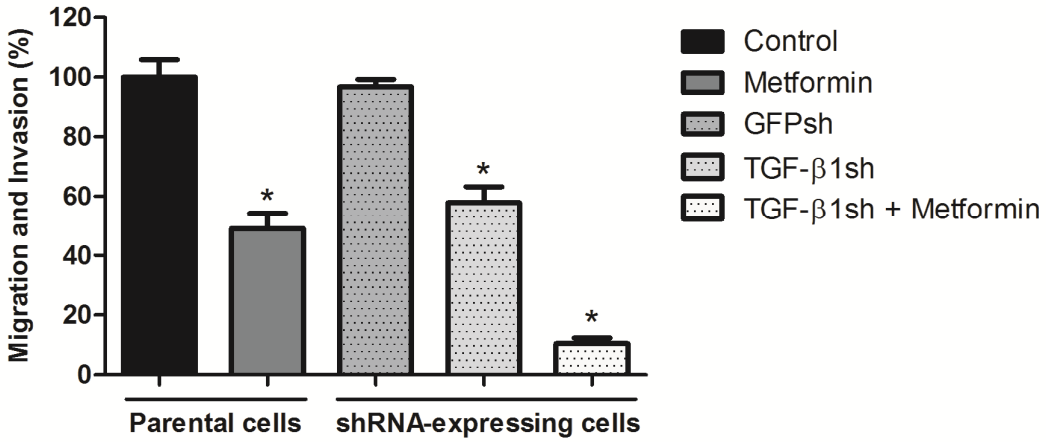

Figure 2. Cell migration and invasion. Cell migration and invasion assay was carried out in CF41 and TGF-β1sh cells after treatment with 5 mM metformin. Statistically significant differences, verified by ANOVA followed by Bonferroni's test. *$p < 0.05$ in comparison to control of untreated parental cells (solid columns) and to control of shRNA-expressing cells (dotted columns).

N-cadherin and E-cadherin expression after treatment with metformin and TGF-β1 shRNA

N-cadherin and E-cadherin are key EMT markers in breast cancer [31]. Therefore, we examined the expression level of these markers to assess possible EMT-suppressing effects of TGF-β1 silencing and metformin treatment, alone or in combination.

Treatment with metformin for 24 hours decreased N-cadherin gene and protein expression. Specifcally, we observed a decrease in N-cadherin mRNA expression in parental cells treated with metformin (-0,1947 ± 0,03820 a.u.; $p = 0.008$) compared with positive control (0,0 ± 0,0110 a.u.), and in TGF-β1sh cells and TGF-β1sh cells plus metformin (-1,023 ± 0,07815 a.u.; $p = 0.0002$) compared to GFPsh control (0.0 ± 0,2077 a.u.). The protein levels were significantly reduced by the same treatments (metformin, 13,62 ± 1,298 a.u.; $p = 0.0001$; TGF-β1sh, 11,40 ± 0,9234 a.u.; $p = 0.0001$ and TGF-β1sh + metformin, 7,633 ± 0,7067 a.u.; $p = 0.0001$), compared with respective control groups (Control, 25,97 ± 2,092 a.u.; GFPsh, 25,62 ± 1,966 a.u.) (**Fig. 3**). E-cadherin protein expression was higher in CF41 parental cells treated with metformin (9,683 ± 0,9668 a.u.; $p = 0.0001$) compared to untreated control cells (7,217 ± 0,6032 a.u.) (**Fig. 4**). In the TGF-β1 silencing cells, E-cadherin expression was significantly higher compared to GFPsh control cells (8,250 ± 0,9378 a.u.) at both the protein (13,78 ± 0,9415 a.u.; $p = 0.03$) and RNA level. Interestingly, when TGF-β1 silenced cells were treated with metformin, E-cadherin expression levels (6,500 ± 0,6362 a.u.; $p = 0.0001$) returned to GFPsh control levels (8,250 ± 0,9378 a.u.) (**Fig. 4**).

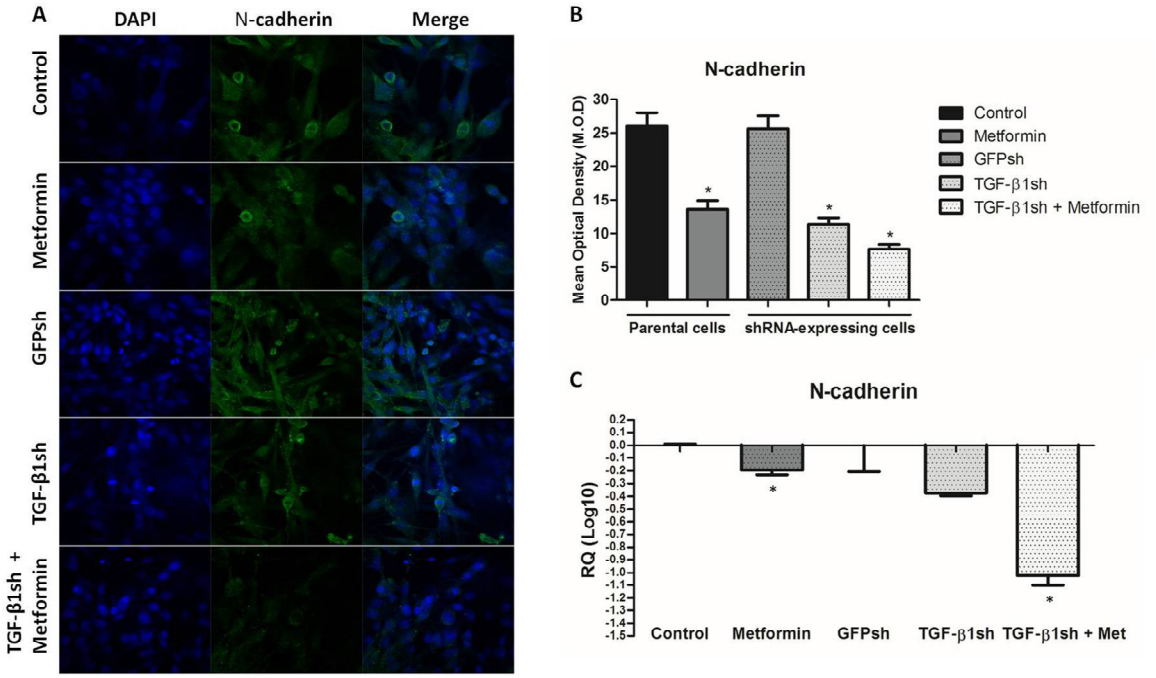

Figure 3. N-cadherin expression in CF41 cell line. A. Photomicrographs of immunofluorescence (IF) staining for N-cadherin (green) and Dapi (blue) in CF41 and TGF-β1sh cells treated with metformin.. ZEISS, 2014, 40X. **B.** Plotted results of immunofluorescence quantification. **C.** Gene expression of N-cadherin after treatment, compared to control of untreated parental cells and control of shRNA-expressing cells, respectively. Each column is the mean ± standard error of triplicates. Significant values from ANOVA followed by Bonferroni's test (*$p < 0.05$).

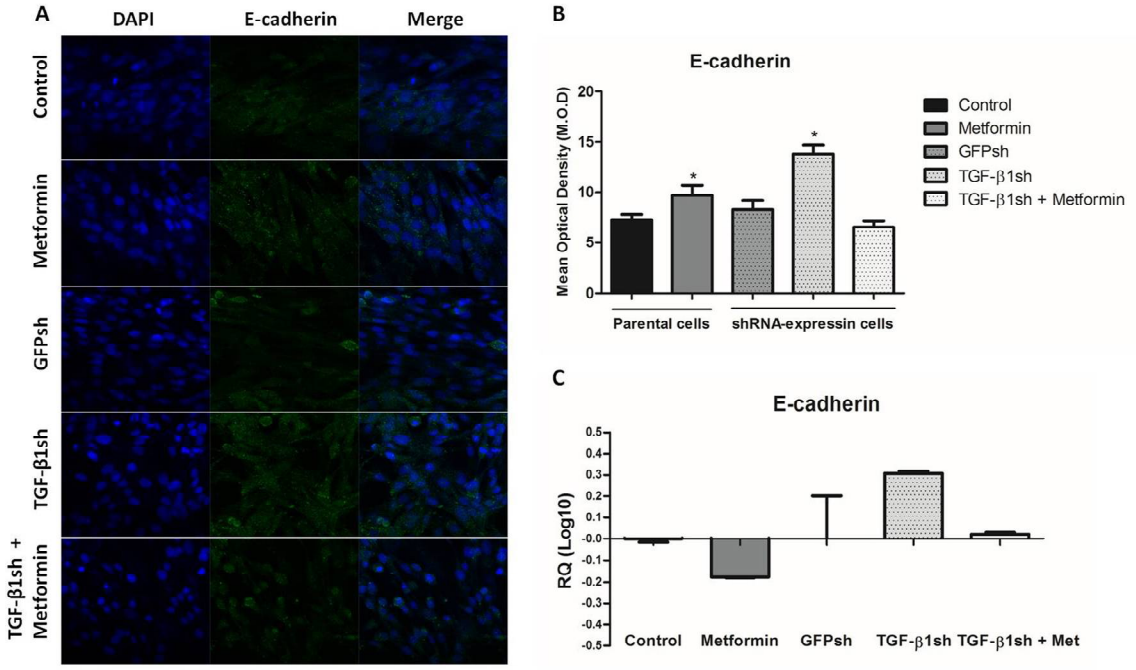

Figure 4. E-cadherin expression in CF41 cell line. A. Photomicrographs of immunofluorescence (IF) staining for E-cadherin (green) and Dapi (blue) in CF41 and TGF-β1sh cells treated with metformin.. ZEISS, 2014, 40X. **B.** Plotted results of immunofluorescence quantification. **C.** Gene expression of E-cadherin after treatment, compared to control of untreated parental cells and control of shRNA-expressing cells, respectively. Each column is the mean ± standard error of triplicates. Significant values from ANOVA followed by Bonferroni's test (*$p < 0.05$).

Effects of metformin and TGF-β1 silencing in animal model of breast cancer metastasis

To determine the impact of combined metformin and TGF-β1 shRNA intervention in metastasis, we employed a CF41 mouse xenograft model of breast cancer metastasis to the lung. All mice remained healthy during the experiment and no clinical or pathologic signs of metformin treatment were found. All groups had weight changes, however, no changes were significant between the treated groups and the control group ($p > 0.05$) [for more details see additional file 2]. After a week of tumor cell implantation, all groups were assessed weekly for blood glucose levels. The animals from the negative control group, who received no tumor

induction, presented the same glycemic variation that animals treated with metformin, and the other evaluated groups ($p > 0.05$) [for more details see additional file 3]. In addition, behavioral indicators such as drowsiness or agitation were not observed.

There was a gradual growth of the primary tumor in the breast region in all groups implanted with tumor cells after 5 weeks, except GFPsh control group, which showed an absolute regression of the tumor size in all animals after the third week of monitoring relative to first week of parental untreated cells ($p = 0.001$) [for more details see additional file 4]. CF41 was shown to be a highly metastatic canine mammary tumor cell line when implanted in athymic nude mice. As shown in **Fig. 5**, animals of the group treated with metformin has less metastasis (1,000 ± 0,3162 a.u.) compared to control group (4,800 ± 0,9695 a.u.; $p = 0.005$). For the GFPsh control group, although no specific gene silencing is expected in these tumors, we noted that they displayed reduced tumor growth [for more details see additional file 4] and a reduced incidence and number of metastasis (20% and 30%, respectively) as compared to the untreated control (parental cells) group (**Fig. 5**), although no statistically significant differences in the number of metastasis/lung were observed (**Fig.5**). However, for TGF-β1sh group treated with metformin or not it was observed higher primary tumor size [for more details see additional file 4] and less pulmonary metastass in both, the TGF-β1sh group (1,000 ± 0,3162 a.u.; $p = 0.05$) and TGF-β1sh group treated with metformin (0,6000 ± 0,2449 a.u.) compared to GFPsh control group (3,000 ± 0,8367 a.u.; $p = 0.02$) (**Fig. 5)**

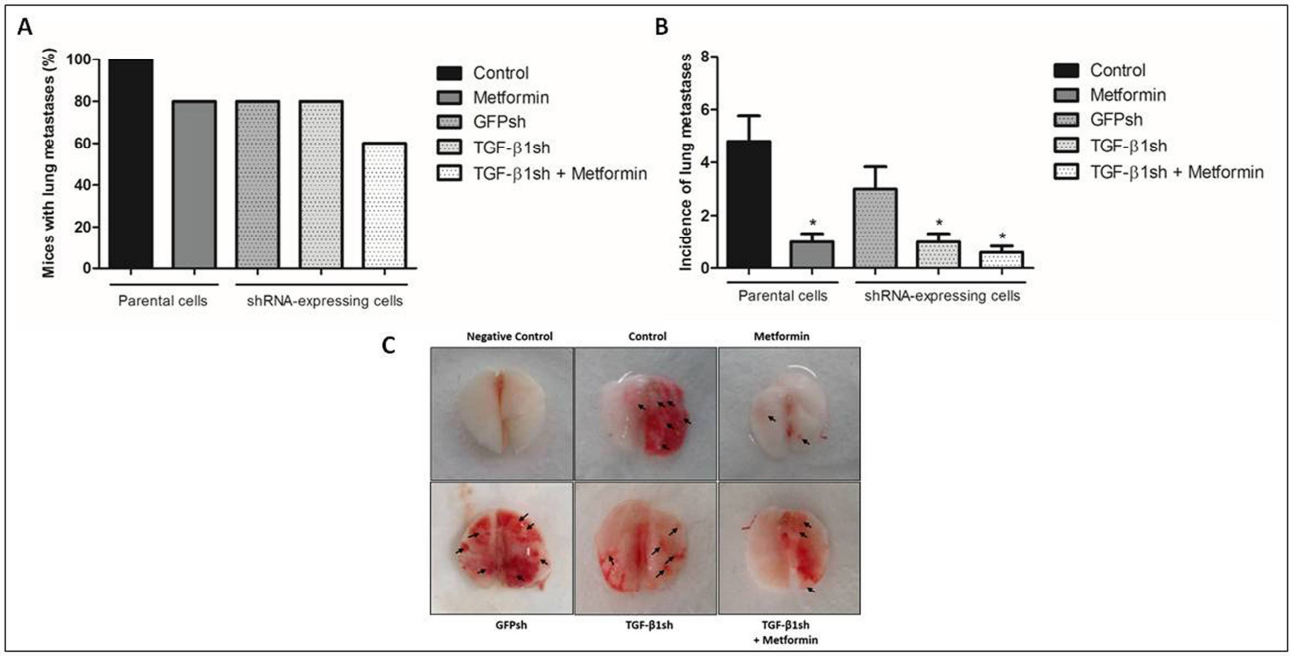

Figure 5. **Analysis of lung metastasis in animal model**. **A.** Percentage of mice who had metastasis in each group. **B.** Incidence of metastases in the lungs of mice in each group. Statistically significant differences, verified by ANOVA followed by Bonferroni's test. * $p < 0.05$ in comparison with control of untreated parental cells and control of shRNA-expressing cells. **C.** Macroscopic lung metastasis. Arrows indicate sites of lung metastasis.

For analysis of protein markers associated to EMT (N-cadherin, E-cadherin, vimentin, and claudin-7) in lung metastatic colonies, IHC was performed, followed by densitometry quantification.

The presence of N-cadherin was observed in lung metastases from all groups, with lesser intensity in the groups treated with metformin and with TGF-β1sh cells**.** Animals implanted with CF41 cells and treated with metformin showed a significant reduction in protein expression of N-cadherin (106,7 ± 1,307 a.u.) when compared to the control group (154,1 ± 1,817 a.u.; $p = 0.0001$). The animals implanted with TGF-β1sh cells (108,1 ± 0,8859 a.u.; $p = 0.0001$) and TGF-β1sh cells plus subsequent metformin treatment (104,8 ± 1,049 a.u.; $p =$

0.0001) also showed a statistically significant reduction in N-cadherin expression in the lungs compared to GFPsh group (140,7 ± 1,853 a.u.) (**Fig. 6**).

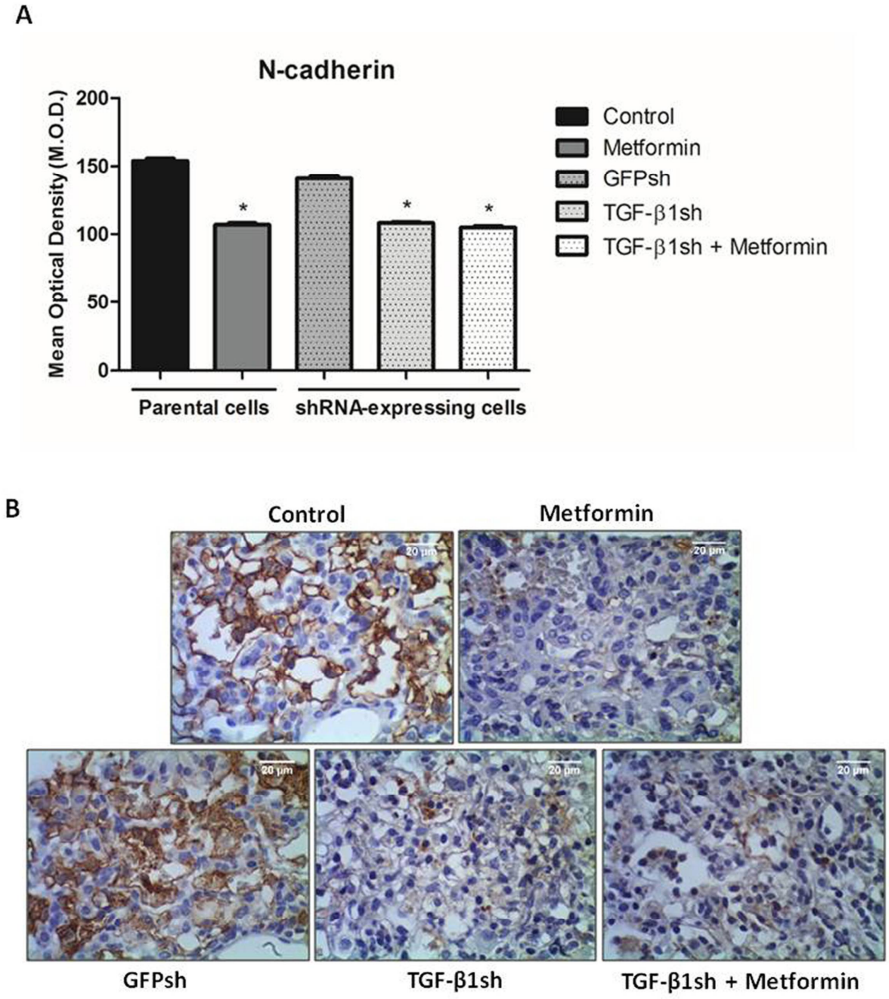

Figure 6. N-cadherin protein expression. A. Semi-quantitative analysis of N-cadherin protein expression by densitometry in metastatic colonies. **B**. N-cadherin immunostaining in the lungs. Statistically significant differences, verified by ANOVA followed by Bonferroni's test. * $p < 0.05$ in comparison with control of untreated parental cells and control of shRNA-expressing.

E-cadherin had increased expression in the lung metastatic lesion of the animals implanted with parental cells and treated with metformin (155,4 ± 1,577 a.u.), compared to the

lungs of untreated animals (105,3 ± 1,383 a.u.; $p = 0.0001$). A similar effect was seenin the animals implanted with with TGF-β1sh cells in the absence (139,0 ± 1,776 a.u.; $p = 0.0001$) or after metformin treatment (143,4 ± 2,013 a.u.; $p = 0.0001$), as compared to the GFPsh group (114,6 ± 1,623 a.u.) (**Fig. 7**).

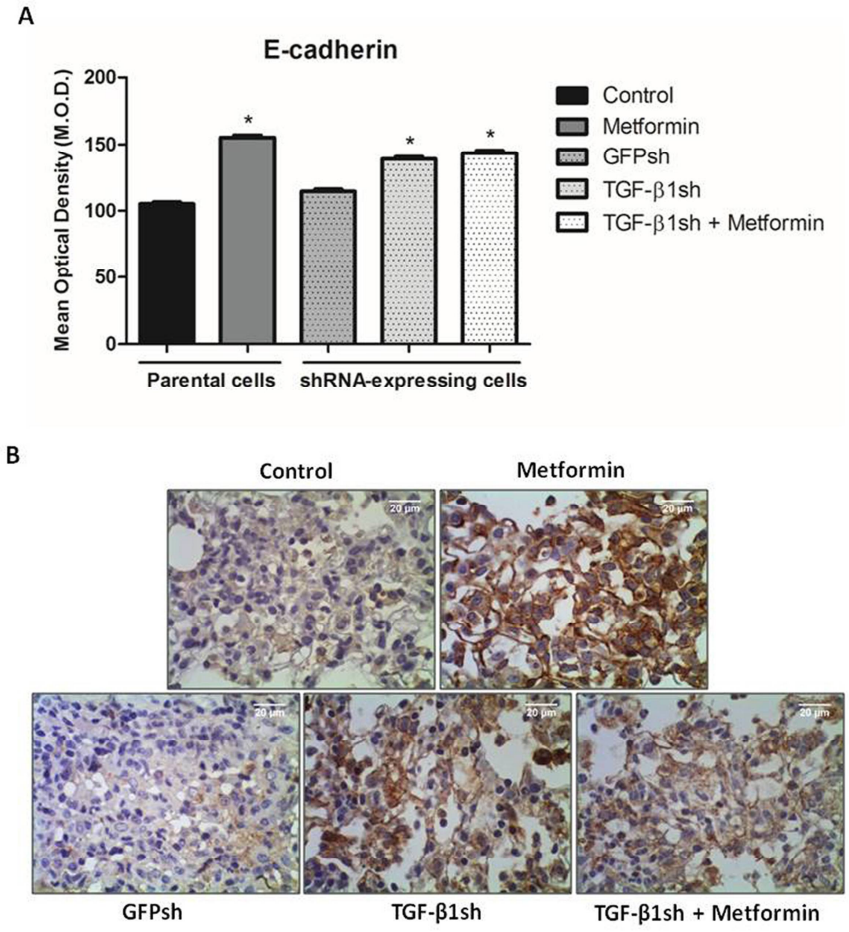

Figure 7. E-cadherin protein expression. A. Semi-quantitative analysis of E-cadherin protein expression by densitometry in metastatic colonies. **B**. E-cadherin immunostaining in the lungs. Statistically significant differences, verified by ANOVA followed by Bonferroni's test. * $p < 0.05$ in comparison with control of untreated parental cells and control of shRNA-expressing.

Treatment with metformin reduced vimentin expression in lung metastatic colonies (126,3 ± 1,378 a.u.) as compared to those formed by control, untreated parental cells (166,5 ± 1,392 a.u.; $p = 0.0001$). Similar results were observed for the TGF-β1sh (118,5 ± 1,408 a.u.; $p = 0.0001$) and TGF-β1sh plusmetformin groups (114,0 ± 1,606 a.u.; $p = 0.0001$), in relation to GFPsh group (166,6 ± 1,895 a.u.) (**Fig. 8**).

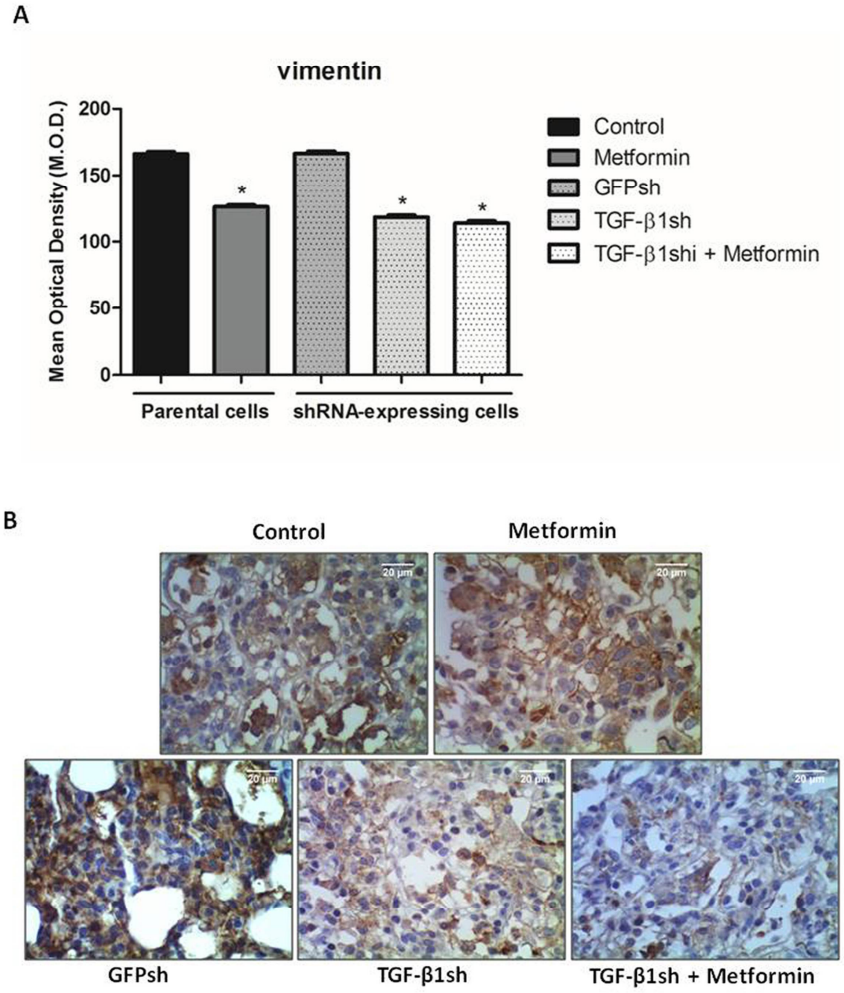

Figure 8. Vimentin protein expression. A. Semi-quantitative analysis of vimentin protein expression by densitometry in metastatic colonies. **B**. Vimentin immunostaining in the lungs. Statistically significant differences, verified by ANOVA followed by Bonferroni's test. * $p < 0.05$ in comparison with control of untreated parental cells and control of shRNA-expressing.

Animals treated with metformin had a statistically significant increase in protein expression of claudin-7 (131,5 ± 1,775 a.u.) compared to the positive control group (116,3 ± 1,326 a.u.; $p = 0.0001$). Similarly, animals transfected with TGF-β1sh cells and treated with metformin had increased protein expression relative to GFPsh control group (131,0 ± 1,786 a.u.; $p = 0.008$) (**Fig. 9**).

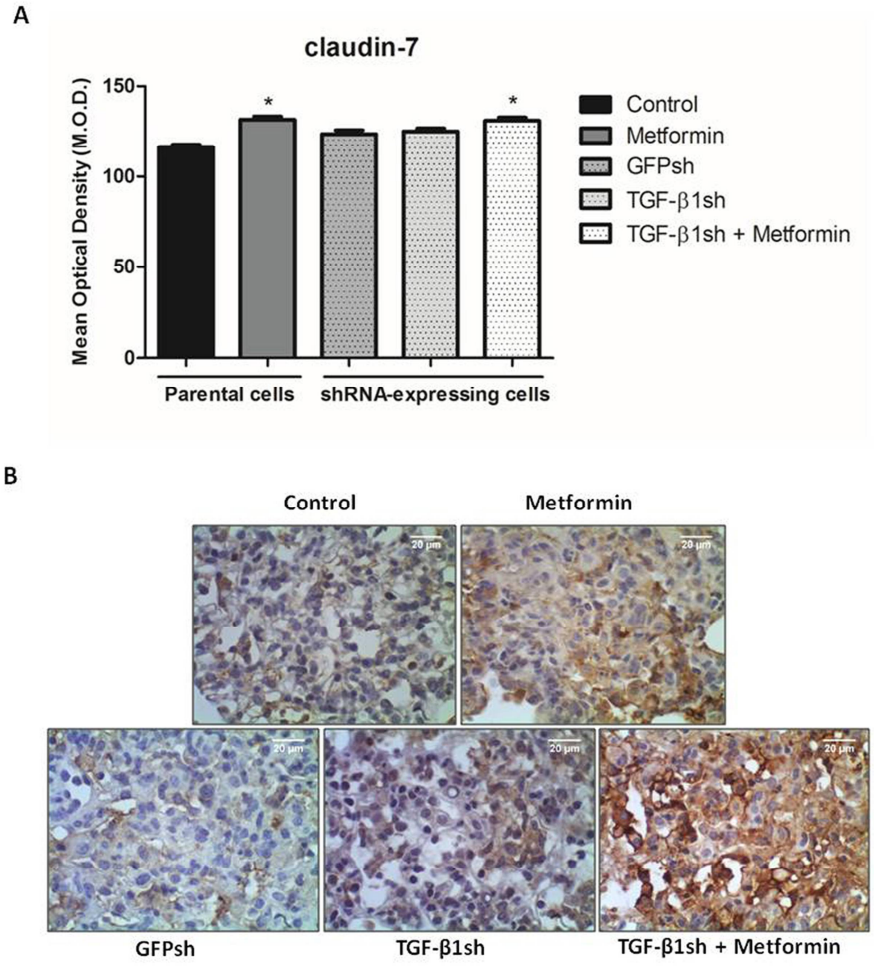

Figure 9. Claudin-7 protein expression. A. Semi-quantitative analysis of claudin-7 protein expression by densitometry in metastatic colonies. **B**. claudin-7 immunostaining in the lungs. Statistically significant differences, verified by ANOVA followed by Bonferroni's test. * $p < 0.05$ in comparison with control of untreated parental cells and control of shRNA-expressing.

DISCUSSION

EMT is well-documented to mediate the process of metastasis, particularly in breast cancer [32]. Developing strategies to maximize inhibiton of EMT to halt cancer progression has high potential to translate into patient benefit. In the present study, treatment with metformin *in vitro* was able to decrease the viability of the mesenchymal/metastatic CF41 cell line, as well as their rate of migration and invasion. Furthermore, as expected [4], metformin reduced mesenchymal marker expression N-cadherin and increased the expression of the epithelial marker E-cadherin.

There are few studies that demonstrate the action of metformin on EMT. In accordance with our results, Qu et al. [25] demonstrated that metformin reversed the EMT phenotype and decreased invasiveness of human breast cancer cell lines MDA-MB-231 and MCF-7. Further, Cerezo et al. [33] demonstrated that metformin was able to inhibit cell invasion of melanoma cell lines, while Hwang et al. [34] showed antimetastatic action of metformin in fibrosarcoma cells, inhibiting cell migration and invasion.

Several mechanisms of antitumor action of metformin have been described, especially a direct effect on AMPK activation, which results in inhibition of mTOR signaling pathway [35]. It has been demonstrated that AMPK activation inhibits EMT induced by TGF-β in breast cancer cells [28]. Of the aforementioned Qu et al. [25] study demonstrated that treatment with metformin activated AMPK signaling pathway in estrogen receptor positive (ER+) breast tumor cells that were chemoresistant to 5-fluorouracil (MCF-7/5-FU), as well as triple-negative MDA-MB-231, reversing the multidrug resistant phenotype beyond effects on the EMT process. Metformin also targets other pathways associated with EMT, such as mTOR signaling pathway-S6K. The high expression of the tyrosine kinase p70S6K has been

associated to low expression of E-cadherin and high expression of N-cadherin and vimentin. Thus, treatment with metformin inhibits the mTOR-S6K pathway, and this could also be one possible mechanism by which metformin inhibits EMT and metastasis [36, 37].

Several signaling pathways resulting from the activity of different growth factors are involved in the EMT process, especially TGF-β signalling [38]. Thus, given the importance of TGF-β1 in the induction of EMT, and the tendency of tumor cells to activate this pathway in an autocrine fashion by secreting their own TGF-β, the present study focused on gene silencing of TGF-β1 in the mesenchymal and metastatic CF41 cell line. As expected, the *in vitro* results showed that silencing of TGF-β1 inhibited cell migration and invasion, and there was a greater reduction with the addition of metformin to gene silencing, suggesting cooperativity of treatments in most experiments. Similar to our results, Moore et al. [18], using metastatic human mammary tumor cells, demonstrated that TGF-β1 silencing by RNAi led to a decrease of 35% of cell migration and 55% of cell invasion.

Analyzes of mRNA and protein expression *in vitro* and *in vivo* studies demonstrated that silencing of TGF-β1 resulted in reduction of the mesenchymal markers N-cadherin and vimentin and enhanced expression of the epithelial markers E-cadherin and Claudin-7, and was equally or more effective in addition to metformin. To the best of our knowledge, this is the first study to investigate the combined action of TGF-β1 silencing and metformin treatment. Zhang et al. [35] demonstrated that *in vitro* treatment with metformin inhibited EMT process induced by TGF-β1 in human prostate cancer cells, reducing the gene and protein expression of N-cadherin and vimentin, and increasing the expression of E-cadherin and β- catenin.

Our *in vivo* study found that there were fewer mice with lung metastases and fewer metastastic colonies/lung in animals that received cells with TGF-β1 silenced or animals that

were treated with metformin, as compared to untreated parental cells. The effect was further enhanced by combined TGF-β1 silencing and metformin treatment, and was significant as compared to animals implanted with shRNA control cells. Our *in vivo* results with TGF-β1 silencing alone are in agreement with a previous study by Moore et al. [18] using a mouse xenograft model of metastatic MDA-MB-435 human breast cancer cells, where a 90% reduction in the number of macroscopic lung metastases was observed in the TGF-β1 shRNA group compared to the control group. Further, the increased tumor growth by TGF-β1 silencing is also in agreement with the previously reported dual function of this factor in breast cancer, whereby it can simultaneously act as a primary tumor supressor and metastasis inducer, as initially observed in seminal studies by Dr. Massague's [39] and Arteaga's [40] groups.

Our findings with metformin treatment also support previous results. The addition of metformin to conventional chemotherapy was observed to inhibit tumor progression and delay relapse in animal models of breast, prostate and lung cancer [30], and Zhang et al. [41] observed that patients with colorectal cancer and type 2 diabetes treated with metformin showed a significantly reduced rate of distant metastasis compared to the untreated group. Interestingly, although we observed a significantly enhanced *in vitro* effect of combined TGF-β1sh and metformin as compared to each treatment alone, with relation to viability, invasiveness and EMT markers, the combined treatment was better that each treatment alone at reverting EMT features. It is possible that other factors produced by the tumor stroma (including TGF-β1 it self) also induce EMT and metformin treatment does not have the capacity to fully inhibit this in addition to the EMT-promoting effect of tumor cell-derived TGF-β1. In this regard, combining metforming with a TGF-β1 blocking antibody that targets both the cell autonomous and the stromal tumor compartments might be more effective at

maximizing the EMT inhibitory effect. More importantly, this will be more feasible in actual patients, as antibodies of this kind has already demonstrated safety in clinical trials [42, 43]. Based on the divergence between these and our *in vitro* findings, one possibility is that metformin treatment *in vivo* promotes stroma-dependent compensatory mechanisms that yield TGF-β1 silencing at the tumor cell compartment not only ineffective at halting, but possibly more permissive of, lung colonization. Alternatively, and based on recent evidence of the involvenmnet of a mesenchymal-to-epithelial reversion in metastatic colonization [4, 5, 44, 45], it is also possible that excessive rescuing of an epithelial phenotype might in part promote lung colonization. We are currently investigating these possibilities.

CONCLUSION

The present study proved the efficacy of metformin at significantly reducing cell viability of the mesenchymal, metastatic CF41 cells. Either metformin treatment or TGF-β1 silencing in CF41cells resulted in enhanced expression of epithelial markers and decreased expression of mesenchymal markers, as well as inhibition of invasiveness and metastases, and the combination of the two approaches was more effective that each treatment alone *in vitro* and *in vivo*. Our data suggests that therapies combining TGF-β1 targeting and metformin may be effective in reducing the process of EMT and metastatic potential. Further studies are necessary to improve our understanding of metformin's effect on the tumor microenvironment *in vivo*, as to be able to design an approach that exploits these findings more effectively.

LIST OF ABREVIATIONS

ATCC, American type culture collection; BSA, Bovine Serum Albumin; cDNA, Complementary DNA; TGF-β1sh, Small hairpin RNA constructs targeting TGF-β1 in CF41 cells; DMEM, Dulbecco's modified Eagle's medium; EMT, epithelial-mesenchymal transition; FBS, fetal bovine serum; GFPsh, green fluorescent protein reporter into CF41 cells; H&E, Haematoxylin and eosin; MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS, Phosphate buffered saline; qRT-PCR, Real time polymerase chain reaction; RNA, Ribonucleic acid; RPS5, Ribosomal Protein S5; RPS19, Ribosomal Protein S19; TBS-T, Tris-Buffered Saline and Tween 20; TGF-β1, Transforming growth factor beta 1.

Competing interests

The authors declare that they have no competing interests.

Author's contributions

CL and DAPCZ conceived, designed and interpreted experiments and drafted the manuscript. MCB and LCF contributed to the realization of gene silencing. TFB, LCF and MGM contributed in the treatment of animals in the *in vivo* study and analysis of immunohistochemistry. AMVP shared infrastructure, provided expertise for IF optimization/implementation, and contributed to both the experimental design and writing of the manuscript. All authors read and approved the final manuscript.

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Additional files

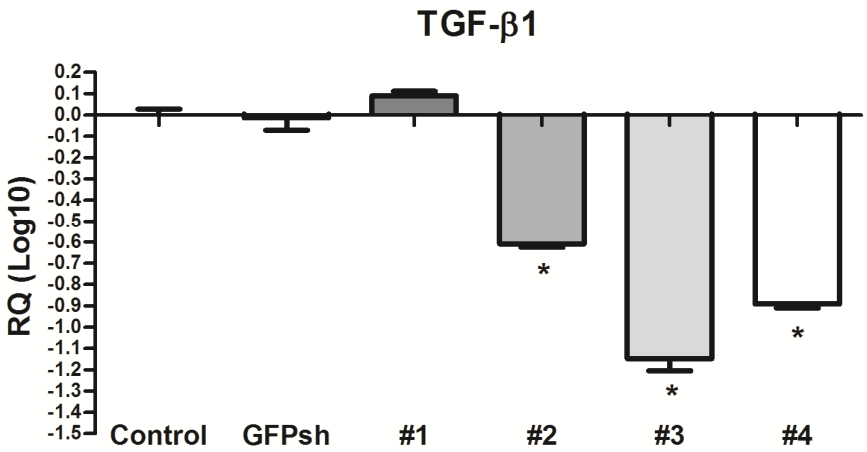

Figure 1. TGF-β1 mRNA expression in cells with TGF-β1 knockdown. Analyses of TGF-β1 mRNA expression in cells with TGF-β1 knockdown (TGF-β1sh cells) to select the best sequence to implant into the animals. The numbers 1, 2, 3 and 4 correspond to different TGF-β1 shRNA sequences. Statistically significant differences were determined by ANOVA followed by Bonferroni's test. * $p < 0.05$ in comparison with untreated parental cells.

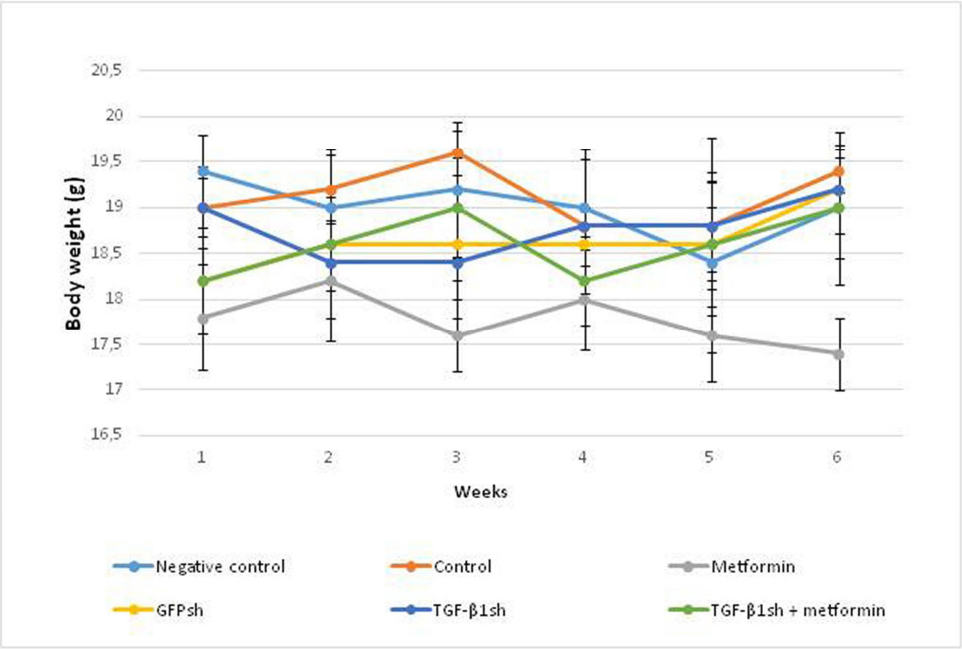

Figure 2. Body weight. Average body weight of animals in the different treatment groups as a function of time. No statistically significant differences were observed ($p>0.05$).

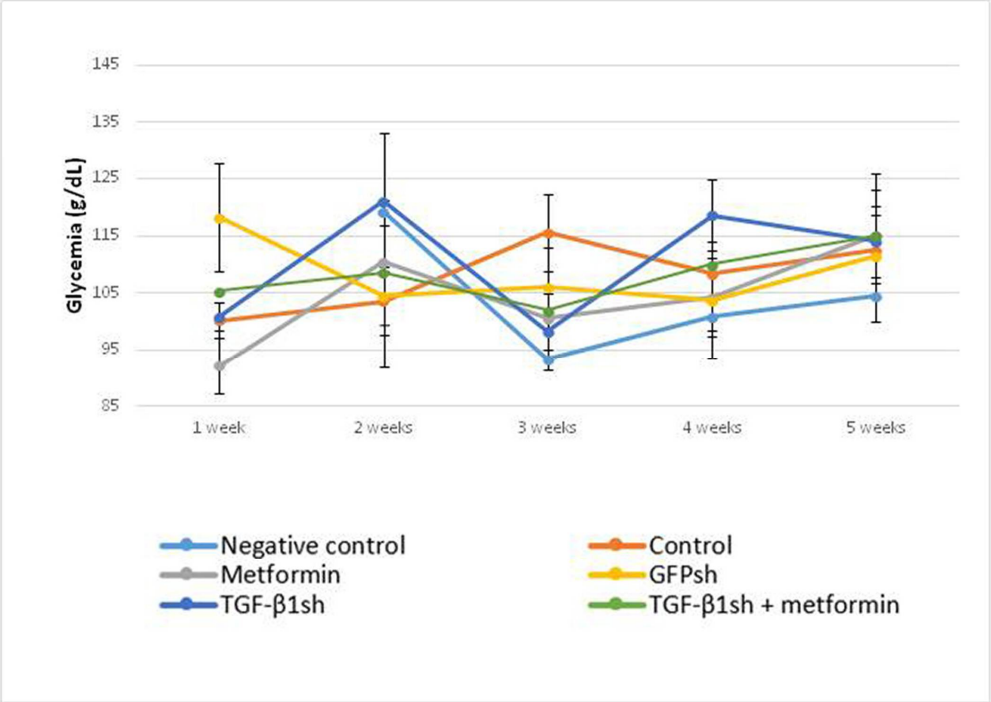

Figure 3. Glycemia. Glucose levels in the different animal groups as a function of time. No statistically significant differences were observed ($p>0.05$).

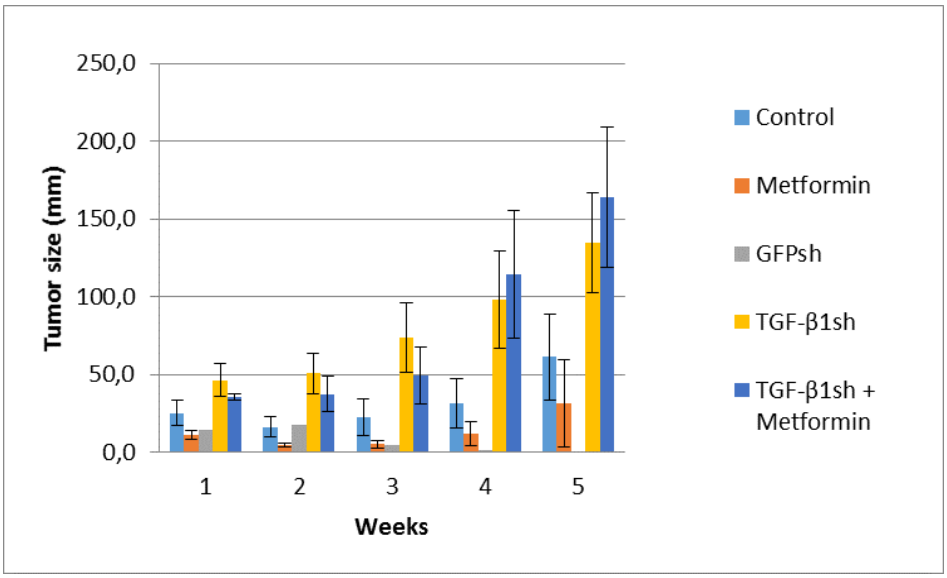

Figure 4. Tumor volume. Average tumor volume in the different animal groups as a function of time. Statistically significant differences, verified by ANOVA followed by Bonferroni's test. *$p < 0.05$ in comparison to control of untreated parental cells or control of shRNA-expressing cells.

Artigo II

Short title: Inhibition of EMT in Breast Cancer

Inhibition of Epithelial-mesenchymal Transition in Response to Treatment with Metformin and Y27632 in Breast Cancer Cell Lines

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Abstract

ROCK-1 expression is associated with the malignant character of tumors, while inhibiting this molecule results in a significant suppression of tumor metastasis. Likewise, transforming growth factor beta (TGF-β) is associated with this malignancy by having the ability to induce epithelial-mesenchymal transition (EMT). Metformin, a drug used in the treatment of diabetes, has previously been shown to inhibit EMT in breast cancer cells. From the TGF-β1 action model for induction of EMT, we evaluated the action of metformin and ROCK-1 inhibitor Y27632. MCF-7 and MDA-MB-231 cell lines were treated with metformin and Y27632, after induction of EMT by TGF-β1, to examine the effects on cell migration as well as the protein expression of the ROCK-1 markers, vimentin, E-cadherin, CD44 and CD24 by immunocitochemistry. There was a lower protein expression of ROCK-1, vimentin, CD44 and CD24 in both cell lines after treatment with metformin and Y27632. In MDA-MB-231 cells, E-cadherin expression was increased in all treatment groups. Treatment of MDA-MB-231 cell line with metformin and Y27632 significantly reduced the invasion of these cells. This study confirms the benefits of metformin and Y27632 as potential therapeutic agents for breast cancer patients, by blocking EMT process and metastatic potential.

Key words: Breast cancer. anticarcinogenic agents, TGF-β, EMT, ROCK1

INTRODUCTION

Breast cancer is a highly malignant carcinoma estimated to be the second most common cause of cancer death among women (TSAI et al., 2016; KOH et al., 2013). The mortality rate is intrinsically related to the occurrence of metastasis, as observed in over 90% of the fatal cases (BORIN et al., 2015). The metastatic process is divided into several steps, starting with the invasion of the basement membrane and extracellular matrix, extravasation into the circulation, survival in blood vessels, spreading to distant tissues, and then the establishment of a secondary tumor (SHI et al., 2010).

The Epithelial-Mesenchymal Transition (EMT) is the underlying mechanism responsible for the invasive and metastatic potential of cancer progression (KOREN et al., 2016; TSAI et al., 2016), being the major mechanism for the conversion of early-stage tumors to invasive malignancies due to the loss of epithelial adherence and tight junctions (BASU et al., 2015). This transitional mechanism occurs during carcinogenesis, in which the cells acquire characteristics of stem cells (self-renewal and tumor initiation), increasing their migratory and invasive capacity. This is characterized by the change in the epithelial to mesenchymal phenotype marked by loss or reduced expression of the epithelial cells of markers, such as claudin, keratins and e-cadherin, and upregulation of mesenchymal markers such as n-cadherin, vimentin and fibronectin (FORONI et al., 2011; SIGURDSSON et al., 2011; RHODES et al., 2015).

The Transforming Growth Factor Beta (TGF-β) is a major growth factor capable of inducing the EMT process, sufficient to generate migrating cancer stem cells by directly linking the acquisition of cellular motility with the maintenance of tumor-initiating capacity (MASSAGUÈ, 2008; YANG and WEINBERG, 2008; CUFI et al., 2010). The TGF-β family controls numerous cellular functions including proliferation, apoptosis, differentiation, migration (SYED et al., 2016) and the deregulation of TGF-β pathway can lead to various pathological conditions, including cancer (PRINCEPE et al., 2014; AL-AZAYZIH et al., 2015). Although studies have demonstrated the tumor suppressive role of

TGFβ1 during the early stages of hyperplasia and tumor development, it switches to a tumor promoter during the advanced metastatic stages of cancer (PRINCEPE et al., 2014 ;ZARZYNSKA, 2014; AL-AZAYZIH et al, 2015). Thus, pharmacological prevention and/or reversal of TGFβ-induced EMT may therefore have important clinical applications in the management of cancer metastasis as well as in the prevention and/or treatment of end-state organ failures (CUFI et al., 2010).

The TGF-β signaling pathway plays an important role in upregulating expressions of hypertrophic gene (ROCK-1, Rho and PKC) (YU et al., 2012) and this activation has mainly been implicated in the process of EMT and cytoskeletal rearrangements (KAMARAJU et al., 2005). However, little is known about TGF-β in regulating Rho-associated protein kinase (ROCK) signal pathway (PEI et al., 2014). Margadant and Sonnenberg (2010) suggested that the activation of TGF-β by integrins can also be initiated by G-protein-coupled receptors; for example, the stimulation of PAR1 with thrombin leads to RhoA-dependent and ROCK-dependent TGF-β activation by integrin αvβ6 in vitro and in vivo. Furthermore, αvβ6-mediated TGF-β activation can be induced by lysophosphatidic acid signaling to RhoA and ROCK, through the lysophosphatidic acid receptor coupled to small Gprotein Gαq. Bhowmick et al. (2001) demonstrated the involvement of RhoA signaling cascade as TGF-β target response, focusing ROCK-1 action as a potential mediator of induction of EMT by TGF-β, regulating the motility of normal epithelial cell lines of mouse mammary glands (NMuMG) by means of cytoskeletal actin and junction and adhesion integrins.

The Rho-associated protein kinase and its isoforms (ROCK-1 and ROCK-2) play a central role in the invasion and migration process by regulating cell cytoskeletal actin rearrangements (Ortiz-Lopez et al., 2009; Bottino et al., 2014). ROCK-1 functions as an oncogene, and possesses a wide range of functions, including cellular migration, invasion, and metastasis (SHIN et al., 2014; XI et al., 2015) being implicated in the regulation of breast cancer metastasis (LIU et al., 2009). Bokobza et al. (2011) demonstrated that an induction in the expression of genes including SNAI1, RhoC, ROCK-1, and N-cadherin is related to the EMT process in prostate cancer cells. Liu et al.

(2009) showed an increased expression of ROCK-1 gene in metastatic tumor cell lines and animal models of breast cancer. In addition, it has found that a specific inhibitor of ROCK-1, Y27632, was able to affect the cell motility *in vitro* and metastasis, *in vivo*.

Several therapies have been found to contain tumor progression and prevent metastasis. In this respect, metformin is a commonly used drug worldwide in the treatment of diabetes mellitus. It belongs to the group of biguanidine drugs, administered orally to reduce the concentration of glucose and to increase insulin sensitivity in patients with type 2 diabetes (ŚMIESZEK et al., 2015; ROJA & GOMES, 2013). This drug has significantly reduced the incidence of cancer and increased survival in type 2 diabetic patients diagnosed with cancer (BEN SAHRA, et al., 2010; DECENSI et al., 2010; JALVING et al., 2010; NOTO et al., 2012).

The action of metformin against breast cancer in patients has been observed in population studies (JIRALERSPONG et al., 2009), animal experiments (RATTAN et al., 2011) and in breast cancer cell lines (PHOENIX et al., 2009; VAZQUEZ-MARTIN et al. 2011). The findings are particularly encouraging, because metformin was able to decrease tumor size and suppress the phenotype CD44+/CD24- stem cells, inhibiting initial tumor formation (VASQUEZ-Martin et al., 2010). Metformin also prevents the loss of the epithelial marker (E-cadherin) promoted by TGF-β in MCF-7 cells and the dissemination and accumulation of mesenchymal markers (vimentin) in canine kidney cells induced by TGF-β, since it keeps the allocation of e-cadherin contact sites between cells and prevents morphological changes associated with mesenchymal status (CUFI et al., 2010).

In this context, we emphasize the importance of studying the EMT process induced by TGF-β, using metformin with or without ROCK-1 inhibitor, as an alternative therapy for inhibiting breast cancer metastasis in vitro, evaluated by ROCK-1 protein expression, vimentin, E-cadherin, CD44 and CD24.

MATERIAL AND METHODS

Cell culture

Triple negative and metastatic (MDA-MB-231) (ATCC, Manassas, VA, USA) and the positive estrogen receptor (ER) and non-metastatic (MCF-7) (ATCC, Manassas, VA, USA) human breast cancer cell lines were cultured in 75 cm^2 culture flasks (Sarstedt, Nümbrecht, Germany) with Dulbecco's modified Eagle's medium (DMEM) (Cultilab, Campinas, SP, Brazil) supplemented with 10% fetal bovine serum (FBS) (Cultilab, Campinas, SP, Brazil), penicillin (100 IU/mL) and streptomycin (100 mg/mL) (Sigma-Aldrich, St. Louis, MO, USA) in a humidified incubator at 5.0 % CO_2 at 37 ºC.

Cell viability by MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay.

For MTT assay, 5 x 10^4 cells/well were plated in 96-well plates in 100 µL DMEM with 2% FBS. Cells were incubated for 24 hours in starving medium containing 2 % FBS with different concentrations (0.01 mM, 0.05 mM, 0.1 mM, 0.5 mM, 1 mM, 3 mM, 5 mM and 8 mM) of metformin (Sigma-Aldrich, St. Louis, MO, USA) diluted in ultrapure water, and different concentrations (2.5 µM, 10 µM, 25 µM, 50 µM e 100 µM) of Y27632 (Sigma-Aldrich, St. Louis, MO, USA) were diluted in phosphate-buffered saline (PBS). 10 µL of MTT solution (Vibrant MTT Cell Proliferation Assay Kit, Invitrogen - Life Technologies, Eugene, OR, USA) was added to each well and the plates were incubated at 37 ºC for an additional 4 hours. Absorbance was measured at 540 nm by ELISA plate reader (Thermo Fisher Scientific - Waltham, MA USA). Medium with 2% FBS was used as background and subtracted from the samples. Cell viability (%) was calculated for all groups and compared to control sample (untreated). All treatments were performed in triplicate.

In vitro experiment groups

The cells were divided into five groups for each cell line and received treatments in the concentration required to inhibit cell growth as determined by the MTT assay. Group I: used as a control containing cells cultured in own culture, no additives. Group II: treated with TGF-β1 5 ng/mL for EMT induction (Schneider et al, 2012; Zhang et al, 2011). Group III: treated with metformin, after EMT induction by TGF-β1; Group IV: treated with ROCK-1 inhibitor, Y27632, 2 hours after the EMT induction by TGF-β1 and Group V: treated with metformin and Y27632 induction of EMT by TGF-β1. The cells were incubated with treatments for 24 hours.

Migration and invasion assay

The migration and invasiveness of MDA-MB-231 and MCF-7 cells was tested in 24 well plates with 8 μm inserts *BDMatrigel*™ membranes (Becton Dickinson Labware®). In the upper compartment of the chamber, approximately 2.5 x 10^4 cells/insert were added into culture medium without serum, while to the other compartment was added 750 μL of culture medium with the chemoattractant, 0.5% and 10% FBS respectively to negative and positive controls. 10% FBS was associated to different concentrations of melatonin.

After these membranes had been washed and impermeabilized, they were stained with hematoxylin to detect the migrant cells. The counting was performed with an inverted optic microscope by putting the insert over a plate containing glycerol at 50%. The invasion rate was calculated by dividing the average number of treated cells that migrated and invaded the matrigel membrane by the average number cells that migrated in positive control.

Immunocytochemistry staining

The cells were plated in adherent individual well of a slide in a concentration of 6 x 10^4 cells and treated for 24 hours. They were then fixed in 4% paraformaldehyde solution. The primary

antibodies ROCK-1 (Santa Cruz Biotechnology, Dallas, TX, USA), Vimentin (DAKO, Carpinteria, CA, USA), E-cadherin (Cell Signaling Technology, Danvers, Massachusetts, USA), CD24 (Merck Millipore, Darmstadt, Germany) and CD44 (Merck Millipore, Darmstadt, Germany) were used to delineate the expression of corresponding antigens. The secondary and tertiary antibodies were provided by kit STARR TREK Universal HRP Detection (Medical BioCare®, Concord, CA, USA). The reveal was performed with chromogenic substrate (3,3'-diaminobenzidine tetrahydrochloride - DAB) (Medical BioCare®, Concord, CA, USA) and the counter-staining with Harry's hematoxylin. All reactions were accompanied by a positive control for the tested antibody and a negative control.

Evaluation of immunocytochemistry

Proteins were quantified in 60 points regarding three different photomicrography and analyzed based on the intensity of the staining by ImageJ Software (NIH, Bethesda, MD, USA). Each photograph was divided into four quadrants, and 20 spots (small circular ROI) were randomly selected (avoiding the nucleus) in each photomicrography comparing the treatment groups with controls. A negative control section of the corresponding staining was used for measuring background activity. The slides were observed under a 40X microscope Nikon Eclipse E200 and photographed. The values were obtained in arbitrary units (a.u) and showed the average optical density (D.O.M.) for each sample.

Statistical Analysis

All data were expressed as mean ± standard error of mean (SEM). The results were previously submitted for descriptive analysis to determine the normal range. For samples with normal distribution Analysis of Variance (ANOVA) was used, followed by Bonferroni's test or Student's t-test with GraphPad Prism4 (San Diego, CA, USA). Values of $p \leq 0.05$ were considered statistically significant.

RESULTS

Metformin and ROCK-1 inhibitor reduce cell viability of breast cancer cells

The MTT assay was performed after incubation for 24 hours with different concentrations of metformin in order to confirm the effect of this treatment on the viability of MDA-MB-231 and MCF-7 cell lines. Our results showed that MCF-7 cells demonstrated more sensitivity to metformin treatment than MDA-MB-231 cells. For MDA-MB-231 cell line only 8 mM of metformin significantly decreased the cell viability compared to the control group ($p < 0.05$; **Figure 1A**). There was a significant reduction of cell viability of MCF-7 cells after treatment with 0.5 mM to 8 mM of metformin ($p < 0.05$; **Figure 1B**). Based on the results of MTT assay, we have selected 8 mM concentration of metformin as the standard dose for subsequent experiments.

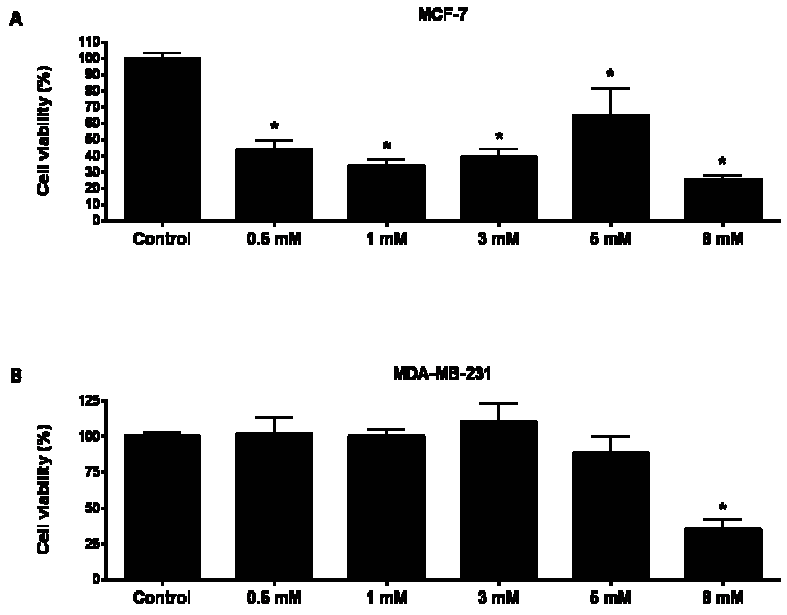

Figure 1. Inhibitory effect of metformin on viability of breast cancer cell lines. A. MCF-7 cells were treated with 0.5 mM to 8mM of metformin for 24 hours and **B.** MDA-MB-231 cells were treated with 0.5 mM to 8mM of metformin for 24 hours and cell viability was measured by MTT assay. The first column corresponds to control group and the remaining columns correspond to cells treated with the metformin as the indicated concentrations. Each column is the mean ± standard error of triplicate. (*$p < 0.05$). Significant values in ANOVA followed by Bonferroni.

Cell viability was also analyzed after treatment with Y27632 at 2.5 µM to 100 µM for 24 hours. For MDA-MB-231 cell line, only the 10 µM concentration was able to produce a statistically significant decrease in cell viability compared to control group ($p < 0.05$; **Figure 2A**). On the other hand, for MCF-7, except the 2.5 µM concentration, all the others significantly reduced the cell viability, specially 10 µM of Y27632 ($p < 0.05$; **Figure 2B**).

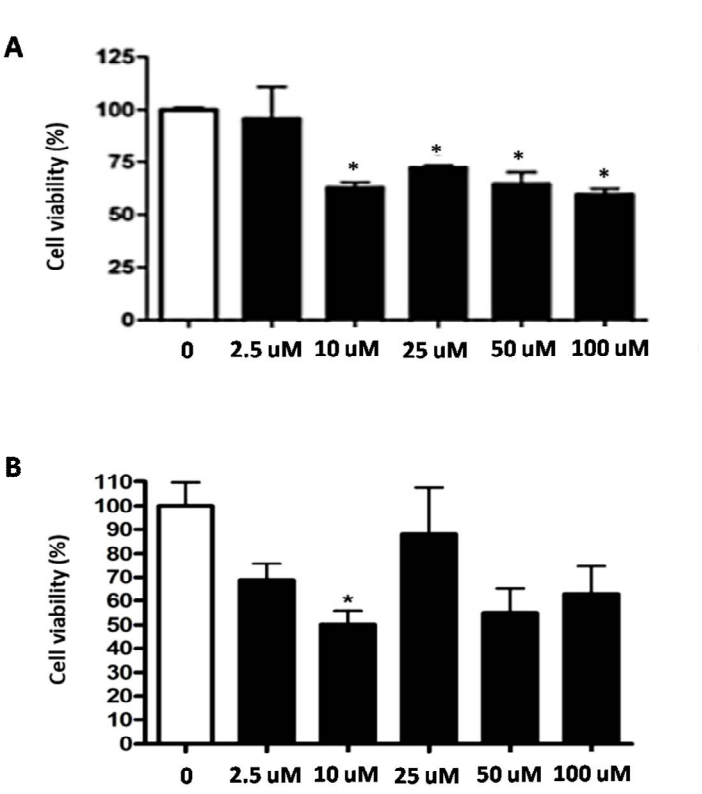

Figure 2. Inhibitory effect of Y27632 on viability of breast cancer cell lines. A. MCF-7 cells were treated with 2,5 µM to 100 µM of Y27632 for 24 hours and **B.** MDA-MB-231 cells were treated with 2.5 µM to 100 µM of Y27632 for 24 hours and cell viability was measured by MTT assay. The first column corresponds to the control group and the remaining columns correspond to cells treated with the ofY27632 as the indicated concentrations. Each column is the mean ± standard error of triplicate. (*p < 0.05). Significant values in ANOVA followed by Bonferroni.

Metformin and Y27632 treatments decrease cell migration and invasion in MDA-MB-231 cell line

To verify whether metformin or Y27632 alone or in combination would decrease the migration and invasive potential of breast cancer cell lines, both cell lines were subjected to migration and invasion assay. The negative control (7.618 ± 0.7557%) of MDA-MB-231 cell line had a significant difference when compared to the positive control, which in turn indicates the validity of the results ($p < 0.05$; **Figure 3A**). For this assay, the positive control was compared with treatment results. Even after the induction of EMT by TGFβ-1 (69.55 ± 1.123%), the metformin treatment was also able to inhibit cell invasion and migration (53.16 ± 3.421%; $p < 0.05$; **Figure 3B**). The same was observed upon treatment with a ROCK inhibitor (45.18 ± 1.225; $p < 0.05$; **Figure 3B**) and especially after treatment with metformin associated Y27632 there was a 59.07% 2.247 ($p < 0.05$) reduction for MDA-MB-231 cells (**Figure 3B**).

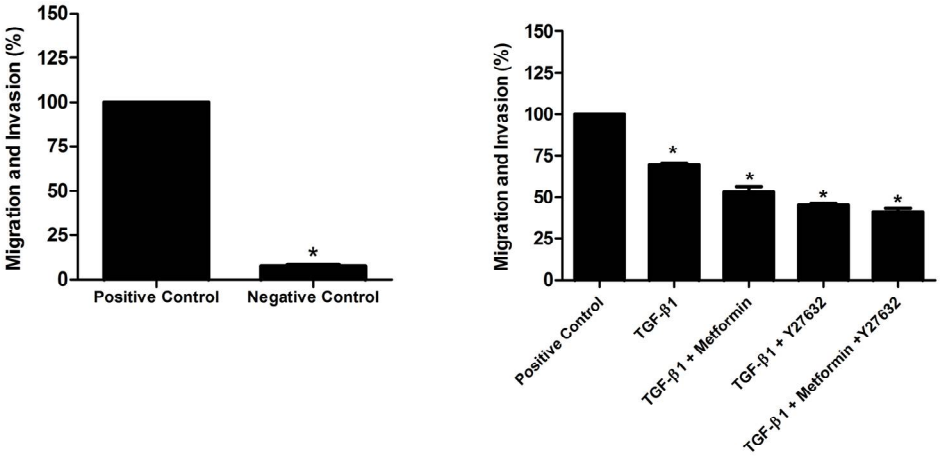

Figure 3. Migration and invasion assay of metastatic cell line MDA-MB-231. A. Comparison between positive and negative control (* p<0.05). **B.** Comparison between the control, with 10% FBS without treatment, and cells treated with TGF-β1, metformin and Y27632 (* p < 0.05). Significant values in ANOVA followed by Bonferroni´s test.

For MCF-7 cell line, a negative control assay showed a reduction in the migration of the cells compared to that of the positive control ($p < 0.05$; data not shown). However, after induction of

EMT by TGFβ-1, the treatment with metformin and Y27632 inhibitor had no response in cell invasion and migration when compared to the positive control group (data not shown).

Metformin and Y27632 treatments decreased ROCK-1 protein expression

Protein analysis showed a statistically significant decrease of ROCK-1 expression after induction of EMT by TGF-β1 (201.0 ± 1.157 a.u.) compared to the control group (212.7 ± 1.576 a.u.; $p < 0.001$; **Figure 4A**) in MDA-MB-231 cell line. In the same way, ROCK-1 protein expression had a statistically significant reduction after metformin (199.9 ± 0.9173 a.u.; $p < 0.001$) and Y27632 (201.7 ± 0.8981; $p < 0.001$) treatments, especially in combination with metformin and Y27632 inhibitor (197.5 ± 0.7615 a.u.; $p < 0.001$; **Figure 4A**). Although protein expression seems to reduce after all treatments compared to the TGF-β1 group, there was no significant difference ($p > 0.05$). The protein expression of ROCK-1 after EMT induction by TGF-β1 and treatment with metformin and specific inhibitor Y27632 in MDA-MB-231 cells is shown in **Figure 4B**.

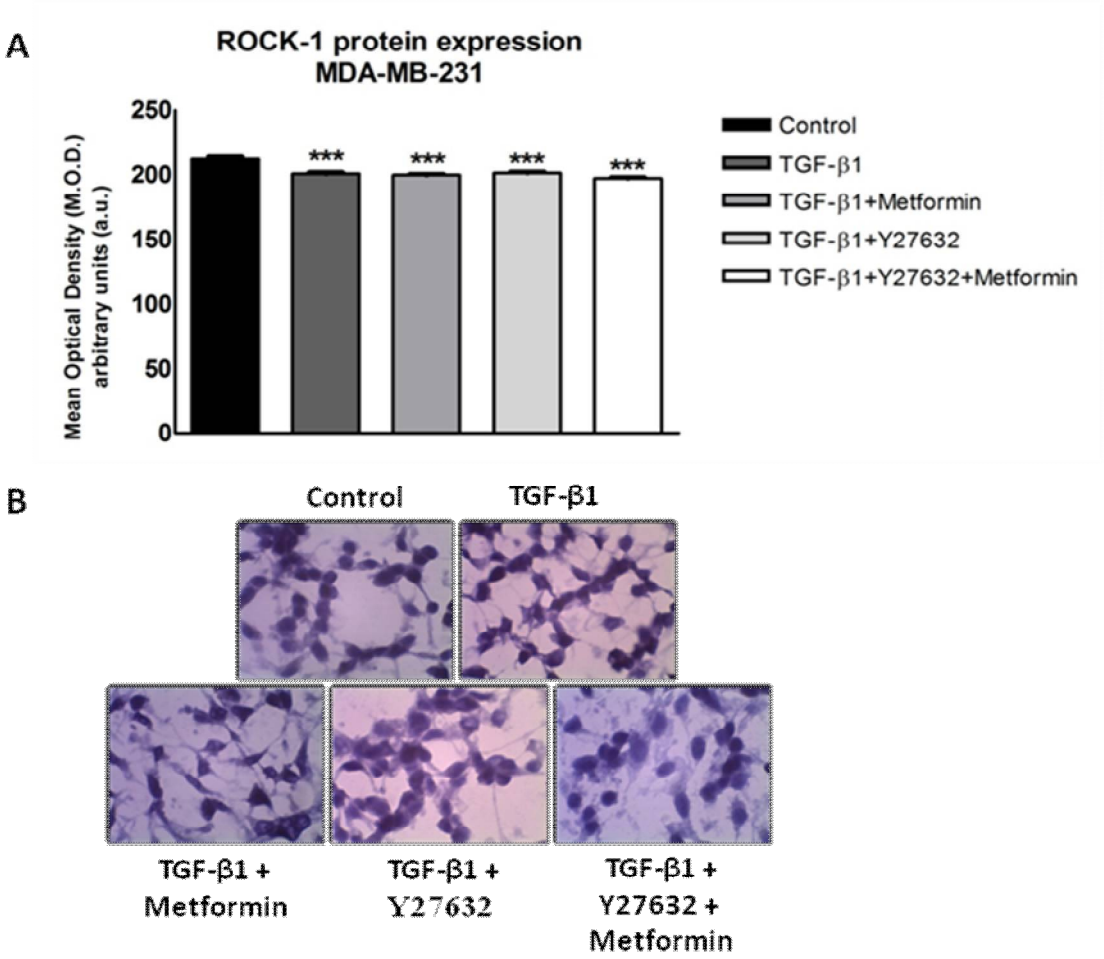

Figure 4. ROCK-1 protein expression in MDA-MB-231 cell line. A. Semi-quantitative analysis of ROCK-1 protein expression by densitometry. **B**. ROCK-1 immunostaining in MDA-MB-231 cell line. Statistically significant differences verified by ANOVA followed by Bonferroni's test. *** p< 0.001 compared to control group.

For MCF-7 cell line, the induction of EMT by TGF-β1 (194.7 ± 1.490 a.u.) significantly increased the protein expression of ROCK-1 compared to the control group (175.4 ± 2.032 a.u.; $p <$ 0.001; **Figure 5A**). However, comparing all treatments with the control group, only the treatment with Y27632 reduced ROCK-1 protein expression (150.6 ± 1.772; $p < 0.001$; **Figure 5A**). Furthermore, the treatment with metformin (174.4 ± 1.440 a.u.; $p < 0.001$) and specific inhibitor Y27632 (150.6 ± 1.772 a.u.; $p < 0.001$) or in combination (176.2 ± 0.9893 a.u.; $p < 0.001$), decreased the protein expression of ROCK-1 after EMT induction by TGF-β1 compared to the TGF-β1 group ($p < 0.001$; **Figure 5A**). The protein expression of ROCK-1 is shown in **Figure 5B**.

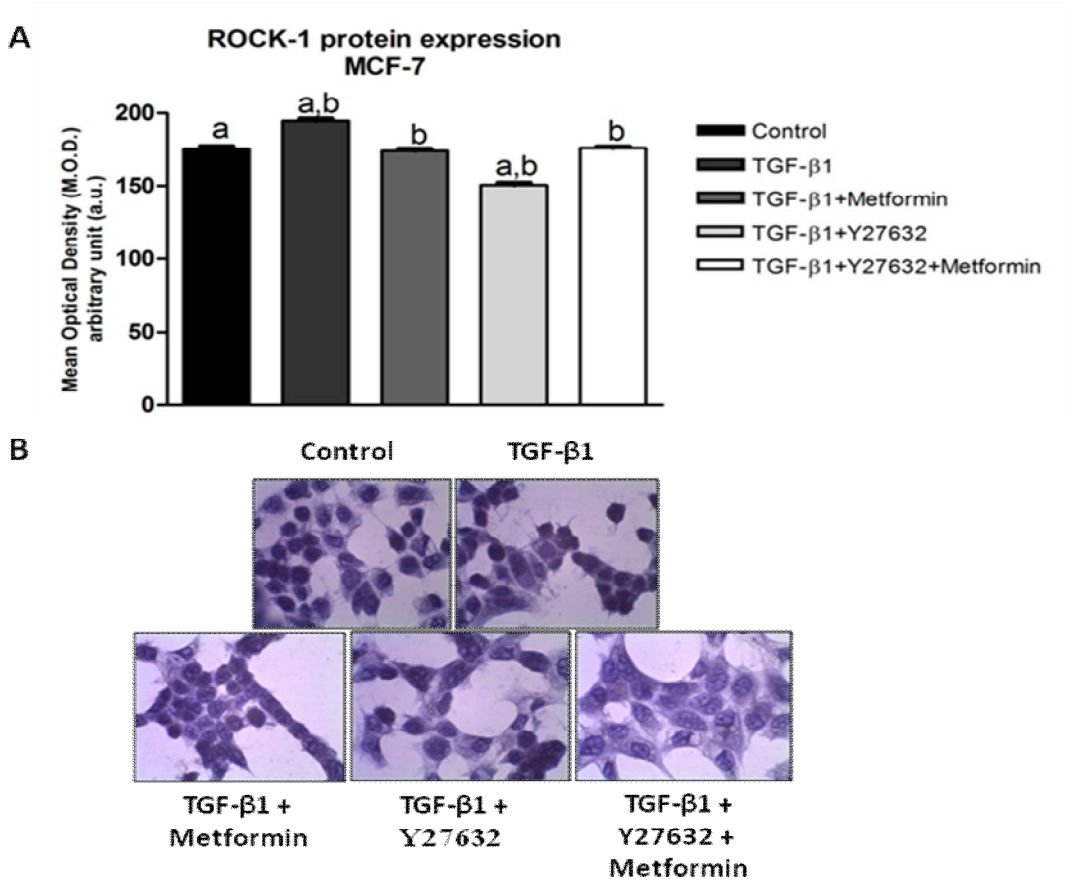

Figure 5. ROCK-1 protein expression in MCF-7 cell line. A. Semi-quantitative analysis of ROCK-1 protein expression by densitometry. **B**. ROCK-1 immunostaining in the MCF-7 cell line. Statistically significant differences, verified by ANOVA followed by Bonferroni's test. **(a)** $p < 0.001$ compared to control group and **(b)** $p < 0.001$ compared to TGF-β1 group.

Metformin and Y27632 treatments decreased vimentin protein expression

The vimentin protein expression was determined by immunocitochemistry to verify the capacity of metformin and Y27632 treatments to inhibit the EMT process in MDA-MB-231 and MCF-7 cell lines. In MDA-MB-231 cell line, there was no difference of vimentin protein expression between control group (206.4 ± 1.154 a.u.) and TGF-β1 (201.2 ± 1.156 a.u.; **Figure 6A**). However, a statistically significant reduction of vimentin protein expression was observed in the groups treated with metformin (163.3 ± 2.441 a.u.; $p < 0.001$), Y27632 (170.5 ± 1.553 a.u.; $p < 0.001$) and

especially metformin and Y27632 associated (156.6 ± 3.055 a.u.; $p < 0.001$) compared to both control and TGF-β1.

In the same way, the vimentin protein expression in MCF-7 cell line decreased after all treatments (169.1 ± 2.356 a.u., 162.9 ± 2.314 a.u., respectively), being more effective when drugs were combined (153.3 ± 2.323 a.u.) compared to both control (178.2 ± 1.226 a.u.; $p < 0.05$) and TGF-β1 (180.5 ± 1.181 a.u.; $p < 0.001$; **Figure 7A**). On the other hand, the induction of EMT process by TGF-β1 did not increase the vimentin protein expression as expected. The protein expression of vimentin after EMT induction by TGF-β1 and treatment with metformin and specific inhibitor Y27632 in MDA-MB-231 and MCF-7 cells are shown in **Figure 6B** and **Figure 7B**, respectively.

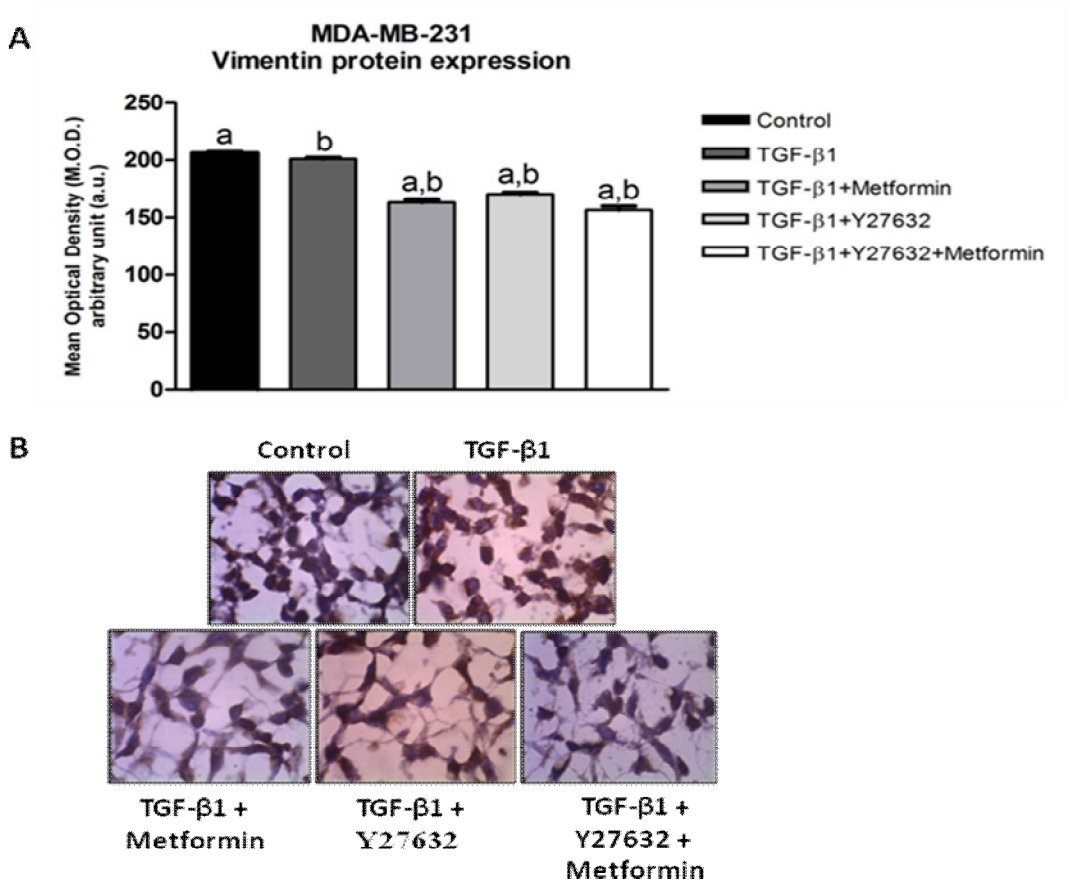

Figure 6. Vimentin protein expression in MDA-MB-231 cell line. A. Semi-quantitative analysis of vimentin protein expression by densitometry. **B**.Vimentinimmunostaining in the MDA-MB-231 cell line. Statistically significant differences, verified by ANOVA, followed by Bonferroni's test. **(a)** $p < 0.001$ compared to control group and **(b)** $p < 0.001$ compared to TGF-β1 group.

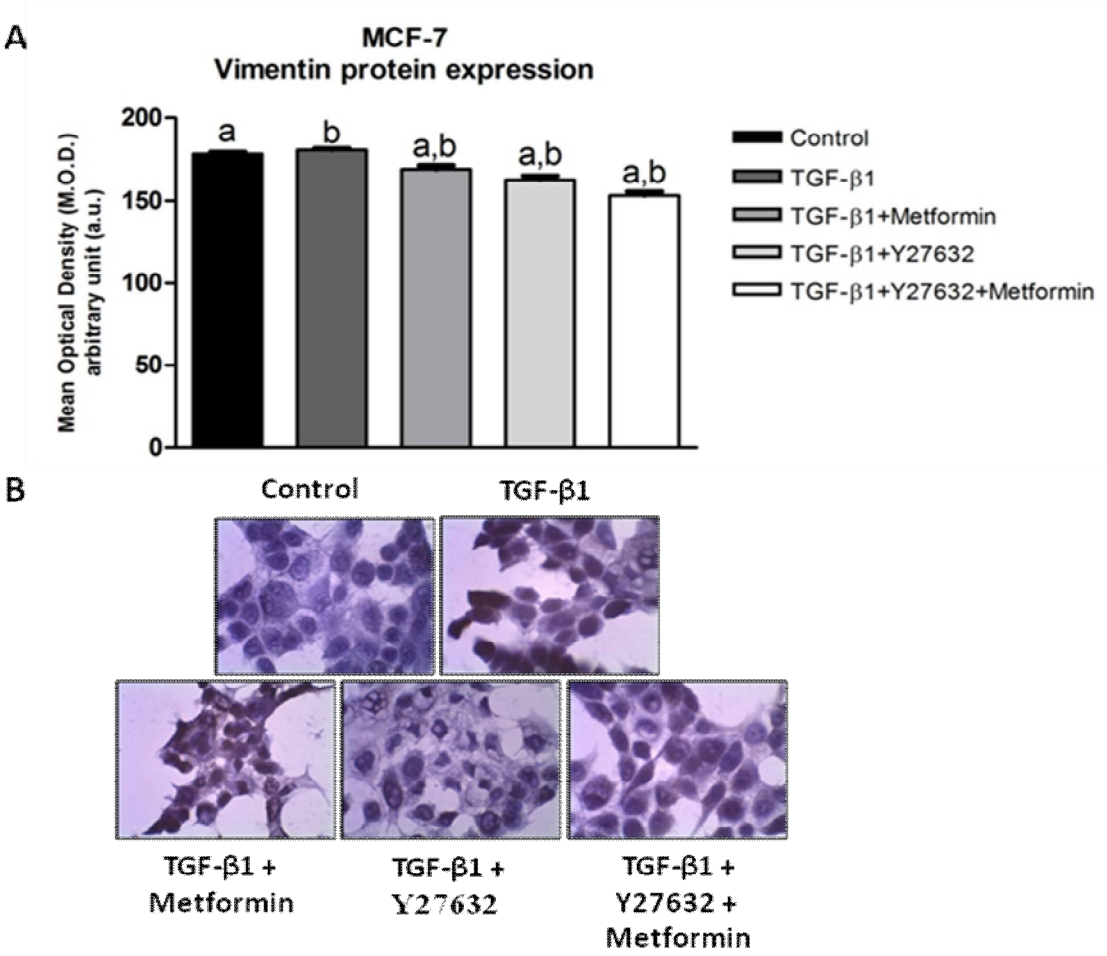

Figure 7. Vimentin protein expression in MCF-7 cell line. A. Semi-quantitative analysis of vimentin protein expression by densitometry. **B**.Vimentinimmunostaining in the MCF-7 cell line. Statistically significant differences, verified by ANOVA followed by Bonferroni's test. **(a)** $p < 0.05$ compared to control group and **(b)** $p < 0.001$ compared to TGF-β1 group.

Metformin and Y27632 treatments increased E-cadherin protein expression

The E-cadherin protein expression in MDA-MB-231 cell line significantly decreased after EMT induction by TGF-β1 (132.7 ± 2.069 a.u.) compared to the control group (145.3 ± 1.853 a.u.; $p < 0.001$; **Figure 8A**). Although protein expression increased only after metformin (143.6 ± 1.774 a.u.; $p < 0.001$) and Y27632 (145.7± 1.961 a.u.; $p < 0.001$) treatments compared to TGF-β1 ($p < 0.001$; **Figure 8A)**, but no significant difference was observed when compared to the control group (*p*

> 0.05). The protein expression of E-caderin after EMT induction by TGF-β1 and treatment with metformin and specific inhibitor Y27632 in MDA-MB-231 cells is shown in **Figure 8B**.

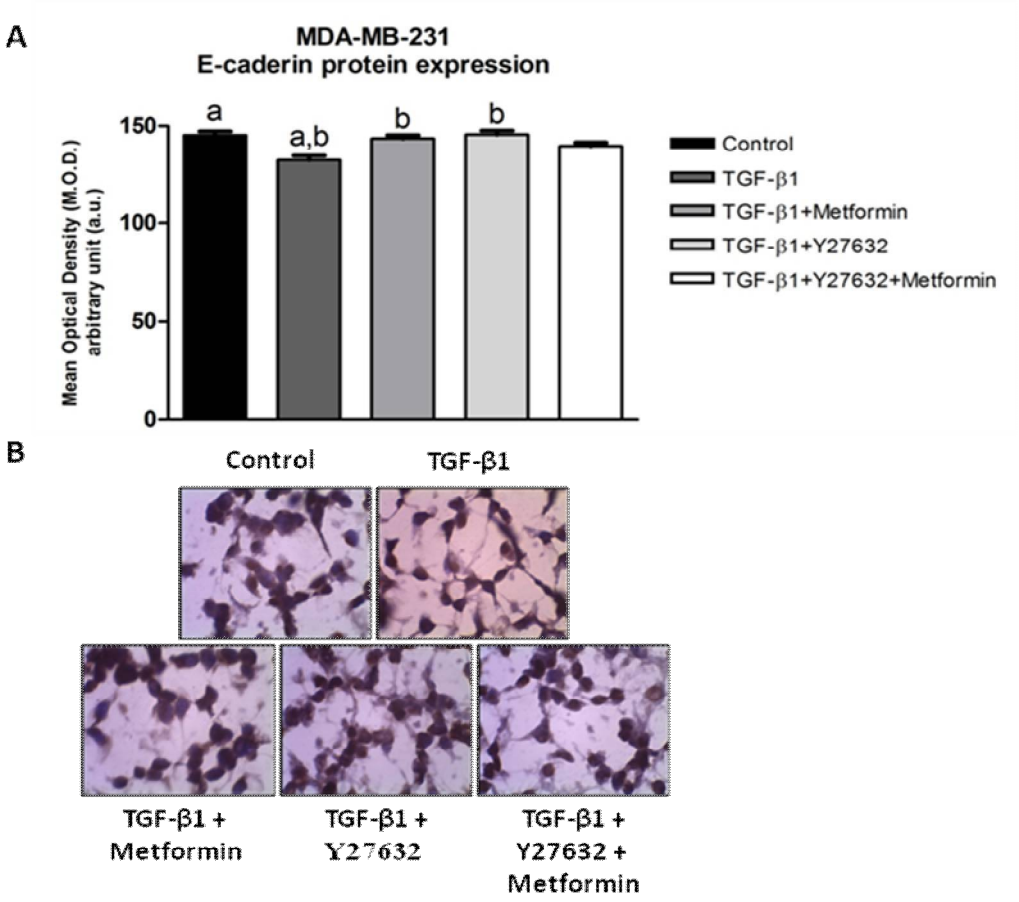

Figure 8. E-cadherin protein expression in MDA-MB-231 cell line. A. Semi-quantitative analysis of vimentin protein expression by densitometry. **B.** E-cadherin immunostaining in the MDA-MB-231 cell line. Statistically significant differences, verified by ANOVA followed by Bonferroni's test. **(a)** $p < 0.001$ compared to control group and **(b)** $p < 0.001$ compared to TGF-β1 group.

For MCF-7 cell line, E-cadherin expression was reduced after all treatments and especially when EMT process was induced with TGF-β1 (123.9 ± 3.221 a.u.) when compared to the control group (177.9 ± 1.286 a.u.; $p < 0.001$). Furthermore, there was a significantly increased protein expression only when treated with Y27632 (160.7 ± 2.183 a.u.; $p < 0.001$) compared to TGF-β. The protein expression of E-caderin is shown in **Figure 9B**.

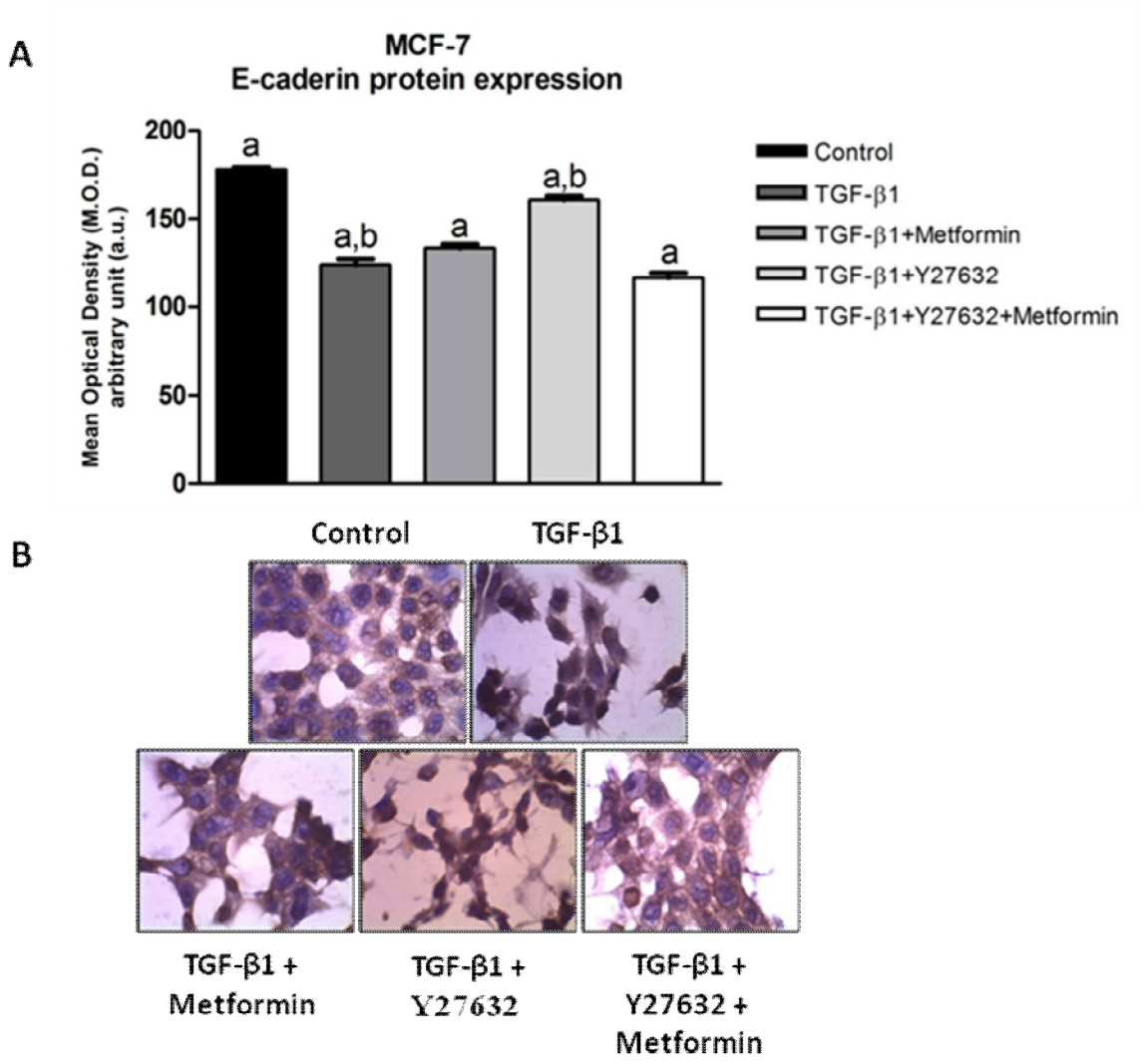

Figure 9. E-cadherin protein expression in MCF-7 cell line. A. Semi-quantitative analysis of vimentin protein expression by densitometry. **B**. E-cadherin immunostaining in the MCF-7 cell line. Statistically significant differences, verified by ANOVA followed by Bonferroni's test. **(a)** $p < 0.001$ compared to control group and **(b)** $p < 0.001$ compared to TGF-β1 group.

Metformin and Y27632 treatments decreased CD44 and CD24 protein expression

Incubation with TGF-β1 (199.9 ± 1.198 a.u.) showed a tendency to increase CD44 protein expression compared to the control group (193.2 ± 1.307 a.u.; $p > 0.05$; **Figure 10A**), confirming the participation of this factor in EMT process, as demonstrated in the literature. When we compared the treatment with metformin (164.5 ± 1.702 a.u.), inhibitor ROCK-1 Y27632 (150.5 ± 2.542 a.u.) and

combined drugs (157.0 ± 1.706 a.u.) to both control and TGF-β1 groups, there was a significant decrease in CD44 protein expression ($p > 0.001$; **Figure 10A)**. For MCF-7 cell lines, there was a decreased CD44 protein expression after all treatments, including the incubation with TGF-β1 (149.7 ± 1.817 a.u.), compared to the control (186.1 ± 0.8198 a.u.; $p < 0.001$; **Figure 11A**). The immunostaining of CD44 protein expression after TGF-β1 induction and treatment with metformin and Y27632 inhibitor in MDA-MB-231 and MCF-7 cells are shown in **Figure 10B** and **Figure 11B**, respectively.

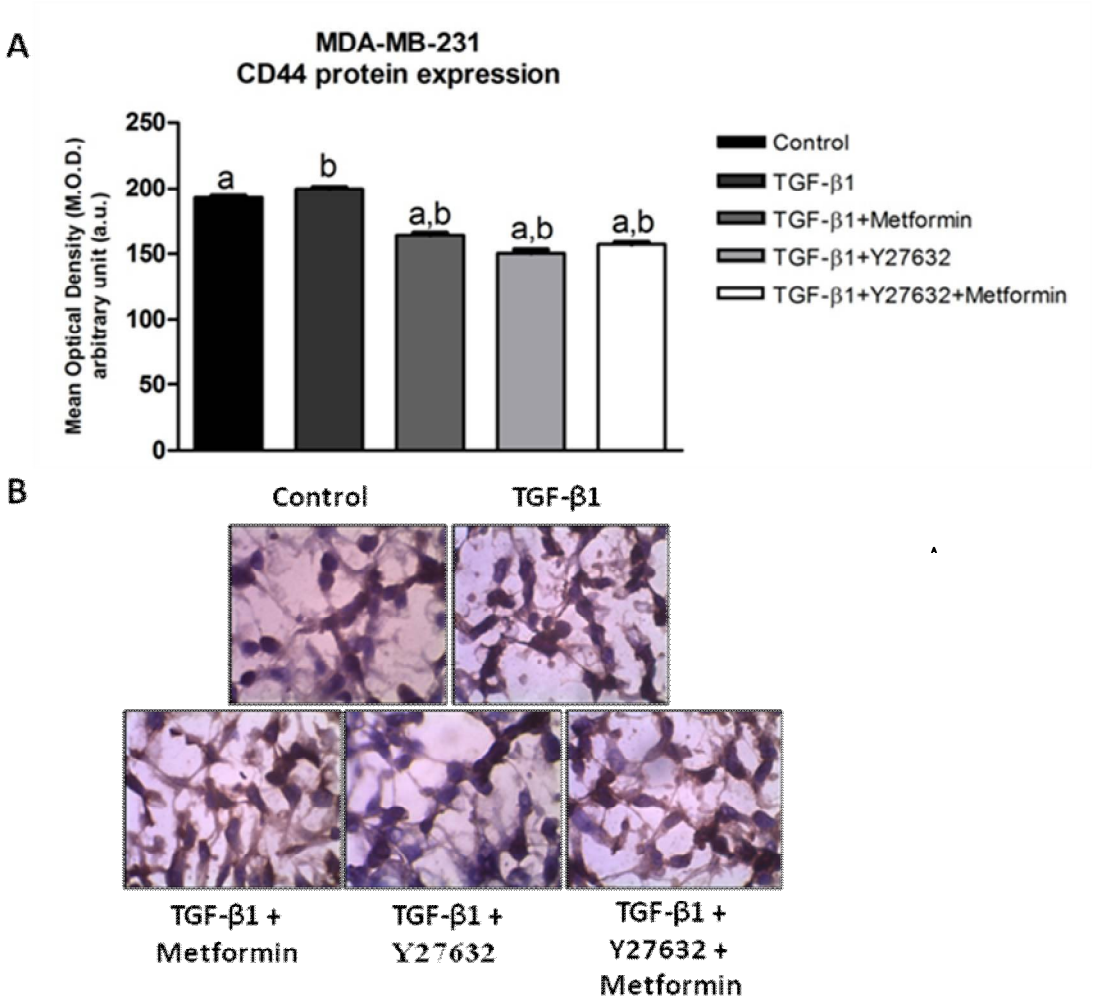

Figure 10. CD44 protein expression in MDA-MB-231 cell line. A. Semi-quantitative analysis of CD44 protein expression by densitometry. **B**. CD44 immunostaining in the MDA-MB-231 cell line. Statistically significant differences, verified by ANOVA followed by Bonferroni's test. **(a)** $p < 0.001$ compared to control group and **(b)** $p < 0.001$ compared to TGF-β1 group.

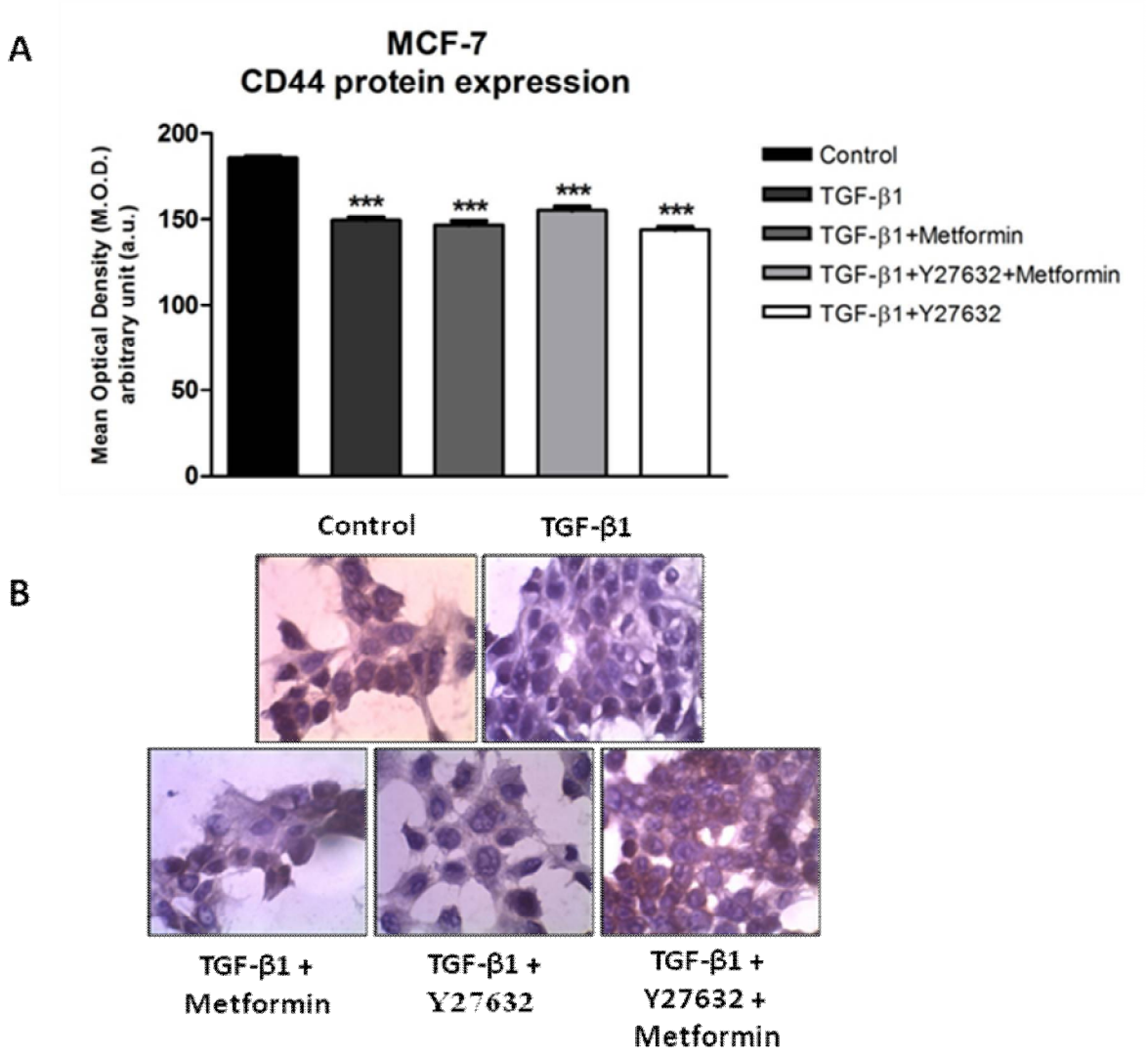

Figure 11. CD44 protein expression in MCF-7 cell line. A. Semi-quantitative analysis of CD44 protein expression by densitometry. **B**. CD44 immunostaining in the MCF-7 cell line. Statistically significant differences, verified by ANOVA followed by Bonferroni's test. *** $p < 0.001$ compared to control group.

The protein expression of CD24 in MDA-MB-231 cell line decreased after all treatments compared to the control group (193.7 ± 1.160 a.u.; $p < 0.001$; **Figure 12A**). However, when compared to the TGF-β1 group (175.6 ± 1.725 a.u.), only the cells treated with metformin (158.3 ± 2.046 a.u.) and Y27632 inhibitor (158.8 ± 2.099 a.u.) separated significantly decreased the CD24 protein expression ($p > 0.001$; **Figure 12A)**. For MCF-7 cell line, CD24 expression was decreased after EMT induction by TGF-β1 (141.4 ± 1.083 a.u.) and treatment with metformin (151.6 ± 2.283

a.u.), Y27632 (157.7 ± 2.149 a.u.) and combined drugs (141.4 ± 1.083 a.u.; $p < 0.001$; **Figure 13A**). The analysis of CD24 protein expression after TGF-β1 induction and treatment with metformin and Y27632 inhibitor in MDA-MB-231 and MCF-7 cells are shown in **Figure 12B** and **Figure 13B**, respectively.

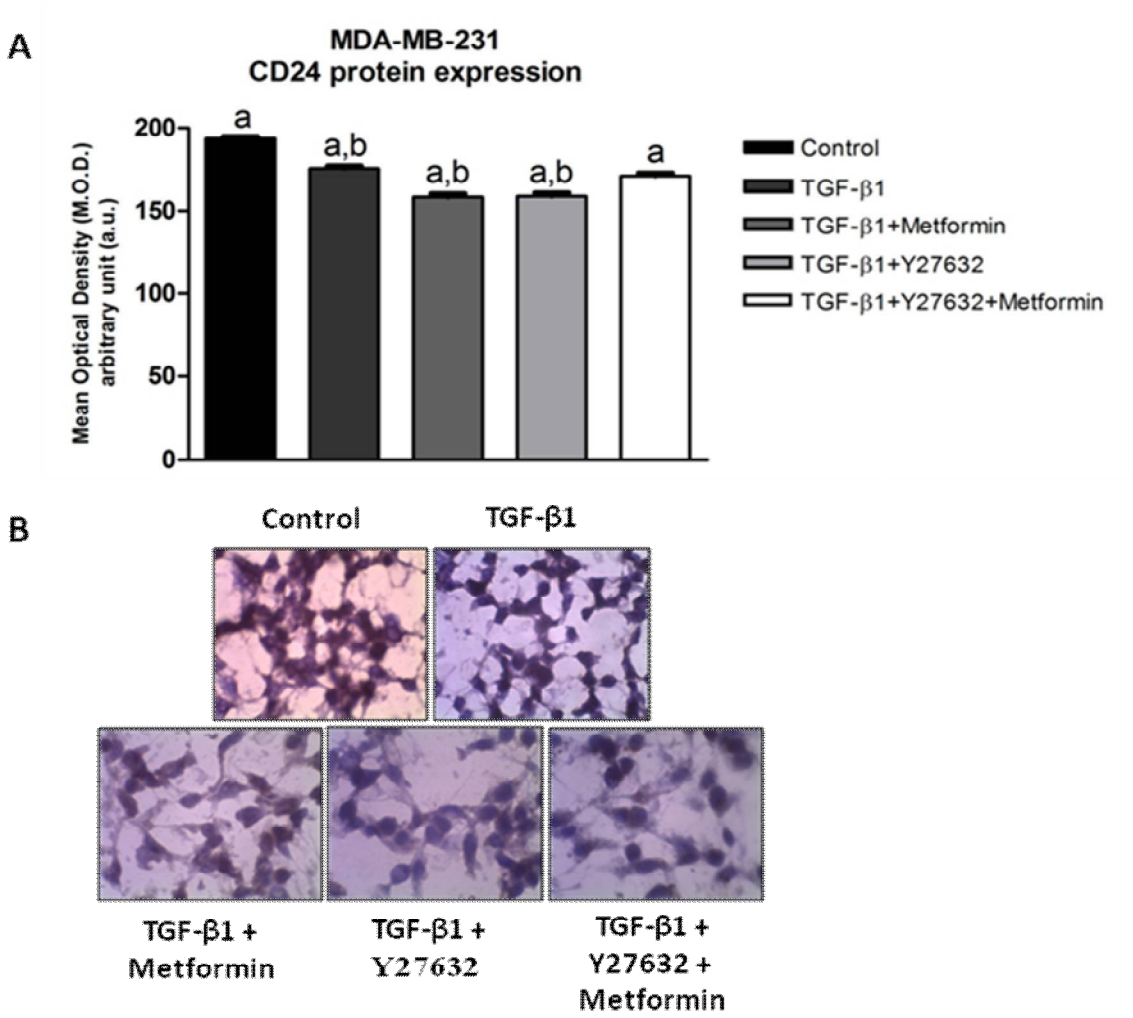

Figure 12. CD24 protein expression in MDA-MB-231 cell line. A. Semi-quantitative analysis of CD24 protein expression by densitometry. **B**. CD24 immunostaining in the MDA-MB-231 cell line. Statistically significant differences, verified by ANOVA followed by Bonferroni's test. **(a)** $p < 0.001$ compared to control group and **(b)** $p < 0.001$ compared to TGF-β1 group.

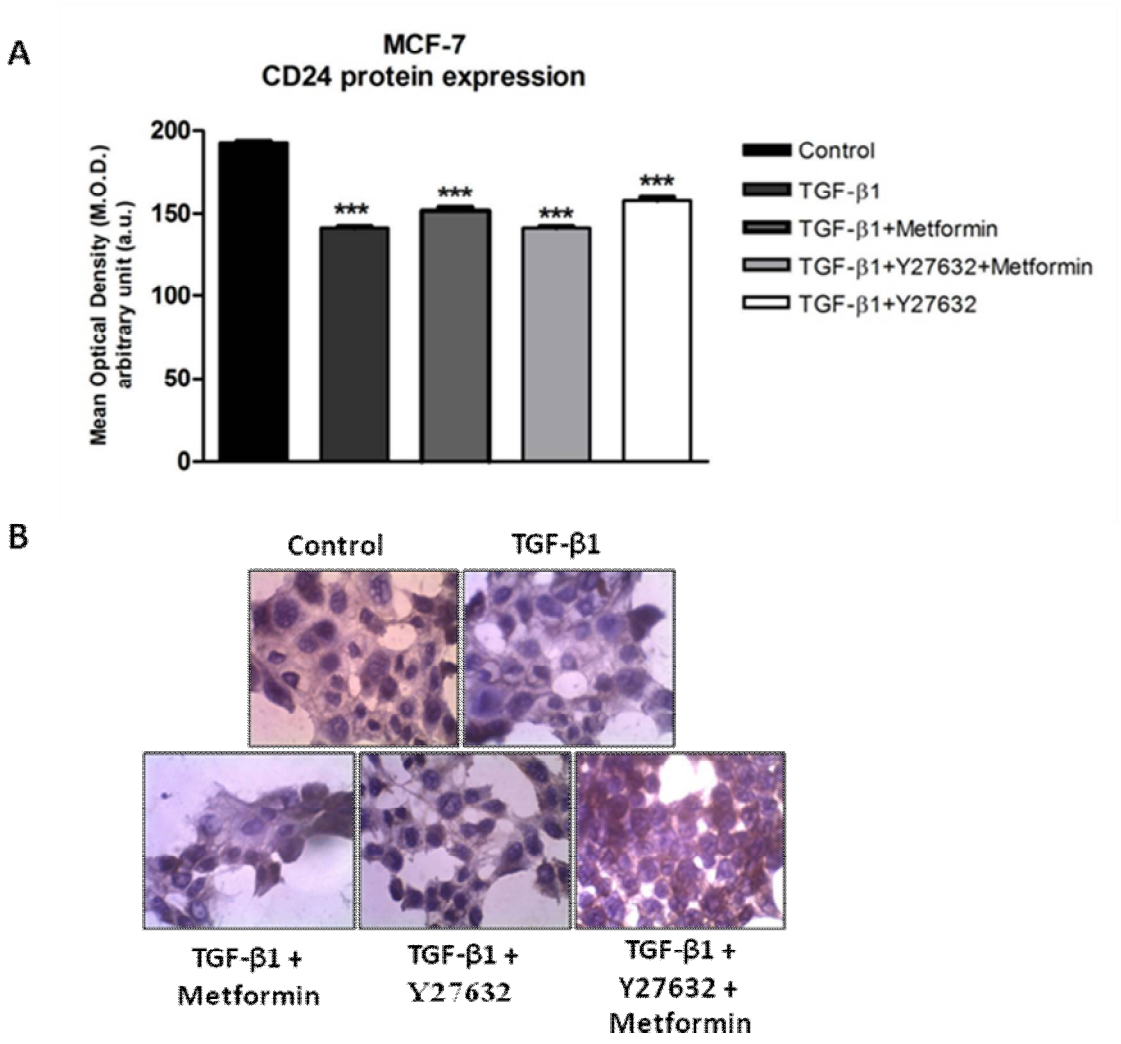

Figure 13. CD24 protein expression in MCF-7 cell line. A. Semi-quantitative analysis of CD24 protein expression by densitometry. **B**. CD24 immunostaining in the MCF-7 cell line. Statistically significant differences, verified by ANOVA followed by Bonferroni's test. *** $p < 0.001$ compared to control group.

DISCUSSION

Cancer cells transit from epithelial cells to mesenchymal cells through EMT, which is a critical step required for metastasis (YANG et al., 2014). In this process, cells lose their polarized epithelial characteristics and acquire mesenchymal properties that consequently confer an aggressive phenotype to cancer cells (LI et al., 2015). The AMPK signaling pathway inhibits cancer cell

migration and EMT via both TGF-β-dependent and-independent mechanisms (LIN et al., 2015) and the activation of AMPK by metformin is known to contribute to its anti-tumor effects in reverse EMT process in human breast cancer cells (LIU et al., 2015). In this respect, our results show that metformin and Y27632 are capable of decreasing cell viability in MDA-MB-231 and MCF-7 cell lines after 24 hours of treatment, demonstrating its important oncostatic role on cancer. Similarly, Phoenix et al. (2010) had already demonstrated the inhibitory effect of metformin in breast cancer on tumor growth in vitro and in vivo. Experiments with MCF-7 and MDA -MB -231 also showed the anti-proliferative effect of metformin is dose and time dependent through by MAPK signaling pathway (HADAD et al., 2014; ZORDOKY et al., 2014).

In addition, Queiroz et al. (2014) demonstrated that treatment with metformin was able to decrease the viability considerably after 72 hours of treatment with 20 mM of metformin in MCF-7 cell line; and this effect was due to cell cycle arrest in the G 0 -G 1 phase, cyclin D1 inhibition and induction of apoptosis. The antiproliferative action of metformin has also been reported in lung cancer cells A549 and HCC827 at concentrations 5 mM and 10 mM (ZHAO et al., 2014); in renal cell carcinoma 786-S, and Caki-2 after treatment with 10 mM metformin for 24, 48 and 72 hours (ZHANG et al., 2015a), and nasopharyngeal carcinoma by inhibiting cell invasion and metastasis via regulation of E-cadherin expression and metalloproteinase matrix 9 (MMP -9) (SUN et al., 2014).

EMT can be induced or regulated by various growth factors, among which TGF-β1 inhibits tumor growth in early stages of tumorigenesis and promotes tumor progression in late stages (LIN et al., 2015). Its activation signaling pathway begins from TGF-β1 binding to and activation of the receptor complex on the cell surface, which consists of two type I and two type II transmembrane serine/threonine kinase receptors that leads to the repression of epithelial genes (e.g. E-cadherin) and activation of mesenchymal genes (e.g. N-cadherin) (GU et al., 2016).

The incubation with TGF-β1 didn`t change the invasion and migration of MDA-MB-231 and MCF-7 cell lines; on the other hand, there was a decrease in the invasion of MDA-MB -231 cell line when treated with metformin and Y27632. In the same way, Kidera et al. (2010) and Borin et al.

(2015) demonstrated that treatment with Y27632 resulted in decreased metastasis, especially in breast cancer in vitro and in vivo. Unbekandt et al (2014) demonstrated that MDA-MB-231 cell invasion through Matrigel was more efficiently blocked by Y27632. Similarly, Mao et al. (2013) showed that metformin was able to inhibit the growth and metastasis in MDA-MB-231 xenograft model. Other authors have also shown that metformin inhibited *in vivo* the growth and metastasis of ovarian cancer (Wu et al., 2012) and Cerezo et al. (2013) demonstrated that metformin inhibited the invasion of melanoma cells through regulatory factors EMT showing the crucial role of AMPK in this process.

Rho-associated kinase (ROCK), a downstream of RhoA small GTPase (RhoA), regulates a wide variety of ubiquitous biological processes including the acquisition of unlimited proliferation potential, survival and evasion from apoptosis, tissue invasion differentiation, gene expression, and in particular, regulation of cell detachment, cell movement and establishment of metastasis (YANG et al., 2014; ZHANG et al., 2014a), playing a central role in EMT process (ORTIZ-LOPEZ et al., 2009).

Little is known about the interaction between ROCK and TGF-β1 in EMT process. Considering the major findings of Jia et al. (2015), the up-regulation of Rho-ROCK, increased Rho activity, and greater susceptibility toward EMT with TGF-β1 treatment. Our results showed that in MCF-7 line there was an increased expression of ROCK- 1 protein after the induction of EMT by TGF-β1 compared to the control group. According to Xu et al. (2009) TGF-β induces activation of ROCK- 1 in different cell systems. However, most studies have focused on the role of RhoA and its effector kinase ROCK in TGF-β-induced EMT. The activation of RhoA in response to TGF-β in turn results in activation of ROCK and complementary to these observations, TGF-β also directly regulates RhoA activity at the tight junctions of epithelial cells. Yet there is a contrast with the overall activation of RhoA by TGF-β, which may be explained by the spatio-temporal regulation of Rho during EMT.

Only MCF-7 cells treated with Y27632 associated with TGF-β decreased ROCK-1 protein expression compared to the control group, while all treatments decreased ROCK-1 protein expression

compared to the group incubated with TGF-β. On the other hand, MDA-MB -231 cell line showed a decrease in ROCK- 1 expression in all treatment groups. A number of recent studies using cancer cell-lines and mouse metastasis models have shown the ability of ROCK inhibitors to reduce the migratory and invasive properties of adherent tumor cells; and consequently, tumor progression and metastasis, which has generated significant interest in using ROCK inhibitors (e.g. Y27632) as cancer therapies (BHANDARY et al., 2015). It has already been demonstrated in a recently published study (BORIN et al., 2015), that the treatment with Y27632 without TGF-β was able to inhibit the ROCK-1 protein expression in MCF-7 non metastatic cell line.

The TGF-β plays multi-facet roles which are still awaiting a better comprehension in order to develop effective anti-metastasis therapies. More specifically, TGF-β can promote tumorigenesis by activating the EMT process (BIONDINI et al., 2015). Regarding the action of TGF-β1 in the expression of epithelial and mesenchymal markers in breast cancer cell lines, our results showed that after the induction of EMT process by TGF-β1 the expression of E-cadherin decreased and contrary to expectations, the expression of vimentin did not change in either cell line MDA-MB-231 and MCF-7. These results are in agreement with the literature, which demonstrated that EMT induced by TGF-β induces changes by decreased E-cadherin expression and prevents the concomitant appearance of expression of vimentin, events that occur when an epithelial cell is converted into cell mesenchymal (CUFI et al., 2010).

Both MDA-MB -231 and MCF -7 cell lines in the presence of metformin and Y27632 treatments decreased vimentin protein expression, especially when metformin was associated with Y27632, indicating that the treatment was effective in reversing the EMT induced by TGF- β. According to Cufi et al. (2010) metformin prevented the expression of EMT marker (vimentin) in the cytoplasm of canine kidney cells induced by TGF-β1. Similarly, Zhang et al. (2014b) showed that metformin could inhibit TGF-β-induced EMT in prostate cancer cells, as manifested by decreasing of N-cadherin, Vimentin and increasing of E-cadherin and β-catenin at mRNA and protein levels. Wang et al. (2014) induced EMT in the prostate cancer cells through the addition of TGF-β1 and verified

the process by examining EMT-related genes, such as vimentin expression. These results indicated that metformin suppresses EMT and provides further evidence of its anticancer effects.

In addition, Zhang et al. (2014a) treated hepatocarcinoma cell line with Y27632 (a specific inhibitor of ROCK) and analyzed the EMT markers: E-cadherin and vimentin. The results showed that all the expression profiles were attenuated by blockage of ROCK.

Among E-cadherin protein expression, the expression decreased after the EMT induction by TGF-β1; however, only the treatment with Y27632 increased the expression of E-cadherin. These results are in accordance with Wang et al. (2010), who showed that Y27632 increased expression of E-cadherin in squamous tumor cells. Furthermore, Ewald et al. (2008) also demonstrated that the inhibition of ROCK by Y27632 resulted in a phenotype with reduced levels of E-cadherin in intercellular surfaces.

The ability of a single cell to move from the primary tumor to metastatic site is facilitated through the transition from an epithelial phenotype (CD44 +/CD24+) to a mesenchymal phenotype (CD44 +/CD24-) (MANI et al., 2008). There was a tendency to increase the expression of CD44 after TGF-β addition compared to the control in MDA-MB-231. On the other hand, its expression decreased in MCF-7 cells and after all treatments in both cell lines. The high expression of CD44 is a marker for EMT induced by TGF-β and a characteristic of cancer stem cells (CSCs) in breast cancer (MANI et al., 2008) and suppression in CD44 expression is associated with tumor suppression (GODAR et al., 2008). Additionally, studies have shown that metformin may repress the EMT transcriptionally and repress a cell phenotype associated with CSCs in breast cancer (ZHANG et al., 2015b)

Our study showed decreased CD24 expression in all treatment groups. Kristiansen et al. (2003) showed that high expression of this protein can be considered a new marker of breast cancer prognosis. Another study reports that CD24 is highly expressed in ovarian, breast, prostate cancer. It is involved in cell adhesion process and metastasis, being a significant marker of prognosis and

tumor diagnosis (JAGGUPILLI et al., 2012). Our results suggested that the treatment with metformin and Y27632 were effective in decreasing CD44 expression and may contribute to a better prognosis.

The results of this study confirm the findings suggested by the literature that considers metformin and Y27632 effective drugs for in vitro treatment of breast cancer, confirming their effects on decreased cell viability, ability to reduce cell invasion and the ability to reduce protein expression of ROCK-1, vimentin, CD44 and CD24 and increase expression of e-cadherin in metastatic and non-metastatic cell lines. These results may represent a new approach to the treatment of breast cancer.

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COMPETING INTERESTS

The authors declare they have no competing interests.

AUTHOR CONTRIBUTIONS

CL and DAPCZ conceived, designed and interpreted experiments and drafted the manuscript, CL, MGM and LCF performed the immunohistochemistry, TFB and GSF contributed to the realization of MTT assay, MRH and JACPR contributed to the realization of migration and invasion assay. All authors have read and approved the final manuscript.

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DISCUSSÃO

IV. DISCUSSÃO

As células tumorais transitam de células epiteliais para células mesenquimais através da EMT, sendo um processo crítico necessário para ocorrência de metástases (Yang et al., 2014). Neste processo, as células perdem suas características epiteliais e adquirirem propriedades mesenquimais que, consequentemente, conferem um fenótipo agressivo para células tumorais (Li et al., 2015). A via de sinalização da AMPK inibe a migração de células tumorais e a EMT através de ambos os mecanismos de TGF-β-dependentes e independentes (Lin et al., 2015) e a ativação da AMPK pela metformina é conhecida por contribuir para os seus efeitos anti-tumorais no processo da EMT inversa em células de câncer de mama (Liu et al., 2015).

O desenvolvimento de estratégias para maximizar a inibição da EMT para deter a progressão do câncer tem alto potencial para o benefício do paciente. No presente estudo, o tratamento com metformina *in vitro* foi capaz de diminuir a viabilidade das células das linhagens CF41, MDA-MB-231 e MCF-7, bem como a taxa de migração e invasão, demonstrando o seu importante papel oncostático. Existem poucos estudos que demonstram a ação da metformina na EMT. De acordo com nossos resultados, Qu et al. (2014) demonstraram que a metformina inverteu o fenótipo EMT e diminuiu a capacidade de invasão das linhagens celulares de câncer de mama MDA-MB-231 e MCF-7. Além disso, Cerezo et al. (2013) demonstraram que a metformina foi capaz de inibir a invasão de células de melanoma, enquanto Hwang et al. (2010) mostraram ação anti-metastática da metformina em células de fibrossarcoma, inibindo a migração e invasão celular.

Além disso, Queiroz et al. (2014) observaram que o tratamento com metformina foi capaz de diminuir consideravelmente a viabilidade celular após 72 horas de tratamento com 20 mM de metformina na linhagem celular MCF-7; e este efeito foi devido a parada do ciclo celular na fase G0 - G1, inibição da ciclina D1 e indução de apoptose. A ação antiproliferativa de metformina também foi avaliada em células do câncer de pulmão A549 e HCC827, em concentrações de 5 mM e 10 mM

(Zhao et al, 2014.) e em carcinoma de células renais 786-S e Caki-2 após o tratamento com 10 mM de metformina por 24, 48 e 72 horas (Zhang et al., 2015a),

Vários mecanismos da ação antitumoral da metformina foram descritos, especialmente um efeito direto sobre a ativação da AMPK, o que resulta na inibição da via de sinalização da mTOR (Zhang et al., 2014). Tem sido demonstrado que a ativação da AMPK inibe a EMT induzida por TGF-β em células de câncer de mama (Cufí et al., 2010). O estudo de Qu et al. (2014) demonstrou que o tratamento com metformina ativou a via de sinalização da AMPK em células tumorais de câncer de mama com receptor de estrógeno positivo (ER+) que foram resistentes à quimioterapia para 5-fluorouracilo (MCF-7/5-FU), assim como a linhagem triplo negativa MDA-MB-231, invertendo o fenótipo resistente a múltiplos fármacos para além dos efeitos sobre o processo de EMT. A metformina também tem como alvo outras vias associadas a EMT, tais como sinalização mTOR-S6K. A expressão elevada da p70S6K tirosina-quinase tem sido associada à baixa expressão de E-caderina e alta expressão de N-caderina e vimentina. Assim, o tratamento com metformina inibe a via mTOR-S6K, e isto pode também ser um mecanismo possível pelo qual a metformina inibe a EMT (Pon et al., 2008; Rattan et al., 2012).

Diversas vias de sinalização que resultam da atividade de diferentes fatores de crescimento estão envolvidos no processo de EMT, especialmente o TGF-β (Zarzynska et al., 2014). Assim, dada a importância do TGF-β1 na indução de EMT, e a tendência de células tumorais para ativar esta via de uma maneira autócrina, segregando o seu próprio TGF-β, o presente estudo realizou o silenciamento do gene TGF-β1 na linhagem canina metastática CF41. Como esperado, os resultados *in vitro* demonstraram que o silenciamento do TGF-β1 inibiu a migração e invasão celular, e houve maior redução com a adição de metformina nas células silenciadas, o que sugere cooperatividade de tratamentos na maioria dos experimentos. Semelhante aos nossos resultados, Moore et al. (2008), utilizando células de tumor mamário humano metastático, demonstraram que o silenciamento do TGF-β1 por RNAi levou a um decréscimo de 35% da migração de células e 55% de invasão celular.

Análises na expressão gênica e proteica em nossos estudos *in vitro* e *in vivo* demonstraram que o silenciamento do TGF-β1 resultou na redução dos marcadores mesenquimais N-caderina e vimentina e aumento da expressão dos marcadores epiteliais E-caderina e claudina-7, e foi igualmente ou mais eficaz com adição da metformina. Para conhecimento, este é o primeiro estudo a investigar a ação combinada de silenciamento gênico do TGF-β1 e tratamento com metformina. Zhang et al. (2014) demonstraram que o tratamento *in vitro* com metformina inibiu o processo de EMT induzida por TGF-β1 em células humanas de câncer de próstata, reduziu a expressão gênica e protéica da N-caderina e da vimentina, e aumentou a expressão de E-caderina e β-catenina.

Nosso estudo *in vivo* constatou que havia menos camundongos com metástases pulmonares e menos colônias metastáticas/pulmonar em animais que receberam células com TGF-β1 silenciado ou animais que foram tratados com metformina, em comparação com as células parentais não tratadas. O efeito foi ainda maior pela combinação do silenciamento do TGF-β1 com o tratamento com metformina, sendo significante quando comparado aos animais implantados com células controle shRNA. Nossos resultados *in vivo* com silenciamento do TGF-β1 estão de acordo com um estudo anterior de Moore et al. (2008) utilizando camundongos que receberam células de câncer de mama MDA-MB-435, que observaram redução de 90% no número de metástases macroscópicas no pulmão no grupo TGF-β1 shRNA em comparação com o grupo controle. Além disso, o aumento do crescimento do tamanho tumoral em camundongos do grupo TGF-β1sh também está de acordo com a previamente relatada função dupla deste fator no câncer da mama, em que ele pode atuar simultaneamente como um supressor de tumor primário e indutor de metástase, como inicialmente observada em estudos dos grupos do Dr. Massague (Siegel et al., 2003) e Arteaga (Muraoka et al., 2013).

Em relação às linhagens MDA-MB-231 e MCF-7, a incubação com TGF-β1 não alterou a invasão e a migração celular; por outro lado, o inibidor de ROCK, Y27632, foi capaz de diminuir a viabilidade celular nestas duas linhagens além da diminuição na migração e invasão celular da linhagem MDA-MB-231 e também quando tratada com metformina. Da mesma forma, Kidera et al.

(2010) e Borin et al. (2015) demonstraram que o tratamento com Y27632 resultou na diminuição da metástase, especialmente em câncer da mama, *in vitro* e *in vivo*. Unbekannt et al. (2014) demonstraram que a invasão de células MDA-MB-231 por meio de Matrigel foi mais eficientemente bloqueada pelo Y27632.

ROCK, regula uma grande variedade de processos biológicos, incluindo a aquisição do potencial de proliferação ilimitada, a sobrevivência e a evasão da apoptose, expressão gênica, e em particular, o deslocamento celular e estabelecimento de metástases (Yang et al, 2014; Zhang et al., 2014a; Ortiz-Lopez et al, 2009), desempenhando um papel central no processo da EMT.

Pouco se sabe sobre a interação entre o ROCK e o TGF-β1 no processo da EMT. Considerando-se as principais conclusões de Jia et al. (2015), a alta expressão de Rho-ROCK, aumenta a atividade de Rho, e gera uma maior susceptibilidade para EMT com o tratamento da TGF-β1. Os nossos resultados mostraram que em células MCF-7, houve aumento da expressão da proteína ROCK-1 após a indução da EMT por TGF-β1 em comparação com o grupo controle. De acordo com Xu et al. (2009), TGF-β induz a ativação de ROCK-1 em diferentes sistemas celulares. No entanto, a maioria dos estudos têm-se centrado no papel da RhoA e a sua efetora quinase ROCK na EMT induzida por TGF-β. A ativação de RhoA em resposta ao TGF-β por sua vez resulta na ativação de ROCK e complementar a estas observações, o TGF-β também regula diretamente a atividade de RhoA nas junções de oclusão das células epiteliais. No entanto, existe um contraste com a ativação global da RhoA por TGF-β, o que pode ser explicado pela regulação espaço-temporal de Rho durante a EMT.

Somente células MCF-7 tratadas com Y27632 associado com TGF-β diminuiu a expressão proteica de ROCK-1 em comparação com o grupo controle, ao passo que todos os tratamentos diminuiram a expressão da proteína ROCK-1 em comparação com o grupo incubado com TGF-β. Por outro lado, a linhagem celular MDA-MB-231 mostrou diminuição na expressão de ROCK-1 em todos os grupos de tratamento. Uma série de estudos recentes utilizando linhagens celulares de câncer e modelos de metástases em ratos têm demonstrado a capacidade dos inibidores de ROCK para

reduzir as propriedades migratórias e invasivas de células tumorais aderentes e, consequentemente, a progressão de tumores e metástases, o que tem gerado interesse significativo no uso de inibidores da ROCK (por exemplo Y27632) como terapias contra o câncer (Bhandary et al., 2015). Já foi demonstrado em um estudo recentemente publicado (Borin et al., 2015), que o tratamento com Y27632 sem TGF-β foi capaz de inibir a expressão da proteína ROCK-1 em células da linhagem MCF-7.

O TGF-β desempenha papéis multi-facetados que ainda estão aguardando uma melhor compreensão, a fim de desenvolver terapias eficazes anti-metástase. Mais especificamente, o TGF-β pode promover a tumorigênese através da ativação do processo de EMT (Biondini et al., 2015). No que diz respeito à ação do TGF-β1 na expressão de marcadores epiteliais e mesenquimais em linhagens celulares de câncer de mama, os nossos resultados mostraram que, após a indução do processo de EMT por TGF-β1 a expressão de E-caderina diminuiu e ao contrário das expectativas, a expressão da vimentina não se alterou em nenhuma das linhagens celulares MDA-MB-231 e MCF-7. Estes resultados estão de acordo com a literatura, o que demonstrou que EMT induzida por TGF-β induz alterações por diminuição da expressão de E-caderina e previne o aparecimento concomitante de expressão de vimentina, eventos que ocorrem quando uma célula epitelial é convertida em células mesenquimal (Cufí et al., 2010). Ambas linhagens MDA-MB-231 e MCF-7 na presença do tratamento com metformina e Y27632, diminuiu a expressão da proteína vimentina, especialmente quando a metformina foi associada com Y27632, indicando que o tratamento foi eficaz para reverter a EMT induzida por TGF-β. De acordo com Cufí et al. (2010), a metformina inibiu a expressão do marcador de EMT (vimentina) no citoplasma de células de rim canino induzidas por TGF-β1. Da mesma forma, Zhang et al. (2014b) mostraram que a metformina poderia inibir EMT induzida por TGF-β em células de câncer de próstata, tal como pela diminuição de N-caderina, vimentina e aumento da expressão gênica e proteica de E-caderina e β-catenina. Wang et al. (2014) induziram a EMT em células de câncer de próstata através da adição do TGF-β1 e verificou o processo examinando genes relacionados com a EMT, tais como a expressão de vimentina. Estes resultados

indicaram que a metformina suprime a EMT e fornece uma evidência adicional dos seus efeitos anti-neoplásicos.

Além disso, Zhang et al. (2014a) trataram a linhagem celular de hepatocarcinoma com Y27632 e analisaram os marcadores EMT: E-caderina e vimentina. Os resultados mostraram que todos os perfis de expressão foram atenuados pela inibição da expressão de ROCK.

A expressão proteica de E-caderina diminuiu após a indução da EMT por TGF-β1; no entanto, apenas o tratamento com Y27632 aumentou a expressão de E-caderina. Estes resultados estão de acordo com Wang et al. (2010), que observaram que Y27632 aumentou a expressão de E-caderina nas células tumorais escamosas. Além disso, Ewald et al. (2008) também demonstraram que a inibição de ROCK-1 por Y27632 resultou em um fenótipo com níveis reduzidos de E-caderina em superfícies intercelulares.

A capacidade de uma única célula mover-se partir do tumor primário para o local metastático é facilitado através da transição de um fenótipo epitelial (CD44+/CD24+) para um fenótipo mesenquimal (CD44+/CD24-) (MANI et al., 2008). Houve aumento da expressão de CD44, após a adição de TGF-β em comparação com o controle nas células MDA-MB-231. Por outro lado, sua expressão foi menor nas células MCF-7 e depois de todos os tratamentos em ambas as linhagens celulares. A elevada expressão de CD44 é um marcador para EMT induzida por TGF-β e uma característica de células-tronco cancerosas (CSCs) no câncer de mama (Mani et al., 2008) e a supressão da expressão de CD44 está associada à supressão tumoral (Godar et al., 2008). Além disso, estudos têm mostrado que a metformina pode reprimir a transcrição de EMT e reprimir um fenótipo celular associada com CSCs no câncer de mama (Zhang et al., 2015b)

Nosso estudo mostrou diminuição da expressão de CD24 em todos os grupos de tratamento. Kristiansen et al. (2003) mostraram que a elevada expressão desta proteína pode ser considerada um novo marcador de prognóstico do câncer de mama. Outro estudo relata que CD24 é altamente expresso no câncer de ovário, de mama e de próstata. Ele está envolvido no processo de adesão celular e metástase, sendo um importante marcador de diagnóstico e prognóstico do tumor (Jaggupilli

et al., 2012). Os nossos resultados sugerem que o tratamento com metformina e Y27632 foram eficazes na diminuição da expressão de CD24 e pode contribuir para um melhor prognóstico.

Conclusões

V. CONCLUSÕES

O trabalho permitiu estabelecer as seguintes conclusões:

- A metformina é capaz de reduzir a viabilidade celular das linhagens tumorais mamárias metastáticas e não-metastáticas humanas e caninas.

- Metformina, Y27632 e TGF-β1sh são capazes de aumentar a expressão de marcadores epiteliais e diminuir a expressão de marcadores mesenquimais, bem como inibir a metástase *in vitro* e *in vivo*.

- Em conjunto, nossos resultados demonstram o potencial terapêutico do silenciamento do TGF-β1 e da metformina, associada ou não ao Y27632 no câncer de mama.

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Anexos

VII. ANEXOS

ANEXO 1: Parecer da Comissão de Ética na Experimental Animal da Faculdade de Medicina de São José do Rio Preto.

Comissão de Ética na Experimentação Animal
CEEA
FAMERP

Faculdade de Medicina de São José do Rio Preto
Comissão de Ética na Experimentação Animal - CEEA
FAMERP Autarquia Estadual, Av. Brig. Faria Lima 5416 CEP 15090.000 Tel. 3201-5700 S.J.Rio Preto/

DELIBERAÇÃO CEEA Nº 007/2012

A Comissão de Ética na Experimentação Animal da Faculdade de Medicina de São José do Rio Preto – CEEA/FAMERP, em reunião nesta data, analisou o projeto de pesquisa intitulado **"Inibição da metástase via transição epitélio-mesenquimal por RNA de interferência e metformina em neoplasia mamária" (Protocolo FAMERP nº 3244/2012)**, sob responsabilidade da Prof.ª Dr.ª Debora Aparecida Pires de Campos Zuccari, e deliberou que de acordo com os princípios éticos estabelecidos na Lei nº 11.794/2008 e na Resolução nº 714/2002 o mesmo foi **aprovado**.

Atenção: **Até 30 dias após a finalização do projeto**, o pesquisador deverá preencher o Formulário do Relatório Final disponível no site e enviar ao CEEA. O descumprimento desta obrigação poderá prejudicar o andamento de futuras solicitações.

São José do Rio Preto, 09 de agosto de 2012.

Profa. Dra. Gloria Elisa Florido Mendes
Vice-Presidente da CEEA
FAMERP

ANEXO II: Comprovante de submissão do artigo: **Inhibition of epithelial-mesenchymal transition and metastasis by combined TGFbeta knockdown and metformin treatment in a canine mammary cancer xenograft model** ao periódico *Journal of Mammary Gland Biology and Neoplasia*.

Journal of
MAMMARY GLAND BIOLOGY and NEOPLASIA

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Submissions Being Processed for Author Debora Aparecida pires de Campos Zuccari Zuccari

Page: 1 of 1 (1 total submissions) Display 10 results per page.

Action	Manuscript Number	Title	Initial Date Submitted	Status Date	Current Status
Action Links	JOMG-D-16-00003	Inhibition of epithelial-mesenchymal transition and metastasis by combined TGFbeta knockdown and metformin treatment in a canine mammary cancer xenograft model	Jan 22, 2016	Jan 23, 2016	Under Review

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APÊNDICE

VIII. APÊNDICE

Para avaliar a ocorrência de metástase, foram coletados órgãos alvos como, fígado, cérebro e pulmão de todos os grupos experimentais **(Figura 1).** Na análise histopatológica foram encontrados formações neoplásicas somente nos pulmões, sendo observada a presença de nódulos metastáticos em maior número e tamanho nos controles positivo e nos controles shGFP quando comparados aos grupos tratados e sem metástase **(Figura 2).**

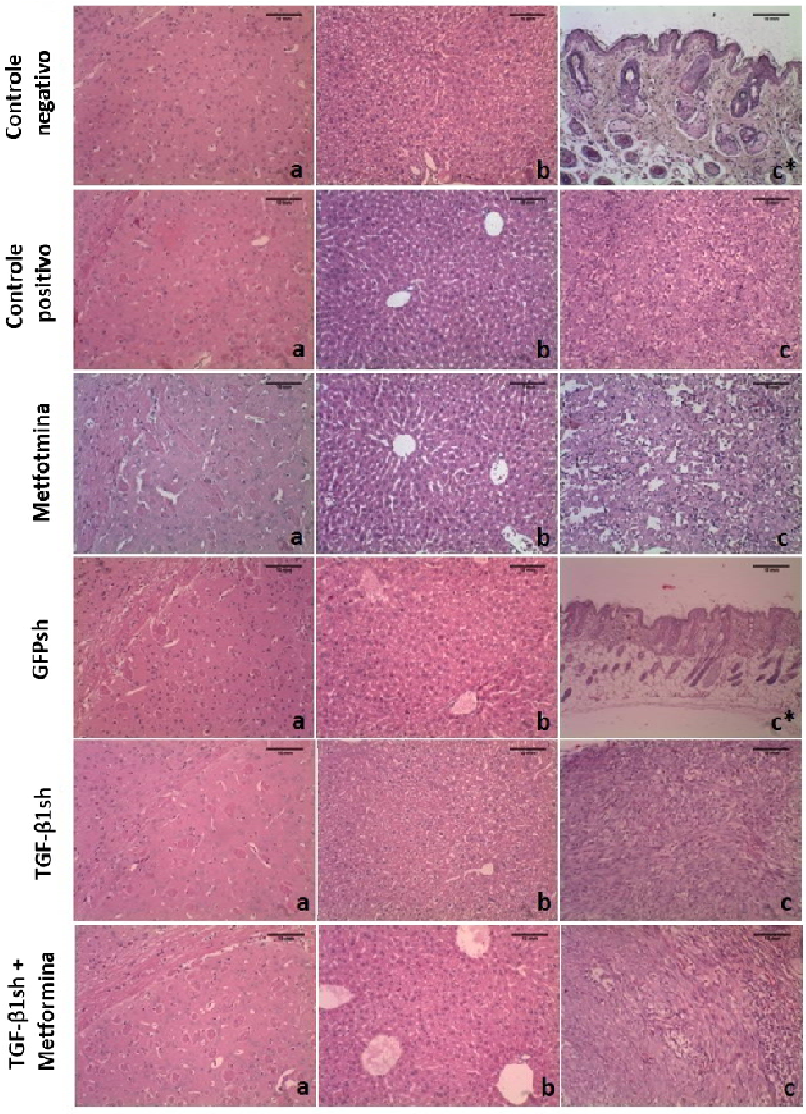

Figura 1. Fotomicrografias em coloração de H.E. representativas dos órgãos avaliados em relação a presença de metástases ou nódulo tumoral, nos grupos de estudo após uma semana de indução e 4 semanas de tratamento. (a**)** Cérebro; (b) Fígado; (c) Tumor primário em Glândula mamária; (c*) Glândula mamária sem tumor. 10x

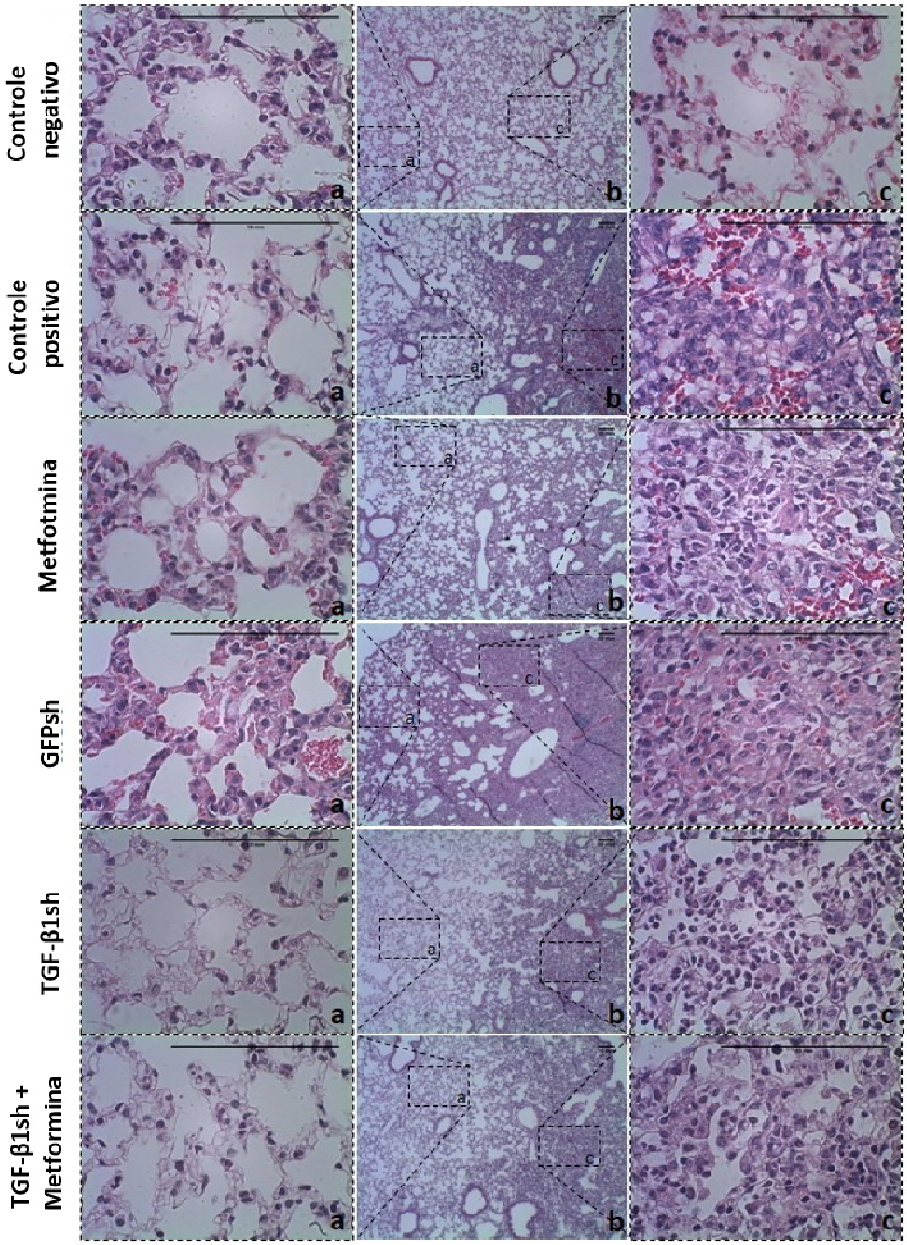

Figura 2. Fotomicrografias de regiões pulmonares representativas dos grupos de estudo avaliados em relação a presença de metástases após uma semana de indução tumoral e 4 semanas de tratamento em coloração de H.E. (**a** e **c**) região com metástases (40x), (**b**) região sem metástases (4x).