**UNIVERSIDADE FEDERAL DE SÃO CARLOS – UFSCar**

CENTRO DE CIÊNCIAS BIOLÓGICAS E DA SAÚDE

**Programa de Pós-Graduação em Genética Evolutiva e Biologia Molecular**

MILENE NÓBREGA DE OLIVEIRA MORITZ

# A INTEGRINA $\alpha_2\beta_1$ NA PROGRESSÃO TUMORAL MAMÁRIA: PAPEL NO MICROAMBIENTE TUMORAL E NA METÁSTASE

SÃO CARLOS - SP

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Tese apresentada ao Programa de Pós-Graduação em Génetica Evolutiva e Biologia Molecular, do Centro de Ciências Biológicas e da Saúde da Universidade Federal de São Carlos, como parte dos requisitos para a obtenção de título de Doutor em Genética e Biologia Molecular.

Orientadora: Profa Dra. Heloisa Sobreiro Selistre de Araujo

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Centro de Ciências Biológicas e da Saúde
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*Dedicado aos meus pais Olavo e Edenise e ao meu irmão Vitor.*

*Dedicado ao meu marido Carlos.*

*Dedicado à memória dos meus avós.*

*Dedicado à memória da minha prima-irmã Fabinha.*

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## RESUMO

O funcionamento harmônico das células envolve uma sincronia orquestrada de sinalização que mantém o equilíbrio entre proliferação e morte celular nos tecidos saudáveis. Eventualmente, mutações em genes podem ocorrer e o acúmulo dessas alterações culminar em divisões celulares descontroladas e, consequentemente, na formação de neoplasias malignas. O câncer de mama é o mais frequente entre as mulheres, sendo a metástase a maior causa de morte entre as pacientes. O subtipo triplo negativo de câncer de mama é um dos tumores mais agressivos e ainda não existe terapia específica para seu tratamento. O tecido ósseo é o destino mais comum para a metástase de câncer de mama e a existência de receptores de membrana, como as integrinas, nas células tumorais auxiliam nesse endereçamento. Neste estudo, dividido em dois capítulos, avaliamos o papel da integrina $\alpha_2\beta_1$ na metástase do câncer de mama. Na primeira etapa, utilizamos a alternagina-C, uma proteína ligante da integrina $\alpha_2\beta_1$ proveniente do veneno da serpente *Bothrops alternatus* e o silenciamento transiente da subunidade $\alpha_2$ a fim de verificar o papel desse receptor na metástase de origem mamária. Os resultados demonstraram que a alternagina-C reduziu em 50% a adesão das células tumorais ao colágeno tipo I e aumentou mais de três vezes a expressão do supressor de metástase 1 (*MTSS1*). Na segunda etapa deste estudo, utilizamos três modelos *in vivo* (*Mammary Fat Pad*-MFP, intracardíaco-IC e intratibial-IT) de metástase óssea de câncer de mama com células tumorais humanas em camundongos nudes atímicos. Os três modelos foram tratados com o inibidor da integrina $\alpha_2\beta_1$, TCI-15, ou injeção de células tumorais superexprepressando a subunidade $\alpha_2$ com o objetivo de avaliar o papel da integrina $\alpha_2\beta_1$ especificamente na metástase óssea de tumor de mama. Com esses modelos verificamos que a integrina $\alpha_2\beta_1$ possui papel relevante no crescimento do tumor primário (modelo MFP), porém não houve resultados significativos nas fases mais tardias da metástase óssea (modelos IC e IT). Além disso, observamos uma expressão bifásica da integrina $\alpha_2\beta_1$, sendo menor a expressão dessa integrina nas células metastáticas para osso comparado à expressão nas células tumorais de mama parentais. Para as células metastáticas, também demonstramos uma alta expressão dos genes PTHrP (*parathyroid hormone related protein*), Gli2 e TGFβRII, os quais contribuem com a destruição óssea. Estes dados sugerem uma relação inversa entre a expressão da integrina $\alpha_2$ e um fenótipo destrutivo ósseo. O tratamento com TGF-β diminuiu a quantidade da integrina $\alpha_2\beta_1$ nas células metastáticas, indicando que o TGF-β pode influenciar a seleção de células tumorais para o osso através da regulação dessa integrina. Em conjunto, esses resultados demonstraram que a integrina $\alpha_2\beta_1$ está envolvida com diferentes funções ao longo da cascata metastática de câncer de mama para o osso. Dessa forma, esse estudo contribuiu para a identificação de um possível alvo molecular para tratamento e prevenção de metástase de câncer de mama triplo negativo.



**Palavras-chave:** Câncer de Mama, Metástase Óssea, Microambiente Tumoral, Integrina, Alternagina-C

## ABSTRACT

The harmonic functioning of cells involves an orchestrated synchronization of signaling that maintains the balance between proliferation and cell death in healthy tissues. Eventually, gene mutations can occur and the accumulation of these alterations culminate in uncontrolled cellular divisions and consequently in the formation of malignant neoplasias. Breast cancer is the most frequent among women, with metastasis being the major cause of death. The triple negative subtype of breast cancer is one of the most aggressive tumors and there is no specific therapy for its treatment. Bone is the most common site for breast cancer metastasis and the existence of membrane receptors such as integrins on tumor cells assist in this addressing. In this study, divided into two chapters, we evaluated the role of $\alpha_2\beta_1$ integrin in the metastasis of breast cancer. In the first chapter, we used alternagin-C, a protein that binds to $\alpha_2\beta_1$ integrin and from the venom of *Bothrops alternatus* snake, and the transient silencing of the $\alpha_2$ subunit to verify the role of this receptor in mammary metastasis. The results demonstrated that alternagin-C decreased in 50% tumor cell adhesion to type I collagen and increased more than three times the expression of the metastasis suppressor 1 (*MTSS1*). In the second chapter, we used three *in vivo* models (Mammary Fat Pad-MFP, intracardiac-IC and intratibial-IT) of breast bone metastasis with human tumor cells in athymic nude mice. All three models were treated with the $\alpha_2\beta_1$ integrin inhibitor, TCI-15, or superexpressing the $\alpha_2$ subunit in tumor cells that were injected into the animals with the aim of evaluate the role of $\alpha_2\beta_1$ integrin specifically in breast tumor bone metastasis. With these models, we observed that $\alpha_2\beta_1$ integrin plays a significant role in primary tumor growth (MFP model), but there were no significant results in later stages of bone metastasis (IC and IT models). In addition, we verified a biphasic expression of $\alpha_2\beta_1$ integrin during the metastasis cascade, with lower expression in the metastatic cells compared to expression in the parental breast tumor cells. We also demonstrated that the metastatic cells express high levels of PTHrP (parathyroid hormone related protein), Gli2, and TGFβRII, which have been shown to contribute to bone destruction. These data suggest an inverse relation between $\alpha_2$ integrin expression and a bone destructive phenotype. Furthermore, TGF-β treatment decreased $\alpha_2\beta_1$ integrin levels in metastatic cells, suggesting that TGF-β may influence the selection of tumor cells to bone through this integrin regulation. Taken together, these results demonstrated that $\alpha_2\beta_1$ integrin is involved with different roles in metastasis cascade of breast cancer to bone. Thus, this study contributed to identify a possible molecular target for the treatment and prevention of triple negative breast cancer metastasis.



**Key words:** Breast Cancer, Skeletal Metastasis, Tumor Microenvironment, Integrin, Alternagin-C

**LISTA DE FIGURAS**

## LISTA DE ABREVIATURAS E SIGLAS

**BSA** - Soro Albumina Bovina (***B**ovine **S**erum **A**lbumin*)

**cDNA** - Ácido desoxirribonucléico complementar

**DAPI** - *4',6-Diamidino-2-Phenylindole Dihydrochloride*

**DCD** - Ácido aspártico - Cisteína - Ácido aspártico

**DMEM** - Meio Dulbecco modificado (***D**ulbecco's **M**odified **E**agle **M**edium*)

**DMSO** - Sulfóxido de Dimetil (*Dimethyl Sulfoxide*)

**DNA** - Ácido desoxirribonucléico

**ECD** - Ácido glutâmico - Cisteína - Ácido aspártico

**EDTA** - Ácido etilenodiamino teracético

**EILDV** - Ácido glutâmico - Isoleucina – Leucina - Ácido aspártico - Valina

**ERK-2** - ***E**xtracellular Signal-**R**egulated **K**inase **2***

**FAK** - ***F**ocal **A**dhesion **K**inase*

**FBS** - Soro Fetal Bovino (***F**etal **B**ovine **S**erum)*

**FITC** - Isotiocianato de fluoresceína (*Fluorescein isothiocyanate*)

**FVB** - ***F**riend Leukemia **V**irus **B** mouse strain*

**FvW** - **F**ator de **v**on **W**illebrand

**GAPDH** - Gliceraldeído 3-fosfato desidrogenase

**Gli2** - *Gli Family zinc finger 2*

**HER2** - Receptor do fator de crescimento humano 2 (***H**uman **E**pidermal Growth Factor **R**eceptor-**2***)

**HIF1α** - **H**ypoxia-**I**nducible **F**actor **1**-alpha

**HPV** - *Human Papilomavirus*

**IDH - Í**ndice de **D**esenvolvimento **H**umano

**IL-6** - Interleucina 6 (*Interleukin* 6)

**IL- 8** - Interleucina 8 (*Interleukin* 8)

**IL-11** - Interleucina 11 (*Interleukin* 11)

**KGD** - Lisina - Glicina - Ácido aspártico

**MAPK** - ***M**itogen-**A**ctivated **P**rotein **K**inase*

**MEC/ECM** - Matriz extracelular (*Extracellular matrix*)

**MMPs** - Metalopeptidases de Matriz (*Matrix Metalloproteinases*)

**MMTV- PyMT** - ***M****ouse* ***M****ammary* ***T****umor* ***V****irus-****P****olyoma* ***M****iddle* ***T***

**PBS** - Tampão fosfato salino (***P****hosphate* ***B****uffered* ***S****aline*)

**PCR** - Reação em cadeia da Polimerase (***P****olymerase* ***C****hain* ***R****eaction*)

**PI3K** - *Phosphoinositide 3-kinase*

**PTHrP** - *Parathyroid hormone-related protein*

**qPCR** - *Quantitative PCR*

**RNA** - Ácido ribonucleico

**RNAm** - Ácido ribonucleico mensageiro

**RANKL** - ***R****eceptor* ***A****ctivator of* ***N****uclear Factor* ***K****appa-β* ***L****igand*

**REDV -** Arginina - Ácido glutâmico - Ácido aspártico - Valina

**RGD** - Arginina - Glicina - Ácido aspártico

**RhoA and C** – *Rho family of GTPases*

**RUNX2** - *Runt-related transcription factor 2*

**SDS-PAGE** - ***S****odium* ***D****odecyl* ***S****ulphate* ***P****olyacrylamide* ***G****el* ***E****lectrophoresis*

**SRC** - ***S****teroid* ***R****eceptor* ***C****oactivator*

***SVMPs*** - Metaloprotease de veneno de serpente (*Snake Venom Metalloproteinase*)

**TEM/EMT** - **T**ransição **E**pitélio-**M**esenquimal (***E****pithelial-****M****esenchymal* ***T****ransition*)

**TME/MET** - **T**ransição **M**esenquimal-**E**pitelial (***M****esenchymal-* ***E****pithelial* ***T****ransition*)

**TGF-β** -***T****ransforming* ***G****rowth* ***F****actor beta*

**TGFβR2** -***T****ransforming* ***G****rowth* ***F****actor beta* **R**eceptor **2**

**TN-** ***T****riple-****N****egative*

**TNBC-** ***T****riple-****N****egative* ***B****reast* ***C****ancer*

**VEGF** - Fator de crescimento vascular endotelial (***V****ascular* ***E****ndothelial* ***G****rowth* ***F****actor*)

**VGD** - Valina – Glicina - Ácido aspártico

**SUMÁRIO**

# 1 CAPÍTULO 1: O PAPEL DA INTEGRINA $\alpha_2\beta_1$ NO MICROAMBIENTE TUMORAL EM CÉLULAS DE TUMOR MAMA

## 1.1 Introdução

### *1.1.1 O ciclo natural celular e a ocorrência de neoplasias malignas*

Células saudáveis crescem, dividem-se, envelhecem e morrem, mantendo assim a integridade dos tecidos por esse delicado equilíbrio de proliferação e morte celular. Um complexo sistema de sinalização atua de forma orquestrada para manter o controle da divisão celular. Uma vez perdida essa sincronia, as células podem proliferar desordenadamente originando tumores, também denominados neoplasias. Caso a população celular permaneça localizada sem caráter invasivo é dita como neoplasia benigna. Ao contrário, se essa população celular adquire grande potencial de crescimento e invasão é definida como neoplasia maligna (INCA, 2018; PIERCE, B. A., 2011).

O câncer é uma neoplasia maligna, considerada uma doença fundamentalmente genética, resultante do acúmulo de mutações em genes relacionados à proliferação, diferenciação e morte celular. De acordo com a teoria da evolução clonal dos tumores, o câncer é proveniente de uma única célula em que mutações geram instabilidade no DNA e consequentemente propiciam mais mutações que culminam em subsequentes clones de células com acentuada proliferação. A frequência com que surgem novas mutações rege a velocidade da evolução clonal nas células tumorais (CAMPBELL; FUTREAL, 2009; STEWART, 2014; STRATTON). Dentre os fatores que podem desencadear ou acentuar a ocorrência dessas mutações no câncer estão, principalmente, fatores hereditários e fatores ambientais. O fator hereditário predispõe as células a sofrerem mutações aumentando a incidência de câncer naquele indivíduo que herdou a alteração genética (PARSA, 2012). Cerca de 95% dos cânceres, porém, são desencadeados por fatores ambientais tais como agentes químicos (benzeno, dimetil sulfato), agentes físicos (luz ultravioleta e radiação ionizante) e agentes biológicos (papilomavírus-HPV e *Helycobacter pylori*) assim como pelo estilo de vida como tabagismo e alcoolismo (MILLER; MILLER, 1981; PARSA, 2012).

As alterações genéticas causadas pelos fatores relacionados ao câncer atuam transformando proto-oncogenes em oncogenes ou inativando genes supressores tumorais. Proto-oncogenes são genes responsáveis por funções básicas em células normais, entretanto quando mutados tornam-se oncogenes que atuam como estimuladores da divisão celular e

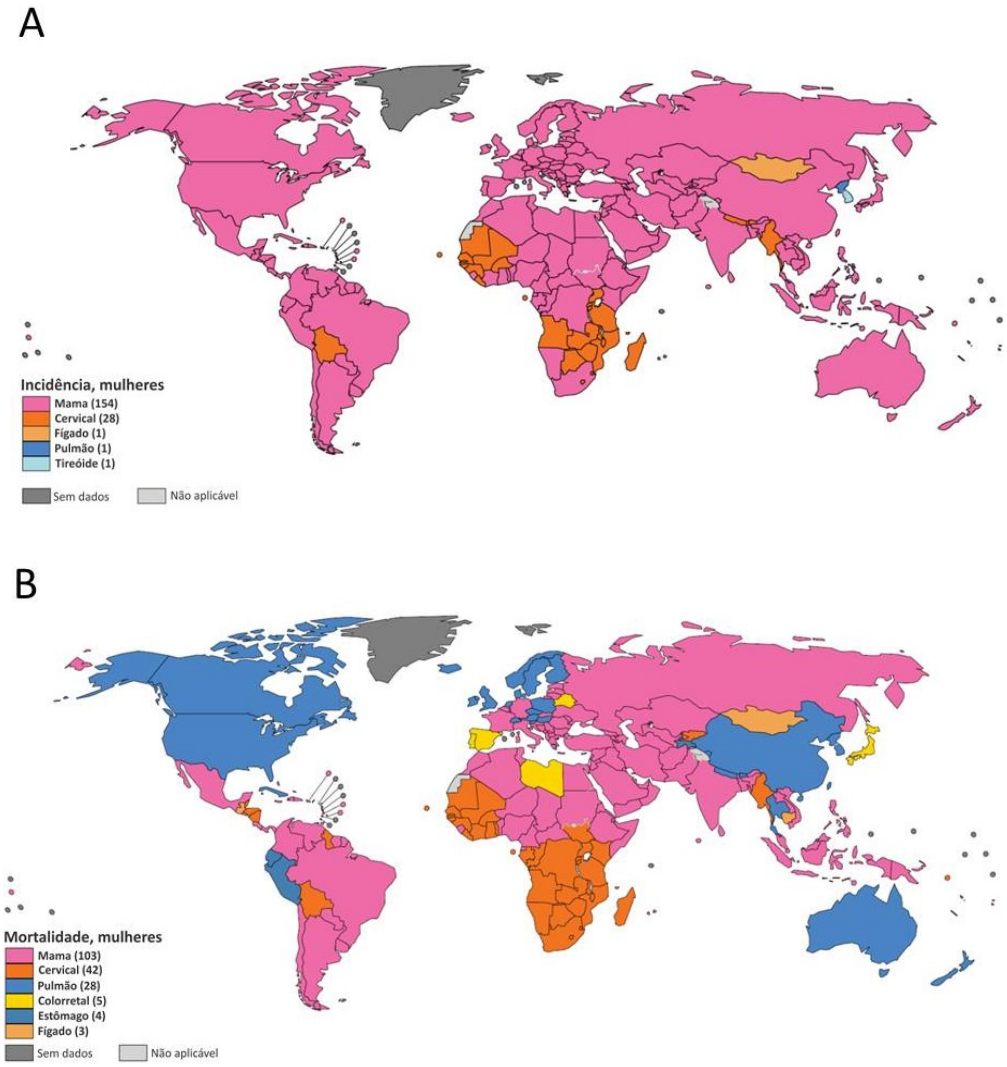
consequentemente da progressão tumoral (STEHELIN et al., 1976). Já os genes supressores tumorais atuam inibindo a proliferação celular e contribuem para o desenvolvimento do câncer quando estão inativados (LEVINE, 1993). Alterações nos genes de reparo do DNA também propiciam o acúmulo de mutações estimulando indiretamente a divisão celular.

A partir do estudo da função dos genes envolvidos no desenvolvimento tumoral, tornou-se possível identificar potencias alvos para a ação de novos fármacos antitumorais (BALMAIN, 2001). Entretanto, ainda não foi possível identificar genes em comum que invariavelmente sejam alterados nos diversos tipos de câncer. De fato, cada câncer apresenta um padrão de alterações genéticas único e complexo (KNUDSON, 2001), além de alterações epigenéticas que estão envolvidas no controle da expressão do fenótipo tumoral (FEINBERG; TYCKO, 2004).

### *1.1.2 O câncer de mama: epidemiologia e seus diferentes aspectos*

O câncer de mama é considerado atualmente um importante problema de saúde pública mundial, sendo a neoplasia maligna mais comumente diagnosticada em mulheres e a principal causa de morte por câncer. Segundo as últimas estimativas mundiais de 2018 (BRAY et al., 2018), o câncer de mama é o mais frequente na maioria dos países, seguido pelo câncer do colo de útero (**figura 1A**). Para o câncer de mama a mortalidade é a mais alta em 103 países comparado ao câncer de colo de útero em 42 países, seguido pelo câncer de pulmão em 28 países (**figura 1B**).

**Figura 1 -** Tipo mais comum de incidência de câncer entre mulheres em cada país em 2018 (A). Tipo mais comum de mortalidade por câncer entre mulheres em cada país em 2018 (B). Fonte: modificado de BRAY et al., 2018.




Os países que apresentam maior índice de desenvolvimento humano (IDH) são os que possuem as taxas de incidência de câncer de mama mais elevadas. Essas taxas também vêm aumentando na maioria dos países nas últimas décadas, com alguns dos aumentos mais proeminentes ocorrendo em países da América do Sul, África e Ásia (BRAY et al., 2018; BRAY; MCCARRON; PARKIN, 2004; STEWART, 2014). Essa tendência provavelmente deve-se a uma combinação de fatores demográficos aliados ao desenvolvimento social e econômico que predispõe ao câncer de mama, incluindo o adiamento da gravidez, menor número filhos, ingestão de hormônio exógeno (uso de contraceptivos orais e terapia de reposição hormonal) e maiores níveis de obesidade (BRAY et al., 2018; D'AVANZO et al., 1996).

Diante da alta incidência de câncer de mama, inúmeros esforços têm sido direcionados na identificação dos subtipos moleculares. O câncer de mama possui um elevado grau de diversidade e heterogeneidade entre os tipos de tumores, além de uma surpreendente plasticidade dinâmica do microambiente tumoral que faz de sua categorização e tratamento uma tarefa desafiadora (POLYAK, 2011). Com base no perfil de expressão gênica, os tumores de mama são classificados em três principais subtipos moleculares: luminal, positivo para o receptor tipo 2 do fator de crescimento epidérmico humano (HER2+) e o triplo negativo (TN) (SØRLIE et al., 2001; TONG et al., 2018). O tipo luminal positivo para os receptores de estrógeno e progesterona é o mais responsivo às intervenções hormonais, enquanto que os tumores HER2+ respondem bem a terapias anti-HER2. Já os tumores TN não possuem os dois receptores hormonais e o receptor HER2. Atualmente, não existe terapia com alvos moleculares para os tumores TN e infelizmente apenas 20 % desses tumores respondem ao tratamento convencional com quimioterápicos.

Vários estudos mostraram que essa pequena classe de tumores de mama (TN) pode apresentar mais seis subtipos de acordo com suas características moleculares e sensibilidade a agentes terapêuticos (LEHMANN et al., 2011; MARKLAND et al., 2000; PRAT et al., 2010; RUSSNES et al., 2010). As células tumorais de mama TN são bastante agressivas e marcadas por altas taxas de metástase, sendo o subtipo com pior sobrevida (CAREY et al., 2006; DENT et al., 2009; KASSAM et al., 2009; RODRIGUEZ-PINILLA et al., 2006). Dessa forma, há uma enorme necessidade de se encontrar novos alvos moleculares objetivando o desenvolvimento de terapias antimetáteses mais eficazes para o tratamento do câncer de mama TN.

### *1.1.3 A metástase e a matriz extracelular no microambiente tumoral*

Atualmente dez marcos foram descritos como característicos da progressão tumoral (HANAHAN; WEINBERG, 2011) sendo estes: autonomia na sinalização de crescimento, evasão de sinais inibidores do crescimento, potencial de replicação ilimitado, inflamação, evasão do sistema imune, angiogênese, instabilidade genômica e mutação, evasão de morte celular, metabolismo de energia reprogramado e invasão e metástase (**figura 2**).

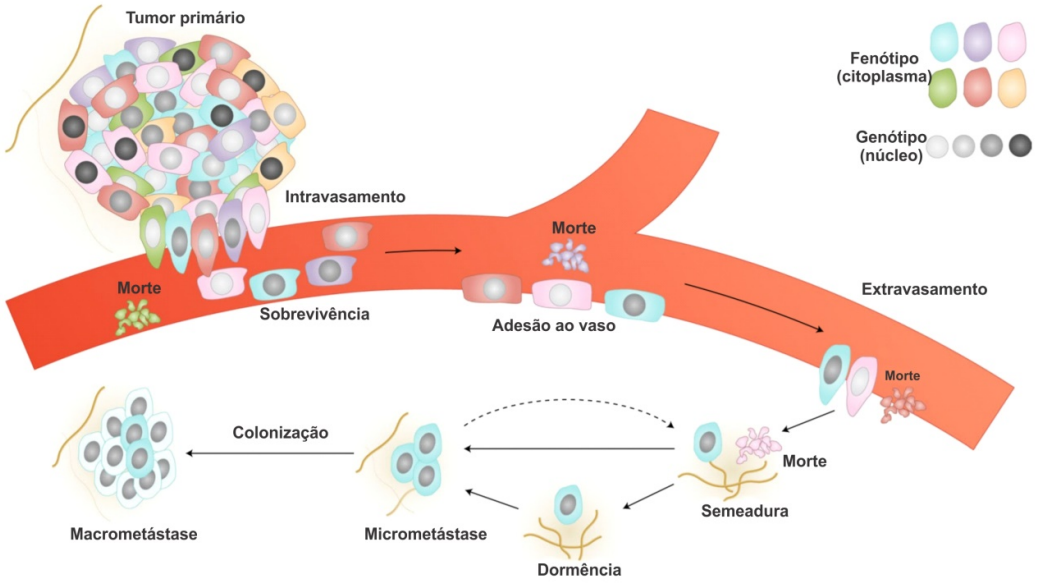
**Figura 2** - Os 10 “marcos” do câncer. Fonte: modificado de HANAHAN; WEINBERG, 2011.


Células tumorais frequentemente exibem a maioria dessas características sendo a metástase a maior causa de morte entre as pacientes com câncer de mama (MARUSYK; POLYAK, 2010). Metástase é a capacidade de invadir tecidos e formar tumores secundários (FOKAS et al., 2007). A metástase ocorre basicamente quando as células tumorais adquirem as seguintes propriedades: invasão e migração para corrente sanguínea ou vasos linfáticos, sobrevivência na circulação, captura e adesão das células tumorais aos vasos, extravasamento, dormência e ressurgimento metastático no sítio secundário (DASGUPTA; LIM; GHAJAR, 2016; LAWSON et al., 2018) (**figura 3**).

Na fase de invasão, as células do tumor primário se dissociam invadindo o tecido adjacente. Esse processo ocorre por meio de interações das células tumorais com a matriz extracelular (MEC) em que os receptores de adesão da família das integrinas, bem como mecanismos proteolíticos, atuam para superar as barreiras teciduais (WOLF; FRIEDL, 2006). Na etapa subsequente, as células tumorais migram através das células endoteliais atingindo o interior dos vasos sanguíneos ou linfáticos. Uma vez na corrente sanguínea, as células que conseguiram intravasar precisam evadir do sistema imunológico para sobreviver. Nesse processo, as plaquetas auxiliam aderindo-se às células tumorais que, protegidas, passam despercebidas pelo sistema imunológico (COUPLAND; PARISH, 2014). Ao chegarem próximo ao sítio secundário, as células tumorais extravasam dos vasos para colonizar o novo tecido (PEINADO; LAVOTSHKIN; LYDEN, 2011). Uma vez fora da corrente sanguínea, as

células tumorais colonizam o sítio metastático, formando micrometástases e subsequentemente macrometástases. Nesse árduo processo de colonização, algumas células podem morrer ou entrar em estágio de dormência, formando micrometástases após encontrarem condições favoráveis (COMINETTI et al., 2019; GHAJAR, 2015; LAWSON et al., 2018).

**Figura 3 -** Propriedades genéticas e fenotípicas de células iniciadoras de metástase no nível celular. A metástase é um evento raro, no qual a maioria das células tumorais não progride através das principais barreiras associadas à invasão, intravasamento, extravasamento, semeadura e colonização para produzir um tumor macrometastático maligno. Neste modelo, as células tumorais são heterogéneas no genótipo (núcleos) e no fenótipo (citoplasma), e as células que iniciam a metástase possuem uma combinação distinta de ambas. A seta tracejada indica que as células tumorais dentro das micrometástases podem morrer. As taxas de mortalidade nas micrometástases podem equilibrar as taxas de proliferação e, assim, impedir a progressão para macrometástase pela incapacidade de produzir crescimento. Fonte: modificado de LAWSON et al., 2018.




Vários eventos que acontecem durante a metástase são favorecidos pela conversão entre dois estágios celulares: o fenótipo epitelial e o mesenquimal. As células epiteliais são mais diferenciadas e não migram, já as células mesenquimais são mais indiferenciadas e migram com maior facilidade. No desenvolvimento embrionário, o processo chamado transição epitélio-mesenquimal (TEM) permite que células epiteliais assumam características de células mesenquimais e migrem. Uma vez no local de destino, essas células voltam ao fenótipo epitelial pelo processo chamado de transição mesenquimal-epitelial (TME) (VAN DENDEREN; THOMPSON, 2013). Tais processos ocorrem de forma semelhante na metástase, porém de maneira mais complexa, visto que as células metastáticas apresentam um

fenótipo misto com a coexpressão de ambas as características fenotípicas (LEE et al., 2006). Essa plasticidade de transição das células metastáticas permite um ajuste do comportamento dependendo das circunstâncias encontradas durante as etapas da metástase. De maneira geral, as células do tumor primário sofrem TEM para invadir e migrar para a corrente sanguínea e ao chegar ao sítio secundário revertem o seu fenótipo por meio da TME estabelecendo-se no tecido alvo (MITTAL, 2018).

Durante todo o processo de metástase, as funções de adesão e migração celular estão relacionadas com a capacidade das células interagirem com a MEC. A MEC é um complexo entrelaçado proteico formado por diversas famílias de proteínas que incluem pelo menos 27 tipos de colágeno e 15 isoformas de laminina, além da elastina, fibronectina e outros componentes. Além do papel mecânico na manutenção da integridade dos tecidos, a MEC controla o fluxo de nutrientes e sinais para as células influenciando o comportamento celular (GEIGER; YAMADA, 2011; HYNES, 2009; TIMPL et al., 2003).

O remodelamento da MEC ocorre através de enzimas que degradam a matriz, especialmente membros da família das metaloproteinases de matriz (MMPs). As MMPs são endopeptidades dependentes de zinco classificadas de acordo com a especificidade ao seu substrato: colagenases, gelatinases, estromelisinas e matrilisinas. Como o número de substratos conhecidos para as MMPs vem aumentando, o sistema numeral passou a ser adotado e as MMPs têm sido classificadas de acordo com as suas estruturas. Existem oito classes baseadas nessa nova classificação: cinco são secretadas e três são *membrane-type* MMPs. Estas são covalentemente ligadas à membrana celular, enquanto que aquelas também podem estar localizadas na superfície celular conectadas a integrinas ou a outras moléculas da membrana (ISAACSON ET AL., 2017).

A expressão inadequada de MMPs pode ocasionar a degradação da membrana basal, interrompendo o tecido adjacente o que contribui para invasão e disseminação celular, por exemplo, das células tumorais (WITTY; WRIGHT; MATRISIAN, 1995). Além de seu papel no remodelamento da MEC e na migração de células tumorais, as MMPs também regulam vias de sinalização que controlam o crescimento celular, inflamação e angiogênese (KESSENBROCK; PLAKS; WERB, 2010). As MMP-2 e -9 foram descritas como importantes no processo de invasão e formação de metástase (AMĂLINEI et al., 2010), e também foram identificadas em soro de pacientes com câncer de mama (DANIELE et al., 2010).

Além das MMPs e dos componentes da MEC, o microambiente tumoral é constituído por células associadas ao tumor, como por exemplo fibroblastos e macrófagos associados ao

tumor, que interagem por meio de fatores locais e sistêmicos influenciando a expressão gênica das células tumorais de modo que seu comportamento é determinado por uma "reciprocidade dinâmica" (NELSON; BISSELL, 2006). No contexto dessa reciprocidade, alterações na MEC podem, por exemplo, promover alterações na expressão de moléculas de adesão como as integrinas e desencadear a progressão tumoral (LU; WEAVER; WERB, 2012).

### *1.1.4 Integrinas como alvos terapêuticos*

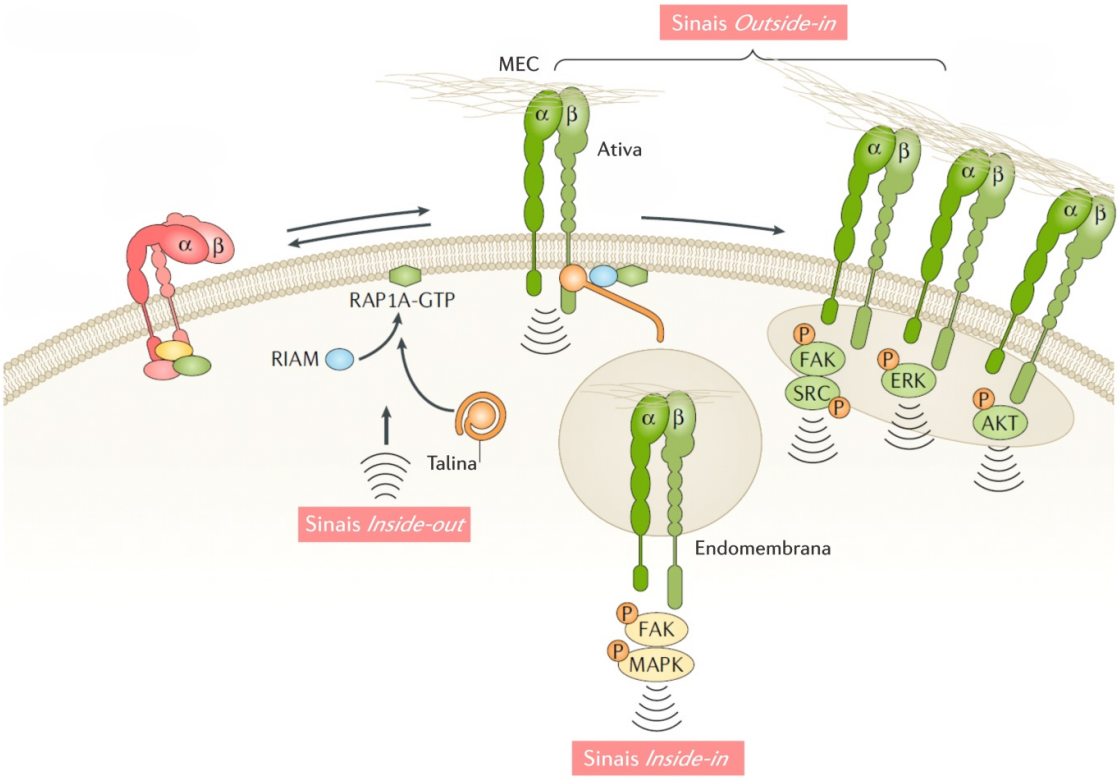
Moléculas de adesão estão frequentemente alteradas em tumores, sendo as integrinas uma das principais moléculas envolvidas nesse contexto. Essas alterações geram uma cascata de sinalização que regula a expressão gênica, a organização do citoesqueleto, a adesão e a sobrevivência celular. Como resultado, as células tumorais tornam-se mais capazes de invadir, de migrar e de sobreviver em diferentes microambientes (HAMIDI; IVASKA, 2018; HOOD; CHERESH, 2002).

As integrinas são receptores heterodiméricos, formados por uma subunidade α e uma subunidade β. Nos mamíferos, foram reconhecidas 24 integrinas formadas a partir da combinação de uma subunidade α das 18 existentes com uma subunidade β, das 8 identificadas (SHATTIL; KIM; GINSBERG, 2010). As diferentes integrinas ligam-se aos componentes da MEC dependendo do sítio do ligante que são capazes de reconhecer. As integrinas $\alpha_v\beta_3$ e a $\alpha_5\beta_1$, por exemplo, reconhecem a sequência RGD (Arg-Gly-Asp) em seus ligantes, sendo classificadas como receptores RGD. Outras, como por exemplo a integrina $\alpha_4\beta_1$, reconhecem as sequências Glu–Ile–Leu–Asp–Val (EILDV) e Arg–Glu–Asp–Val (REDV) presente no colágeno, sendo classificadas como receptores de colágeno. Existem também integrinas que reconhecem outras sequências como os receptores de laminina e os receptores leucócito-específico (DESGROSELLIER; CHERESH, 2010).

O repertório de integrinas presente numa célula determina o grau com o qual essa célula irá aderir e migrar em diferentes matrizes. A ligação da célula à MEC desencadeia a sinalização "*outside-in*", que envolve o agrupamento de integrinas e o recrutamento de proteínas de adesão focal que as ligam fisicamente ao citoesqueleto de actina. Esta organização matricial estimula um *feedback* positivo que promove mais agrupamentos de integrinas com fosforilação de proteínas quinases como a de adesão focal (FAK). Durante a sinalização "*inside-out*", a proteína intracelular talina se liga à cauda citoplasmática das subunidades β, resultando em uma mudança conformacional do domínio extracelular para uma forma ativa que tem alta afinidade por componentes da MEC (GIANCOTTI, 1999; QIN;

VINOGRADOVA; PLOW, 2004). Recentemente, foi relatada uma sinalização multidirecional das integrinas (**figura 4**) em que integrinas em endomembranas desencadeiam sinalização "*inside-in*" (ALANKO et al., 2015; BARROW-MCGEE et al., 2016; NADER; EZRATTY; GUNDERSEN, 2016).

**Figura 4 -** Sinalização multidirecional das integrinas. As integrinas são moléculas de sinalização que existem em diferentes estados conformacionais que determinam a afinidade do receptor para proteínas da matriz extracelular (MEC): uma integrina dobrada (fechada) representa a forma inativa, com baixa afinidade por ligantes da MEC, enquanto uma integrina estendida (aberta) representa a forma ativa e capaz de provocar sinalização a jusante e respostas celulares. A consequência do envolvimento integrina-ligante (adesão) e agrupamento na membrana plasmática fornece uma plataforma para a montagem de complexos multiméricos que provocam sinalização de adesão a jusante (sinalização *outside-in*). Esse sinal externo envolve recrutamento e autofosforilação de quinase de adesão focal (FAK) com subsequente recrutamento e ativação do SRC. A adesão à integrina também ativa, outras vias de sinalização RAS-MAPK e PI3K-AKT. As integrinas também respondem a sinais *inside-out*, em que a estimulação da atividade de pequenas GTPase na membrana plasmática desencadeia o recrutamento da moléculas adaptadoras que interage com RAP1-GTP para ativar o talina. A ligação de talina à cauda da subunidade β-integrina desencadeia uma conformação do receptor aberto. Também foi demonstrado que as integrinas são funcionais em locais subcelulares como as encontradas nos endossomos que podem desencadear a sinalização *inside-in*. Fonte: modificado de HAMIDI; IVASKA, 2018.

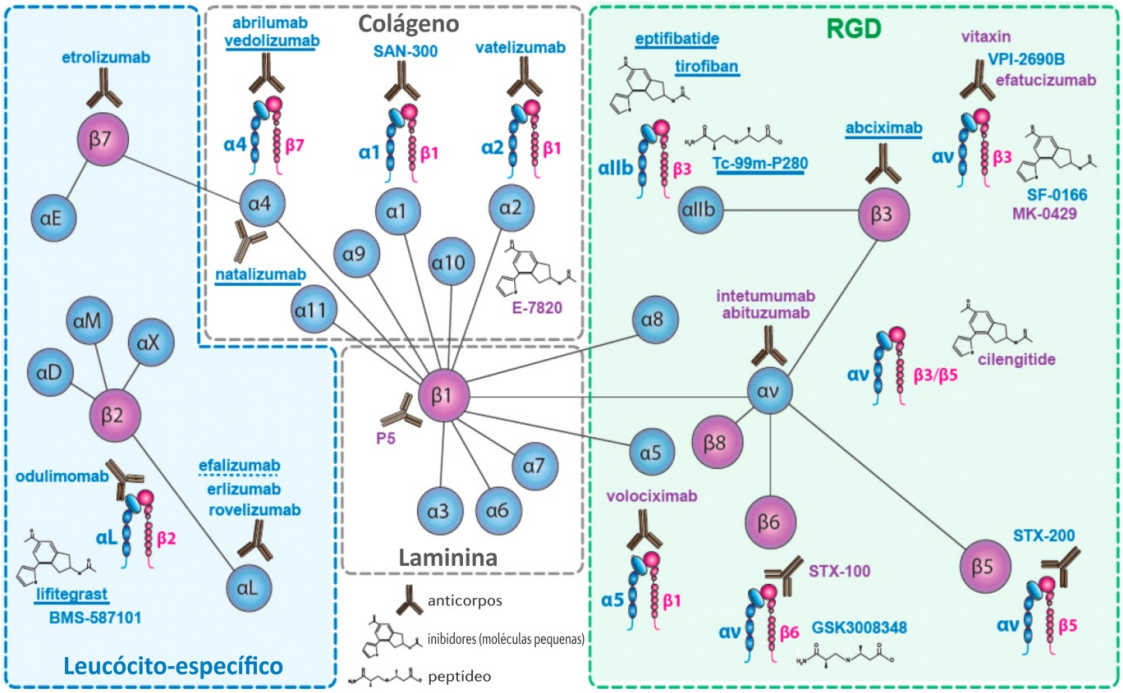



As integrinas não possuem atividade quinase intrínseca, mas indiretamente recrutam proteínas tais como talina, paxilina, FAK e pequenas GTPases. Esses efetores regulam os

níveis de ciclinas e de fatores de transcrição de genes necessários para regulação do ciclo celular e proliferação (WALKER; ASSOIAN, 2005). As integrinas podem também mediar a proliferação celular e a motilidade por meio da ativação de proteína quinase ativada por mitógeno (MAPK), SRC, fosfatidilinositol-3-quinase (PI3K) e RhoA, desencadeando propagação e invasão celular (ARTHUR; PETCH; BURRIDGE, 2000; COSTA et al., 2013; LONGHURST; JENNINGS, 1998).

Por suas posições estratégicas na membrana plasmática e por interagir em locais regulatórios extracelulares, as integrinas se tornam potenciais alvos sensíveis à intervenção farmacológica, como comprovado pelo sucesso clínico de sete (vedolizumab, natalizumab, abciximab, eptifibatide, tirofiban, Tc-99m-P280 e lifitegrast) candidatos a fármacos direcionados a elas . Dezenove dos 24 heterodímeros de integrinas têm sido alvos terapêuticos para a descoberta de fármacos, como indicado na figura 5 (COX; BRENNAN; MORAN, 2010; GOODMAN; PICARD, 2012; RAAB-WESTPHAL et al., 2017).

**Figura 5 -** Integrinas alvos em ensaios clínicos. Em violeta: substâncias usadas em testes de câncer. Em azul: substâncias usadas em testes não tumorais. Os fármacos comercializados estão sublinhados com linha contínua; fármacos retirados do mercado estão sublinhados com linha pontilhada. Fonte: modificado de RAAB-WESTPHAL et al., 2017.



Embora estudos pré-clínicos e ensaios clínicos em câncer de mama tenham avaliado inibidores farmacológicos para integrinas (BURKE et al., 2002; LI et al., 2015), mais estudos são necessários para elucidar se os inibidores de integrina podem bloquear a metástase. Terapias baseadas em proteínas têm sido amplamente testadas devido à sua capacidade de se ligar diretamente às integrinas e afetar alvos a jusante dentro das células. Essas terapias incluem anticorpos, peptídeos que simulam a MEC, antagonistas de moléculas pequenas e desintegrinas (GOODMAN; PICARD, 2012; MILLARD; ODDE; NEAMATI, 2011; RAAB-WESTPHAL et al., 2017).

### *1.1.5 As desintegrinas no estudo do papel das integrinas*

As desintegrinas de veneno de serpente são principalmente produtos de proteólise de precursores contendo um domínio metaloprotease, chamadas de *Snake Venom Metalloproteases* (SVMPs). Membros dessa família de proteínas SVMPs têm sido classificados de acordo com as estruturas de seus multi-domínios nas classes: P-I, P-II e P-III. Os membros da classe P-I são formados por pro-domínio e um domínio metaloprotease, enquanto que as proteínas P-II além desses domínios possuem também um domínio desintegrina. As proteínas P-III têm um pro-domínio, um domínio metaloprotease e um domínio tipo-desintegrina seguido de um domínio rico em cisteína e, em alguns casos, um domínio adicional lectina (FOX; SERRANO, 2005, 2008). O domínio desintegrina da classe P-II geralmente contém os motivos adesivos RGD, VGD ou KGD. Já as proteínas tipo-desintegrina provenientes da classe P-III, normalmente possuem as sequências DCD (Asp-Cys-Asp) ou ECD (Glu-Cys-Asp) (FOX; SERRANO, 2008; MARKLAND, 2013; RAMOS; SELISTRE-DE-ARAUJO, 2006; SELISTRE-DE-ARAUJO et al., 2010). A classe P-III é caracterizada por possuir uma grande diversidade de atividades biológicas, sendo subdividida nas subclasses P-IIIa, P-IIIb, P-IIIc e P-IIId. Essa subdivisão baseia-se em suas modificações pós-traducionai, como pela homo-dimerização (P-IIIc) ou proteólise entre os domínios metaloprotease e domínio tipo-desintegrina (P-IIIb). Aclasse P-IV está incluída agora no grupo P-III como uma subclasse (P-IIId). A classe P-IIId é considerada um derivado pós-traducional da classe P-III (P-IIIa) de SVMPs (TAKEDA, et al., 2012).

A ação das SVMPs envolve propriedades catalíticas e antiadesivas observadas na interferência das funções plaquetárias por hidrólise do fator de von Willebrand (FvW) e por perturbar a interação da integrina $\alpha_2\beta_1$ com o colágeno, por exemplo. As SVMPs tem como

alvo importantes elementos como proteínas da membrana basal, da coagulação, de plaquetas e de células endoteliais (MOURA-DA-SILVA; BUTERA; TANJONI, 2007; TANJONI et al., 2010). O domínio desintegrina e o domínio tipo-desintegrina mais especificamente despertam interesse terapêutico por se ligarem às integrinas e desencadearem respostas celulares como inibição da adesão celular, migração, angiogênese e metástase (HUANG, 1998; MARCINKIEWICZ, 2005; MCLANE; JOERGER; MAHMOUD, 2008; YANG et al., 2005). Dessa forma, os domínios desintegrinas e tipo-desintegrinas das SVMPs podem, além de nos permitir uma melhor compreensão das atividades celulares via integrinas, ser considerados potenciais agentes antimetástase.

### *1.1.6 A ALT-C e a integrina $\alpha_2\beta_1$ como potencial alvo antimetástase*

A alternagina-C (ALT-C), proveniente do veneno da serpente *Bothrops alternatus*, é produto do processamento proteolítico da alternagina, uma metaloprotease da classe PIII com massa molecular de 55 kDa. Nesse processamento proteolítico a alternagina libera o domínio tipo-desintegrina associado ao domínio rico em cisteína, formando assim a ALT-C (29 kDa) (SOUZA et al., 2000). O sequenciamento parcial dos aminoácidos da ALT-C confirmou a presença do motivo ECD semelhante a outras proteínas tipo-desintegrina (SELISTRE-DE-ARAUJO et al., 2005).

Estudos utilizando a ALT-C mostraram que essa proteína é capaz de inibir a quimiotaxia celular em neutrófilos. A ALT-C também induziu significativamente mudanças no citoesqueleto com polimerização da F-actina, ativação de FAK e PI3K, assim como translocação de ERK-2. Esses efeitos foram reproduzidos por um peptídeo com domínio ECD derivado da sequência primária da ALT-C, sugerindo o domínio ECD como relevante aos efeitos celulares observados (MARIANO-OLIVEIRA et al., 2003; RAMOS et al., 2007; SOUZA et al., 2000). ALT-C inibiu a adesão celular ao colágeno tipo I em várias linhagens celulares tumorais e não tumorais provavelmente pela interação com a integrina $\alpha_2\beta_1$ (COMINETTI et al., 2004; SOUZA et al., 2000).

O efeito da ALT-C apresenta certa dualidade que é dependente da concentração utilizada. Observa-se que em baixas concentrações a ALT-C induz proliferação, angiogênese e migração enquanto que em altas concentrações inibe tais processos celulares (RAMOS et al., 2007). Recentemente, confirmou-se a ALT-C como um ligante da integrina $\alpha_2\beta_1$, sendo esta capaz de diminuir a atividade das metaloproteinases – 2 (MMP-2) e -9 (MMP-9) em

células endoteliais e células tumorais de mama, demonstrando a ALT-C como um potencial agente antimetástase (MORITZ et al., 2018).

## 1.2 Justificativa

A adesão celular e a migração são passos cruciais para o desenvolvimento de metástases, processos em que as integrinas estão fortemente envolvidas. Tem sido demonstrado que proteínas de veneno de serpente inibem metástase experimental por efetivamente bloquearem a atividade de integrinas. Diante disso, estudos que determinem o papel das integrinas na modulação de metástase tumoral são fundamentais para se encontrar novos alvos farmacológicos destinados à prevenção de metástase (SELISTRE-DE-ARAUJO et al., 2010).

O tratamento para o câncer de mama, nos últimos anos, vem aprimorando-se e várias terapias podem levar com sucesso à remissão completa do tumor, elevando a sobrevida dos pacientes. No entanto, há ainda uma grande porcentagem de morte ligada ao câncer mamário devido à metástase, sendo urgente para a sociedade o desenvolvimento de novos fármacos antimetastáticos. Estudos recentes têm demonstrado uma relação entre a integrina $\alpha_2\beta_1$, um receptor de colágeno, e o desenvolvimento de metástases. Estudos anteriores do grupo mostraram que a alternagina-C (ALT-C) inibiu a adesão celular ao colágeno tipo I (Col I) em linhagens celulares tumorais de colo de útero e de bexiga, assim como modulou a expressão de genes relacionados à angiogênese e metástase (COMINETTI et al., 2004; SELISTRE-DE-ARAUJO et al., 2005). Dessa forma, a principal hipótese deste estudo é que a integrina $\alpha_2\beta_1$ tem um relevante papel para o desenvolvimento de metástase em câncer de mama e que a ALT-C interfere na adesão celular ao colágeno por ligar-se à integrina $\alpha_2\beta_1$, desencadeando respostas celulares que diminuem o surgimento de metástases. Essa hipótese foi investigada por meio do tratamento de células mamárias com ALT-C, um ligante da integrina $\alpha_2\beta_1$, assim como por meio do silenciamento da subunidade $\alpha_2$.

## 1.3 Objetivos

O objetivo geral deste estudo foi investigar a influência da integrina $\alpha_2\beta_1$ na migração e adesão celular para possíveis intervenções farmacológicas, visando a prevenção de metástases de origem mamária, utilizando células tumorais e não tumorais mamárias, tratadas com ALT-C ou silenciadas para a subunidade $\alpha_2$.

### *1.3.1 Objetivos específicos*

- Analisar os efeitos da ALT-C ou do silenciamento da subunidade $\alpha_2$ sobre células tumorais e não tumorais mamárias em relação às suas atividades adesivas e migratórias.
- Verificar o perfil de expressão de genes relacionados à metástase em células tumorais de mama tratadas com ALT-C (1000 nM) por PCR *array*.

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## 1.5 Manuscrito 1

### Alternagin-C stimulates the metastasis suppressor 1 (*MTSS1*) expression and attenuates collagen-based adhesion in triple-negative breast tumor cells

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**ABSTRACT**

Triple-negative breast cancer has an aggressive clinical course and its treatment has been challenging due to high metastatic risk. In order to overcome this type of cancer, molecular targets have been sought to provide better strategies for therapies. Integrins are cell adhesion receptors involved in tumor progression and $\alpha_2\beta_1$ integrin, a collagen receptor, has a key role in breast metastasis. In addition, breast cancer patients with a poor prognosis for metastasis have been shown reduced expression of the metastasis suppressor 1 (*MTSS1*). Alternagin-C (ALT-C), a $\alpha_2\beta_1$ integrin ligand, attenuates imflamation andangiogenesis and decreases metalloprotease levels in tumor microenvirment, suggesting anti-metastasis effects. However, its mechanisms of action in metastasis have not been fully explored. In this study, we investigated the effects of ALT-C in a triple-negative breast cancer cell line to elucidate how $\alpha_2\beta_1$ integrin affects cellular adhesion and migration and tumor metastasis pathways. For that, we performed adhesion and migration assays *in vitro* and a PCR array for 84 genes related to metastasis. In addition, we evaluated the $\alpha_2$ integrin subunit relevance in tumor cell behavior using transient silence for this subunit.. Alternagin-C attenuatted cell adhesion of triple-negative breast cancer cells (MDA-MB-231) to collagen I, but this effect was not observed in non-tumor breast cells (MCF 10A). The silencing of $\alpha_2$ integrin subunit in MDA-MB-231 cells did not inhibit cell adhesion and migration to collagen I, indicating that $\alpha_2$ integrin subunit did not play a crucial role in cell motility for this cell line. We also demonstrated that ALT-C stimulated the tumor suppressor 1 (*MTSS1*) gene expression and previously upregulated c-Myc mRNA level. Furthermore, we suggest that ALT-C may contribute to impair tumor establishment in secondary sites rich in collagen I.

## INTRODUCTION

Triple-negative breast cancer (TNBC) which lacks estrogen receptor (ER), progesterone receptor (PR) and human epithelial growth factor 2 receptor (HER2) has been challenging to treat (LEHMANN et al., 2011). A patient who was identified in this subgroup has a poorer prognosis and a higher risk of metastasis compared to non-TNBCs (DENT et al., 2007; PODO et al., 2010). Despite advances in TNBC therapies, little is known about molecular targets that could be pharmacological addressed to impair its progression. In this context, integrins have been investigated as a new strategy to triple-negative breast cancer therapy (PARVANI et al., 2015; TRUONG et al., 2014). Integrins comprise a family of transmembrane adhesion receptors that mediate attachment to each specific extracellular matrix (ECM) components such as collagen I (Col I) and fibronectin (FN). Integrins play different roles during breast cancer progression and mammary tumorigenesis (DAS et al., 2018; WHITE et al., 2004). These cell adhesion receptors regulate several steps during the metastatic cascade such as cell proliferation, invasion, migration and consequently tumor growth and survival (GUO; GIANCOTTI, 2004; SEGUIN et al., 2015; YAO et al., 2007; ZAHIR et al., 2003).

Among other adhesion receptors, Col I has recognition sites for $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_{10}\beta_1$ and $\alpha_{11}\beta_1$ integrins (GULLBERG et al., 1992; HEINO, 2007). Studies have been shown $\alpha_2\beta_1$ integrin as a novel therapeutic target for breast cancer (ADORNO-CRUZ; LIU, 2019; RAMIREZ et al., 2011). Recently, our group showed that Alternagin-C (ALT-C), a $\alpha_2\beta_1$ integrin ligand purified from *Bothrops alternatus* snake venom, decreases MMP-9 expression in breast tumor cells and MMP-2 in endothelial cells (MORITZ et al., 2018). In addition, ALT-C presented a potent chemotactic effect in neutrophils, activating intracellular signaling proteins such as focal adhesion kinase (FAK) and phosphatidylinositol 3-kinase (PI3K) without inducing cell death (COMINETTI et al., 2005; RAMOS et al., 2007). Besides, high concentration of ALT-C attenuates imflamation andangiogenesis in a murine models (RAMOS et al., 2007; RABELO et al., 2019). While previous results have suggested that ALT-C contributes to impair metastasis, the mechanisms involved remain unclear.

In this study we investigated cell adhesion and migration as well as gene expression related to metastasis signaling. To understand how ALT-C interferes in metastasis signaling, we performed a PCR array that demonstrated an increase more than three times in the metastasis suppressor 1 (*MTSS1*) expression in a triple-negative cell line (MDA-MB-231).

MTSS1 known as " missing in metastasis" was first identified in bladder cancer (LEE, et al., 2002), but it loss is also related to metastasis in breast cancer (LEI et al., 2014). This metastasis suppressor has the ability to regulate actin and plasma membrane dynamics. MTSS1 links cytoskeleton to plasma membrane, binding actin and phosphatydilinositol 4,5-bisphosphate (MATTILA, et al., 2007). Here, we investigate the effects of an $\alpha_2\beta_1$ integrin ligand in breast cancer cells and specifically in metastasis-related gene expression. To the best of our knowledge, this study was the first to explore the functional role between $\alpha_2\beta_1$ integrin and the *MTSS1* expression.

## MATERIALS AND METHODS

### *Alternagin-C purification*

ALT-C from *Bothrops alternatus* venom (donated by the Butantan Institute, São Paulo, Brazil) was purified as described by Souza *et al* (MORITZ et al., 2018; SOUZA et al., 2000). Briefly, two gel filtration followed by anion exchange chromatography was performed and mass spectroscopy was used to confirm the purity of ALT-C samples.

### *Cell culture*

Human triple-negative breast adenocarcinoma cell line (MDA-MB-231) from American Type Culture Collection (ATCC HTB-26, Manassas, USA) was maintained in Dulbecco's Modified Eagle Medium (DMEM – Vitrocell, Brazil) supplemented with 10% fetal bovine serum (FBS) and glycose (3.5 g/L). Human non-tumor breast cell line (MCF 10A) from ATCC (CRL-10317) were cultured in DMEM-F12 (DMEM – Vitrocell, Brazil) supplemented with 5% horse serum, EGF (20 ng/mL), hydrocortisone (0.5 mg/mL) and insulin (10 µg/mL). Human dermal microvascular endothelial cells (HMEC-1) from ATCC (CRL-3243) were cultured in MCDB-131 Medium (Sigma, Brazil) supplemented with 10% FBS. All cell lines were cultured in the presence of penicillin (100 IU/mL) and streptomycin (100 µg/mL) and L-glutamine (2 mM) in a humidified environment with 5% $CO_2$ at 37 °C.

***Transient $\alpha_2$ integrin subunit silencing***

The $\alpha_2$ integrin subunit transient silencing, MDA-MB-231 cells was seeded ($10^5$ cells/well) into 6-well plate with regular media overnight. Then, the cells were washed with phosphate-buffered saline (PBS) and media without antibiotics was added for the followed groups: i) non transfected control cells ii) lipofectamine control (Lipo), iii) scramble-EGFP (Sigma-Aldrich, EHUEGFP) control and iv) esi$\alpha_2$-MDA-MB-231 cells. The transfection was performed according tolipofectamine™ RNAiMAX reagent fabricant instructions (ThermoFisher, USA) and it was used 28 nM of MISSION®esiRNA (Sigma-Aldrich, EHU040531) to silence $\alpha_2$ integrin subunit (esi$\alpha_2$-MDA). The experiments were carried out after three days of transfection. Western Blot and flow cytometry was performed to confirm $\alpha_2$ integrin subunit silencing (**Supplementary figure 1**).

***Flow cytometry for integrin profile***

Flow cytometry was performed to check if ALT-C and $\alpha_2$ integrin subunit silencing change the integrin profile from tumor cells. Briefly, after dissociate cells and wash them with PBS, primary antibodies (anti-$\alpha_v$:SC-9969; anti-$\alpha_v\beta_5$:SC-81632; anti-$\alpha_v\beta_3$:LSBio 13734) were incubated for 1 hour and then cells were washed twice with PBS and incubated for more 1 hour with secondary antibodies (goat anti-mouse IgG-FITC: SC-2010), following dilutions indicated by fabricant. For the conjugated antibodies (PE mouse anti-human $\alpha_2$: BD-558759; PE mouse anti-human $\alpha_5$: BD-555617; FITC rat anti-human $\alpha_6$: BD-555735; PE mouse anti-human $\beta_1$: BD-555669) just one incubation during 1 hour was carried out. All incubations were performed at $4^0$C in absence of light and before analysis; cells were washed with PBS, centrifuged and acquisition was processed using Accuri c6 flow cytometer (BD Bioscience, USA).

***Morphology assay***

ALT-C treatment (1000 nM) for 24h in MDA-MB-231 cel line was performed. Cells ($10^4$ cells/well) were plated on Col I (10 μg/well) precoated 96-well plate and Col I coatings were blocked with 1% BSA (Sigma, Brazil) for 1 hour before cells plate. After 24h, cells were stained with Alexa fluor 488 Phalloidin (A12379, Thermo Fisher Scientific) as described by manufactures and nuclei stained with DAPI.

***Inhibition of adhesion to collagen I assay***

ALT-C (10, 100 and 1000 nM) treatments and $\alpha_2$ integrin subunit silencing was carried out in inhibion adhesion assay. Col I (10 μg/well) was added previously to a 96 plate and incubated overnight at $4^0$C. Col I coatings was blocked with 1% BSA (Sigma, Brazil) for 1h and, then, MDA-MB-231 and MCF 10A cells were added to the wells ($10^4$ cells/well) in serum free media. Cells were treated before with ALT-C (10, 100 and 1000 nM) for 30 min at $37^0$C. Then, cells were added into 96-well precoated Col I plate and incubated during 45 minutes at $37^0$C. After, wells were washed three times with PBS and cells were fixed with 4% paraformaldehyde and nuclei stained with DAPI for cellular counting in automated fluorescence microscopy (ImageXpress, Molecular Device), using MetaXpress software (Molecular Device). For $\alpha_2$ integrin subunit silenced MDA-MB-231 cells, assays were performed after 3 days of transfection.

***Haptotaxis migration assay***

Inserts with 8 μm pore size for 24-well plate were precoated on the underside with Col I (10 μg/mL) in cold room overnight. Then, inserts with Col I was placed inside wells with serum free media. MDA-MB-231 cells were treated previously with ALT-C (10, 100 and 1000 nM) in serum free media for 30 min at $37^0$C. Then, 2 x $10^5$ cells were added to the upper chambers in serum free media. After 24 hours of incubation, inserts were cleaned inside with swabs and cells that migrated to collagen-bound chemoattractant were fixed with 4% paraformaldehyde and nuclei stained with DAPI. Images were acquired in automated fluorescence microscopy (ImageXpress, Molecular Device) and nuclei counted by MetaXpress software (Molecular Device). For $\alpha_2$ integrin subunit silencing in MDA-MB-231 cells, the migration assay was performed after 3 days of transfection. Migration assays were not performed for MCF 10A cell line due to its incapability to migrate.

***Transmigration assay***

HMEC-1 cells (8 x $10^4$ cells/insert) were 48 hours previously seeded into an 8 μm pore size inserts for 24 well plate with 10% FBS supplemented media. Under the inserts was added serum free media to avoid migration. When HMEC-1 cells formed a monolayer on

inserts, esi$\alpha_2$-MDA cells were stained with PKH26 Red fluorescent cell linker kit (Sigma) accordingly with fabricant instructions after 3 days of transfection. Labeled cells (5 x $10^4$ cells/insert) were, then, added into inserts with serum free media to transmigrate for 16 hours toward chemoattractant (5% FBS media under inserts). After 16 h, inserts were washed and cleaned inside and cells were fixed with 4% paraformaldehyde and nuclei stained with DAPI. Images were capture with fluorescence microscopy (AXI0 Zeiss, 20x objective). Transmigration assay was not performed with MCF 10A cell line due to its cell incapability to migrate.

***PCR array for*** **metastasis related gene expression analysis**

Cells were plated ($10^6$ cells/well) in 6 cm cell culture dish and incubated overnight. After, it was added ALT-C treatment (1000 nM) in serum free media for 24 hours at $37^0$C. Then, total RNA was extracted using RNeasy MiniKit (Qiagen), following fabricant instructions. Samples were quantified using Nanodrop (Thermo Scientific); the 260nm/280nm and 260nm/230nm ratios were examined for protein and solvent contamination. RNA integrity number (RIM) analysis was performed to confirm RNA quality, using 2100 Bioanalyser (**Supplementary figure 2**). The $RT^2$ First Strand Kit (Qiagen) was used for cDNA synthesis reaction from 1 μg RNA. $RT^2$ RNA Quality Control-QC (Qiagen) was also performed before PCR array as indicated by manufactures. The RT2 RNA Quality Control-QC (Qiagen) plate contains controls for RNA integrity, DNA contamination, inhibitors of reverse transcription and of PCR amplification. The validated $RT^2$ Profiler PCR array (PAHS-0287, Qiagen) was carried out for 84 gene expression related with tumor metastasis. Data analysis was accessed using the GeneGlobe Data Analysis Center from Qiagen website (https://www.qiagen.com/us/shop/genes-and-pathways/data-analysis-center-overview-page/?akamai-feo=off). CT values were exported to an Excel file and then uploaded to the company's website for analysis (http://www.qiagen.com/geneglobe). CT values were normalized based on an Automatic selection from housekeeping genes (HKG) panel with ACTB; B2M; GAPDH; HPRT1 and RPLP0 reference genes. The data analysis web portal calculates fold change using delta delta CT method. Fold change is then calculated using 2^ (-delta delta CT) formula.

### *Statistics*

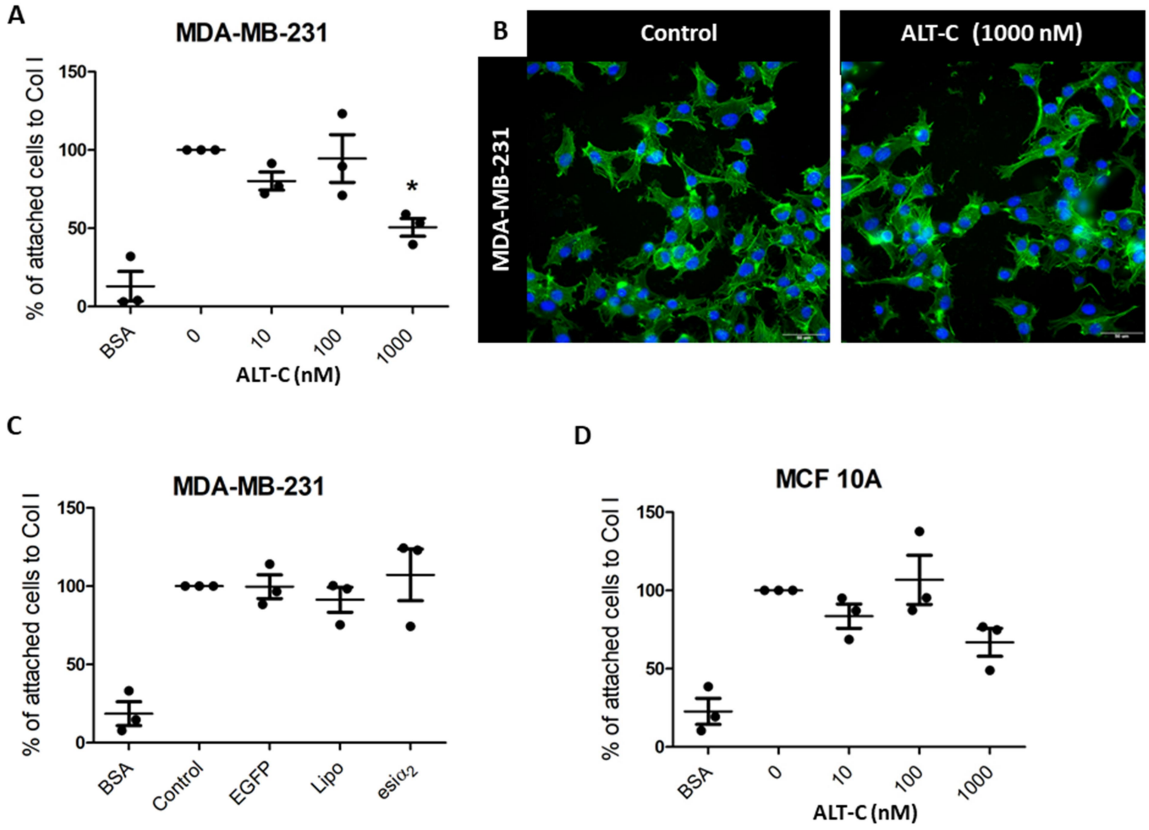
*In vitro* experiments were done in triplicates with three independent assays. After normality Shapiro-Wilk test, one-way analysis of variance (ANOVA) and Tukey's post-hoc test was used in GraphPad Prism 6 Software. Values are presented as mean ± SEM where * denotes $p<0.05$. For the PCR array, the p values are calculated based on a Student's t-test of the replicate 2^ (- Delta CT) values for each gene in the treatment group compared to control group.

## RESULTS

### *ALT-C attenuated cell adhesion to Col I in tumor cells, but not in $\alpha_2$* integrin *subunit silenced cells*

We first investigated whether ALT-C could inhibit cell adhesion in breast tumor cells and non-tumor breast cells. ALT-C decreased around 50% of tumor cells (MDA-MB-231) attachment to Col I at 1000 nM (**figure 1A**), however thistreatment did not change actin fiber stress and cell morphology (**figure 1B**). Considering the importance of $\alpha_2\beta_1$ integrin in breast cancer metastasis and that ALT-C binds to this integrin (MORITZ et al., 2018; SOUZA et al., 2000), we also silenced $\alpha_2$ integrin subunit in order to confirm its specific relevance in tumor cells. The results showed that $\alpha_2$ integrin subunit silencing did not inhibit MDA-MB-231 adhesion to Col I (**figure 1C**). In addition, ALT-C did not significantly inhibited non-tumor cells (MCF 10A) attachment to Col I at any used concentrations (**figure 1D**).

**Figure 1.** ALT-C (1000 nM) inhibited cell adhesion of MDA-MB-231 cells to collagen I but did not inhibit for MCF 10A cells as well as for esi$\alpha_2$-MDA cells. **A**: MDA-MB-231 cell percentage attached to collagen I (Col I) after treatments with ALT-C (10, 100 and 1000 nM). **B**: ALT-C did not change morphology of MDA-MB-231 cells when attached to Col I. Actin was stained with Alexa fluor 488 Phalloidin in green and nuclei stained with DAPI in blue. Images were obtained using fluorescence microscopy (20x objective, Image Xpress, Molecular Device). Scale bar: 50 μm. **C**: MDA-MB-231 cell percentage attached to Col I. Control means non transfected cells. EGFP control means Enhanced Green Fluorescent Protein MISSION® esiRNA (Sigma) which do not target any human genes. Lipo means cells treated with transfecting agent only. esi$\alpha_2$ means MDA-MB-231 cells transfected for $\alpha_2$ silencing. **D**: MCF 10A cell percentage attached to Col I after treatments with ALT-C (10, 100 and 1000 nM). BSA means negative control which wells was blocked with 1% BSA during 1 hour. These assays were performed in triplicate with three independent experiments (n=3). * denotes $p<0.05$.

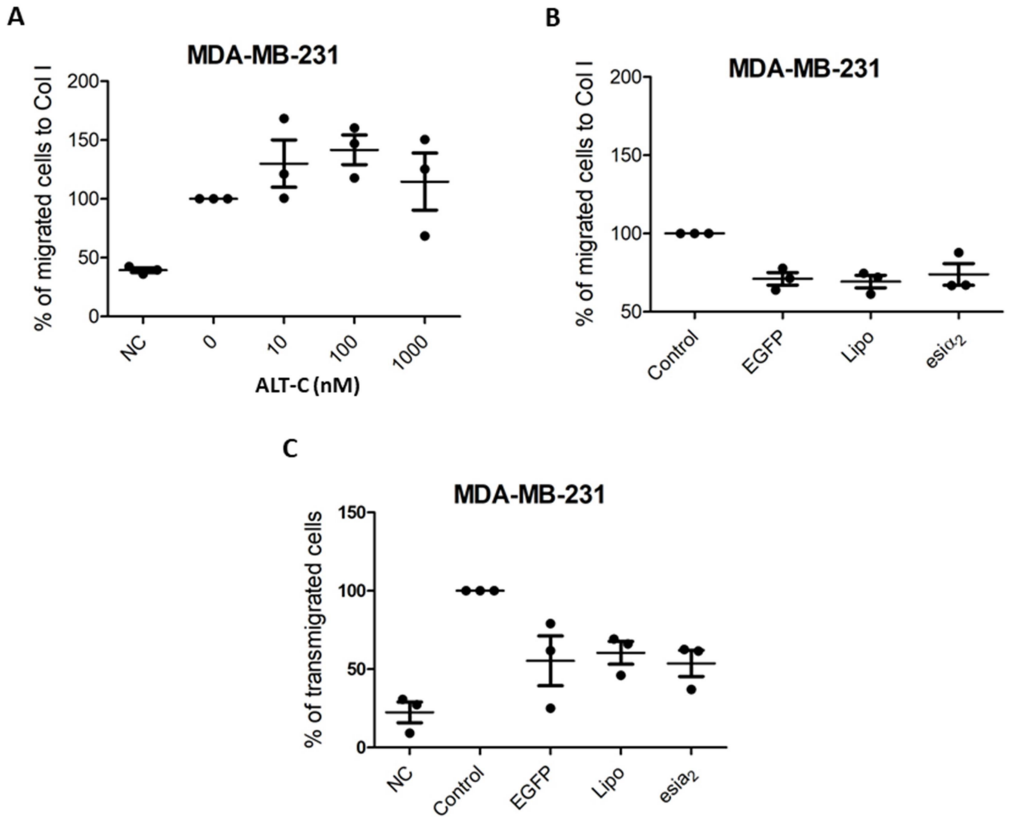



***ALT-C treatment and $\alpha_2$ integrin subunit silencing did not inhibit cell migration behavior***

To further investigate ALT-C treatment and $\alpha_2$ integrin subunit silencing in cell migration to Col I, it was performed haptotaxis migration assay. ALT-C treatments (10, 100 and 1000 nM) or $\alpha_2$ integrin subunit silencing did not affect migration in MDA-MB-231 cells to Col I (**figure 2A and B**). As recently demonstrated, ALT-C stimulated cell transmigration through HMEC-1 cells at low concentrations (MORITZ et al., 2018). To elucidate if $\alpha_2$ integrin subunit has a role in this process we also performed transmigration assay with esi$\alpha_2$-MDAcells. However, $\alpha_2$ integrin subunit silencing did not affect transmigration through endothelial cells (**figure 3C**).

**Figure 2.** ALT-C did not affect migration of MDA-MB-231 cells to Col I. **A**: MDA-MB-231 cell percentage that migrated to Col I after treatments with ALT-C (10, 100 and 1000 nM). Negative control (NC) means inserts without Col I. **B**: esi$\alpha_2$-MDA cell percentage that migrated to Col I in haptotaxis assay. **C**: Percentage of MDA-MB-231 cell transmigrated through HMEC-1 cells. The chemoattractant used was 5% of fetal bovine serum (FBS) in the lower chamber. Negative control (NC) means absence of FBS in the lower chamber. Control means non transfected cells. EGFP control means Enhanced Green Fluorescent Protein MISSION® esiRNA (Sigma)

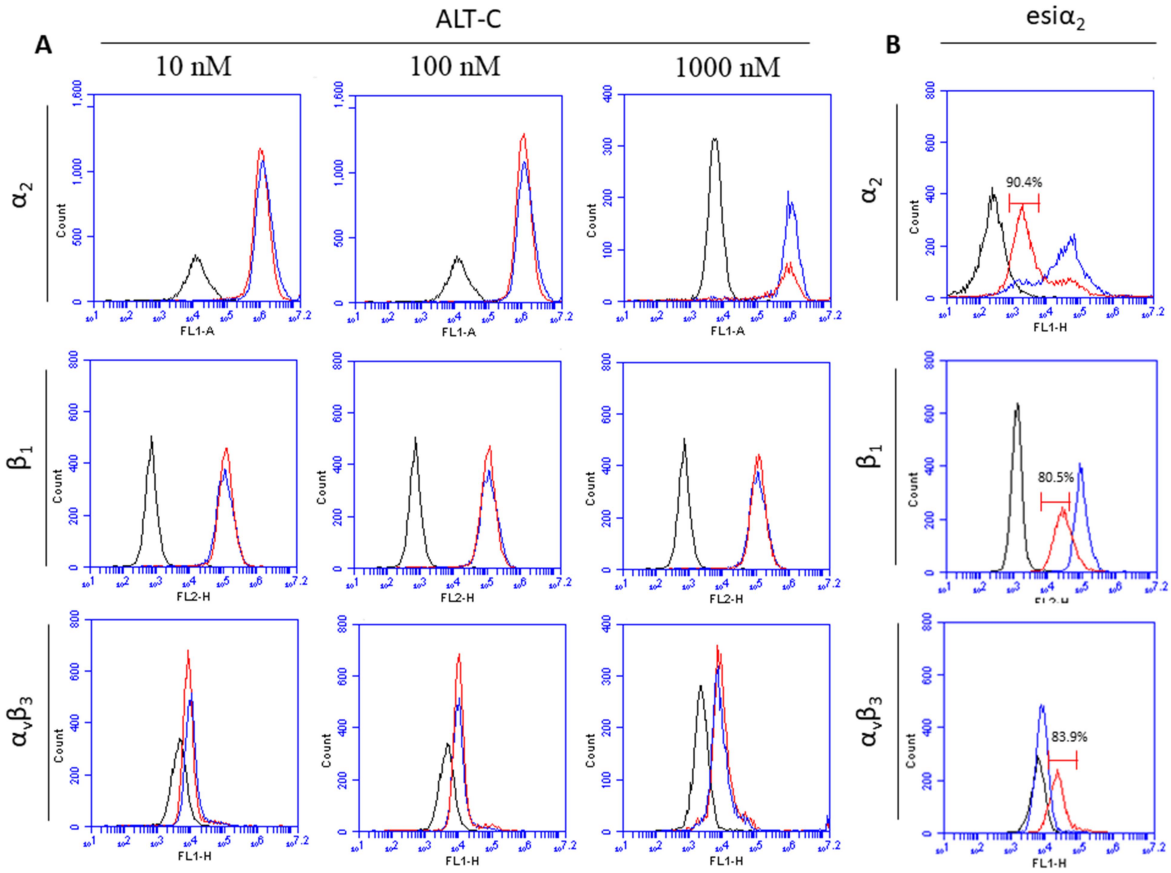
which do not target any human genes and Lipo means cells treated with transfecting agent only. esi$\alpha_2$ means cells transfected for $\alpha_2$ silencing. Assays were performed in triplicate with three independent experiments (n=3).




***Flow cytometry for integrin profile***

Since esi$\alpha_2$-MDA cells did not show any significant results and ALT-C interfered in cell behavior (MORITZ et al., 2018), we hypothesized that another integrin could play a role on ALT-C response and some integrin could be compensating the $\alpha_2$ integrin subunit silencing effect. In order to confirm these hypotheses we carried out flow cytometry for integrin profile changes analysis. ALT-C (10, 100 and 1000 nM) did not interfered in MDA-MB-231 integrin profile (**figure 3A**). Indeed, esi$\alpha_2$-MDA cells have increased $\alpha_v\beta_3$ (83.9%) integrin levels and decreased $\alpha_2$ (90.4%) and $\beta_1$ (80.5%) integrin subunits levels (**figure 3B**).

**Figure 3**. Flow cytometry for integrin profile. **A**: ALT-C (10, 100 and 1000 nM) did not interfere on the MDA-MB-231 cells integrin profile. Blue lines represent MDA-MB-231 cells labeled for integrin $\alpha_2$, $\beta_1$ or $\alpha_v\beta_3$; red lines represent MDA-MB-231 cells treated with ALT-C (10, 100 and 1000 nM) and labeled for integrin $\alpha_2$, $\beta_1$ or $\alpha_v\beta_3$. **B**: $\alpha_2$ integrin subunit silencing decreased $\alpha_2$ and $\beta_1$ integrin subunits and increased $\alpha_v\beta_3$ integrin levels in MDA-MB-231 cells. Blue line represents MDA-MB-231 labeled for integrin $\alpha_2$, $\beta_1$ or $\alpha_v\beta_3$; red line represents esi$\alpha_2$-MDA cells and labeled for integrin $\alpha_2$, $\beta_1$ or $\alpha_v\beta_3$. Black lines represent non-labeled MDA-MB-231 cells for cellular autofluorescence control.

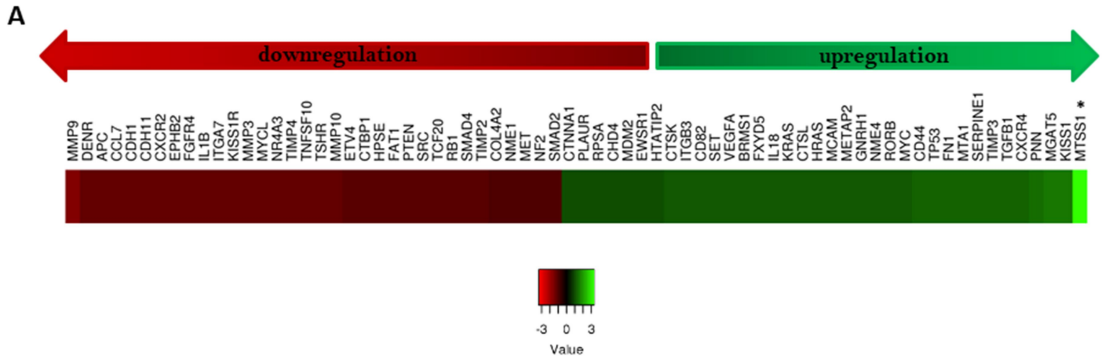



$\alpha_v$, $\alpha_5$ and $\alpha_6$ integrin subunits, as well as $\alpha_v\beta_5$ integrin were also screened by flow cytometry, however no differences were observed for ALT-C treatments (data not shown). $\alpha_v$, $\alpha_5$ and $\alpha_6$ integrin subunits and $\alpha_v\beta_5$ integrin had no differences for $\alpha_2$ integrin subunit silenced in MDA-MB-231 cells (data not shown).

### *ALT-C increased MTSS1 gene expression*

ALT-C (1000 nM) treatment upregulated *MTSS1* expression after 24 hours, showing that $\alpha_2\beta_1$ binding can increase a metastasis suppressor in TNBC cells (**figure 4**).

**Figure 4.** ALT-C increased the metastasis suppressor 1 (*MTSS1*) gene expression in a triple-negative breast cancer cell line (MDA-MB-231). A: Tumor cells were treated with 1000 nM ALT-C for 24 hours. Data analysis was performed using the GeneGlobe Data Analysis Center from Qiagen website and heat map performed by Morpheus online software. Negative values mean downregulated genes and positive values mean upregulated genes compared to non-treated control. * denotes $p<0.05$. Values represent magnitude of $\log_{10}$ (Fold Change).




*CDH6*, *CDKN2A*, *CST7*, *CXCL12*, *FLT4*, *HGF*, *IGF1*, *MMP11*, *MMP13*, *MMP2*, *MMP7*, *SKTR2*, *SYK* and *TRPM1* genes from RT$^2$ Profiler PCR array (PAHS-0287, Qiagen) were not detected, showing that MDA-MB-231 cell line has extremely low mRNA levels for these genes. Reference primer numbers, gene symbols and descriptions for the 84 genes screened were presented at Supplementary table 1.

## DISCUSSION

During the metastastic process, tumor cells spread from primary sites, migrate and attach to secondary sites by means of adhesion receptors such as integrins that provide interactions between cells and ECM. Integrin expression is frequently altered during all steps of the metastatic cascade (RATHINAM; ALAHARI, 2010). In breast cancer, the collagen receptor $\alpha_2\beta_1$ integrin has been studied as a molecular target for anti-metastasis therapies. In this study we investigated ALT-C, a $\alpha_2\beta_1$ integrin ligand, as well as $\alpha_2$ integrin subunit silencing effects in a metastatic breast tumor cell line.

Previous studies of our group showed that ALT-C strongly inhibited cell adhesion in erythroleukemia cell line (K562) transfected with $\alpha_2\beta_1$ integrin, however it did not inhibit K562 cells transfected with $\alpha_{IIb}\beta_3$, $\alpha_1\beta_1$, $\alpha_5\beta_1$, $\alpha_4\beta_1$, $\alpha_v\beta_3$, and $\alpha_9\beta_1$ integrins (SOUZA et al.,

2000), indicating the ALT-C specificity for tumor cells expressing $\alpha_2\beta_1$ integrin . Besides, ALT-C inhibited adhesion to Col I of other tumor cells such as human cervix epithelioid carcinoma (HeLa) and human bladder epithelioid carcinoma (ECV-304/T24) (Cominetti et al, 2005). In this study, we demonstrated that ALT-C inhibited MDA-MB-231 breast tumor cells adhesion to Col I, but did not in non-tumor breast cell line (MCF-10A).

The pattern of integrin expression changes during cell migration along tumor progression and metastasis. Cell motility is commonly related with $\alpha_v\beta_3$ and $\alpha_3\beta_1$ integrins in several metastatic tumor cells as melanoma, breast and prostate carcinomas (FELDING-HABERMANN, 2003; LI et al., 2001; SLOAN et al., 2006). We suggest that the increase of $\alpha_v\beta_3$ integrin in esi$\alpha_2$-MDA cells compensated tumor cell migration and this increase impaired the motility inhibition by $\alpha_2$ subunit silencing. On the other hand, $\alpha_2$ integrin subunit silencing decreased $\beta_1$ integrin as expected, since it is a partner of $\alpha_2$ integrin subunit. It was previously demonstrated that TNBC cells increased $\beta_3$ integrin levels after knockdown of $\beta_1$ integrin enhancing dissemination of breast cancer cells to lung (TRUONG et al., 2014). The switch between $\beta_1$ and $\beta_3$ integrins were also described by Parvani *et al* (PARVANI et al., 2013) that observed a compensatory increase in $\beta_3$ integrin upon $\beta_1$ integrin decrease in breast cancer. Our results suggest that $\alpha_2$ integrin subunit silencing did not inhibit cell migration due to an increase $\alpha_v\beta_3$ integrin which compensates the cell migration response in agreement with previous studies showed for $\beta_1$ knockdown (MADAMANCHI; ZIJLSTRA; ZUTTER, 2015). Data from cytometry analysis also showed that ALT-C treatments did not change integrin profile in MDA-MB-231 cells, demonstrating that ALT-C induced cellular response without interfering in the expression of other integrins. In addition, it was previously demonstrated that ALT-C interacts directly with $\alpha_2\beta_1$ integrin, showing that the main integrin involved in ALT-C cellular effects should be this integrin (MORITZ et al., 2018). ALT-C was unable to inhibit cell migration, although it was able to stimulate MDA-MB-231 transmigration at low concentrations through endothelial cells (MORITZ et al., 2018), indicating that ALT-C (10 nM) also interacts with endothelial cells to stimulates tumor cell transmigration. The $\alpha_2$ integrin subunit silencing did not inhibit cell migration and curiously neither cell adhesion to Col I. Ziaee *et al* (ZIAEE; CHUNG, 2014) demonstrated that a highly bone metastatic RANKL-overexpressing LNCaP prostate cancer cell line (LNCaP) induced $\alpha_2$ integrin expression in 3D model but not in 2D model, suggesting that a spatial role for $\alpha_2\beta_1$ integrin in cell adhesion. Taking all these findings, we indicate that $\alpha_2\beta_1$ integrin could act as a secondary player in cell migration. Besides, considering that ALT-C binds to $\alpha_2\beta_1$ integrin (MORITZ et

al., 2018; SOUZA et al., 2000) and $\alpha_2$ integrin subunit silenced cells did not presented any significant change in tumor cell behavior, we suggest that ALT-C may acts by interfering with $\beta_1$ integrin subunit. Similar observations were made for DisBa-01, a RGD-disintegrin that inhibits $\alpha_v\beta_3$ integrin by binding to the $\beta_3$ subunit (DANILUCCI et al., 2019; MONTENEGRO et al., 2012, 2017). *MTSS1*, also known as *missing in metastasis* (*MIM*) has been related to a poor prognosis when at low levels in several cancer types such as glioblastoma, bladder cancer, gastric cancer, lung cancer and breast cancer (LEI et al., 2014; LING et al., 2016; LIU et al., 2010; WANG et al., 2007; ZHANG; QI, 2015). Otherwise, high expression of *MTSS1* is also associated with an increased survival rate in patients with breast cancer (LEI et al., 2014) and a decreased cell invasion, migration, tumor growth and adherence in human breast cancer cells (PARR; JIANG, 2009). Studies demonstrated that MTSS1 enhances cell-cell junction strength in tumor metastasis and is associated with actin cytoskeleton reorganization (DAWSON et al., 2012; MERTZ et al., 2014), however MTSS1 was not related with adhesion to ECM components before. Moreover, *MTSS1* plays a key role in cell-cell adhesion stability and its loss promotes epithelial-to-mesenchymal transition in cancers during tumor progression (DAWSON et al., 2012; VADAKEKOLATHU et al., 2018; XIE et al., 2018; ZHANG; QI, 2015). Although several integrin-mediated cell signaling are related to breast cancer metastasis (COX; BRENNAN; MORAN, 2010; QIN; VINOGRADOVA; PLOW, 2004; SEGUIN et al., 2015), *MTSS1* was not described in integrin downstream signaling yet. Integrins are closely related to actin dynamics through focal adhesion (COSTA et al., 2013) and FAK was also demonstrated as a major mediator of the regulatory effects of MTSS1 (LING et al., 2016). Once ALT-C was involved in FAK activation (MARIANO-OLIVEIRA et al., 2003; MORITZ et al., 2018) and in this study we observed that ALT-C impared cell adhesion to collagen I and increased *MTSS1* expression, we suggest that this metastasis suppressor gene could also be involved in cell-ECM adhesion via $\alpha_2\beta_1$ integrin. Despite MTSS1 may be involved with lamellipodia formation and interacts with actin-associated protein (LIN et al., 2005), ALT-C did not change actin fiber stress and cell morphology of MDA-MB-231 cells. In this way, we also suppose that ALT-C may be able to increase the formation of cellular junctions, overexpressing *MTSS1* instead of interferes in actin directly.

In conclusion, the present study demonstrated that ALT-C, after binding to $\alpha_2\beta_1$ integrin, inhibited cell adhesion to Col I and stimulated *MTSS1* expression in breast tumor cells. These findings suggest that ALT-C may impair tumor cell adhesion in secondary sites such as in bone which has high collagen I percentage. Besides, we indicate that ALT-C could

be a potential anti-metastasis agent due to stimulation of *MTSS1* expression in breast cancer cells. Overall, these results provide new insights into the role of $\alpha_2\beta_1$ integrin in metastasis and can also contribute to help the development of new therapies for triple-negative breast cancers.

## ACKNOWLEDGMENTS

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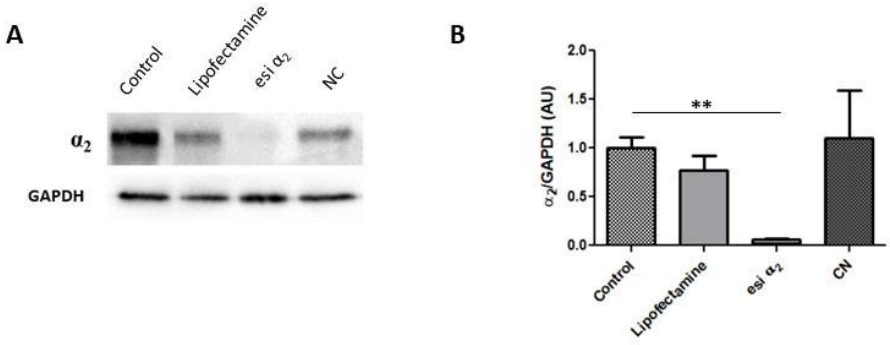
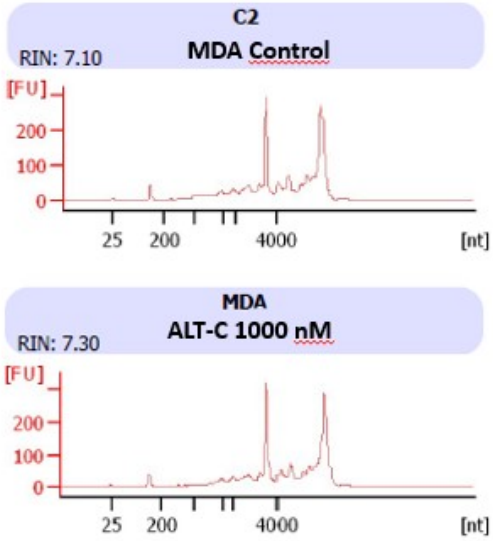
## Supplementary Material

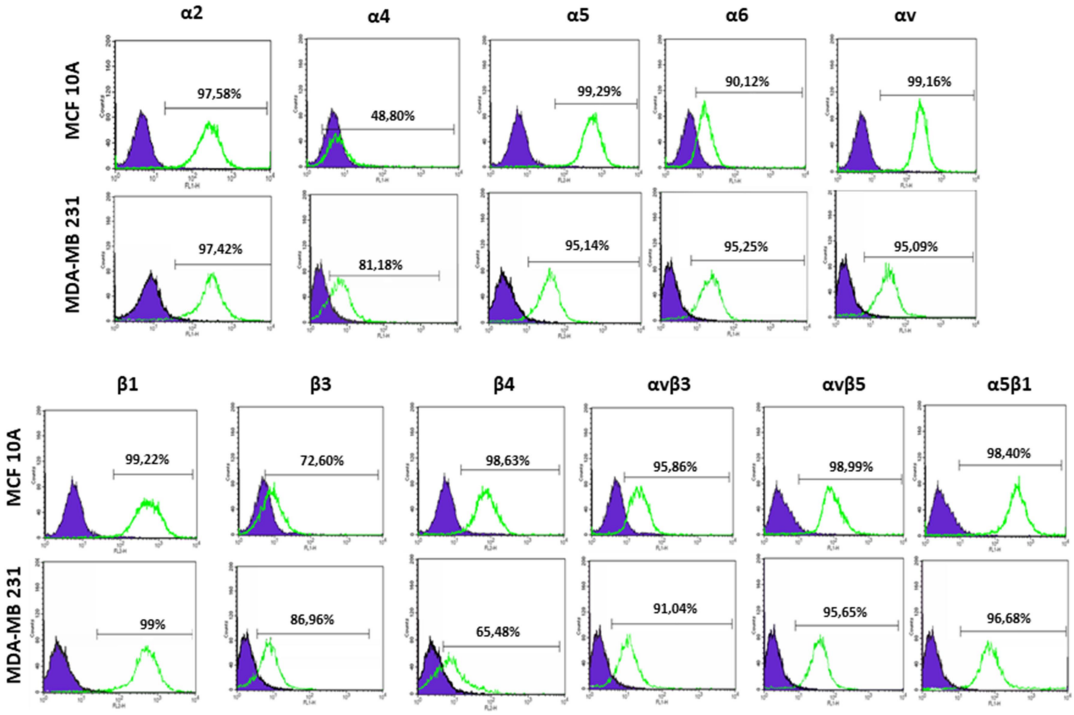
**Supplementary figure 1.** A: MDA-MB-231 cells transfected for $\alpha_2$ subunit integrin silencing. $\alpha_2$ subunit integrin protein level revealed by Western blotting. B: Values represent the normalized densitometry ratio in three independent assays. **p < 0.01 compared to control.




**Supplementary figure 2.** RNA quality from MDA-MB-231 treated with ALT-C at 1000 nM for 24 hours and MDA-MB-231 control samples RNA integrity number (RIN) samples analysis using 2100 Bioanalyser.



**Supplementary figure 3.** Flow Cytometry integrin profile for MDA-MB-231 and MCF 10A cell lines. Green lines represent cells labeled for integrins and blue represent non-labeled cells for cellular autofluorescence control.



**Supplementary table 1.** Reference sequence numbers, gene symbols and descriptions from $RT^2$ Profiler PCR array (PAHS-0287, Qiagen).

| RefSeq Number | Symbol | Description |
|---|---|---|
| NM_000038 | APC | Adenomatous polyposis coli |
| NM_015399 | BRMS1 | Breast cancer metastasis suppressor 1 |
| NM_006273 | CCL7 | Chemokine (C-C motif) ligand 7 |
| NM_000610 | CD44 | CD44 molecule (Indian blood group) |
| NM_002231 | CD82 | CD82 molecule |
| NM_004360 | CDH1 | Cadherin 1, type 1, E-cadherin (epithelial) |
| NM_001797 | CDH11 | Cadherin 11, type 2, OB-cadherin (osteobla |
| NM_004932 | CDH6 | Cadherin 6, type 2, K-cadherin (fetal kidney) |
| NM_000077 | CDKN2A | Cyclin-dependent kinase inhibitor 2A (melan |
| NM_001273 | CHD4 | Chromodomain helicase DNA binding prote |
| NM_001846 | COL4A2 | Collagen, type IV, alpha 2 |
| NM_003650 | CST7 | Cystatin F (leukocystatin) |
| NM_001328 | CTBP1 | C-terminal binding protein 1 |
| NM_001903 | CTNNA1 | Catenin (cadherin-associated protein), alpha |
| NM_000396 | CTSK | Cathepsin K |
| NM_001912 | CTSL | Cathepsin L1 |
| NM_000609 | CXCL12 | Chemokine (C-X-C motif) ligand 12 |
| NM_001557 | CXCR2 | Chemokine (C-X-C motif) receptor 2 |
| NM_003467 | CXCR4 | Chemokine (C-X-C motif) receptor 4 |
| NM_003677 | DENR | Density-regulated protein |
| NM_004442 | EPHB2 | EPH receptor B2 |
| NM_001986 | ETV4 | Ets variant 4 |
| NM_005243 | EWSR1 | Ewing sarcoma breakpoint region 1 |
| NM_005245 | FAT1 | FAT tumor suppressor homolog 1 (Drosophil |
| NM_002011 | FGFR4 | Fibroblast growth factor receptor 4 |
| NM_002020 | FLT4 | Fms-related tyrosine kinase 4 |
| NM_002026 | FN1 | Fibronectin 1 |
| NM_014164 | FXYD5 | FXYD domain containing ion transport regul |
| NM_000825 | GNRH1 | Gonadotropin-releasing hormone 1 (luteiniz |
| NM_000601 | HGF | Hepatocyte growth factor (hepapoietin A; sc |
| NM_006665 | HPSE | Heparanase |

| | | |
|---|---|---|
| NM_002425 | MMP10 | Matrix metallopeptidase 10 (stromelysin 2) |
| NM_005940 | MMP11 | Matrix metallopeptidase 11 (stromelysin 3) |
| NM_002427 | MMP13 | Matrix metallopeptidase 13 (collagenase 3) |
| NM_004530 | MMP2 | Matrix metallopeptidase 2 (gelatinase A, 72kDa |
| NM_002422 | MMP3 | Matrix metallopeptidase 3 (stromelysin 1, progel |
| NM_002423 | MMP7 | Matrix metallopeptidase 7 (matrilysin, uterine) |
| NM_004994 | MMP9 | Matrix metallopeptidase 9 (gelatinase B, 92kDa |
| NM_004689 | MTA1 | Metastasis associated 1 |
| NM_014751 | MTSS1 | Metastasis suppressor 1 |
| NM_002467 | MYC | V-myc myelocytomatosis viral oncogene homolo |
| NM_005376 | MYCL | V-myc myelocytomatosis viral oncogene homolo |
| NM_000268 | NF2 | Neurofibromin 2 (merlin) |
| NM_000269 | NME1 | Non-metastatic cells 1, protein (NM23A) express |
| NM_005009 | NME4 | Non-metastatic cells 4, protein expressed in |
| NM_006981 | NR4A3 | Nuclear receptor subfamily 4, group A, member |
| NM_002659 | PLAUR | Plasminogen activator, urokinase receptor |
| NM_002687 | PNN | Pinin, desmosome associated protein |
| NM_000314 | PTEN | Phosphatase and tensin homolog |
| NM_000321 | RB1 | Retinoblastoma 1 |
| NM_006914 | RORB | RAR-related orphan receptor B |
| NM_002295 | RPSA | Ribosomal protein SA |
| NM_000602 | SERPINE1 | Serpin peptidase inhibitor, clade E (nexin, plasmi |
| NM_003011 | SET | SET nuclear oncogene |
| NM_005901 | SMAD2 | SMAD family member 2 |
| NM_005359 | SMAD4 | SMAD family member 4 |
| NM_005417 | SRC | V-src sarcoma (Schmidt-Ruppin A-2) viral oncoge |
| NM_001050 | SSTR2 | Somatostatin receptor 2 |
| NM_003177 | SYK | Spleen tyrosine kinase |
| NM_005650 | TCF20 | Transcription factor 20 (AR1) |

# 2 CAPÍTULO 2: O PAPEL DA INTEGRINA $\alpha_2\beta_1$ NA METÁSTASE ÓSSEA DE CÂNCER DE MAMA

## 2.1 Introdução

### 2.1.1 *O tecido ósseo: um solo fértil e atrativo para as células metastáticas de câncer de mama*

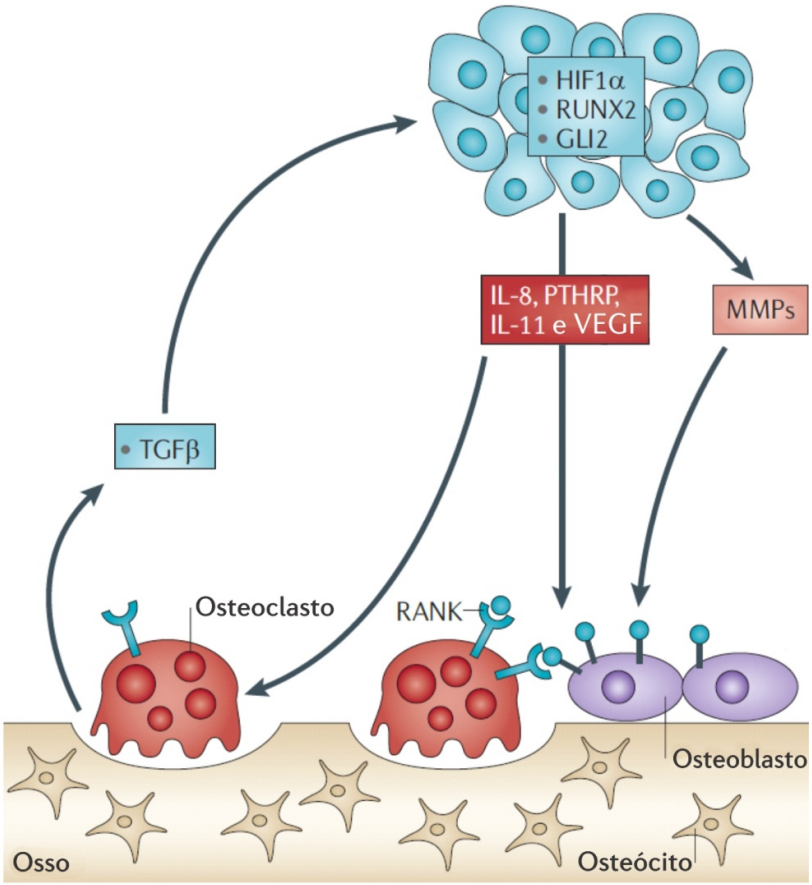
O tecido ósseo é um destino frequente para metástase em diversos tipos de câncer, sendo a incidência mais alta nos casos de câncer de mama (65-75%) (SUVA et al., 2011). Existem muitas características do tecido ósseo que o tornam um ambiente ideal para migração e colonização de células tumorais. Encontrada nas extremidades dos ossos longos, nas costelas, e nas vértebras, a metáfise é uma região composta por osso trabecular com uma rica vasculatura, adaptada para facilitar a passagem de células na medula óssea. Além disso, essa região do osso é constantemente remodelada por processos que liberam fatores de crescimento da matriz. Durante o remodelamento ósseo, citocinas e quimiocinas fornecem um meio de comunicação entre osteoblastos e osteoclastos, entretanto, ocasionalmente, também podem atrair células metastáticas (BUSSARD; GAY; MASTRO, 2008). Esse evento foi primeiro descrito em 1989 por Stephen Paget, que reconheceu o movimento não aleatório de células tumorais dentro do corpo pelo fluxo sanguíneo. Paget (1989) comparou as células tumorais a sementes que são levadas em todas as direções, mas só podem crescer se cair em solo fértil como, por exemplo, o tecido ósseo (FOKAS et al., 2007; PAGET; S, 1989).

Certos tumores sólidos migram para o osso, causando destruição óssea desregulada (metástase osteolítica) e/ou formação óssea anormal (metástase osteoblástica), sendo comum a ocorrência simultânea de ambas lesões. Os tumores de próstata possuem maior propensão a metastizarem para o osso e formarem lesões osteoblásticas, já os tumores de mama costumam formar lesões osteolíticas, causando dor e fraturas (GUISE et al., 2006). Os mecanismos responsáveis pelo crescimento do tumor no osso são complexos e envolvem a estimulação de osteoclastos e osteoblastos, bem como a liberação de fatores do microambiente ósseo.

Nas lesões osteolíticas, células tumorais secretam fatores como o *parathyroid hormone-related protein* (PTHrP), interleucina 11 (IL-11) e interleucina 8 (IL-8), os quais estimulam os osteoblastos a ativar osteoclastos via *receptor activator of nuclear factor kappa-β ligand* (RANKL), induzindo a reabsorção óssea. Fatores de crescimento como o *transforming growth factor beta* (TGF-β) são, então, liberados da matriz óssea o que

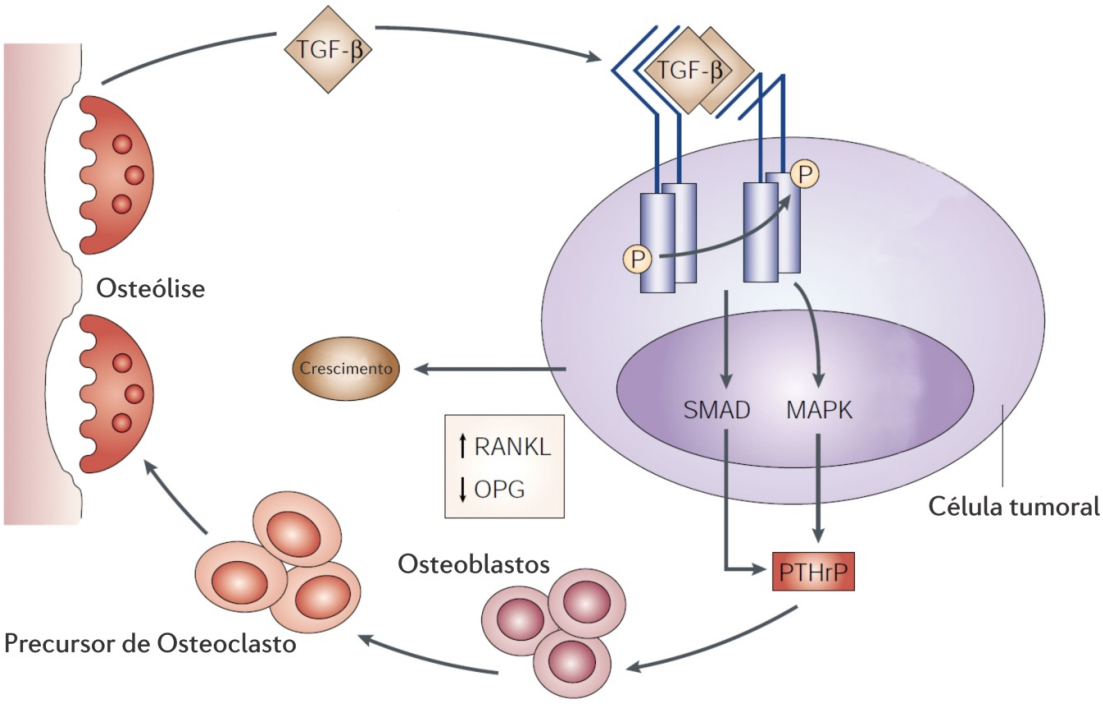
retroalimenta as células tumorais, estabelecendo um ciclo de perda óssea (**figura 6**) (STERLING et al., 2011; STERLING; EDWARDS, 2011; WEILBAECHER; GUISE; MCCAULEY, 2011). No microambiente ósseo, a liberação de TGF-β, a partir de matriz óssea mineralizada, suporta a proliferação de células tumorais através da ativação do receptor TGF-β tipo II e das proteínas Smad reguladoras (**figura 7**) (ROBERTS; SPORN, 1993; SHI; CELL; 2003). Essa ativação das células tumorais pelo TGF-β, via Smad, aumenta a produção de PTHrP, reiniciando assim o ciclo (**figura 7**) (WEILBAECHER; GUISE; MCCAULEY, 2011; WU et al., 2018). Além disso, o TGF-β também suprime a proliferação de células T e a atividade de células natural *killer*, inibindo o sistema imunológico (FOURNIER; 2006).

**Figura 6** - Ciclo da metástase osteolítica. Os tumores secretam fatores osteolíticos (como a proteína relacionada ao hormônio da paratireóide (PTHRP), interleucina-11 (IL-11), IL-6, IL-8, fator de crescimento endotelial vascular (VEGF) que estimulam a reabsorção óssea osteoclástica via RANKL. A reabsorção óssea osteoclástica causa a liberação do fator de crescimento transformador-β (TGFβ) da matriz óssea mineralizada. A hipóxia associada ao tumor e o fator indutível à hipóxia 1a (HIF1α) em conjunto com TGFβ podem aumentar a produção tumoral de VEGF. As metaloproteinases da matriz produzida por tumor (MMPs) podem clivar RANKL ligado à membrana (bolas azuis) o que pode favorecer a osteoclastogênese. Fonte: modificado de WEILBAECHER et al., 2011.



Outro fator regulador transcricional superexpresso nas células tumorais de mama é o Runx2. Esse fator é tradicionalmente conhecido como específico para os osteoblastos e mostrou-se altamente expresso em células tumorais metastáticas para o osso. As células tumorais de mama mimetizam o microambiente ósseo expressando Runx2, que regula a expressão de metalopeptidase de matriz 9 (MMP-9) e o fator de crescimento vascular endotelial (VEGF) aumentando a migração e invasão das células tumorais. Runx2 ativa também a sinalização Hedgehog, que regula PTHrP mediada por TGF-β, levando ao aumento da osteoclastogênese e consequentemente, a osteólise (DRABSCH; TEN DIJKE, 2011; PRATAP et al., 2005, 2008; WEILBAECHER; GUISE; MCCAULEY, 2011). Também tem sido demonstrada a importância do fator de transcrição Gli2, da via de sinalização Hedgehog, como regulador da expressão de PTHrP em linhagens celulares de câncer de mama metastático que causam lesões osteolíticas *in vivo* (STERLING et al., 2006). Tais fatores cooperam em conjunto no microambiente ósseo contribuindo com as lesões osteolíticas (STERLING et al., 2006).

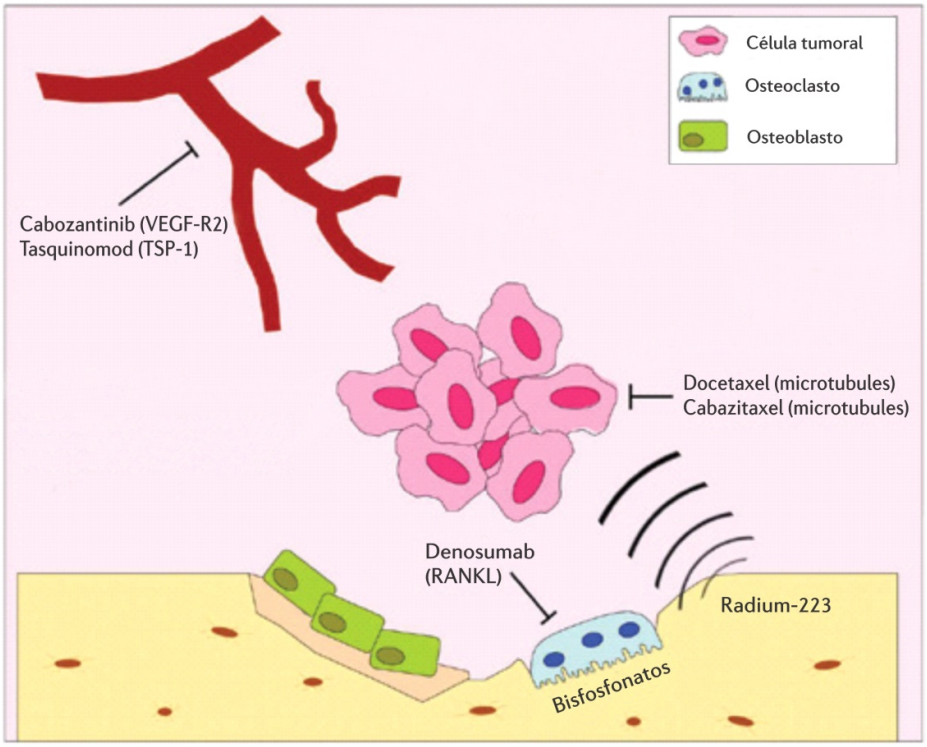
**Figura 7** - Via do TGF-β no ciclo da metástase osteolítica. As interações entre células tumorais e osteoclastos causam não apenas a ativação dos osteoclastos e subsequente destruição óssea, mas também o crescimento e o comportamento agressivos das células tumorais. A produção do peptídeo relacionado ao hormônio da paratireóide (PTHrP) pelas células tumorais ativa os osteoblastos para produzir RANKL e regular a osteoprotegrina (OPG). A osteólise leva à liberação de fatores de crescimento derivados do osso, incluindo fator de crescimento transformador-β (TGF-β). O TGF-β se liga aos seus receptores na superfície da célula tumoral e ativa a autofosforilação (P) e a sinalização por vias que envolvem SMAD (mediadores citoplasmáticos da maioria dos sinais de TGF-β) e proteína quinase ativada por mitogênio (MAPK). A sinalização por essas vias promove a proliferação de células tumorais e a produção de PTHrP. Outras citocinas também podem estar envolvidas, como interleucina (IL) -1, IL-6, IL-11 e IL-18 (não mostradas). Fonte: modificado de MUNDY *et al*., 2002.




### *2.1.2 Abordagens terapêuticas moleculares para o tratamento de metástase óssea*

A identificação dos mecanismos moleculares pelos quais as células dos tumores de mama desencadeiam a metástase óssea tem auxiliado no desenvolvimento de fármacos que previnam ou diminuam a progressão das lesões osteolíticas. Com o conceito do ciclo da metástase osteolítica, novas abordagens para o tratamento de metástases ósseas surgiram (**figura 8**), indicando também que inibidores de osteólise podem diminuir as lesões osteolíticas derivadas de metástases (DU et al., 2017; FRIELING; BASANTA; LYNCH, 2015). Esse conceito foi comprovado por estudos *in vivo* com fármacos usados para o tratamento de osteoporose, os bisfosfonatos. Quando administrado em camundongos com lesões osteolíticas após inoculação de células tumorais de mama MDA-MB-231, os bisfosfonatos diminuíram as lesões ósseas e a carga tumoral (SASAKI et al., 1995). Da mesma forma, camundongos que foram tratados com anticorpos neutralizantes para PTHrP experimentaram uma diminuição na carga tumoral (GUISE et al., 1996; YIN et al., 1999).

**Figura 8** - Terapias emergentes para o tratamento de metástase óssea. Uma melhor compreensão dos mecanismos de progressão tumoral permitiu a melhoria de opções de tratamentos como o uso de anticorpos, inibidores e agentes radioativos para o desenvolvimento de novas terapias direcionadas ao microambiente ósseo. Fonte: modificado de FRIELING et al., 2015.




Após sua administração, os bisfosfonatos ligam-se avidamente ao mineral ósseo, através de sua estrutura central, exposto em torno da região de absorção dos osteoclastos. Os bisfosfonatos são internalizados pelo osteoclastos, onde causam a interrupção dos processos bioquímicos envolvidos na reabsorção óssea (KOHNO, 2008; ROGERS et al., 1997). Os bisfosfonatos também causam apoptose de osteoclastos e estudos sugerem que possam ter efeito apoptótico direto nas células tumorais (JAGDEV et al., 2001; ROGERS; WATTS; RUSSELL, 1997). Dados pré-clínicos indicam que os bisfosfonatos não têm efeito sobre as metástases de tecidos moles, se forem administrados após metástases já estabelecidas. No entanto, se forem administrados a partir do momento da inoculação da célula tumoral, podem aumentar a metástase nos tecidos moles, embora previnam metástase óssea. Esses dados indicam que os bisfosfonatos podem ser administrados com segurança após o estabelecimento de metástases ósseas, entretanto devem ser usados com grande cautela para prevenção de outras metástases (MUNDY, 2002).

Outro inibidor de osteoclastogênese é o anticorpo monoclonal denosumab que se liga ao RANKL impedindo-o de se ligar ao seu receptor nos osteoclastos e consequentemente diminui a reabsorção óssea. Como terapia emergente tem-se também o rádio-223 (alpharadina) que é um emissor de radiação alfa que visa especificamente metástases ósseas devido às suas propriedades químicas e físicas semelhantes ao cálcio. Esse agente é capaz de inibir a diferenciação de osteoclastos *in vitro* e em modelos de metástase óssea em camundongos (DU et al., 2017; SO et al., 2012).

Numerosos estudos buscam compreender melhor a fisiopatologia da metástase óssea, entretanto ainda não existem métodos clínicos estabelecidos e eficientes para sua cura e prevenção. Atualmente, a gestão médica de metástases ósseas baseia-se em abordagens locais (isto é, cirurgia, terapia de radiação) ou estratégias sistêmicas, tais como terapias dirigidas a alvos moleculares (LIEPE; KOTZERKE, 2011; T. BUIJS; KUIJPERS; VAN DER PLUIJM, 2010). Nesse contexto, o conceito do ciclo da metástase osteolítica mudou a abordagem terapêutica e as estratégias de bloqueio de reabsorção óssea emergiram como novos tratamentos potenciais (MUNDY, 2002), juntamente com várias outras abordagens farmacológicas, incluindo terapias direcionadas a alvos em células tumorais e à vasculatura tumoral (**figura 8**) (ZOU et al., 2008). Entretanto, apesar dos avanços para o tratamento da metástase óssea, as terapias atuais frequentemente causam danos ao osso e à medula óssea, aumentando o risco de fraturas e a morbidade (LEE et al., 2019; MIGLIORATI et al., 2005; PSYRRI et al., 2014). Assim, é fundamental identificar novos alvos antitumorais para o tratamento da metastática óssea, sem agravar a doença em outros tecidos ou danificar o osso ou a medula óssea.

### *2.1.3 Integrinas como alvos para terapias de metástase óssea*

Atualmente, o câncer de mama triplo-negativo (TNBC) carece de alvos moleculares para terapias, sendo a quimioterapia padrão pouco eficaz no tratamento de TNBC. Dessa forma, as integrinas têm emergido como alvos estratégicos para a prevenção e tratamento de metástase óssea (SCHNEIDER; AMEND; WEILBAECHER, 2011) de câncer de mama triplo-negativo (PARVANI et al., 2015; TRUONG et al., 2014).

Evidências de estudos pré-clínicos apontam a integrina $\alpha_v\beta_3$ como uma das principais integrinas relacionadas à metástase óssea de câncer de mama (KWAKWA; STERLING, 2017), sendo descrita como importante tanto nas células tumorais (LI et al., 2015; ZHAO et al., 2007) como nos osteoclastos (BAKEWELL et al., 2003). A superexpressão da integrina $\alpha_v\beta_3$ aumenta a metástase óssea, assim como a inibição desse receptor por antagonistas diminui a habilidade das células tumorais de colonizar o osso e de causar osteólise (HARMS et al., 2004; MCCABE et al., 2007). Porém, os mecanismos moleculares pelos quais a integrina $\alpha_v\beta_3$ promove a osteólise ainda foram pouco explorados.

Estudos preliminares mostraram que a sinalização via $\alpha_v\beta_3$ resultou na localização nuclear de fatores de transcrição, como Runx2, e atuou no recrutamento e diferenciação de osteoclastos (AKECH et al., 2010). Além disso, a integrina $\alpha_v\beta_3$ pode aumentar a sinalização via TGF-β (GALLIHER; SCHIEMANN, 2006) que estimula a expressão de PTHrP pelas células tumorais e a expressão de RANKL pelos osteoblastos, promovendo a destruição óssea mediada por osteoclastos (JOHNSON et al., 2011; STERLING; EDWARDS, 2011). Dessa forma, a integrina $\alpha_v\beta_3$ demonstra ser um potencial alvo molecular no tratamento e prevenção de metástase óssea (KWAKWA; STERLING, 2017).

O TGF-β desempenha um papel supressor tumoral nos estágios iniciais em carcinomas, entretanto atua como promotor de crescimento, invasão e metástase nos estágios posteriores da progressão tumoral (BIERIE; MOSES, 2006). Esse comportamento do TGF-β é conhecido como o "paradoxo do TGF-β" e representa um aspecto fisiopatológico complexo dessa citocina no desenvolvimento e progressão de cânceres de mama (TAYLOR; PARVANI; SCHIEMANN, 2010; TIAN; NEIL; SCHIEMANN, 2011). O TGF-β tem sido relacionado à aquisição de fenótipo metastático em tumores mamários por estimular a transição epitelial-mesenquimal (TEM), processo no qual as integrinas participam de forma ativa (SIEG et al., 2000). Estudos demonstraram que a estimulação por TGF-β regula positivamente a integrina $\beta_3$, formando um complexo com TGFβR2 e a quinase de adesão focal (FAK). Tal complexo contribui para a amplificação da ativação da proteína ativada por mitógeno p38 quinase (MAPK), necessária na condução de programas de TEM e consequentemente na promoção de metástase de câncer de mama (GALLIHER; SCHIEMANN, 2006; TSUBAKIHARA; MOUSTAKAS, 2018). Também foi demonstrado que a integrina $\beta_1$ é necessária para ativação de MAPK durante a TEM ativada por TGF-β e está envolvida na tumorigênese e metástase mamária (BHOWMICK et al., 2001; HUCK et al., 2010; LAHLOU; MULLER, 2011).

Outra integrina que tem chamado a atenção por sua atividade em promover invasão e metástase de células tumorais é a integrina $\alpha_2\beta_1$. A inibição das subunidades $\alpha_2$ e $\beta_1$ com anticorpos diminuiu significativamente a ligação de células tumorais de próstata ao tecido ósseo (LANG et al., 1997). Hall *et al*. demostraram que uma linhagem celular de câncer de próstata metastática ao osso liga-se ao colágeno I de forma dependente de $\alpha_2\beta_1$ *in vivo* (HALL et al., 2006). A adesão da célula tumoral, via $\alpha_2\beta_1$, ao colágeno I do estroma da medula óssea ativa a GTPase RhoC que desencadeia uma cascata de sinalização responsável pela reorganização do citoesqueleto, migração e invasão, estimulando metástase esquelética preferencial (HALL et al., 2008). Curiosamente, estudos demonstraram que a integrina $\alpha_2\beta_1$ atua como supressora tumoral em câncer de mama. Ao contrário do relatado para o câncer de próstata, a perda da integrin $\alpha_2\beta_1$, e não seu aumento, caracterizou um fenótipo mais metastático em modelo murino de câncer de mama (RAMIREZ et al., 2011; ZUTTER et al., 1995).

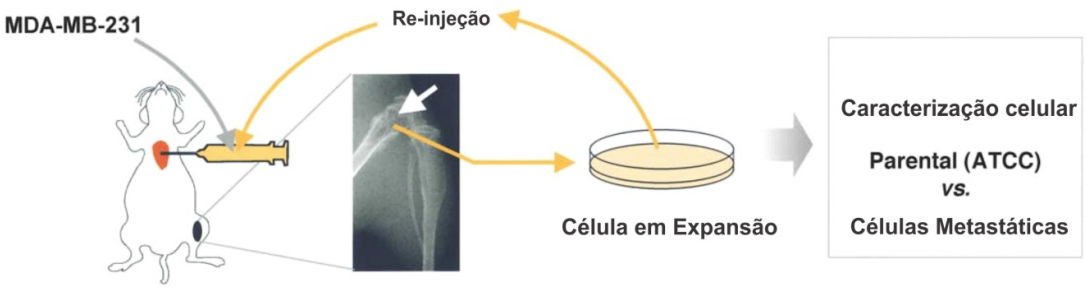
### *2.1.4 Modelos in vivo para estudo da metástase óssea*

Modelos animais fornecem um recurso essencial para investigar mecanismos de metástase. A pesquisa de metástase de câncer de mama, em geral, utiliza modelos de metástase experimental, tipicamente envolvendo a injeção de células tumorais humanas de mama diretamente no sistema circulatório de camundongos, resultando em metástases em qualquer um dos pulmões (após injeção da veia caudal) ou osso (após injeção intracardíaca) (ARGUELLO et al., 1988; DUIVENVOORDEN et al., 2002; KANG et al., 2003; PRICE et al., 1990). Esses modelos têm sido valiosos para o estudo dos estágios finais da metástase e, quando combinados à análise de expressão gênica em larga escala, podem contribuir com a identificação de genes que regulam a colonização de tecidos específicos (KAKIUCHI et al., 2003; KANG et al., 2003).

Uma melhor compreensão dos vários estágios da progressão metastática foi obtida pela determinação do perfil de expressão gênica em modelos pré-clínicos de camundongos com metástase de câncer de mama para o osso. Algumas dessas abordagens, a partir de culturas heterogêneas de células de câncer de mama, envolvem a derivação de sub-populações que preferencialmente se espalham para os ossos. Essas populações celulares são isoladas diretamente a partir de metástases ósseas estabelecidas que são obtidas por meio de injeções

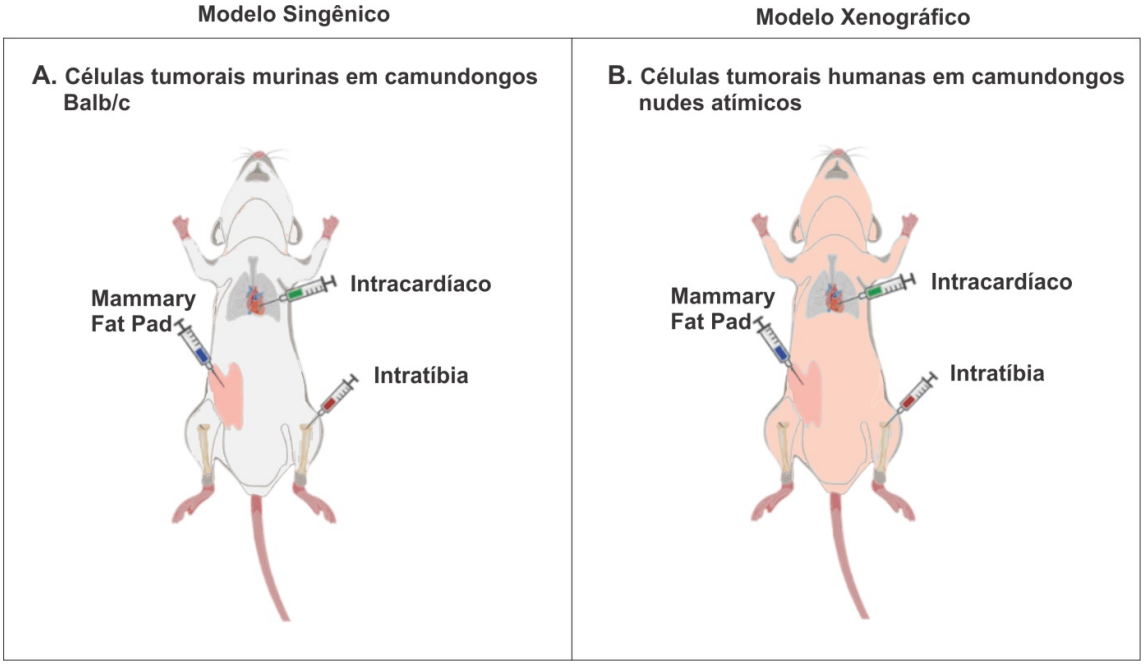
de células tumorais parentais no ventrículo esquerdo cardíaco (**figura 9**). As sucessivas seleções *in vivo* resultam em células de câncer de mama que metastatizam agressivamente para o tecido ósseo quando comparadas à população de células originais (KANG et al., 2003; SCHMID-ALLIANA et al., 2018). Essa abordagem facilitou a identificação de mediadores moleculares individuais de processos metastáticos, como IL-8 (WAUGH; RESEARCH; 2008), bem como de conjuntos de genes que trabalham cooperativamente, incluindo a metalopeptidase de matriz 1 (MMP-1) e os genes regulados por TGF-β, IL-6 e IL-11 (GOERGE et al., 2006; JACOBSON; CUNNINGHAM, 2012; SOTIRIOU et al., 2001; WEIDLE et al., 2016).

**Figura 9** - Esquema representativo para obtenção da sub-populações de células tumorais de mama seletivas para osso. Fonte: modificado de KANG et al., 2003.




Tipicamente, são empregados dois modelos animais para estudar a metástase de câncer para o osso: modelo singênico e o xenográfico (**figura 10**). O modelo singênico consiste na utilização de células tumorais da mesma espécie do animal. Já no modelo xenográfico a espécie da célula tumoral difere do animal. Experimentalmente, a via mais frequente de injeção de células tumorais é a intracardíaca (no ventrículo cardíaco esquerdo), que permite a colonização de células tumorais principalmente na região de metáfise (região de crescimento ósseo entre a epífise e a diáfise) dos ossos longos. A injeção intratibial (intraóssea) de células tumorais diretamente no espaço medular é frequentemente utilizada para examinar as interações do estroma tumoral durante o crescimento das lesões ósseas. Já a injeção de células tumorais na quarta mama de camundongos fêmea (modelo *mammary Fat Pad*) utiliza-se para avaliar o crescimento tumoral no sítio primário assim como, para o modelo singênico, avaliar as subsequentes etapas finais da metástase (WRIGHT et al., 2016).

**Figura 10** - Esquema representativo dos modelos singênico e xenográfico para estudo de metástase óssea de câncer de mama. No modelo Mammary Fad Pad (MFP) células tumorais de mama são injetadas no tecido adiposo da quarta mama de camundongos fêmea. No modelo intracardíaco (IC) as células tumorais são injetadas no ventrículo esquerdo de camundongos fêmea. No modelo intratíbia (IT) as células tumorais são injetadas diretamente nas tíbias de camundongos fêmea. Fonte: O autor (2019).




Como a metástase espontânea para o esqueleto a partir de tumores primários em animais é rara e nenhum modelo único reproduz todas as alterações genéticas e fenotípicas da metástase óssea do câncer de mama humano, os pesquisadores devem selecionar um modelo ou uma combinação de modelos que melhor se adapte ao aspecto da doença metastática que eles desejam investigar. Inoculação de células tumorais com tropismo para o osso diretamente no fluxo sanguíneo fornece uma ferramenta útil para investigar os processos associados ao câncer de mama como endereçamento, colonização e crescimento tumoral metastático ósseo e formação de lesão osteolítica (WRIGHT et al., 2016). Em modelos de tumor singênico onde linhagens celulares derivadas de murino são inoculadas em um hospedeiro murino, um perfil metastático ósseo pode ser observado com vários graus de sucesso após injeção intracardíaca de células metastáticas de câncer mamário murino (ex. 4T1) em camundongos Balb/c (LEE et al., 2014; WERBECK et al., 2014).

Metástases viscerais, particularmente no pulmão, podem encurtar o tempo de vida de um camundongo e, assim, limitar o período durante o qual as metástases esqueléticas podem

ser estudadas *in vivo*. As linhagens de células MDA-MB-231 e 4T1 foram, portanto, manipuladas em laboratório para produzir sub-linhagens seletivas ao osso que favorecem o direcionamento e a colonização de tíbias e fêmures de camundongos com propensão reduzida à metastatização para o pulmão (KANG et al., 2003; NUTTER et al., 2014; WETTERWALD et al., 2002). Injeção intratibial de várias linhagens celulares de câncer de mama incluindo MDA-MB-231, MDA-MB-436 e SUM1315 em camundongos imunocomprometidos e linhagens de células 4T1 e MMTV- PyMT em camundongos Balb/c e FVB imunocompetentes resultam no desenvolvimento de tumores mamários osteolíticos com impacto mínimo fora da medula óssea (FATHERS et al., 2012; OTTEWELL et al., 2009; WERBECK et al., 2014).

Os modelos intracardíaco, intra-arterial e intratibial fornecem ferramentas úteis para examinar os estágios posteriores da metástase óssea do câncer de mama; no entanto, esses modelos não permitem investigar os estágios que precedem a colonização do câncer de mama no osso, incluindo o crescimento do tumor primário ou a disseminação de células tumorais por meio da intravazação. Embora as células MDA-MB-231 não metastatizem ao osso murino a partir do sítio primário em camundongos nudes atímicos, o crescimento tumoral primário pode ser observado (WRIGHT et al., 2016).

Após o período do desenvolvimento de cada modelo, técnicas histológicas e imuno-histoquímicas são empregadas para estudar os tecidos excisados, enquanto cada vez mais, modalidades de imagens *in vivo* (sistemas de imagem *in vivo* - IVIS), radiografia, micro tomografia computadorizada (μCT) e imagem por ressonância magnética (MRI) têm sido utilizadas para avaliar o crescimento de lesões metastáticas ósseas e o efeito na reabsorção óssea. Com o desenvolvimento e aprimoramento dessas técnicas hoje é possível obter-se análises de qualidade para o estudo da metástase óssea (FERNANDES et al., 2016).

## 2.2 Justificativa

O câncer de mama é o mais comum entre as mulheres, sendo a metástase a principal causa de morte nesses pacientes. Células metastáticas de câncer de mama possuem maior preferência para o tecido ósseo (65-75%) (COLEMAN, 2002; SUVA et al., 2011), entretanto a razão dessa preferência não é completamente compreendida. Uma vez estabelecidos no osso, os tumores induzem complicações sérias, incluindo dor, aumento de fraturas e redução da qualidade de vida nos pacientes (COLEMAN et al., 2014; COLEMAN, 2002; SUVA et al., 2011; TONG et al., 2018). Dessa forma, é fundamental identificar novos alvos terapêuticos para a prevenção e tratamento de metástase óssea de câncer de mama.

Já foram identificadas diversas integrinas, ao longo da cascata metastática, exercendo funções fundamentais para invasão, migração e colonização do sítio secundário (DESGROSELLIER; CHERESH, 2010; SCHNEIDER; AMEND; WEILBAECHER, 2011; SÖKELAND; SCHUMACHER, 2019). Estudos mostraram que integrinas contribuem em diferentes etapas na metástase óssea (KWAKWA; STERLING, 2017; SCHNEIDER; AMEND; WEILBAECHER, 2011), sendo identificada a integrina $\alpha_2\beta_1$ como um marcador de progressão maligna no câncer de próstata (HALL et al., 2006, 2008; SOTTNIK et al., 2013). Por outro lado, a supressão dessa integrina aumenta o fenótipo metastático em câncer de mama (RAMIREZ et al., 2011). No entanto, existem poucos estudos abordando o papel deste receptor na metástase do câncer de mama para o osso. A hipótese desse estudo é que a integrina $\alpha_2\beta_1$ desempenha papéis diferentes, dependendo do estágio na metástase óssea em que as células tumorais de mama se encontram. Dessa forma, justificou-se investigar essa hipótese com estudos *in vivo* em colaboração com o grupo da Dr. Julie Rhoades na Vanderbilt University (USA) que possui uma vasta experiência com modelos animais de metástase óssea. O papel da integrina $\alpha_2\beta_1$ foi avaliado por meio do tratamento com o inibidor da integrina $\alpha_2\beta_1$ (TCI-15) em três modelos *in vivo* (*mammary fat pad*-MFP, intracardíaco-IC e intratibia-IT) com camundongos nudes atímicos, assim como por meio da superexpressão da subunidade $\alpha_2$ nas células tumorais injetadas nos animais. Além disso, as linhagens tumorais parentais e metastáticas utilizadas foram caracterizadas por PCR em tempo real e Western Blot. Também foi investigado o efeito do TGF-β sob a integrina $\alpha_2\beta_1$ nas células tumorais metastáticas.

## 2.3 Objetivos

O objetivo geral deste estudo foi investigar o papel da integrina $\alpha_2\beta_1$ em células tumorais de mama humana triplo-negativo (MDA-MB-231) durante as seguintes etapas da metástase óssea: crescimento do tumor primário (modelo *in vivo* MFP), extravasamento e colonização tumoral no sítio secundário (modelo *in vivo* IC) e lesões óssea após o estabelecimento tumoral no sítio secundário (modelo *in vivo* IT), utilizando camundongos nudes atímicos.

### *2.3.1 Objetivos específicos*

- Estabelecer uma sub-linhagem tumoral de mama metastática para osso (MDA-Bone) a partir da linhagem tumoral parental (MDA-Parental), utilizando o modelo *in vivo* IC.
- Estabelecer a sub-linhagem tumoral de mama metastática para osso superexpressando a subunidade de integrina $\alpha_2$ (OE- $\alpha_2$).
- Caracterizar as sub-linhagens estabelecidas por PCR em tempo real e por Western Blot para algumas subunidades integrinas ($\alpha_2$, $\beta_1$ e $\beta_3$), fatores de transcrição (RUNX2, FAK e Gli2) e genes (*PTHLH*, *TGFβR2*) relacionados com a promoção da osteólise.
- Avaliar o efeito do tratamento com o inibidor da integrina $\alpha_2\beta_1$ (TCI-15) nos três modelos *in vivo*.
- Avaliar o efeito da superexpressão da subunidade de integrina $\alpha_2$ nos três modelos *in vivo*.
- Avaliar o efeito do TGF-β sob a expressão da integrina $\alpha_2\beta_1$ nas células tumorais metastáticas.

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## 2.5 Manuscrito 2

### Biphasic α2β1 integrin expression modulated via TGF-β indicates this integrin as a potential target for primary breast tumor treatment

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**Abstract**

Integrins participate in the epithelial-mesenchymal transition (EMT) during tumor progression and are involved in the pathogenesis of metastatic cascade at many levels. Despite substantial evidence suggesting that α2β1 integrin loss enhances breast cancer metastasis, other studies suggest this integrin as essential for tumor establishment in bone. Thus, we hypothesized that the role of α2β1 integrin varies during the metastatic cascade to bone. MDA-MB-231 bone-derived cells (MDA-Bone) expressed lower levels of α2β1 integrin compared to MDA-MB-231 parental (MDA-Parental) cells, indicating that the loss of this integrin contributes to bone metastasis. MDA-Bone cells also express higher levels of PTHrP (parathyroid hormone related protein), Gli2, and TGFβR2, which have been shown to contribute to bone destruction. Furthermore, TGF-β treatment decreased α2β1 integrin expression in MDA-Bone cells, indicating that TGF-β may influence the selection of tumor cells to bone through integrin regulation. The role of α2β1 integrin was also evaluated in *in vivo* assays using a potent α2β1 integrin inhibitor (TCI-15) in three athymic nude mouse models (intratibial-IT, intracardiac-IC and mammary fat pad-MFP) to simulate breast tumor metastasis to bone. Mice treated with TCI-15 showed decreased primary tumor size in the MFP model, but no changes were detected in bone destruction after tumor establishment in bone (IT and IC models). Conversely, MDA-Bone cells overexpressing α2 integrin subunit (OE-α2) increased primary

tumor growth (MFP model); with no change in bone destruction (IC and IT model). These data suggest $\alpha_2\beta_1$ integrin as a therapeutic target to limit tumor expansion of primary tumors, but not metastasis to bone.

**Introduction**

Breast cancer is the most common cancer among women and metastasis is the leading cause of this cancer death (BRAY et al., 2018). Metastasis involves fundamental alterations in tumor cell morphology that contributes to an invasive and metastatic phenotype. During tumor progression, cells need to alter their anchored stage and detach from primary sites, invade tissue surroundings, migrate through blood vessels or lymphatic system to achieve secondary sites and form a secondary tumor (NGUYEN; BOS; MASSAGUÉ, 2009). For this process to happen, tumor cells had to undergo epithelial-mesenchymal transition (EMT) to disseminate and then they had to go back through mesenchymal-epithelial transition (MET) to colonize secondary sites (THIERY, 2002).

Defects in the expression of cell surface adhesive receptors or in extracellular matrix (ECM) components may result in altered cell activity contributing to tumor progression (HYNES, 2002; PLANTEFABER; HYNES, 1989). Integrins are αβ heterodimeric transmembrane receptors that support cell adhesion to ECM and trigger intracellular signaling that can modify cellular behavior (SCHWARTZ; SCHALLER; GINSBERG, 1995; SHATTIL; KIM; GINSBERG, 2010). Due its critical role in metastasis, integrins have been considered attractive as therapeutic targets (ALBELDA, 1993; GOODMAN; PICARD, 2012). Studies have shown that α2β1 integrin is a marker of malignant progression in prostate cancer (HALL et al., 2006, 2008; SOTTNIK et al., 2013). In addition, loss of α2β1 integrin has been shown to increase metastatic phenotype in breast cancer (RAMIREZ et al., 2011). However, there are very few studies addressing the role of this receptor in breast cancer metastasis to bone.

Metastatic breast cancer cells have preference for bone and the reason of the highest risk of skeletal metastasis (65-75%) in these patients (COLEMAN, 2002; SUVA et al., 2011) is not completely understood. In the bone microenvironment, breast tumor cells secrete parathyroid hormone-related protein (PTHrP) and other factors that stimulate osteoblast expression of Receptor Activator of NF-kb Ligand (RANKL)(POWELL et al., 1991;

SOUTHBY et al., 1990). RANKL then promotes osteoclast differentiation, thereby stimulating osteoclast activity. This increased osteoclast activity leads to bone destruction and release of matrix-derived proteins such as transformation growth factor β (TGF-β), which then feeds back on the tumor cells to promote further production of PTHrP (YIN et al., 1999) that is regulated by the transcription factor Gli2 (STERLING et al., 2006).

TGF-β can also interfere with integrin expression (MARGADANT; SONNENBERG, 2010) and its signaling causes reversible EMT in carcinomas (PORTELLA et al., 1998), contributing to the regulation of tumor initiation, progression and metastasis (BIERIE; MOSES, 2006; (LIBRARY; MEULMEESTER; TEN DIJKE, 2011). Additionally, αβ1 integrins can be modulated by TGF-β (HEINO et al., 1989), however the paradigm of how integrins, ECM and TGF-β functions are related was still unclear (MUNGER; SHEPPARD, 2011). This study aimed to investigate the role of α2β1 integrin in breast cancer metastasis to bone and the influence of TGF-β on breast bone metastatic cells. We hypothesize that α2β1 integrin expression varies during the breast cancer metastatic progression to bone and MET occurs under TGF-β stimulus. To test this hypothesis we used three mouse models (mammary fat pad-MFP, intracardiac-IC, intratibial-IT) in order to compare the metastasis stages using a potent α2β1 integrin inhibitor (TCI-15) or the overexpression of α2 subunit (OE-α2). We demonstrate that α2β1 integrin was crucial to tumor development at primary tumor site, but it was not relevant in bone destruction after tumor establishment in bone. We also show that bone derived metastatic cells had low levels of α2β1 integrin with high levels of osteolytic genes such as PTHrP (parathyroid hormone related protein), Gli2, and TGFβRII, suggesting an inverse correlation between α2 integrin expression and a bone destructive phenotype. Furthermore, we verify that TGF-β treatment decreased α2β1 integrin, indicating that TGF-β may influence the selection of tumor cells to bone through integrin regulation.

## Materials and methods

### *Cell culture*

Human MDA-MB-231 breast cancer adenocarcinoma cells were obtained from American Type Tissue Culture Collection (ATCC) and bone derivative cells (MDA-Bone) were selected from bone marrow after MDA-MB-231 parental (MDA-Parental) via

intracardiac inoculation (WRIGHT et al., 2016). Both cell lines were maintained in Dulbecco's Modified of Eagle's Medium- DMEM from Corning (#10-013-CV, Manaassas, VA) and cultured in a humidified incubator at 37˚C under 5% $CO_2$. All media were supplemented with 10% fetal bovine serum- FBS from Peak (#PS-FB1, Colorado, USA) from Hyclone Laboratories (Logan, UT) and 1% Penicillin and Streptomycin from Mediatech (Manassas, VA).

***Transfection***

Cells (1.0 x $10^5$ cells/well) were plated in a 6-well plate on the day before transfection. MDA bone derived cells were stable transfected with 5 µg of m-Emerald-Integrin-Alpha2-N-18 plasmid (#54128, Addgene, Watertown, MA, USA) or 5 µg of p-Dest m-Cherry N1 plasmid control (#31907, Addgene, Watertown, MA, USA). All transfections were done using Lipofectamine LTX with plus reagent (#15338030, Invitrogen) per manufacturer's instructions. Cells were selected in medium containing 700 µg/mL of geneticin (G418). Colonies were isolated, expanded and maintained in medium with 700 µg/mL G418.

***In vitro drug treatments***

To investigate the influence of TGF-β signaling on integrin expression, cells were treated with 10 ng/mL of recombinant human TGF-β1 (R&D), 20 µg/mL of anti-TGF-β antibody 1D11 (Genzyme), 100 nM of inhibitor of TGF β receptor 1 kinase SD208 (Sigma) or DMSO control for 72h under 2% serum media condition.

***Quantitative real-time PCR***

To measure gene expression changes, cells were harvested with trypsin and total RNA was extracted using the RNeasy Mini Kit (Qiagen). For mRNA reverse transcription, the qScript cDNA synthesis kit, (Quanta, VWR) was used according to the manufacturer's instructions. To synthesize cDNA, 1µg of total RNA was used. The expression of *PTHrP (Hs00174969), ITGA2 (Hs04332845), ITGB1 (Hs00559595), ITGB3 (Hs01001469) and TGFβRII (Hs00234253)* was measured in triplicate by quantitative PCR (qPCR) using validated TaqMan primers from Applied Biosciences (Carlsbad, CA) with the 7500 Real-Time PCR System (Applied Biosciences) using the following cycling conditions: 95°C for 15 seconds and 60°C for 1 minute, preceded by an initial incubation period of 95°C for 10

minutes. Quantification was performed using the absolute quantitative for human cells method using *18S* or *GAPDH* as an internal control.

***Western Blot analysis***

Cells were harvested in RIPA buffer containing a cocktail of proteases and phosphatase inhibitors from Thermo Scientific (#78442, Rockford, USA). Equal protein amount (20 µg) was prepared for a 4-20% SDS-PAGE. Proteins were transferred to a nitrocellulose membrane and blocked with 5% BSA for 1h at room temperature, followed by incubation with primary antibodies at dilutions of 1:500 (anti-phosphoFAK, Abcam #ab81298, anti-gli2 noveus NB600-874); 1:1000 (anti-FAK, Abcam #ab40794, anti-integrin β3, Santa cruz #sc 13579, anti-phospho integrin β1, Abcam #ab5189, anti-runx2, Abcam #ab76956) and 1 1:5000 (anti-integrin α2, Abcam #ab133557, anti-integrin-β1, Santa cruz #sc-8978) overnight at $4^0$C. Membranes were then incubated with a secondary antibody at 1:5000 (anti-mouse, Santa cruz #sc-2005 or anti-rabbit, Santa cruz #sc-2004) and bands were detected by chemiluminescence using a Chemidoc Touch gel imager (BioRad). Loading control was performed using antibody for GAPDH.

***In vivo studies***

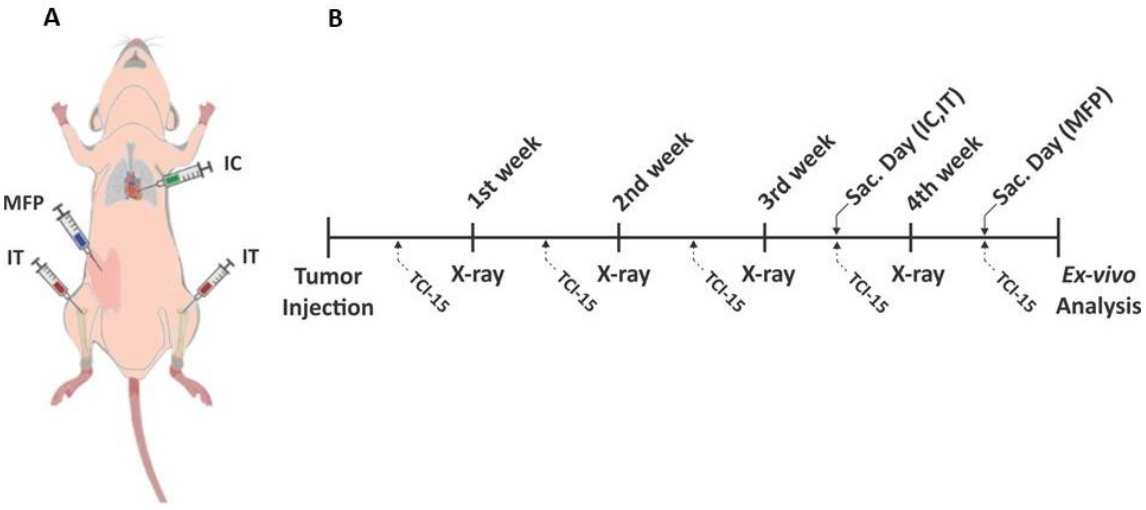
All animal experiments were carried out in compliance with the Vanderbilt University Institutional Animal Care and Use Committee and the National Institute of Health guidelines (Protocol # M1600153-00). Four-six weeks-old athymic nude mice were injected with a bone metastatic clone of MDA-MB-231 cells.

***Mouse models of breast cancer metastasis to bone***

To investigate primary tumor growth, $5x10^5$ tumor cells were injected into the $4^{th}$ inaugural mammary fat pad (MFP model, n=16). To investigate tumor extravasation and colonization to bone, $1x10^5$ tumor cells were injected into the left cardiac ventricle (intracardiac, IC model, n=24). To investigate tumor growth at metastatic site, $1x10^5$ tumor cells were injected into the right tibia and PBS into the left tibia as control (intratibial, IT model, n=16). Each mouse model place of tumor injections was represented in **figure 1A**. Procedure details of animal models were described by Wright, L. E., *et al* (WRIGHT et al., 2016). Mice were treated with the selective integrin α2β1 inhibitor, TCI-15 (BORZA et al.,

2012; MILLER et al., 2009) (Tocris) at 20 mg/kg via intraperitoneal (IP), 3x per week beginning 48 hours post tumor inoculation and continuing for the remainder of the study. To compare the effects of α2β1 inhibition, MDA-MB-231 bone derived cells stably transfected with either shα2 (#54128, m-Emerald-Integrin alfa2-N18, Addgene) or shControl plasmid (#31907, P-Dest-m-cherry N1, Addgene) were also injected using these three (MFP, IC and IT) mouse models.

**Figure 1.** Athymic nude mice models for breast cancer bone metastasis. Bone metastatic MDA-MB-231 breast cancer cells (MDA-bone) were injected via Intratibial (IT) model, Intracardiac (IC) model or mammary fat pad (MFP) model (**A**). Time line of *in vivo* experiments. Mice were treated every 3 days with an $\alpha_2\beta_1$ integrin inhibitor (TCI-15) or cell transfected for overexpress α2 integrin subunit were injected in these three mouse models. Bone destruction was monitored with weekly x-ray analysis. Sacrifice day was abbreviated as Sac. Day (**B**).




***Radiography analysis and quantification of tumor growth***

After 1 week from tumor cell inoculation, bone destruction was monitored by weekly X-ray imaging on a XR-60 digital radiography system (Faxitron) at 35kVp for 8 sec and mice were sacrificed at 25 days (IT or IC models) or 32 days (MFP model) post tumor cells inoculation (**figure 1B**). Osteolytic lesions were quantified bilaterally in the humeri, femora, and tibiae and quantified using image analysis software (Metamorph; Molecular Devices). Primary tumor size was monitored by 3x weekly caliper measurements. Mice were sacrificed after 3-4 weeks after tumor injections and post primary tumor and hindlimbs were harvested for *ex vivo* analysis.

***Microcomputed tomography analysis***

For trabecular bone volume analysis, formalin-fixed tibiae were scanned by micro-computed tomography analysis using a Scanco mCT 40 (Scanco Medical) at 70 kVp and 114 mA with a 12 μm voxel size and an integration time of 300 ms or VivaCT 80 (Scanco Medical) at 70 kVp and 114 mA with a 10.4 μm voxel size and an integration time of 350 ms.

***Histologic analysis of mouse tissue***

Primary tumor and bones were fixed in 10% formalin and tibiae were decalcified in 20% EDTA for 3-4 days at room temperature. Decalcified bones were embedded in paraffin and serial sections were performed to confirm tumor burden in tibiae, using hematoxylin and eosin staining (H&E). For osteoclast measurements, it was carried out a standard tartrate-resistant acid phosphatase (TRAP) stain protocol. Immunohistochemistry (IHC) staining for $\alpha_2$ integrin (1:250, Abcam #ab13355) was also performed on primary and secondary tumor sections to evaluate the expression of this receptor. The standard pH 6.0 citrate buffer was used to antigen retrieval followed by blocking with 2% goat serum for 1h and primary antibody incubation overnight at 4 $^0$C. Day after, anti-rabbit secondary antibody (Santa cruz, #sc2004) was incubated on tumor sections for 2h at room temperature. For H&E and IHC analysis it was used quantitative image analysis software (Metamorph; Molecular Devices).

***Statistical analysis***

Statistical analyses were performed using one-way ANOVA for multiple comparisons and two-tailed Student's t-tests. All statistical analysis were performed using GraphPad Prism software and values were presented as mean ± SEM. Statistical tests were considered significant when *$p < 0.05$; **$p < 0.01$; and, ***$p < 0.001$.

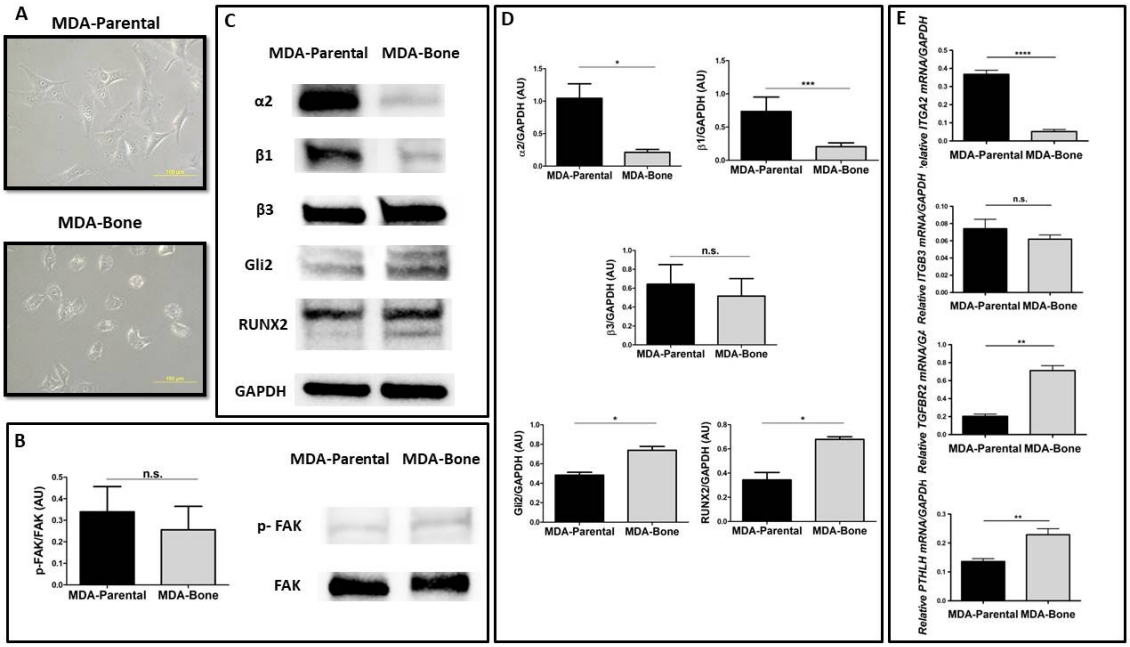
## Results

***Breast tumor cells that migrated to bone have lost integrin α2β1 and enhanced osteolytic genes expression.***

Cells images showed that MDA-Parental cells from primary site had a spreaded phenotype, comparing to MDA-Bone cells from secondary site that were smaller and less spreaded (**figure 2A**). This morphological plasticity was also described during tumor metastasis stages (THOMPSON; HAVIV, 2011). In order to investigate the differences between MDA-Parental and MDA-Bone morphology, we characterized both cell lines for integrins and osteolytic factors, using Western blotting and qPCR to measure protein (**figure 2B, C and D**) and mRNA (**figure 2E**) expression, respectively.

The expression of integrins subunits $\alpha_2$ and $\beta_1$ was decreased in MDA-Bone cells (**figure 2C and D**), suggesting that the loss of $\alpha_2\beta_1$ integrin expression may be correlated to a more aggressive phenotype for tumors that grow in bone. These metastatic cells form osteolytic tumor in the secondary site. Interestingly, the expression of integrin β3 in both cell lines did not change significantly, indicating that mesenchymal-epithelial transition (MET) is more related with $\alpha_2\beta_1$ integrin expression. MDA-Bone cells also expressed increasing amounts of Gli2 and RUNX2 (**figure 2C and D**) as well as *PTHLH* and *TGFβR2* (**figure 2E**) genes which are related to bone destruction through vicious cycle.

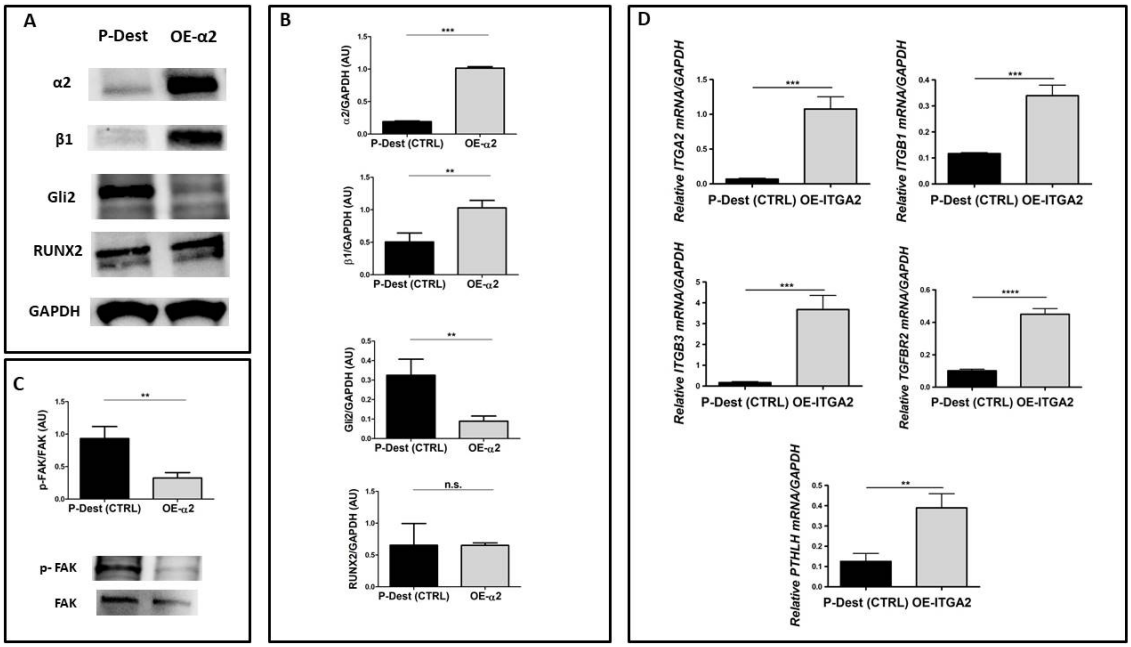
**Figure 2.** Differences between MDA-Parental and MDA-Bone cells. **(A)** Cell morphology by light microscopy (40X). **(B)** Western blot analysis of FAK phosphorylation (p-FAK). Values represent the normalized densitometry ratio of p-FAK and FAK. **(C)** Western blot representative images of integrin and osteolytic protein levels. **(D)** Densitometry analysis were performed normalizing protein levels by GAPDH. **(E)** qPCR analysis of mRNA expression of integrin and osteolytic genes. Three independent assays were performed and p value determined using student`s T-test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.




***Breast tumor bone derived cells overexpressing α2 integrin subunit presented decreased Gli2 and FAK phosphorylation.***

MDA-Bone stable transfected with shα2 or shControl plasmid were utilized to restore the α2 expression in these cells. To determine if the OE-α2 altered β1 and β3 integrins and osteolytic genes expression Western blot and qPCR were performed. β1 and β3 integrins expression has been related to metastasis and to high aggressive tumors (YAO et al., 2007; KWAKWA; STERLING, 2017). OE-α2 cells presented an increase in β1 and β3 integrin levels (**figure 3A and B**) as well as a reduction in the expression of FAK phosphorylation (**figure 3C**) suggesting a lower aggressive phenotype, once FAK activation was described as contributing to metastasis (MITRA; SCHLAEPFER, 2006). On the other hand, OE-α2 cells showed *PTHLH* and *TGBβR2* mRNA levels upregulated (**figure 3D**) and a reduction in the expression of Gli2 (**figure 3A and B**).

**Figure 3.** Differences between MDA-Bone cells transfected with shα2 and shControl plasmids. **(A)** Western blot representative images of integrin and osteolytic protein levels. **(B)** Densitometry analysis were performed normalizing protein levels by GAPDH**. (C)** Western blot analysis of FAK phosphorylation (p-FAK). Values represent the normalized densitometry ratio of p-FAK and FAK. **(D)** qPCR analysis of mRNA expression of integrin and osteolytic genes. Three independent assays were performed and p value determined using student`s T-test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

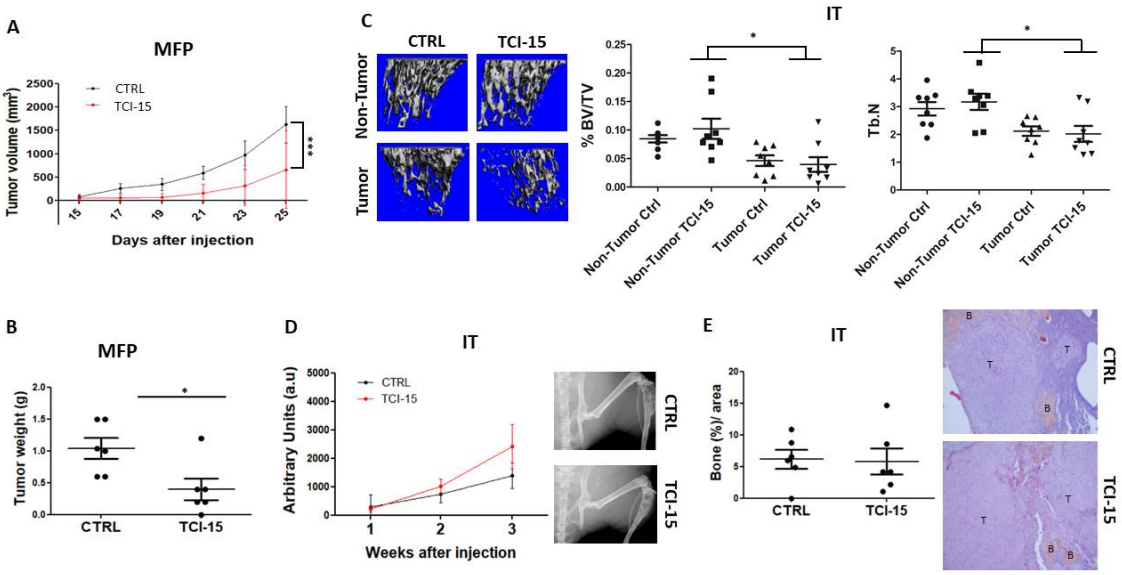



***Inhibition of α2β1 integrin impairs breast primary tumor growth, but showed no effect on bone metastasis.***

Depending on the cancer type, studies have been shown that α2β1 integrin can be considered a tumor suppressor as in breast metastatic cancer (ZUTTER et al., 1995) or a tumor promoter as in prostate cancer (HALL et al., 2006; SOTTNIK et al., 2013). Furthermore, studies elucidating the role of that α2β1 integrin in primary tumor growth were scarce. Here, three mouse models (mammary fat pad-MFP, intracardiac-IC and intratibial-IT) were used to investigate the effect of a potent α2β1 integrin inhibitor, TCI-15, in order to verify which tumor progression stage the repression of α2β1 integrin could be more relevant. We found that inhibition of α2β1 integrin decreased tumor growth in the primary site (MFP model), but showed no effect on colonization in the bone (IC model, data not shown) or subsequent bone destruction (IT model). Tumor volume (**figure 4A**) and tumor weight (**figure 4B**) were significantly inhibited in mice treated with TCI-15 compared to untreated control. After the mice were sacrificed, we performed *ex vivo* μCT analysis of the tibias, which demonstrated a significant decrease in the bone volume and trabecular number between the non-tumor and tumor mice, but no significant difference with TCI-15 treatment (**figure 4C**). Furthermore, there was no significant difference between treated and untreated non-tumor mice, suggesting that inhibition of α2β1 integrin by TCI-15 does not affect normal bone remodeling (**figure 4C**). X-ray analysis revealed no significant difference in lesion area in an

IT model of mice treated with TCI-15 (**figure 4D**). Additionally, TCI-15 treatment had no significant effect on tumor burden once tumors have established in the bone (**figure 4E**) as well as on osteoclast number in the three mouse models (data not shown).

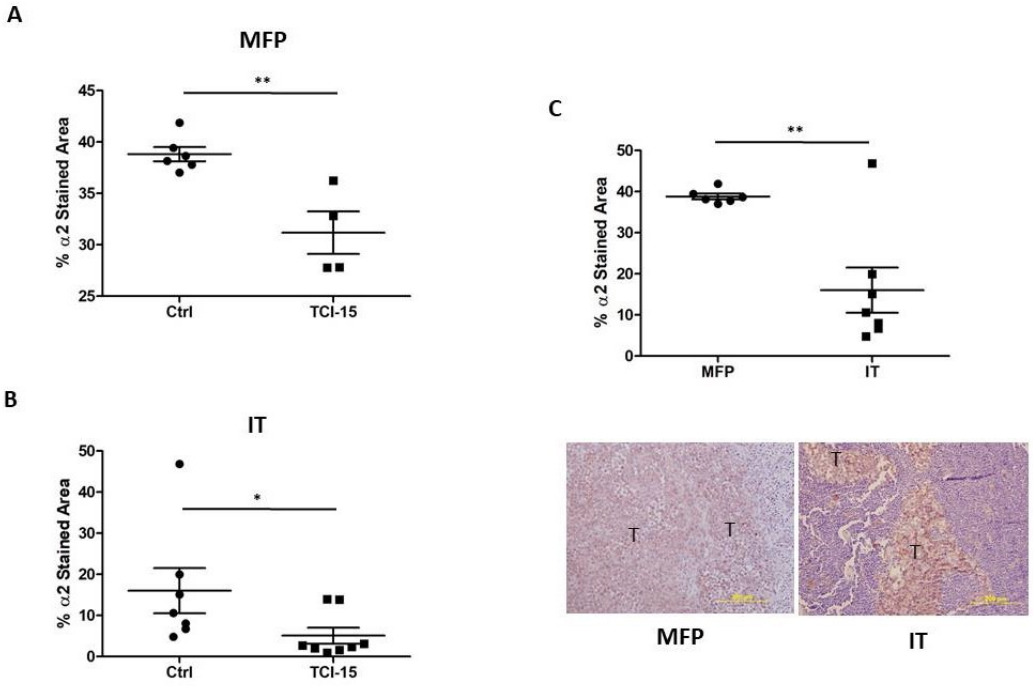
**Figure 4.** α2β1 integrin inhibition decreased primary breast tumor growth, but had no effect on bone destruction. Primary tumor volume **(A)** and tumor weight **(B),** analysis compared TCI-15 treatment to untreated control. µCT analysis for bone volume fraction (BV/TV) and trabecular number (Tb.N) **(C)**. X-ray analysis in lesion area between TCI-15 treatment and control **(D)**. H&E staining of tibia sections. The letter T represents tumor and B bone in the stained sections **(E)**. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. student`s T-test.




***Inhibition of α2β1 integrin with TCI-15 treatment decreased α2 integrin expression.***

IHC analysis on primary tumor sections and tibia sections from the IT model showed a significant decrease in α2 expression, indicating that the TCI-15 treatment inhibited α2β1 integrin as expected (**figure 5A and B**). Additionally, the secondary tumor model (IT) has less α2 integrin expression by percent of the total area than the primary tumor model (MFP) (**figure 5C**), giving further evidence that the loss of the α2 integrin subunit contributes to a more aggressive metastatic phenotype. These data suggest that the loss of the α2 integrin subunit can be used as a biomarker for breast cancer metastasis to bone.

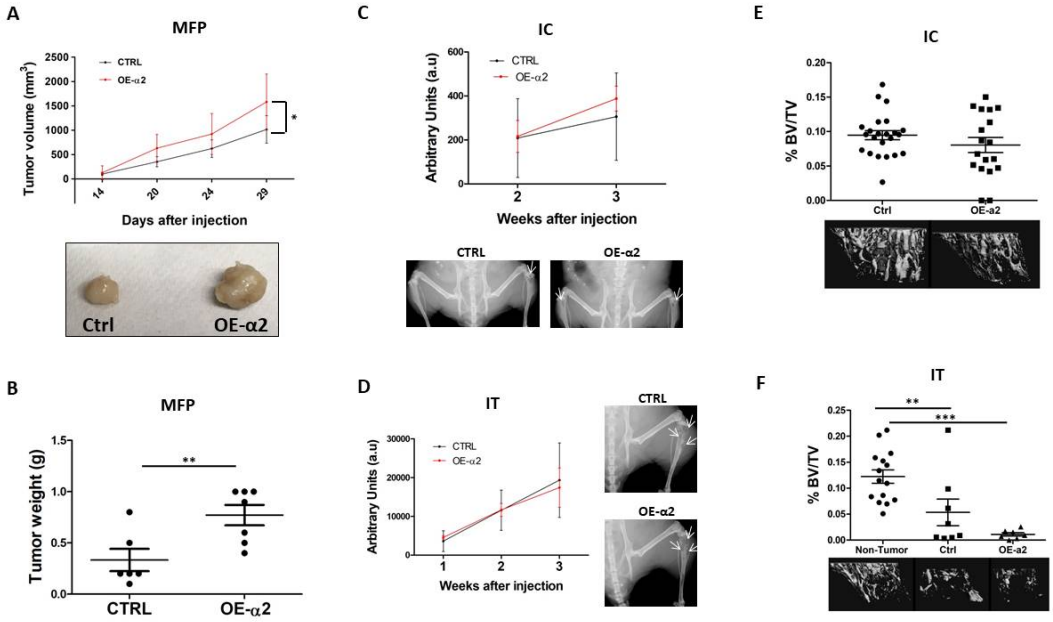
**Figure 5.** Immunohistochemistry (IHC) for α2 integrin in MFP model **(A)** and IT model **(B)**. Images represent IHC for α2 subunit in primary tumor versus in secondary site (right tibia) **(C)**. Quantifications were performed using Image J software. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. student`s T-test.




***Overexpression of α2 integrin increased breast primary tumor growth.***

The role of α2β1 integrin in tumor initiation has not been elucidated (NACI; VUORI; AOUDJIT, 2015). To investigate if α2 integrin could contribute to tumor initiation and tumor growth, we overexpressed α2 integrin in MDA-Bone cells and performed the MFP model in nude athymic mice. In addition, we also performed IC and IT *in vivo* models to check if this subunit integrin would have effect in tumor bone colonization and bone destruction. OE- α2 MDA-Bone cells increased primary tumor growth (MFP model) (**figure 6A and B**), and had no effect in bone destruction (IT model) (**figure 6D and F**). In agreement with the results from MFP model treated with TCI-15, the results from OE-α2 cells showed that this integrin contributes to breast tumor growth in primary site. The overexpression of α2 integrin had also no effect in tumor colonization (**figure 6C and E**).

**Figure 6.** α2 integrin subunit overexpression (OE-α2) increased primary breast tumor growth, but had no effect on bone destruction. Primary tumor volume **(A)** and tumor weight **(B)**, analysis compared TCI-15 treatment to untreated control. X-ray analysis in lesion area between OE-α2 and control in IC model **(C)** and IT model **(D)**. µCT analysis for bone volume fraction (%BV/TV) for OE-α2 in IC model **(E)** and IT model **(F)**. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. student`s T-test.

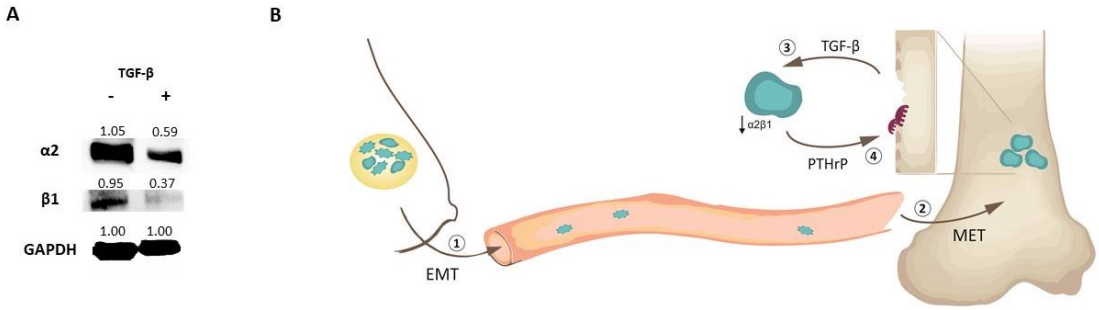



***TGF-β decreased α2β1 integrin in breast bone metastatic cells which enhances osteolytic phenotype.***

Studies have been reported that TGF-β regulates β1 integrin subunits and modify adhesion and migration behaviors (HEINO et al., 1989; HEINO; MASSAGUE, 1989) as well as had influence in EMT (BHOWMICK et al., 2001; PARVANI et al., 2013). Metastatic cells appear to have an epithelial phenotype and lack of mesenchymal markers (TARIN; THOMPSON; NEWGREEN, 2005; VAN DENDEREN; THOMPSON, 2013). α2β1 integrin has been also shown as a stemness marker (SEGUIN et al., 2015). To verify if TGF-β could change α2β1 integrin expression we treated MDA-Bone cells with recombinant TGF-β (10 ng/mg) and after 24 h we analyzed protein levels by western blot. TGF-β treatment decreased α2β1 integrin expression in MDA-Bone cells (**figure 7A**), indicating that TGF-β may influence the selection of tumor cells to bone through integrin regulation. We suggest that (**figure 7B**). The treatments with anti-TGF-β antibody (1D11) and the inhibitor of TGF β receptor 1 kinase (SD208) showed no change in α2β1 integrin expression (data not shown),

indicating that the mechanism reported here (**figure 7B-3**) is independent from TGF-β signaling.

**Figure 7.** Matrix-derived proteins like transformation growth factor β (TGF-β) decreased α2β1 integrin that contributes to mesenchymal epithelial transition and tumor osteolytic phenotype. Effect of TGF-β on MDA-Bone cells after 24h of treatment. Cells were harvested and the subunits α2 and β1 levels analyzed by Western Blot **(A)**. Schematic diagram of metastasis steps with the potential mechanism of α2β1 integrin and TGF- β that enhances osteolytic pathways in breast cancer bone metastatic cells **(B)**. Dissemination of tumor cells from primary site involves epithelial-mesenchymal transition-EMT (1). Once tumors cells achieve secondary site, mesenchymal-epithelial transition-MET is need for revert back to epithelial phenotype and form body structures in metastasis (2). Osteoclast activity leads to bone destruction and release of TGF-β, which decreases α2β1 integrin promoting, MET (3). TGF-β also induces osteolytic genes such as PTHrP that contributes to osteoclast-mediated bone lesions (4).




## Discussion

To understand better the various stages of metastasis progression in breast cancer, sub-populations that preferentially spread to bones has been isolated to compare differences in protein and gene profiles (SCHMID-ALLIANA et al., 2018). The biphasic α2β1 integrin expression found in this study relates a higher expression of this integrin in the primary site and a lower expression in the secondary site, which is related to a bone metastatic phenotype. This results corroborates Ramirez *et al* (RAMIREZ et al., 2011) that described by the first time the α2β1 integrin as a suppressor tumor, correlating the lack of this integrin and increased breast cancer metastasis (RAMIREZ et al., 2011). In agreement, our results showed that MDA-Parental cells from primary breast tumor had higher levels of α2β1 integrin compared to MDA-Bone cells, indicating that tumor cells have to lose this integrin to

establish in bone. Additionally, we suggest that this lack is associated with an increase in osteolytic genes as *PTHLH*, *TGFβR*2 as well as some factor such as Gli2 and RUNX2, which are involved in metastatic properties of cancer cells in bone microenvironment (GUELCHER; STERLING, 2011; PRATAP et al., 2008; STERLING; GUELCHER, 2011).

Integrins display different profiles on cell surface, depending what step tumor cells are in the metastasis context. Due to this dynamic process, target integrins become a challenge (HAMIDI; IVASKA, 2018). To investigate the role of α2β1 integrin, we performed three mouse breast bone metastatic models (MFP, IC and IT) to compare the stages in the metastasis cascade. Treating mice with the inhibitor of α2β1 integrin TCI-15, we found that only primary tumor growth was affected. TCI-15 was not able to avoid tumor bone destruction. One reason to explain this result may be the fact that some drugs are difficult to reach the bone and thus the dose used may not have been enough to elicit a response. Other reason that must be considered is related to the lack of α2β1 integrin in the cells that reach the bone. In this case, the α2β1 integrin inhibitor treatment would not have any effect in these tumor cells and consequently in bone lesions. We also treated MDA-Bone cells *in vitro* with TCI-15 and we observed no change in cell proliferation, α2 and β3 integrins expression as well as in osteolytic genes (**supplementary figure 1**).

To verify specifically if the α2 subunit is more related to the *in vivo* response found with TCI-15 treatment, we overexpressed this subunit in MDA-Bone cells and injected it in the three mice models (MFP, IC, IT). The results confirmed that α2 integrin has a crucial role in primary tumor grow, but no effect in bone metastasis. Despite OE-α2 cells still had high levels of *PTHLH* and *TGFβR2* that are related with bone destruction, the lesions caused by these cells were not worse compared to control cells (**figure 6 C-F**). These *in vitro* paradoxical results (**figure 3**) together with *in vivo* study (**figure 6**) indicate that the osteolytic phenotype is independent from α2 integrin overexpression. As MDA-Bone cells had low expression of α2 integrin and high expression of *PTHLH* and *TGFβR2* genes, we also conclude that changes in this subunit was not enough to contribute to decrease osteolytic lesions. Despite OE-α2 cells had decreased levels of Gli2, it was not sufficient to decrease bone destruction *in vivo*, suggesting that the role of α2 in bone metastasis is independent of this osteolytic transcriptional factor. Once FAK activation is associated with cancer and metastasis (MITRA; SCHLAEPFER, 2006), OE-α2 cells presenting low levels of phosphorylated FAK indicates that α2 integrin contributes to a less aggressive bone metastatic phenotype, compensating the high expression of *PTHLH* and *TGFβR2* genes.

β3 integrin has been associated with breast cancer bone metastasis and its overexpression increased bone metastasis incidence (ZHAO et al., 2007). Interestingly, in this study the overexpression of α2 integrin subunit also increased β3 integrin, however did not change bone metastasis in IC and IT mouse models. This finds suggest that the decrease in α2β1 integrin is critical for osteolytic lesion by MDA-Bone cells. It has also been shown that αvβ3 overexpression does not affect cell proliferation in mouse tibia (SLOAN et al., 2006), corroborating our findings for IT model. Taking all together, we suggest that α2β1 integrin helps tumor progression depending of metastasis stage and the tumor microenvironment. In this study, we observed that MDA-Parental from primary tumor treated with TGF-β had no change in α2β1 integrin levels (data not shown), but MDA-Bone selected from MDA-Parental that migrated to secondary site decreased α2β1 integrin after 24h of TGF-β treatment (**figure 7A**), indicating different TGF-β sensibility. TGF-β has a dual role in mammary tumorigenesis and metastatic progression and depending in each tumor site this factor has a different response (MURAOKA-COOK; DUMONT; ARTEAGA, 2005). Based on this, we suggest that integrin profile together with TGF-β receptors orchestrate different TGF-β responses in primary and secondary sites.

Evidences that circulating tumor cells (CTCs) had both epithelial and mesenchymal markers coexpression (ARMSTRONG et al., 2011) were presented in breast cancer patients. Studies with metastatic animal models have suggested TGF-β as a link between EMT and metastasis (TSUBAKIHARA; MOUSTAKAS, 2018). Besides, bone resorption induced by breast tumor cells was related with TGF-β, PTHrP, integrins and hedgehog signaling pathways which are important to EMT and MET facilitating bone metastasis formation (DEMIRKAN, 2013). In this study, we suggest that some more sensitive CTCs to TGF-β were stimulated and have decreased α2β1 integrin selecting them to bone (**figure 7B-3**) with PTHrP and Gli2 hedgehog factor participation. Other evidence that rebuild our hypothesis that the lack of α2β1 integrin occurs in bone microenvironment is that the MDA-Bone was obtained from MDA-Parental injected via intracardiac, indicating us the difference between both cells happens after tumor cell reach blood vessels.

In conclusion, despite mouse model limitation, we suggest that α2β1 integrin expression is involved in EMT and MET during breast tumor progression to bone and TGF-β regulates α2β1 integrin expression differently in primary site and secondary site. In primary site, TGF-β was not able to change α2β1 integrin expression. On the other hand, at secondary site tumor cells, which present low levels of α2β1 integrin, stimulate bone resorption secreting

PTHrP and TGF-β, released from bone matrix after osteoclast resorption, enhances tumor cell selection decreasing α2β1 integrin levels. This study reveals α2β1 integrin as a potential target to limit tumor initiation and expansion in primary breast tumors early on, but not once metastasis has occurred.

**Acknowledgments**

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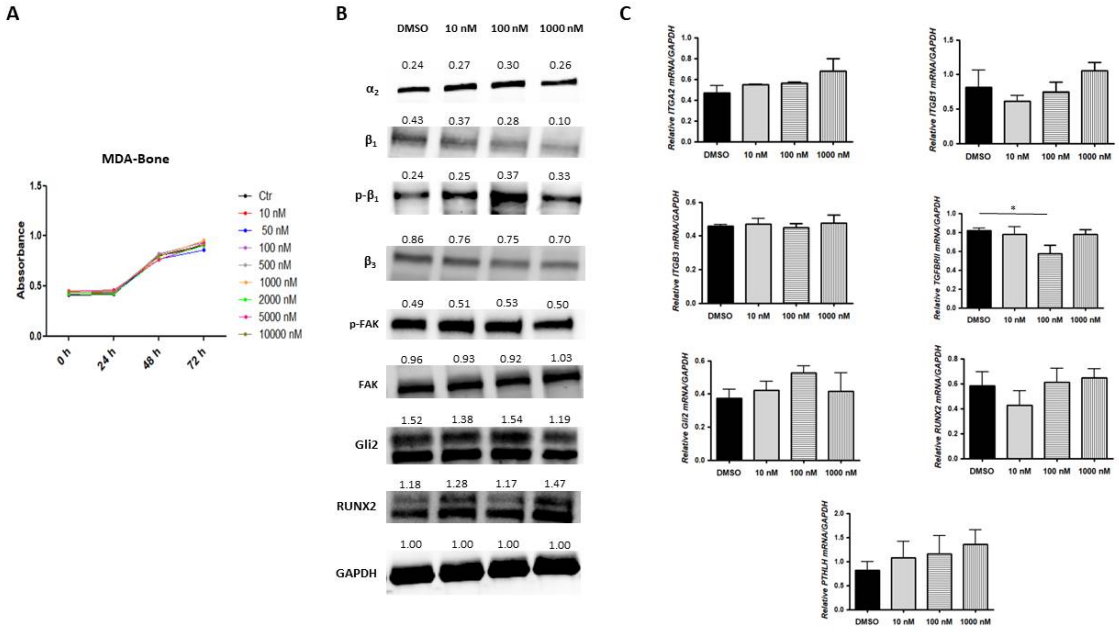
## Supplementary material

**Figure 1.** TCI-15 inhibitor did not change MDA-Bone cell proliferation as well as α2β1 and β3 integrins and osteolytic genes. Cell proliferation assay showed that TCI-15 did not change cell viability at 10- 10000 nM after 24, 48 and 72h **(A)**. TCI-15 (10, 100 and 1000 nM) treatment also did not change protein levels **(B)** and mRNA (C) after 24h. Only 100 nM of TCI-15 decreased *TGFβR2* gene expression. *p < 0.05. student`s T-test.



# 3 CONCLUSÕES

Os resultados apresentados nos capítulos 1 e 2 desse estudo permitem concluir que:

- A ALT-C, um ligante da integrina $\alpha_2\beta_1$, possui potencial antimetástase visto que promoveu o aumento da expressão do supressor de metástase 1 (*MTSS1*) e a inibição da adesão ao colágeno tipo I nas células tumorais de mama.
- O silenciamento da subunidade $\alpha_2$ foi compensado pelo aumento da expressão da integrina $\alpha_v\beta_3$ o que providenciou competência metastática às células tumorais.
- A expressão da integrina $\alpha_2\beta_1$ apresenta-se diminuída nas células que estabeleceram metástase óssea, sugerindo uma perda da expressão dessa integrina nas fases tardias da metástase óssea.
- Para essas células metastáticas houve aumento na expressão dos genes osteolíticos (*TGFβ2* e *PTHLH*) assim como na quantidade do fator de transcrição Gli2, o qual está relacionado à degradação do tecido nas lesões ósseas, indicando um fenótipo mais agressivo para metástase óssea nessas células.
- Apesar de não reverter a metástase óssea, o inibidor da integrina $\alpha_2\beta_1$, TCI-15, foi eficiente para diminuir o crescimento do tumor primário de mama.
- Corroborando os resultados do tratamento com o TCI-15, a superexpressão da subunidade $\alpha_2$ aumentou o crescimento do tumor primário de mama e não alterou a quantidade de lesões ósseas.
- O TGF-β diminuiu os níveis da integrina $\alpha_2\beta_1$ nas células tumorais, sugerindo que o estabelecimento da metástase óssea é mediado por esse fator via integrina $\alpha_2\beta_1$.

Dessa forma, a integrina $\alpha_2\beta_1$ apresenta-se com um papel chave durante o crescimento tumoral no sítio primário, sendo a perda de sua expressão importante durante as fases de estabelecimento tumoral na metástase óssea. Os resultados desse estudo sugerem a integrina $\alpha_2\beta_1$ como potencial alvo terapêutico para o tratamento e prevenção de metástase em câncer de mama triplo-negativo.

# 4 PRODUÇÃO CIENTÍFICA

## 4.1 Artigos publicados

- Alternagin-C binding to $\alpha_2\beta_1$ integrin controls matrix metalloprotease-9 and metalloprotease-2 in breast tumor cells and endothelial cells. Moritz, M. N. O. et al. **Journal of Venomous Animals and Toxins including Tropical Diseases**, 2018.

- Purification of a fragment obtained by autolysis of a PIIIb-SVMP from *Bothrops alternatus* venom. Van de Velde, A. C. et al. **International Journal of Biological Macromolecules**, 2018.

- ADAM9 silencing inhibits breast tumor cells transmigration through blood and lymphatic endothelial cells. Micocci, K. C., et al. **Biochimie**, 2016.

## 4.2 Artigo em fase de submissão

- **Manuscrito revisão:** Integrin regulation of breast cancer metastasis to bone. Moritz, M. N. O. et al. **Cancers**.