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Identificação de um *tandem* CD24/p38MAPK envolvido na plasticidade celular e na resistência à quimioterapia na linhagem agressiva de câncer de mama MDA-MB-231

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Orientadores: Prof Dr Alfredo Goes
Dra Catherine Ropert

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ATA DA DEFESA DE TESE DE DOUTORADO DE

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Às **quatorze horas** do dia **13 de dezembro de 2019**, reuniu-se, no Instituto de Ciências Biológicas da UFMG, a Comissão Examinadora da Tese, indicada pelo Colegiado do Programa, para julgar, em exame final, o trabalho final intitulado: **"IDENTIFICAÇÃO DE UM TANDEM CD24/P38MAPK ENVOLVIDO NA PLATISCIDADE CELULAR E NA RESISTÊNCIA À QUIMIOTERAPIA EM LINHAGEM DE CÂNCER DE MAMA AGRESSIVO"**, requisito final para obtenção do grau de Doutor em Biologia Celular, Abrindo a sessão, o Presidente da Comissão, **Dr. Alfredo Miranda de Góes**, após dar a conhecer aos presentes o teor das Normas Regulamentares do Trabalho Final, passou a palavra ao candidato, para apresentação de seu trabalho. Seguiu-se a arguição pelos examinadores, com a respectiva defesa do candidato. Logo após, a Comissão se reuniu, sem a presença do candidato e do público, para julgamento e expedição de resultado final. Foram atribuídas as seguintes indicações:

Prof./Pesq.	Instituição	Indicação
Dr. Alfredo Miranda de Góes	UFMG/ICB	APROVADA
Dra. Catherine Maryvette Ropert	UFMG	APROVADA
Dr. Ênio Ferreira	UFMG	APROVADO
Dr. José Morgado Diaz	INCA	APROVADA
Dr. Humberto Gravina	FMRP	Aprovada
Dr. Cláudio Antônio Bonjardin	UFMG	Aprovado

Pelas indicações, o candidato foi considerado: Aprovado

O resultado final foi comunicado publicamente ao candidato pelo Presidente da Comissão. Nada mais havendo a tratar, o Presidente encerrou a reunião e lavrou a presente ATA, que será assinada por todos os membros participantes da Comissão Examinadora. **Belo Horizonte, 13 de dezembro de 2019.**

Dr. Alfredo Miranda de Góes (Orientador) _____

Drª. Catherine Maryvette Ropert (Dupla Orientação) _____

Dr. Ênio Ferreira _____

Dr. José Morgado Diaz _____

Dr. Humberto Gravina Humberto Boriguello Gravina

Dr. Cláudio Antônio Bonjardin _____

Obs: Este documento não terá validade sem a assinatura e carimbo do Coordenador

Prof. Vanessa Pinho da Silva
Sub-Coordenadora do Programa de
Pós Graduação em Biologia Celular ICB/UFMG

Vanessa Pinho da Silva

Dedicatória

Dedico essa tese à minha mãe Vera, que recentemente me disse: "Eu me vejo realizada em você!"

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E a todos que de alguma forma torceram e vibraram com mais esta conquista.

Resumo

A Heterogeneidade Intratumoral (HIT) é uma das principais responsáveis pelo fenômeno de resistência à quimioterapia. Uma das moléculas relacionadas a HIT é a Cluster Differentiation 24 (CD24), uma glicoproteína que fica ancorada à membrana celular por uma GPI, sendo correlacionada ao fenótipo de células tronco tumorais (CSC). Nos cânceres de mama invasivos, a CD24 está expressa de maneira elevada em cerca de 84,6% dos casos. Sendo assim, o objetivo desse trabalho foi investigar o papel da CD24 na sensibilidade à doxorrubicina na linhagem de câncer de mama agressiva MDA-MB-231, estabelecendo uma correlação entre expressão de CD24 e ativação diferencial de vias de sinalização. Nós identificamos uma utilização diferencial de MAPKs de acordo com a presença de CD24 na membrana das células MDA-MB-231. Mais especificamente, células $CD24^{+}$ ativam preferencialmente a MAPK p38, correlacionando-se a uma atividade proliferativa em cultura. Durante o tratamento quimioterápico *in vitro*, nós observamos a translocação de CD24 do citosol para a membrana, que ocorre poucas horas após adição da droga, devido à presença de um reservatório intracelular dessa glicoproteína. Essa mudança de fenótipo conduziu a uma reprogramação celular caracterizada pela ativação constitutiva de p38, tri-metilação da histona H3K9 e aumento da expressão da proteína anti-apoptótica Bcl-2. As células que resistiram ao tratamento apresentaram um fenótipo de senescência que foi revertido após várias semanas em cultura correspondendo a uma entrada no ciclo celular e aumento significativo da quimiorresistência. Fato importante, a capacidade do inibidor da atividade de p38 de sinergizar com a doxorrubicina na indução de morte celular está de acordo com sua capacidade de reduzir a expressão de Bcl-2. Por fim, nossos dados nos permitem inferir que a estratégia de impedir quimiorresistência utilizando inibidores de vias de sinalização associadas a CSCs é factível.

Palavras chave: Câncer de mama; Células tronco tumorais; CD24; MAPK; p38; Apoptose; Bcl-2; Quimiorresistência

Abstract

Intratumor Heterogeneity (ITH) is one of the main responsible for chemoresistance. The Cluster Differentitation 24 (CD24) is a GPI-anchored glycoprotein closely related with ITH and it is identified as a Cancer Stem Cell (CSC) marker. In invasive breast cancer, CD24 is overexpressed in 84,6% of patients. Hence, the aim of our study was to investigate the role of CD24 in the sensibility of the aggressive MDA-MB-231 cell line to doxorubicin, establishing a correlation between CD24 expression and differential activation of signaling pathway networks. A relatioship was established between the differential use of MAPKs and the presence of CD24 on cell membrane. More specifically, a preferential activation of p38 MAPK was constantly observed in $CD24^+$ cells which correlated with a higher proliferative activity of these cells. During *in vitro* chemotherapy, we have shown a rapid translocation of CD24 towards membrane, few hours after treatment, thanks to an intracellular reservoir of CD24 immediately available. This phenotype switching was associated with a constitutive activation of p38MAPK leading to cell reprogramming exemplified by the trimethylation of H3K9 and the overexpression of the anti-apoptotic protein Bcl-2. Importantly, the use of p38 inhibitor sensitized cells to doxorubicin that was in accord with its ability to reduce Bcl-2 levels and consequently to overcome drug resistance. Besides, we accompanied the journey of cells after treatment with doxorubicin starting from their switching to $CD24^+$ state and continuing through their entry into senescence-like state and reawakening after several weeks of culture. We presented an insight into the molecular underpinnings of the “reawakened” phenotypes that correlated with their higher chemoresistance. This may be put in parallel with cancer relapse. In conclusion, our data indicate that strategy to prevent drug resistance controlling signaling network associated with CSC marker is workable in aggressive breast cancer.

Key words: Breast cancer; Cancer Stem Cell; CD24; MAPK; p38; Apoptosis; Bcl-2; Chemoresistance

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Abreviaturas

Bax - *Bcl-2-associated X protein*

Bcl-2 - *B-cell lymphoma 2*

CD24 - *Cluster Differentiation 24*

CD24$^+$/DxR - Células CD24$^+$ resistentes à doxorrubicina senescentes

CD24$^+$/DxR30 - Células CD24$^+$ resistentes à doxorrubicina proliferativas

CSC - *Cancer Stem Cells*

ER - *Estrogen Receptor*

ERK - *Extracellular signal-regulated kinases*

FDA - *Food and Drug Administration*

GPI - *Glygosylphosphatidylinositol*

HDAC - *Histone deacetylase*

HER2 - *Human Epidermal growth factor Receptor 2*

HIT - Heterogeneidade Intratumoral

HMT - *Histone Methyltransferase*

INCA - Instituto Nacional do Câncer

JNK - *c-Jun N-terminal kinase*

MAPK - *Mitogen-Activated Proteins Kinase*

NCSC - *Non-Cancer Stem Cells*

OMS - Organização Mundial de Saúde

p38 - *p38 mitogen-activated protein kinase*

PR - *Progesterone Recptor*

SiC - *Control-Silenced Cells*

SiCD24 - *CD24-Silenced Cells*

SLT - Síndrome da Lise Tumoral

TNBC - *Triple Negative Breast Cancer*

TNF-α - *Tumor Necrosis Factor alfa*

Sumário

1 - Introdução

1.1 - O Câncer

O câncer é a segunda causa de morte em todo o mundo, atrás somente de doenças cardiovasculares (Banerji *et al.*, 2013). Segundo dados da Organização Mundial de Saúde (OMS) de 2017, há uma estimativa de cerca de 14 milhões de novos casos por ano, com 8,8 milhões de morte em todo o mundo. Vale ressaltar que o câncer pode ser considerado uma doença de caráter econômico/social, visto que a proporção de novos casos/morte é maior em países de baixo a médio desenvolvimento, como o Brasil, por exemplo.

Segundo dados levantados pelo Instituto Nacional do Câncer (INCA, 2018) para o biênio 2018 e 2019, estima-se que o câncer de pele do tipo não melanoma 1 é o mais incidente na população brasileira (174 mil casos novos por ano), seguido pelos tumores de próstata nos homens (68 mil novos casos por ano) e câncer de mama nas mulheres (59 mil novos casos por ano). Ainda segundo o INCA, o câncer de mama é mais frequente em mulheres, respondendo por 29% dos novos casos registrados anualmente (Fig 1). Segundo a OMS, em 2010, o câncer de mama foi responsável por 12,8% das mortes em decorrência do câncer em todo o mundo.

Distribuição proporcional dos dez tipos de câncer mais incidentes estimados para 2018 por sexo, exceto pele não melanoma*

Localização Primária	Casos	%			Localização Primária	Casos	%
Próstata	68.220	31,7%	Homens	Mulheres	Mama Feminina	59.700	29,5%
Traqueia, Brônquio e Pulmão	18.740	8,7%			Cólon e Reto	18.980	9,4%
Cólon e Reto	17.380	8,1%			Colo do Útero	16.370	8,1%
Estômago	13.540	6,3%			Traqueia, Brônquio e Pulmão	12.530	6,2%
Cavidade Oral	11.200	5,2%			Glândula Tireoide	8.040	4,0%
Esôfago	8.240	3,8%			Estômago	7.750	3,8%
Bexiga	6.690	3,1%			Corpo do Útero	6.600	3,3%
Laringe	6.390	3,0%			Ovário	6.150	3,0%
Leucemias	5.940	2,8%			Sistema Nervoso Central	5.510	2,7%
Sistema Nervoso Central	5.810	2,7%			Leucemias	4.860	2,4%

*Números arredondados para múltiplos de 10.

Figura 1: Estimativa dos tipos de câncer mais incidentes para o biênio 2018/2019. Com exceção do câncer de pele não melanoma, o câncer mais frequente na população masculina é o de próstata e na população feminina o câncer de mama (INCA, 2018).

O termo câncer abrange um conjunto de mais de 100 tipos de doenças que podem ser resultantes de mutações gênicas induzidas pelo ambiente ou herdadas (Hoskin and Ramamoorthy, 2008). Entretanto, apesar da grande variabilidade existente entre os diferentes tipos de câncer, as células tumorais são caracterizadas por comportamentos específicos comuns, como: potencial replicativo ilimitado; angiogênese intensa; autossuficiência em sinais

de crescimento; inibição da morte celular e evasão ao sistema imunológico (Bhutia and Maiti, 2008).

Baseando-se nas características moleculares, os tumores de mama são classificados, desde 1970, como detentores ou não de receptores de estrógeno (ER), receptores de progesterona (PR) e como tendo amplificação ou não dos receptores para fator de crescimento epidermal 2 (HER2). Além dessa classificação quanto aos receptores hormonais, os tumores de mama também podem ser classificados como luminais ou basais. Os tumores luminais são ER^+/PR^+ enquanto os tumores basais, em geral, são $ER^-/PR^-/HER2^-$, sendo chamados de câncer de mama triplo negativo (*triple negative breast cancer* - TNBC) (Norum, Andersen and Sørlie, 2014).

Atualmente, a escolha dos métodos de tratamento do câncer de mama envolvem o estágio e o tipo do tumor, baseando-se na combinação de fatores clínicos e patológicos incluindo: presença de metástase, tamanho do tumor, envolvimento de vasos linfáticos e subtipo imuno-histoquímico (Goldhirsch *et al.*, 2007; Matsen and Neumayer, 2013), mas de modo geral, as estratégias de tratamento incluem cirurgia, radioterapia e terapia sistêmica (quimioterapia, terapia hormonal e terapia biológica) (Hammond *et al.*, 2010).

Os tumores luminais respondem bem a hormonoterapia. Os tumores basais, por sua vez, somente 20% normalmente respondem bem à quimioterapia convencional (Tabela 1) (Polyak, 2011). A linhagem metastática MDA-MB-231, por exemplo, é do tipo basal ou TNBC. Pacientes com tumores caracterizados como TNBC têm mau prognóstico e recebem quimioterapia, tratamento mais comumente utilizado no câncer de mama (Goldhirsch *et al.*, 2007; Matsen and Neumayer, 2013).

Devido a essa grande variabilidade, a existência de um tratamento único para o câncer de mama parece ser utópica. Isso nos leva a introduzir o conceito de terapia personalizada. De fato, essa subclassificação de tumor de mama foi um grande avanço na orientação do tratamento e deve ser levada em consideração, mas a resistência à quimioterapia permanece como o maior problema a ser combatido. Uma das grandes dificuldades enfrentadas durante o tratamento do câncer de mama é a variabilidade de células que compõem o tumor. À essa variabilidade dá-se o nome de Heterogeneidade Intratumoral (HIT). O termo HIT refere-se à coexistência de várias subpopulações celulares com “vida própria” e com capacidade de cooperação entre elas (Tabassum and Polyak, 2015). Entender a origem da HIT, bem como a sua dinâmica durante o tratamento quimioterápico, pode ser

considerado um passo essencial no entendimento de como controlar a resistência das células tumorais aos tratamentos quimioterápicos convencionais além de poder fornecer novos alvos terapêuticos.

Subtipo intrínseco	Definição imunohistoquímica	Tratamento recomendado
Luminal A	ER+ e/ou PR+ HER2- Ki-67 baixo	Terapia hormonal
Luminal B	ER+ e/ou PR+ HER2- (ou HER2+) Ki-67 alto	Terapia hormonal, quimioterapia, anti-HER2 se HER2+
HER2 amplificado	HER2+ (amplificado ou superexpresso) ER-	Anti HER2, quimioterapia
Basal	ER-, PR-, HER2- (triplo negativo)	Quimioterapia
Normal	Sem relevância	Sem relevância
ER, receptores de estrógeno; PR, receptores de progesterona; HER, Receptor de fator de crescimento humano;		

Tabela 1. Definições imunohistoquímicas e seus respectivos tratamentos para os subtipos de câncer de mama. Adaptado de Norum, 2014.

1.2 - Heterogeneidade Intratumoral

Os tumores sólidos, incluindo o câncer de mama, representam um conjunto de doenças que demonstram heterogeneidade a nível molecular, histopatológico e clínico, podendo sofrer tanto variações intertumorais (variações de tumores entre pacientes diferentes) quanto variações intratumorais (subpopulações celulares em um mesmo tumor) (Norum, Andersen and Sørlie, 2014). Apesar dos tumores sólidos surgirem a partir de uma única célula neoplásica, subsequentes alterações são geradas ao longo da progressão da doença, originando subpopulações cada vez mais agressivas (Swanton, 2013). A Heterogeneidade Intratumoral (HIT) está intimamente relacionada à tumorigênese, angiogênese, invasão, potencial metastático e resistência ao tratamento quimioterápico. (Agarwal and Kaye, 2003; Norum, Andersen and Sørlie, 2014). Devido a isso, a HIT favorece o a recidiva do tumor após um período de remissão, muito comumente encontrado após o tratamento quimioterápico (Fig 2).

Figura 2: A HIT aumenta a probabilidade de existir uma subpopulação de células resistentes à droga quimioterápica utilizada no tratamento do tumor, aumentando a probabilidade de relapso da doença. Adaptado de Aktipis & Nesse, 2012 (Aktipis and Nesse, 2012)**.**

Dois modelos foram propostos para explicar a origem e como a HIT contribui para a progressão do câncer (Fig 3). O primeiro modelo se baseia na seleção clonal, a qual sugere que subpopulações celulares evoluem progressivamente por meio da aquisição de diferentes fenótipos, originários de sucessivas alterações genéticas e epigenéticas. Tais fenótipos são então selecionados seguindo o modelo Darwiniano de evolução. O outro modelo proposto para explicar a HIT envolve a existência de células tronco tumorais (CSC) identificadas em tumores sólidos. Tal conceito é espelhado sobre o conceito de células tronco embrionárias, ou seja, elas são caracterizadas pela capacidade de autorrenovação, capacidade de se diferenciarem em células não-tronco tumorais (NCSC) e de induzirem a formação de tumores após injeção de células em suspensão em camundongos imunossuprimidos. Al-Hajj e colaboradores demonstraram que o implante de cerca de 100 células classificadas como CSCs foi capaz de formar tumores primários e secundários de mama, enquanto o mesmo efeito não foi obtido com o implante de dezenas de milhares de NCSC (Al-Hajj *et al.*, 2003). Outros estudos clínicos e pré-clínicos verificaram que após tratamento quimioterápico ou radioterápico, NCSCs foram praticamente eliminadas, enquanto um considerável número de células com características de CSCs sobreviveram (Bao *et al.*, 2006; Levina *et al.*, 2008; Dallas *et al.*, 2009; Shibue and Weinberg, 2017). Sendo assim, as CSCs estão diretamente associadas à tumorigênese, metástase, resistência ao tratamento antitumoral e relapso da doença.

Devemos acrescentar mais um parâmetro à HIT: o dinamismo das subpopulações tumorais, definido como plasticidade celular. A plasticidade celular é geralmente ligada à mudança de fenótipo das células tumorais (ex: expressão de receptores de membrana, utilização diferencial de vias de sinalização), ou seja, um fenômeno transitório, possivelmente reversível e independente de mutação gênica (Shackleton *et al.*, 2009; Pisco and Huang, 2015; Gao *et al.*, 2016). Estudos recentes relataram que NCSCs também podem gerar CSCs, seja de forma espontânea ou sob o estresse causado pelo tratamento quimioterápico (Shackleton *et*

al., 2009; Yang *et al.*, 2012). De maneira interessante, a proporção de CSCs se mantém estável ao longo do tempo entre as diferentes linhagens de células tumorais em condições ótimas de cultura, sugerindo um possível mecanismo de controle entre as subpopulações de CSC e NCSC (Yang *et al.*, 2012). Tal processo envolve a capacidade das células tumorais de alterarem seu fenótipo devido a mudanças no microambiente tumoral em prol de sua própria sobrevivência. Alguns estudos já descreveram que a transição de fenótipo de NCSC para CSC favorece invasão e metástase das células tumorais (Biddle *et al.*, 2016; O'Brien-Ball and Biddle, 2017) outros demonstraram que NCSCs podem adquirir propriedades de CSC sob tratamento quimioterápico convencional, induzindo o fenômeno de quimiorresistência (Liu *et al.*, 2014; Goldman *et al.*, 2015). Fato importante, a aquisição de propriedades de CSC induzidas por drogas está correlacionada com o aumento da expressão de modificadores epigenéticos, como histona desacetilase (HDAC) e histona metiltransferase (HMT), sugerindo que essa transição fenotípica pode ser transitória (Doherty *et al.*, 2016). Inclusive, diversas drogas moduladoras de epigenética estão sendo testadas em ensaios clínicos (Toh, Lim and Chow, 2017).

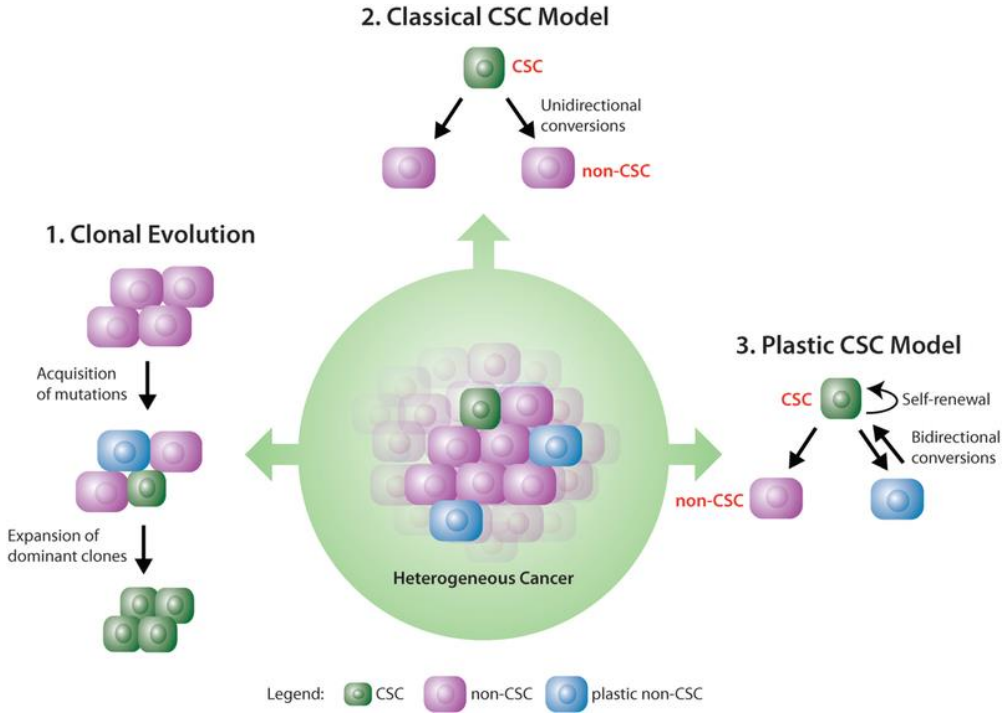

Figura 3: Modelos heterogeneidade tumoral. (1) Evolução clonal: Neste modelo, distintas populações de células tumorais evoluem progressivamente durante as etapas de formação do tumor por meio de sucessivas alterações genéticas e epigenéticas. Os clones dominantes são então selecionados por meio de pressões seletivas e colonizam grande parte do tumor. **(2)** Teoria clássica das CSC: este modelo propõe que a heterogeneidade intratumoral surge por meio da diferenciação de CSCs residentes no tumor em diferentes subclones de NCSCs de maneira unidirecional. **(3)** Modelo plástico das CSC: este modelo propõe a existência de uma diferenciação bidirecional entre CSC e NCSC, na qual CSC pode~~m~~ dar origem a NCSC e vice-versa. Figura retirada e adaptada de Marjanovic 2013 (Marjanovic, Weinberg and Chaffer, 2013).

O caráter agressivo das CSCs, sua capacidade de autorrenovação, mas principalmente a geração de novas CSCs a partir de NCSC, abre um leque importante para o estudo da plasticidade celular, e a avaliação das suas consequências diretas sobre o tratamento do câncer (Yang *et al.*, 2012). Um grande campo de estudo está se abrindo a respeito desse *shift* de fenótipo de forma a melhor entender os mecanismos envolvidos e, consequentemente, na descoberta de novos alvos terapêuticos.

1.3 - Marcadores de CSC: *Cluster Differentiation* 24 (CD24)

Na busca de melhor entender os fenômenos que englobam o surgimento e manutenção da HIT, uma série de marcadores de CSC foram identificados – como CD24, CD244, CD133 – e estão sendo utilizados em clínica como classificação de prognóstico. Vários, senão todos, tumores sólidos contêm células tronco tumorais, podendo ser identificadas por meio da expressão de marcadores moleculares localizados na membrana dessas células (Jaggupilli and Elkord, 2012).

CD24 é uma glicoproteína ancorada à membrana celular por uma glicosil-fosfatidil-inositol (GPI). Historicamente, a CD24 foi identificada como um marcador de células hematopoiéticas. No câncer, a CD24 vem sendo relacionada à HIT e pode ajudar na classificação tumoral. Nos cânceres de mama invasivos, CD24 está expressa de maneira elevada em 84,6% dos casos, o que indica uma possível relação entre a presença dessa molécula e agressividade do câncer de mama (Kristiansen *et al.*, 2003). Além disso, CD24 demonstrou ser altamente expressa em vários tumores malignos comuns, sendo relacionado ao fenótipo metastático (Jiao, Zhao, Niu, & Chen, 2013; Vazquez-Martin et al., 2011). O fato de CD24 ser considerado um ligante de p-selectina, permitindo o rolamento das células tumorais em células endoteliais, reforça essa hipótese. Entretanto, apesar de ser frequentemente encontrada em células tumorais agressivas, a função de CD24 e os possíveis mecanismos regulados por essa glicoproteína ainda são pouco elucidados.

Apesar de não apresentar domínio intracelular como a maioria das moléculas sinalizadoras, a CD24 é capaz de mediar a transdução de sinais pela ativação de proteínas cinases, controlando o desenvolvimento e apoptose de células-B e -T (Fang *et al.*, 2010). No câncer, vários estudos já relacionaram o envolvimento da CD24 em vias de proliferação/morte celular. Em um estudo feito em 2010, Wang e colaboradores mostraram que células de câncer colorretal proliferam de maneira CD24/p38 dependente. Para estabelecer essa relação, os autores observaram que o inibidor seletivo da via p38 reduziu a proliferação celular em células

que superexpressavam CD24 (Wang *et al.*, 2010). Outro estudo demonstrou que a expressão de CD24 está associada à ativação da via ERK1/2, aumentando a capacidade invasiva de uma linhagem de tumor colorretal (Su *et al.*, 2012). Baumann et al demonstraram que CD24, em associação com balsas lipídicas, aumenta a atividade da c-src cinase em câncer de mama, elevando a capacidade de adesão e metastática dessas células (Baumann *et al.*, 2012).

Recentemente foi demonstrada uma ligação entre CD24 e p53. Wang e colaboradores demonstraram que o silenciamento de CD24 contribui para a restauração da função da p53 mutada nas células tumorais, sugerindo que CD24 poderia controlar a morte celular através da inativação de p53 (Wang, Gerstein and Snyder, 2009). De fato, em condições normais, a proteína p53 é capaz de induzir apoptose em células que exibem anormalidades ou stress por mecanismos dependentes ou independentes de transcrição gênica (Livesey *et al.*, 2012). Apesar da relação entre CD24 e p53 já estar sendo descrita, a relação entre CD24 e proteínas da família Bcl-2 ainda não foi estabelecida. No câncer, a regulação da expressão dessas proteínas é importante, pois pode interferir no sucesso de tratamentos antitumorais que geralmente induzem apoptose das células neoplásicas.

Evidências apontam que a mudança de fenótipo associada a expressão membranar de marcadores de CSC pode estar correlacionada à quimiorresistência. Por exemplo, a expressão de CD24 parece ser um marcador molecular significativo em células de câncer de laringe resistentes à cisplatina. Goldman *et al* observaram um enriquecimento da população $CD24^{+}$ após tratamento com docetaxel em diferentes linhagens de tumor de mama, indicando a geração de novas CSCs a partir de NCSCs. Barkal *et al* demonstraram que tumores que expressam CD24 têm atividade antifagocítica contra macrófagos. Além disso, o bloqueio de CD24 por anticorpo resultou na redução do tumor de maneira macrófago-dependente *in vivo* e aumentou a sobrevida dos camundongos (Barkal *et al.*, 2019). Em estudo liderado por Liu, foi demonstrado que CD24 em interação com SiglecG (camundongo) ou Siglec10 (humano) favorece o escape das células tumorais ao sistema imune (Liu, Chen and Zheng, 2009).

1.4 - Vias de sinalização envolvidas na vida e morte das células tumorais

O ciclo celular em organismos multicelulares é rigorosamente controlado por uma rede complexa de vias de sinalização que assegura que as células proliferem apenas quando exigido pelo organismo como um todo, como por exemplo, durante o desenvolvimento embrionário ou a cicatrização de feridas (Kyriakis and Avruch, 2012). O câncer ocorre quando a regulação de crescimento normal das células se desestabiliza, geralmente por causa de

defeitos nestas vias de sinalização. Várias vias de sinalização envolvendo cinases e fatores de transcrição já foram descritas como pontos chaves no desregulamento da proliferação de células tumorais (Pearson and Fabbro, 2004; Wada and Penninger, 2004; Degirmenci, Wang and Hu, 2020; Pushpam and Bakhshi, 2020).

1.4.1 - Mitogen Activated Proteins Kinases (MAPKs)

As *Mitogen Activated Proteins Kinases* (MAPKs) são um grupo de serina-treonina cinases responsáveis por modular uma diversa gama de sinais intracelulares, incluindo proliferação, morte, diferenciação e transformação celular, além de possuírem um papel fisiológico importante no controle de processos inflamatórios. As MAPKs mais estudadas encontradas nos mamíferos são *extracellular signal-regulated kinase* (ERK), *p38 mitogen-activated protein kinase* (p38) e *c-Jun N-terminal kinase* (JNK) (McCubrey *et al.*, 2006; Kim and Choi, 2010; Zhang and Selim, 2012; Koul, Pal and Koul, 2013). Cada uma dessas MAPKs possuem diferentes isoformas: ERK1-8; p38-$\alpha/\beta/\gamma/\delta$; JNK1-3 (Kyriakis and Avruch, 2012). A via de sinalização das MAPKs é composta de pelo menos 3 componentes: MAPK *kinase kinase* (MAP3Ks), MAPK *kinase* (MAP2Ks) e MAPK (Figura 4). As MAP3Ks são responsáveis por fosforilar e ativar as MAP2Ks, que por sua vez fosforilam e ativam as MAPKs. Uma vez ativadas, as MAPKs podem translocar-se para o núcleo, onde ativam fatores de transcrição e induzem resposta biológica (McCubrey *et al.*, 2006; Kim and Choi, 2010; Zhang and Selim, 2012; Koul, Pal and Koul, 2013).

Como esperado, as MAPKs são utilizadas pelas células tumorais e têm envolvimento na progressão tumoral. Isto levou diversos inibidores de MAPKs, principalmente inibidores da via ERK, a serem testados em estudos clínicos como novas alternativas no combate ao câncer (Wagner and Nebreda, 2009; Akinleye *et al.*, 2013). A descrição das vias das MAPKs, os efeitos fisiológicos desencadeados pela ativação delas, o papel das MAPKs no câncer e as drogas que estão sendo testadas em ensaios clínicos estão detalhados no capítulo intitulado "New challenges in cancer therapy: MAPK inhibitors from bench to bedside" anexado na seção 4.1 (Ropert and Huth, 2017).

Além dos inibidores de MAPKs, outros inibidores de cinases também estão sob investigação, como inibidores específicos de ciclina cinase. Dentre eles o ribociclibe, um inibidor de CDK4/6 aprovado pela FDA em 2017 para o uso no tratamento do câncer de mama classificados como $ER^+/HER2^-$ (Otto and Sicinski, 2017; Fiorillo, Sotgia and Lisanti, 2019).

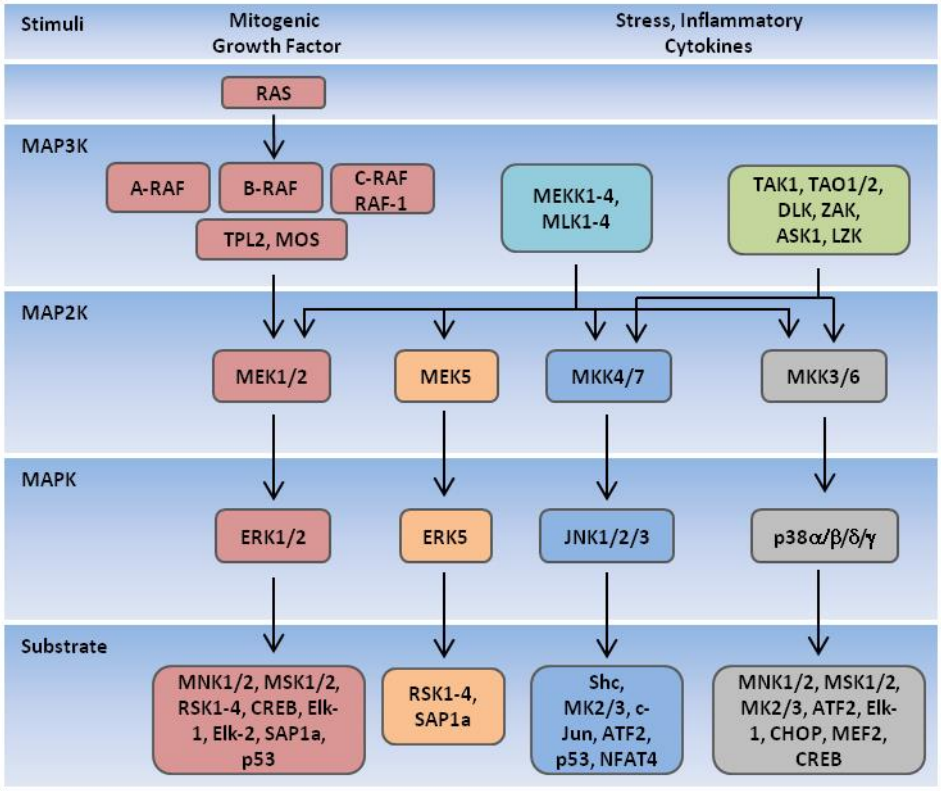

Figura 4: Representação esquemática das diferentes vias das MAPKs. Os vários ativadores de ERKs, JNKs, e p38 MAPKs, como os membros da família MAP2K e MAP3K, estão descritos na figura. A via ERK é principalmente ativada por meio do estímulo de fatores de crescimento, enquanto a via JNK e p38 são definidas como vias de estresse são ativadas por fatores ambientais de estresse e citocinas inflamatórias. A fosforilação das MAPKs leva a ativação de fatores de transcrição no núcleo. Notavelmente, o mesmo fator de transcrição pode ser ativado por diferentes MAPKs. Todas as três MAPKs podem estar envolvidas em atividade proliferativa, sobrevivência ou morte celular. Tais funções variam de acordo com o tipo de célula, isoforma da MAPK e fator de transcrição ativado. Figura adaptada de (Ropert and Huth, 2017).

1.4.2 - Via apoptótica

O termo apoptose é utilizado como um distinto e importante mecanismo de "morte celular programada" que envolve a morte de células geneticamente determinadas. A apoptose ocorre normalmente durante o desenvolvimento e o envelhecimento como um processo de homeostase, mantendo o equilíbrio entre células sadias e células que sofreram algum tipo de dano, seja por doença ou por um agente nocivo (Elmore, 2007).

Existem duas vias descritas em mamíferos pela qual a apoptose pode ser induzida, a via extrínseca e a via intrínseca. A via extrínseca envolve a ativação de receptores de morte celular encontrados na membrana plasmática por um ligante. Dentre esses ligantes o mais conhecido é o *tumor necrosis factor alfa* (TNF-α). Ao ligar-se ao seu receptor, o TNF-α ativa

uma série de proteínas intracelulares sinalizadoras que, por sua vez, ativam a via das caspases e, consequentemente, induzem apoptose. Já a via intrínseca envolve mecanismos de controle da via mitocondrial, que podem ser ativados por meio de danos provocados ao DNA por drogas citotóxicas ou irradiação (Agarwal and Kaye, 2003).

As proteínas envolvidas no balanço morte/sobrevivência na via intrínseca pertencem a uma família ampla, a família Bcl-2. Os membros pró-apoptóticos mais estudados incluem Bax, Bak, Bad, enquanto os membros anti-apoptóticos incluem Bcl-2, Bcl-XL e Bcl-XS (Alam, 2003; Hassan *et al.*, 2014; Fickova, Macho and Brtko, 2015). Os membros anti-apoptóticos exercem sua função mantendo a integridade da membrana mitocondrial, inibindo a indução da apoptose. Por outro lado, os membros pró-apoptóticos agem desestabilizando a membrana mitocondrial e induzindo a liberação de citocromo C no citosol, promovendo a ativação de caspases e desencadeando o processo de apoptose. Em um estudo de caso, foi verificado que Bcl-2 está expressa em aproximadamente 53% dos casos de câncer de mama em humanos e correlaciona-se com mau prognóstico, com exceção para o sub-tipo luminal A (Eom *et al.*, 2016). Sendo assim, Bcl-2 é considerado como alvo terapêutico importante na busca de novas estratégias de tratamento.

A regulação da expressão das proteínas da família Bcl-2 está sob influência direta ou indireta de p53, uma das proteínas mais mutadas no câncer. p53 foi primeiramente descrita como um importante fator de transcrição, capaz de regular a transcrição gênica de importantes proteínas reguladoras do ciclo celular e apoptose. Quando no citosol, p53 induz a morte celular formando complexos inibitórios com proteínas como Bcl-2 e Bcl-XL, o que leva à permeabilização da mitocôndria e consequentemente à liberação de citocromo c. Além disso, p53 pode ativar proteínas pró-apoptóticas, como Bax e Bak, por meio de ligações protéina-proteína (Youle and Strasser, 2008; Hassan *et al.*, 2014; Labi and Erlacher, 2015).

Drogas reguladoras de vias apoptóticas também estão sendo desenvolvidas e testadas em clínica. Venetoclax, um antagonista específico de Bcl-2, provou ser altamente eficaz no combate da leucemia linfocítica crônica. Porém, estudos recentes demonstraram que o uso do venetoclax pode provocar a "síndrome da lise tumoral" (SLT), podendo levar o paciente a morte (Roberts *et al.*, 2016). Com isso, apesar de Bcl-2 ser considerado um alvo potencial no combate ao câncer, novas drogas devem ser desenvolvidas a fim de diminuir os efeitos colaterais dos fármacos já testados em clínica.

2 - Justificativa

Um dos grandes desafios no tratamento contra o câncer é superar a resistência que as células tumorais adquirem contra a quimioterapia convencional. Essa resistência pode se originar por meio do surgimento da HIT, em que um mesmo tumor apresenta diferentes subtipos celulares caracterizados com diferente sensibilidade aos quimioterápicos. Um dos eventos ligados à HIT envolve a utilização de vias de sinalização alternativas pelas células tumorais, tornando essas células mais resistentes à apoptose.

Dentro do contexto da HIT, os marcadores de CSC estão sendo estudados, dentre eles, a glicoproteína de membrana CD24. A presença de CD24 nos tumores correlaciona-se ao mau prognóstico da doença, mas o seu papel a nível molecular não está bem esclarecido. Nos nossos estudos anteriores observamos que a subpopulação $CD24^+$ da linhagem MDA-MB-231 se mostrou mais proliferativa sob diferentes estímulos, como, o TNF-α ou um peptídeo derivado de veneno de aranha. O aumento da incorporação de BrdU (base análoga a Timina) indicando aumento da atividade replicativa, juntamente com o aumento da viabilidade celular da subpopulação $CD24^+$ sugeriram que a presença de CD24 pode representar um marcador de agressividade tumoral nessa linhagem.

A correlação próxima entre marcadores de CSC e agressividade tumoral já está bem descrita na literatura, porém a exata função desses marcadores ainda não foi bem esclarecida. A doxorrubicina é o tratamento de primeira linha em indivíduos com câncer de mama, mas infelizmente observa-se com frequência resistência a esse fármaco ao longo do tratamento. Sendo assim, torna-se relevante estudar o papel da CD24 na indução de resistência às drogas antitumorais, como a doxorrubicina, e correlacionar a sensibilidade à essa droga com as diferentes vias de sinalização utilizadas pelas células $CD24^+$.

3 - Objetivos relacionados ao projeto original da tese (paper 3 - anexo 4.4)

3.1 - Objetivo Geral

Avaliar o papel de CD24 na resistência da linhagem MDA-MB-231 à doxorrubicina e comparar a expressão de proteínas ligadas, direta ou indiretamente, às vias apoptóticas após tratamento com doxorrubicina das células $CD24^+$ e $CD24^-$.

3.2 - Objetivos Específicos

1. Avaliar a expressão e a localização de CD24 nas células da linhagem MDA-MB-231 sob tratamento com doxorrubicina em diferentes tempos
2. Avaliar a viabilidade celular das subpopulações SiCD24, $CD24^-$ e $CD24^+$ da linhagem MDA-MB-231, sob tratamento com doxorrubicina
3. Avaliar a proliferação das células $CD24^-$ e $CD24^+$ da linhagem MDA-MB-231
4. Correlacionar o estado de fosforilação das MAPKs (ERK1/2, JNK e p38) com a presença de CD24 nas células da linhagem MDA-MB-231
5. Quantificar a expressão de proteínas relacionadas à via apotótica (p53, Bax, Bcl-2) na linhagem MDA-MB-231 após tratamento com doxorrubicina
6. Correlacionar a expressão de proteínas da via apotótica (p53, Bax, Bcl-2) na linhagem MDA-MB-231 com a expressão de CD24
7. Correlacionar a inibição de p38 com a sensibilidade à doxorrubicina e com a expressão de proteínas da via apoptótica (p53, Bax, Bcl-2) na linhagem MDA-MB-231

4 - Anexos

4.1 - Capítulo

New Challenges in Cancer Therapy: MAPK Inhibitors from Bench to Bedside

4.2 - Paper 1

MEK2 controls the activation of MKK3/MKK6-p38 axis involved in the MDA-MB-231 breast cancer cell survival: Correlation with cyclin D1 expression.

4.3 - Paper 2

Upregulation of p38 pathway accelerates proliferation and migration of MDA-MB-231 breast cancer cells

4.4 - Paper 3 (referente ao projeto original da tese)

Translocation of intracellular CD24 constitutes a triggering event for drug resistance in breast câncer

4.5 - Paper 4 (manuscrito em preparação)

Imiquimod controls aggressive breast cancer cell growth without stemness induction

4.6 - Revisão (manuscrito em preparação)

Heterogeneity, plasticity and cancer stem cells: the three musketeers of breast cancer drug resistance

4.1 - Capítulo

New Challenges in Cancer Therapy: MAPK Inhibitors from Bench to Bedside

Ammad Ahmad Farooqi
Muhammad Ismail *Editors*

Molecular Oncology: Underlying Mechanisms and Translational Advancements

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New Challenges in Cancer Therapy: MAPK Inhibitors from Bench to Bedside

Catherine Ropert and Hugo W. Huth

Abstract The mitogen-activated protein kinases (MAPKs) belong to a family of serine/threonine kinases which transduce the signals by post-translational modifications of different downstream effectors and transcription factors involved in different aspects of the cell life, such as proliferation, migration, differentiation and death. The deregulation of MAPK pathways is well correlated with cancer development. Recent advancements made in genetics, genomics and proteomics have provided a better understanding of the mechanisms involved in tumor progression and have confirmed the relevance of aiming MAPKs as novel therapeutic targets in cancer. Indeed, different MAPK inhibitors are currently under investigation demonstrating the potential of such compounds in cancer therapy. Here, we present the latest advances involving MAPK inhibitors in clinical trials, and we summarize key parameters for the translation of results from bench to bedside.

Keywords Cancer therapy • MAPK • MAPK inhibitor • Signaling pathway • Tumor heterogeneity

Introduction

Cancer is a multifaceted and genomically complex disease and rapidly expanding information related to subtype-specific molecular abnormalities has revolutionized the development of molecular diagnostics for cancer subtypes and rationally designed therapeutics tailored to patients based on the molecular profiles of their tumors [1, 2]. Recent breakthroughs in unbiased genome-wide association studies

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have helped in the identification of previously unrecognized genetic predisposing factors which are contributory to an individual's risk of developing cancer [2, 3]. Furthermore, over the past several years, analyses of data from high-throughput studies of somatic alterations of the genomic and transcriptomic landscapes in tumors have provided convincing evidence related to intracellular signaling cascades which are characteristically dysregulated in cancer subtypes [4, 5]. Tumor cells have evolved different mechanisms to escape from apoptosis. Imbalance of pro-apoptotic and anti-apoptotic proteins also plays instrumental role in cancer development [4, 6–8]. p53 protein is involved in positive regulation of apoptotic pathway [9–11]. Substantial fraction of information has been added into the pre-existing pool of knowledge and it is now more understandable that dysregulations of spatio-temporally controlled signaling cascades, existence of significant inter-tumoral and intra-tumoral heterogeneity,genetic/epigenetic mutations are some of the most widely studied mechanisms which underlie cancer development. Moreover, different mechanisms including loss of apoptosis, Darwinian evolution in response to therapeutic pressures and highly intricate multi-directional spread of metastatically competent cancer cells from primary site to the metastatic sites are also being widely studied.

Many of the signals transduced intracellularly through different receptors and environmental cues converge on mitogen-activated protein kinases (MAPKs), which phosphorylate and activate different downstream effectors and transcription factors. MAPKs are members of a dynamic and hierarchially organized protein kinase network through which signals from the respective MAPK are transduced to the specific spatio-temporal cellular loci.

In this context, a growing interest in a better understanding of the signaling pathways involved in cancer has created new avenues for the identification and development of new anticancer drugs at molecular level. Here, we present challenges and opportunities in the targeting of MAPKs in cancer.

MAPK Signaling Pathway in Cancer

Mitogen Protein Activated-Kinases (MAPKs), that comprise a serine/threonine kinase group, are among most deeply studied signal transduction pathways. The MAPK pathways present in all eukaryotic cells respond to a plethora of extracellular stimuli and control many biological activities like gene expression, mitosis, metabolism, migration, survival, apoptosis, and differentiation [12–14]. A canonical MAPK pathway consists of a three-kinase module in which a MAPK is activated upon phosphorylation on threonine and tyrosine residues by a mitogen-activated protein kinase kinase (MAPKK), when phosphorylated by a MAPKKK (Fig. 1). Once activated, the MAPK may translocate to the nucleus to activate transcription factors and induce a biological response [15]. This assembly of MAPK pathways in a three-component module has been conserved from yeast to humans. The MAPK network includes extracellular signal-regulated kinase (ERK), p38 mitogen-activated protein kinase (p38), c-Jun N-terminal kinase (JNK). MAPK pathways play

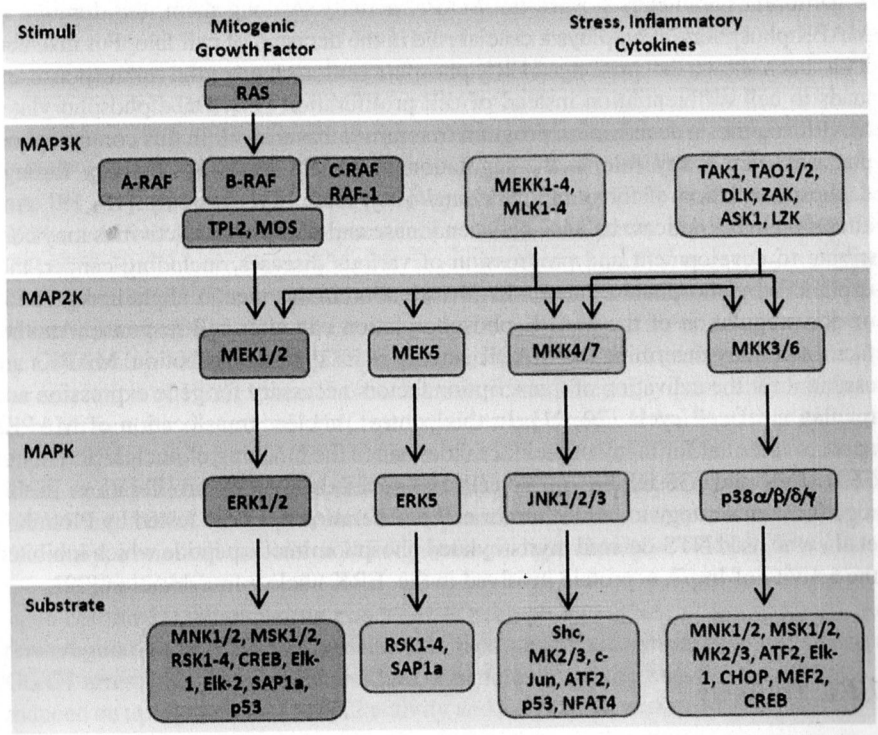

Fig. 1 Schematic representation of the different MAPK signaling pathway. The various upstream activators of ERKs, JNKs and p38 MAPKs, such as MAP2K and MAP3K family members, are depicted in the figure. ERK pathway is mainly activated through growth factors while p38 and JNK pathways are defined as stress pathways and consequently activated by environmental stress and inflammatory cytokines. The phosphorylation of MAPK leads to transcription factor activation in the nucleus. Notably, the same transcription factor can be activated by different MAPKs. All the three MAPKs may be involved in the proliferative activity, cell survival or proapoptotic function depending on cell type, MAPK isoform and transcription factor activated

important roles in normal cellular physiology, but the tumorigenesis process "captures" and also uses MAPKs to continuously transmit survival signals to the nucleus [16]. Besides, ERK5 and other atypical MAPKs like ERK3/4, ERK7 have been described. But, ERK1/2, JNKs, and p38 isoforms represent the most studied mammalian MAPKs that is why we focused on these three later MAPKs.

While ERK pathway links preferentially to cell proliferation, differentiation, and death, JNK and p38 MAPK pathways participate in stress signaling thus promoting an inflammatory response as depicted in the Fig. 1. As the association between chronic inflammation and cancer development has been evidenced, an important part for these stress-activated kinases in cancer has emerged. However, besides their involvement in inflammation, the role of JNK and p38 as a regulator of cell proliferation and apoptosis must be considered.

Different parameters govern the MAPK activity. Among them, the duration of MAPK phosphorylation plays a crucial role in the decision of cell fate. For instance, it has been shown that prolonged ERK phosphorylation changes the cell response and leads to cell differentiation instead of cell proliferation [17]. Once phosphorylated MAPK requires a deactivation program to return to basal level. In this context, phosphatases play a key role in the regulation of MAPK pathways activity through dephosphorylation of threonine (Thr) and/or tyrosine (Tyr) residues [18, 19]. Any alteration in the delicate balance between kinase and phosphatase activities may contribute to development and progression of various diseases, including cancer, that explains why phosphatases are under investigation in this area. A slight upregulation or downregulation of the MAPK phosphorylation can alter cell response. Another factor that may determine the MAPK activity is its spatial distribution. MAPKs are essential for the activation of transcription factors necessary for gene expression and regulation of cell cycle [20, 21]. In this context, nuclear translocation of MAPKs appears essential for many of their activities, since the blocking of nuclear activity of ERK, JNK and p38 inhibits many cellular processes like cell proliferation. In this regard, such strategy to reduce tumor cell proliferation has been tested by Plotnikov et al., who used NTS-derived myristoylated phosphomimetic peptide which inhibited the activity of Imp7, a protein involved in the ERK nuclear translocation [22].

ERK Pathway

ERK1 was the first mammalian MAPK to be cloned and characterized [23–26]. It was originally found to be phosphorylated on Tyr and Thr residues in response to growth factors [25, 27, 28]. The ERK signalling pathway is activated by an array of receptor types, including receptor tyrosine kinases (RTKs), G protein-coupled receptors and cytokine receptors [29, 30]. The core of this pathway is involved in activation of the cascade of RAF, MEK, and ERK. Although this cascade is classically shown as linear, its representation should take into account the complexity of the signaling network. Indeed, this is illustrated by the convergence of signaling at MEK1/2 level as presented in the Fig. 1. Besides the RAF proteins (A-RAF, B-RAF and C-RAF) that are the best-studied MEK activators, a number of other MAP kinase kinase kinases (MAP3Ks) like MEKK1or MEKK2 may activate MEK1/2.

The RAF proteins have been intensely studied over the past years, and most of the studies have been focused on C-RAF. Then, with the discovery of B-RAF mutations involved in a large number of tumors, B-RAF has received more attention, specifically in melanomas. However, very little is known about A-RAF. Differences in function and tissue expression among RAF isoforms have been evidenced. For example, B-RAF is frequently overexpressed in cells of neuronal origin [31]. The activation of RAF triggered by various signals like RAS GTPases [21, 32] appears complex and involves phosphorylation/dephosphorylation cycle before reaching the activated state. Importantly, it has been shown that B-RAF requires fewer phosphorylation events than A-RAF and C-RAF for maximal activation [33]. The ability to activate MEK in vitro also differs depending on the isoform: B-RAF

is the strongest inducer of MEK activation, followed by C-RAF and A-RAF whose MEK activity is merely detected [34]. Later, it has been reported that heterodimerization was crucial to promote the RAF activity. Indeed, Rushworth et al. have demonstrated that B-RAF and C-RAF heterodimerize in multiple cell lines in response to mitogens [35]. This supports the hypothesis of Matallanas et al. suggesting that C-RAF and A-RAF may act as modulators to enhance the ability of B-RAF to activate the ERK pathway [36]. This pathway is highly regulated as illustrated by the ERK-mediated feedback phosphorylation of B-RAF reducing the lifetime of C-RAF/B-RAF heterodimers [35, 37]. In some other way, ERK may act directly on RAS reducing the efficiency of the RAS/C-RAF association and renders ERK activation transient [38].

Concerning MEK1 and MEK2 kinase isoforms that are directly responsible for the activation of ERK, it has also been assumed that they may have different functions. In fact, the absence of MEK1 provoked an embryonic lethality of the *MEK1*$^{-/-}$ mice [39]. By contrast, *MEK2*$^{-/-}$ mice are viable and fertile [40]. But, more recently, Aoidi et al. [41] have changed this equation by describing the contribution of both MEK1 and MEK2 in the placenta development and embryo survival. According to this study, a minimal amount of MEK protein, independently of which isoform, was required for embryo survival.

In cell model, by depleting either MEK subtype by siRNA, it was reported that downregulation of MEK1 expression inhibited pancreatic cancer cells and induced G0/G1 arrest [42]. In colon cancer line or pancreatic cancer line, the lack of MEK1 induced an upregulation of MEK2 activity and a sustained strong ERK activity leading to growth arrest. By contrast, when MEK1 activity became predominant as in the absence of MEK2, ERK pathway activation induced cell proliferation [43]. Recently, a differential function for MEK1 and MEK2 has been evidenced in breast cancer cells. The authors have shown that the depletion of MEK2 but not MEK1 reduced the cyclin D1 expression leading to decreased cell survival. Further, the authors have described a link between MEK2 and p38 pathway never described in normal cells [44]. MEK1 and MEK2 are phosphorylated by RAF or MAP3K at Serine (Ser) 218 and Ser 222 and Ser 222 and Ser 226, respectively. Both isoforms have a strategic position in ERK pathway since they are considered the only activators of ERK1/2. MEK1 and MEK2 have been noted to exist as heterodimers. ERK is involved in phosphorylation of MEK1 on Thr292 that impedes the formation of MEK1/MEK2 heterodimers, and promotes dephosphorylation of activating residues Ser 218 and Ser 222 [45, 46]. The direct consequence of the MEK1 phosphorylation is a reduction of MEK1/2 activity and consequently of ERK activation illustrating another negative feedback of ERK on this pathway.

Concerning ERK1 and ERK2 isoforms, different debates are still in progress about functional difference or functional redundancy between them. As inhibitors of B-RAF and MEK1/2 have been approved for the treatment of cancer and they are expected to inhibit their downstream effectors ERK1 and ERK2, it is crucial to understand whether the two isoforms exert specific or redundant functions. Several studies have claimed a redundant role for both isoforms, in which generally, the methodological approach was the reduction of ERK1 and/or ERK2 to clarify their role in cells. Single and combined silencing of ERK1 and ERK2 have shown that

their decisive role in growth signaling depend on their expression levels [47]. In other way, Vantaggiato et al. have suggested that ERK1 and ERK2 may assume opposite functions in RAS-mediated signaling since ERK1 negatively regulates the signal induced by ERK2 [48]. Shin et al. have defined that downregulation of ERK2 but not ERK1, abolish oncogenic RAS-induced senescence [49]. In the same way, other groups have demonstrated that ERK2 but not ERK1 depletion significantly reduces global ERK activity that corroborates with the absence of effect on ERK activity after ERK1 knockdown [50, 51]. Importantly, ERK2 is generally more expressed than ERK1 in most mammalian tissues. Such observations address the use of silencing methods to define ERK1 or ERK2 role due to their differential expression in tissues. In addition, this may explain why the impact of ERK2 silencing appears more pronounced in different models; however, the role of ERK1 cannot be neglected as suggested by Frémin et al. [52]. They have demonstrated that the loss of functions due to ERK2 deficiency may be rescued by overexpression of ERK1 and have concluded that ERK1 and ERK2 exert redundant functions in mouse development. The same remains to be demonstrated in cancer or in others diseases. Interestingly, Buscà et al. propose a new concept where total ERK activity and not isoform itself is the critical parameter to achieve ERK functions [53].

The ERK cascade plays a crucial role in multiple cellular processes involved in cell fate [30, 54, 55]. Importantly, the RAS-RAF-MEK-ERK1/2 pathway is one of the most dysregulated signaling pathways in human cancer. For instance, B-RAF mutation frequently occurs in certain types of cancers such as melanoma (50–80% of cases), papillary thyroid carcinoma (~45%), hepatocellular carcinoma (~40%) and colorectal cancer (~10%) [56, 57]. The direct consequence of RAF mutation might be the constitutive activation of ERK1/2. But, constitutive ERK activation is not necessarily linked to mutated component of this pathway. Other factors like epigenetic factors may also play a role in the dysregulation of ERK pathway. The high incidence of human cancers with a constitutively active ERK pathway has encouraged the research of pharmacological inhibitors able to efficiently target different regulators of this cascade.

JNK Pathway

JNK was described, almost 20 years ago, as the protein kinase responsible for activating c-Jun which is a component of the activating protein-1 (AP-1) transcription factor family that may be involved in malignant transformation [58]. Besides, JNK was defined as a stress-activated kinase responding to a large panel of stimuli leading to the production of cytokine, and consequently to the development of chronic inflammation [59]. JNK can be phosphorylated by two distinct MAPKK, MKK4, and MKK7, which cooperate in the JNK activation. In turn, MKK4/7 are phosphorylated by a plethora of MAKKKs, including MEKK1 to −4, MLK1 to −3, Tpl-2, DLK, TAO1/2, TAK1 and ASK1/2 [13, 14]. It has been described, up to now, three JNK isoforms, JNK1/2/3, which have about 85% of homology, but their tissue distribution may vary [60]. While JNK1 and JNK2 are distributed in different

organs, JNK3 is mostly expressed in neuronal tissues, testis, and cardiomyocytes, probably predicting functional differences [61]. It has been stated by Gupta et al. that JNK isoforms may act individually on specific transcription factors in vivo. Indeed, JNK2 seems to have a stronger affinity for c-Jun and induces a higher proliferative activity when compared to JNK1 [62].

JNK has been reported to play a significant role in various diseases. In cancer, for instance, JNK seems to have an ambiguous position, acting like a proliferative or death inducer, depending on cell type, tumor stage, and isoform [59]. For example, activation of JNK1 was essential for tumorigenesis in response to RAS activation induced by UV radiation. Furthermore, in childhood sarcoma, JNK1 appeared crucial for cell proliferation, while JNK2 might induce apoptosis [63]. In the same way, another study has suggested that JNK2, in contrast to JNK1, downregulated cellular proliferation in a wide range of cell models [64]. Also, JNK3 was described as an apoptosis inducer in chemoresistant human ovarian cancer cells [65]. In mouse models, the lack of JNK1 or JNK2 favored the development of breast cancer. However, JNK1-deficient mice presented a decrease in gastric carcinogenesis compared to their wild-type counterparts [66]. Mice lacking of JNK1 in the liver were less susceptible than wild-type animals to hepatocellular carcinoma [67]. Considering the different studies cited above it clearly appears that there is no consensus concerning the role of JNK isoforms in tumor cell physiology. These discrepancies must be explained by the use of gene silencing technique which may bias the interpretation of the results. This underlines the necessity to use diverse tools of investigation to better define kinase functions [68] before targeting JNK in cancer. In any case, a selective role of different JNK isoforms depending on tumor types should be further investigated.

p38 Pathway

The p38 MAPK was first identified in 1994 and reportedly triggered by stress stimuli like inflammatory cytokines, UV irradiation, hypoxia, and ischemia. The activation of p38 occurs via its upstream kinase MKK3/MKK6, but MKK4 may also activate p38. MKK3/6 are activated by a plethora of MAPKKKs, including MEKK1 to −3, MLK2/3, ASK1, Tpl2, TAK1, and TAO1/2. The MAPKKK responsible for activating the p38 MAPK pathways appears to be cell type and stimulus-specific [13, 14]. After the identification of p38 (also known as p38α), three other isoforms were also described: p38β/δ/γ, [69]. MKK6 can activate all isoforms of p38, while MKK3 are responsible for phosphorylating preferentially p38α, γ and δ. An important fact is that the expression of these isoforms varies from tissue to tissue: p38α is found in almost all cell types, while p38β is more brain-tissue specific, p38γ is expressed in skeletal muscle and p38δ in endocrine glands [70].

p38 has a key role in the inflammatory response [71, 72]. Once activated, p38 pathway induces the synthesis of pro-inflammatory cytokines by modulating transcriptional factors implicated in cancer development [70]. More precisely, α and β isoforms of p38 are predominant in chronic inflammation [73]. Interestingly, these

same isoforms are involved in breast cancer progression, maybe due to their inflammatory properties [74]. Besides, p38 is also involved in cell cycle, controlling its proliferation and survival. In fact, p38 may regulate both the G2/M as well as G1/S cell cycle checkpoint [75–77]. In cancer, the role of p38 is not completely understood [78]. There are several studies describing p38 as a proliferative agent [74, 79]; on the other hand, some studies have defined p38 as an apoptosis inducer [80–82]. Notably, p38 may directly affect tumor growth independently of its role in inflammation. In most of the studies concerning prostate cancers, cancer cells require the activity of p38 to proliferate, indicating that p38 may act as proto-oncogenic protein [83, 84]. Furthermore, overexpression of p38 in its phosphorylated form is also associated with a bad prognosis and with high morbidity rate in colorectal cancer [85]. Besides, analysis of the phenotype of MKK3/6$^{-/-}$ and p38$\alpha^{-/-}$ mice has led to the conclusion that p38 can function as a tumor suppressor. The tumor suppressive effects of p38 may be mediated in different ways since p38 is involved both in the induction of p53-dependent apoptosis and in the negative control of cell cycle progression. A reduction of p38 activity has been shown in hepatocellular carcinomas, where an inverse correlation was established between tumor size and p38 activity. This ambiguous role of p38 pathway may occur due to the existence of four isoforms (p38α/β/δ/γ) acting independently and sometimes leading to opposite effects. The absence of selective p38 isoform inhibitors adds another layer of complexity to intricate crosstalk between proteins of different networks in cancer cells. The heterogeneity of cancer cells and its variable genomic mutations may also contribute to the ambiguous p38 pathway function in cancer cells.

As related above, the properties of ERKs, JNKs, and p38s to provoke cell death or to induce cell proliferation are tumor-dependent indicating that there are no rules concerning their role in cancer.

What Is a "Good" MAPK Inhibitor?

Current efforts to discover kinase inhibitors are concentrated on fundamental hallmarks of cancers like excessive cell proliferation, increased survival or tumor angiogenesis. As reported above, an extensive literature supported a role for MAPKs in these different cellular events and opened new horizons to explore their potential as a therapeutic target in cancer.

Specificity Towards Binding Site

The development of specific MAPK inhibitors themselves has been less successful and only a few compounds have been identified. Interestingly, the idea that MAPK pathways would represent drug targets in cancer therapy emerged in the mid-1990s by the characterization of SB203580 as a selective p38 inhibitor that downregulated TNF-α [71]. The first step towards kinase inhibitor development was selection of ATP

cleft as target site. Indeed, binding of ATP to a protein kinase is essential for the kinase's phosphotransferase activity, and thus, the ATP binding pocket was the "target" of most inhibitor screens [86, 87]. The majority of MAPK inhibitors belongs to class of ATP-competitive inhibitors that recognizes the active conformation of the kinase. Accordingly, many compounds have been tested in vitro using enzymatic assays in which kinases were in their active conformation and the strategy to synthesize MAPK inhibitors was to mimic ATP structure. The common problem with kinase inhibitors that compete with ATP binding is their lack of specificity since the ATP-binding pocket is highly conserved among members of the kinase family. This is illustrated by the anthrapyrazole SP600125, one of the earliest and most studied ATP-competitive JNK inhibitor, that has been shown to inhibit 13 other protein kinases [88]. SB203580 represents the other most famous MAPK inhibitor that binds to ATP-binding pocket [71]. But, interestingly, this inhibitor may recognize the active and inactive conformation of the kinase [89]. The binding of a drug to the inactive conformation of the kinase is becoming an attractive approach. The major advantage of this strategy is that the inhibitor will face weaker competition from cellular ATP and even for those classified as ATP-competitive inhibitors. They may act by shifting the equilibrium between inactive and active kinase conformation impeding kinase activation. It has been proposed that SB203580 could stabilize p38α in its inactive conformation that reduces the rate of p38 phosphorylation by MAPKK. This additional mechanism of action of SB203580 may explain its relative specificity towards p38α.

Alternative strategies to inhibit MAP kinase function are currently under investigation. For example, compounds that bind outside the ATP-binding site—at an allosteric site (regulatory site)—and modulate kinase activity in an allosteric manner may represent an attractive alternative. Inhibitors from this category generally exhibit the greatest kinase selectivity because they bind to sites that are specific to a particular kinase. The most well described allosteric kinase inhibitor is CI-1040 (PD184352), previously described by Sebolt-Leopold et al., which decreases MEK1 and MEK2 activity by occupying a pocket adjacent to the ATP binding site [90]. Interestingly, a lot of new MEK1/2 inhibitors belong to this family [91, 92].

Small molecule and peptide-based inhibitors that target the docking site of downstream substrates or the scaffold proteins involved in MAPK cascade are a promising alternative to the traditional ATP-competitive inhibitors with improved efficacy and specificity. For example, JNK-interacting protein-1 (JIP1), a scaffolding protein that promotes JNK activity by facilitating the interaction between JNK and upstream kinases [93, 94] may be a relevant target in cancer. With this focus, a peptide corresponding to the minimal region of JIP1 (pepJIP1) has been developed as an inhibitor of JNK activity by reducing the interaction between JIP1 and JNK [95, 96]. It has been shown that cell-permeable JIP1-based peptides may help to resolve some JNK-dependent diseases. But, several limitations have interfered with the development of peptide-based inhibitors due to physiochemical instability and unsatisfactory pharmacological properties. Other compounds that inhibit the interaction between JIP1 and JNKs have also been characterized that may represent a promising strategy in therapy [97].

Another different way to inhibit protein kinase activity may be targeting of protein substrate binding site to inhibit the activation of downstream kinase or

transcription factor phosphorylation. This strategy may have many advantages because this type of inhibitor is specific for selected kinase substrates contrasting with the ATP-competitive inhibitors that would inhibit phosphorylation of all substrate proteins. The same specificity may be expected from the new approaches which include the use of inhibitors of kinase dimerization, as proposed by Herrero et al. In this study, efficiency of a peptide was evaluated which inhibited ERK dimerization and reduced tumor growth in xenografted mice [98]. Besides, as commented above, inhibitor of ERK translocation may represent prototype for the development of new cancer drug.

The challenge of making selective inhibitors with good pharmacological properties remains daunting due to the 518 kinases encoded in the human genome and over 2000 other nucleotide-dependent enzymes, including chaperones, polymerases, reductases, motor proteins and methyltransferases, that may provide potential binding sites [99]. In addition, natural variation in the expression levels of kinases can indirectly alter inhibitor efficiency by changing the total kinase activity in the cell. Kinase inhibitors have been classically tested at the protein level for their potential to inhibit kinase-catalysed phosphotransfer from ATP to a substrate protein or peptide that may sometimes explain their low selectivity. Due to the constraints encountered in cells, the screening of new potential inhibitors may involve not only kinase assays but also critical analysis of downstream effectors and substrates of these kinases in cells where specificity and sensitivity parameters may be evaluated before clinical trials.

Specificity Towards MAPK Isoform

The existence of different isoforms for the same MAPK put into question their redundant or independent activities that may directly influence the MAPK inhibitor efficiency in cancer. This is the case of ERK1 and ERK2 functions, as related above. Such discrepancies may be explained by different cell response evaluated (proliferation, survival, inflammatory response, drug resistance). There are some outstanding questions related to biological outcome associated with the use of ERK isoform selective inhibitors. Up to now, inhibitors targeting ERK1 and ERK2 in an indiscriminate way are yet under investigation in the preclinical stage. The notion that global ERK inhibition would not be recommended in all situation is support by the fact that different studies have reported opposite roles for ERK1 and ERK2 in tumor biology [48, 49, 100, 101]. Interestingly, Aceves-Luquero et al. have involved ERK2 but not ERK1 in resistance to Imatinib Mesylate in a model of chronic myelogenous leukemia sustaining the use of therapeutic approaches based on ERK2 inhibition. ERK inhibitors have been tested in phase I and are ongoing in phase II of clinical trials. So, more related clinical studies should be performed in more advanced stages before the validation of inhibitors of both ERK isoforms as chemotherapeutic agent in cancer treatment.

Existing data clearly suggested that different JNK isoforms may have different or redundant functions in cancer cell [61, 62, 64, 68]. So it appears essential to define

Table 1 p38 pathway inhibitor in clinical trials

Target	Drug	Cancer type	Phase	Interpretation	Sponsor
p38α/β	Ralimetinib	Ovarian	2	Study is ongoing	Lilly Oncology (NCT01663857)
p38α/β	Ralimetinib	Advanced cancer	1	Completed	Eli Lilly and Company (NCT01393990)

when JNK isoforms may cooperate or play opposite role depending on cancer type and cancer stage. This reinforces the necessity to develop inhibitors with more selectivity towards JNK and its isoforms. Recently, it has been shown that JNK3 can be selectively targeted [102]. These results are encouraging and future studies must converge on identification and evaluation of JNK isoform inhibitors with minimum off target effects.

The preservation of the four p38 MAPK isoforms suggests a functional difference between them [69, 88, 103]. The broad body of literature has reported findings related to p38α and p38β. This is due to the fact that generally p38 inhibitors are more selective to α and β isoforms. This is well illustrated by the capacity of SB203580 to inhibit p38α and p38β but not p38γ and p38δ. The differences in chemical inhibitor sensitivity towards isoforms could be attributed to their different substrate specificities and expression patterns [73, 88]. There is only one global inhibitor of p38 described up to now, BIRB 796, that inhibits efficiently α and β isoforms and moderatly γ and δ isoform. But unfortunately, this compound also inhibits JNK2 isoform at the same concentration [88]. The recent interest in the role of p38δ in cancer was based on the fact that p38δ expression and activation were significantly increased in a variety of carcinoma cell lines such as human primary cutaneous squamous carcinoma cells [104], neck and head squamous carcinoma cells and tumors [105], cholangiocarcinoma, and liver cancer cell lines [106]. In the absence of specific inhibitor of p38 isoform, p38δ knocked-down mice were used to confirm the importance of this p38 isoform in the model of chemically induced skin carcinogenesis. These findings paved the way towards development of specific p38 inhibitor [107]. Unfortunately, until now, there are fewer studies related to the use of p38 inhibitors in clinical trials, as depicted in Table 1.

All together, these data show the pertinence to seek new selective inhibitors towards MAPK isoforms in order to clarify their redundant or different functions.

Use of MAPK Inhibitors in Clinical Trials: A Cycle of Hope and Disappointment

The possible use of MAPK pathway inhibitor in cancer has ushered in a new era. Nearly 50% of human malignancies exhibit dysregulated RAS-ERK signaling [32, 108, 109] that justifies the prevalence of clinical trials investigating RAF-ERK

pathway inhibitors as shown in Table 2. Targeting of RAS protein was the initial strategy to control signaling pathway involved in tumor progression [110, 111]. However, RAS inhibition did not reach the expected results in clinical trials probably because these inhibitors were unable to target selective proteins. It is well known that mutations in RAS happen quite often in cancer patients. Mutation in RAS induced constitutive activation. In such context, the inhibition of RAS remains challenging.

Completed clinical trials of MEK inhibitors include some disappointments as well as some promising signs of the value of these compounds [92]. The potential for MEK inhibitors as monotherapy and their use in drug combination with RAF inhibitors are currently under clinical investigation and more frequently in melanoma (Table 2). At first, randomized Phase III clinical trial was conducted with the RAF inhibitor sorafenib (Nexavar; Bayer/Onyx Pharmaceuticals) in patients with metastatic melanoma [112, 113], including patients with tumors that carry the B-RAF^{V600E} mutation, but no antitumor activity was observed [114]. This may be because sorafenib, originally developed as a C-RAF and wild-type B-RAF inhibitor, appeared less efficient towards *B-RAF*^{V600E} [115]. Further, B-RAF^{V600E}-selective inhibitors like vemurafenib and dabrafenib entered in clinical trials. But, rapidly paradoxical effects have been noted particularly the activation of RAF instead of its inhibition in cells with wild-type B-RAF, when used at non-saturating concentrations [116].

The MAPK inhibitor saga continued with the use of MEK inhibitors [91]. Indeed, unlike B-RAF, activating mutations in MEK are more rarely encountered in human tumors. Selumetinib (AZD6244; AstraZeneca/Array Biopharma), another allosteric MEK1 and MEK2 inhibitor that is highly selective for MEK1 and MEK2 has been tested using different protocols in patients with biliary and pancreatic cancer [117, 118]. At first, a modest clinical activity of this compound has been reported. In metastatic melanoma, the therapy impact was also moderate but, the trials were not carried out in patients selected for activating mutations in B-RAF [119]. In fact, a correlation has been drawn between sensitivity to MEK inhibitor and the presence of mutations in B-RAF, since MEK inhibitors are more efficient in cells which carry B-RAF mutations [120, 121]. Reconsidering the first results obtained from studies that have been performed without patient selection and after further evaluation, trametinib (Mekinist, GSK1120212; GSK) became the first MEK1/MEK2 inhibitor to be approved by the US Food and Drug Administration (FDA) for the treatment of metastatic melanoma with the B-RAF$^{V600E/K}$ mutation [122]. Going downstream on ERK signaling pathway, there are few ERK inhibitors reported in clinical trials, but tests are just at the beginning and still ongoing. Carlino et al. have reported that inhibition of ERK, rather than MEK, was more efficient at reducing MAPK activity and inhibiting the proliferation of multiple B-RAF inhibitor resistant melanoma cell models but, without inducing cell death [123]. The efficiency of ERK inhibitors has been shown in cell lines in B-RAF/MEK inhibitor resistance model [124, 125]. But, the use of ERK inhibitor in monotherapy may represent a double edge sword. Indeed, the inhibition of ERK may cause the relief of ERK-dependent negative feedback, provoking a sustained activation of ERK cascade and the cell survival. One strategy to inhibit ERK-induced proliferation without affecting negative

Table 2 RAF-ERK pathway inhibitors in clinical trials

Target	Drug	Cancer type	Phase	Interpretation	Sponsor
B-RAF[a]	Vemurafenib	Melanoma	3	Inhibition of B-RAF with vemurafenib improves survival in patients with the most common B-RAF^{V600E} mutation and in patients with the less common B-RAF^{V600K} mutation	Hoffman (NCT01307397)
B-RAF[a] MEK1 and MEK2	Dabrafenib + trametinib vs. vemurafenib	Melanoma	3	Study is ongoing	GlaxoSmith Kline (NCT01597908)
B-RAF[a] MEK1 and MEK2	Dabrafenib in combination with trametinib	Melanoma	3	Modest clinical efficacy in patients with B-RAF inhibitor-resistant melanoma. Increased survival benefit	GlaxoSmith Kline (NCT01072175)
B-RAF[a] MEK1 and MEK2	Dabrafenib in combination with trametinib	Melanoma stage III–IV	2	Study is ongoing. Testing of the superiority of intermittent dosing of dabrafenib and trametinib compared to continuous dosing with these two same agents	National Cancer Institute (NCI) (NCT02196181)
MEK1 and MEK2	Trametinib	Melanoma	3	Increased survival in ongoing trials for B-RAF-mutated melanoma	GlaxoSmith Kline (NCT01245062)
MEK1 and MEK2 immunotherapy	Selumetinib in combination with durvalumab (MEDI4736)	Advanced solid tumors	1	Study is ongoing	AstraZeneca (NCT02586987)
MEK1 and MEK2	Binimetinib	Melanoma	3	Successful trials for mutant-N-RAS melanoma but lack efficacy in ovarian cancer	Array Biopharma (NCT01763164)
ERK1/2	BVD-523	Myelogenous Leukemia or Myelodysplastic Syndromes	1 and 2	Study is ongoing	BioMed Valley Discoveries (NCI201402600) (NCT02296242)

(continued)

Table 2 (continued)

Target	Drug	Cancer type	Phase	Interpretation	Sponsor
MEK1 and MEK2	Selumetinib	Thyroid	2	Well tolerated but the study was negative with regard to the primary outcome. Disappointing results	AstraZeneca (NCI 7918)
MEK1 and MEK2	RO4987655	B-RAF-mutated and non-mutated Melanoma, small cell lung, K-RAS-mutated colorectal	1	Manageable toxicity, a favorable pharmacokinetics/pharmacodynamics profile, and promising preliminary antitumor activity	Hoffmann-La Roche (NCT00817518)
B-RAF RAF and MEK1/2	Vemurafenib Cobimetinib	Melanoma	3	Improvement in progression-free survival among patients with *BRAF* V600–mutated metastatic melanoma, at the cost of some increase in toxicity	Hoffmann-La Roche (NCT01689519)
B-RAF RAF and MEK1/2	Binimetinib Encorafenib	Melanoma	3	Improvement in several clinically relevant endpoints was well tolerated and may offer a new treatment against *N-RAS*-mutant melanoma	Novartis Pharmaceuticals and ArrayBioPharm (NCT01909453)
MEK1 and MEK2	Trametinib	Low-grade ovarian and peritoneal cavity	2 and 3	Study is ongoing	National Cancer Institute (NCT02101788)
MEK1 and MEK2	Refametinib (BAY 86-8766)	Carcinoma hepatolceular	2	This study has been completed. No results published yet	Bayer/Ardea Biosciences (NCT01915589)
MEK1 and MEK2	Pimasertib (AS703026)	Metastatic solid tumors or locally advanced	1	This study has been completed	Merck KGaA (NCT01713036)

Target	Drug	Cancer type	Phase	Interpretation	Sponsor
MEK1 and MEK2	AZD8330	Patients with advanced malignancies with no described treatment	1	This study has been completed. No results published yet.	AstraZeneca (NCT00454090)
ERK	MK-8353/SCH900353	Metastatic melanoma or metastatic corectal cancer	1	This study has been completed. No results published yet	Merck Sharp & Dohme Corp. (NCT01358331)
ERK1/2	RG7842 (GDC0994)	Solid tumors	1	Study is ongoing	Genentech (NCT01875705)
ERK	CC-90003	Metastatic solid tumors or locally advanced	1	This study has been completed. No results published yet	Celgene Corporation (NCT02313012)

[a]Patients with B-RAF^{V600E} mutation-positive have been screened for eligibility

feedback loops, is to avoid ERK1/2 nuclear translocation [22]. This may offer a double advantage in reducing the activation of ERK substrates and at the same time maintaining ERK cytosolic effect like negative feedback loops.

The association of compounds acting at different levels on a same pathway has been tested as shown in the Table 2. The drug association was expected to present a superior efficiency than a single compound but, in long term treatment, resistance emerged either way. So, intermittent therapy has been considered as a strategy to delay the development of resistance mechanisms. Therefore, studies comparing intermittent dosing schedule and continuous treatment with patients treated with B-RAF and MEK inhibitors are ongoing. Other approach to delay resistance includes B-RAF and MEK targeted therapies in association with immunotherapy.

Resistance to MAPK Inhibitor Treatment

Resistance is still an important issue that restrains the long-term responsiveness of the majority of the patients to MAPK inhibitors. Different events may originate drug-resistance phenomenon like target mutation, intratumor heterogeneity, and crosstalk.

As many kinase inhibitors exert their apoptotic properties primarily by inhibiting a specific kinase, there is a strong selective pressure for cells to acquire resistance through mutations in the kinase gene that abolish drug binding. This is illustrated by resistance to the MEK inhibitor AZD6244 reported in melanoma patients that was associated with mutations in MEK1 [126]. MEK1 mutations have been identified to confer resistance to B-RAF inhibitor according to different studies [126, 127]. Recently, MEK2 mutations have also been involved in drug resistance [128–130]. The same occurred concerning ERK1 after long-term treatment with the ERK1/2 inhibitor SCH772984 [131]. Various strategies may be adopted to surmount the problem. One possibility is to develop kinase inhibitor that can tolerate mutation of one or two amino acids in the target. A second possibility is to target the kinase with an association of inhibitors that bind at alternative binding sites. Besides mutation, other change in drug target may occur like its expression that may be increased as exemplified in the study by Wang et al. which described an increase of K-RAS and MEK after long-term treatment with the MEK inhibitor CI-1040 [132] indicating that the inefficiency of a drug may be related to the overexpression of its target.

Resistance may also lead to the upregulation of alternative pathway. In the case of B-RAF inhibitor after long-term treatment, the acquired resistance has been associated with pathway switching, where MAPK signaling is forwarded from B-RAF to C-RAF [133]. In addition to MAPK pathway activation, resistant tumors often show the activation of PI3K/AKT/mTOR pathway [134, 135], this may explain why PI3K inhibitors was classically associated with MAPK inhibitors or other conventional drugs [136].

Surprisingly, some kinase inhibitors have presented paradoxical effects. This is the case of RAF inhibitors that has been reported to increase RAF activities. In the first study describing such paradoxical effect of RAF inhibitor, the authors concluded that RAF kinases suppress their own activation by engaging feedback loops

in a MEK-dependent manner [137]. Thereafter, the increased RAF activity in the presence of RAF inhibitor in tumors with wild-type RAF isoforms has been explained by RAF dimerization, membrane localization and increased interaction of RAF with G-RAS [116]. However, it appears that the next generation of RAF inhibitors might evade paradoxical MAPK pathway activation [138]. In the same line, the paradoxical effect of ERK pathway inhibitors observed might be due to the existence of ERK negative feedback loop. The inhibition of ERK may cause the relief of ERK-dependent negative feedback, that may also provoke tumor resistance due to the reactivation of ERK pathway [35, 37].

Crosstalk between Signaling Pathways

Crosstalk between signaling pathways is a common event in cell regulation, which generally depends on cell context and plays a major role in the regulation of biological responses. This may be one of the greatest challenges of using MAPK inhibitors in cancer therapy, once crosstalk between different pathways may implicate an effect that, sometimes, cannot be predicted. A recent study published by Huth et al., described a new connection between MEK2 and p38, suggesting a crosstalk between ERK and p38 pathway [44]. Such phenomenon reveals a greater complexity of the ERK signaling cascade and may explain why ERK inhibition may not be an efficient strategy in all the cases. This illustrates the necessity to develop inhibitors of other MAPK pathways in cancer. In a similar way, Shimo et al. have also described a crosstalk between ERK and p38 pathway, wherein MEK1 inhibition by using PD98059 induced an upregulation of p38 activation [139]. Another interconnection between p38 and JNK pathways is often described due to the fact that both are stress pathways and share upstream activators [140]. Furthermore, crosstalk exists between MAPK and PI3K signaling pathways as cited above [136]. More precisely, MEK downregulation induced by the inhibitor PD0325901 enhances PI3K signaling [141]. Hence, crosstalk could be one of the most common cellular event responsible for drug-resistance phenomenon in cancer therapy. In such context, the association of different MAPK and PI3K inhibitors has been suggested to overcome drug resistance. Indeed, a synergy between both classes of inhibitor has been reported in different studies [142–144]. This kind of association could usefully be extended to the combination of different MAPK pathway inhibitors.

Tumor Heterogeneity

Up to now, research in cancer considers tumors as single entities and underestimates molecular diversity among the various cell types. Indeed, although solid tumors appear from a unique neoplastic cell, consequent mutations occur over time increasing tumor heterogeneity and its resistance to conventional chemotherapeutic drugs [145]. Tumor heterogeneity refers to the existence of subpopulations of cells with

distinct genotypes and phenotypes that may harbor different biological behaviors, within a primary tumor and its metastasis, or between tumors of the same histopathological subtype (inter- and intratumor, respectively) [146, 147]. Even within a given tumor, individual cells can display substantial variations at the genetic [148, 149], epigenetic [150, 151] and phenotypic levels [152, 153]. This heterogeneity might be particularly beneficial for the tumor, when cancers are under selective pressures of chemotherapy, by the enrichment of drug resistant tumor cells [154, 155]. The mechanisms, aside from genetic mutations, that mediate phenotypic heterogeneity generation in driving cancer progression remain poorly understood. Indeed, Nguyen et al. have proposed that variability in subpopulation may be achieved at the transcriptional level, generating phenotype diversity within the same population [156]. Furthermore, heterogeneity phenomenon suggests that key signaling pathways may be used by the various cell subpopulations in different ways in order to regulate proliferation, migration and cytokine production. This is another argument in favor of the use of the association of drugs in cancer therapy.

Perspectives: Predicting the Effect of MAPK Inhibitors

It is relevant to mention that inhibitors developed against MAPK pathways have a narrow spectrum of activity. Noteworthy efficacy is observed in patients with N-RAS-mutant and B-RAF-mutant melanoma and other types of tumors which harbor similar mutations. Some clinical results have provided evidence of efficacy of MAPK pathway inhibitors in therapy. This is illustrated by the fact that to date more than ten allosteric MEK1/2 inhibitors have made their entry into various phases of clinical trials and one of them, trametinib, has been approved by the US FDA for the treatment of B-RAF$^{V600E/K}$ mutated melanoma either as monotherapy or in combination with the B-RAF inhibitor dabrafenib [157]. But, the involvement of MAPKs in cancer is ambiguous, provoking cell death or inducing cell proliferation, depending on cancer type. It is now more understandable that the role of MAPKs in oncology signaling is cell type dependent, tissue specific, isoform-specific, and dependent on the tumor stage, and that it might vary according to stress signals and microenvironment. Identification of new biomarkers will be helpful to distinguish and stratify patients and predict therapeutic responses associated with MAPK inhibitor therapy in different tumor types.

There is a variety of biomarkers, which can include proteins (e.g., an enzyme or receptor), nucleic acids (e.g., a microRNA or other non-coding RNA), antibodies, and peptides, or a group of alterations, such as gene expression, metabolomic, and proteomic signatures. B-RAF^{V600E} the most frequent (>90%) *B-RAF* gene mutation in melanoma [158] has been used for cancer diagnosis and development of therapeutic molecules. However, some controversial results have been encountered like the development of resistance or the activation of alternative pathways when cells are under pressure of B-RAF inhibitors treatment. The kinase signaling cascades involve a complex network of interconnected pathways; so, it appears necessary to

develop more sophisticated modeling to predict how these pathways are reprogrammed during the tumor progression and in the presence of inhibitors. In this chapter we have provided an overview of recently suggested concepts of resistance to MAPK pathway inhibitors in cancers with particular focus on intra and inter-individual as well as intra-tumor heterogeneity. In this way, Majumder et al. have implemented a platform that may capture the real heterogeneity of the tumor and the tumor microenvironment to validate the treatment predictions [159].

Keeping in view, the known variables that may interfere with tumor progression, drug association sounds a reasonable concept to limit the resistance development that may originate from cross-talk, heterogeneity, and compensatory pathway. The combination strategy may include the association between a conventional drug and MAPK inhibitor or between different MAPK inhibitors. In such context, effort should be devoted to the development of specific inhibitors of the other signaling pathways out of ERK pathway.

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4.2 - Paper 1

MEK2 controls the activation of MKK3/MKK6-p38 axis involved in the MDA-MB-231 breast cancer cell survival: Correlation with cyclin D1 expression.

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MEK2 controls the activation of MKK3/MKK6-p38 axis involved in the MDA-MB-231 breast cancer cell survival: Correlation with cyclin D1 expression

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ABSTRACT

The Ras-Raf-MEK-ERK1/2 signaling pathway regulates fundamental processes in malignant cells. However, the exact contributions of MEK1 and MEK2 to the development of cancer remain to be established. We studied the effects of MEK small-molecule inhibitors (PD98059 and U0126) and MEK1 and MEK2 knock-down on cell proliferation, apoptosis and MAPK activation. We showed a diminution of cell viability that was associated with a downregulation of cyclin D1 expression and an increase of apoptosis marker in MEK2 silenced cells; by contrast, a slight increase of cell survival was observed in the absence of MEK1 that correlated with an augment of cyclin D1 expression. These data indicate that MEK2 but not MEK1 is essential for MDA-MB-231 cell survival. Importantly, the role of MEK2 in cell survival appeared independent on ERK1/2 phosphorylation since its absence did not alter the level of activated ERK1/2. Indeed, we have reported an unrevealed link between MEK2 and MKK3/MKK6-p38 MAPK axis where MEK2 was essential for the phosphorylation of MKK3/MKK6 and p38 MAPK that directly impacted on cyclin D1 expression. Importantly, the MEK1 inhibitor PD98059, like MEK1 silencing, induced an augment of cyclin D1 expression that correlated with an increase of MDA-MB-231 cell proliferation suggesting that MEK1 may play a regulatory role in these cells. In sum, the crucial role of MEK2 in MDA-MB-231 cell viability and the unknown relationship between MEK2 and MKK3/MKK6-p38 axis here revealed may open new therapeutic strategies for aggressive breast cancer.

1. Introduction

Breast cancer is the most common cancer in women both in the developed and developing countries. One in 8 women will be diagnosed with breast cancer in their lifetime [1]. Despite the great advance in the treatment of cancer, many factors remain to be studied in order to better understand the key mechanisms used by the cancer cells to proliferate, migrate and invade other tissues.

Specific targeted-based therapies are widely considered to be the future of cancer treatment and much attention has been focused on developing inhibitors of mitogen-activated protein kinase (MAPKs). The MAPKs are serine/threonine protein kinases that participate in intracellular signaling during proliferation, differentiation, cellular stress responses, and apoptosis [2]. To date six distinct groups of MAPKs have been characterized in mammals; extracellular signal-regulated kinase (ERK)1/2, ERK3/4, ERK5, ERK7/8, Jun N-terminal kinase (JNK)1/2/3 and the p38 isoforms, p38α/β/γ/δ [2–5]. Deregulations of the MAPK cascades are often observed in cancer [6]. The ERK pathway plays dominant roles in regulating diverse cellular processes, including proliferation and survival, in breast cancer. More precisely, this pathway consisting of Raf, MEK1/2, and ERK1/2, regulates cell proliferation via its impact on cell cycle control [7]. For all the above reasons, the Raf-MEK-ERK pathway is a potential pathway to be targeted for therapeutic intervention. Recently, next-generation of Raf and MEK inhibitors entered clinical trials [8]. Although certain small molecule inhibitors of MEK1/2 have been tested as potential candidates for cancer therapy, the response rates are highly variable and the efficacy of these drugs is primarily limited by the development of resistance [9]. Understanding the failure in such treatments implies a better knowledge of the ERK pathway and more specifically of MEK1/2 function. Because of their strong homology [10] disconnecting the role of MEK1 and MEK2 appeared more complex and little is known about the functional specificity of these isoforms. As an example, MEK1 and ERK1 depletion causes a cell cycle arrest at the G2 phase in FT210 mouse cells whereas loss of

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MEK2 or ERK2 arrests the cycle at the G1 phase [11]. However, the functions of MEK1 and MEK2 might not be entirely redundant. MEK1 gene knock-out in mice is lethal due to failure of the placenta vascularization while MEK2 is not necessary for mouse embryo development [12,13]. By depleting MEK1 or MEK2, Ussar and Voss demonstrated that MEK isoforms have distinct ways to contribute to ERK activity and cell cycle progression [14].

Here, we aimed to define the functional specificities of MEK1 and MEK2 isoforms in the regulation of MDA-MB-231 aggressive breast tumor cell growth and survival. Testing different MEK inhibitors, we have shown a differential impact of these compounds on MDA-MB-231 cell proliferation and apoptosis. Furthermore, by using siRNA, we have demonstrated that MEK1 or MEK2 silencing impacted differentially on MDA-MB-231 cell biology. More precisely, it was observed a downregulation of cyclin D1 expression and a decrease of MDA-MB-231 cell viability after knock-down of MEK2. By contrast, an upregulation of cyclin D1 and an increased cell viability was noted in MEK1-knocked-down cells. Importantly, an unrevealed link between MEK2 and MKK3/MKK6-p38 axis has been established in MDA-MB-231 cells that impacted directly on cyclin D1 expression.

2. Results

2.1. Differential impact of PD98059 and U0126 on MDA-MB-231 and MCF-7 breast tumor cell survival

To determine the role of MEK in breast tumor cell proliferation, we used PD98059 and U0126, two specific inhibitors of MEK [15,16] and consequently of ERK pathway activation. Viability assay by using these inhibitors was performed on MDA-MB-231 aggressive breast tumor cell and, in parallel, similar experiments have been performed using MCF-7 non-aggressive breast tumor cells. As shown in Fig. 1A, an

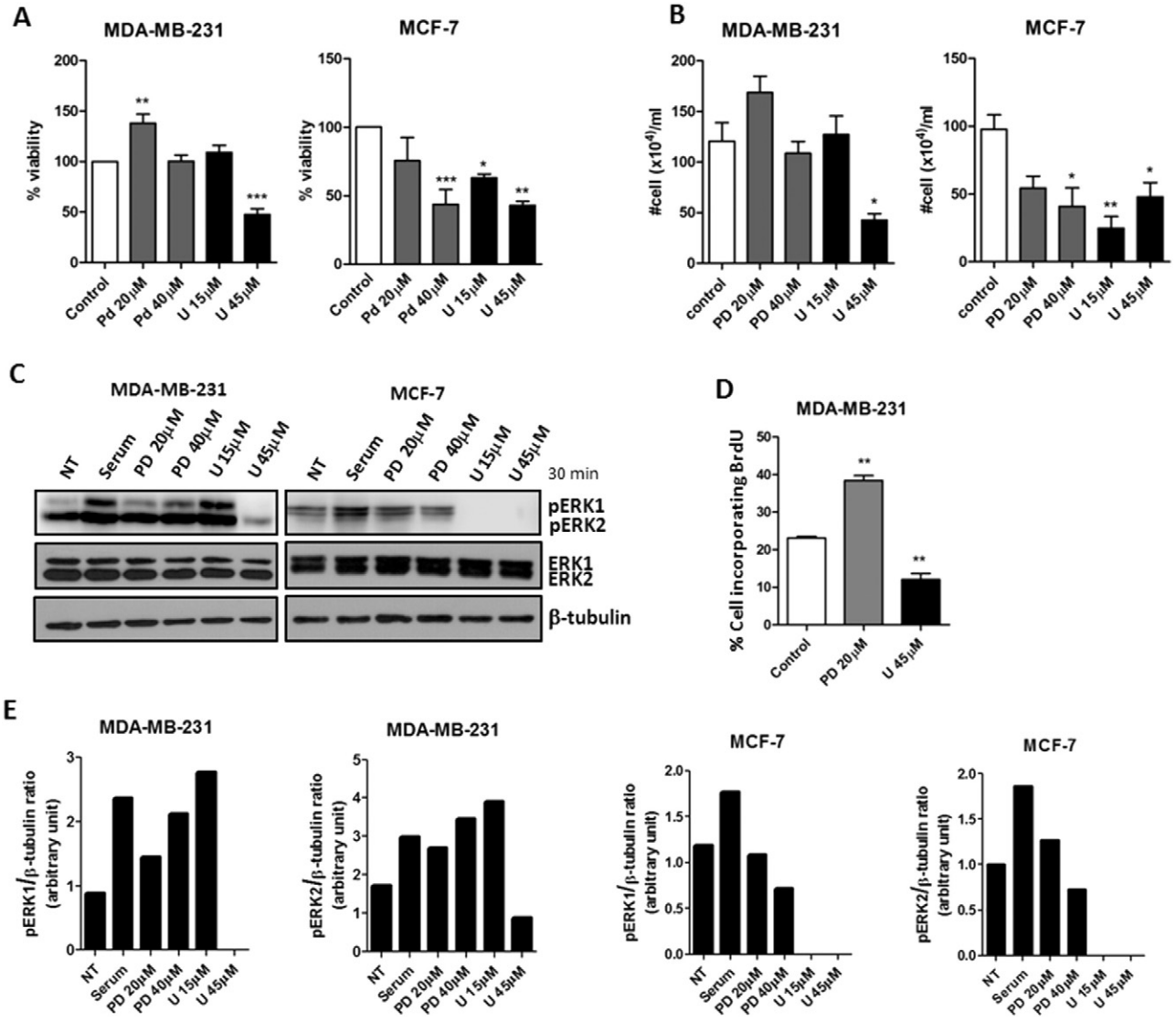

Fig. 1. ERK pathway inhibitors PD98059 and U0126 have different effects on MDA-MB-231 and MCF-7 breast cancer cell viability. Cell viability was measured by (A) MTT assay and (B) cell counting after incubation for 96 h with MEK inhibitors PD98059 (20 μM and 40 μM) or U0126 (15 μM and 45 μM). Control group represents cells incubated only with medium supplemented with 10% FBS. (C) To evaluate the phosphorylation of ERK1/2, cells were stimulated for 30 min with medium supplemented with 10% FBS in the presence or not of PD98059 or U0126. Cell lysates were immunoblotted with anti-phospho ERK1/2, total ERK and β-tubulin antibody. NT group represents cells non-stimulated with serum. (D) For the proliferative assay, MDA-MB-231 cells were incubated with medium supplemented with 10% FBS and pretreated with PD98059 or U0126 for 30 min before the addition of BrdU. Control group represents cells incubated only with medium supplemented with 10% FBS and BrdU. After 8 h of incubation cells were fixed, permeabilized, stained with anti-BrdU and analyzed by FACS. The graphic shows the percentage of $BrdU^+$ cells after treatment (average ± SD). The MTT assay and cell counting were performed three times and western-blotting and FACS were performed twice, in triplicate. (E) The level of phospho ERK1 and phospho ERK2 were quantified and normalized to the level of β-tubulin. $^*p < 0,05$; $^{**}p < 0,01$; $^{***}p < 0,001$ comparing to control group. Statistical analyses were performed using one-way ANOVA followed by Bonferroni post-test.

unexpected increase of viability was observed when MDA-MB-231 cells were treated with PD98059 at 20 μM. This phenomenon was not observed in MCF-7 cells. In fact, PD98059 at 20 or 40 μM reduced the viability of MCF-7 cells (Fig. 1A). Concerning the second MEK inhibitor used, U0126, a decrease of viability was observed at concentration of 45 μM in both cell lines.

As MTT assay measures viable cell metabolism and not specifically cell proliferation, we have confirmed MTT assay data by cell counting (Fig. 1B). As expected, PD98059 and U0126 impacted differentially on MDA-MB-231 cell number after 96 h of incubation. An increase of cell number was noted in the presence of PD98059 at 20 μM while U0126 induced a reduction of cell number when compared with untreated cells. Concerning MCF-7 cells, both MEK inhibitors induced a reduction of cell number.

Considering that ERK is recognized as the only physiologic substrate of MEK up to now, we evaluated the effect of MEK inhibitors, PD98059 and U0126, on the phosphorylation of ERK1/2. According to our data, PD98059 was able to reduce the phosphorylation of ERK1 without altering significantly the phosphorylation of ERK2 in MDA-MB-231 cells (Fig. 1C). By contrast, U0126 diminished dramatically phosphorylation of both isoforms in these cells at 45 μM. PD98059 and U0126 reduced the phosphorylation of ERK1 and ERK2 in MCF-7 cells being that U0126 effect was more pronounced (Fig. 1C). This constitutes a relevant observation showing that the same inhibitor may affect differentially the signaling pathway engaged depending on the cell line.

The discrepancies between U0126 and PD98059 effect on MDA-MB-231 cells were unexpected and need to be better characterized. To evaluate whether PD98059 and U0126 effects were due to an impact on proliferative activity in MDA-MB-231 cells, we used BrdU assay that can discriminate proliferating cells from arrested cells. PD98059 was able to significantly increase the number of cells incorporating BrdU indicating that PD98059 treatment augmented the DNA replicative activity in these cells while U0126 disrupted the cell ability to incorporate this nucleotide analogue as shown in Fig. 1D. These results were consistent with the MTT assay and cell counting.

2.2. Effect of MEK1 and MEK2 knock-down on MDA-MB-231 cell proliferation

The fact that PD98059 and U0126 have distinct binding sites on MEK and exhibit different mechanisms of action, might explain the differential impact of these inhibitors on MDA-MB-231 viability. Specifically, PD98059 inhibits the activation of MEK by upstream activators [17] while U0126 inhibits the activated phospho-MEK1/2 to phosphorylate downstream ERK1/2 [18]. Furthermore, PD98059 may possess a greater affinity for MEK1 [15] suggesting that the use of PD98059 may interfere preferentially with MEK1 activation while U0126 is able to affect the activity of both isoforms [16].

In this context, it appeared relevant to explore separately the function of MEK1 and MEK2 in MDA-MB-231 cells. For this purpose, MEK1 and MEK2 were knocked-down in MDA-MB-231 cells by siRNA. At first, transfection conditions have been optimized (Fig. 2A). In the selected conditions (4.5 μl of HiPerfect) as described in the Materials and methods section, transfection of cells with MEK1 #2 (named as MEK1 in the subsequent experiments) or MEK2 siRNA diminished the expression of its target kinase, as demonstrated by western blotting (Fig. 2A). The silencing of MEK1 did not affect the MEK2 expression and vice versa. When we measured the impact of the knock-down of MEK1 or MEK2 on MDA-MB-231 cell response, it has been shown that the silencing of MEK1 induced a slight increase of MDA-MB-231 cell viability while silencing MEK2 led to a significant reduction of viability. These opposite roles for MEK1 and MEK2 were confirmed by MDA-MB-231 cell counting (Fig. 2C). These data indicate that MEK1 and MEK2 did not have redundant role in MDA-MB-231 cells.

To verify whether the reduction of MEK2 silenced (si-MEK2) cell viability was linked to apoptosis, cells were stained against cleaved poly (ADP-ribose) polymerase (PARP) that has been served as one hallmark of apoptosis [19]. In parallel, H2AX in its phosphorylated form (Ser 139) has been used as marker of the DNA doubled stranded breaks, that may indicate DNA damage, to define the population of our interest after flow cytometry analysis. According to the data presented in Fig. 2D, the reduction of si-MEK2 cell proliferation involved apoptotic process since a significant increase of $\gamma H2AX^+$/cleaved $PARP^+$ population was noted within this cell population. The same was not observed during the silencing of MEK1. Furthermore, an increase of $\gamma H2AX^+$/cleaved $PARP^+$ population was noted in the presence of MEK1/2 inhibitor U0126 but not MEK1 inhibitor PD98059. These data support the involvement of MEK2 in survival of MDA-MB-231 cells.

2.3. Effect of MEK1 and MEK2 downregulation on MAPK activation in MDA-MB-231 cells

Given the requirement of MEK for ERK1/2 activation, we determined whether downregulation of MEK1 or MEK2 would affect phosphorylation of ERK1/2. As shown in Fig. 3A, the MEK1 silencing was able to affect the ERK1 phosphorylation, and to a lesser extent, the ERK2 phosphorylation in response to serum. Indeed, the determinant role of MEK1 in serum-induced ERK1/2 phosphorylation has been previously reported in LS174T colon carcinoma cells by Shama et al. [20]. By contrast, MEK2 alone did not seem to be required for the serum-induced activation of ERK1/2, since the ERK phosphorylation did not differ between si-MEK2 cells or its control group. As shown in Fig. 3B, it was possible to establish a parallel between the effect of PD98059 and MEK1 knock-down since in both conditions the reduction of ERK1 phosphorylation correlated with an increase of cell proliferation. While U0126 treatment and MEK2 silencing induced a reduction of cell viability, the impact of both treatments on ERK1/2 phosphorylation was different, suggesting that ERK would not be involved in cell death. This led us to explore the activity of another MAPK in si-MEK cells.

The involvement of p38 MAPK has been previously discussed in cancer without real consensus but, a correlation has been established between high levels of p38 and highly invasive breast tumor and poor prognostic [21,22]. In this context, we have evaluated a possible cross-talk between MEK2 involved in MDA-MB-231 cell survival and p38 activation. We assessed the p38 phosphorylation kinetic in the different conditions that included the silencing of MEK and the use of MEK inhibitors. As observed in Fig. 3A, MEK2 knock-down led to a dramatic reduction of p38 phosphorylation. According to the data presented in Fig. 3C, a canonical p38 pathway activation by MKK3/MKK6 phosphorylation was detected in MDA-MB-231 cells. This led us to investigate the relationship between MEK2 and MKK3/MKK6. As shown in Fig. 3A, MEK2 silencing reduced significantly the MKK3/MKK6 phosphorylation like the p38 activation after serum stimulation. Importantly, it was confirmed that this reduction of p38 phosphorylation observed in si-MEK2 cells was not due to a reduction of p38 protein levels as shown in Fig. 3A. This allowed to establish a link between the diminution of p38 activation and the reduction of MKK3/MKK6 phosphorylation observed in si-MEK2 cells. All together these data strongly indicate that MKK3/MKK6 remained responsible of the phosphorylation of p38 and that these events are under the control of MEK2 [23,24]. Furthermore, as shown in Fig. 3B, U0126 was also able to significantly inhibit MKK3/MKK6 and p38 phosphorylation that is in agreement with the fact that the knock-down of MEK2 impacted on the activation of both kinases. By contrast, in si-MEK1 cells, a significant p38 phosphorylation was detected after 15 min of serum incubation followed by a rapid dephosphorylation. This phosphorylation pattern was also observed concerning MKK3/MKK6. The same was noted in the presence of PD98059 (Fig. 3B). So, this led to conclude that a change in the kinetic of p38 pathway activation was induced by the absence of MEK1. In sum, these data indicate that MEK2 plays a crucial role in MKK3/MKK6-p38 axis activation in MDA-MB-231 cells. In addition, these

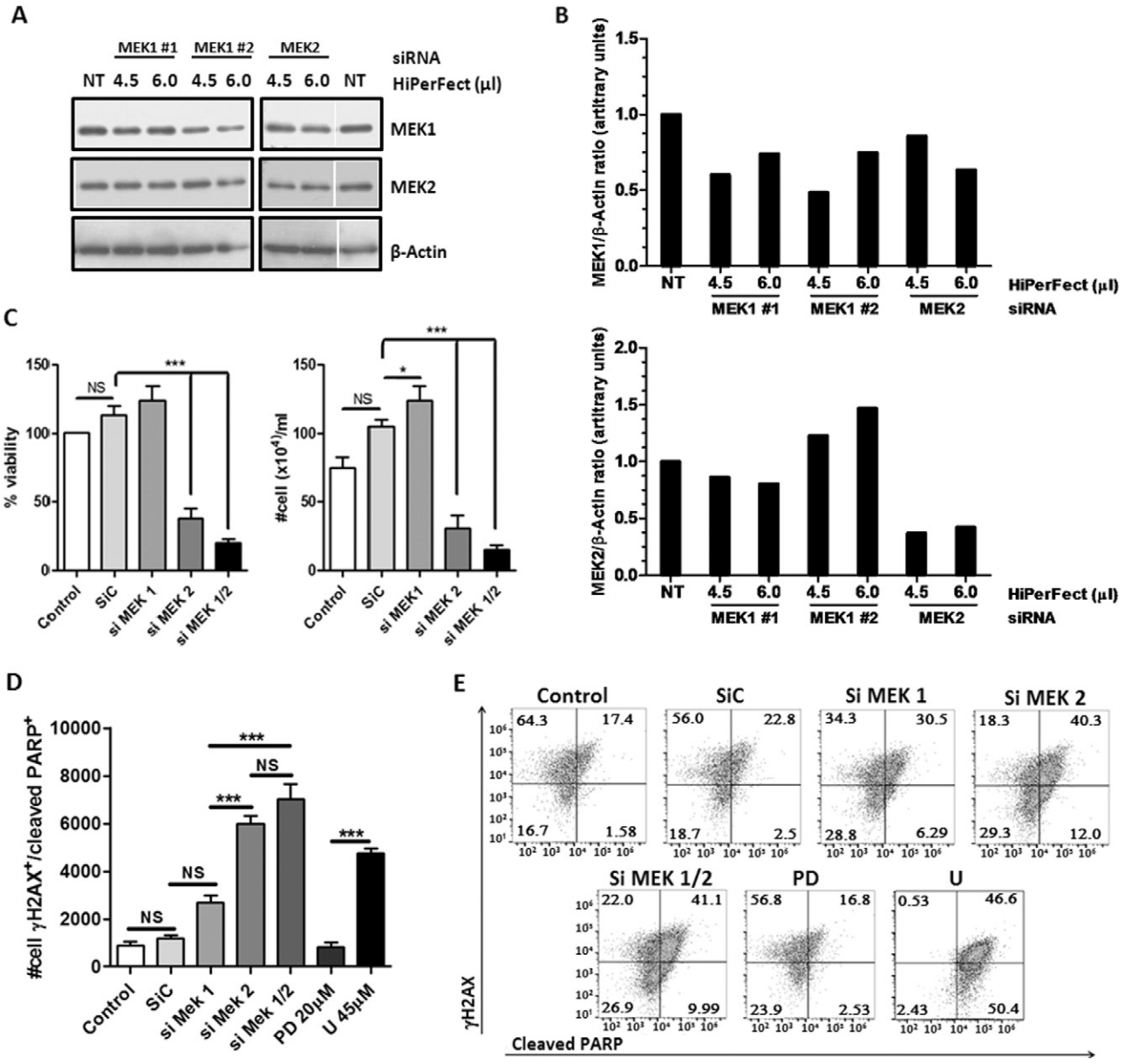

Fig. 2. The presence of MEK2 is essential for MDA-MB-231 survival. (A) MEK-siRNA (MEK1 #1 and MEK1 #2) and MEK2-siRNA were tested at the concentration of 100 nM. The silencing by siRNA was confirmed by evaluating the expression of both isoforms of MEK by western blotting. MDA-MB-231 cells were incubated with siRNA for 48 h in different conditions (HiPerFect 4.5 or 6 µl/well), then cell lysates were immunoblotted with anti-MEK1 or anti-MEK2 antibodies. NT group represents MDA-MB-231 cells treated with medium supplemented with 10% FBS. (B) The level of MEK1 and MEK2 after silencing were quantified and normalized to the level of β-actin. (C) Cells were submitted to MTT assay and counting after 96 h of incubation with medium supplemented with 10% FBS. (D, E) Cell death was measured by cleaved PARP level. MEK1- and MEK2-knocked-down cells were incubated with medium supplemented with 10% FBS for 8 h. In parallel, MDA-MB-231 cells were treated with PD98059 (20 µM) or U0126 (45 µM) for 8 h. Then, cells were stained with fluorescent antibodies anti-cleaved PARP and anti-γH2AX and analyzed by FACS. The graphic (D) presents the number of cleaved $\gamma H2AX^+/PARP^+$ cells in the different experimental group and the representative flow cytometry plots (E) shows the percent of cleaved $\gamma H2AX^+/PARP^+$ cells within the total population. SiC represents the group that received a non-specific siRNA sequence. Control group represents cells incubated only with medium supplemented with 10% FBS. These data are representative of two independent experiments in triplicate. NS = non-significant; $^*p < 0,05$; $^{**}p < 0.01$; $^{***}p < 0.001$. Statistical analyses were performed using one-way ANOVA followed by Bonferroni post-test.

results allow to reaffirm that MEK1 and MEK2 do not play a redundant role in MDA-MB-231 cells.

2.4. Silencing MEK1 or MEK2 differentially affects cyclin D1 expression

The cyclin-dependent kinases (CDKs) are a family of serine/threonine kinases controlling progression through the cell cycle [25]. Cyclin D1 is one of the cyclins that controls CDKs and it is considered as a driver of proliferation and it is overexpressed in about 50% of breast cancer [26,27]. The importance of cyclin D1 in cancer makes it an attractive target for anti-cancer therapy and several conventional and experimental anti-cancer agents have been observed to induce cyclin D1 degradation in a wide range of cancer cell lines [28–30]. Here, we have compared the expression of this protein in si-MEK1 and si-MEK2 cells under normal growth culture for 6 and 24 h. As presented in Fig. 4A, a significant reduction of the expression of cyclin D1 was observed after downregulation of MEK2. Furthermore, the reduction of cyclin D1 expression was noted in si-MEK1/2 cells, confirming that MEK2 is important for the expression of this protein. Interestingly, in the absence of MEK1 an upregulation of cyclin D1 expression was observed after serum treatment which corroborates with the increased proliferation observed at the same condition. In parallel, the influence of the two MEK inhibitors

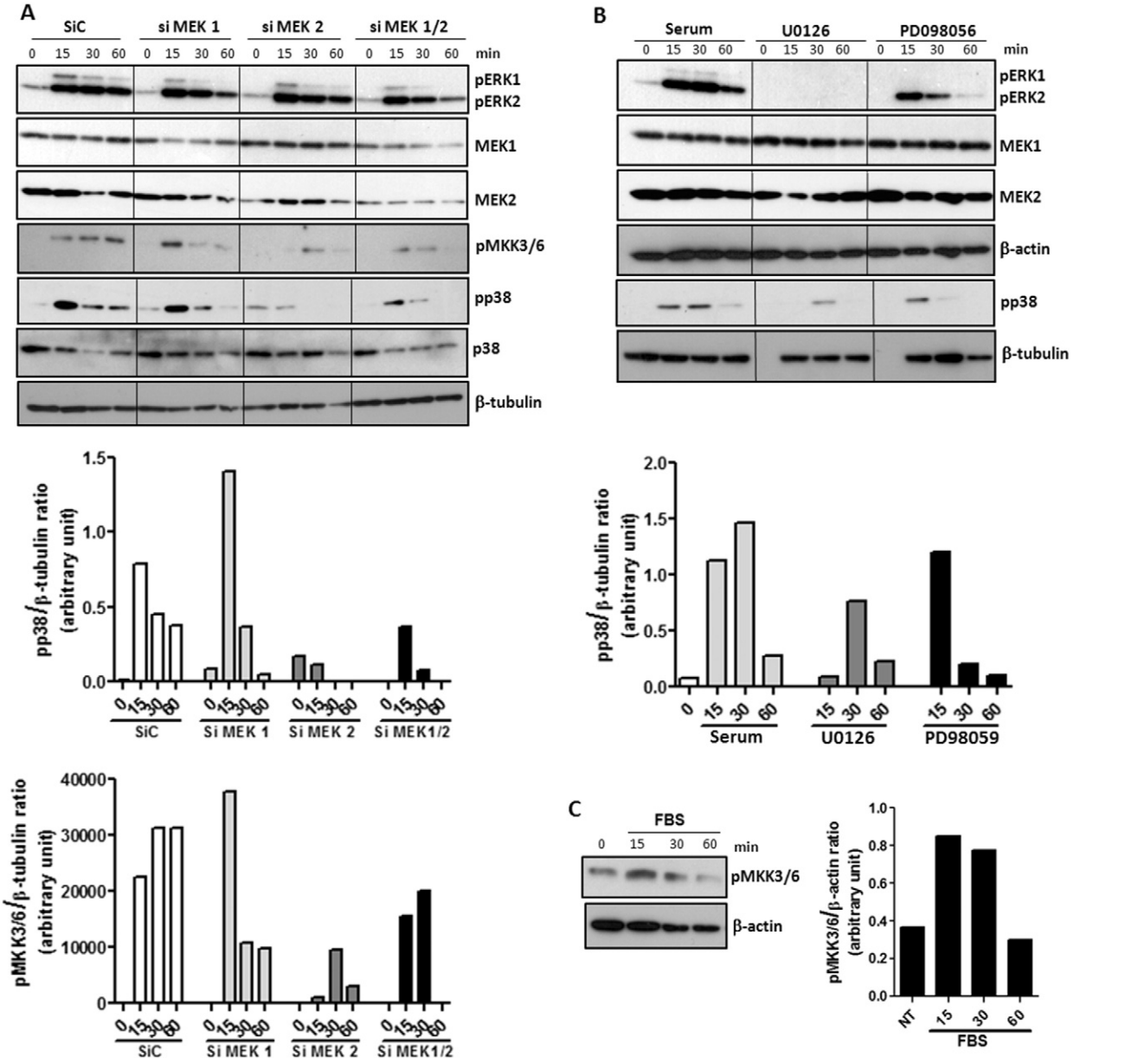

Fig. 3. MEK2 knock-down results in decreased p38 activation but does not affect ERK1/2 phosphorylation. To evaluate the kinetic of ERK1/2, MKK3/MKK6 (MKK3/6) and p38 phosphorylation induced by serum, cells were previously serum-starved for 2 h. Then, (A) silenced MDA-MB-231 cells, (B) MDA-MB-231 cells pre-treated or not with PD98059 (20 μM) or U0126 (45 μM) for 30 min and (C) MDA-MB-231 cells were stimulated for 15, 30 and 60 min with medium supplemented with 10% FBS. Then, cells were harvested and cell lysates were immunoblotted with the indicated antibodies. The graphics represent the level of phospho MKK3/MKK6 and phospho p38 quantified and normalized to the level of β-tubulin or β-actin.

(PD98059 and U0126) on cyclin D1 expression was evaluated. As noted in Fig. 4B, the MEK1/2 inhibitor downregulated cyclin D1 level, that contrasted with the elevated expression level of this protein detected in cells treated with PD98059. These later data are in accord with the results presented in Fig. 4A indicating that MEK2 plays a crucial role in cyclin D1 expression.

Considering that we have shown a relationship between MEK2 and p38 phosphorylation, and MEK2 and cyclin D1 expression as well, we sought to establish a direct connection between p38 activity and cyclin D1 expression in MDA-MB-231 cells. When SB203580, inhibitor of p38α/β isoforms [16] was used at concentration that reduced cell viability (Fig. 4D), a reduction of cyclin D1 expression was observed in cells incubated with serum (Fig. 4C).

Taken together, these results have shown a link between MEK2 activation, p38 phosphorylation and level of cyclin D1 expression that correlates with the capacity of MEK1/2 inhibitor U0126 or p38 inhibitor SB203580 to affect cell proliferation and viability.

3. Discussion

Despite the prevalence of aberrant Ras/Raf/MEK/ERK signaling in human cancers, MEK inhibitors have not shown strong activity in early stage of clinical trials [31,32]. Indeed, the role played by MEK1/2 in this pathway has not been completely defined. It has been suggested that MEK1 and MEK2 may not have fully redundant functions [12,13,33], that may explain the high variability and poor efficiency of response rates to MEK inhibitors.

Here, we sought to define the role of MEK1 and MEK2 separately in the proliferation and survival of MDA-MB-231 breast cancer cells. For this purpose, we knocked-down MEK1 and MEK2 expression to

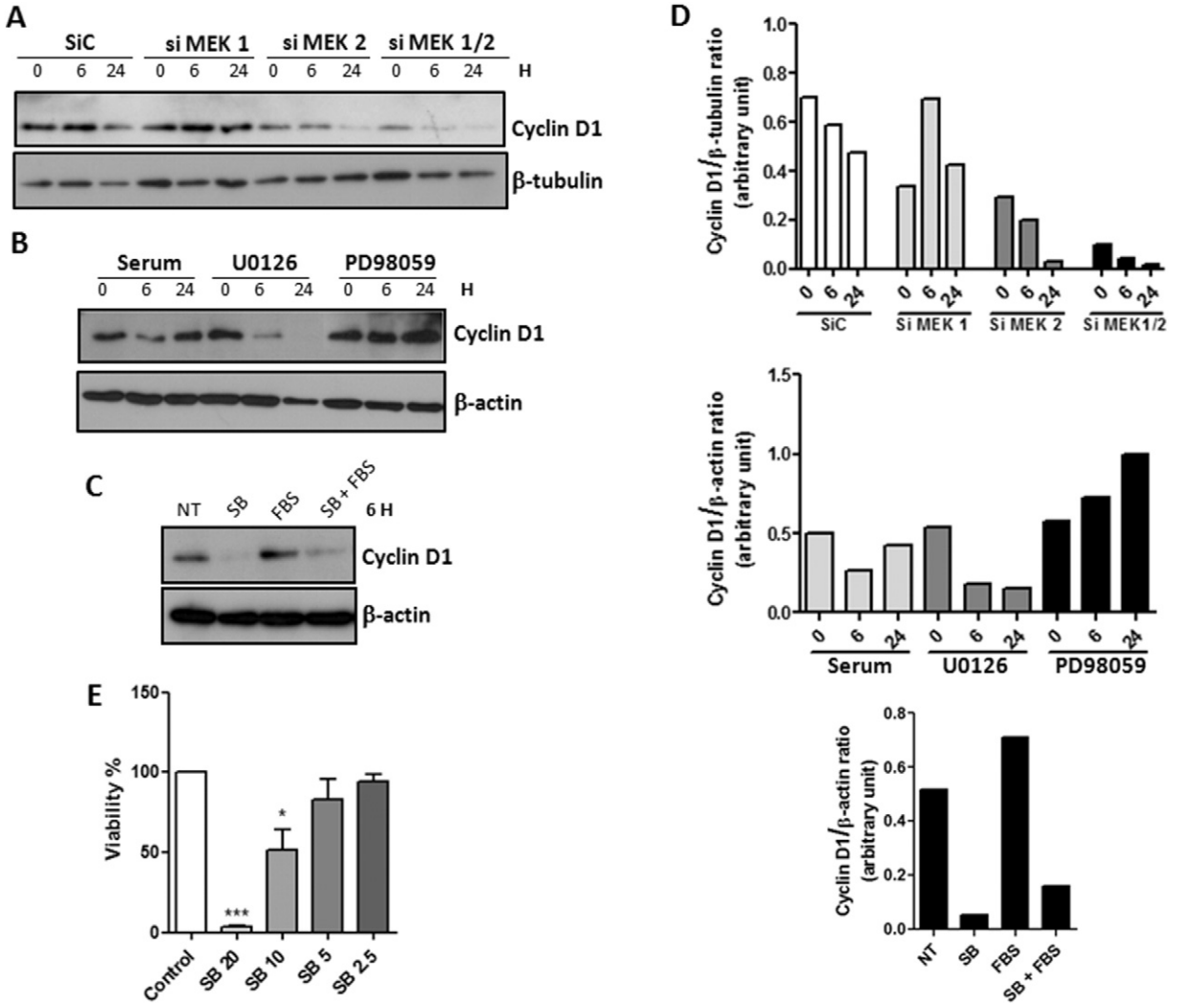

Fig. 4. MEK2 knock-down decreases cyclin D1 expression. To evaluate the kinetic of cyclin D1 expression induced by serum, cells were previously serum-starved for 2 h. Then, (A) silenced MDA-MB-231 cells, (B) MDA-MB-231 cells pre-treated or not with PD98059 (20 μM) or U0126 (45 μM) for 30 min were stimulated for 6 and 24 h with medium supplemented with 10% FBS. (C) MDA-MB-231 cells pre-treated or not with SB203580 (SB) at 10 μM for 30 min were stimulated for 6 h with medium supplemented with 10% FBS. Then, cells were harvested and cell lysates were immunoblotted with the indicated antibodies. (D) The graphics represent the level of phospho MKK3/MKK6 (pMKK3/6) and phospho p38 quantified and normalized to the level of β-tubulin or β-actin. (E) MDA-MB-231 cells were treated with different concentrations (μM) of SB203580 (SB) or not (non-treated (NT)) as indicated and after 96 h MTT assay was performed. The experiments were performed at least twice in triplicate. *$p < 0,05$; **$p < 0,01$; ***$p < 0,001$ comparing to control group. Statistical analyses were performed using one-way ANOVA followed by Bonferroni post-test.

examine their functions in MDA-MB-231 aggressive breast cancer cells. The results showed that MEK2 but not MEK1 was effective in inducing cell proliferation and impeding cell death. We correlated the pro-survival role of MEK2 with the increased expression level of cyclin D1 that is a key regulator of cell proliferation [34,35] and has been recently described as cytoprotective against cell death [36,37]; the increased number of $\gamma H2AX^+$/cleaved $PARP^+$ cells, a hallmark of apoptosis, was observed when cells were knocked-down for MEK2, indicating an antiapoptotic function for MEK2. An inverse correlation was demonstrated in si-MEK1 cells, where an increased cyclin D1 expression was associated with an augment of proliferative capacity. The fact that disabling MEK1 activity increased cyclin D1 expression and cell proliferation might mean that MEK1 controls MEK2 activation suggesting a regulatory role for MEK1 as proposed by Catalanotti et al. [38] in a mouse embryonic fibroblast model. This may explain why the MEK1 inhibitor PD98059 induced an increased cell proliferation at the lower concentration when the inhibitor effect was more specific and restricted to MEK1 activity, whereas at higher concentrations, PD98059 could affect MEK1 and MEK2 activity. According to our data, the role of MEK1 and MEK2 in MDA-MB-231 cells did not appear to be interchangeable in controlling different aspects of the cell fate. This is in agreement with other studies that have argued for a non-overlapping function of MEK1 and MEK2 function. The disconnected role of each kinase in the regulation of cell cycle and proliferation has been reported in HeLa cells [10] and colon cancer line [14].

The Raf/MEK/ERK may have different effects on growth and prevention of apoptosis in tumor cells of various lineages. Different reports have involved the ERK pathway in apoptosis induced by various antitumor compounds [39]. Our data may disagree with this statement since they indicate that ERK1/2 activity is not involved in the balance apoptosis/survival in MDA-MB-231 cells stimulated by serum. This is supported by the fact that MEK2 silencing induced cell death while having no impact by itself on phosphorylation of ERK1/2. Furthermore, the MEK1 inhibitor PD98059, like MEK1 silencing, induced a decreased ERK1/2 activation but led to an increase of cell proliferation. So, the level of ERK activation may not be considered as a reliable marker for monitoring the effectiveness of MEK inhibitors in some cell lines. Interestingly, Cagnol and Chambard have suggested that tumor cells with high ERK activity might also have re-modeled the ERK signaling to escape ERK-mediated cell death. Considering that MDA-MB-231 cells were

characterized by an ERK hyperactivity compared to others lineages [40], we have hypothesized that ERK remodeling may exist in MDA-MB-231 leading to the use of other pathway to control the balance proliferation/death. It is within this context that we have explored p38 pathway in si-MEK cells.

The effect of p38 is still heavily debated in cancer. Some studies have shown that p38 pathway functions as a tumor suppressor by regulating tumor cell proliferation whereas others have reported that p38 contributes to tumorigenesis [21,22]. In fact, we have defined in MDA-MB-231 cells that p38 is involved in cyclin D1 expression and cell survival by using SB203580 inhibitor of p38α/β isoforms activity. In addition, we were able to correlate the effect of the lack of MEK2 on cell survival and its role on p38 phosphorylation. To investigate the relationship between MEK2 and p38, the phosphorylation of MKK3/MKK6, the well-known upstream kinase of p38, was evaluated in silenced cells. Our data have revealed that MKK3/MKK6 and p38 activation were down-regulated in si-MEK2 cells indicating that MKK3/MKK6 remains responsible for p38 activation and that MEK2 controls the MKK3/MKK6-p38 axis activation in MDA-MB-231 cells. This cross-talk between ERK and p38 pathway has never been described until now. Such event reveals a greater complexity to the once simple linear Raf/MEK/ERK signaling cascade.

Cells treated with PD98059 showed a change in the phosphorylation kinetic of p38. The same is observed in a more pronounced way concerning si MEK1 cells. But, we cannot draw a parallel between this change in p38 phosphorylation kinetic, the cyclin D1 expression level and the increased cell proliferation observed in both conditions (MEK1 silencing and PD98059 use). More experiments will be necessary that concern the regulatory mechanisms of p38 phosphorylation and its impact on transcription factor activation.

Besides, our data directly call into question the real mechanism of action of kinase inhibitor. Indeed, the MEK1/2 inhibitor U0126 effect, that conducted to MDA-MB-231 cell death, is probably due to the impact on p38 rather than ERK1/2 activation. This opens up an important debate concerning the specific effect of signaling pathway inhibitor when cells use non-canonical pathway and constitutes a plea for a better knowledge of signaling pathway network before the use of such inhibitor in tumor cells.

An interesting finding of this study was the divergence of cellular response between the non-aggressive MCF-7 and aggressive MDA-MB-231 tumor cells to MEK inhibitors. PD98059, MEK1 inhibitor, induced an increased number of MDA-MB-231 cells but a diminution of MCF-7 cell population indicating a different role of MEK1 in MDA-MB-231 and MCF-7 cells that was confirmed when the silencing of MEK1 in MCF-7 induced a decrease of cell viability (data not shown). This reinforces the idea that the two cancer cell lines are behaving differentially with respect to the use of signaling pathways in order to proliferate.

In conclusion, we have shown that aggressive MDA-MB-231 tumor cells use a non-canonical connection between ERK and p38 pathway through MEK2 and that such pathway governs the expression of cyclin D1 protein and consequently cell survival. Identifying key signaling molecules like MEK2 involved in the cross talk between signaling pathways can be useful to understand the mechanisms that render tumor cells more aggressive and drug resistant.

4. Materials and methods

4.1. Reagents

DMEM and fetal bovine serum (GIBCO) were from Thermo Fisher Scientific (Waltham, MA, USA). Thiazolyl blue tetrazolium blue (MTT) and RIPA buffer were purchased from Sigma-Aldrich (St. Louis Co., MO, USA). MEK inhibitors PD98059, U0126 and p38 inhibitor SB203580 were obtained from InvivoGen (San Diego, CA, USA). Protease cocktail inhibitor and phosphatase cocktail inhibitor were acquired from Roche (Mannheim, Germany). Monoclonal antibodies against phospho-p44/42 (ERK1/2), phospho-p38 MAPK, p38 MAPK, cyclin D1, MEK1 and MEK2 isoforms, β-tubulin, β-actin and peroxidase-conjugated goat anti-mouse IgG and goat anti-rabbit IgG were obtained from Cell Signaling Technology (Danvers, MA). Apoptosis, DNA Damage and Cell Proliferation Kit was obtained from BD Biosciences (Franklin Lakes, NJ, USA). HiPerFect Transfection Reagent was purchased from Qiagen (Hilden, Germany). siRNAs were synthetized by Sigma-Aldrich: two MEK1 siRNAs were tested (MEK1 #1 siRNA: 5′ GCUUCUAUGGUGC GUUCUA 3′ and MEK1 #2 siRNA: 5′ GUUAGCAUUGCUGUAAUAA 3′) and a prevalidated sequence was used for silencing MEK2 (MEK2 siRNA: 5′ CUGCAAUGGCCAUCUUUGA 3′). For control an AllStars Negative Control siRNA from Qiagen was used. Immobilon-P membranes and Luminat were acquired through Millipore Merck.

4.2. Cell culture conditions

We obtained the breast tumor cell lines MDA-MB-231 and MCF-7 cells from ATCC (USA) (gift from Institute Curie - Paris - France). Cells were grown at 37 °C in a humidified atmosphere of 5% CO_2 in DMEM supplemented with 10% heat inactivated fetal bovine serum (FBS). For starvation conditions, cells were incubated with serum-free medium for 2 h before the western blotting assay.

4.3. MTT assay

Breast cancer cells (10^4/well) were plated in 24-well plates in DMEM with 10% serum. Cells were incubated for a further 96 h in the following conditions: medium alone, or with DMSO (0.1% v/v), or U0126 (15 μM and 45 μM), or PD98059 (20 μM and 40 μM), or SB203580 (10 μM). After 96 h, the medium was removed and cells were incubated with l00 μl/well of MTT at 5 mg/ml for 2 h at 37 °C. After removing the medium, 200 μl of DMSO were added to dissolve the crystals of formazan and the absorbance was measured in a microplate reader at a wavelength of 595 nm. A value of 100% was assigned to untreated control cultures. Results were derived from at least three independent sets of triplicate experiments.

4.4. Cell counting

Cells were seeded in a density of 1×10^4 cells/well in 24 wells plate. Cells were treated with PD98059 (20 μM and 40 μM) and U0126 (15 μM and 45 μM) in DMEM containing 10% FBS and incubated for 96 h. Then, cells were harvested, stained with Trypan Blue (GIBCO) and counted in Neubauer plate. The same was realized concerning the counting of silencing cells. Number of cells = media of three counting $\times 10^4$/ml.

4.5. RNA interference

MDA-MB-231 cells were seeded in 24-well plates at a density of 1×10^4 cells per well or in 6-well plates at a density of 5×10^4 cells per well. After 4 h of culture, the cells were transiently transfected with short interfering RNA (siRNA) specific for MEK1 (MEK1 #1 and MEK1 #2), MEK2, or a control siRNA at a final concentration of 100 nM by using HiPerFect Transfection Reagent according to manufacturer's instructions. Briefly, siRNA was diluted in serum-free OPTI MEM, mixed with 4.5 μl/well (24-well plates) or 18 μl/well (6-well plates) of HiPerFect Transfection Reagent by vortexing. Then, the mixture was incubated for 5–10 min at room temperature to allow the formation of transfection complexes and was added drop-wise onto the cells under their normal growth conditions and after 48 h used in the different assays. The best performance for silencing MEK1 was observed in the presence of the oligonucleotide MEK1 #2, named as MEK1 in the subsequent experiments.

4.6. Western blotting

Cells seeded in 6-well plates (10^6 cells/well), transfected with MEK1/2-specific and control siRNAs as described, were serum-starved for 2 h. Then, cells were treated or not with MAPK inhibitors (PD98059 20 μM, U0126 45 μM, SB203580 10 μM) for 30 min and stimulated with medium containing 10% FBS for the indicated time-points. Cells were harvested and lysed in RIPA buffer supplemented with phosphatase inhibitor and protease inhibitor cocktail according to the manufacturer's instructions. Protein lysates were separated by polyacrylamide gel electrophoresis on 12% gels, and electrotransferred to Immobilon-P membranes. Membranes were incubated with primary antibodies. After incubation with peroxidase-conjugated secondary antibody, protein expression was detected using Luminat reagent.

4.7. Measurement of cell proliferation and apoptosis with BrdU, γH2AX, and cleaved PARP

This assay was realized using the Apoptosis, DNA Damage, and Cell Proliferation Kit from BD Biosciences in accord with the manufacturer's instructions. For proliferation assay, MDA-MB-231 cells were pretreated for 30 min with MAPK inhibitors (PD98059 20 μM and U0126 45 μM) and incubated with BrdU (10 μM) for 8 h and cells were fixed and permeabilized. After several washing, cells were treated with DNase (30 μg/10^6 cells) for 1 h and stained with PerCP-Cy™5.5 anti-BrdU for 20 min at room temperature. For the apoptotic assay, MDA-MB-231 silenced cells or cells pre-treated for 30 min with MAPK inhibitors (PD98059 20 μM and U0126 45 μM) were fixed and stained with PE anti-cleaved PARP and Alexa647 anti-γH2AX for 20 min at room temperature after cell permeabilization. After washing, cells were suspended in staining buffer and analyzed by flow cytometry. The data were collected by the cell analyzing LSRFortessa (BD Biosciences – Immunocytometry Systems) using "BD FACSDiva™ Software" (BD Biosciences) and analyzed with "FlowJo (Tree Star) Software". For setting the gate for $BrdU^+$ cells, cells were incubated with rhTNF-α (20 pg/ml) that is a strong inducer of MDA-MB-231 cell proliferation at this concentration. For setting the gate of $\gamma H2AX^+$/cleaved-$PARP^+$ cells, cells were treated with U0126 at 45 μM that induced a high mortality of these cells after 24 h of incubation. The number of $H2AX^+$/cleaved PARP + cells in the different experimental groups presented in the graphic 2C represents the media of four measures realized by FACS and analyzed with FlowJo Cell Analysis Software. This number was derived from percent value defined by $H2AX^+$/cleaved PARP + gate (Fig. 2D) multiplied by the number of cells in the direct ancestor population calculated by FlowJo software.

4.8. Statistical analysis

Statistical analysis was performed using the GraphPad Prism 5 program (GraphPad Software, San Diego, CA, USA).

Disclosures

The authors declare to have no financial conflicts of interest.

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4.3 - Paper 2

Upregulation of p38 pathway accelerates proliferation and migration of MDA-MB-231 breast cancer cells

Upregulation of p38 pathway accelerates proliferation and migration of MDA-MB-231 breast cancer cells

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Abstract. Tumor cells capture the signaling pathways used by normal tissue to promote their own survival and dissemination and among them, the NF-κB and MAPK pathways (ERK, JNK and p38). MAPK activation has ambiguous effects on tumor cell fate depending on cell type, cancer stage and the engaged MAPK isoforms. A synthetic peptide named LyeTx II, derived from the venom of the Brazilian spider *Lycosa erythrognatha*, was capable of increasing MDA-MB-231 aggressive breast cancer cell proliferation as indicated by MTT and BrdU (5-bromo-2'-deoxyuridine) incorporation assay and cell migration. A correlation has been established between the accelerated proliferation and migration observed in the presence of LyeTx II and the upregulation of p38 MAPK phosphorylation. The use of the selective inhibitor of p38α/β (SB203580) abrogated the peptide effect in MDA-MB-231 cells. Besides, an augment of the canonical NF-κB pathway activation considered as crucial in cancer progression was noted after cell incubation with LyeTx II. Importantly, activation of p38 and NF-κB pathways was dependent on TAK1 activity. Together, these data suggest that TAK1-p38 pathway may represent an interesting target for treatment of aggressive breast cancers.

Introduction

Cancer is the second most common cause of death worldwide, preceded only by heart diseases (1). Breast cancer is one of the most common in women, with high morbidity. Generally, treatment occurs via surgery, chemotherapy, radiotherapy and immunotherapy. However, despite the various therapeutic strategies that can be adopted, the mortality rate and the side effects of the treatment remain a challenge (2,3). Therefore, the search for new alternative treatments with less side effects is under investigation.

In such context, there is great interest in the pharmaceutical industry to target pathways that regulate cell proliferation and apoptosis in cancer. These pathways are initiated from various cell surface receptors, and may converge on the MAPK cascade, a module consisting of MAP kinase kinase (MEK) and MAPK (4-6). The MAPKs are serine/threonine protein kinases that participate in different intracellular processes such as proliferation, differentiation, cellular stress responses, and apoptosis (7,8). ERK1/2, JNK1/2/3 and p38α/β/γ/δ constitute the main mammalian MAPKs studied in cancer area. ERK pathway is activated by mitogen factors, and this pathway is one of the most mutated in cancer often leading to an increase of cell proliferation and generally a decrease of apoptosis (9). On the other hand, p38 and JNK pathways are activated by stress factors, but their roles in cancer remain unclear, depending on cancer stage, cell type or MAPK isoforms (10-13).

Another pathway that called attention and has also been described as crucial in cancer progression is the NF-κB pathway (14,15). The transcription factor NF-κB controls many intracellular signals including cell cycle (e.g. cyclin D1), suppression of apoptosis (e.g. Bcl-2 and Bcl-xL) and inflammation (e.g. IL-6) (16). In response to a wide variety of stimuli, NF-κB becomes active via canonical or non-canonical pathways in cancer. Generally, in the canonical pathway NF-κB activation is preceded by the phosphorylation of IKK-αβ and IκBα (17,18).

Nowadays, the major interest is to identify relevant molecular targets that may offer a specific therapeutic alternative treatment to cancer. In this context, MAPKs and NF-κB pathways represent an interesting signaling network for investigation. In the present study, we used a peptide (named LyeTx II) derived from the venom of the spider *Lycosa erythrognatha*, that induced an exacerbated proliferation of MDA-MB-231 breast cancer cells, to evaluate the importance of the MAPKs and NF-κB pathways in proliferation and migration of this breast cancer cell line considered as aggressive. According to our data, the proliferative and migratory

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Abbreviations: MAPK, mitogen-activated protein kinase

Key words: breast cancer, MDA-MB-231, MAPK, p38, proliferation, migration

effect induced by the peptide occurred mainly through upregulation of p38 pathway activation, without involvement of ERKs and JNKs. This study provides new insights into the role of p38 in aggressive breast tumor indicating that p38 may be a suitable target in this cancer type.

Materials and methods

Reagents. DMEM and fetal bovine serum (FBS) (Gibco) were from Thermo Fisher Scientific (Waltham, MA, USA). Thiazolyl Blue Tetrazolium Blue (MTT) and RIPA buffer were purchased from Sigma-Aldrich (St. Louis, MO, USA). p38 inhibitor SB203580 were obtained from InvivoGen (San Diego, CA, USA). Protease cocktail inhibitor and phosphatase cocktail inhibitor were acquired from Roche (Mannheim, Germany). Monoclonal antibodies against phospho-p44/42 (ERK1/2), phospho-p38 MAPK, phospho-JNK MAPK, β-actin and peroxidase-conjugated goat anti-mouse IgG and goat anti-rabbit IgG were obtained from Cell Signaling Technology (Danvers, MA, USA). Apoptosis, DNA Damage and Cell Proliferation Kit were obtained from BD Biosciences (Franklin Lakes, NJ, USA). Immobilon-P membranes and Luminat were acquired through Merck Millipore (Darmstadt, Germany).

Peptide information, synthesis and purification. LyeTx II is a 19 amino acid peptide, which includes four lysine residues conferring positive charge to it. LyeTx II synthesis and purification was performed at Professor J.M. Resende's Laboratory, from the Chemistry Department of UFMG, Brazil, according to methodology described by Santos *et al* (19). The purity of LyeTx II was checked by mass spectometry.

Cell culture conditions. The breast tumor cell lines MDA-MB-231 and MCF-7 were gifts from the Laboratory of Prof. A.M. Goes. MACL-1 and MGSO-3 cell lines were derived from primary tumor samples in the same laboratory (20). Cells were grown at 37˚C in a humidified atmosphere of 5% CO_2 in DMEM supplemented with 10% heat inactivated FBS. For starvation conditions, cells were incubated with serum-free medium for 2 h before the western blot assay.

MTT assay. Breast cancer cells (10^4/well) were plated in 24-well plates in DMEM with 10% serum. Cells were incubated for a further 96 h in the following conditions: medium alone, LyeTx II (from 0.1 to 100 nM), SB203580 (10 μM). After 96 h, the medium was removed and cells were incubated with 100 μl/well of MTT at 5 mg/ml for 2 h, at 37˚C. After removing the medium, 200 μl of DMSO was added to dissolve the formazan crystals and the absorbance was measured in a microplate reader at a wavelength of 595 nm. A value of 100% was assigned to untreated control cultures. Results were derived from at least three independent sets of triplicate experiments.

Western blotting. Cells seeded in 6-well plates (10^6 cells/well) were serum-starved for 2 h. Then, cells were treated or not with LyeTx II (100 nM) in DMEM containing 10% FBS for the indicated time-points. 5z-7-oxozeaenol was added 30 min before the addition of LyeTx II. Cells were harvested and lysed in RIPA buffer supplemented with phosphatase inhibitor and protease inhibitor cocktail according to the manufacturer's instructions. Protein lysates were separated by polyacrylamide gel electrophoresis on 12% gels, and electrotransferred to Immobilon-P membranes. Membranes were incubated with primary antibodies. After incubation with peroxidase-conjugated secondary antibody, protein expression was detected using Luminat reagent.

Measurement of cell proliferation by BrdU incorporation. This assay was realized using the Apoptosis, DNA Damage, and Cell Proliferation kit from BD Biosciences in accord with the manufacturer's instructions. Briefly, cells were treated or not with LyeTx II (100 nM) in DMEM containing 10% FBS and incubated with BrdU (10 μM) for 8 h. When used, SB203580 was added 30 min before the addition of LyeTx II. After labeling, cells were fixed and permeabilized. After several washing, cells were treated with DNase (30 μg/10^6 cell) for 1 h. Following this treatment, cells were simultaneously stained with PerCP-Cy™5.5 anti-BrdU and Alexa-647 anti-γH2AX for 20 min at room temperature. After washing, cells were suspended in staining buffer and analyzed by flow cytometry. The data were collected by the cell analyzing LSRFortessa (BD Biosciences - Immunocytometry Systems) using BD FACSDiva™ software (BD Biosciences) and analyzed with FlowJo (Tree Star) software.

Cell counting. Cells were seeded in a density of $1x10^4$ cells/well in 24-well plates. Cells were treated with LyeTx II (100 nM) in DMEM containing 10% FBS and incubated for 24, 48, 72 and 96 h. In each time, cells were harvested, stained with Tripan Blue (Gibco) and then counted in Neubauer plate. Number of cells = media of three counting $x10^4$/ml.

Transwell assay. The 24-well Boyden chamber with 8 μm pore size polycarbonate membrane Millipore (Millicell Hanging Cell Culture Inserts, Millipore, EUA) was used to analyze cell motility. For invasion assay, the membrane was pre-coated for 1 h with 300 μl of free serum DMEM. Cells (10^6)were seeded on the upper chamber with serum-free medium with or without LyeTx II (100 nM). When used, SB203580 (10 μM) was added 30 min before LyeTx II addition also in the upper chamber. Medium 700 μl (or until the volume reaches the upper chamber membrane) with 10% serum was added to the lower chamber as a chemoattractant. After 24 h of incubation, the non-motile cells at the top of the membranes were removed with cotton swabs, then the membranes were fixed and stained with 0.5% crystal violet (Sigma). Five visual fields of x200 magnification of each membrane were randomly selected and counted.

Results

Peptide LyeTx II enhances MDA-MB-231 cell population growth. Without initial information about LyeTx II activity, we predicted that this peptide, derived from the venom of the Brazilian spider *Lycosa erythrognatha*, could interact with the anionic membrane of the cancer cells due to its amino acid composition (four lysine residues conferring positive charge) and its secondary structure (α-helix). Therefore, we have evaluated the effect of the peptide on four different breast cancer

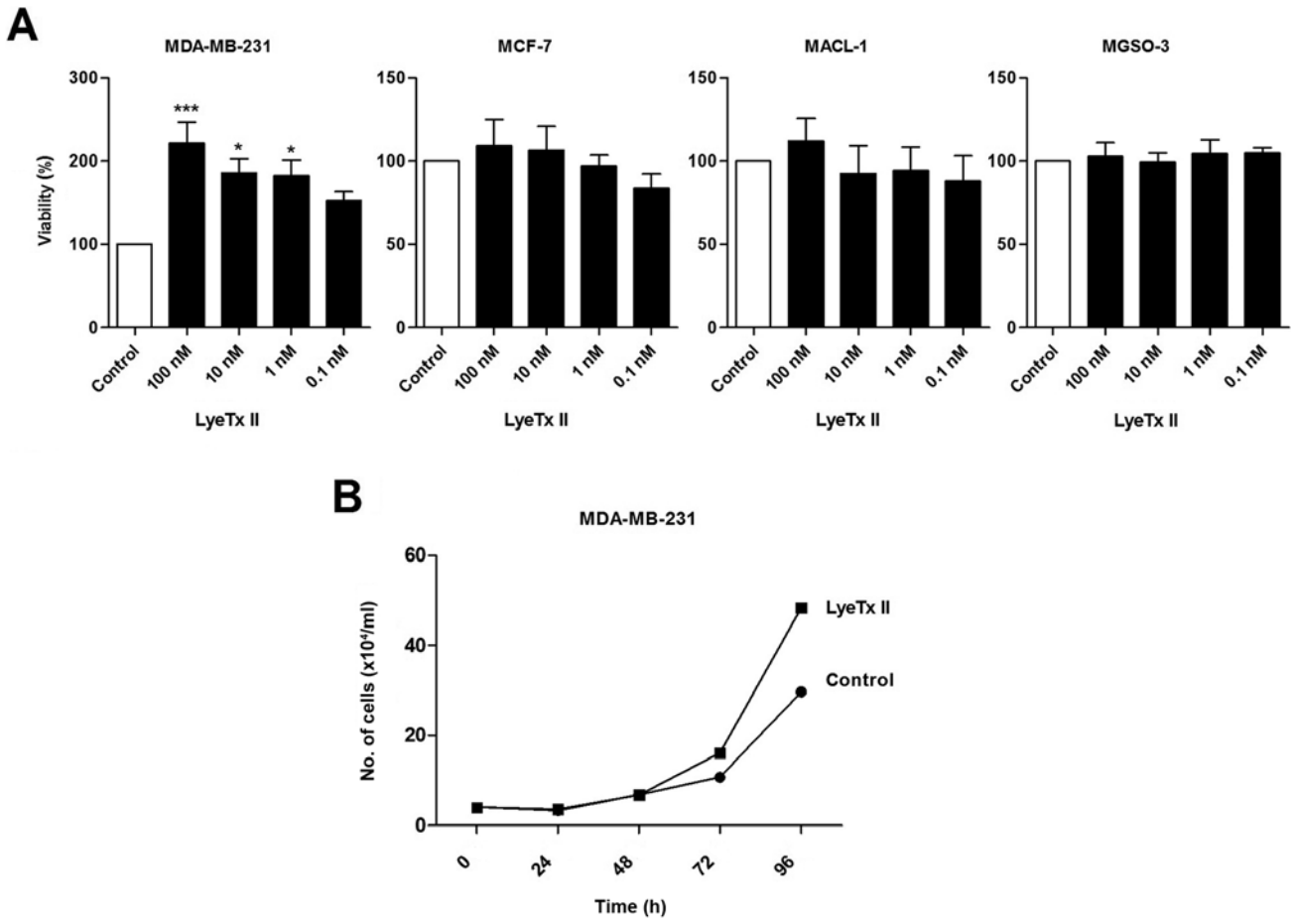

Figure 1. The peptide LyeTx II increases the proliferation of MDA-MB-231 cells. (A) Cell viability of breast cancer cell lines (MDA-MB-231, MCF-7, MACL-1, MGSO-3) was measured by MTT assay. $^{*}p<0.05$; $^{***}p<0.01$. MTT assay was performed after 96 h of incubation in DMEM supplemented with 10% of FBS in the presence or not of LyeTx II at different concentrations. (B) Growth curve of MDA-MB-231 cells was realized in the presence of 100 nM of LyeTx II during 96 h and cell counting was performed at each 24 h. Statistical analyses were performed using one-way ANOVA followed by Bonferroni post test.

cell lines (MDA-MB-231, MCF-7, MACL-1 and MGSO-3). Cells were incubated with different peptide concentrations varying from 0.1 to 100 nM and an MTT assay was performed to evaluate cell viability (Fig. 1A). Of note, LyeTx II induced an increase of MDA-MB-231 cell viability after 4 days and this effect was more marked at 100 nM. No effect induced by the peptide was observed on the other breast cancer cell lines indicating that only the most aggressive cell line was responsive to LyeTx II. As MTT assay measures viable cell metabolism and not specifically cell proliferation, we confirmed our data by cell counting. According to Fig. 1B, an increased cell number was noted after 72 and 96 h of treatment with LyeTx II when compared to the control group.

Peptide LyeTx II enhances MDA-MB-231 cell proliferation. We sought to define whether the increased cell number observed in the presence of LyeTx II after 96 h was due to a direct activation of the proliferative machinery or liberation of secondary compounds. For this purpose, a BrdU assay was performed, that allowed us to correlate the proliferative effect induced by the peptide and the incorporation of BrdU that is a synthetic nucleoside analog of thymidine. Firstly, we pregated cells according to size (SSC) and granulometry (FSC). To select the population in division, H2AX in its phosphorylated form (γH2AX) has been used as a marker of the DNA doubled stranded breaks. The detection of the incorporation of BrdU and the presence of γH2AX has been achieved by using antibody stained with PerCP-Cy5.5 and Alexa-647, respectively. As shown in the Fig. 2A, by double immunodetection, after 8 h of incubation with LyeTx II and BrdU, augmentation of the $BrdU^{+}/\gamma H2AX^{+}$ population was noted when compared with the control cell population. The $BrdU^{+}/\gamma H2AX^{+}$ population increased from 6.9 to 11.0% (Fig. 2B). Taken together, these data suggest that LyeTx II is able to interfere directly on the proliferative machinery of MDA-MB-231 cells. These findings led us to investigate the intracellular signaling pathways involved in the increased proliferation induced by LyeTx II.

p38 and NF-κB pathway activation are upregulated in the presence of LyeTx II. Considering that MAPKs are frequently involved in the balance between cell proliferation and cell death, we sought to evaluate the phosphorylation state of the three main MAPKs (ERKs, JNKs and p38s) in the presence of LyeTx II (Fig. 3A). LPS (1 μg/ml) and TNF-α (20 pg/ml) were used as positive control for the MAPK phosphorylation. At first, we observed that MDA-MB-231 cells did not use the JNK pathway, not in the presence of serum or in the presence of the peptide. However, we verified, that MDA-MB-231 cells

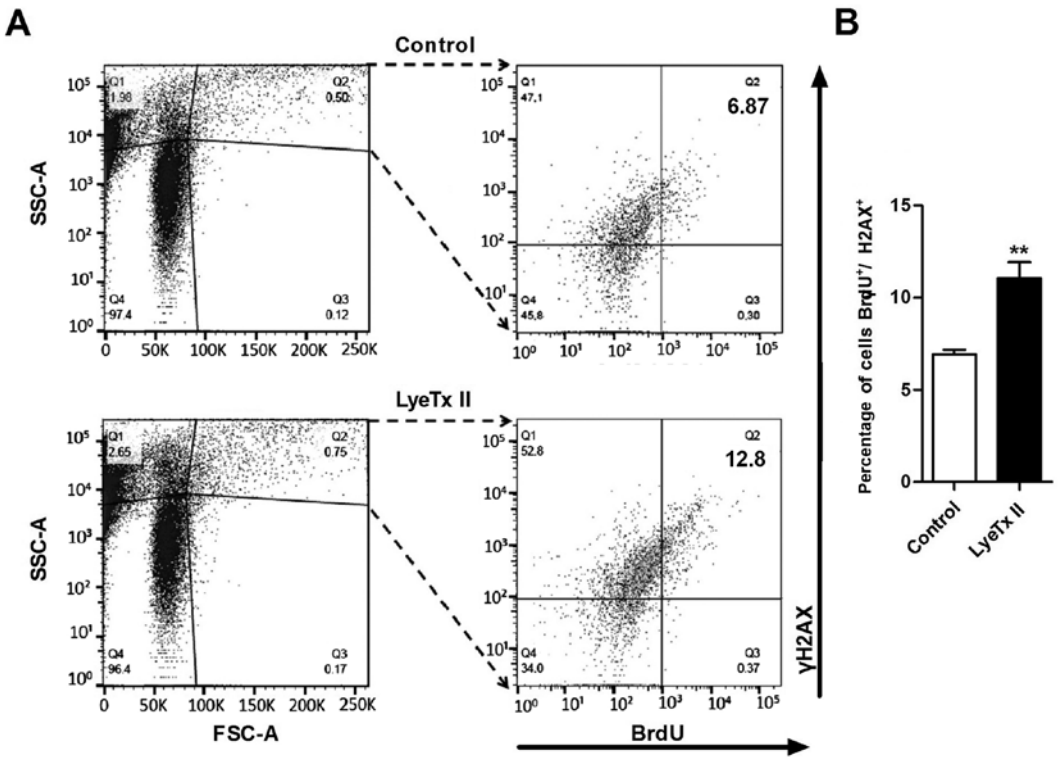

Figure 2. The peptide LyeTx II augments BrdU incorporation in MDA-MB-231 cells. MDA-MB-231 cells were incubated in DMEM with 10% of FBS and treated with LyeTx II (100 nM) and BrdU (10 μM) for 8 h. Then, cells were fixed, permeabilized and stained with anti-BrdU and anti-γH2AX and analyzed by FACS. Data are presented as (A) the representative dot plots of the frequency of individual data values and (B) the frequency of $BrdU^+/\gamma H2AX^+$ cells (average ± SD). Data are representative of two independent experiments in triplicate. Statistical analyses were performed using t-test. $^{**}p<0.01$.

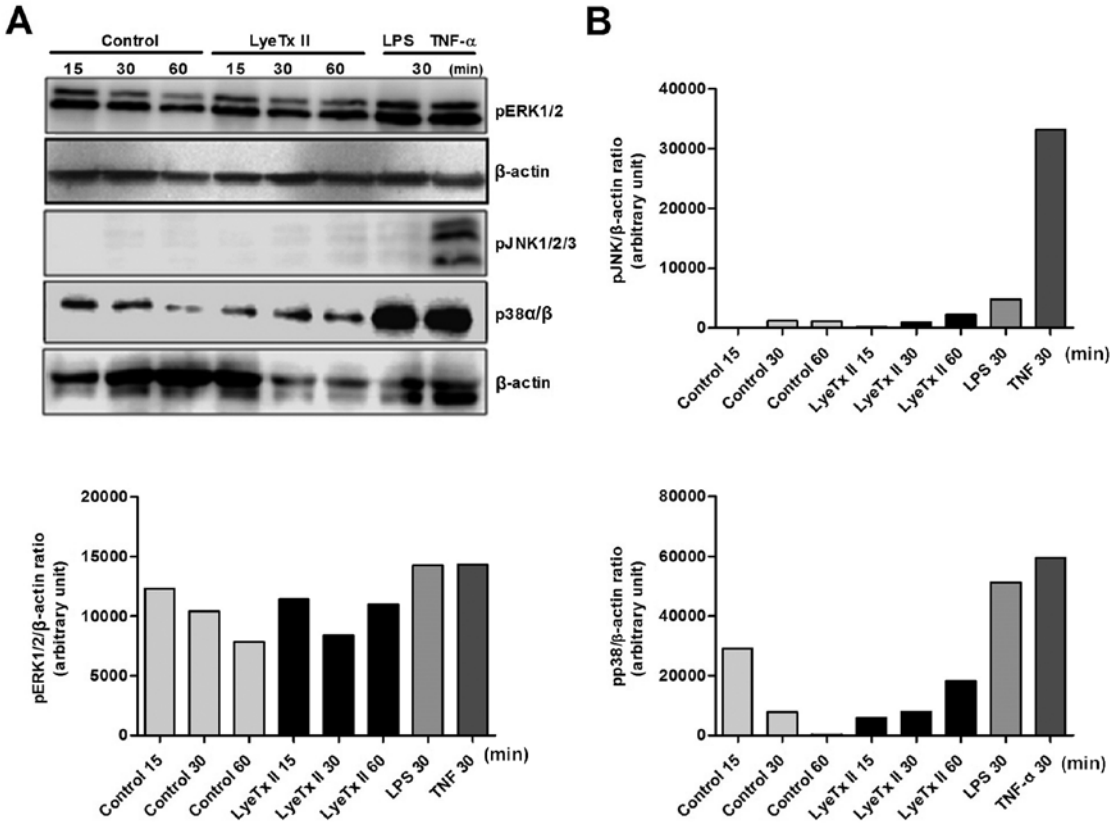

Figure 3. The peptide LyeTx II modulates p38 pathway activation in MDA-MB-231 cells. (A) MDA-MB-231 cells were incubated for 15, 30 and 60 min with LyeTx II at 100 nM in DMEM supplemented with 10% FBS (Control = medium 10% FBS). Then, cells were harvested and cell lysates were immunobloted with the indicated antibodies. LPS (1 μg/ml) and TNF-α (20 pg/ml) were used as positive controls. (B) The graphics represent the level of proteins quantified and normalized to the level of β-actin.

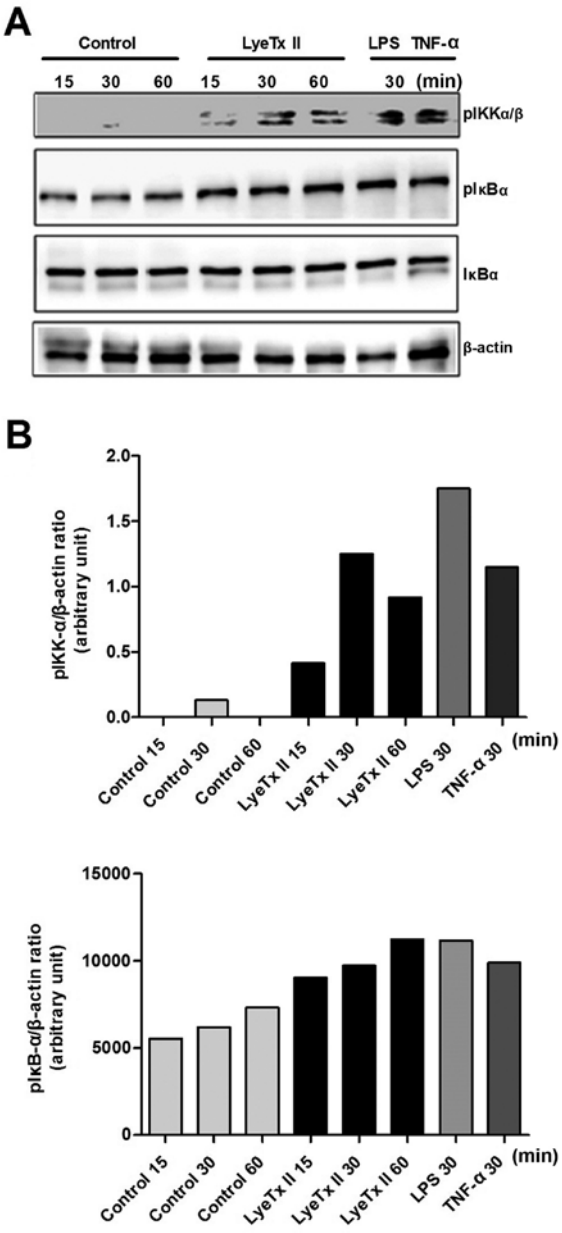

Figure 4. The peptide LyeTx II increases NF-κB pathway activation in MDA-MB-231 cells. (A) MDA-MB-231 cells were incubated for 15, 30 and 60 min with LyeTx II at 100 nM in DMEM supplemented with 10% FBS (Control = medium 10% FBS). Then, cells were harvested and cell lysates were immunobloted with the indicated antibodies. LPS (1 μg/ml) and TNF-α (20 pg/ml) were used as positive control. (B) The graphics represent the level of proteins quantified and normalized to the level of β-actin.

were able to phosphorylate JNK1/2/3 under stress stimulation when TNF-α induced activation of the three isoforms of JNK. This constitued a direct evidence that LyeTx II did not require JNK activation to induce its proliferative effect. Concerning ERK1/2 pathway, no significant change in the kinetic phosphorylation of this MAPK was detected in the presence of LyeTx II. The most significant impact induced by the peptide was the modulation of p38 phosphorylation. We observed that the phosphorylation of p38 was upregulated during stimulation with LyeTx II, suggesting that the peptide effect might be related to p38 pathway activation. As shown in the Fig. 3A, phosphorylation of p38 remained elevated when compared to control group, even 60 min after peptide stimulation.

In parallel, we explored the NF-κB pathway, another pathway well described as having an important role in cancer development. As shown in Fig. 4A, the peptide was able to enhance IκBα phosphorylation as well as IKK-αβ, two of the main kinases involved in the canonical NF-κB pathway activation. These data suggest a possible involvement of NF-κB pathway in MDA-MB-231 cell proliferation under stimulation with LyeTx II.

Upregulation of p38 pathway is associated with the accelerated proliferation of MDA-MB-231 in the presence of LyeTx II. To evaluate whether the peptide effect was dependent on IκBα and p38 activation, we used two selective inhibitors: BAY 11-7082 for NF-κB pathway (21) and SB203580 for p38 pathway (22). It was not possible to define the involvement of NF-κB pathway in the peptide activity, since IκBα inhibitor induced MDA-MB-231 cell death in the concentration range used (2.5-20 μM), that represent the concentrations able to inhibit IκBα activity as previously reported (21). To correlate the peptide effect with the p38 pathway activation, we performed an MTT assay using SB203580 at concentration that did not affect the basal cell proliferation in our system. As shown in Fig. 5C, SB203580 was able to abrogate the proliferative effect induced by the peptide. To confirm the relationship between p38 activation and the proliferative effect induced by the peptide, we evaluated the BrdU incorporation in the presence of LyeTx II, SB203580 and SB203580 plus LyeTx II (Fig. 5A and B). Corroborating with the MTT assay, SB203580 was able to abolish the peptide effect maintaining the proliferative rate at a basal level. More precisely, in the presence of SB203580, the frequency of $BrdU^+$ cells was significantly reduced in LyeTx II group comparing with LyeTx II group pretreated with SB203580. Collectively, the data confirmed that p38 plays a role in the increase of MDA-MB-231 cell proliferation when stimulated with LyeTx II.

p38 pathway upregulation is associated with the increased migration of MDA-MB-231 cells in the presence of LyeTx II. We hypothesized that accelerated proliferation and migration events might share the same signaling pathways in MDA-MB-231 cells. In such context, we evaluated whether the peptide could increase MDA-MB-231 cell migration by using the transwell assay. By placing the cells on one side of the membrane and using FBS as a chemoattractant on the other side, migration was determined by counting those cells that traversed the cell-permeable membrane. As shown in Fig. 6A and B, LyeTx II at 100 nM was also capable of enhancing cell migration in a significant way when compared to the control group. It is important to note that MDA-MB-231 cell line is considered as invasive, that explains the high number of cells that can pass through the membrane pores observed in serum conditions. The involvement of p38 pathway in MDA-MB-231 cell migration was confirmed when it was shown that SB203580 reduced the peptide effect on cell migration.

p38 and IκBα phosphorylation induced by LyeTx II is dependent on TAK1. According to our data, LyeTx II was able to upregulate p38 and NF-κB pathway activation, so we sought to determine possible crosstalk between these pathways. TAK1 has been previously described as a common upstream kinase

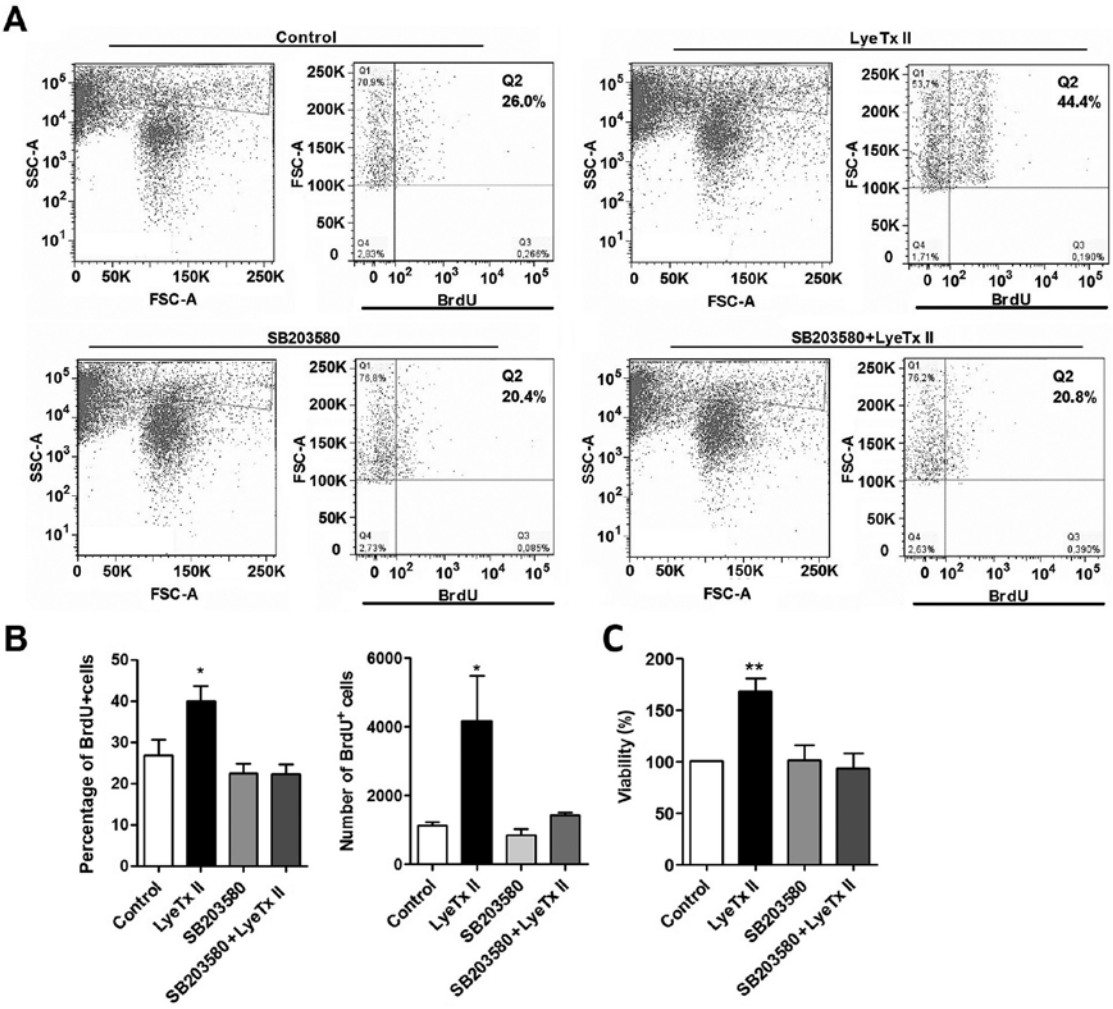

Figure 5. p38 inhibitor abrogates LyeTx II effect on MDA-MB-231 cell proliferation. (A) Cells incubated in DMEM supplemented with 10% of FBS were treated with LyeTx II (100 nM) and BrdU (10 μM) for 8 h, pretreated or not with p38 inhibitor SB203580 (10 μM) for 30 min. Then, cells were stained with fluorescent antibodies anti-BrdU and analyzed by FACS. Data are presented as (A) the representative dot plots of the frequency of individual data values and (B) the frequency and the number of $BrdU^+$ cells (average ± SD). (C) Cell viability was measured by MTT assay after incubation for 96 h with LyeTx II pretreated or not with SB203580 (10 μM) for 30 min. Control group (NT) represents cells incubated with medium supplemented with 10% FBS. MTT data are representative of three experiments. Statistical analyses were performed using one-way ANOVA followed by Bonferroni post test. $^*p<0.05$; $^{**}p<0.01$.

for both pathways (23). Further, other groups have previously described the involvement of TAK1 in the MDA-MB-231 cell survival (24,25). Thus, we explored the impact of TAK1 inhibition on the peptide effect. As shown in Fig. 7, when cells were stimulated with the peptide, they were still able to phosphorylate p38 and IκBα in the presence of TAK1 at 300 nM. However, at 500 nM, TAK1 inhibitor reduced significantly p38 and IκBα phosphorylation in LyeTx II-treated cells. Taken together, these data indicate that LyeTx II upregulates p38 and NF-κB pathways in a TAK1-dependent manner.

Discussion

A peptide derived from the venom of the Brazilian spider *Lycosa erythrognatha* was used to correlate activated signaling pathways and aggressive breast cancer cell proliferation. There are several studies describing p38 as a proliferative agent (26,27); on the other hand, some studies have defined p38 as an apoptosis inducer (28-30). Herein, we clearly defined that p38 was involved in the peptide induced-MDA-MB-231 cell gain functions such as proliferation and migration. Further, these data show a direct role for p38 in the cell cycle since the use of p38 inhibitor SB203580 abrogates the peptide effect on the BrdU incorporation in the first hours. In fact, it has been previously reported that p38 may regulate both the G2/M as well as G1/S cell cycle checkpoints (31-33). Different hypothesis may be advanced like the upregulation of cyclin D1 expression in a p38-dependent manner (34) or an additional mechanism that may contribute to augmentation of proliferation in the presence of the peptide. As p38 has also a well-known role in inflammation (35), we investigated a possible role of inflammatory mediators in the peptide effect. Since no augmentation of cytokine secretion was observed in the presence of LyeTx II, we were able to discard the correlation between the pro-inflammatory property of p38 and the peptide activity (data not shown). In addition, the use of rhTNF-α at a very low concentration (20 pg/ml) enhanced MDA-MB-231 proliferation (data not shown) and MAPK phosphorylation (including ERK and JNK) in a more pronounced way than the peptide. Taken together, these data support the idea that the proliferation effect induced by LyeTx II is independent on inflammatory processes.

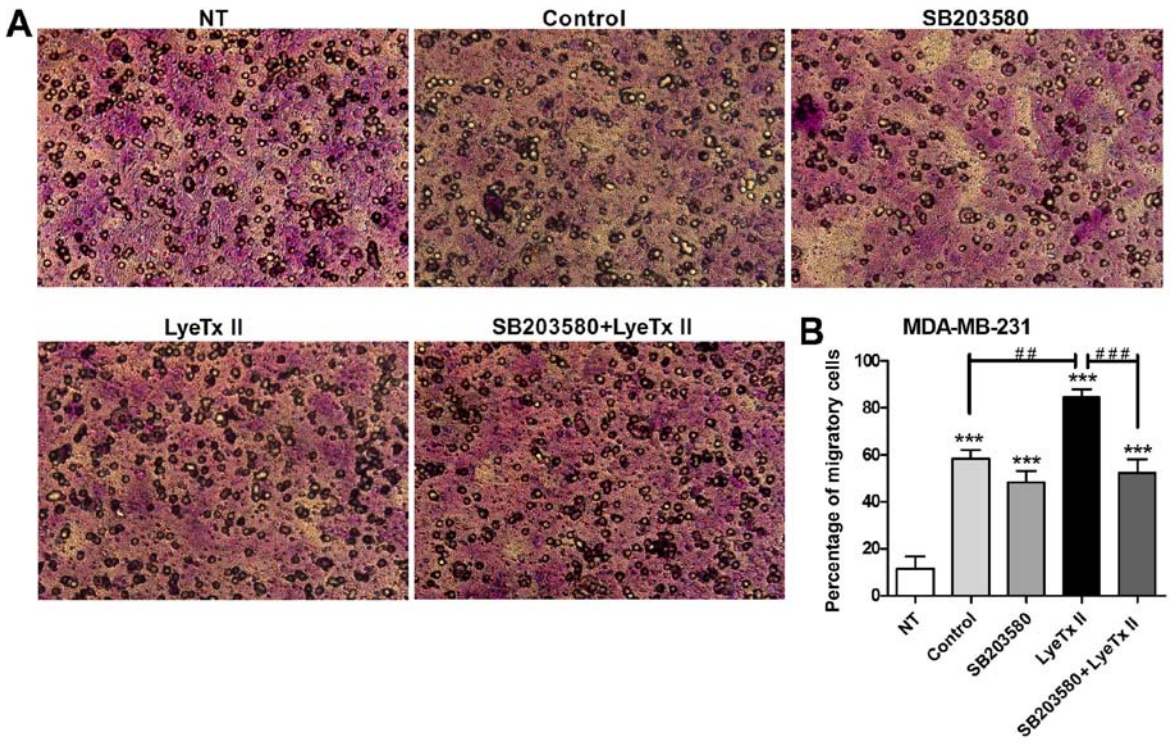

Figure 6. p38 inhibitor abrogates LyeTx II effect on MDA-MB-231 cell migration. (A) Cells were seeded on the upper chamber with serum-free medium in the presence or not of LyeTx II (100 nM). SB203580 (10 μM) was added in the upper chamber 30 min before LyeTx II treatment. DMEM with 10% FBS was added to the lower chamber as a chemoattractant. After 24 h of incubation, cells that passed through the membrane were counted in 5 visual fields of x200 magnification of each membrane. (B) Graphic represents the media ± SD of the percentage of cells that passed through the membrane after different treatments. Statistical analyses were performed using one-way ANOVA followed by Bonferroni post test. ***p<0.001 compared with non-treated (NT) group. $^{\#\#}$p<0.01; $^{\#\#\#}$p<0.001.

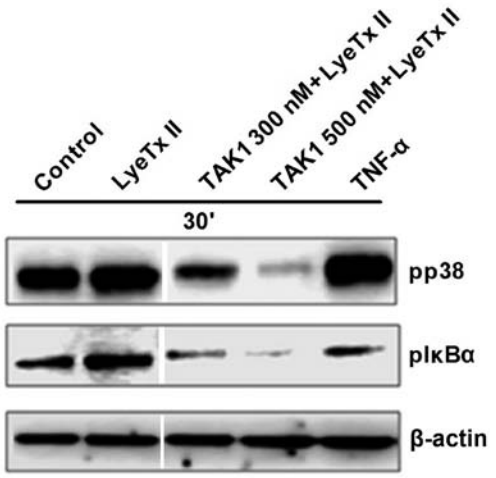

Figure 7. Impact of TAK1 inhibitor on p38 and IκBα phosphorylation induced by LyeTx II peptide. MDA-MB-231 cells were pretreated or not with TAK1 inhibitor [(5Z)-7-oxozeaenol] and incubated for 30 min with LyeTx II at 100 nM in DMEM supplemented with 10% FBS (Control = medium 10% FBS).

It has also been reported that p38 may play an important role in many steps of metastasis, such as invasion/migration, in pancreatic, hepatocellular and head and neck squamous carcinoma cell lines on the basis of the use of SB203580 and dominant-negative mutants (36). Herein we demonstrated that LyeTx II, through upregulation of p38 phosphorylation, enhanced MDA-MB-231 cell migration, reinforcing the involvement of p38 in the augmentation of the aggressive character of the tumor cells.

We sought to define whether the increased proliferation was due to a combination of events involving proliferation and/or anti-apoptotic pathways. Given the role of the transcription factor NF-κB in prosurvival pathways (37), we analyzed the capacity of the peptide to upregulate the NF-κB pathway. At first, it was of interest to note that while MDA-MB-231 cells were capable of phosphorylating IKK-αβ and IκBα in the presence of serum, MCF-7 did not (data not shown). This is in accord with a model proposed by Lee *et al* (38) where ER$^-$ breast cancer cells, such as MDA-MB-231, use constitutively NF-κB pathway and ER$^+$ breast cancer cells, such as MCF-7, do not. The peptide activity fits this model by enhancing IKK-αβ and IκBα phosphorylation only in MDA-MB-231 cells. This suggests that LyeTx II, besides its proliferative activity, might play a pro-survival role through upregulation of NF-κB pathway. This might explain the significant impact of this peptide on MDA-MB-231 cell proliferation and migration at low concentration (approximately 100 nM). Peptides used in cancer studies act generally at concentration superior to 1 μM (39,40).

Considering that LyeTx II modulated p38 and NF-κB pathways activation, we hypothesized that a common molecule could regulate both pathways in response to the peptide. TAK1 has been reported as an upstream kinase of p38 and NF-κB (41-43), so it appeared relevant in our model to correlate p38 and IκBα activation with TAK1 activity. Our data suggest that the phosphorylation of p38 and IκBα is dependent

on TAK1 activation. Noteworthy, it has been reported that p38 may exert a positive feedback on TAK1 phosphorylation (44), which may occur in our model since a sustained phosphorylation of p38 has been observed in presence of LyeTx II. Such feedback mechanism may contribute to the peptide activity.

An interesting finding in this study was the divergence of cellular response between the non-aggressive MCF-7 and aggressive MDA-MB-231 tumor cells to LyeTx II, probably due to the differential use of signaling pathways by cell lines. Comparative phosphoproteosome analysis reveals differences in levels of various phosphoproteins in these two cell lines (45). This indicates that the sole presence or lack of receptors (ER, PR, HER2) may not be sufficient to classify tumor cells and to predict treatment.

In conclusion, we were able to associate a dysregulation of MAPK signaling pathways with a specific breast tumor cell function. We identified, by using the synthetic peptide LyeTx II, that upregulation of the p38 pathway in a TAK1-dependent manner led to an accelerated proliferation and migration rate in MDA-MB-231 cells. This study highlights the importance of using compounds derived from animal venoms that may contribute to identify new targets for breast cancer treatment. Furthermore, these data open new perspectives for the use of p38 and TAK1 inhibitors as a targeted-treatment in cancer.

Acknowledgements

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4.4 - Paper 3 (referente ao projeto original da tese)

Translocation of intracellular CD24 constitutes a triggering event for drug resistance in breast cancer

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Translocation of intracellular CD24 constitutes a triggering event for drug resistance in breast cancer

Hugo Werner Huth[1], Thiago Castro-Gomes[2], Alfredo Miranda de Goes[3] & Catherine Ropert[3]✉

The capacity of tumor cells to shift dynamically between different states could be responsible for chemoresistance and has been commonly linked to the acquisition of stem cell properties. Here, we have evaluated the phenotype switching associated with drug resistance in breast cancer cell lines and cell lineage obtained from Brazilian patients. We have highlighted the role of the cancer stem cell marker CD24 in the dynamics of cell plasticity and the acquirement of drug resistance. We showed that the translocation of CD24 from cytosol to cell membrane is a triggering event for the phenotype change of breast tumor cells exposed to drug stress. Here, we provide evidence that the phenotype switching is due to the presence of a cytosolic pool of CD24. Importantly, the cellular localization of CD24 was correlated with the changes in the dynamics of p38 MAPK activation. A strong and continuous phosphorylation of the p38 MAPK led to the overexpression of Bcl-2 after treatment in persistent cells presenting high density of CD24 on cell membrane. This phenotype enabled the cells to enter in slow-down of cell cycle, after which several weeks later, the dormant cells proliferated again. Importantly, the use of a p38 activity inhibitor sensitized cells to drug treatment and avoided chemoresistance.

Drug resistance continues to be the main limiting factor to cure patients with cancer that can be imputed to the adaptation capacity of tumor cells that corresponds to the emergence of resistant cells after anti-tumor therapy. Indeed, this phenomenon has been commonly associated with stemness when the acquisition of Cancer Stem Cell (CSC) properties by non-CSCs has been reported under several conditions, including drug treatment. CSCs would be responsible for tumor initiation, maintenance, metastasis formation, phenotypic plasticity and drug resistance[1–7]. CD24, a cell surface adhesion glycoprotein, was identified as a CSC marker and is present in various types of cancer including breast, pancreatic and lung[8] and has often been associated with more aggressive diseases in ovarian, breast, lung and prostate cancers[9,10]. This explains the considerable interest for this marker in tumor biology and also in treatment outcome. In this line, relevant studies have revealed that the phenotype switching associated with the detection of surface CD24 could be responsible for chemoresistance[11,12]. For instance, CD24 expression level has appeared to be a significant molecular phenotype of cisplatin-resistant residual cells in laryngeal carcinoma lines, which corresponds to a differential expression of critical apoptotic and drug resistance[13]. Goldman et al. have observed an enrichment of $CD24^+$ cells following treatment with docetaxel in different breast tumor cell lineages, which corresponded to the generation of new CSCs from non-CSCs[12]. Importantly, the mechanism leading to an increased surface CD24 expression in tumor cells under drug stress remains unknown.

Recent findings support the hypothesis that epigenetic mechanisms are key players in the phenotypic transition of tumor cells[14,15]. These phenotype changes that do not involve alterations in the DNA sequence can result in drastic changes not only in cell plasticity but also in drug resistance[16,17]. Besides, MAPK signaling pathways, which are deregulated in tumor[18,19], have been constantly associated with chemoresistance in different type of cancer[20–22]. For instance, it has been reported that p38 and JNK MAPK pathways play a role in the control of the balance between autophagy and apoptosis in response to genotoxic stress[20]. Others have explored the role of ERK and p38 MAPK in breast cancer chemotherapy[21]. But, little is known concerning the involvement of MAPKs in tumor plasticity. Indeed, the molecular me chanisms that control cellular plasticity upon drug treatment remain to be fully established.

Here, using different breast cancer cell lines, we have evaluated the impact of drug stress on the immediate phenotype change by tracing the CSC marker CD24. We have shown that the rapid translocation of CD24 from

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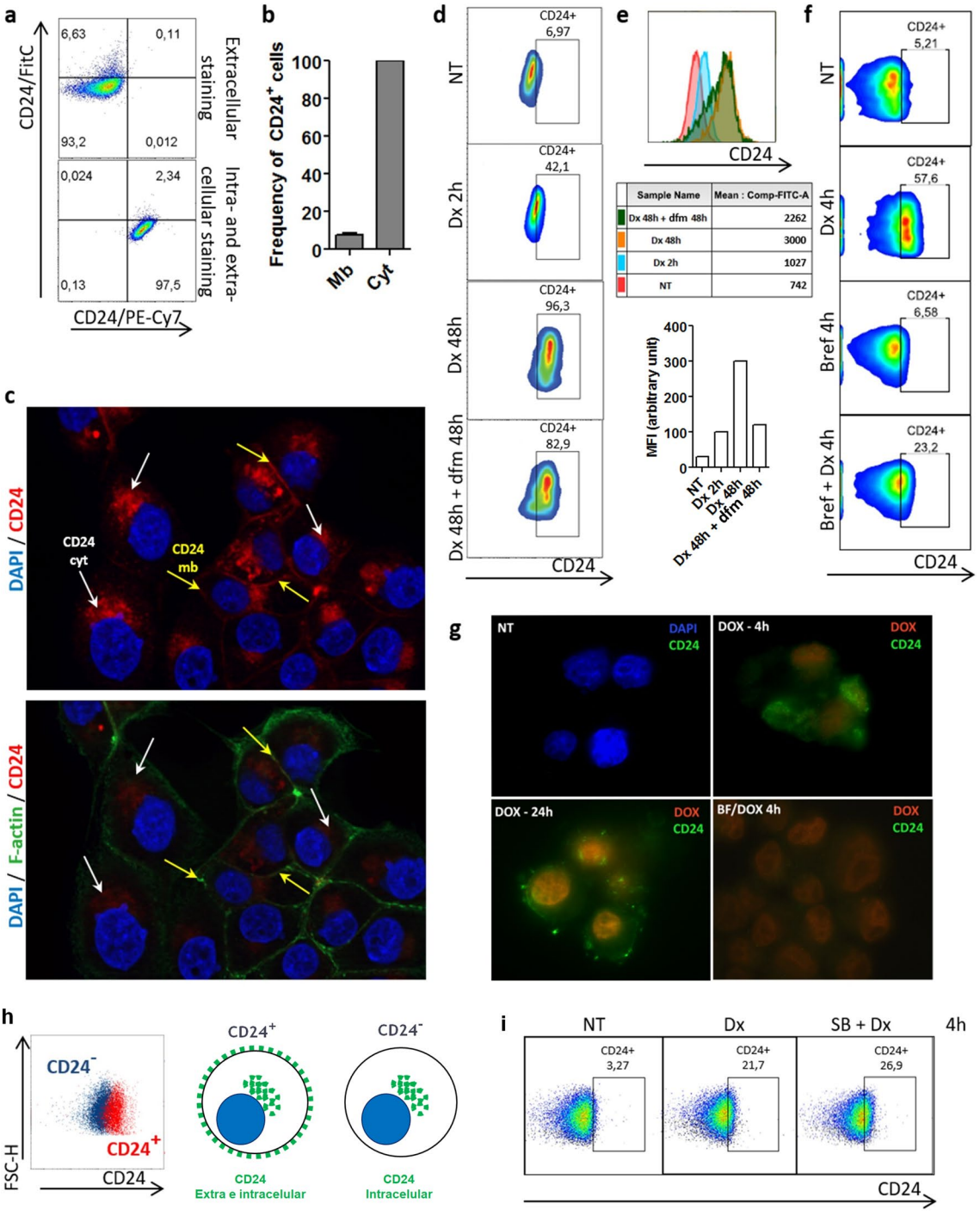
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DOX - 24h
DOX
CD24
BF/DOX 4h
DOX
CD24
h
FSC-H
CD24
CD24-
CD24+
CD24+
CD24
Extra e intracelular
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i
NT
Dx
SB + Dx
4h
CD24+ 3,27
CD24+ 21,7
CD24+ 26,9
CD24

◂**Figure 1.** Translocation of CD24 from cytosol to cell membrane is an early event in MDA-MB-231 cells under drug stress. (**a**) Evaluation of the localization of CD24 in MDA-MB-231 cell line. MDA-MB-231 cells were stained with anti-CD24/FITC (extracellular staining) and with anti-CD24/PE-Cy7 (intracellular staining). Pseudocolor plots are representative of triplicates. (**b**) Frequency of $CD24^+$ cells according to the detection of CD24 in membrane (mb) or in cytosol (cyt). The results are representative of four independent experiments (means ± SD of triplicates). (**c**) Visualization of CD24 localization by fluorescence microscopy. MDA-MB-231 cells were fixed, permeabilized and then stained using anti-CD24/PE-Cy7, anti-F-actin/Alexa488 and the nuclear dye DAPI. The yellow and white arrows indicate the mb CD24 and cyt CD24, respectively. (**d**) $CD24^+$ cell enrichment in MDA-MB-231 population during doxorubicin (Dx) treatment. MDA-MB-231 cells were treated with Dx (0.6 µM) according to the indicated times followed or not by a drug-free medium (dfm) incubation. Then, an extracellular staining was performed using anti-CD24/FITC. The pseudocolor plots are representative of four independent experiments. (**e**) Histograms represent the shift of CD24 on x axis under different treatments. Table shows the MFI (median fluorescence intensity) values of surface CD24 expression calculated from figure d pseudocolor plots. Graph represents the means of triplicates obtained from the MFI calculation. (**f**) Reduction of CD24 translocation by brefeldin A in Dx-treated cells. MDA-MB-231 cells were pretreated or not with brefeldin A (1 µg/ml) 30 min before the addition of Dx (0.6 µM). Extracellular staining was performed using anti-CD24/PE-Cy7. The pseudocolor plots are representative of two independent experiments. (**g**) Effect of brefeldin A on CD24 localization in doxorubicin-treated cells visualized by fluorescence microscopy. MDA-MB-231 cells were treated with Brefeldin A prior to doxorubicin (0.6 µM). Then, cells were stained using anti-CD24/Alexa488 and DAPI without fixation and permeabilization. (**h**) Representative merged pseudoclor plots of $CD24^+$ (red) and $CD24^-$ (blue) subpopulations obtained from parental MDA-MB-231 cells after magnetic sorting. Schematic representation of CD24 localization in $CD24^+$ and $CD24^-$ suubpopulations. (**i**) Translocation of CD24 in $CD24^-$ cells during doxorubicin treatment. $CD24^-$ cells were treated with Dx (0.6 µM) for 4 h. Then cells were stained (extracellular staining) with anti-CD24/Pe-Cy7 antibody. The pseudocolor plots are representative of triplicates.

cytosol to cell membrane was the triggering event for the acquisition of chemoresistance. In drug-resistant MDA-MB-231 cells, we have identified a tandem constituted by CD24 and p38 MAPK, where the continuous p38 activation controlled the overexpression of the survival marker Bcl-2. This phenotype enabled the cells to enter in slow-down of cell cycle, after which several weeks later, the dormant cells retrieved their capacity to proliferate. These cells were characterized by an increased resistance to drug and migratory capacity. Importantly, the use of p38 inhibitor was able to block the acquisition of drug resistance by impeding the upregulation of the anti-apoptotic protein Bcl-2. The correlation between translocation of CD24 and increased expression of Bcl-2 made in doxorubicin model was supported by the use of the TLR7 agonist Imiquimod that reduced cell proliferation without affecting phenotype switching nor Bcl-2 expression. Finally, we propose that the association of the p38 inhibitor, SB203580, with doxorubicin, a leading drug in clinic, could open up a new strategy in the fight against cancer.

Results

The translocation of CD24 from cytosol to membrane is an early event in breast tumor cells under drug stress. Phenotype switching, also commonly referred to as cell plasticity, is an important process observed during treatment of cancer, which was repeatedly associated with stemness. Here, using different breast cancer cell lines, we explored the dynamics of the CSC marker CD24 after doxorubicin treatment. At first, we sought to define the localization of CD24 in MDA-MB-231 cells by extra and intracellular staining. As shown by our flow cytometry results only about 5% of cells expressed CD24 in cell membrane (Fig. 1a,b). These finding corroborates other study[23] and explains why MDA-MB-231 is considered $CD24^{low/-}$. By contrast, a significant intracellular pool of CD24 was encountered in all the cells. Fluorescence microscopy confirmed the presence of extracellular (yellow arrows) and intracellular CD24 (white arrows) (Fig. 1c). After the treatment with doxorubicin at 0.6 µM—concentration representing the EC50 after 24 h of treatment calculated in MDA-MB-231 cell line—a cell phenotype switching occurred, which corresponded to an enrichment of the $CD24^+$ subpopulation (Fig. 1d). Notably, MFI analysis showed an increase of surface CD24 density during drug treatment (Fig. 1e). This phenomenon occurred rapidly since ~ 42% of cells converted into $CD24^+$ after 2 h to finally reach ~ 96% after 48 h of treatment, as visualized by flow cytometry (Fig. 1d). Importantly, the majority of cells remained positive even after a pause in the treatment (incubation in drug-free medium for 48 h after treatment) as visualized by the last pseudocolor plot in the Fig. 1d. The fact that this event was detected in the first hours of treatment excludes the possibility of a Darwinian selection of $CD24^+$ cells. These results led us to hypothesize that the intracellular pool of CD24 immediately available might play a role in the CD24 translocation to cell surface. To support these data, MDA-MB-231 cells were sorted into $CD24^+$ and $CD24^-$ subpopulations (Fig. 1h) by using magnetic beads considering that $CD24^+$ subpopulation expresses CD24 in both membrane and cytosol while $CD24^-$ subpopulation lacks membrane CD24 expression as schematized in Fig. 1h. Then, CD24 localization was evaluated in $CD24^-$ cells after doxorubicin treatment. As shown in the Fig. 1i, the translocation of CD24 occurred even in $CD24^-$ population obtained after cell sorting since $CD24^-$ cells were able to rapidly convert in $CD24^+$ cells. Such data reinforce the idea that $CD24^+$ cells enrichment during drug treatment does not correspond to a pre-selection of clones but to a drug-induced phenotype switching. In order to confirm this theory, we took the opportunity of using brefeldin A, an inhibitor of protein transport from endoplasmic reticulum to Golgi apparatus, to disturb the CD24 traffic after drug treatment. After flow cytometry analysis, we observed that when MDA-MB-231 cells were treated with brefeldin A prior to doxorubicin, the translocation of CD24

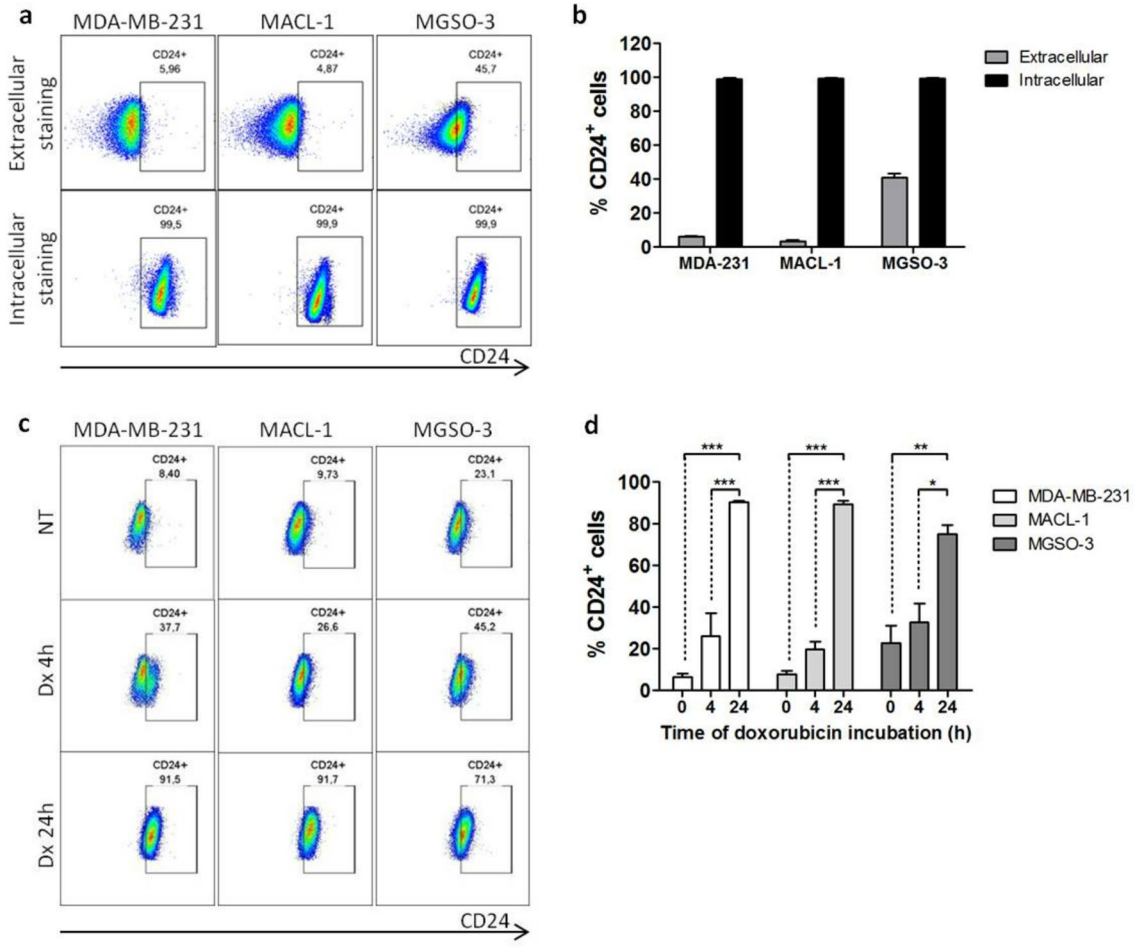


Figure 2. Translocation of CD24 from cytosol to membrane is observed in patient-derived breast cancer cells under drug stress. (**a**) Localization of CD24 in MDA-MB-231, MACL-1 and MGSO-3 cells after staining with anti-CD24/Pe-Cy7 (extracellular and intracellular staining). Dot-plots are representative of triplicates. (**b**) Frequency of $CD24^+$ cells according to the detection of CD24 in membrane (extracellular) or in cytosol (intracellular). (**c**) $CD24^+$ cell enrichment in breast cancer cell lines during Dx treatment. MDA-MB-231, MACL-1 and MGSO-3 cells were treated with Dx (0.6 μM) according to the indicated times and stained using anti-CD24/FITC. The dot-plots are representative of triplicates. (**d**) Frequency of $CD24^+$ cells under Dx treatment according to the detection of CD24 in membrane. Data were plotted as means of triplicates ± SD. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$ (one-way ANOVA with bonferroni post-test).

was reduced (Fig. 1f). These results were consistent with the fluorescence microscopy images obtained from doxorubicin-treated cells, which were stained with anti-CD24 without permeabilization to solely detect surface CD24 (Fig. 1g). To discard the hypothesis that the increased membrane CD24 expression could be partially due to an increased protein synthesis, we used actinomycin D, a DNA-transcription inhibitor. The incapability of actinomycin D to reduce the $CD24^+$ phenotype enrichment in presence of doxorubicin indicated that the intracellular pool of CD24 was the main source of CD24 traffic in the presence of doxorubicin (data not shown). Importantly, using staurosporine, an inhibitor of protein kinase, a same phenomenon was observed resulting in the almost complete conversion of MDA-MB-231 cells into $CD24^+$ cells after few hours (Supplementary Fig. 1).

Concerning MACL-1 and MGSO-3 breast cancer cell lines, obtained from Brazilian patients, the percentage of $CD24^+$ cells was about 5% and 46% respectively, corroborating their classification by another study[24]. The presence of intracellular CD24 was also detected in the whole population of both cell lines, as observed in MDA-MB-231 population (Fig. 2a,b). In the same way, phenotype switching occurred in both cell lines which corresponded to an enrichment of the $CD24^+$ subpopulation after 4 h doxorubicin treatment. The conversion rate in $CD24^+$ cells reached ~ 90% for MDA-MB-231 and MACL-1 and ~ 70% for MGSO-3 cells (Fig. 2c,d) in cell lines treated for 24 h.

Therefore, we propose a new dynamic model of cell transition phenotype under drug stress which involves the translocation of intracellular CD24 that allows each breast cancer cell to convert into $CD24^+$ cell.

Translocation of CD24 in MDA-MB-231 cells treated with doxorubicin correlates with the upregulation of Bcl-2 expression and chemoresistance. Here, we investigated the molecular identity of the survival cells after 48 h of doxorubicin treatment, which we have named $CD24^+$/DxR cells. The Fig. 3a

represents the methodology used to obtain CD24⁺/DxR cells and schematizes the steps leading to MTT and western blot assays. At first, we tested the sensibility of the CD24⁺/DxR cells to respond to a second doxorubicin treatment using MTT assay. As shown in Fig. 3b, only a slight reduction of CD24⁺/DxR cell number was noted after a second treatment with doxorubicin. Meanwhile, the viability of naïve cells that received the treatment for the first time, declined below 40%. These data indicated that CD24⁺/DxR cells became tolerant. So, we sought to identify whether this phenotype change corresponded to a putative cell reprogramming in response to drug stress. By using western blot assay, we compared the protein profile of naïve and CD24⁺/DxR cells. When we focused on protein expression involved in cell proliferation or death, it was detected a significant increase of Bcl-2 expression, which was inversely correlated with Bax expression in CD24⁺/DxR (Fig. 3c). In addition, we observed a remarkable decrease of cyclin D1, a regulator of cell cycle progression, suggesting that acquisition of drug tolerance controlled by Bcl-2 expression may also require cells to exit the cell cycle (Fig. 3c). In previous studies, we have focused on the role of p38 MAPK and ERK1/2 in the proliferation of MDA-MB-231 cells[25,26], so we analyzed their activation profile in the resistant CD24⁺/DxR cells. Interestingly, the phenotype switching was accompanied by a strong and continuous activation of p38 MAPK at the detriment of ERK1/2 MAPK (Fig. 3d). Then, we verified the relationship between CD24 and Bcl-2 by silencing CD24 using interference-RNA. In CD24-silenced cells (SiCD24), a decreased Bcl-2 and p38 expression was observed (Fig. 3e). This may explain the reduced capacity of SiCD24 cells to resist to drug treatment in all the doxorubicin concentrations tested when compared to control-silenced (SiC) and parental MDA-MB-231 cells (Fig. 3f). Interestingly, CD24⁻ subpopulation, obtained from magnetic sorting, presented a drug sensibility similar to CD24⁺ subpopulation and parental MDA-MB-231 cells. This is in accord with the fact that even CD24⁻ converted into CD24⁺ after drug treatment (Fig. 1i).

Next, we sought to treat MDA-MB-231 cells with a drug capable to reduce cell proliferation without inducing immediate death. We tested the efficacy of the TLR7 agonist Imiquimod, previously used in skin cancer cutaneous metastatic breast cancer treatment[27–29], in reducing MDA-MB-231 cell proliferation. At the concentration of 1 µM, a significant decrease of cell proliferation was observed while a total blocking of cell replication was noted at 10 µM during the period of experiment (Fig. 3g). No significant cell death was observed in the first 48 h in the presence of both concentrations of Imiquimod. In such context, we tested the capacity of cells to respond to a second treatment 96 h after the first dose of Imiquimod. As shown in Fig. 3h, a similar pattern of cell viability was observed when a unique dose or two subsequent treatments with the TLR7 agonist were used indicating the absence of chemoresistance. To correlate phenotype switching and chemoresistance, we traced the CD24 marker in Imiquimod treated cells. We confirmed that no enrichment of CD24⁺ cells occurred during the treatment (Fig. 3i). In accordance with this, no upregulation of Bcl-2 expression was detected in cells treated with Imiquimod (Fig. 3j). These data reemphasize a link between CD24 translocation, upregulation of Bcl-2 expression and chemoresistance.

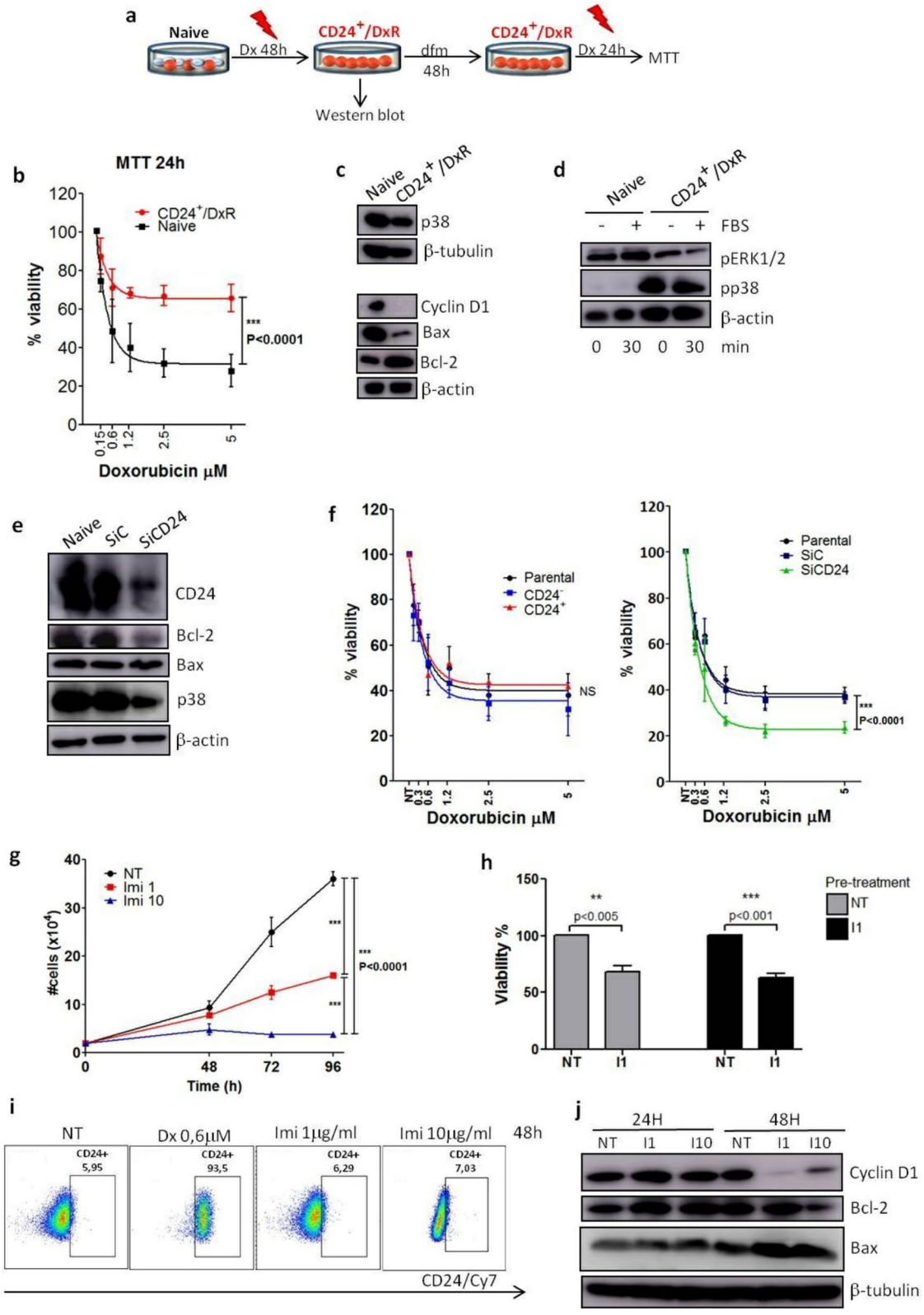
Association between CD24 translocation and activation of p38 in the chemoresistance acquisition phenotype of MDA-MB-231 breast cancer cells. According to the Fig. 3d, p38 phosphorylation was stronger, constitutive and independent on serum in CD24⁺/DxR cells which contrasts with the serum-dependent activation of p38 in MDA-MB-231 cells under proliferative conditions. This suggests that p38 activation in diverse configurations can cause different outputs. This led us to investigate its role in the phenotype switching of MDA-MB-231 cells in the presence of doxorubicin.

So, our next question was whether there was a privileged relationship between CD24 and p38. At first, we explored this in MDA-MB-231 cells under growing culture conditions. After magnetic sorting, we evaluated the status of MAPK activation with western blotting after cell stimulation with serum according to the kinetic presented in the Fig. 4a. As clearly shown, CD24⁺ cells phosphorylated p38 in a more pronounced way than CD24⁻ subpopulation. In contrast, a higher phosphorylation of ERK1/2 was observed in the CD24⁻ and parental MDA-MB-231 cells. The results obtained by flow cytometry confirmed the correlation between surface CD24 expression and preferential p38 phosphorylation. About 70% of the CD24⁺ cells phosphorylated p38, while the activation of this MAPK was observed in only ~ 15% of the CD24⁻ cells (Fig. 4b).

Another piece of evidence that demonstrates a link between CD24 and p38 is presented in the Fig. 4c. According to the western blotting, siRNA-mediated knockdown of CD24 decreased the phosphorylation of p38 when cells were submitted to doxorubicin treatment, which indicated that the absence of CD24 jeopardized the cell capacity to induce activation of p38 MAPK.

The sustained p38 activation in CD24⁺/DxR cells makes it a prime target. In this context, we used SB203580, a p38 activity inhibitor[30] to evaluate its impact on drug resistance. According to the results obtained by cell counting, the combination of SB203580 and doxorubicin was more efficient in reducing cell number than doxorubicin alone. Importantly, SB203580 alone was unable to impact on cell viability (Fig. 4d). Concerning the results observed by MTT assay, the inhibition of p38 was benefit from two aspects: first, SB203580 sensitized MDA-MB-231 cells to the therefore doxorubicin treatment (blue line vs black line). Second, SB203580 disrupted the resistant-phenotype acquired by the cells that received two consecutives doxorubicin treatments (green line vs red line) (Fig. 4e). The effect of the drug association may be considered as synergistic since the total effect of combined SB203580 and doxorubicin was greater than the sum of the individual effects of each drug.

To visualize these results, we performed the capture of light microscopy images of cells treated with the drug pair. These experiments were performed under sub-confluence or confluence conditions to exclude the influence of fluctuating environment. A direct impact of doxorubicin on MDA-MB-231 cells was observed after 24 h of treatment marked by a decreased cell number and changes in morphology. The association of SB203580 and doxorubicin exacerbated the cell phenotype changes under confluent and sub-confluent conditions. The results


a
Naive
Dx 48h
CD24+/DxR
dfm
48h
CD24+/DxR
Dx 24h
MTT
Western blot
b
MTT 24h
CD24+/DxR
Naive
% viability
***
P<0.0001
Doxorubicin µM
c
Naive
CD24+ /DxR
p38
β-tubulin
Cyclin D1
Bax
Bcl-2
β-actin
d
Naive
CD24+ /DxR
- + - + FBS
pERK1/2
pp38
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0 30 0 30 min
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SiC
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CD24
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Parental
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P<0.0001
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Imi 1
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***
P<0.0001
Time (h)
h
Viability %
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p<0.005
***
p<0.001
Pre-treatment
NT
I1
NT
I1
NT
I1
i
NT
Dx 0,6µM
Imi 1µg/ml
Imi 10µg/ml
48h
CD24+
5,95
CD24+
93,5
CD24+
6,29
CD24+
7,03
CD24/Cy7
j
24H
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NT I1 I10 NT I1 I10
Cyclin D1
Bcl-2
Bax
β-tubulin

◂**Figure 3.** Translocation of CD24 is associated with chemoresistance and overexpression of Bcl-2. (**a**) Schematic shows experimental design used to obtain cell populations for MTT (**b**) and western blot assays (**c**,**d**). *dfm* drug-free medium, *Dx* doxorubicin, *CD24*$^+$*/DxR* CD24$^+$ doxorrubicin-resistant cells. (**b**) CD24$^+$/DxR cells become tolerant to a second Dx treatment. Parental MDA-MB-231 cells were cultured in the presence of Dx at 0.6 µM for 48 h leading to the obtention of CD24$^+$/DxR cells, which were cultured in a drug-free medium (dfm) for 48 h. CD24$^+$/DxR and naïve MDA-MB-231 cells were submitted to Dx treatment for 24 h and cell viability was measured by MTT. The curves were fitted with non-linear regression as means of triplicates ± SD. ***$p < 0.001$ (two-way ANOVA with Bonferroni post-test). (**c**) CD24$^+$/DxR cells are characterized by a reduction of cyclin D1 and Bax expression and increase of Bcl-2 level. Representative image of western blotting are shown and the data have been reproduced two times. (**d**) CD24$^+$/DxR cells are characterized by a constitutive activation of p38 MAPK. Cells were starved for 2 h and then stimulated with serum (FBS) for 30 min. Cell lysates were immunoblotted with antibodies against the phosphorylated form of p38 (pp38) or ERK (pERK). (**e**) Downregulation of Bcl-2 and p38 expression in CD24 silenced (SiCD24) cells. SiC (control silenced) and SiCD24 (CD24 silenced) cell lysates were immunoblotted with the depicted antibodies. Representative images of western blotting are shown and the data have been reproduced three times. (**f**) Correlation between surface CD24 expression and sensitivity to Dx. Cell subpopulations were submitted to Dx treatment for 24 h and then cell viability was measured by MTT assay. Curves were fitted with non-linear regression as means of triplicates ± SD. ***$p < 0.001$ (two-way ANOVA with Bonferroni post-test). (**g**) Imiquimod reduces MDA-MB-231 cell proliferation. MDA-MB-231 cells were treated with Imiquimod. Then cells were counted and the curves were fitted as means of triplicates ± SD. ***$p < 0.001$ (two-way ANOVA with Bonferroni post-test). (**h**) Imiquimod does not induce chemoresistance to subsequent treatments on MDA-MB-231 cells. MDA-MB-231 cells were pre-treated with Imiquimod (1 µg/ml) for 96 h. After that, cells were incubated with a drug free medium for 48 h and then resubmitted to a second treatment with Imiquimod (1 µg/ml) for 96 h. Cell viability was evaluated by MTT. Data were plotted as means of triplicates ± SD. **$p < 0.01$ and ***$p < 0.001$ (T test). (**i**) Imiquimod does not induce CD24 translocation. MDA-MB-231 cells were treated with Dx or Imiquimod (1 µg/ml and 10 µg/ml) for 48 h. Then, an extracellular staining was performed using anti-CD24/Cy7. The pseudocolor plots are representative of two independent experiments. (**j**) Imiquimod reduces Bcl-2 expression. MDA-MB-231 cells were treated with Imiquimod (1 µg/ml or 10 µg/ml) in DMEM supplemented with fetal bovine serum (FBS) 10% for 24 h and 48 h. Cell lysates were immunoblotted with the depicted antibodies. The results are representative of two independent experiments.

confirmed that the efficiency of the drug pair constituted by doxorubicin and SB203580 was superior in killing cells in both plating conditions (Fig. 4f).

Consistent with the above results, western blots showed that SB203580 prevented the increase in Bcl-2 expression induced by doxorubicin (Fig. 4g). Further, in MDA-MB-231, which has high levels of a mutant p53, it has been described that mutant p53 can contribute to the suppression of apoptosis[31]. In line with this, SB203580 was also able to reduce the expression of p53 in doxorubicin treated cells (Fig. 4g).

Taken together, these results suggest that targeting p38 can overcome adaptive resistance to doxorubicin treatment.

CD24/DxR cells become proliferative after a long-lasting period in dormancy. The capacity of slow-cycling cells to reentry into cell cycle has been a topical debate for quite some time. As reported above, CD24$^+$/DxR cells have adopted a slow-down cell cycle after doxorubicin treatment. This was evidenced by a reduction of cyclin D1 (Fig. 3c).

So, we sought to monitor CD24$^+$/DxR cells to evaluate the reversibility of their dormant state according to the scenario presented in Fig. 5a. As shown in Fig. 5b by fluorescence microscopy, CD24$^+$/DxR cells have acquired an enlarged cell morphology, which is a hallmark of dormant cells (Fig. 5b). CD24$^+$/DxR cells were cultured in drug-free medium for a long period, and then, submitted to a serum deprivation for 2 days followed by culture in medium with 10% of serum. In such conditions, we observed the emergence of revertant cells (named DxR/30) which reacquired the ability to proliferate as confirmed by their capacity to incorporate BrdU (Fig. 5c).

The percentage of CD24$^+$ cells in the revertant-population recovered the levels observed in naïve MDA-MB-231 cell population (Fig. 5d). Importantly, even in the absence of the drug, the phosphorylation of p38 in DxR/30 remained strong and constitutive, which indicates that DxR/30 cells have conserved some features of their precedent states while eliminated others (Fig. 5e).

When we evaluated the drug resistance of DxR/30 cells, more than 1 month after the first treatment, the cells remained tolerant to doxorubicin. As shown in the light microscopy images, the morphology of these cells appeared little affected after treatment when compared to naïve MDA-MB-231 treated cells. More surprisingly, DxR/30 cells retained their capacity to proliferate even in the presence of drug (5 days). After 12 days of treatment, DxR/30 cells have re-colonized the plastic dishes (Fig. 5f). One of the hypothesis is that DxR/30 cells might have a competitive advantage over naïve cells under drug stress due to their constitutive phosphorylation of p38. Finally, we evaluated the migratory capacity of these cells by using the in vitro wound healing assay, which is based on the creation of an artificial gap, so called "scratch", on a confluent cell monolayer. Images were captured every 24 h during cell migration, the scratch was measured and a comparison of time required to close the scratch between naïve cells and DxR/30 was performed. The incubation time was determined at 48 h when the faster moving cells DxR/30 were just about to close the scratch. The confirmation of the migratory capacity of both cells was made with the Image J software (Fig. 5g). These data confirm that DxR/30 cells have acquired a new identity, conserving some traces of naïve cells and acquiring markers of chemoresistance.

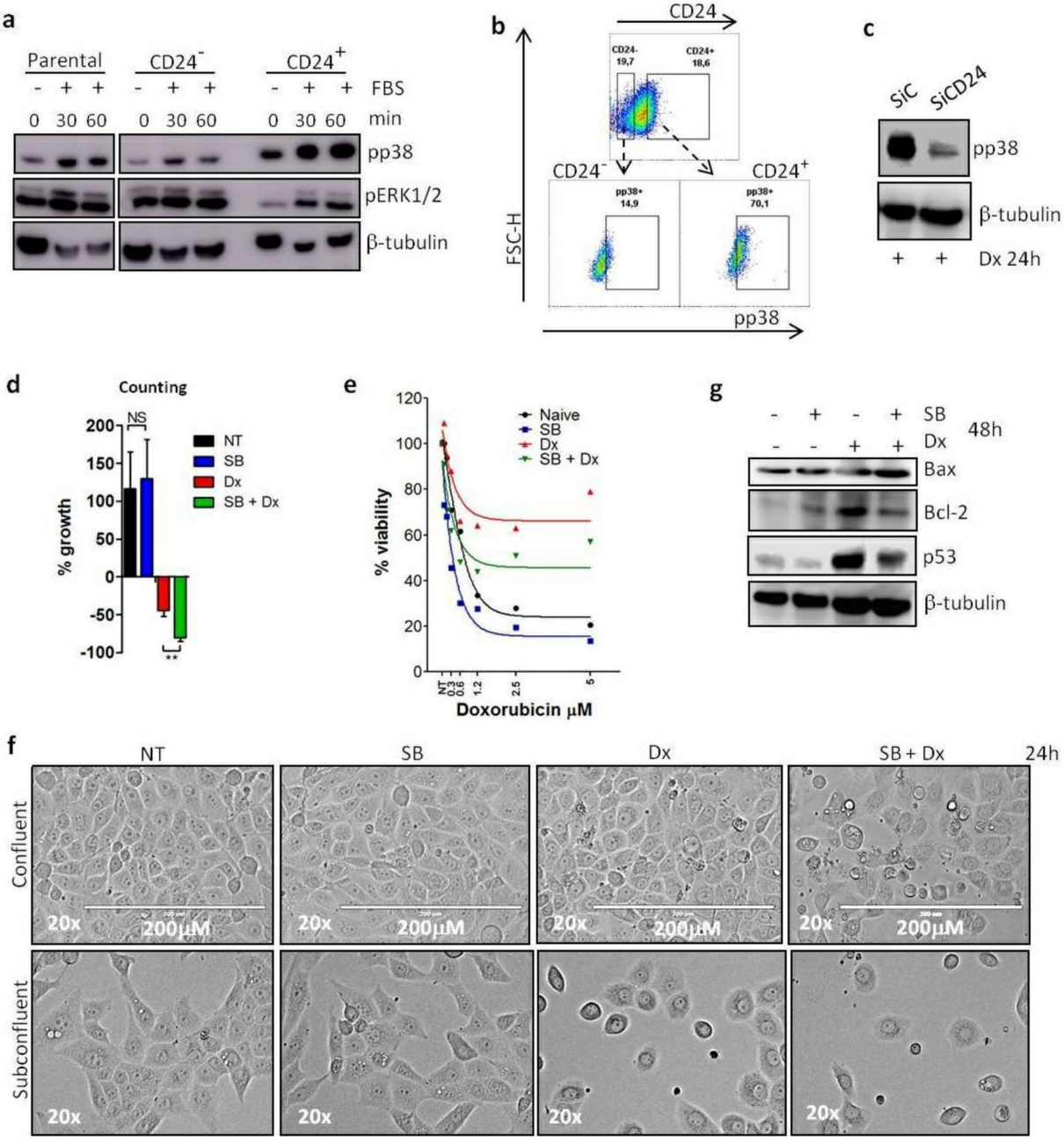


Figure 4. Inhibition of p38 activity impedes phenotype switching of MDA-MB-231 cells under doxorubicin treatment. (**a**) Differential activation of MAPKs in $CD24^+$ and $CD24^-$ subpopulation. Parental and the magnetic sorted $CD24^+$ and $CD24^-$ cells were starved for 2 h. Then, cells were submitted to time kinetic using fetal bovine serum (FBS) as stimulus. Cell lysates were immunoblotted with the depicted antibodies. The results are representative of two independent experiments. (**b**) Higher p38 phosphorylation (pp38) MAPK in $CD24^+$ cells. Parental MDA-MB-231 cells were stained with anti-CD24/Pe-Cy7 (extracellular staining) and anti-pp38/FITC (intracellular staining). The pseudocolor plots are representative of triplicates. (**c**) Decreased phosphorylation of p38 (pp38) in SiCD24 cells treated with Dx. SiC and SiCD24 cells were treated with Dx (0.6 μM) for 24 h. Cell lysates were immunoblotted with the depicted antibodies. All blots were performed at least twice. (**d**) Increased efficiency of Dx when associated with the p38 inhibitor SB203580. Cells were treated with SB203580 (SB) at 10 μM or/and Dx at 0.6 μM for 48 h. Then, cells were counted and the growth percentage was calculated based on the initial number of cells before each treatment (% growth = $[fn/in - 1] \times 100$)[#]. Countings were performed in three independent experiments. (**e**) Reduction in the acquisition of Dx resistance in the presence of the p38 inhibitor SB203580. MDA-MB-231 cells were pre-treated with SB203580 (10 μM) and/or Dx (0.6 μM) during 48 h before the addition of Dx at different concentrations for 24 h and then cell viability was measured by MTT assay. Curves were fitted with non-linear regression as means of triplicates. (**f**) Representative light microscopy images of cell population at confluence (10^6 cells) or subconfluence (5×10^5 cells) density treated with SB203580 (10 μM) and/or Dx (0.6 μM) for 24 h. Cell density and morphology were evaluated by microscopy (Evos Skedda). The microscopy images are representative of triplicates. (**g**) Increased expression of Bax but decreased expression of Bcl2 and p53 after cell treatment with the association of SB203580 and Dx. MDA-MB-231 cells were treated with SB203580 (10 μM) and/or Dx (0.6 μM) and cells were lyzed after 48 h treatment. Representative western blotting is shown and the data have been reproduced twice. [#]*fn* final number of cells, *in* initial number of cells.

According to our data, slow-cycling cells under stress may reentry into cell cycle, which gave to cells new properties, including higher drug resistance and higher migratory capacity, reaffirming that they have acquired a new identity.

Discussion

Solving the drug resistance problem in cancer remains a big challenge, which includes the need for a better tracking of "persistent" tumor cells after treatment. Robust evidence showed that the CSC subpopulation is enriched after chemotherapy, suggesting that this subset is responsible for the majority of treatment failure[32,33]. In this context, the relevant question is how can we summarize the tumor heterogeneity using a few pertinent markers to predict cell behavior under drug treatment. Here, using CD24 as marker, we provide new insight about the mechanisms leading to the emergence of "persistent" subpopulation during chemotherapy. By monitoring the CSC marker CD24 during doxorubicin treatment in MDA-MB-231 breast cancer cells, we have shown the uniform conversion of $CD24^-$ population into $CD24^+$ cells, which we have identified as drug-resistant cells.

However, the translocation of CD24 observed in MDA-MB-231 cells under drug stress is not restricted to doxorubicin treatment since the cell incubation with staurosporine, another anticancer drug, induced a similar cell phenotype switching. Further, when we treated other breast cancer cell lines like MGSO-3 and MACL-1 with doxorubicin an increased expression of CD24 at cell surface was also detected. Importantly, MDA-MB-321, MGSO-3 and MACL-1 cell lines share some important features like metastatic properties, aggressive character, the presence of an intracellular pool of CD24 and the capacity to modulate membrane CD24 expression under stress. According to our data, CD24 translocation could represent a more global phenomenon linked to tumor cell plasticity that may overtake specific mechanism of resistance. These different considerations highlight the relevance of the use of CD24 as a marker of cell plasticity and transition state in aggressive breast tumor cells.

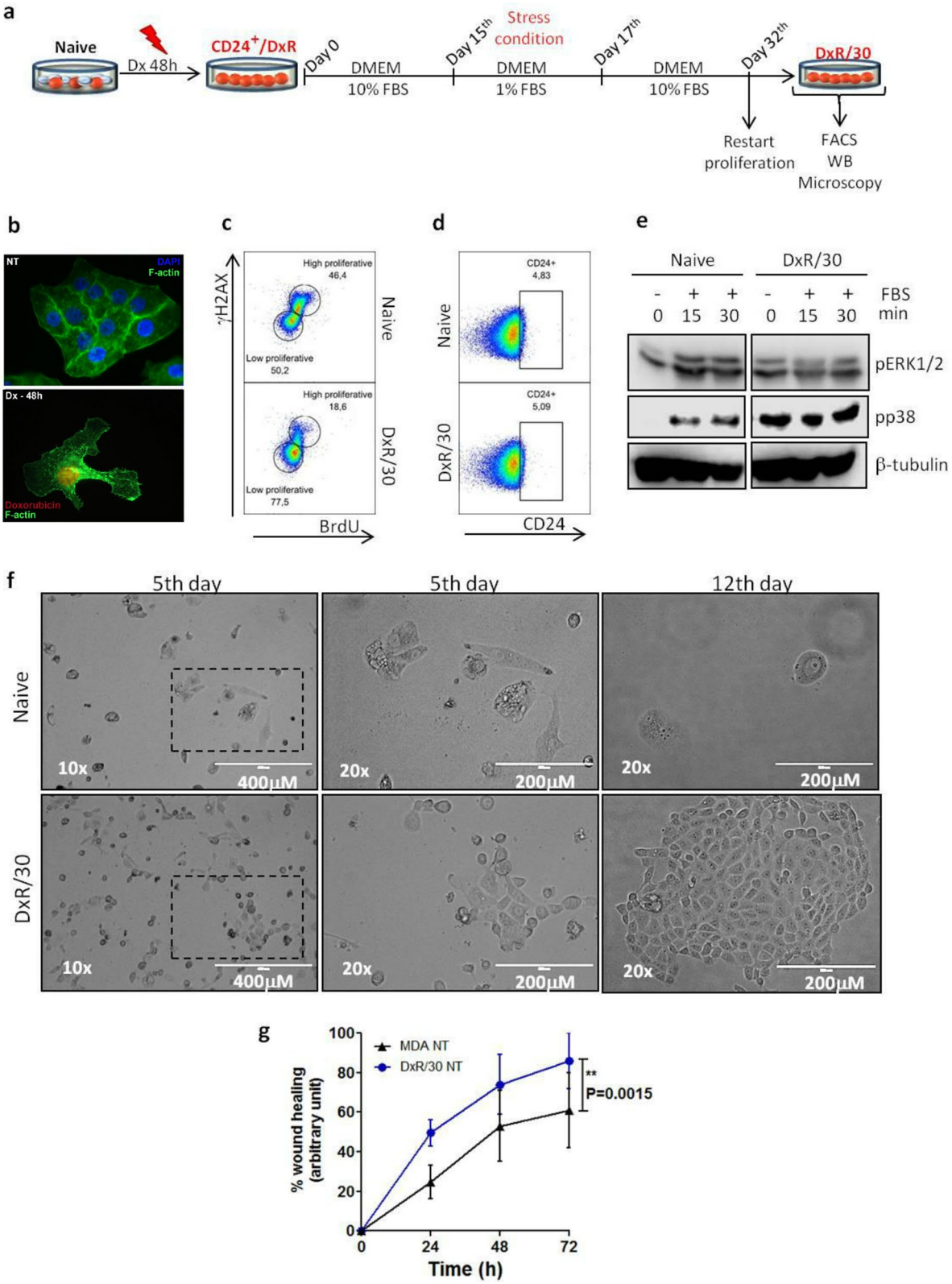
In the same line, Goldman et al. have reported that the treatment of breast or ovarian cancer cells with high concentration of taxanes results in the generation of "persistent" cells, which are defined by a transition towards a $CD44^{Hi}CD24^{Hi}$ expression status[12]. Others studies have demonstrated an enrichment for CSC-like phenotype after chemotherapy in glioblastoma[34] indicating how the phenomenon is ubiquitous.

Here, we provide evidence that translocation is the most reasonable hypothesis to explain a rapid appearance of CD24 (within 2 h) at the cell surface under drug stress**.** According to that, each given breast tumor cell may convert into $CD24^+$ phenotype made possible by the presence of an intracellular pool of CD24. Such findings suggest that the presence of CSC marker in cell membrane does not correspond to a clonal identity but rather to a state as also claimed by Dirske et al.[34]. The systematic conversion of $CD24^-$ into $CD24^+$ after chemotherapy demonstrates that cell plasticity emerges as an important contributor to therapy escape. This was verified when we used the TLR7 agonist Imiquimod, capable of reducing MDA-MB-231 cell proliferation without inducing translocation of $CD24^+$, nor changes in the expression of the anti-apoptotic protein Bcl-2. We were able to correlate these data with the absence of resistance to a second Imiquimod treatment. In this regard, great efforts have been directed towards finding small molecules to inhibit these anti-apoptotic Bcl-2 family proteins, and thus, to tackle anti-apoptotic adaptation of tumor cells[35].

Hence, targeting cell plasticity should provide a unique opportunity to improve the efficiency of existing therapies. Tumor cells have been shown to hijack signaling pathways involved in reprogramming to become plastic and evolve towards drug-refractory cells. So, the identification of these signaling pathways may be a key to hamper chemoresistance. The tandem constituted by CD24 and the p38 MAPK appears crucial in the cell fate of MDA-MB-231 population. A preferential use of p38 in $CD24^+$ cells have been noted under normal conditions, which has been amplified under drug treatment in accord with the dynamics of CD24. The relationship between CD24 and p38 is supported by the incapability of SiCD24 cells to activate p38 under drug stress. Indeed, we hypothesized that the change in the dynamics of p38 activation, which turned sustained early after CD24 translocation, is linked to the increased surface CD24 expression observed in doxorubicin treated cells. In this regard, a very recent paper reported that MAPK cascade signaling dynamics (transient to sustained activation) may be controlled by the activation kinetics of a given membrane receptor and not necessarily by the intracellular topology of the kinase networks. In the same study, it was proposed that redirecting signal dynamics may be a more fruitful and effective approach than controlling receptor activation in pathologic situation[36].

In other tumor models, doxorubicin resistance mechanism seems to use some common molecular features involving p38 signaling pathway. Downregulation of stemness/EMT pathways like Notch-1 and Wnt/β-catenin, alongside of downregulation of STAT3, a well-known p38 downstream target, avoided doxorubicin-resistance in enriched $CD44^-/CD24^+$ subpopulation of MCF-7[37]. Pharmacological downregulation of AKT, another p38 downstream target, also reduced doxorubicin resistance in non-small cell lung cancer[38]. Yet, downregulation of STAT3 and Bcl-2 by miRNA reduced doxorubicin-resistance in breast cancer[39,40]. This is why we considered the profile of p38 activation as an important marker of the resistant cell identity ($CD24^+$/DxR) and a key target to hinder phenotype switching during doxorubicin treatment.

The relevance of this strategy was verified when we compared the protein expression pattern of $CD24^+$/DxR cells and naïve MDA-MB-231 cells when they were treated with doxorubicin in the presence of the p38 inhibitor SB203580. The overexpression of Bcl-2, indicator of adaptative cellular reprogramming and also marker of premature senescence[41–43] observed in $CD24^+$/DxR was not detected in cells treated with the combination of SB203580 and doxorubicin. The benefit of this association was translated into a synergism of the cytotoxic effect of the drug pair. The impact of SB203580 on cell fate may not only correlate with Bcl-2 expression but also with p53 levels since we have observed a modulation of p53 expression in the SB203580 treated cells. Recently, p53 was also considered as a marker of cell reprogramming and acquisition of stemness[44]. These data sustain the idea that p38 is an active player in drug resistance by inducing cell identity changes, notably increasing cell survival marker expression.


a
Naive
Dx 48h
CD24+/DxR
Day 0
DMEM
10% FBS
Day 15th
Stress condition
DMEM
1% FBS
Day 17th
DMEM
10% FBS
Day 32th
DxR/30
Restart proliferation
FACS
WB
Microscopy
b
NT
DAPI
F-actin
Dx - 48h
Doxorubicin
F-actin
c
γH2AX
High proliferative
46,4
Low proliferative
50,2
Naive
High proliferative
18,6
Low proliferative
77,5
DxR/30
BrdU
d
CD24+
4,83
Naive
CD24+
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DxR/30
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0 15 30 0 15 30 min
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pp38
β-tubulin
f
5th day
5th day
12th day
Naive
DxR/30
10x
400μM
20x
200μM
g
MDA NT
DxR/30 NT
% wound healing (arbitrary unit)
Time (h)
**
P=0.0015

◂**Figure 5.** CD24⁺/DxR cells become proliferative and more invasive than naïve cells after a long-lasting period in drug-free environment. (**a**) Schematic shows the journey of CD24⁺/DxR cells from their switching to CD24⁺ phenotype to their entry in slow- cycling state and reversion into proliferative cells. CD24⁺/DxR cells were exposed to a stress condition (fetal bovine serum starvation = 1% FBS) during 48 h. (**b**) Differences in morphology between naïve MDA-MB-231 and CD24⁺/DxR cells. Naïve MDA-MB-231 and CD24⁺/DxR cells were fixed, permeabilized and stained using anti-F-actin/Alexa488 and the nuclear dye DAPI. (**c**) CD24⁺/DxR cells retrieve their capacity to proliferate (named as DxR/30 or revertant). Naive MDA-MB-231 and DxR/30 cells were incubated with 10 µM of BrdU (thymine analogue base) for 4 h. Then, cells were stained with anti-BrdU/PerCP-Cy5.5 and anti-γH2AX/Alexa647 (intracellular staining). The low and high proliferative subpopulations were defined and analyzed. The pseudocolor plots are representative of triplicates. (**d**) DxR/30 cell population returns to the basal ratio of CD24⁺ cells. Cells were stained with anti-CD24/Pe-Cy7 (extracellular staining). Pseudocolor plots are representative of triplicates. (**e**) Constitutive activation of p38 MAPK in DxR/30 cells. After starvation for 2 h, cells were stimulated with serum in different times. Cell lysates were immunoblotted with the depicted antibodies. All blots were performed at least twice. (**f**) Higher resistance of DxR/30 cells to Dx treatment. MDA-MB-231 and DxR/30 cells were treated with Dx (0.6 µM) for 24 h. Then cells were counted, re-seeded in the same density and cultured in normal conditions (DMEM 10% FBS). Cell density and morphology were analyzed at 5 and 12 days post-Dx treatment. (**g**) DxR/30 cells are more invasive than MDA-MB-231 cells. MDA-MB-231 and DxR/30 cells were seeded (8×10^5) in 6 wells plate and a scratch performed according to Mat and Med. Closing-time percentage was calculated based on the initial scratch size of each cell type. Curves were fitted as means of duplicates ± SD. $^{**}p < 0.01$ (two-way ANOVA with Bonferroni post-test).

To better assess the long term consequences of the rapid phenotype switching after drug treatment we have evaluated whether the slow-cycling state of CD24⁺/DxR cells was reversible after several weeks in drug-free medium since several studies have demonstrated the reversibility of senescence[45–48]. In fact, the revertant cells (named DxR/30), recovered their capacity to proliferate and importantly, have gained higher drug resistance and stronger migratory properties. This could be attributed to the fact that p38 is constitutively activated in these cells. Interestingly, in the absence of the drug pressure and in proliferative conditions, the DxR/30 population retrieved a basal level of CD24⁺ cells. This follows mathematical models that tend to establish that tumor cell populations always maintain its heterogeneity at fixed ratio in dynamic conditions like proliferation[49].

Thus, here we have established a model to accompany the journey of MDA-MB-231 cells after drug treatment starting from their switching to CD24⁺ phenotype to their entry into slow-cycling state and reversion into proliferative cells. Notably, the sustained p38 phosphorylation observed in all the post-treatment stages may also indicate the participation of this MAPK in the network that successfully promotes the formation of metastasis at distant organs. Previous studies have shown that the EMT (Epithelial Mesenchymal Transition) program has been involved in the distant metastases frequently detected following chemotherapy[50] which could also include p38. The monitoring of cell evolution throughout this journey has shown that they have conserved some features of previous state while eliminated others. In other words, they have acquired hybrid properties corresponding to a new identity.

This study reaffirms how a better understanding of the biology and molecular drivers of the cellplasticity will enable identification of new anticancer targets. The pertinence of using a leading drug with an inhibitor of cell reprogramming was illustrated by the high efficiency of the combination of p38 inhibitor and doxorubicin on the killing of aggressive MDA-MB-231 breast cancer cell line as schematized in the Fig. 6.

Materials and methods

Reagents. *Thiazolyl Blue Tetrazolium Blue* (MTT), Doxorubicin, Staurosporine and RIPA buffer were purchased from Sigma-Aldrich. p38 inhibitor SB203580 and Imiquimod were obtained through InvivoGen. Protease cocktail inhibitor and phosphatase cocktail inhibitor (Roche) were acquired from Sigma Aldrich. Monoclonal antibodies against phospho-p44/42 (ERK1/2), phospho-p38 MAPK, p38 MAPK, cyclin D1, β-tubulin, β-actin and peroxidase-conjugated goat anti-mouse IgG and goat anti-rabbit IgG were obtained from Cell Signaling Technology and anti-Bcl-2 (Novex) and anti-Bax (Invitrogen) from Thermo Fisher Scientific. Apoptosis, DNA Damage and Cell Proliferation Kit from BD Biosciences and *Lipofectamin 300 transfection reagent* (Invitrogen) from Thermo Fisher Scientific. SiRNAs for CD24 silencing (Cat# Hs01 00232148) *and for control (Cat# SIC001)* were synthetized by Sigma-Aldrich. *PVDF membranes (0.45 µM) and Luminat (Millipore) were acquired from Merck and nitrocellulose membrane (022 µM) from Bio-Rad.*

Cell culture conditions. MACL-1 and MGSO-3 cell lines were previously established by Goes et *al.* from Brazilian patients with breast cancer[51]. MDA-MB-231 cell line was obtained from ATCC (USA). Cells were grown at 37 °C in a humidified atmosphere of 5% CO_2 in DMEM supplemented with 10% heat inactivated fetal bovine serum (FBS). For starvation conditions, cells were incubated with serum-free DMEM for 2 h before the addition of the respective stimuli.

Obtaining of the resistant cell clones from parental MDA-MB-231. The procedures to obtain MDA-MDA-231 cells resistant to doxorubicin (named CD24⁺/DxR cells) are schematized in Fig. 4a. Briefly, MDA-MB-231 cells were incubated with doxorubicin at 0.6 µM for 48 h. The remaining cells, named CD24⁺/DxR, were trypsinized and used in the respective experiments. These cells were characterized as non-proliferative. DxR/30 achievement is schematized in Fig. 5a. After obtaining CD24⁺/DxR cells, these cells were cultured

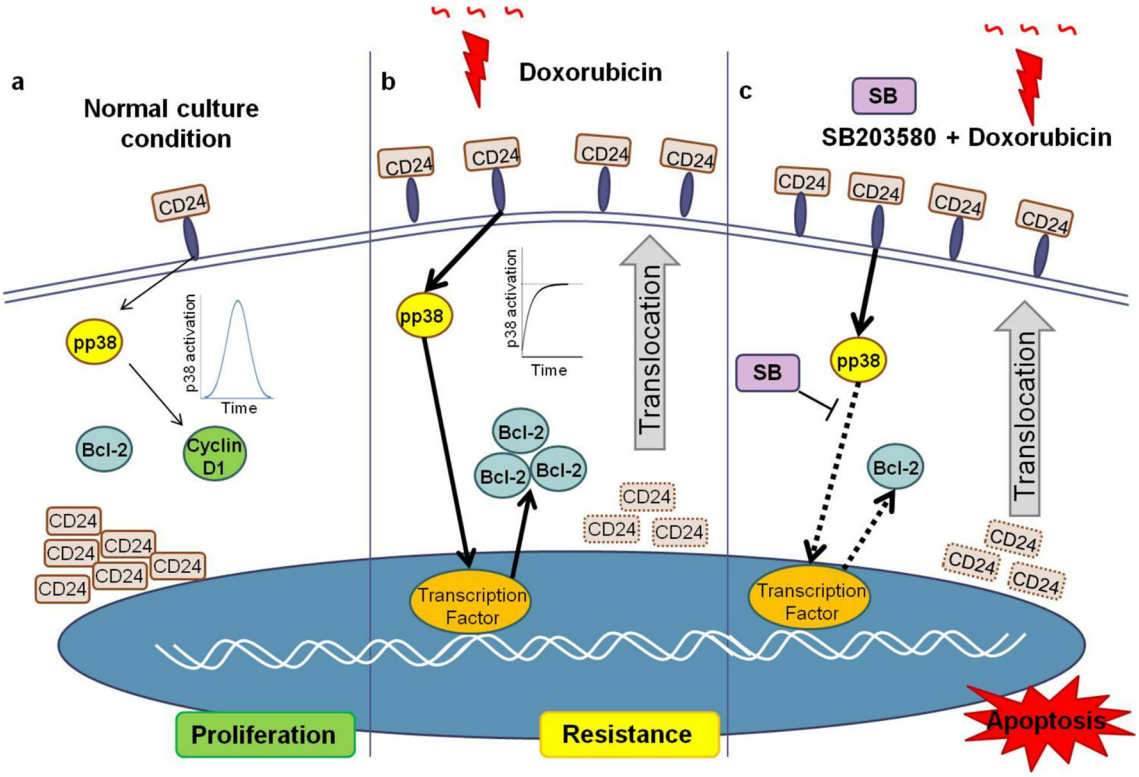

Figure 6. Schematic shows breast cancer cell population phenotype changes under drug stress toward a drug tolerant state arising through translocation of CD24 and sustained p38 activation. (**a**) In proliferative conditions, CD24 is mainly detected in the cytosol of all cells and the population is characterized by a low frequency of $CD24^+$ cells (cells carrying CD24 in membrane). The transient phosphorylation of p38 leads to cyclin D1 expression and cell cycle progression. (**b**) Under drug treatment, CD24 translocation associated with an increased CD24 density on cell membrane occurs in every given cell. In accord with this, a sustained and strong p38 activation, an increased Bcl-2 expression are observed indicating a phenotype switching. (**c**) This transition state is sensitive to p38 inhibition since the use of a specific inhibitor, like SB203580, in association with doxorubicin reduces Bcl-2 expression leading to an increased cell death.

with DMEM 10% of fetal bovine serum for 15 days, then cells were cultured under stress condition (DMEM 1% of fetal bovine serum for 48 h) following by incubation in normal culture conditions until they recover their proliferative activity. From then, cells were named DxR/30. In order to obtain MDA-MDA-231 cells resistant to Imiquimod, MDA-MB-231 cells were incubated with Imiquimod at 1 µg/ml for 96 h. The remaining cells were trypsinized and used in the respective experiments.

MTT (viability assay). For doxorubicin assay, MDA-MB-231 cells (10^4/well) were plated in 96-well plates in DMEM with 10% serum. Cells were incubated for 24 h with different stimuli according to the figures. For Imiquimod assay, MDA-MB-231 cells (10^4/well) were plated in 24-well plates in DMEM with 10% serum. Cells were incubated for 96 h with different stimuli according to the figures. After the respective treatment time, the medium was removed and cells were incubated with 50 µl/well of DMEM 10% FBS plus of 40 µl/well of MTT (5 µg/ml) for doxorubicin assay or µl/well of DMEM 10% FBS plus of 40 µl/well of MTT (5 µg/ml) for Imiquimod assay for 2 h at 37 °C. After removing the medium, 50 µl (for doxorubicin) or 200 µl (for Imiquimod) of DMSO were added to dissolve the crystals of formazan and the absorbance was measured in a microplate reader at a wavelength of 595 nm. A value of 100% was assigned to untreated control cultures. Results were derived from at least three independent sets of triplicate experiments.

RNA silencing. MDA-MB-231 cells were seeded in 24-well plates at a density of 2×10^4 cells/well. After 24 h of incubation, the cells were transiently transfected with short interfering RNA (siRNA) specific for CD24 or control siRNA at a final concentration of 100 nM by using Lipofectamin 3000 Transfection Reagent according to manufacturer's instructions. Briefly, siRNA and Lipofectamin were diluted separately in serum-free OPTI MEM. Then, the diluted Lipofectamin and siRNA were mixed (1:1 v/v). After 5 min, the mix was added drop-wise onto the cells under their normal growth conditions and after 48 h silenced cells were used in the different assays. CD24 silencing was confirmed by western blotting.

Western blotting. For protein expression, cells were harvested, counted and lysed in RIPA buffer supplemented with phosphatase and protease inhibitor cocktail according to the manufacturer's instructions. For the

evaluation of the phosphorylated form of MAPKs, cells were seeded in 6-well plates (10^6 cells/well) and were starved for 2 h in free-serum medium (SFM). Then, cells were treated for 30–60 min with medium containing 10% FBS as stimuli. At the end of treatment cells were lysed in RIPA buffer supplemented with phosphatase and protease inhibitor cocktail according to the manufacturer's instructions. Protein lysates were separated by polyacrylamide gel electrophoresis on 10% or on 15% gels when concerned histone extract, and electrotransferred to PVDF-0.45 µM membranes (Milipore) or nitrocellulose-0.22 µM (BioRad). Membranes were blocked overnight with 5% dry milk and were incubated with primary antibodies in 5% BSA also overnight. After incubation with the peroxidase-conjugated secondary antibody for 1 h, protein expression was detected using Luminat HRP reagent (Milipore) and analyzed using LAS-4000 imaging system (Fuji) or C-DiGit Blot Scanner (Li-Cor).

Magnetic sorting. The different cell subpopulations ($CD24^+$ and $CD24^-$) were sorted from parental MDA-MB-231 cells using *CD24 MicroBead Kit* (*MACS Miltenyi Biotec*) following manufacturer's instructions. Briefly, the $CD24^+$ cells were indirectly magnetically labeled with CD24-Biotin antibodies and Anti-Biotin MicroBeads. Then the cell suspension was loaded onto a MACS Column, which is placed in the magnetic field of a MACS Separator. The magnetically labeled $CD24^+$ cells were retained within the column. The unlabeled cells ($CD24^-$ cells) ran through; this cell fraction was thus depleted of $CD24^+$ cells. After removing the column from the magnetic field, the magnetically retained $CD24^+$ cells can be eluted as the positively selected cell fraction. The purity of the sorted populations was verified by Flow Cytometry.

Flow cytometry analysis. To evaluate the proliferative activity of cells, an assay using the Apoptosis, DNA Damage, and Cell Proliferation Kit from BD Biosciences was realized in accord with the manufacturer's instructions. Cells were cultured as indicated in the figure legends and washed/blocked in staining buffer (PBS 4% fetal bovine serum—v/v). Cells were fixed and permeabilized using *CitoFix/CitoPerm* reagent for 20 min on ice, nucleus was permeabilized using *CitoFix/CitoPerm* Plus for 10 min on ice and finally treated with DNAse (30 µg/10^6cells) for 1 h at 37 °C in humidified chamber. Then, cells were simultaneously stained with anti-BrdU/PerCP-Cy5.5 and anti-γH2AX/Alexa647 (BD Biosciences) fluorescent antibodies for 20 min at room temperature. Between every step cells were washed with 1 × PBS/PermWash (BD Biosciences). Concerning CD24 and p38 staining**,** the following step by step was realized**.** For extracellular staining, MDA-MB-231 cells were washed/blocked in staining buffer (PBS 4% fetal bovine serum—v/v) and were labeled using anti-CD24/Pe-Cy7 (eBioscience) or anti-CD24 (BD Pharmigen) for 20 min on ice, and when necessary, followed by FITC-conjugated equivalent secondary antibody (BD Pharmigen) for more 20 min on ice. For intracellular staining, after extracellular labeling, cells were fixed and permeabilized using *CitoFix/CitoPerm* reagent for 20 min on ice. Then, cells were incubated with anti-CD24/Pe-Cy7 (eBioscience) and/or with anti-pp38 (Cell Signaling) for 20 min at room temperature, and when necessary, followed by FITC-conjugated secondary antibody (BD Pharmigen) for 20 min at room temperature. Between every step cells were washed with 1 × PBS/PermWash (BD Biosciences). After labeling protocols, cells were fixed in PFA 4% overnight (4 °C lightless). Cells were re-suspended in isoton buffer and analyzed by flow cytometry. Single-stain controls were used to set gating parameters and any compensation. All flow cytometry results were analyzed by FlowJo software following a rigorous doublet discrimination based on FSC-A versus FSC-H. Data were collected by the cell analyzing LSRFortessa (BD Biosciences-Immunocytometry Systems) using "BD FACSDivaTM Software" (BD Biosciences) and analyzed with "FlowJo (Tree Star) Software".

Wound healing assay. MDA-MB-231 and DxR/30 cells were seeded (8×10^5) in 6 wells plate and let in growing conditions (DMEM 10%FBS, at 37°, 5%CO_2) for 24 h or until cells get confluent. Then, using a 100 µl tip, a scratch was performed in the cell monolayer and cells were cultured in DMEM supplemented with 2% of FBS in order to avoid cell proliferation. To obtain the same field during the image acquisition, pen markings were performed at the bottom of the culture plates as reference points close to the scratch. Cell migration was registered by light microscopy (Evos Skedda) at 24, 48 and 72 h post scratch. Using ImageJ software the wound healing was measured and a closing-time percentage was calculated based on the initial scratch size of each cell type.

Statistical analysis. The data were presented as mean of triplicates ± SD or as means of triplicates. Statistical significance was determined using Student's t test, or two-way ANOVA followed by Bonferroni post-test. The criterion for statistical significance was $p < 0.05$.

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Author contributions

H.W.H. and C.R. conceived, designed and performed the experiments, analyzed the data and wrote the paper. A.M.G. contributed with reagents/materials/analysis tools. T.C.G. designed and analyzed the data of fluorescence microscopy experiments.

Competing interests

The authors declare no competing interests.

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4.5 - Paper 4 (manuscrito em preparação)

Imiquimod controls aggressive breast cancer cell growth without stemness induction

Imiquimod controls aggressive breast cancer cell growth without stemness induction

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Introduction

The resistance to chemotherapy is a major problem facing current cancer research. The mechanisms of resistance to "classic" cytotoxic chemotherapeutics share many features, such as alterations in the drug target, mechanisms of drug efflux or activation of prosurvival pathways leading to ineffective induction of cell death (Agarwal and Kaye, 2003; Housman *et al.*, 2014).

Indeed, cancer cells often adopt anti-apoptotic defense mechanisms in response to oncogenic stress or anti-cancer therapy which corresponds to a disbalanced expression of Bcl-2 family protein, which Bcl-2 is the founding member (Evan and Vousden, 2001). In this context, the anti-apoptotic protein Bcl-2 became a sensor of drug resistance and stands as an attractive therapeutic target. Recently, we have correlated the overexpression of Bcl-2 and the presence of the Cancer Stem Cell (CSC) marker CD24 with drug resistance in a model of aggressive breast cancer (Huth *et al*, 2020 – paper 3). In this study, the dynamics of the acquisition of drug resistance associated with elevated levels of Bcl-2 corresponded to a translocation of CSC marker from cytosol to membrane after chemotherapy. The fact that cancer cells often present anti-apoptotic adaptation to ensure survival against oncogenic stress or anti-cancer therapy explains the great efforts have been directed towards finding small molecules to inhibit the anti-apoptotic Bcl-2 family proteins and promote apoptosis in cancer. Generally, this new class of cancer drugs mimiks the natural inhibitors of Bcl-2, the BH3 proteins (Yang *et al.*, 2015; Davids, 2017).

However, the use of the anti Bcl-2 strategy is complexe due to several side effects reported like the tumor lysis syndrome (TLS) – that occurs most commonly after treatment with lymphomas and leukemia – and the absence of markers for monitoring the treatment (Roberts *et al.*, 2016). The search for new compounds able to control Bd-2 family without inducing TLS represents a challenge. Interestingly, some studies have revealed the apoptotic activity of Imiquimod – a TLR7 agonist – in cancer cells. In this line, the treatment of cutaneous metastatic breast cancer with topical Imiquimod treatments showed promising results (Schön and Schön, 2008; Adams *et al.*, 2012; Krishnasamy *et al.*, 2018; Sharma *et al.*, 2019; Verga, Chohan and Verdolini, 2019). From the numerous TLR agonists, only TLR7 agonists have been approved by Food and Drug Administration (FDA) as a therapeutic agent for basal cell carcinoma and genital warts (Galluzzi *et al.*, 2012). The TLR7 agonist Imiquimod was originally included in cancer therapy strategy to activate dendritic cells and is currently restricted to topical use in skin cancer.

The different studies exploring the efficacy of Imiquimod in cancer put into question the mechanism of action of this compound. Here, we sought to define whether Imiquimod may directly interact with MDA-MB-231 aggressive tumor cells derived from breast cancer which represents the second most common type of cancer diagnosed worldwide (WHO 2018).

According to our results, a reduction of the tumor cell proliferative activity was observed in the presence of Imiquimod at immunostimulatory concentrations leading to a significant reduction of cell number after treatment. Importantly, MDA-MB-231 tumor cells remain responsive to successive treatments with Imiquimod that we have correlated with the lack of CD24 translocation to membrane. These findings are in accord with a decreased expression of Bd-2 in MDA-MB-231 cells, the opposite situation observed with conventional chemotherapy drugs.

Results

Imiquimod inhibits MDA-MB-231 breast cancer cell growth

Here, we used MDA-MB-231 aggressive breast cancer cell line to evaluate the capacity of Imiquimod, a TLR7 agonist, to directly impact on aggressive tumor cell growth. If, one of the most appreciated functions of TLRs in cancer therapy is the stimulation of the adaptive immune system, TLRs may also act directly on tumor cells where a link between TLR signaling and cell cycle control has been addressed in previous studies. As shown in the figure 1A, a reduction of cell viability was observed in the presence of the TLR7 agonist in a concentration dependant way. The different curves comparing cell proliferation incubated with different

Imiquimod concentrations during several days confirmed the impact of Imiquimod on cell proliferation. A slowdown effect of Imiquimod at 1ug/ml on cell proliferation was noted while the effect of Imiquimod at the higher concentration was more pronounced reducing significantly the cell growth (Fig 1B). Results obtained from light microscopy showed that Imiquimod delayed the colony formation in a concentration and time dependent manner. In addition, the morphology of the cells treated with the higher drug treatment losted their original morphology and presented cytoplasmic vacuolization, which may predict a future cell death program (Fig 1C).

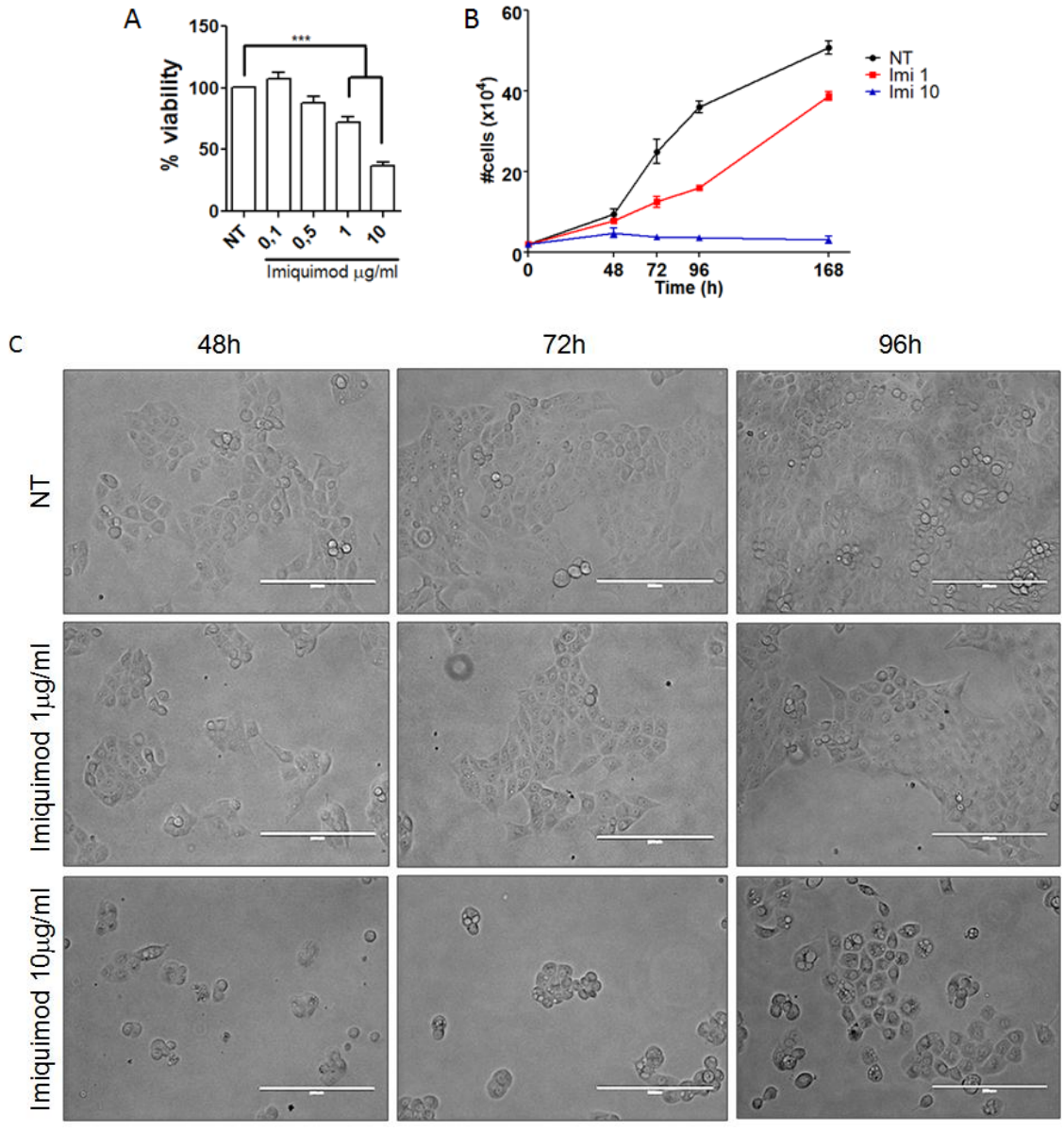

Figure 1: Imiquimod inhibits MDA-MB-231 breast cancer cell growth. (A) MDA-MB-231 cells were treated with Imiquimod at different concentrations for 96h and cell viability was measured by MTT assay. *** $p < 0,001$ (One-way ANOVA followed by Bonferroni post-test). **(B)** MDA-MB-231 cells were treated with Imiquimod (1µg/ml or 10µg/ml) for different time periods. Then, cells were trypsinized, counted and the curves were fitted as means of triplicates. **(C)** MDA-MB-231 cells were treated with

Imiquimod (1µg/ml or 10µg/ml) for different time periods. Light microscopy images were performed using Evos Skedda Microscopy to evaluate cell density and morphology. Images are representative of triplicates.

Imiquimod induces cell cycle arrest without inducing cell death in the first stage

To clarify the mechanisms underlying the growth reduction of MDA-MB-231 cells under Imiquimod treatment, we evaluated the impact of the TLR7 agonist on cell DNA replication using the incorporation of BrdU and phosphorylation of the histone H2AX (γH2AX) as proliferative markers. According to the results obtained by FACS, Imiquimod significantly reduced the incorporation of BrdU at 4h post treatment indicating that a switch from cell proliferation to cell cyde arrest occurred earlier in the presence of the drug . In addition, Imiquimod did not induce apoptosis after 4h and 18h of treatment as shown by the absence of cleaved PARP, an apoptosis marker. This is in accord with the MTT assay evaluating the cell viability of Imiquimod after 24h of treatment (Fig 1C).

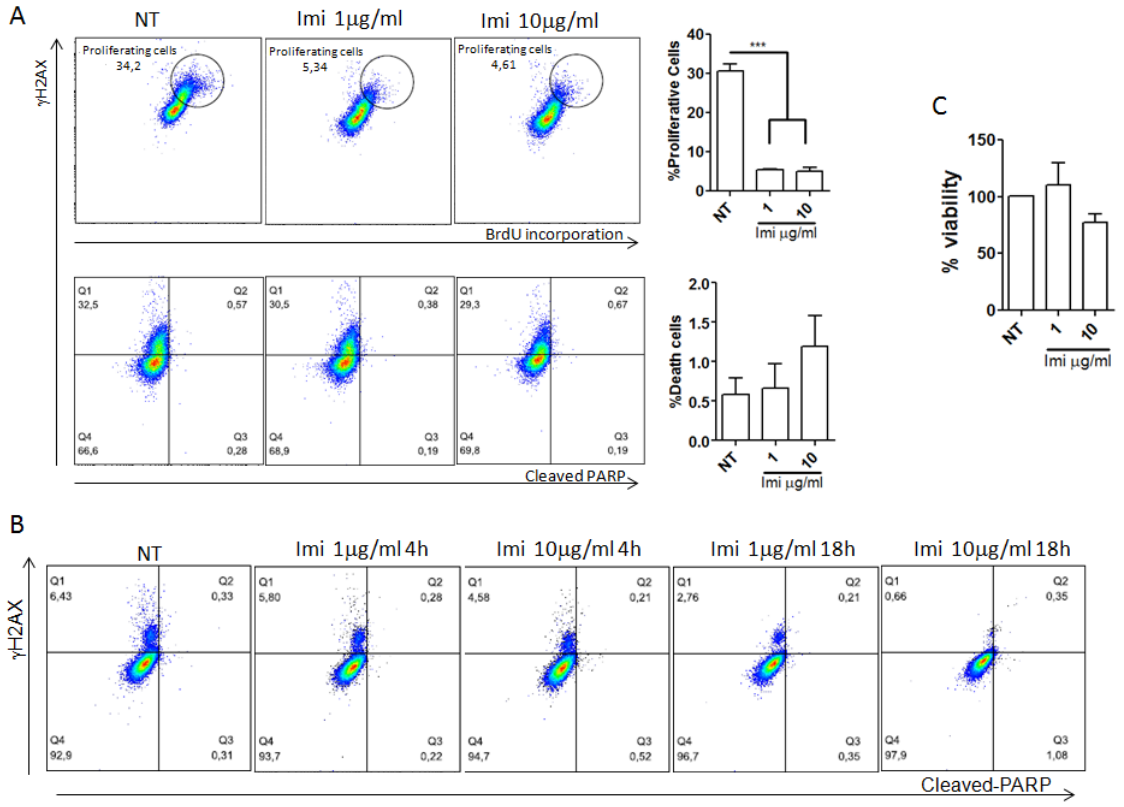

Figure 2: Imiquimod induces cell cycle arrest without inducing cell death in the first stage (A) MDA-MB-231 cells were treated with Imiquimod (1µg/ml or 10µg/ml) and incubated with 10µM of BrdU (thymine analogue base) for 4h. Then, cells were fixed, permeabilzed and stained with anti-BrdU/PerCP-Cy5.5, anti-γH2AX/Alexa647 and anti-cleaved-PARP/PE. The pseudocolor plots are representative of triplicates. Frequency of proliferative cells and dead cells are represented as means +/- SD of triplicates. t test *** $p < 0{,}001$. **(B)** MDA-MB-231 cells were treated with Imiquimod (1µg/ml or 10µg/ml) for 4h and 18h. Then, cells were fixed, permeabilzed and stained with anti-γH2AX/Alexa647 and anti-

cleavedPARP/PE. The pseudocolor plots are representative of triplicates. **(C)** MDA-MB-231 cells were treated with Imiquimod (1μg/ml or 10μg/ml) for 24h and cell viability was measured by MTT assay.

Imiquimod regulates proliferative and apoptotic pathways in MDA-MB-231 cells

NF-κB is an active player in human cancers where its constitutive activation has been shown in most cancers (ref). In this line, the involvement of NF-κB pathway has been reported in the proliferation of MDA-MB-231 cells. Here, we investigated whether Imiquimod impacted on the NF-κB signaling pathway, composed by different components like IKK, IκB, NF-κB (p65). The western blot analysis of the short kinetic of cells treated with Imiquimod at both concentrations showed a reduction of the phosphorylation of IKK, IκB and p65 (Fig 3A).

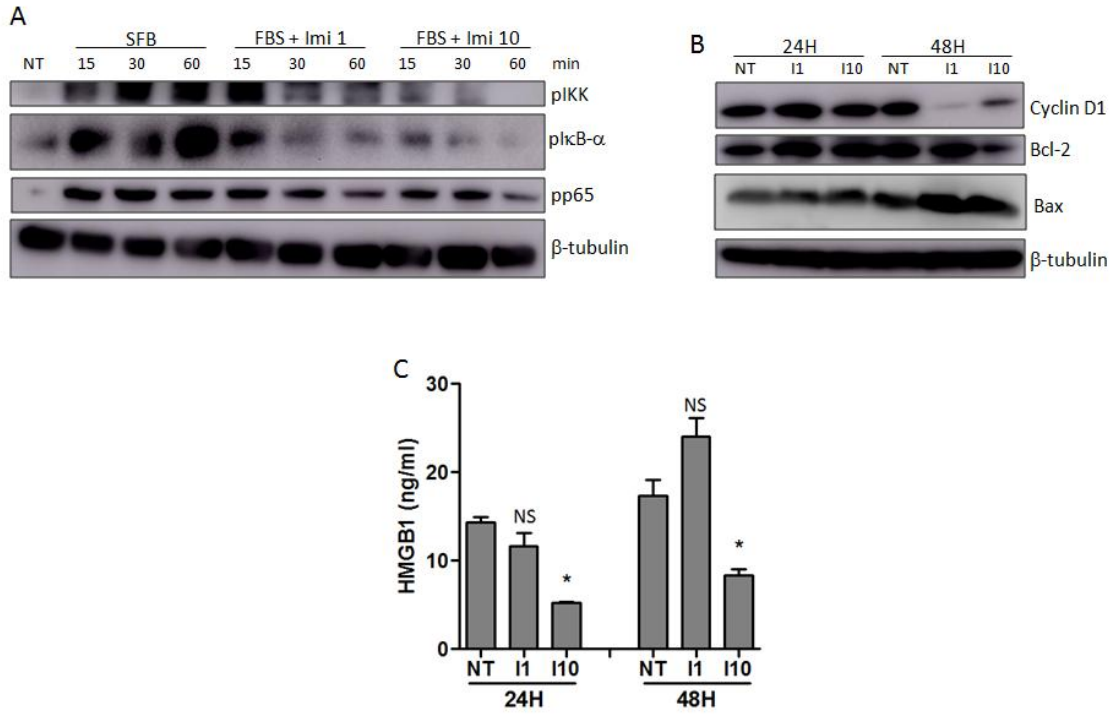

Figure 3: Imiquimod regulates proliferative and apoptotic pathways in MDA-MB-231 cells. (A) MDA-MB-231 cells were starved for 2h. Then, cells were submitted to a short time kinetic using Imiquimod (1μg/ml or 10μg/ml) in DMEM supplemented with fetal bovine serum (FBS) 10% as stimulus. Cell lysates were immunobloted with the depicted antibodies. The results are representative of two independent experiments. **(B)** MDA-MB-231 cells were treated with Imiquimod (1μg/ml or 10μg/ml) in DMEM supplemented with fetal bovine serum (FBS) 10% for 24h and 48h. Cell lysates were immunobloted with the depicted antibodies. The results are representative of two independent experiments. **(C)** MDA-MB-231 cells were treated with Imiquimod (1μg/ml or 10μg/ml) in DMEM supplemented with fetal bovine serum (FBS) 10% for 24h and 48h. Then, supernatant were collected and HMGB1 secretion were measured by ELISA.

In addition, we evaluated the capacity of Imiquimod to control the expression of proteins involved in cell fate. Concerning the cell cycle progression, it was observed a decrease of cell cyclin D1 expression after 48h in Imiquimod-treated cells, at both concentrations, when

compared with the control group. This may be explained by the diminution of BrdU incorporation and cell cycle arrest promptly after Imiquimod treatment. Such data are in accord with the capacity of Imiquimod to reduce NF-κB pathway activation. Further, we sought to determine the consequences in the longer term of the cell cycle arrest on cell survival by evaluating the molecular markers of apoptosis like Bax and Bcl-2, Bcl-2 promoting cell survival, whereas Bax antagonizing this effect. According to our data, the TLR7 agonist at the higher concentration downregulated Bcl-2 and increased expression of the pro-apoptotic protein Bax after 48h. Consequently, the deregulated balance Bax/Bcl-2 may promote cell death (Fig 3B). These results indicate that Imiquimod may induce cell cycle arrest followed by cell death at a late stage.

Next, we have evaluated the impact of Imiquimod on the microenvironment exemplified by the cytokine production and secretion by MDA-MB-231 cells. As shown by ELISA, Imiquimod at 10μg/ml reduced the secretion of HMGB1 (Fig 3C), a pro-inflammatory agent well described with a tumorigenic effect. This may be in accord with the fact that Imiquimod downregulated the NF-κB pathway, which is one of the responsible to induce the production and secretion of pro-inflammatory components.

Imiquimod did not induce CD24 translocation, H3K9me3 expression and drug resistance.

As reported by our group in another study, highly cytotoxic chemotherapy induces stemness-related chemoresistance, characterized by the conversion of non cancer stem cells (NCSC) into CSC after translocation of CD24 towards cell membrane. Taking this in account, we wondered whether Imiquimod was capable to induce translocation of CD24 and consequently to induce phenotype switching. As shown by our FACS results, Imiquimod treatment did not provoke CD24 translocation after 48 hours of treatment, maintaining the proportion of CSCs/NCSCs similar to the control group (Fig 4A). In addition, Imiquimod did not induce tri-methylation of the histone H3K9 which was already correlated with stemness properties (Fig 4B).

So, considering that Imiquimod did not induce stemness in MDA-MB-231 cells, we evaluated whether Imiquimod retained its efficacy after successive doses. By MTT assay, we verified that a pre-treatment of Imiquimod did not induced resistance to a further dose. Interestingly, cells that were pre-treated with Imiquimod at 10μg/ml did not recover their normal proliferation rate even after drug removal. This indicates that Imiquimod might induce a prolonged effect through cell reprogramming, keeping them on a lesser extent proliferation rate (Fig 4C).

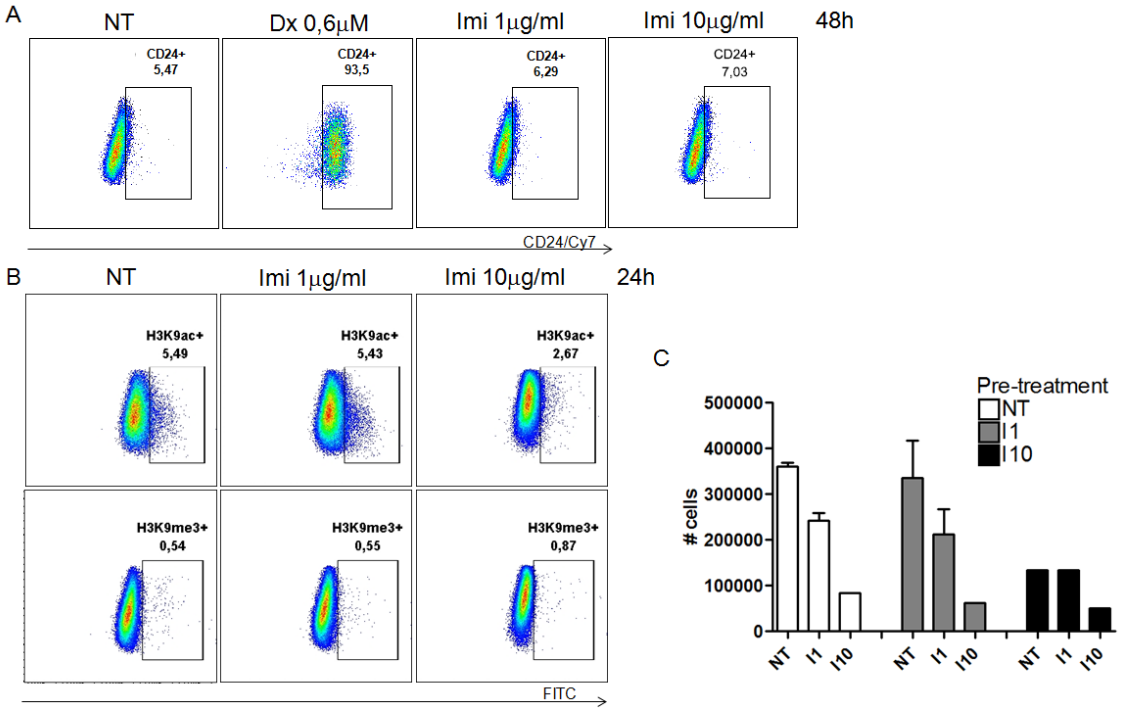

Figure 4: Imiquimod did not induce CD24 translocation, H3K9me3 expression and drug resistance. (A) MDA-MB-231 cells were treated with doxorubicin (0.6µM) or Imiquimod (1µg/ml) for 48h. Then, an extracellular staining was performed using anti-CD24/Cy7. The pseudocolor plots are representative of two independent experiments. **(B)** MDA-MB-231 cells were treated with Imiquimod (1µg/ml or 10µg/ml) for 24h. Then, cell were fixed, permeabilzed and stained with anti H3K9ac/FITC or H3K9me3/FITC. **(C)** MDA-MB-231 cells were pre-treated with Imiquimod (1µg/ml or 10µg/ml) for 96h. Then, the medium was changed for a drug-free medium (dfm). After 48h, cells were trypsinized, counted and treated with a second dose of Imiquimod (1µg/ml or 10µg/ml) for 96h. Then cells were counted and the final number of cells were plotted as means of triplicates +/- SD.

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4.6 - Revisão (manuscrito em preparação)

Heterogeneity, plasticity and cancer stem cells: the three musketeers of breast cancer drug resistance

Heterogeneity, plasticity and cancer stem cells: the three musketeers of breast cancer drug resistance

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Key words: Breast cancer, cancer stem cell, drug resistance, plasticity, epigenetics, signalling pathway.

Abstract

Intratumor heterogeneity arises amongst breast cancer as a challenge in chemotherapy. As a result of this heterogeneity, the bulk tumor might include different cells harbouring different phenotype and molecular signatures with differential levels of sensitivity to treatment. The scientific community is divided concerning heterogeneity origin that might be acquired through clonal evolution or might be derived from the Cancer Stem Cell (CSC) model. Here, we reported the last advances in this field exploring the last theories concerning tumor cell plasticity. Finally, we discuss the different therapeutic strategies integrating heterogeneity, plasticity and CSCs, which are in the spotlight.

Introduction

In accord with the World Health Organization (WHO) the global cancer burden is estimated to have risen to 18.1 million new cases and 9.6 million deaths in 2018. Breast cancer is the top cancer in women worldwide and is increasing particularly in developing countries where the majority of cases are diagnosed in late stage. This is one of the highly heterogeneous kind, having morphologically distinct subtypes with different molecular and biochemical signatures (Polyak, 2011) indicating that breast cancer corresponds to multiple diseases. These are several reasons for tumor heterogeneity. At first, tumors originate from different patients, different tissues and cell types that vary in terms of their genomic landscapes. This first type of heterogeneity refers to intertumoral heterogeneity that has obvious clinical implications since it excludes the possibility of an unique treatment for all patients. For example, the heterogeneous breast cancer is categorized into four distinct molecular subgroups (Perou *et al.*, 2000; Sorlie *et al.*, 2001) which influence standard breast cancer treatment: luminal A and luminal B breast cancers are broadly defined as those with estrogen receptor (ERs) positive expression, which response to the anti-estrogen therapy. The HER2 positive breast cancer is the subtype with high amplification of HER2 gene. The triple negative breast cancer (TNBCs, usually basal-like), lacks the expression of ER, progesterone receptor (PR), and HER2 and is considered as the more aggressive.

The other level of heterogeneity arises among cell population within a same tumor as a consequence of genetic change, environmental differences such as access to nutrients, reversible changes in cellular phenotype and properties. This corresponds to the intratumoral heterogeneity (Marusyk, Almendro and Polyak, 2012; Swanton, 2013; Beca and Polyak, 2016; Januškevičienė and Petrikaitė, 2019). As such, it is believed that intratumor heterogeneity may underlie incomplete treatment responses, acquired and innate resistance, and disease relapse observed in the clinic in response to conventional chemotherapy and targeted agents (Dexter and Leith, 1986; Pribluda, de la Cruz and Jackson, 2015; Tabassum and Polyak, 2015). This explains the crucial need to understand mechanisms driving heterogeneity, so that therapeutic approaches to limit cancer diversity, adaptation and drug resistance can be developed. In this review, we present the different systems of intratumor heterogeneity proposed in cancer including the cancer stem cell model and its plasticity with a focus on breast cancer. Due to the high prevalence of drug resistance in breast cancer, we will discuss the necessity to define new markers able to predict the best therapeutic approach in order to anticipate drug resistance.

The term "anticipation", means developing "adaptive therapies" prior to the emergence of resistance.

1. Darwinism and cancer stem cell (CSC) theory: the two actors of a same story

Genetic diversity within a single tumor has been recognized for a long time. Many established tumors are genetically unstable, creating a tendency to accumulate additional mutations and epigenetic events generating heterogeneity. Indeed, cancer cells exhibit a wide range of genetic modifications, from point mutations to massive chromosomal aberrations (Bièche and Lidereau, 1995; Stephens *et al.*, 2012; Alexandrov *et al.*, 2013). This results in many non-functional mutant cells, but a few whose genetic changes enhance their fitness, proliferate. The darwinian forces have been associated to this phenomenon where the fittest clones survive and expand (Merlo *et al.*, 2006; Greaves and Maley, 2012). Indeed, in 1976 Peter Nowell (Nowell, 1976) analyzed the cancer as an evolutionary process, driven by somatic cell mutations with sub-clonal selection that paralleled Darwinian natural selection (Fig 1A). In this scenario, in accord with the Darwinian selection, during treatment the drug-resistant phenotype results from selection of resistant clones, which were produced by genetic mutations. This conceptual evolutionary model implicitly assumes that tumor cells never achieve a fitness maximum so that new mutations can always generate a novel (and fitter) genotype and phenotype (Parker *et al.*, 2019). In their paper, Lloyd *et al*. propose an alternative model in which cancer cells may evolve to an evolutionary stable state and, thus, cannot be displaced if the environment remains stable (Lloyd *et al.*, 2016). This means that the spatial context like vessel localization or nutrients access would be another key to predict genotype heterogeneity. According to this vision, a regionalization of gene mutations rather than random mutations inside the tumor would be expected.

But, some elements have jeopardized the Darwin theory in cancer. At first, it is considered that the only genetic mutations cannot take into account the immense variability in cell phenotype observed in tumors; further, the reapparence of early clones in tumor that would have been eliminated by fitter clones in the darwinian model, is probably the most relevant point that defy the darwinian theory. Recently, Ling S *et al*. by genotyping more than 200 regions from a same tumor have addressed the Darwinian model (Ling *et al.*, 2015). According to them, the extreme genetic diversity (more than 100 millions of mutations) implies evolution under the non-Darwinian mode. A largely under-investigated aspects in this general scheme is in to what extent the genomic diversity present in a tumor contributes to phenotypic heterogeneity. The study by Ma *et al*. reveals a phenotipic convergence between different genetic tumor cells

from lung cancer suggesting that it exists common oncogenic pathways in distinct genetic population in cancer (Ma *et al.*, 2017). It might be possible that certain circumstance and constraints limit genetic options, the different cells may lead to a same evolution of gene, protein or signalling pathway. So, the existence of recurrent pattern of abberent signalling pathways in tumor promises the identification of common therapeutic targets in different genetic cell population.

In this context, the cancer stem cell (CSC) theory appeared as an additional model to support the existence of intratumor heterogeneity. Such non-genetic theory is based on the parallels with normal stem cells (SC) that are endowed with increased xenobiotic resistance, more efficient DNA repair and can influence tissue change during wound healing (Dean, 2005; Donnenberg and Donnenberg, 2005; Medema, 2013). According to CSC model, many cancers may be hierarchically organized in much the same manner as normal tissues where CSCs are at the top of the pyramide. The CSCs have differentiation potential while still retaining the property of self-renewal, and are possibly involved in drug resistance phenomenon (Fig 1B). Corresponding to the characteristics of CSCs mentioned above, bioinformatics-based studies have shown that a worse prognosis of the patient with leukemia correlates with higher expression of the molecular signatures related to CSCs (Eppert *et al.*, 2011). Generally, CSCs represent a distinct population that can be isolated from the remainder of the tumor cells (Clarke *et al.*, 2006; Nguyen *et al.*, 2012). The most common but not the sole way to identify CSCs is through the expression of characteristic cell surface proteins. The leukemia stem cell (LSC), which specifically displays a $CD34^{+}CD38^{-}$ surface marker phenotype, was the first studied (Bonnet and Dick, 1997). In order to experimentally verify the “stemness” of a given cancer cell subset, researchers until recently had predominantly relied on xenotransplantation assays in mice. Regarding solid cancers, Al-Hajj *et al* have identified $CD44^{+}CD24^{-/low}$ cells as breast CSCs characterized by a greater capacity for tumor formation in NOD/SCID mice (Al-Hajj *et al.*, 2003). In addition, Singh *et al.* have found that transplantation of a very small number of $CD133^{+}$ brain cancer cells can generate tumors in NOD/SCID mice (Singh *et al.*, 2004). Therefore, the CSCs are said to be the drivers of tumor progression, eventhough they are just a minority. When they undergo symmetric division, they either produce two identical daughter cells that are replicas of them (CSCs) and this is known as ‘self-renewal’; or two identical daughter cells that are progenitors (non-CSCs) and can subsequently differentiate to form any of the types of cancer cells within the tumor. There is also asymmetric division in which the stem cell divides to produce two non-identical daughter cells. The CSC model has received

wide attention because it provides an explanation for resistance to both radiation and chemotherapy and eventual tumor relapse.

Figure 1

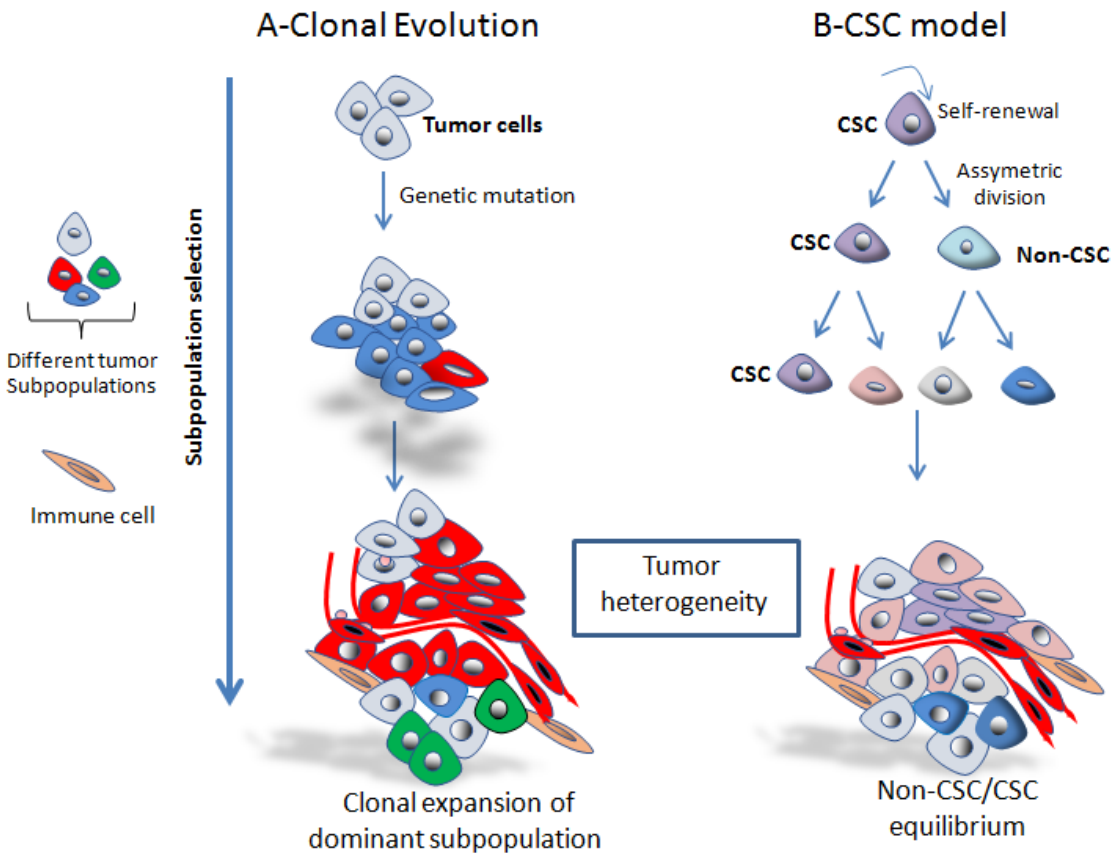

Figure 1 - Two models for intratumor heterogeneity. (A) Evolutionary model: in this model, the intratumor heterogeneity arises from mutated cells, accumulating additional mutations during divisions. This heterogeneity may lead to subclones that possess an evolutionary advantage over the others within the tumour environment, and these subclones may become dominant in the tumour over time. **(B)** Cancer Stem cell model: this model proposes that tumors are hierarchically organized in which the cancer stem cells (CSC)s are at the top of the pyramide identified as drivers of tumor growth. When they undergo symmetric division, they either produce two identical daughter cells that are replicas of them (CSCs) and this is known as 'self-renewal' or there is also asymmetric division in which the stem cell divides to produce two non-identical daughter cells.

Different groups offer a new approach integrating genetic and non genetic phenomenon to explain cancer heterogeneity. Indeed, it appeared that the different populations, CSC and non-CSC, will suffer constantly genetic mutations during their life. It is very likely that the two theories of tumor heterogeneity may apply to human cancers of different types, and in some tumors, both models may apply.

As a result of this heterogeneity, the bulk tumor might include a diverse collection of cells harbouring distinct molecular signatures with differential levels of sensitivity to treatment. This

heterogeneity is expressed in a non-uniform distribution of genetically and phenotipically distinct tumor cell subpopulations within tumor (spatial heterogeneity) and temporal variations in the molecular signature of cancer cells (temporal heterogeneity) which is the result of tumor cell plasticity.

2. Heterogeneity as a dynamic phenomenon: plasticity of tumor cells.

Plasticity between cell types is an important part of normal biology, both in development and in adult homeostasis to respond to diverse situations. In this non genetic phenomenon, cells of the same genotype can exist in different states that may influence their behavior like drug resistance. Generally, plasticity is reversible and non-randomness. During cancer progression, tumor cells undergo molecular and phenotypic changes collectively, which corresponds to cellular plasticity (da Silva-Diz *et al.*, 2018). This phenomenon will impact on the pre-malignant progression, tumor evolution and adaptation to therapy that explains in which targeting plasticity could lead to novel anticancer treatments. Although genetic mutations are closely associated with cancer, the plasticity of cancer is more affected by the microenvironment rather than mutation during the reprogramming process. This is illustrated by the requirement of CSC niches to maintain the CSC properties and cell reprogramming into CSCs (Fessler *et al.*, 2013; Plaks, Kong and Werb, 2015). Indeed, cancer plasticity is driven by reciprocal interactions between a cancer cell and its microenvironment which permits this cell to, on the one hand, adapt its response to the environment and on the other, actively remodel the microenvironment to facilitate its survival and proliferation. In turn, the environment also influences cancer cell phenotypes by providing selective pressure through different mechanisms, including nutrient supply via adjacent blood vessels, immune regulation, and tissue remodeling (Weinberg, 2008; Junttila and de Sauvage, 2013; Greaves, 2015). The different examples of transition in cancer include, but are not restricted, to metabolic plasticity and the state changes from non-CSC to CSC. Indeed, a direct link between phenotypic plasticity and stemness in cancer have been commonly reported (Biddle *et al.*, 2011; Grosse-Wilde *et al.*, 2015; Jolly *et al.*, 2015). Interestingly, stemness and metabolic phenotype may be coupled since it has been reported that the acquisition of CSC properties was associated with metabolic transitions. This is supported by the study of Fiorillo et al where they purified distinct sub-populations of CSCs of breast tumor lineage, based solely on their energetic profile. A high degree of spatial metabolic heterogeneity within tumors has been reported by Hensley *et al*. 2016 (Hensley *et al.*, 2016). The metabolic differences were dynamically quantified for the first time in tumor cells with perfusion experiments that closely mimic the *in vivo* tumor

microenvironment providing evidence that environment will directly influence metabolic cell state (Simões *et al.*, 2015).

The difference between CSCs and non-CSCs is likely to be attributable to the cell-biological program termed epithelial-to-mesenchymal transition (EMT) (Polyak, 2011; Medema, 2013). This program imparts heritable phenotypic changes to tumor cells without introducing new genetic alterations. Upon activation of the program, tumor cells lose many of their epithelial characteristics, and instead acquire an increased capacity for migration and invasion. Interestingly, the signalling pathways involved in embryonic development, such as Wnt/β-catenin, Hedgehog and Notch have also been identified to regulate the non-CSC/CSC transition program (Dissanayake *et al.*, 2007; Vincan and Barker, 2008). We still possess relatively few insights into why these two programs — the CSC phenotype and EMT — are so intimately interconnected.

It is remarkable that CSCs and non-CSCs exist in a dynamic equilibrium in homeostasis, which means that the proportion of both cell types within the population remains constant over many generations. It seems highly likely that the balanced interconversion between these distinct cell types is biologically important. The prediction of a return to phenotypic equilibrium is consistent with the finding that CSCs give rise to tumors that recapitulate the differentiation-state heterogeneity present in the parental tumors from which they were derived. Signals from the tumour microenvironment (Pattabiraman and Weinberg, 2014) and interactions between cells within the tumor could induce and regulate the level of tumor stemness. On this basis, the notion of transition from non-CSCs to CSCs has appeared supporting the fact that cancer cells are highly plastic in their response to microenvironmental cues and can be induced by the infiltration of inflammatory cells, cytokines, chemokines and hypoxia (Chen *et al.*, 2016). In this regard, the pro-inflammatory cytokine IL-6 appeared to play an active role in the in the dynamic state-switching of CSCs (Iliopoulos *et al.*, 2011) evidencing a link constantly reported between inflammation and tumor growth and progression.

Accumulating evidence has shown that CSCs are more resistant than non-CSCs to various types of conventional therapies (Bao *et al.*, 2006; Diehn *et al.*, 2009). This resistance has been attributed to various mechanisms, including: elevated expression of anti-apoptotic proteins; increased levels of ATP-binding cassette (ABC) transporters, transmembrane protein transporters that are known to mediate drug efflux and, thus, to confer multidrug resistance on cancer cells (Bouwens and De Blay, 1996; Zhou *et al.*, 2001). According to this and given

their tumor-initiating capacity, the surviving CSCs might serve as the precursors of new tumors, ultimately leading to clinical relapse.

An enrichment of CSC populations in different cancer after treatment has been reported in several studies including glioblastoma (Bao *et al.*, 2006; Chen *et al.*, 2012) – a highly aggressive form of brain cancer characterized by a high frequency of CSCs – non-small cell lung cancer and breast cancer. At least two main different models have been proposed to account for CSC enrichment: (1) the capability of tumor cells to give rise to new tumors by converting non-CSCs into a CSC phenotype, in a dynamic way in response to drug or (2) a selection of pre-existing CSCs, considered more resistant. For instance, the model of transition state between non-CSCs and CSCs after drug treatment has been proposed in gioblastoma where temozolomide induced a significant increase in the pool of CSCs (Auffinger *et al.*, 2014). Concerning the non-small cell lung cancer, it has been reported that cisplatin, but not doxorubicin nor paditaxel, was able to provoke an enrichment in CSCs leading to multidrug resistance (Wang *et al.*, 2017).

The concept of CSCs, theoretically, implies that all cancer cells are genetically equivalent and differ only transcriptionally and/or epigenetically. However, according to Dirske *et al.*, the CSC phenotype in glioblastoma could be acquired by any given cancer cell independently of its genetic background through intrinsic plasticity . The authors showed that cells expressing CSC markers did not represent a clonal entity defined by distinct functional properties and transcriptomic signatures, but rather a cellular state that is determined by environmental conditions (Dirkse *et al.*, 2019). Such conclusions supported the idea that cell plasticity is probably the sinews of war in cancer. This highlights the critical importance of the understanding of the mechanisms underlying the transitions from non-CSCs to CSCs and vice-versa.

3. Epigenetics: key events in tumor cell plasticity

The functional link between the EMT program and the CSC phenotype has enabled to partially understand the CSC transition phenomenon. According to this, it seems clear that this cross talk between CSC and non-CSC populations involve epigenetic modifications without introducing new genetic alterations (Roesch *et al.*, 2010; Yamazaki *et al.*, 2013) (Fig 2). Epigenetics refer to a change in gene expression without modifications in the primary sequence where chromatin can adopt active and repressive states. Epigenetic mechanisms are primarily mediated by changes in DNA methylation patterns and by the modification of histone, the chromatin packaging. Acetylation and methylation of lysines and arginines at the

histone tails are commonly analyzed; in contrast to DNA methylation, histone modification can be either activating or inhibiting. These epigenetic modifications are combinatorial and occur at histone H3 and H4 moieties through acetylation, methylation, phosphorylation, sumoylation and ubiquitination. For example, H3K4 methylation activates gene expression, whereas H3K9 methylation, H3K27 tri-methylation, and H3K20 tri-methylation induce gene repression (Kouzarides, 2007). The latter two modifications together constitute the two main silencing mechanisms in mammalian cells, H3K9me3 working in concert with DNA methylation and H3K27me3 largely working exclusive of DNA methylation. For example, EZH2, which is the H3K27 Histone Methyl Transferase (HMT), is overexpressed in breast and prostate cancer (Valk-Lingbeek, Bruggeman and van Lohuizen, 2004). Increased levels of G9a, the H3K9 HMT, has been found in liver cancer and is implicated in perpetuating malignant phenotype (Kondo *et al.*, 2008).

The importance of DNA methylation in maintaining CSC properties have been reported in leukemic, lung and colon stem cells (Bröske *et al.*, 2009; Morita *et al.*, 2013; Liu *et al.*, 2014). But, there is growing evidence that CSCs can harbor key epigenetic states defined not just by DNA methylation alterations but also by the histone modifications discussed above. The most compelling evidence supporting a key role of chromatin in the acquisition of CSC phenotypes comes from studies in GBM. The different studies have led to the conclusions that an histone protein may play the role of key driver in GBM demonstrating a direct and major role of chromatin in the emergence of CSCs (Sturm *et al.*, 2012; Wu *et al.*, 2012). In glioma, the histone methyltransferase G9a and the related global increase in H3K9me2 inhibit self-renewal of CSCs *in vitro.* In acute myeloid leukemia (AML), the CSCs and non-CSCs differ in their histone modification patterns (H3K4me3 and H3K27me3) but not DNA methylation patterns (Yamazaki *et al.*, 2013).

The transient and reversible nature of cellular reprogramming during chemotherapy support the involvement of dynamic epigenetic regulatory network in governing the drug resistance. Alterations in genome-wide histone modification on H3 and H4 including atypical acetylation (of H3K9, H3K18, H3K56, H4K12 and H4K16), phosphorylation (of H3 serine 10) and methylation (of H3K9 and H4K20) have been reported to occur in naive breast cancer, upon treatment with DNA-damaging cytotoxic drugs, indicating poor prognosis (Chekhun *et al.*, 2007; Seligson *et al.*, 2009).

A new understanding is growing from recent studies that drug exposure — or perhaps any significant stress — encourages epigenetic “switching” that promotes a transient phenotype

resistant to apoptosis. Given the importance of methylation, methyltransferases, demethylases in development, reprogramming of cancer, targeting histone methylation regulators has been in the spotlight as a therapeutic strategy.

Figure 2

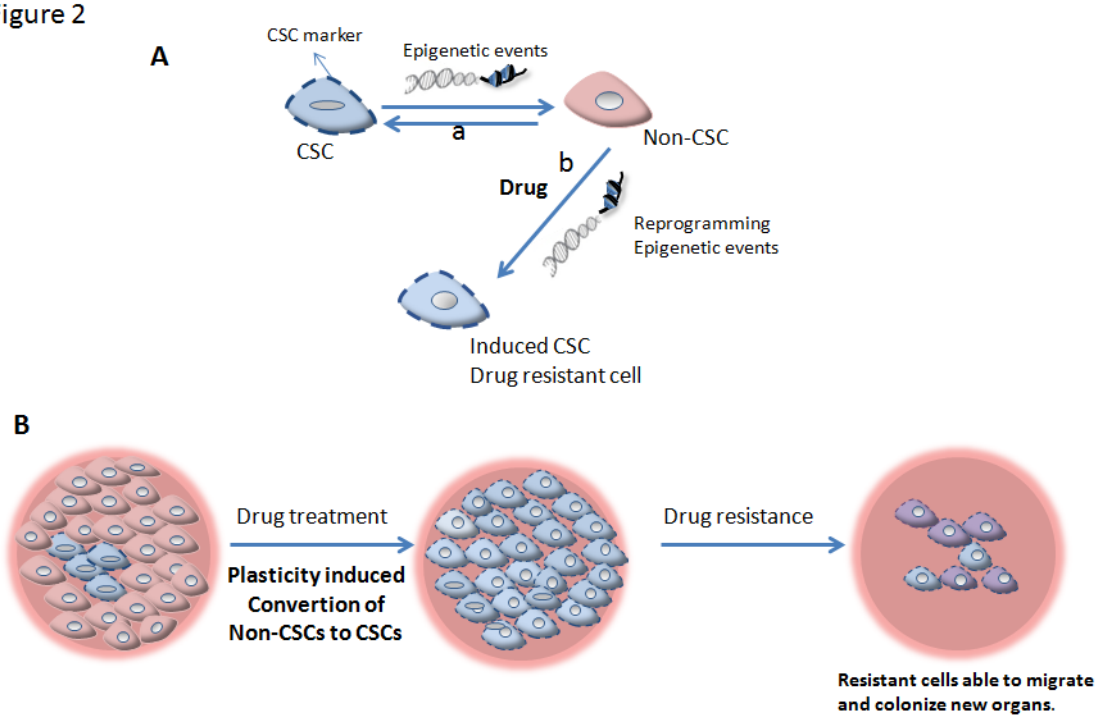

Figure 2 - Tumor cell plasticity. (A) a-Plasticity as a dynamic ability of shifting from a non-CSC to a CSC state and vice versa due to change in microenvironment or to maintain an equilibrium between CSC/non-CSCs, mainly under epigenetic control. **b-**The emergence of induced CSCs observed in the presence of drug or stress that leads to a global convertion of tumor cell population into CSCs after epigentic changes as shown in **(B)**. The resulting cells are the root of cancer relapse.

4. The CSC marker CD24 is a front player in breast cancer

Breast cancer was the first human solid tumor proven to consist of heterogeneous populations of cells constituted by non-CSCs and CSCs. The development of biomarkers to identify breast CSCs as well as validation of *in vitro* and mouse models has facilitated the isolation and characterization of these cells from murine and human tumors. The initial description of human breast CSCs involved the prospective isolation of these populations by the expression of the cell surface markers CD44 (+), CD24 (-/low) and Lin (-) (Al-Hajj *et al.*, 2003). This $CD44^{+}/CD24^{-/low}$ subpopulation of cells appears to be more resistant to chemotherapy in paired primary human breast cancer biopsies (Li *et al.*, 2008). Generally, CD24 has been investigated in combination with CD44. But, the prognostic value of these markers in breast cancer has remained a controversy (Abraham *et al.*, 2005). In MDA-MB-468 rich in $CD44^{+}/CD24^{+}$ cells that are not invasive, it is believed that few $CD44^{+}/CD24^{-}$ cells in this cell line are progressed to

318 $CD44^+/CD24^+$ phenotype after metastasis (Sheridan *et al.*, 2006). Recent studies are evidence
319 for migration, colony formation, and invasion in MDA-MB-468 cell line by $CD44^+$ cells
320 supported the possibility of interconversion between the phenotypes and suggested that
321 $CD44^+/CD24^+$ can give rise to $CD44^+/CD24^-$ cells during tumor initiation (Croker *et al.*, 2009;
322 Meyer *et al.*, 2010). As related the ambiguity of CSC markers in breats cancer is persisting
323 despite of large body of literature and information. Indeed, the strategy that consists in the
324 isolation of CSCs to evaluate their function may have excluded one important aspect of CSC life
325 that is cooperation and interconversion between subpopulations. In a recent review,
326 Tabassum and Polyak made an analogy between tumor and « village » and described the
327 different types of interactions that may occur between different entities within tumor,
328 remembering the quote of Aristotle « *The whole is more than the sum of its parts »* that
329 represents the modern concept of synergy (Tabassum and Polyak, 2015). Further, to maintain
330 the intrinsic homeostatic state, a stable balance between the rates of CSC self-renewal,
331 differentiation, and asymmetric division and the rates of interconversion between non-CSCs to
332 CSCs must be maintained within the individual tumor (Yang *et al.*, 2012). According to this, the
333 isolation of one subpopulation will inevitably lead to the partial conversion to the other.

334 The recent papers support the ideia that CD24 may have its own dynamic inside a same cell
335 population. More recently, CD24 assumed a front-line player in different studies where it
336 gained independence regarding for CD44 cooperation. For instance, using MDA-MB-231 breast
337 cancer cell line, we have shown a differential use of signalling pathway in $CD24^+$ cells where a
338 stronger activation of p38 MAPK was observed that correlated with a more proliferative
339 phenotype (Huth *et al.*, 2016, 2017). Further the role of CD24 has been underlined in the study
340 by Biddle *et al*. where a link between phenotypic plasticity associated with CD24 in cancer cells
341 and drug resistance was established (Biddle *et al.*, 2016). This is also exemplified by the study
342 by Goldman *et al* (Goldman *et al.*, 2015), where the authors have reported that the treatment
343 of breast or ovarian cancer cells with high concentration of taxanes results in the generation of
344 'persistent'cells, which are defined by a transition towards a $CD24^{Hi}$ expression status. More
345 recently, Barkal et al have shown that CD24 may promote the immune evasion in breast and
346 ovarian cancer (Barkal *et al.*, 2019).

347 In our study, we have accompanied the day of MDA-MB-231 aggressive breast cancer cells
348 during doxorubicin treatment monitoring CD24 localization, p38 MAPK activation and the anti-
349 apoptotic Bcl-2 expression. The CSC enrichment detected by FACS in different studies was
350 described as an increase of cell population bearing CSC-surface markers. The mechanism

leading to CD24 emerging at cell surface remains largely unexplored. Our study provides evidence that a translocation of CD24 from cytosol to membrane originated the rapid acquisition of stemness properties (less than four hours), characterized by constitutive p38 activation and overexpression of Bcl-2, in the presence of cytotoxic drugs. The presence of an intracellular pool of CSC marker detected by microscopy and flow cytometry in any given cells of this breast cancer cell line allows this phenomenon (Huth *et al*, 2020 - manuscript in submission). This supports the idea that stemness acquired during drug treatment, corresponds to a behavior and not to a clonal identity (Dirkse *et al.*, 2019). The second lesson that we can draw from this study is the high plasticity of CSCs that are able to acquire a senescence-like program after drug treatment and to re-entry into a cell cycle several weeks later in drug-free medium. These revertant cells have conserved some features of senescent cells while having eliminated others. This phenomenon parallels the relapse sometimes observed in patients with cancer.

Figure 3

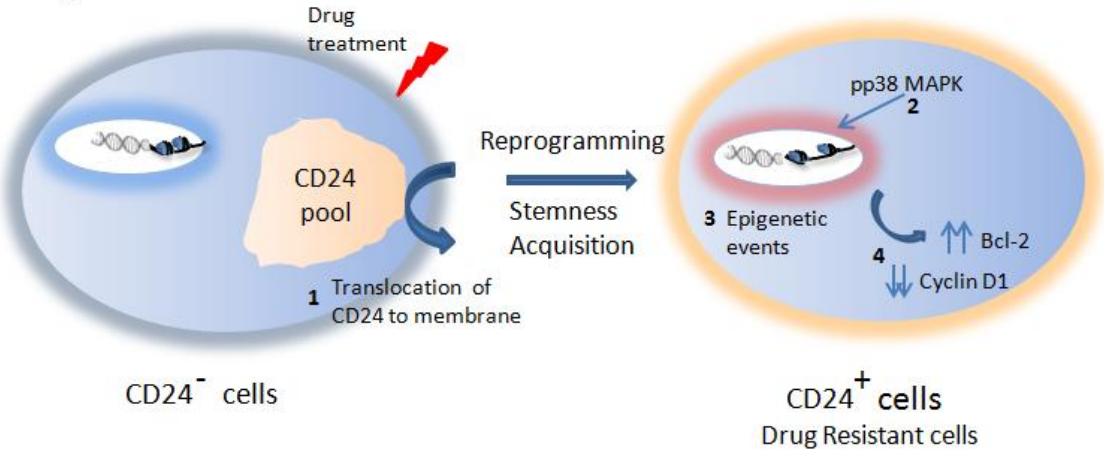

Figure 3 - Molecular model for the enrichment of CSCs during therapy leading to drug resistance. The translocation of the CSC marker CD24 to membrane cell induces stemness acquisition after drug treatment. Several hours after drug addition **(1)** the pool of CD24 immediately available allows a rapid translocation of CD24 to membrane **(2)** followed by a constitutive activation of the p38MAPK **(3)** an increased of the tri methylation of H3K9 that correlates with a diminution of the acetylated form **(4)** a reduction of cyclin D1 expression and an increase of the expression of the anti-apoptotic protein Bcl-2 that is marker of drug resistance.

Avoiding cell reprogramming, to discourage resistant cells to come forward appears crucial in therapeutic strategy. In this context, cancer cell plasticity emerges as a novel relevant target for treatment. To test the hypotesis, we have targeted signalling network associated with CD24 exemplified by p38 MAPK. The combination of SB203580, inhibitor of p38 MAPK activity, and doxorubicin efficiently impeded stemness-related chemoresistance in MDA-MB-231 breast cancer (Huth *et al*, 2020 - manuscript in submission). One of the effects of the p38

inhibitor was the inhibition of epigenetic events like the tri-methylation of H3K9 and the down-regulation of Bcl-2 expression. A schematic of the principal results obtained in our study is presented in the figure 3.

Perspectives

Because CSCs have been shown to have a higher potential to resist conventional therapy and possesses inherent self-renewal properties, targeted CSC therapies appeared as the best approach toward an efficient treatment. However, as reported above, targeting CSCs would only be temporarily effective in eliminating this population since new CSCs may arise from non-CSCs left untargeted. So, new therapeutic strategies counteracting stemness in cancer have been proposed.

Indeed, the tumor plasticity represents a challenge to catch a target. In this context, a variety of therapeutic approaches to inhibit CSC-related pathways are currently being tested in pre-clinical and clinical trials. Amongst them, they are the critical evolutionarily conserved pathways Wnt, Notch, Hedgehog (Katoh, 2007). Notch signalling affects self-renewal and lineage-specific differentiation of normal human breast stem cells (Dontu *et al.*, 2004). Moreover, Notch4 activity is elevated in breast CSCs, and inhibiting Notch4 activity can reduce the breast CSC population, thereby suppressing tumor initiation (Harrison *et al.*, 2010). In the same line, the blockade of Wnt/β-catenin signaling suppresses breast cancer metastasis by inhibiting CSC-like phenotype (Jang *et al.*, 2015). However, an important consideration regarding the effect of inhibiting one (or more) of these CSC pathways is the potential therapeutic escape through compensatory up-regulation of an interconnected pathway. Different crosstalk among Notch, Hedgehog, Wnt, and other signalling pathways have been reported in a variety of cell types. In addition, crosstalk among MAPKs have also been identified in aggressive breast cancer cells (Huth *et al.*, 2016; Ropert and Huth, 2017). So, understanding the crosstalk among these signaling pathways is critical for the development of safe therapies targeting CSCs.

As related in the review, epigenetic mechanisms regulate intratumor heterogeneity either promoting or inhibiting CSC state. Considering the reversible nature of these mechanisms, regulators of epigenetics have therapeutic potential in cancer. We are still in the infancy concerning this field. More *in vitro* experiments are required to evaluate pharmacological parameters such kinetics of cell phenotype changes in the presence of selected inhibitors like histone methyltransferase or deacetylase inhibitors. Another therapeutic strategy that aims to

target CSCs may consist in the combination of a drug leader associated with an inhibitor of CSC plasticity. This is exemplified by our study where the association of SB203580, inhibitor of p38, and doxorubicin was efficient in impeding stemness-acquisition and reducing drug resistance (Huth *et al*, 2020 – manuscript in submission). Identification of a tandem constituted by a stem cell marker and signalling pathway in cancers may guide strategies for therapeutic application in targeted patient populations.

As shown here, the development and use of drugs in combination in biomarker-defined populations enables a more personalized approach to cancer treatment and has the potential to reduce the cost of cancer care.

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634

5 - Discussão

Embora o câncer tenha sido classificado como uma “doença clonal” por várias décadas, é clara a existência de subpopulações celulares dentro do tumor caracterizadas por fenótipos diferentes, que variam em taxa de crescimento, imunogenicidade e resistência ao tratamento quimioterápico. Essa diferença de comportamento entre subpopulações de células cancerígenas dentro do mesmo tumor recebeu o nome de Heterogeneidade Intratumoral (HIT). Vários marcadores tumorais podem ser associados à HIT, como marcadores de células tronco tumorais (CSC) (CD24, CD44, CD133, CD166) (Jaggupilli and Elkord, 2012), marcadores hormonais (ER, PR) e marcadores de fator de crescimento (EGFR, HER2). Inclusive, a presença ou ausência desses marcadores, influencia diretamente na escolha do melhor tratamento para o paciente. Entretanto, o *hotspot* na busca por novos tratamentos do câncer tem se voltado para as CSCs.

Ser ou não ser CSC, eis a questão! O presente trabalho levanta um ponto de discussão ao redor da utilização sistemática de CD24 membranar (dentre outros marcadores de CSC) como marcador para a identificação de CSCs no câncer de mama, células estas que ultimamente se tornaram alvos terapêuticos, pois serviriam de motor para o crescimento tumoral e resistência à quimioterapia. A presença de um reservatório intracelular de CD24 encontrado nas células MDA-MB-231 coloca em cheque a utilização dessas protéinas na classificação do tumor. De fato, estudos recentes confirmam que alguns marcadores de CSCs, como CD133, também podem estar localizados no citosol das células tumorais (Tirino et al. 2008). A localização intracelular de marcadores de CSCs nos permitiu elaborar algumas hipóteses como: (1) a possibilidade dessas proteínas desempenharem funções reguladoras intracelulares; (2) possibilitarem a existência de uma dinâmica populacional, permitindo que as NCSC se tornem CSC em situações de estresse, de forma rápida, por meio da simples mudança da localização do marcador. Importantemente, nós confirmamos esta segunda hipótese quando, pela primeira vez, foi descrita a translocação de um marcador de CSC do citosol para a membrana durante o tratamento quimioterápico. Tal achado pode ser o ponto chave para o melhor entendimento do enriquecimento populacional de CSCs observado durante o tratamento quimioterápico em diversos tipos de câncer como, glioblastoma, câncer de mama, câncer de pulmão, câncer fígado e câncer de cabeça e pescoço (Auffinger *et al.*, 2014; Wang *et al.*, 2017; Pandit *et al.*, 2018).

Outro ponto discutido que pode interferir no caráter transitório entre NCSC e CSC é a epigenética. A metilação, acetilação e ubiquitinação de histonas pode influir diretamente

na expressão diferential de proteínas sem que haja o envolvimento de mutação gênica. Yamazaki e col, demonstraram que a tri-metilação da histona H3K4 e H3K27 estão diretamente correlacionadas à conversão de CSC em NCSC (Yamazaki *et al.*, 2013). Já o nosso estudo demonstrou que a conversão de NCSC em CSC induzida pela doxorrubicina está relacionada à tri-metilação de H3K9 (paper 3). Sendo assim, a classificação das células tumorais como CSC ou NCSC parece estar diretamente relacionada à plasticidade celular cujo controle está sob comando epigenético.

Dentro do contexto da quimioterapia, essa conversão de fenótipo parece ser tempo-dependente, podendo acontecer tanto como um evento precoce - poucas horas após tratamento - (paper 3) quanto como um evento tardio - dias após tratamento (Auffinger *et al.*, 2014). O que poderia explicar essa diferença no tempo de conversão seria a presença de reservatórios intracelulares de marcadores de CSC, prontamente habéis a se translocarem para a membrana das células, quando estas estão sobre algum tipo de estresse. Mais especificamente, quando a conversão do fenótipo ocorreu rapidamente, como no caso do câncer de mama, reservatórios intracelulares de CD24 foram observados nas células da linhagem utilizada; já em células de glioblastomas, a conversão é tardia e poderia envolver mecanismo de síntese *de novo*.

Tais levantamentos refletem diretamente nos testes clínicos já em andamento. Como levantado por Yang et al, existem dois modos de se combater as CSCs: (1) utilizar inibidores específicos de vias de sinalização frequentemente detectadas em CSCs, como Wnt, porcupina e Hedgehoge; (2) utilizar anticorpos contra marcadores de superfície de CSC, como CD24, CD44 e CD133. Diversas drogas têm sido testadas em clínica tento essas duas abordagens como modelo (tabelas 1 e 2), mas poucos são os progressos reportados na literatura (Yang *et al.*, 2019). Dois fatores podem contribuir para esse insucesso: primeiro, a plasticidade encontrada nas células tumorais, uma vez que NCSC podem se tornar CSC e vice-versa o que dificulta a identificação e erradicação dessa subpopulação; segundo, o uso de monoterapias não tem se mostrado muito satisfatório devido a capacidade de adaptação das células tumorais. Isso pode ocorrer pelo fato de células tumorais também apresentarem plasticidade no uso de vias de sinalização, de modo que a inibição de uma via pode levar a ativação de outra (*crosstalk*) como um fenômeno de compensação (Huth *et al.*, 2016).

Devido a isso, cada vez mais tem se pregado o conceito de combinação de drogas de modo a evitar ou diminuir os efeitos dessa adaptação apresentado pelas células tumorais (Ropert and Huth, 2017). A combinação de droga proposta no nosso estudo consiste na ideia

de se associar uma droga líder, já utilizada em clínica, com uma secundária atuando sobre plasticidade e, consequentemente, inibindo a aquisição de resistência. Sendo assim, a identificação de vias de sinalização associadas à plasticidade celular e aos marcadores de CSC se mostra de grande importância, e podem assim, abrir novos horizontes na busca de tratamentos mais eficientes.

TABLE 2 | Potential drugs targeting CSCs in clinical trials.

Drug	Mechanism of action	Condition or disease	Phase	References
WNT-974	PORCN inhibitors	Colorectal cancer and melanoma	I	(10)
ETC-159	PORCN inhibitors	Advanced solid tumors	I	(11, 12)
CGX-1321	PORCN inhibitors	Refractory solid tumors and advanced gastrointestinal cancers	I	(13)
RXC-004	PORCN inhibitors	Solid tumors	I/II	(14)
BC-2059	β-catenin inhibitors	Desmoid tumors	I	(15)
E-7386	CREB-binding protein (CBP)/β-catenin interaction inhibitors	Solid tumors	I	(16)
AL-101	γ-secretase inhibitors	Adenoid cystic carcinoma	II	(17)
Vismodegib	p-glycoprotein inhibitors Breast cancer-resistant protein inhibitors Smo receptor antagonists	Basal cell carcinoma, other cancers	Launched in 2012	(18)
Sonidegib phosphate	Smo receptor antagonists	Basal cell carcinoma, other cancers	Launched in 2015	(19)
Patidegib	Smo receptor antagonists	Sarcoma, basal cell carcinoma	III	(20, 21)
Taladegib	Smo receptor antagonists	Adenocarcinoma, solid tumors	I/II	(22, 23)

TABLE 3 | Potential drugs targeting CSC surface marker in clinical trials.

Drug	Mechanism of action	Condition or disease	Phase	References
P5	Anti-CD49a/CD29 (integrin α5β1)	Non-small cell lung cancer (NSCLC)	III	(32)
ALM-201	Microtubule inhibitors (binds CD44)	Advanced ovarian cancer and other solid tumors	I	(33)
RO-5429083	Anti-CD44	Acute myeloid leukemia	I	(34)
RG-7356	Anti-CD44	Acute myeloid leukemia	I	(35)
AMC-303	CD44 Antigen Exon 6 (CD44v6) inhibitors	Advanced or metastatic malignant solid tumors of epithelial origin	I/II	(36)
CX-2009	Tubulin polymerization inhibitors Anti-ALCAM (CD166)	Solid tumors	I/II	(37)
Chrysin	ABCG2 inhibitors	Chronic lymphocytic leukemia (CLL)	II	(38)

Tabela 2: Drogas potenciais em testes clínicos que têm como alvo vias de sinalização utilizadas pelas CSCs. **Tabela 3:** Drogas potenciais em testes clínicos que têm como alvo marcadores de superfície das CSCs. As referências contidas nessas tabelas devem ser consultadas na bibliografia do artigo ao qual elas foram retiradas (Yang *et al.*, 2019).

Seguindo essa linha de pensamento, a identificação de p38 como um importante elemento na sinalização proliferativa, migratória (Huth *et al.*, 2017) e indutora de resistência no câncer de mama agressivo (paper 3), nos levou a testar o inibidor específico de p38, SB203580, em associação com a doxorrubicina, droga quimioterápica já estabelecida em clínica. Tal associação não só se mostrou mais eficiente em citotoxicidade, como ainda evitou o surgimento de uma população resistente. Além disso, o tandem CD24/p38 identificado nesse trabalho nos ajudou a identificar os padrões moleculares envolvidos no fenômeno de aquisição de resistência apresentado pelas CSCs. Dentre esses padrões moleculares, foi mostrado diminuição de ciclina D1, aumento da expressão de Bcl-2 e tri-metilação da histona H3K9 nas

células tratadas. Tais eventos já foram correlacionadas aos fenômenos de reprogramação celular e resistência em outros estudos (Oltersdorf *et al.*, 2005; Roué *et al.*, 2008; Lu *et al.*, 2018). Fato importante, todas essas alterações são dependentes de p38, abrindo perspectivas para novos tratamentos utilizando inibidores dessa via. Isso se mostra de grande importância, visto que diversos inibidores da via das MAPKs já foram testados em clínica, sem muito sucesso, principalmente inibidores da via ERK. Porém, pouco foi demonstrado com relação a inibidores da via p38 (tabela 1) (Ropert and Huth, 2017). Nesse caso, a utilização do SB203580 poderia trazer diversas vantagens no tratamento do câncer, principalmente pelo baixo custo do tratamento além da citotoxicidade reduzida. Inclusive, a aspirina, um anti-inflamatório utilizado em larga escala, foi relatado como potencializador do tratamento quimioterápico por meio da redução da ativação de p38 (Hu *et al.*, 2018), salientando a possibilidade de se testar o SB203580 ou derivados em clínica.

Como falado acima, um dos principais responsáveis pelo aparecimento de uma subpopulação celular resistente ao tratamento com doxorrubicina é o aumento significativo da expressão de Bcl-2 durante o tratamento quimioterápico. Devido a isso, Bcl-2 é considerado um alvo importantíssimo no combate à resistência à quimioterapia. Em um estudo liderado por Roberts et al, verificou-se que o inibidor de Bcl-2, venetoclax, se mostrou eficiente no combate à recidiva da leucemia linfocítica crônica. Porém, relatos de que o uso do venetoclax aumentam as chances de provocar o fenômeno da "síndrome de lise tumoral", levando a um quadro de insuficiência renal e consequente a morte do paciente, coloca em cheque a biossegurança do uso dessa droga (Roberts *et al.*, 2016). Sendo assim, intensas pesquisas têm sido realizadas na busca por novas drogas que têm como alvo Bcl-2. O fato do SB203580, por si só, reduzir a expressão de Bcl-2 em testes *in vitro* coloca esse inibidor como um grande candidato a ser testado *in vivo* e posteriormente em clínica.

Outra filosofia que pode ser seguida foi proposta por Gatenby, em que sugere que controlar o câncer pode ser mais benéfico do que tentar erradicá-lo (Enriquez-Navas *et al.*, 2016). Dentro deste contexto, nós utilizamos o imiquimode, um agonista toll 7 utilizado no combate ao câncer de pele melanoma (Sharma *et al.*, 2019; Verga, Chohan and Verdolini, 2019). Foi observado que o imiquimode foi capaz de controlar significativamente a proliferação de células agressivas de câncer de mama. Mais especificamente, quando utilizado em baixas concentrações, o imiquimode foi eficaz em reduzir a proliferação de células MDA-MB-231. Já em concentrações mais elevadas, o imiquimode se mostrou citostático, mantendo o número dessas células próximo ao número basal do início do tratamento. O que acrescenta grande valor a esse achado, é o fato de que o imiquimode não gerou resistência a um segundo

tratamento, e em contraste à doxorrubicina, não induziu translocação de CD24, diminuiu a expressão de Bcl-2 e aumentou a expressão de Bax (paper 4). Tal evidência serve de controle para nosso modelo, no qual nós propusemos que reduzir a expressão de Bcl-2 contribui para o controle do tumor sem induzir resistência relacionada ao aparecimento do fenótipo *stemness*. Além disso, o fato do imiquimode ser um agonista toll 7, poderia contribuir para ativação de células do sistema imune, de forma a melhorar a resposta do próprio organismo no combate às células tumorais. Obviamente, novos testes são necessários para comprovar essa eficácia e melhor descrever os mecanismos de ação relacionados ao imiquimode. Mas, de maneira positiva, imiquimode surge como uma importante droga no combate ao câncer de mama, principalmente pelo fato de não induzir resistência.

Uma lição a ser tirada a partir desse trabalho é que se você agredir as células tumorais, elas se tornam mais agressivas, seguindo a máxima "o que não mata, engorda!". A identificação de CD24 como um alarme para as células tumorais se reprogramarem por meio da atividade de p38, nos permitiu propor novas estratégias de tratamento, seja associando uma droga citotóxica convencional a um inibidor de plasticidade ou utilizando uma droga citostática de forma a controlar o crescimento do tumor. Independente de qual seja a estratégia escolhida, evitar o aparecimento do fenótipo *stemness* parece ser uma estratégia pertinente.

6 - Conclusões

- O estresse provocado pelo tratamento quimioterápico induz o enriquecimento de células $CD24^+$ na linhagem MDA-MB-231 por meio da translocação de CD24 do citosol para a membrana, fenômeno nunca descrito até então

- A proliferação, migração e a resistência à quimioterapia das células MDA-MB-231 estão sob controle da atividade da MAPK p38, que por sua vez regula a expressão de ciclina D1, Bcl-2 e H3K9me3 nas células MDA-MB-231

- A atividade de p38 está diretamente relacionada à expressão de CD24 na membrana das células MDA-MB-231

- O inibidor da atividade de p38, SB203580, aumenta a eficácia do tratamento com a doxorrubicina de maneira sinérgica

- A utilização do inibidor da atividade de p38, SB203580, em associação com doxorrubicina reduz a reprogramação celular e, consequentemente, a aquisição de resistência relacionada a *stemness*

- A inibição da via ERK1/2 pode ativar a via p38 devido a um cross-talk existente entre essas duas vias modulado pela atividade de MEK2

- O imiquimode controla o crescimento das células MDA-MB-231 sem induzir translocação de CD24 e, consequentemente, sem induzir resistência

7 - Perspectivas

- Testar a associação entre SB203580 e doxorrubicina, em linhagens celulares de diferentes tipos de câncer - MTT e FACS

- Avaliar as diferenças proteômicas e fosfo-proteômicas das diferentes subpopulações celulares (MDA-MB-231 parental, $CD24^+$/DxR e DxR/30) - RNAseq e espectometria de massa

- Avaliar a utilização das vias metabólicas das diferentes subpopulações celulares (MDA-MB-231 parental, $CD24^-$, $CD24^+$, SiCD24, $CD24^+$/DxR e DxR/30) - Metaboloma

- Avaliar a capacidade tumorigênica das diferentes subpopulações celulares (MDA-MB-231 parental, $CD24^-$, $CD24^+$, SiCD24, $CD24^+$/DxR e DxR/30) em camundongos normais e imunodeficientes - Morfometria

- Avaliar *in vivo* a eficácia da associação entre SB203580 e doxorrubicina bem como a utilização do Imiquimode como novas alternativas farmacológicas no controle do crescimento tumoral - Morfometria

- Avaliar qualitativamente a presença de CSCs em cortes histológicos de tumores xenográficos após tratamento com SB203580 + doxorrubicina ou Imiquimode - Imunohistoquímica ou imunofluorescência

8 - Referências Bibliográficas

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