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Avaliação do impacto de células tronco adiposo-derivadas em células de glioma e no microambiente tumoral

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Avaliação do impacto de células tronco adiposo-derivadas em células de glioma e no microambiente tumoral

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Dedico este trabalho aos meus pais: Erny e Elaine

Aos meus irmãos: Gaile e Marcelo

A meu marido: Bernardo.

Graças aos quais, a vida tem mais sabor.

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"... and now, as you graduate to begin anew, I wish that for you.

Stay Hungry. Stay Foolish".

Steve Jobs

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RESUMO

Glioblastoma multiforme (GBM) é o mais agressivo e maligno dos tumores primários cerebrais. Essa agressividade se deve, em parte, à capacidade invasiva desse tipo de tumor, o que torna a sua completa ressecção cirúrgica praticamente impossível. Acredita-se que um dos fatores responsáveis pela alta malignidade desses tumores seja a capacidade das células tumorais passarem por um processo conhecido como transição epitélio-mesenquimal (EMT). Durante a EMT, as células do tumor apresentam aumento de sua capacidade migratória e invasiva. Além disso, ocorrem alterações na morfologia das células, acompanhadas de aumento da expressão de marcadores mesenquimais. Entretanto, os mecanismos e estímulos que levam as células de glioma a sofrerem EMT ainda não foram completamente elucidados e ainda permanece em aberto o real papel das células que compõem o estroma tumoral em promover ou inibir EMT. Células tronco mesenquimais (MSCs) têm chamado a atenção de pesquisadores devido à sua aplicabilidade em terapia genica e celular, principalmente devido à sua capacidade de *homing* a sítios tumorais. Entretanto, devido a sua alta plasticidade e capacidade secretória, não se sabe ao certo como essas células podem influenciar o crescimento de tumores. A fim de elucidar algumas dessas questões, nosso objetivo neste trabalho foi investigar como o meio condicionado (CM) de células tronco adiposo-derivadas (ADSCs) pode influenciar a biologia de células de glioma de rato C6 *in vitro* e *in vivo*. Nossos resultados mostraram que o tratamento de células C6 com ADSC-CM induz alterações na morfologia celular e nuclear de células de glioma, bem como aumento da capacidade migratória *in vitro*. Além disso, as células C6 tratadas com ADSC-CM apresentaram reduzida capacidade de adesão e aumento na expressão de genes relacionados com EMT. Esses resultados indicam que o CM de ADSCs induz um processo de EMT-*like* em células de glioma C6 *in vitro*. Quando analisamos os efeitos das células C6 tratadas com ADSC-CM *in vivo*, observamos que o tratamento de células C6 com CM não não alterou o padrão de crescimento ou malignidade dos gliomas gerados. Entretanto, quando células C6 foram co-injetadas com ADSCs, verificamos o surgimento de tumores maiores. Surpreendentemente, esse tumores não apresentaram alteração nos níveis de expressão do marcador de proliferação Ki67 e do marcador de astrocitose reativa GFAP. Desse modo, sugerimos a hipótese de que as células tumorais poderiam induzir uma espécie de transformação maligna nas ADSCs. Em conjunto, nossos resultados ressaltam a importância de se ter muita cautela na utilização de ADSCs como veículos terapêuticos no tratamento de doenças.

ABSTRACT

Glioblastoma multiforme (GBM) is the most aggressive and malignant of the brain primary tumors. This aggressiveness is, in part, due to the invasive capacity of this type of tumor, making impossible its complete surgical resection. One of the reasons for the high malignancy of these tumors is the ability of the tumor cells go through a process known as epithelial to mesenchymal transition (EMT). During EMT, tumor cells show a reduction in its adhesion capacity while presenting increased migratory and invasive ability. Additionally, are observed changes in cell morphology, accompanied by increased expression of mesenchymal markers. However, the mechanisms and stimuli that lead glioma cells to undergo EMT have not yet been fully understood and still uncertain if cells of tumor stroma are able to induce or inhibit EMT. Mesenchymal stem cells (MSCs) have attracted attention of researchers due to its applicability for gene and cell therapy, mainly because of their ability to migrate to tumor sites. However, due to its high plasticity and secretory capacity, it was not disclosed how these cells can influence tumor growth. In order to clarify some of these questions, our goal in this work was to investigate how the conditioned medium (CM) from adipose-derived stem cells (ADSCs) can impact on rat C6 glioma cell biology *in vitro* and *in vivo*. Our results showed that treatment of C6 cells with ADSC-CM induces alterations in cellular and nuclear morphology of the glioma cells and promotes increased migratory ability *in vitro*. In addition, the treatment of C6 cells with ADSC-CM induced decreased adhesion ability and increased expression of EMT-related genes. These results indicate that the ADSC-CM induces an EMT-like process in C6 glioma cells *in vitro*. When we analyzed the effects of C6 cells treated with ADSC-CM *in vivo*, we found that the treatment with CM do not change the pattern of growth or malignancy of gliomas. However, when C6 cells were co-injected with ADSCs, we observed the development of larger tumors. Surprisingly, the tumors showed no changes in Ki67 and GFAP expression. Thus, we suggest the possibility that tumor cells could induce a kind of malignant transformation in ADSCs. Together, our results highlight the importance to be very careful in using ADSCs as therapeutic vehicles to treat cancer.

LISTA DE ABREVIATURAS

ADSC – células tronco adipose-derivadas
ASC – célula tronco adulta
B2M – beta 2 microglobulina
CD – *cluster of differentiation*
CD45 – antígeno comum de leucócitos
CFU-F – unidade formadora de colônia de fibroblasto
CM – meio condicionado
CSC – célula tronco tumoral
ECM – matriz extracelular
EGF – fator de crescimento epidermal
EMT – transição epitélio-mesenquimal
ESC – célula tronco embrionária
GAPDH – gliceraldeído 3-fosfato desidrogenase
GBM – glioblastoma multiforme
GFAP – proteína ácida fibrilar glial
GSK-3β – glicogênio sintase quinase 3β
HGF – fator de crescimento de hepatócitos
HIF-1 – fator induzido por hipóxia
HLA – antígeno leucocitário humano
HPRT – hipoxantina-guanina fosforibosiltransferase I
IGF – fator de crescimento semelhante a insulina
IL-6 – interleucina 6
IL-8 – interleucina 8
MAPK – proteína quinase ativada por mitógeno
MMP – metaloproteinase de matriz
MSC – células tronco mesenquimais
mTOR – proteína alvo da rapamicina em mamíferos
OMS – organização mundial da saúde
PDGF – fator de crescimento derivado de plaquetas
PI3K- fosfatidilinositol 3-quinase
qRT-PCR – reação em cadeia da polimerase com transcrição reversa em tempo real
SDF-1/CXCL12 – fator derivado do estroma

SNC – sistema nervoso central

TAF – fibroblasto associado ao tumor

TAM – macrófago associado ao tumor

TBP – proteína de ligação TATA

TGFβ – fator de transformação do crescimento beta

VEGF – fator de crescimento endotelial vascular

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APRESENTAÇÃO DA TESE

A presente tese de doutorado é composta de: uma **Introdução** apresentando os principais conceitos envolvidos na parte experimental, que são Gliomas (seção 1), Microambiente tumoral (seção 2), Células tronco (seção 3), Transição epitélio-mesenquimal (seção 4) e Normalização de qRT-PCR (seção 5). Na sequência, são apresentados os seguintes Capítulos:

CAPíTULO I – Artigo Original (publicado no periódico *Biomedicine and Pharmacotherapy*) – *Identification of valid endogenous control genes for determining gene expression in C6 glioma cell line treated with conditioned medium from adipose-derived stem cell.* Artigo original, no qual foi feita uma precisa validação de candidatos a genes de referência para uma confiável análise de expressão gênica em amostras de linhagem de glioma C6 tratadas ou não com meio condicionado (CM) de células tronco adiposo-derivadas (ADSCs) de rato. Os dados obtidos nesse capítulo foram utilizados para a realização do capítulo II desta Tese.

CAPíTULO II – Artigo Original (publicado no periódico *Molecular Neurobiology*) *Conditioned medium from adipose-derived stem cells (ADSCs) promotes epithelial-to-mesenchymal-like transition (EMT-like) in glioma cells in vitro.* Artigo original que analisa os efeitos celulares do tratamento de células de linhagem de glioma C6 com CM de ADSCs derivadas de rato. Nesse artigo, são analisados mecanismos celulares que podem ser influenciados por fatores contidos no meio condicionado de células tronco.

CAPíTULO III – Artigo Original em preparação (a ser submetido) -Analysis of the impact of rat adipose-derived stem cells (ADSCs) and their conditioned medium (CM) on C6 glioma tumor microenvironment. Artigo original que analisa os efeitos do pré-tratamento de células de glioma C6 com CM de ADSCs derivadas de rato, bem como da co-injeção de ADSCs derivadas de rato com células de glioma C6 no desenvolvimento tumoral *in vivo*.

CAPíTULO IV – Artigo de Revisão (publicado no periódico *Medicinal Research Reviews*) - *The epithelial to mesenchymal transition in glioblastoma: An updated systematic review.* Artigo de revisão sistemática sobre o papel da transição epitélio-mesenquimal na

progressão de glioblastomas. Esse artigo sumariza os mais recentes achados disponíveis na literatura científica sobre os principais mecanismos e vias de regulação que estão envolvidas no processo de transição epitélio-mesenquimal (EMT) em glioblastomas. Por unir um completo e atualizado conteúdo teórico sobre o tema, este capítulo, além de compilar os principais achados presentes na literatura sobre EMT em glioblastoma, ainda acessou intrigantes questões envolvendo o tema, que ainda não haviam sido abordadas anteriormente por outros pesquisadores.

Ao final, é apresentada uma **Discussão Integrada** dos dados obtidos nos quatro capítulos desta tese, bem como as **conclusões** e **perspectivas** geradas por este trabalho.

INTRODUÇÃO

1. Gliomas

Gliomas são os tumores primários mais comuns do sistema nervoso central (SNC), representando 30% de todos os tumores cerebrais. Aproximadamente 30.000 pacientes são diagnosticados com glioma nos Estados Unidos da América (EUA) a cada ano (Holland 2001, Westphal and Lamszus 2011).

Por serem originados no SNC e afetarem as estruturas cerebrais que circundam o tumor, pacientes com glioma, comumente desenvolvem sintomas que incluem dor de cabeça, tontura e alterações neurológicas, como fraqueza e dificuldade na fala. A maioria dos gliomas tem origem esporádica, mas pacientes com esse tipo de tumor geralmente possuem histórico familiar de algum tipo de cancer (Holland 2001, Westphal and Lamszus 2011).

O tipo celular que dá origem aos gliomas ainda não foi comprovado e tem gerado muita discussão, entretanto, enquanto alguns pesquisadores acreditam que astrocitomas são derivados de astrócitos e oligodendrogliomas derivados de oligodendrócitos, outros sugerem que gliomas malignos são derivados de células tronco neurais ou de células precursoras de oligodendrocitos ((Holland 2001, Alderton 2011, Liu, Sage et al. 2011).

Os gliomas são classificados de acordo com as células cerebrais com as quais eles mais se assemelham morfologicamente, como os astrócitos (astrocitomas), oligodendrócitos (oligodendrogliomas) e células ependimais (ependimomas). Além dessa classificação, os gliomas são divididos em diferentes graus clínicos (graus I, II, III e IV para astrocitomas e graus II e III para oligodendrogliomas e oligoastrocitomas), de acordo com a sua histologia e prognóstico (Wen and Kesari 2008, Westphal and Lamszus 2011) (Tabela 1).

Tabela 1. Classificação dos Gliomas de acordo com a OMS.

Glioma type	Grades	Name	Incidence (% of all brain tumors)	Age	Sex
Astrocytic tumors	Grade I	Pilocytic astrocytoma	5-6%	First 2 decades	1:1
		Subependymal giant cell astroctyoma	<1%	2-20	equal
	Grade II	Diffusely infiltrating astrocytoma	10-15%	30-40	1.18:1
	Grade III	Anaplastic astrocytoma	10-15%	45-50	1.1:1
	Grade IV	Glioblastoma multiforme	12-15%	45-75	1.26:1
Oligodendroglial tumors	Grade II	Oligodendroglioma	2.5%	40-45	1.1:1
	Grade III	Anaplastic oligodendroglioma	1.2%	45-50	1.1:1
Oligoastrocytic tumors	Grade II	Oligoastrocytoma	1.8%	35-45	1.3:1
	Grade III	Anaplastic oligoastrocytoma	1%	40-45	1.15:1
Ependymal tumors	Grade I	Subependymoma	0.7%	50-60	2.3:1
		Myxopapillary ependymoma	0.3%	20-35	2.2:1
	Grade II	Ependymoma	4.7%	<16, 30-40	1:1
	Grade III	Anaplastic ependymoma	1%	<16	1:1

(Sarkar, Jain et al. 2009).

Os astrocitomas são ainda subdivididos na seguinte classificação (ver Tabela 1):

1. Glioblastoma multiforme (GBM), conhecido como o mais maligno dos gliomas (grau IV). Pacientes com esse tipo de tumor possuem uma sobrevida de aproximadamente 1 ano.
2. Astrocitoma anaplásico, de grau III. Pacientes sobrevivem em média 2-3 anos.
3. Astrocitoma de baixo grau, ou grau II. Pacientes podem sobreviver entre 10-15 anos.
4. Astrocitoma pilocítico, grau I. São curáveis através de cirurgia.

Uma característica interessante de gliomas de baixo grau é que, após ressecção cirúrgica, esses tumores frequentemente recorrem como lesões de alto grau (glioblastoma secundário). Esse fenômeno é conhecido como progressão e ocorre na maioria dos casos (Wen and Kesari 2008, Westphal and Lamszus 2011).

1.1 Glioblastoma Multiforme (GBM)

GBM é o mais comum e agressivo tipo de glioma. A incidência de GBM nos EUA é de aproximadamente três casos em cada 100.000 habitantes ao ano. O pico de incidência

desse tipo de tumor é por volta dos 40 anos de idade, sendo predominante em homens (Jhanwar-Uniyal, Labagnara et al. 2015).

Apesar dos esforços que têm sido feitos nos últimos anos a fim de aumentar a nossa compreensão a respeito dos mecanismos celulares e moleculares que governam a pato-fisiologia dos GBM, não foi atingida uma melhora significante na sobrevida dos pacientes acometidos por essa doença. Muitas modalidades terapêuticas têm sido empregadas com a finalidade de conter o avanço do tumor, como por exemplo, ressecção cirúrgica, radioterapia, quimioterapia, entretanto, a taxa de sobrevida em cinco anos permanece menor que 5% (Westphal and Lamszus 2011, Jhanwar-Uniyal, Labagnara et al. 2015).

GBMs são tumores com características altamente agressivas, sendo muito proliferativos e infiltrativos, o que impossibilita sua completa ressecção cirúrgica, justificando assim, pelo menos em parte, o fracasso dos tratamentos. Além disso, apresentam intensa proliferação vascular, grandes áreas de necrose, altas taxas de mitose e presença de atipias nucleares (Holland 2001) (Figura 1).

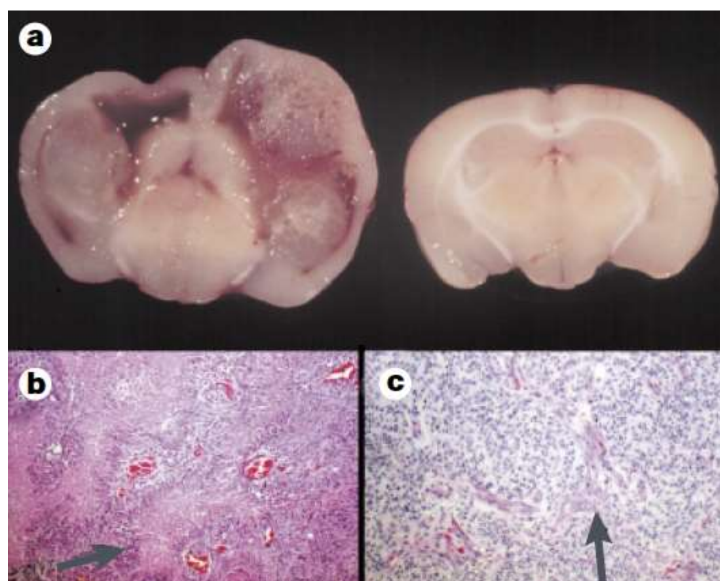

Figura 1. Características histológicas que definem o GBM. (a) Aparência de glioblastoma de camundongo (esquerda) em comparação com cérebro normal (direita). (b) Necrose em pseudopaliçada (flecha) e (c) proliferação microvascular (flecha). Essas características são muito similares às presentes em GBM de humanos (Holland 2001).

GBMs, assim como a maioria dos demais tipos de câncer, se originam a partir de um processo de múltiplos passos que é marcado por profundas alterações genéticas nas células que dirigem a transformação de células humanas normais em células malignas.

Ainda não se sabe ao certo a partir de quais células os GBMs são originados, mas acredita-se que eles possam surgir a partir de células tronco neurais, células progenitoras ou células diferenciadas, como astrócitos e oligodendrócitos. Atualmente, a hipótese que propõe a origem dos GBMs a partir de células indiferenciadas ou “tronco” é a que têm recebido maior atenção por parte da comunidade científica (Zhou, Zhang et al. 2009) (Figura 2).

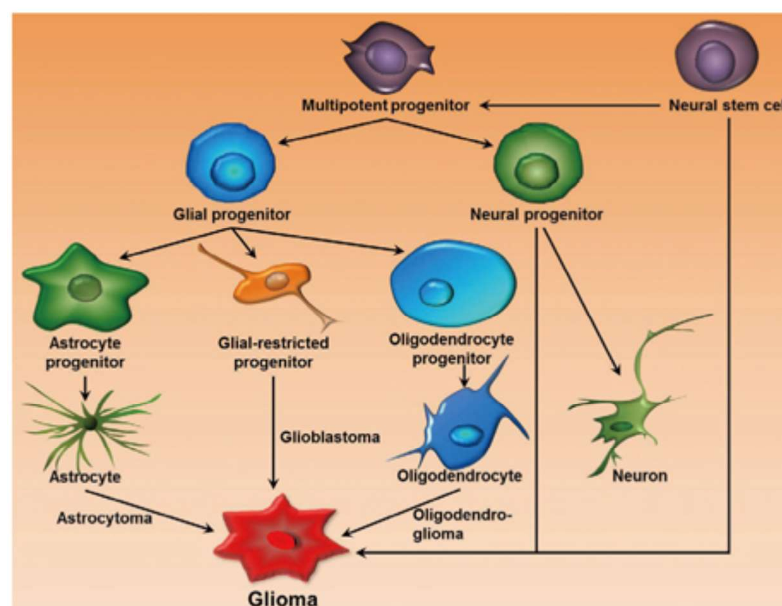

Figura 2. Processo de tumorigênese em GBM (Jha and Suk 2013).

2. O microambiente tumoral

Tumores são compostos não apenas por células tumorais, como se pensava há alguns anos atrás, mas também por células estromais, que dão suporte ao tumor e formam o microambiente tumoral. Em condições normais, as células estromais não possuem características de malignidade, atuando na manutenção estrutural e funcional de tecidos sadios. Entretanto, quando em contato direto com células tumorais ou através de sinalização parácrina, as células estromais normais podem adquirir fenótipos alterados e passar a suportar a progressão tumoral (Adjei and Blanka 2015) (Figura 3).

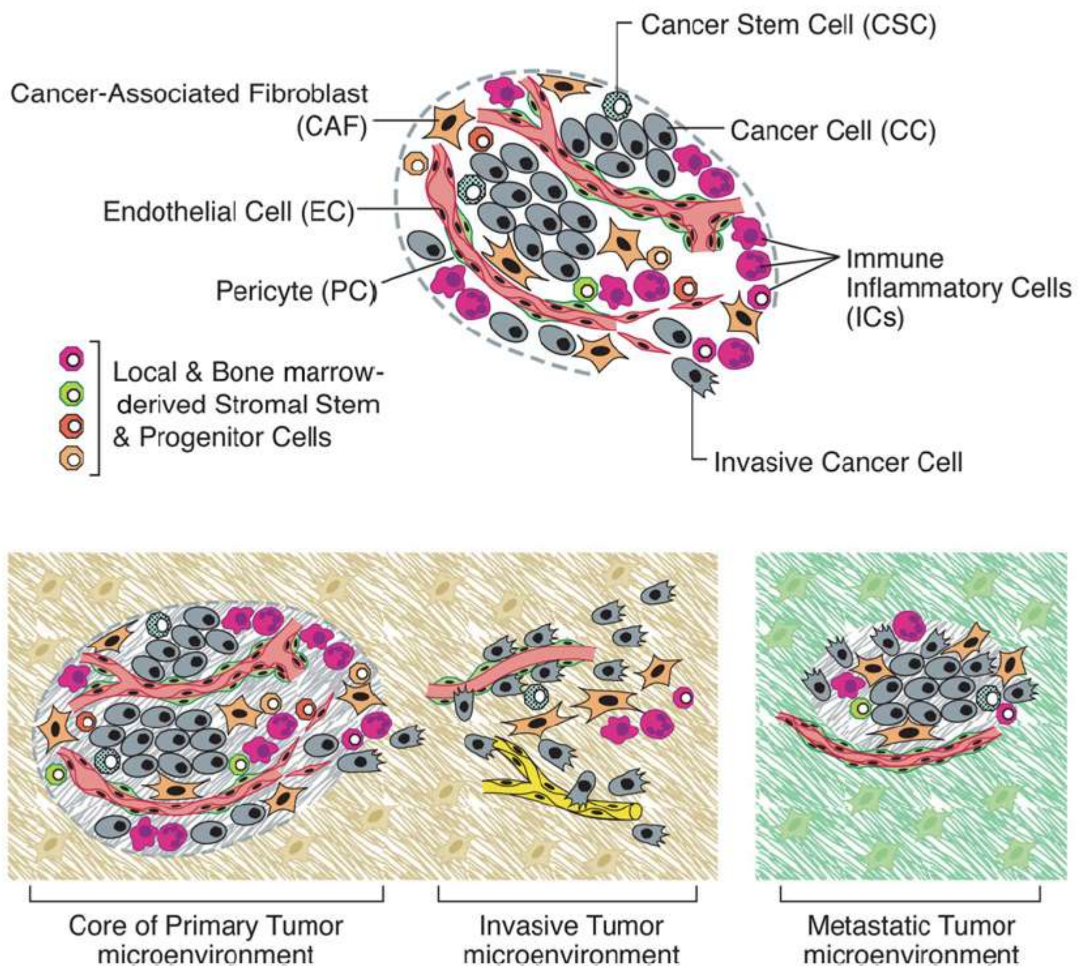

Figure 3. Microambiente tumoral (Hanahan and Weinberg 2011).

Entre as células que compõem o microambiente tumoral, estão fibroblastos, células imunes, células endoteliais e células tronco mesenquimais (MSCs). Além do componente celular, o estroma tumoral também é composto por matriz extracelular (ECM) e moléculas extracelulares secretadas tanto por células do microambiente, como pelas próprias células tumorais, como fatores de crescimento, citosinas e quimosinas, entre elas IL-6, IL-8, EGF, TGF-β, VEGF e HGF. Esses fatores liberados auxiliam no recrutamento de outras células ao tumor, e assim, dão suporte ao crescimento tumoral (Byun and Gardner 2013, Adjei and Blanka 2015).

Outra molécula que está presente no microambiente tumoral e cujo papel vem sendo alvo de estudo por parte do nosso grupo de pesquisa é o ATP extracelular. Trabalhos do nosso grupo sugerem que há um acúmulo de ATP no microambiente tumoral, principalmente devido a ineficiência das células tumorais em degradar essa molécula quando comparadas com astrócitos normais. Esse excesso de ATP na região do tumor, pode levar à morte do tecido adjacente normal e induzir proliferação celular em diferentes linhagens de glioma (Wink, Lenz et al. 2003, Morrone, Horn et al. 2005, Bavaresco, Bernardi et al. 2008).

Em geral, tumores sólidos são marcados por elevada formação de vasos sanguíneos, ou seja, neoangiogênese, o que possibilita o suporte de nutrientes e oxigênio necessários para o crescimento tumoral. Esse processo é facilitado pela presença de diferentes tipos celulares associados ao tumor, principalmente, células endoteliais. Células endoteliais normais podem ser recrutadas ao sítio do tumor e formar vasos sanguíneos associados ao tumor, que servirão tanto para o aporte energético, quanto para que as células tumorais possam entrar na circulação e iniciar o processo de metástase. Por terem papel crucial na progressão tumoral, células endoteliais tumorais têm sido constantes alvos de agentes terapêuticos desenvolvidos para bloquear o suprimento sanguíneo dos tumores (Chouaib, Kieda et al. 2010).

Tumores são classicamente conhecidos por se assemelharem a "feridas que nunca saram", principalmente por formarem um ambiente inflamatório e infiltrado por células imunes que são recrutadas pelas células tumorais (Dvorak 1986). As células imunes que irão migrar ao sítio do tumor (entre elas macrófagos, linfócitos T e células natural *killers)* com o intuito de impedir o crescimento tumoral serão subvertidas dentro do estroma tumoral, passando a recrutar outras células imunes e estromais, liberando fatores solúveis e, contribuindo assim, para a sustentação do tumor (Whiteside 2008). Entre as células imunes mais importantes recrutadas ao sítio tumoral estão os macrófagos. Uma vez dentro do microambiente tumoral, essas células passam a ser chamadas de macrófagos associados ao tumor (TAMs) e mudam o seu fenótipo de M1 para M2. Macrófagos do tipo M1 são considerados como inibidores tumorais, enquanto que macrófagos M2 são associados com angiogênese, atenuação da resposta imune antitumoral, remodelamento da ECM e progressão tumoral (Byun and Gardner 2013).

Fibroblastos associados ao tumor (TAFs) podem tanto ser recrutados ao sítio tumoral, como se originar a partir de outras células como, pericitos, células epiteliais, endoteliais, e

MSCs. Além de produzirem fatores de crescimento e citosinas, os TAFs também são responsáveis pelo remodelamento da ECM e da arquitetura dos tecidos, principalmente por produzirem metaloproteinases de matriz (MMPs), enzimas responsáveis por degradar a ECM, suportando, assim, a invasão das células tumorais através de tecidos sadios (Li, Fan et al. 2007).

MSCs são recrutadas da circulação sanguínea, ou de outros órgãos vizinhos ao tumor e sua presença no ambiente tumoral permanece indefinida. Essas células podem se diferenciar em várias outras células estromais, incluindo células inflamatórias, pericitos e TAFs, bem como permanecer indiferenciadas ou parcialmente diferenciadas. Apesar de não se ter definido ao certo se MSCs são pró ou anti-tumorais, devido ao seu intenso tropismo a sítios de injúria, essas células têm sido muito utilizadas em estratégias terapêuticas como veículos para terapia gênica (Barcellos-de-Souza, Gori et al. 2013).

3. Células tronco

Células tronco podem ser classificadas em dois tipos básicos: células tronco embrionárias (ESC) e células tronco adultas (ASC). Teoricamente, as células tronco embrionárias parecem ser mais versáteis para aplicação terapêutica, entretanto, esse conceito tem sido questionado (Rossant 2001).

As ESCs são originadas da massa celular interna do blastocisto e são chamadas de pluripotentes por serem capazes de se especializar para formar as três camadas germinativas que darão origem ao organismo adulto (Rossant 2001) (Figura 4).

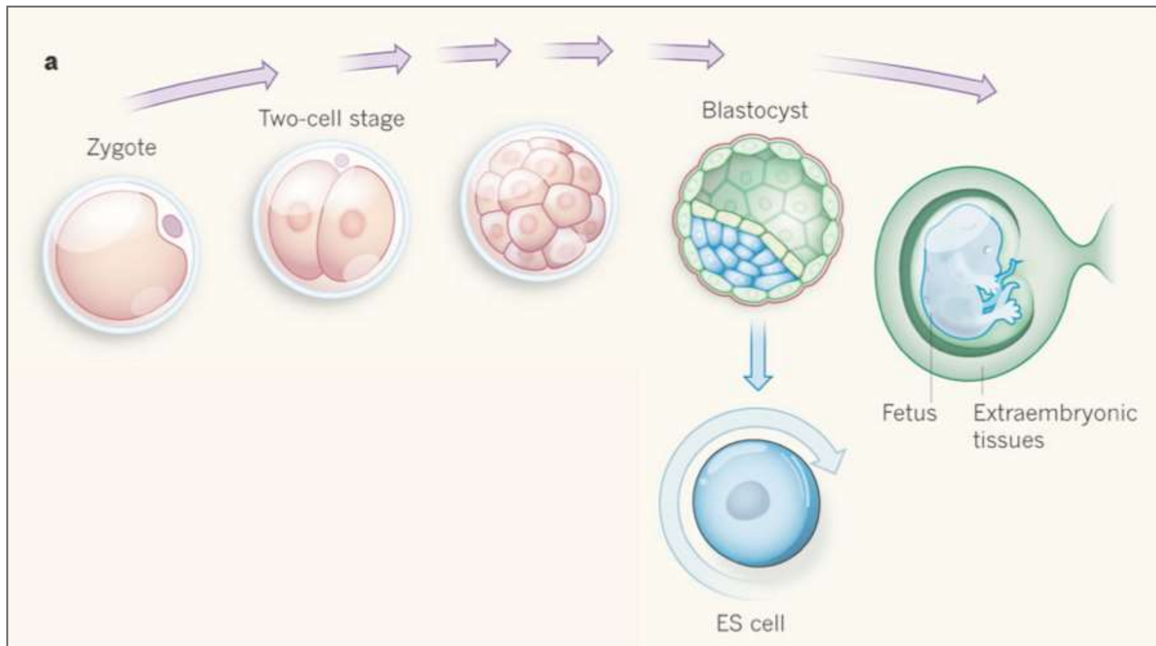

Figura 4. Origem das células tronco embrionárias. Adaptado de: (Surani and Tischler 2012). ES = embryonic stem cell.

As questões éticas que envolvem o uso de ESCs, a falta de conhecimento sobre os mecanismos que regulam a sua diferenciação e sua capacidade tumorigênica (Blum and Benvenisty 2008) têm estimulado os pesquisadores a procurarem outras fontes de células tronco que possam ser utilizadas de forma mais segura.

As ASCs são células multipotentes encontradas em tecidos e órgãos adultos totalmente desenvolvidos. Essas células são capazes de se diferenciar em muitos tipos celulares distintos, em geral, originados a partir da camada germinativa da qual elas próprias são originadas. A função fisiológica das ASCs é manter a integridade de órgão e tecidos, bem como, repará-los quando eles sofrem algum dano (Gomillion and Burg 2006).

ASCs têm sido encontradas em uma ampla variedade de tecidos adultos, como cérebro, tecido adiposo, coração, pulmão, rim e fígado. Entretanto, a mais estudada e bem caracterizada fonte de ASCs é a medula óssea. A medula óssea contem uma população heterogênea de células composta por macrófagos, eritrócitos, fibroblastos, adipócitos e células endoteliais. Além dessas células, a medula óssea contem um conjunto de células tronco não hematopoiéticas, conhecidas como células tronco estromais ou MSCs (Salem and Thiemermann 2010).

3.1 Células tronco mesenquimais (MSCs)

Friedenstein e colaboradores foram os primeiros a reportar e existência de células semelhantes a fibroblastos que poderiam ser isoladas da medula óssea e que possuíam a capacidade de aderir ao plástico em cultura (Friedenstein, Petrakova et al. 1968). Essas células foram posteriormente isoladas também por outros grupos que descreveram uma população de células capazes de se diferenciar em múltiplos tipos celulares de origem mesenquimal e com capacidade de originar clones. Essas células foram definidas como unidades formadoras de colônias de fibroblastos (CFU-F) e mais tarde, foram classificadas como células com capacidade de aderência, morfologia fibroblastóide e capacidade de se diferenciar em osteoblastos, adipócitos e condrócitos *in vitro* (Bianco, Robey et al. 2008, Salem and Thiemermann 2010, Caplan and Correa 2011). Com o passar dos anos, MSCs foram isoladas de diversos outros órgãos e tecidos, como musculo esquelético (Gharaibeh, Lu et al. 2008), tecido adiposo (Arana, Mazo et al. 2013), cordão umbilical (Hong, Gang et al. 2005), pulmão (Summer, Fitzsimmons et al. 2007), fluido amniótico (Fei, Jiang et al. 2013), entre outros. Em 2006, Da Silva Meirelles e colaboradores mostraram que MSCs residem em virtualmente todos os órgãos e tecidos adultos (da Silva Meirelles, Chagastelles et al. 2006). Entretanto, como já mostrado por nosso grupo (Iser, Bracco et al. 2014), diferentes populações de MSCs não são funcionalmente e biologicamente iguais, sendo que células derivadas de diferentes órgãos exibem particularidades, principalmente relacionadas com potencial terapêutico (Akimoto, Kimura et al. 2013), e capacidade de diferenciação (Phinney and Prockop 2007).

MSCs ainda intrigam muito os pesquisadores principalmente no que diz respeito a sua plasticidade. Inicialmente acreditava-se que essas células eram capazes de dar origem a tipos celulares da mesma camada germinativa da qual elas são originadas, ou seja, mesoderme. Entretanto, muitos estudos surgiram, mostrando que MSCs podem também dar origem a células neurais (Munoz-Elias, Marcus et al. 2004) e epiteliais (Nakagawa, Akita et al. 2005, Fu, Fang et al. 2006), ou seja, células de outras camadas germinativas. Apesar dos esforços para definir qual a real capacidade de diferenciação dessas células, essa questão ainda permanece sem resposta (Figura 5).

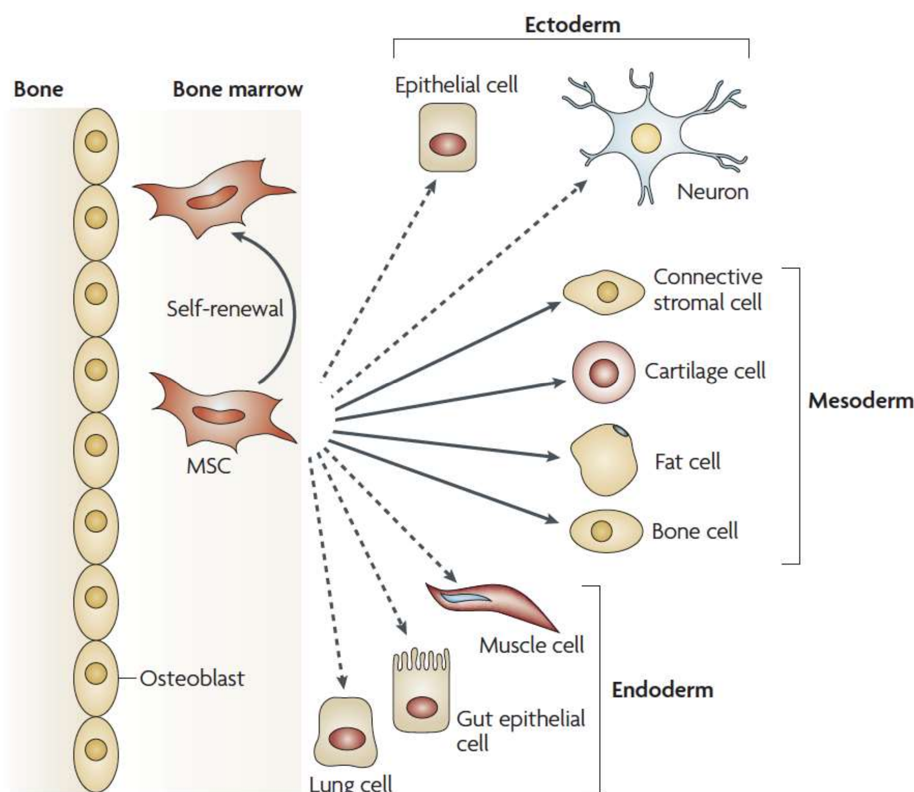

Figura 5. Capacidade de diferenciação das MSCs (Uccelli, Moretta et al. 2008).

A denominação de MSCs foi primeiramente proposto por Arnold Caplan (1991) (Caplan 1991), mas ainda não foi atingido um consenso a respeito da terminologia mais adequada para designar esse tipo celular. Muitas nomenclaturas foram propostas, como *multipotent stromal cells* (Dominici, Le Blanc et al. 2006), *mesenchymal stromal cells* (Horwitz, Le Blanc et al. 2005), ou ainda *medicinal signaling cells* (Caplan and Correa 2011), entretanto, todas elas procuram preservar a sigla MSC. De acordo com a *Mesenchymal and Tissue Stem Cell,* da *International Society for Cellular Therapy (ISCT)*, "*multipotent mesenchymal stromal cells*" é a nomenclatura recomendada (Horwitz, Le Blanc et al. 2005).

Além da natureza ambígua das MSCs, principalmente devido às diferenças na capacidade proliferativa e plasticidade de células originadas de diferentes órgãos, as variações metodológicas entre laboratórios dificultam a comparação entre as propriedades biológicas relatadas em diferentes abordagens experimentais. Para resolver essa questão, o *ISCT* propôs um conjunto de critérios para definir MSCs humanas (Dominici, Le Blanc et al. 2006). Esses

critérios incluem:

- Capacidade de aderência ao plástico em condições padrões de cultura;
- Expressâo dos marcadores de superfície CD105, CD73 e CD90 e ausência de expressão dos antígenos de superfície CD45, CD34, CD14 ou CD11b, CD79a, CD19 e HLA classe II.
- Sob condições específicas de diferenciação, devem ser capazes de se diferenciar em osteoblastos, condrócitos e adipócitos.

O grande potencial de expansão em cultura, seu alto grau de plasticidade, facilidade de isolamento, suas propriedades imunossupressoras, e capacidade de *homing* a sítios de injuria têm atraído a atenção de muitos investigadores ao longo dos anos e muitos estudos tem sido feitos utilizando células tronco para a terapia de diversas doenças (Salem and Thiemermann 2010).

Não se sabe ao certo quais os mecanismos responsáveis pela migração das MSCs a tecidos e órgãos injuriados, entretanto, acredita-se que essas células sejam recrutadas através de sinalização parácrina. MSCs possuem receptores para diversas citosinas e quimosinas, como por exemplo, os receptores CXCR4, CCR1, CCR4, CCR7, CXCR5 e CCR10, podendo assim, responder a diversas moléculas liberadas pelas células localizadas nos tecidos lesados, como CCL25, CXCL1, fator de crescimento epidermal (EGF), fator de crescimento derivado de hepatócito (HGF), interleucina-8 (IL-8), fator de crescimento derivado de plaquetas (PDGF), fator-1 derivado de células estromais (SDF1/CXCL12), fator de crescimento transformador-β (TGFβ), fator de crescimento vascular endotelial (VEGF), além de muitos outros (Chamberlain, Fox et al. 2007, Zimmerlin, Park et al. 2013). Em contrapartida, MSCs também são capazes de liberar uma infinidade de fatores, como HGF, SDF-1/CXCL12, VEGF, CCL2, CCL-5 (RANTES), TGFβ, fator de crescimento semelhante a insulina (IGF), fator de crescimento epidermal (EGF), IL-10, IL8, IL-6 (Meirelles Lda, Fontes et al. 2009, Zimmerlin, Park et al. 2013). Juntamente com o contato direto célula-a-célula, acredita-se que essa comunicação cruzada entre células lesadas ou injuriadas e MSCs seja a principal responsável pelos potenciais efeitos relacionados com as MSCs (Meirelles Lda, Fontes et al. 2009).

Até o momento, existem mais de 500 *trials* clínicos usando MSCs para o tratamento das mais diversas doenças (www.clinicaltrials.gov). MSCs têm sido muito utilizadas para terapia gênica, principalmente no campo da oncologia, uma vez que utilizando-se da capacidade que essas células possuem de migrar aos sítios de injuria, elas funcionam como carreadoras de genes suicidas, vírus oncolíticos e moléculas terapêuticas com a finalidade de inibir ou bloquear o avanço da doença (Dwyer, Khan et al. 2010). Entretanto, apesar de todos os esforços, nenhum avanço significativo, ou molécula com atividade terapêutica foi aprovada até o momento. Diante disso, os pesquisadores tem focado em estudar os fatores liberados pelas MSCs e compreender como elas se comunicam com células de outros tecidos, como tumores, por exemplo. A compreensão dos mecanismos que possibilitam essa comunicação poderia levar ao bloqueio de vias de sinalização e quem sabe, a uma terapia eficaz contra o câncer.

3.2 Células tronco adiposo-derivadas

Um tipo de MSC que tem sido bastante estudada nos últimos anos, é a célula tronco mesenquimal adiposo-derivada (ADSC). Com a crescente onda de procedimentos estéticos na população, entre eles, lipoaspirações, o tecido adiposo tem se tornado uma fonte fácil e abundante de ADSCs. Somente nos EUA, em torno de 400.000 lipoaspirações são realizadas a cada ano, produzindo ao total entre 40.000 a 1,2 x 10^5 L de tecido lipoaspirado que normalmente é descartado (Bunnell, Flaat et al. 2008). Embora muitas denominações têm sido empregadas para designar as células derivadas de tecido adiposo com capacidade de aderência ao plástico, segundo a *International Fat Applied Technology Society,* o termo *adipose-derived stem cell* é o recomendado (Mitchell, McIntosh et al. 2006). ADSCs são multipotentes e podem se diferenciar em, pelo menos, adipócitos, condrócitos e osteoblastos, entretanto, já tem sido relatada capacidade de diferenciação em células endoteliais, neuronais, hepatócitos, entre outras (Gimble and Guilak 2003).

A capacidade das ADSCs de secretar fatores, assim como as demais MSCs, tem recebido enorme atenção. Fatores secretados por hADSCs e contidos em seu meio condicionado foram os principais responsáveis por induzir angiogênese em um modelo de isquemia em camundongos. Entre esses fatores, TGFβ, HGF e VEGF, foram as mais

abundantes moléculas detectadas. É importante notar que muitos desses fatores parácrinos liberados pelas ADSCs além de serem angiogênicos, são anti-apoptóticos e que sua secreção aumenta significativamente sob condições de hipóxia (Rehman, Traktuev et al. 2004). ADSCs parecem possuir capacidade imunossupressora. Dela rosa e colaboradores mostraram que o meio condicionado de ADSCs diminuiu a proliferação de células sanguíneas mononucleares, bem como linfócitos T citotóxicos CD8+. Esse efeito paracrino foi mediado por HGF, TGFβ, entre outros fatores (DelaRosa, Lombardo et al. 2009).

Por fim, ADSCs também são conhecidas por possuírem importante papel na biologia de tumores, principalmente por, juntamente com MSCs derivadas da medula óssea (BM-ADSC), serem recrutadas aos sítios tumorais e lá exercerem ações pro ou anti-proliferativas, como será discutido no tópico abaixo (Karnoub, Dash et al. 2007, Kucerova, Altanerova et al. 2007).

3.3 Células tronco x câncer

Como revisado na “seção 1.2” desta Tese, durante a progressão tumoral, as interações entre as células que compõem o tumor e as células que fazem parte do estroma tumoral, resultam na formação de um microambiente alterado e reativo, constituído principalmente de células inflamatórias e fibroblastos ativados. MSCs, principalmente originadas do tecido adiposo adjacente ao tumor e da medula óssea, são recrutadas a esses sítios de tumorigênese. Esse potencial das MSCs têm estimulado o seu uso como veículos celulares de agentes antitumorais (Fritz and Jorgensen 2008). Entretanto, a falta de compreensão a respeito de como as MSCs interagem com as células que compõem o estroma tumoral, bem como as próprias células do tumor, nos alerta para que estudos abordando esse tipo de interação sejam conduzidos com cautela.

Acredita-se que a função das MSCs nos nossos órgãos e tecidos seja substituir células danificadas, seja por agentes estressores exógenos ou endógenos. Assim, essas células funcionam como reparadoras teciduais, responsáveis pela manutenção da integridade e homeostasia dos órgãos. Quando as MSCs exercem essas funções, elas exibem algumas características, que irão ser definitivas no seu papel durante a tumorigênese (Klopp, Gupta et al. 2011).

A primeira dessas características é a capacidade de *homing* que as MSCs possuem, permitindo que elas sejam mobilizadas e se integrem aos tecidos danificados. Vale lembrar que os tumores são sítios de inflamação, sendo por isso definidos como "*wounds that not will heal*", portanto, as MSCs são fortemente atraídas para esses sítios (Byun and Gardner 2013). Uma vez no ambiente tumoral, as MSCs irão exercer uma segunda característica importante, que é a sua atividade como imunossupressoras. Elas irão inibir as funções da imunidade adaptativa e estimular células T-regulatórias (T-regs), contribuindo assim, com o ambiente inflamatório. A terceira, e talvez a mais importante característica das MSCs, é a sua capacidade de liberar fatores tróficos que poderão estimular ou inibir células vizinhas, incluindo as células do tumor. Por fim, a quarta habilidade das MSCs é a sua capacidade de se diferenciar e substituir as células perdidas durante a injuria, fazendo com que MSCs possam se diferenciar em células do próprio estroma tumoral, como fibroblastos, por exemplo (Cuiffo and Karnoub 2012).

Entretanto, se MSCs promovem ou inibem o processo de formação do tumor e formação de metástases, ainda permanece um mistério. Curiosamente, foi demonstrado que o ambiente inflamatório pode favorecer a diferenciação de ADSCs em células endoteliais, através da indução de agentes angiogênicos, o que poderia favorecer o suprimento sanguíneo dos tumores (Ye and Gimble 2011).

Karnoub e colaboradores (2007) mostraram que hBM-ADSCs foram capazes de induzir a formação de metástases pulmonares de câncer de mama, quando co-injetadas com células tumorais em camundongos. Esses autores viram também que esse efeito foi, em parte, mediado pela quimosina CCL5, que foi liberada pelas hBM-ADSC após indução pelas células tumorais (Karnoub, Dash et al. 2007, Kucerova, Altanerova et al. 2007). Em outro estudo, foi mostrado que tumores cerebrais de camundongo são infiltrados por MSCs e que a presença dessas células favorece a progressão do tumor. *In vitro*, os autores viram que MSCs aumentam a proliferação de células GL261, uma linhagem de GBM de camundongo (Behnan, Isakson et al. 2014).

Entretando, dependendo do estímulo do microambiente, MSCs também podem ser anti-tumorais. Akimoto publicou um estudo que reflete bem este paradigma. Nesse trabalho, foi mostrado que MSCs derivadas de cordão umbilical (UCB-MSCs) foram capazes de inibir o crescimento de tumores cerebrais malignos, tanto *in vitro*, quanto *in vivo*, já as ADSCs

promoveram o crescimento tumoral. Enquanto que UCB-MSCs estimularam vias relacionadas com apoptose nas células de GBM, as ADSCs liberaram altos níveis de fatores como SDF-1, VEGF e IGF, promovendo, assim, angiogênese, o que resultou em tumores altamente vascularizados (Akimoto, Kimura et al. 2013). Outros estudos mostraram que o meio condicionado de hADSC é capaz de induzir apoptose e parada no ciclo celular de células de linhagens humanas de câncer de bexiga e GBM (Yang, Lei et al. 2014, Yu, Su et al. 2015).

Diante desses resultados contraditórios, é difícil definir qual o papel das MSCs no processo de tumorigênese, entretanto, uma coisa parece clara: MSCs podem exercer profundo efeito no microambiente tumoral.

4. Transição epitélio-mesenquimal (EMT)

Em 2011, Hanahan e colaboradores publicaram um artigo no qual eles concluíram que embora existam muitos tipos diferentes de tumores, em geral, eles dividem algumas características em comum e revisaram os seis *hallmarks of cancer*. Essas características possibilitam que as células tumorais sobrevivam, proliferem e se disseminem.

A primeira delas é a manutenção da sinalização proliferativa tumoral, principalmente por liberação de fatores solúveis pelas próprias células tumorais ou pelas células estromais associadas ao tumor. A segunda e terceira marcas do câncer são a capacidade de evadir a supressores tumorais e resistir a morte celular, principalmente por estimular a expressão de fatores anti-apoptóticos, como Bcl-2, ou inibir a expressão de fatores pro-apoptóticos, como as proteínas Bax e Bak. Apesar de a apoptose ser evitada pelas células tumorais, a necrose não é inibida, talvez pelo fato de que células necróticas liberam diversas moléculas pro-inflamatórias, o que poderia favorecer o tumor, enquanto que células apoptóticas não liberam tais fatores. A quarta característica dos tumores é a imortalidade replicativa, atingida através da contínua expressão da enzima telomerase, que possibilita as células fazerem ilimitadas divisões celulares sem que ocorra o encurtamento dos telômeros. Outro pré-requisito para a progressão tumoral é a indução de angiogênese, que gera o desenvolvimento de novos vasos sanguíneos, permitindo maior suprimento sanguíneo ao tumor em franca expansão e possibilita a eliminação de resíduos metabólicos e dióxido de carbono. Por fim, a última e

crucial marca dos tumores é a ativação de invasão e metástases (Hanahan and Weinberg 2011).

Esse ultimo *hallmark* dos tumores só e possível graças ao acionamento de um programa celular que é crucial ao processo de invasão e disseminação tumoral: a transição epitélio mesenquimal.

EMT foi primeiramente descrita como um processo que ocorre durante a embriogênese, quando células derivadas da crista neural (ectoderme) passam por EMT e adquirem características mesenquimais que lhes levarão a formar a mesoderme. No final dos anos 80 esse processo celular começou a ganhar atenção de pesquisadores que começaram a relaciona-lo com progressão tumoral (Thiery and Sleeman 2006).

EMT e um processo caracterizado pela diminuição da expressão da molécula de adesão E-caderina, resultando na dissolução de junções de aderência entre as células, aumento da expressão de moléculas relacionadas com o fenótipo mesenquimal e invasivo, como N-caderina, vimentina, fibronectina e MMPs. Além disso, as células em EMT perdem a polarização celular apico-basal e adquirem morfologia fibroblastóide. O citoesqueleto sofre reorganização, marcada pela superexpressão de vimentina ao invés das citoqueratinas. Essas características aumentam a mobilidade das células, facilitando a sua migração. Resistencia a apoptose e aquisição de características de células mais indiferenciadas também são processos importantes durante EMT (Figura 6) (Lee, Dedhar et al. 2006, Talbot, Bhattacharya et al. 2012). A concomitante perda funcional de E-caderina e a expressão de N-caderina é conhecida como *switch* entre E-caderina e N-caderina, e permite que as células tumorais se dissociem das células vizinhas e possam migrar (van Zijl, Krupitza et al. 2011).

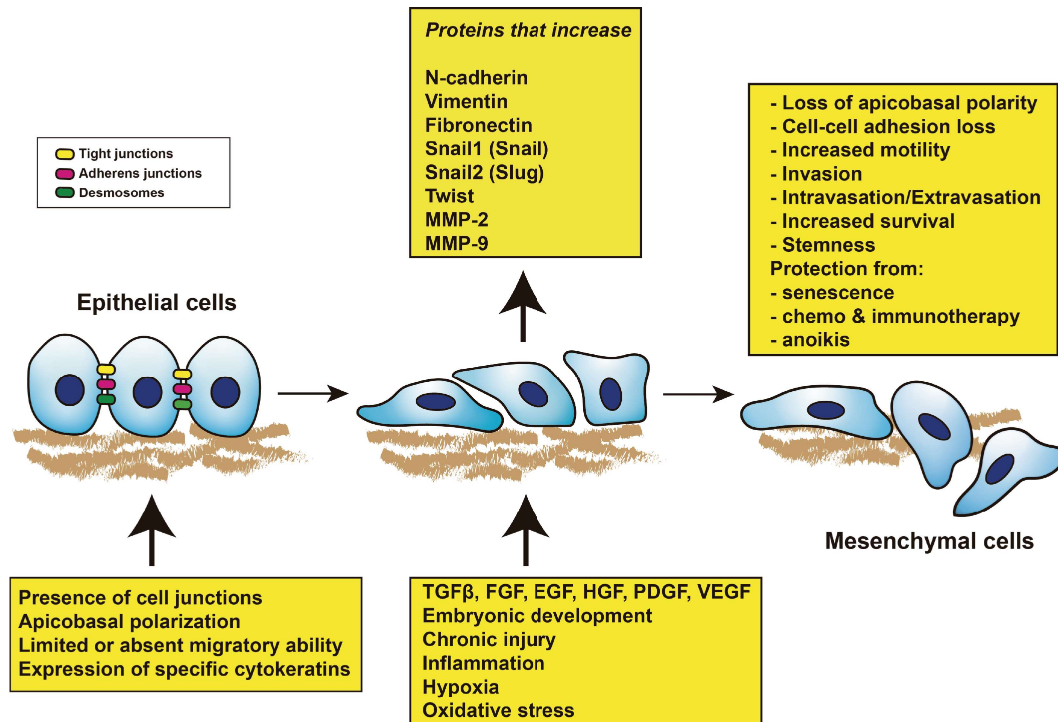

Figura 6. EMT em células tumorais. (Isabele C. Iser, dados pessoais).

EMT pode ser induzida por diferentes estímulos, incluindo fatores de crescimento (ex: EGF, FGF, HGF, IGF), citosinas (ex: TGFβ), vias de sinalização, como Wnt/β catenina, PI3k/Akt, hipóxia e oncogenes, como Ras e Src (Sanchez-Tillo, Liu et al. 2012).

EMT é orquestrada por fatores de transcrição, que incluem as famílias ZEB, Snail e Twist. Esses fatores induzem EMT não só por reprimir E-caderina, mas também por inibir outros marcadores epiteliais, como citoqueratinas. Além disso, esses fatores de transcrição ativam a expressão de marcadores mesenquimais e genes pro-invasivos, como vimentina, N-caderina e MMPs. A aquisição de características "tronco" de algumas células tumorais, resistência a morte celular e indução de angiogênese também são características reguladas por esses fatores (Christiansen and Rajasekaran 2006).

A regulação que rege EMT é muito fina e envolve ainda a participação de miRNAs (micro-RNAS), que seletivamente se ligam em específicos mRNAs (RNAs mensageiros), podendo assim, inibir a sua tradução ou promover a sua degradação. Alguns dos alvos desses miRNAs são justamente esses fatores de transcrição ou outros fatores relacionados com a regulação de EMT (Lamouille, Xu et al. 2014).

Após as células tumorais conseguirem invadir os tecidos circundantes, ou atingir órgãos distantes, elas podem readquirir o seu fenótipo epitelial inicial. Esse processo de reversão é conhecido como transição mesenquimal epitelial (MET) (Christiansen and Rajasekaran 2006).

4.1 Transição epitélio-mesenquimal x GBM

EMT é um processo que tem sido descrito em uma grande variedade de tipos tumorais, incluindo GBM. Apesar de metástases não serem comuns nesse tipo de tumor, EMT tem sido relacionada com agressividade, mau prognostico e invasão de células de GBM.

Durante a execução desse trabalho de doutorado, percebemos que havia uma carência na literatura de revisões que reunissem informações completas e atualizadas sobre EMT em GBM. Diante disso, elaboramos uma revisão sistemática que abordou o tema de forma ampla e completa e reuniu a grande maioria dos trabalhos relacionando EMT e GBM disponíveis na literatura.

Portanto, o tema que engloba esta seção da introdução está apresentado no Capítulo I da presente Tese.

5. Normalização da reação em cadeia da polimerase quantitativa em tempo real (qRT-PCR)

qRT-PCR é uma técnica estabelecida para quantificar mRNA em amostras biológicas. Essa metodologia é a técnica de maior sensibilidade e especificidade para avaliar a expressão gênica entre diferentes grupos experimentais, pois permite determinar o número exato de cópias produzidas a cada ciclo. Por isso, tem se tornado o método de escolha para detecção de mRNA (Bustin, Benes et al. 2005). Contudo, devido à alta sensibilidade desta técnica, a etapa da normalização dos dados obtidos, vem sendo bastante discutida em trabalhos recentes (Andersen, Jensen et al. 2004, Dheda, Huggett et al. 2004, Meller, Vadachkoria et al. 2005, Rubie, Kempf et al. 2005, Zhang, Ding et al. 2005).

A normalização dos níveis de expressão de um gene alvo é essencial para controlar erros experimentais entre as amostras. As maiores fontes dessas variações são a qualidade da

extração e pureza do RNA total, além da eficiência da síntese de DNA complementar (cDNA) (Silver, Best et al. 2006). Assim, a escolha de um gene de referência apropriado para cada experimento é um ponto crucial na análise da expressão gênica para ajustes compensatórios dessas variações experimentais.

Genes de referência, comumente chamados de genes constitutivos ou genes normalizadores, são caracterizados por regularem funções básicas e essenciais para as células, e por tal razão, a expressão destes genes deveria ser estável em diferentes indivíduos, tipos de tecidos e células em diferentes estados fisiológicos ou não (She, Rohl et al. 2009). No entanto, recentemente alguns pesquisadores têm mostrado que os "clássicos" genes de referência podem variar muito entre indivíduos ou em resposta a tratamento (Huggett, Dheda et al. 2005). Genes convencionais, como gliceraldeido-3-fosfato desidrogenase (GAPDH) e β-actina, que são amplamente utilizados como normalizadores em muitos laboratórios, deveriam ser empregados com mais cautela, pois muitas vezes seu uso é inapropriado devido a sua alta variabilidade (Dheda, Huggett et al. 2004). Essas variações são explicadas principalmente porque genes constitutivos não estão relacionados somente com o metabolismo celular basal, mas participam também de outras funções dentro das células (Thellin, Zorzi et al. 1999). Devido a isso, genes de referência selecionados devem ser validados para cada configuração experimental e a escolha deve ser baseada na maior estabilidade do gene entre as amostras analisadas.

Algoritmos computacionais como *BestKeeper* (Pfaffl, Tichopad et al. 2004), *GeNorm* (Vandesompele, De Preter et al. 2002) e *Normfinder* (Andersen, Jensen et al. 2004), entre outros, têm ajudado na escolha do gene de referência mais estável dentre um grupo de genes candidatos para a normalização individualizada de cada experimento. O programa *NormFinder*, calcula um valor de estabilidade para cada gene candidato, baseado na análise da expressão dos diferentes subgrupos do experimento, permitindo fazer uma estimativa da variação da expressão do gene em análise tanto intra e intergrupo. A estabilidade dos genes é mostrada como valor de estabilidade, sendo que os genes com menor valor de estabilidade possuem alta estabilidade de expressão (Andersen, Jensen et al. 2004).

HIPÓTESE DE TRABALHO

Como apresentado na introdução desta Tese, muitas perguntas ainda permanecem em aberto à respeito de como se dão as interações entre células tumorais e o seu microambiente. Dentre as células que compõem o estroma tumoral, a que mais tem atraído a atenção é a MSC, principalmente devido ao seu potencial de aplicabilidade em terapia gênica e celular, além de sua surpreendente plasticidade e versatilidade. Entretanto, existem evidências que apontam para o potencial de MSCs em estimular angiogênese e proliferação, induzir CSCs, regular o sistema imune e inibir apoptose em diferentes tipos tumorais. Diante disso, a hipótese de trabalho desta Tese é que **o meio condicionado de ADSCs possa influenciar a biologia e o microambiente de gliomas, possivelmente através da indução de transição epitélio-mesenquimal.**

OBJETIVOS

Objetivo Geral

Investigar os efeitos do meio condicionado (CM) de células tronco adiposo derivadas (ADSCs) em células C6 de glioma de rato .

Objetivos específicos

i. Avaliar o gene de referência adequado em cultura de células de glioblastoma de rato C6, expostas ou não a tratamento com meio condicionado de ADSCs;
ii. Avaliar os efeitos do CM de ADSCs na biologia e mecanismos celulares de células de glioblastoma de rato *in vitro*;
iii. Avaliar os efeitos das ADSCs e do seu meio condicionado no crescimento de glioblastoma *in vivo*;
vi. Discutir, através de um artigo de revisão, o papel da transição epitélio mesenquimal (EMT) na progressão de glioblastomas.

CAPÍTULO I

Identification of valid endogenous control genes for determining gene expression in C6 glioma cell line treated with conditioned médium from adipose-derived stem cell.

Artigo publicado no periódico ***Biomedicine and Pharmacotherapy***

APRESENTAÇÃO

Durante a execução da parte experimental dessa Tese, nos deparamos com a necessidade de validarmos o melhor gene de referência para ser usado nas nossas análises de qRT-PCR. Essa necessidade surgiu principalmente devido a nossa preocupação em garantir a confiabilidade dos nossos resultados. Entretanto, quando fizemos uma busca na literatura a fim de verificar se algum gene já havia sido validado, nos deparamos com a escassez de análises e resultados confiáveis. Embora existam inúmeros artigos publicados que utilizam tratamentos com CM de células tronco, surpreendentemente, nenhum desses trabalhos descrevia o métodos de escolha e validação do gene de referência utilizado. Mais surpreendente ainda, a maioria dos artigos que utilizam células de glioma C6, independentemente do tratamento, não validam seus genes de referência para análises moleculares.

Diante deste alarmante quadro, este artigo se propõe a apresentar os métodos e análises feitos para validarmos o mais estável e adequado gene de referência para ser utilizado em experimentos de qRT-PCR em amostras de células C6 tratadas com CM de ADSCs de rato. Essas análises também podem ser utilizadas em amostras oriundas do co-cultivo de células C6 e ADSCs.

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Original article

Identification of valid endogenous control genes for determining gene expression in C6 glioma cell line treated with conditioned medium from adipose-derived stem cell

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ABSTRACT

Introduction: There is growing evidence that mesenchymal stem cells (MSCs) can be important players in the tumor microenvironment. They can affect the glioma progression through the modulation of different genes. This modulation can be evaluated through a very useful model, treating the tumor cells with MSC-conditioned medium. However, for an accurate and reliable gene expression analysis, normalization of gene expression data against reference genes is a prerequisite.
Methods: We performed a systematic review in an attempt to find a reference gene to use when analyzing gene expression in C6 glioma cells lines. Considering that we were not able to find a reference gene originated by an appropriate validation, in this study we evaluated candidate genes to be used as reference gene in C6 cells under different treatments with adipose-derived stem cells conditioned medium (CM-ADSCs). β-actin (ACTB); glyceraldehyde-3-phosphate dehydrogenase (GAPDH); hypoxanthine-guanine phosphoribosyltransferase I (HPRT-1); TATA box binding protein (TBP) and beta-2-microglobulin (B2 M) were evaluated by real-time reverse transcription PCR (RT-qPCR). The mean Cq, the maximum fold change (MFC) and NormFinder software were used for reference gene evaluation and selection.
Results: The GAPDH and ACTB genes have been the most widely used reference genes to normalize among the different investigated genes in our review, however, controversially these genes underwent a substantial variability among the genes evaluated in the present work. Individually, TBP gene was more stable when compared with other genes analyzed and the combination of TBP and HPRT-1 was even more stable.
Conclusion: These results evidence the importance of appropriate validation of reference genes before performing qPCR experiments. Besides, our data will contribute with researchers that work analyzing the role of ADSCs in glioma microenvironment through gene expression.
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1. Introduction

Glioblastoma multiforme (GBM), classified as grade IV, is the most aggressive and lethal intracranial human tumor. This cancer is characterized by widespread invasion throughout the brain, marked angiogenesis, resistance to traditional and newer targeted therapeutic approaches, destruction of the functional architecture of the brain and death. Despite the available treatments, the high-grade gliomas remain fatal [1–3].

C6 glioma cell is a rat cell line of GBM that has the ability to form tumors in vivo and share several malignant characteristics with the human GBM [4]. Moreover, C6 glioma model has been widely characterized in the literature, being used in experimental neuro-oncology to evaluate chemotherapeutic [5], new therapies [6], gene therapy [7] and anti-angiogenic therapy [8].

Mesenchymal stem cells (MSCs) are multipotent adult stem cells with capacity to differentiate along many lineages of adult cells, such as adipocytes, chondrocytes, neuronal cells, and osteoblasts [9]. Currently, it is known that MSCs can be found in multiple tissue sites, including adipose tissue [10]. The plastic adherent cell population isolated from adipose tissue has been described by a variety of names, such as adipose-derived stem/

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stromal cells (ASCs), adipose derived adult stem (ADAS) cells, adipose mesenchymal stem cells (AMSC), among others [11]. However, the International Fat Applied Technology Society reached a consensus to adopt the term "adipose-derived stem cells" (ADSCs) to identify the plastic-adherent multipotent cell population isolated from adipose tissue [12]. Therefore, in this work, ADSCs terminology will be adopted.

Among the current new strategies that have been searched to successfully treat GBM, the use of MSCs has gained special attention, since they exhibit marked ability to preferentially engraft into tumor microenvironment [13]. MSCs have been described to support or suppress malignant cell growth depending on the tumor model tested, route of administration, nature of the tumor cells, location of the primary tumor, type of stem cell injected and interaction with the environment [14–18]. Moreover, MSCs have been used as a tool in gene therapy, since these cells can act as gene delivery vehicles for anticancer agents and appear to offer hope for a promising solution. [13,19–21]. Although many studies have been conducted for these purposes, scanty information about the mechanisms of bi-directional crosstalk between tumorigenic cells and MSCs are still available [22]. For this purpose, the conditioned medium (CM) from both, stem cells and cancer cells have been studied and many genes have been described to be up- or down- regulated by CM treatment. For instance, Zhang et al. [23] showed that CM from a human prostate cancer cell line enhances the gene expression of pro-angiogenic factors in human bone marrow derived-MSC. Furthermore, Hernanda et al. [24] demonstrated that CM from human MSC up-regulated gene expression of cell growth and proliferation-related pathways, whereas down regulated cell death-related genes in a human hepatocellular carcinoma cell line.

Gene expression analysis using quantitative real-time reverse-transcription PCR (RT-qPCR) has been shown to be a promising tool to study molecular markers related with cell malignancy, chemo-resistance and response to treatment [25–29] besides being a fast, sensitive, reproducible and specific method to analyze gene expression [30]. The normalization of this method is an important step to control the experimental errors introduced during the process, such as variability of RNA, variability of extraction protocols, RNA purity, inefficiency of reverse transcription, inaccurate quantification of RNA sample and errors of pipetting [31,32]. However, RT-qPCR is frequently applied without appropriate validation and selection of the most suitable reference gene [31].

A perfect reference gene should be stably expressed in all samples investigated, but considering the difficulty to find a perfect housekeeping gene, reference genes have been selected by RT-qPCR analysis for each individual cell type studied [33]. Furthermore, popular software programs such as Normfinder, GeNorm, and Bestkeeper, have been used to help select the best reference gene [34–36]. The advantage of using NormFinder is that this software focuses on calculating a stability value based on intra- and inter-group expression variations for candidate reference genes and employs a model allowing the elimination of systematic error [37].

Thus, in order to contribute with many researchers that work investigating the role of ADSCs in the glioma microenvironment and considering that a reliable gene expression analysis by RT-qPCR depends on the correct housekeeping gene selection, in the present study, we evaluate the most stable reference gene in C6 glioma cell line treated and untreated with conditioned medium from adipose derived- stem cells (ADSCs-CM).

2. Materials and methods

2.1. Ethical aspects

The protocols used in this study were approved by the Ethics Committee on Animal Use (CEUA) of Universidade Federal de Ciências da Saúde de Porto Alegre (UFCSPA), under the number 104/11, following the resolutions of the the CONCEA (Conselho Nacional de Controle de Experimentação Animal). The NIH "Guide for the Care and Use of Laboratory Animals" (NIH publication No. 80–23, revised 1996) was followed in all experiments. The surgery of ADSC isolation was performed with all efforts to minimize the animal suffering.

2.2. Literature review

We carried out a systematic review using PubMed database to search publications available between January 2009 and February 2015 that analyze possible reference genes in RT-qPCR for C6 cells. The search strategy comprised the Medical Subject Heading (MeSH) terms 'reference genes' or 'housekeeping genes' or 'RT-qPCR' or 'quantitative PCR' or 'gene expression' AND "C6 cells". All full-text studies in English were included. Publications were evaluated according to the type of samples, reference gene used for normalization of target genes and the utilized RT-qPCR chemistry (SYBRgreen or TaqMan).

2.3. C6 glioma cell line culture

All materials were supplied by Sigma-Aldrich Corporation (St. Louis, MO, USA) unless otherwise stated. The rat C6 glioma cell line was kindly provided by Dr. Ana Maria Oliveira Battastini (UFRGS, Brazil). The cells were grown (passage 10–20) in Dulbecco's modified Eagle's medium (DMEM) low glucose supplemented with 10% (v/v) FBS containing 8.39 mM HEPES (pH 7.4), 23.8 mM $NaHCO_3$, 0.1% fungizone, 0.5 U/ml penicillin/streptomycin (Gibco BRL, Grand Island, NY, USA). The cultures were maintained in 5% CO_2/95% air at 37 °C.

2.4. Isolation, culture and characterization of ADSCs

ADSCs were isolated from adult Wistar rats as described previously [10]. Briefly, rats (6–8 week-old male Wistar rats, weighing approximately 200 g) were euthanized by intraperitoneal (i.p.) administration of an overdose of ketamine/xylazine followed by cervical dislocation. Then, adipose tissue was collected, rinsed in phosphate-buffered saline (PBS) and subsequently digested with collagenase type I (2 mg/ml in DMEM/10 mM HEPES) for 30 min at 37 °C. After collagenase digestion, the supernatant was transferred to a new tube and centrifuged at 198 g for 10 min at room temperature (RT), the pellets were resuspended in complete medium and seeded in six-well dishes. ADSCs cultures were grown in culture flasks and maintained in DMEM low glucose, containing 8.39 mM HEPES (pH 7.4), 23.8 mM $NaHCO_3$, 0.1% fungizone, 0.5 U/ml penicillin/streptomycin (Gibco BRL, Grand Island, NY, USA and supplemented with 10% (v/v) fetal bovine serum (FBS; Gibco BRL, Grand Island, NY, USA). Cells were kept at a temperature of 37 °C, humidity of 95%/5% CO_2 in air. Cultured ADSCs were tested for their ability to differentiate into adipogenic and osteogenic cell lineages as described by Da Silva Meirelles et al. [10].

The cultures were characterized to confirm the presence or absence of MSC surface markers using the flow cytometry technique. The cells were trypsinized, centrifuged, and incubated for 25 min at 4 °C with phycoerythrin (PE)- or peridinin-chlorophyll protein (PerCP-Cy5.5)-conjugated antibodies anti-rat CD45, CD29 and CD90.2 (Life Technologies, Carlsbad, CA, USA). The unconjugated antibody CD11b (Life Technologies, Carlsbad, CA, USA) was incubated with the secondary anti-mouse IgG2 FITC-conjugated antibody (Sigma–Aldrich, St. Louis, MO, USA) for an additional 25 min. Excess of antibody was removed by washing cells in PBS (phosphate buffered saline). The cells were analyzed using a FACScalibur cytometer equipped with 488 nm argon laser (Becton Dickinson, San Diego, CA, USA) with the CellQuest

software. At least 10,000 events were collected. In each analysis, gating was set using unstained cells.

2.5. Medium conditioning procedure

For all experiments, cells between the 4th and 10th passages were seeded in T75 tissue culture flask in DMEM with 10% (v/v) FBS low glucose, containing 8.39 mM HEPES (pH 7.4), 23.8 mM $NaHCO_3$, 0.1% amphotericin B, 0.5 U/mL penicillin/streptomycin and supplemented with 10% (v/v) FBS at a density of 249,000 cells. After twenty-four hours the non-adherent cells were removed by changing the medium that was conditioned for the next 24 h or 48 h. Conditioned medium (CM) from flasks was collected, filtrated on 0.22 μm filters (Millipore), and stored at −80 °C until use.

2.6. Treatment of C6 cells with ADSC-CM

C6 cell cultures were plated with complete DMEM medium and were allowed to attach overnight. Then, the medium was replaced by ADSC-CM and cells were incubated for 24 h, 48 h or 5 days. Cells control were grown in DMEM with 10% (v/v) FBS during the same period.

2.7. RNA extraction

Total RNA from ADSCs cultures was isolated with Trizol LS reagent (Life Technologies, Carlsbad, CA, USA) in accordance with the manufacturer's instructions and stored at −80 °C. RNA concentration and purity were assessed by Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Rockland, DE, USA). RNA purity was considered appropriate when the ratio of measurements at A260:A280 was from 1.8 to 2.1.

2.8. cDNA synthesis

The cDNA species were synthesized with M-MLV Reverse Transcriptase (Promega, Madison, WI, USA) from 3 μg total RNA in a final volume of 20 μL with a random hexamer primer in accordance with the manufacturer's instructions. cDNA was diluted to 1:10 in diethyl pyrocarbonate (DEPC) water and stored at −20 °C.

2.9. Quantitative real-time PCR analysis

RT-qPCR amplification was carried out with five genes commonly used as reference gene in the literature [33,38–41] (Table 1): beta-actin (ACTB); glyceraldehyde-3-phosphate dehydrogenase (GAPDH); hypoxanthine-guanine phosphoribosyl-transferase 1 (HPRT-1); TATA box binding protein (TBP) and beta-2-microglobulin (B2 M). Primers were designed using the Primer-BLAST program (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) and were analyzed using Integrated DNA Technologies OligoAnalyzer 3.1 software (http://www.idtdna.com/calc/analyzer/) (Table 2). Real-time PCR samples were prepared in a 12.5 μL final volume composed of 6.25 μL of Fast SYBR® Green Master Mix (Applied Biosystems, Foster City, CA, USA), 0.4 μL of primer pair solution (0.2 μM final concentration of each primer), 3.85 μL of water and 2 μL of diluted cDNA. Real-time PCRs were carried out in an Applied-Biosystem StepOnePlus™ Real-Time PCR cycler and performed in duplicates. Reaction settings were composed of an initial enzyme activation step of 20 s at 95 °C, followed by 40 cycles of 3 s at 95 °C and 30 s at 60 °C for data aquisition. The melting curve analysis was performed in single cycle by heating samples from 65 °C to 95 °C at a ramp rate of 0.5 C/s with continuous fluorescent acquisition.

Table 2
Primer sequences, annealing temperatures, and fragment size.

Gene	Primer sequence	*T* (°C)	Fragment size (bp)
ACTB F	5′-CAGGATGCAGAAGGAGATTAC-3′	60	115
ACTB R	5′-CAGTGAGGCCAGGATAGA-3′		
B2M F	5′-CCACCCACCTCAGATAGAA-3′	60	113
B2M R	5′-TGTGAGCCAGGATGTAGAA-3′		
HPRT-1 F	5′-AGCCAAGTACAAAGCCTAAA-3′	60	101
HPRT-1 R	5′-ATGGCCACAGGACTAGAA-3′		
GAPDH F	5′-GCATCTCCCTCACAATTCC-3′	60	99
GAPDH R	5′-GGGTGCAGCGAACTTTAT-3′		
TBP F	5′-CGTGACGATAACCCAGAAAG-3′	60	115
TBP R	5′-GGTGGAAGGCTGTTGTTC-3′		

bp: base pairs; °C: celsius degree, B2M: β-2-microglobulin; GAPDH: glyceraldehydes 3-phosphate dehydrogenase; TBP: TATA box binding protein; HPRT-1: hypoxanthine-guanine phosphoribosyltransferase 1.

2.10. Quantification and data analysis

The relative standard curve method was used for quantification of mRNA expression. cDNA standard curves were constructed using the threshold cycles with five successive tenfold dilution points of a pool of cDNA samples and relative gene expression of each sample was calculated from its respective standard curve.

2.11. Statistical analysis

To evaluate the stability of the candidate reference genes in C6 cells treated or not with ADSC-CM, the NormFinder algorithm was used [34]. In addition, the analysis of raw quantification cycle (Cq) values of each gene was used to evaluate their stability. Mean Cq values, standard deviation (SD), coefficient of variation (CV), and maximum folds change (MFC, the ratio of the maximum and minimum values observed within the dataset) were calculated.

3. Results

3.1. Systematic review

In 2009, Bustin published guidelines, also called 'MIQE Guidelines', to help the research community with criteria to achieve the

Table 1
Candidate normalization genes evaluated in this study.

Symbol	Gene name	Accession number[a]	Function
ACTB	Actin, beta	NM_031144.2	Cytoskeletal structural protein
B2M	beta-2 Microglobulin	NM_012512.1	Component of MHC-I[b]
HPRT-1	Hypoxanthine-guanine phosphoribosyltransferase 1	NM_012583.2	Catalyzes conversion of hypoxanthine to inosine monophosphate and guanine to guanosine monophosphate
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	NM_017008.9	Glycolysis and gluconeogenesis
TBP	TATA box binding protein	NM_001004198.1	Transcription factor

[a] Primer design based on this sequence. The database is the Ensembl database (http://www.ensembl.org).
[b] MHC-I major histocompatibility complex class I.

Table 3
Characteristics of RT-qPCR in C6 cell line.

Author; year	Method of quantification	Reference gene	Method of reaction
Bensalma S.; 2015 [51]	Relative standard curve	GAPDH	SYBR
Ma W.W.; 2015 [52]	ND	GAPDH	SYBR
Azevedo H.; 2014 [53]	Relative standard curve	GAPDH	SYBR
Kumar P.; 2014 [54]	ND	ACTB	ND
kwon M.S.; 2014 [55]	ND	GAPDH	SYBR
Yu Z.Q.; 2014 [56]	Relative quantification (2 -deltadeltaCt)	GAPDH	SYBR
Bernier M.; 2013 [57]	Relative quantification (2 -deltadeltaCt)	GAPDH	SYBR
Chen M.L.; 2013 [58]	Relative standard curve	Mean of peptidylprolylisomerase A (PpiA) and 18S rRNA	SYBR
Fang K.M.; 2013 [59]	Relative quantification (2 -deltadeltaCt)	Cyclophilin A (CyPA)	SYBR
Wu Q.; 2013 [60]	ND	ND	ND
Biegańska K.; 2012 [61]	Relative standard curve	GAPDH	SYBR
Hattermann K.; 2012 [62]	Relative quantification (2 -deltadeltaCt)	GAPDH	nd
Lin Y.; 2012 [63]	ND	ACTB	SYBR
Liu J.; 2012 [64]	Relative quantification (2 -deltadeltaCt)	ACTB	SYBR
Zu H.; 2012 [65]	Relative quantification (2 -deltadeltaCt)	GAPDH	ND
Adach-Kilon A.; 2011 [66]	Relative quantification (2 -deltadeltaCt)	18S rRNA	SYBR
Escartin C.; 2011 [67]	Relative quantification (2 -deltadeltaCt)	GAPDH	SYBR
Gatson J.W.; 2011 [68]	ND	GAPDH	SYBR
Shu M.; 2011 [69]	ND	U6 snRNA	SYBR
Sugimoto T.; 2011 [70]	Relative quantification (2 -deltadeltaCt)	GAPDH	SYBR
Tsuchioka M.; 2011 [71]	Relative quantification (2 -deltadeltaCt)	GAPDH	TaqMan
Wang T.; 2011 [72]	ND	ACTB	SYBR
Yang L.; 2011 [73]	ND	ACTB	ND
Chen Y.Y.; 2010 [74]	Relative quantification (2 -deltadeltaCt)	GAPDH	SYBR
Jung S.E.; 2010 [75]	Relative quantification (2 -deltadeltaCt)	ACTB	TaqMan
Tnabe K.; 2010 [76]	ND	GAPDH	SYBR
Chu S.H.; 2009 [77]	ND	18S rRNA	TaqMan
Hsuchou H.; 2009 [78]	ND	GAPDH	SYBR
Zhang S.; 2009 [79]	Relative quantification (2 -deltadeltaCt)	ACTB	ND

high-quality in RT-qPCR analysis [42]. Then, we performed a literature review after MIQE Guidelines publication and identified 109 full-text studies from January 2009 to February 2015 analyzing reference genes in C6 cell line. After the analysis of these studies, 26.6% was considered eligible for this review (Table 3). Surprisingly, no study reported any data about the tools or the methods employed in the choice of reference gene in their analysis. Most of these studies analyzed used GAPDH as reference gene (55.17%), followed by the ACTB gene (24.13%). One study used cyclophilin A (CyPA), two studies used 18S rRNA, one study used U6 small nuclear ribonucleic acid (snRNA) and one another study used the mean of peptidylprolylisomerase A (PpiA) and 18S rRNA as reference gene. SYBR® Green, a fluorescent binding dye, was the most frequently used (68.96%), while three studies used TaqMan

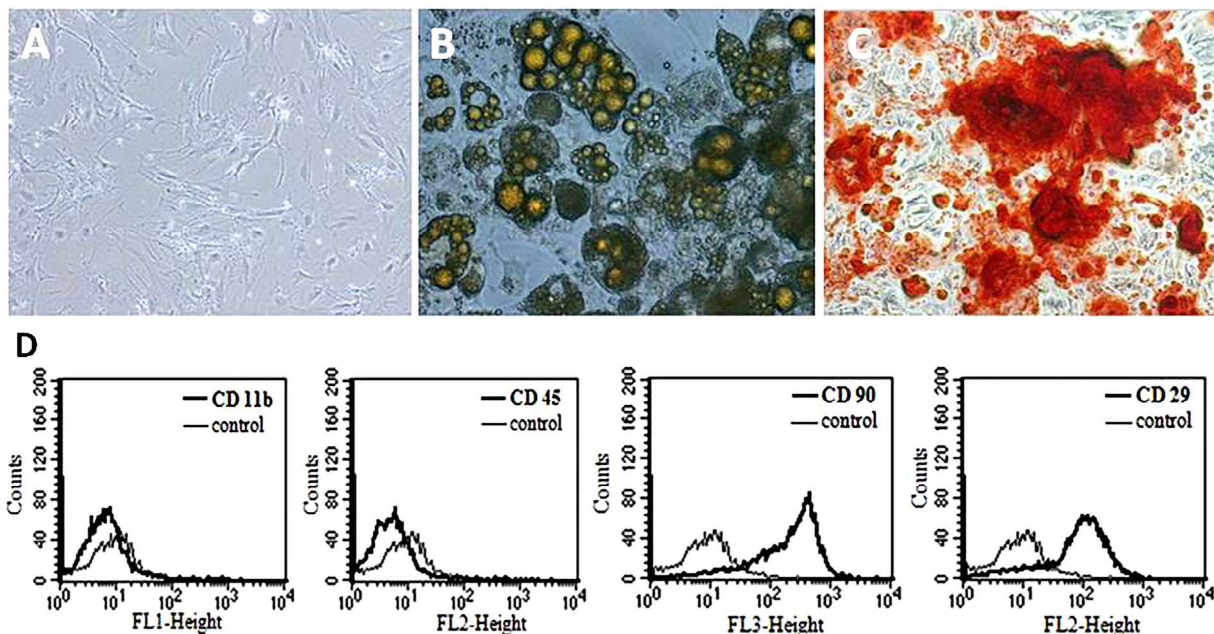


Fig. 1. ADSC characterization. The differentiation of ADSCs is shown in light microscopy at 200× magnification. (A) Morphology of rat ADSCs cultures. (B) Cells differentiated into adipocytes showing the lipid vacuoles stained with Oil Red. (C) Cells differentiated into osteoblasts showing the bone matrix stained by Alizarin Red. (A–C) Magnifications, ×200. (D) Immunophenotyping analysis of surface marker expression in ADCSs. Flow cytometry histograms show the expression (bold line) of selected molecules (CD90, CD29, CD11b and CD45) by ADSC populations compared with controls.

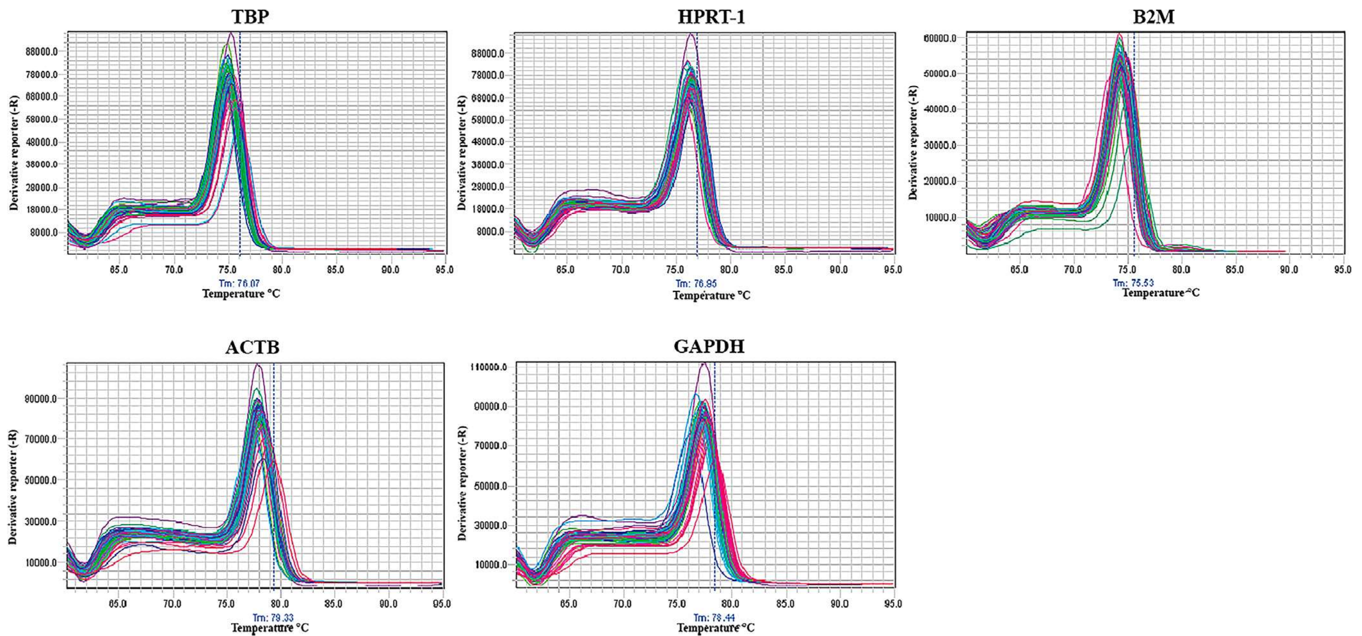


Fig. 2. Melting curves of the 5 reference genes. Melt curve peak chart collected using the StepOnePlus™ (Applied Biosystems).

assay (10.34%) and six studies did not report chemistries utilized in RT-qPCR assay (20.68%). For quantification of mRNA levels, the most frequently used was the comparative Ct method (44.82%), followed by relative standard curve (13.79%), while twelve studies (41.37%) did not report any data about quantification method used.

3.2. ADSC characterization

ADSCs adhered to plastic and exhibited fibroblast-like spindle-shaped morphology as can be expected for mesenchymal stem cells (Fig. 1a). In order to examine whether ADSCs show differentiation potential *in vitro*, adipogenic and osteogenic induction were performed. The cells studied were committed toward adipogenic lineage, acquiring intracellular lipid droplets, evidenced by Oil Red O staining (Fig. 1b). The osteogenic induction was also successful as shown by the alizarin red staining result, with calcium-rich mineralized matrix deposits in culture (Fig. 1c).

The analysis of surface markers indicated that the ADSCs populations studied expressed CD90.2 (Thy1.2) and CD29 (integrin β-1) as demonstrated by flow cytometry analysis. The hematopoietic markers CD45 (leukocyte common antigen) and CD11b (integrin αM) were not expressed by ADSCs (Fig. 1d). All experiments were performed using cells between fourth and tenth passages.

3.3. Reference genes validation in C6 cells treated or untreated with ADSCs-CM

Five reference genes were amplified in 9 groups of C6 cells (treated or untreated with ADSCs-CM). All RT-qPCR assays produced a single peak of fluorescence in the melting curve (Fig. 2). The absorbance ratios (A_{260}/A_{280}) which provide an indication of RNA purity are presented in Table 4. We found that the absorbance ratios ranged from 1.89 to 2.06. Also, primer specificity was checked by PCR and subsequent agarose gel electrophoresis (not shown). The quality of the standard curve can be evaluated from the slope and the correlation coefficient (R^2). A good reaction should reach efficiency between 90% and 110%, which corresponds to a slope between −3.10 and −3.58 [43,44]. In our samples, the efficiency of studied reference genes ranged from 97.16% to 106.16% and slope from −3.16 to −3.39 (Table 5). The correlation coefficient confirm the linear relationship between the Ct and the logarithm of cDNA concentration. Standard curves should be led to a high linearity ($R^2 \geq 0.99$) [43,44]. In this work, the standard curves had a R^2 ranging between 0.979 and 0.997 (Table 5).

In the current report, NormFinder was used to select the most stable candidate genes. NormFinder software ranks all reference gene candidates based on intra- and inter-group variations and combines both results into a stability value for each candidate gene [45]. Lower stability values are referred to as higher expression stability. In Table 6, stability values obtained are shown, already considering both variations. The most stable to the least stable genes were TBP > HPRT-1 > GAPDH > ACTB > B2M.

The most stable gene was TBP (0.363). However, the stability is higher when TBP and HPRT-1 are used combined (0.297). The mean Cq cycle values, SD, CV and MFC obtained for each gene in control and treated C6 are described in Table 7. Genes that exhibit the lowest CV and SD are considered as the most stable genes [46]. Moreover, an ideal normalizer gene should have the MFC < 2 [47,48]. In our study, all 5 genes had a MFC below 2. However, the lowest values were found to HPRT-1 (1.21) and TBP (1.23). Indeed, HPRT-1 and TBP also showed the lowest values of CV, 4.1% and 4.2%, respectively.

Table 4
Absorbance ratios (A_{260}/A_{280}) of C6 cell line treated and untreated with ADSC-CM.

	G1	G2	G3	G4	G5	G6	G7	G8	G9
1	1.97	2	1.94	2.02	1.99	2.02	2.01	1.99	2.06
2	1.94	2	1.94	1.96	2.03	2.02	1.95	2.02	2.02
3	1.97	1.98	1.95	2.02	1.89	2	1.91	1.99	2.03

G1: ADSCs after 24 h of exposure to unconditioned DMEM medium; G2: ADSCs after 24 h of exposure to ADSC-conditioned medium (conditioned during 24 h); G3: ADSCs after 24 h of exposure to ADSC-conditioned medium (conditioned during 48 h); G4: ADSCs after 48 h of exposure to unconditioned DMEM medium; G5: ADSCs after 48 h of exposure to ADSC-conditioned medium (conditioned during 24 h); G6: ADSCs after 48 h of exposure to ADSC-conditioned medium (conditioned during 48 h); G7: ADSCs after 5 days of exposure to unconditioned DMEM medium; G8: ADSCs after 5 days of exposure to ADSC-conditioned medium (conditioned during 24 h); G9: ADSCs after 5 days of exposure to ADSC-conditioned medium (conditioned during 48 h). 1, 2 and 3 are the different C6 cultures treated and analyzed ($n = 3$).

Table 5
qPCR parameters providing the standard curve for each primer pair on 5 reference genes.

Gene	Efficiency	Slope	R^2
TBP	106.86	−3.16	0.997
HPRT-1	105.22	−3.20	0.996
ACTB	99.52	−3.33	0.990
B2M	98.81	−3.35	0.979
GAPDH	97.16	−3.39	0.985

Correlation coefficient (R^2). Efficiencies (E) were calculated based on the standard curve according to the equation $[E = 10(-1/\text{slope}) - 1] \times 100$ and are expressed as a percentage.

Table 6
Candidate reference genes for normalization of RT-qPCR in C6 cells treated and untreated with ADSC-CM, according to their stability, as calculated by NormFinder.

Ranking order	Gene	Stability value
1	TBP	0.363
2	HPRT-1	0.403
3	GAPDH	0.691
4	ACTB	0.928
5	B2M	0.999

The candidates are listed from the most stable gene (TBP) to the least stable (B2M).

Table 7
Dispersion data of raw Cq values for candidate reference genes in C6 cells treated and not treated with CM.

Symbol gene	Mean Cq	SD	CV (%)	Minimum	Maximum	MFC
TPB	24.54	1.03	4.2	22.85	28.19	1.23
HPRT-1	26.36	1.08	4.1	24.60	29.89	1.21
ACTB	18.10	1.63	9.0	15.99	24.58	1.53
GAPDH	18.85	1.05	5.6	17.41	22.66	1.30
B2M	21.18	0.96	4.5	20.05	24.90	1.24

SD: standard deviation; CV: coefficient of variation; and MFC: maximum fold change (the ratio of the maximum and minimum values).

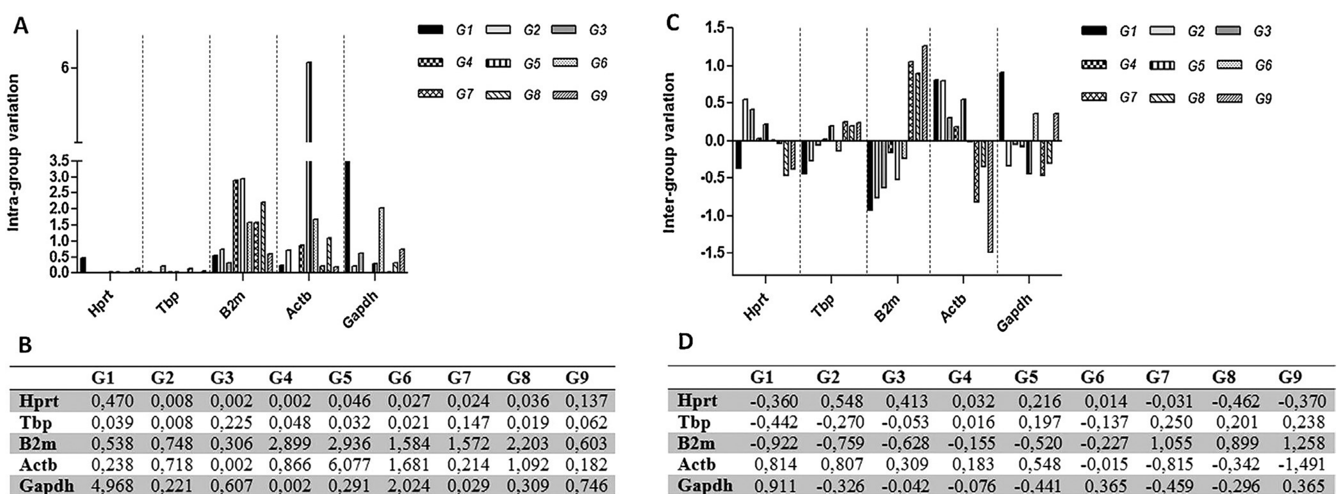
These results reflect the minor variation in expression of those candidate housekeeping genes within the dataset. The evaluation of inter- and intra-group expression variations is also very important because it allows evaluating the heterogeneity between different cultures and treatments. In Fig. 3 it can be seen that TBP and HPRT-1 had the lowest variation both, intra- (Fig. 3a and b) and inter-group (Fig. 3c and d) when compared to the other evaluated genes.

4. Discussion

Gene expression analysis using RT-qPCR has been shown to be a promising approach to improve the current knowledge about cancer progression [33]. However, the PCR efficiency can vary over time and across samples [49]. Therefore, the choice of an appropriate reference gene is fundamental to avoid an erroneous interpretation of the results. After a systematic review of the literature becomes clear the necessity of more attention from scientific community to the importance of the correct choice of housekeeping genes to RT-qPCR experiments. It is surprising that among the studies included in our search, none has sufficiently evaluated the appropriateness of the applied normalization strategy. The GAPDH and ACTB genes have been the most widely used housekeeping genes to normalize the different investigated genes in our review (Table 3). However, it seems to be the choice adopted by researchers without taking into account the different treatments used in these many studies.

A recently published report has identified that "classic" genes widely used in the current literature, such as GAPDH and ACTB, are inappropriate, due to their variability [31] in the biological samples. In agreement, the stability levels of these commonly used genes fluctuated dramatically among the genes evaluated in the present work (0.691 and 0.928, respectively) (Table 1). Moreover, these genes showed the highest CV values, ranged from 5.6% (GAPDH) to 9.0% (ACTB) (Table 2), reflecting the high variable levels of those commonly used housekeeping genes within our analysis.

In spite of few studies found in the literature about choice of housekeeping genes in glioblastomas, according to our findings, Velente, et al. analyzed seven housekeeping genes in 30 human glioblastoma samples and also found that TBP and HPRT-1 were the best combination of reference genes for expression studies in human GBM [50]. Although this work corroborate with our findings, it is always important considerer the conditions of each experimental assay such as the different treatments used.



	G1	G2	G3	G4	G5	G6	G7	G8	G9
Hprt	0,470	0,008	0,002	0,002	0,046	0,027	0,024	0,036	0,137
Tbp	0,039	0,008	0,225	0,048	0,032	0,021	0,147	0,019	0,062
B2m	0,538	0,748	0,306	2,899	2,936	1,584	1,572	2,203	0,603
Actb	0,238	0,718	0,002	0,866	6,077	1,681	0,214	1,092	0,182
Gapdh	4,968	0,221	0,607	0,002	0,291	2,024	0,029	0,309	0,746

	G1	G2	G3	G4	G5	G6	G7	G8	G9
Hprt	-0,360	0,548	0,413	0,032	0,216	0,014	-0,031	-0,462	-0,370
Tbp	-0,442	-0,270	-0,053	0,016	0,197	-0,137	0,250	0,201	0,238
B2m	-0,922	-0,759	-0,628	-0,155	-0,520	-0,227	1,055	0,899	1,258
Actb	0,814	0,807	0,309	0,183	0,548	-0,015	-0,815	-0,342	-1,491
Gapdh	0,911	-0,326	-0,042	-0,076	-0,441	0,365	-0,459	-0,296	0,365

Fig. 3. Intra- and inter-group variation. Intra- (a and b) and intergroup variation (c and d) of five reference genes in C6 glioma cell line treated or untreated with ADSC-CM for 24 or 48 h, as determined by NormFinder, showing TBP and HPRT-1 as the genes with the slowest variation (most stability). G1: ADSCs after 24 h of exposure to unconditioned DMEM medium; G2: ADSCs after 24 h of exposure to ADSC- conditioned medium (conditioned during 24 h); G3: ADSCs after 24 h of exposure to ADSC- conditioned medium (conditioned during 48 h); G4: ADSCs after 48 h of exposure to unconditioned DMEM medium; G5: ADSCs after 48 h of exposure to ADSC- conditioned medium (conditioned during 24 h); G6: ADSCs after 48 h of exposure to ADSC- conditioned medium (conditioned during 48 h); G7: ADSCs after 5 days of exposure to unconditioned DMEM medium; G8: ADSCs after 5 days of exposure to ADSC- conditioned medium (conditioned during 24 h); G9: ADSCs after 5 days of exposure to ADSC- conditioned medium (conditioned during 48 h)

Considering the growing evidences about the importance of the tumor microenvironment, it is possible to assume that interactions between ADSCs and tumor cells could become a new target in cancer therapy. Thus our results could be potentially helpful, contributing with future studies addressing how ADSCs affect brain tumors, using a C6 glioma cell line as a model.

5. Conclusion

In conclusion, our data show the relevance of previous validation of candidate housekeeping genes for each specific application. The results of the present study suggest that TBP or TBP plus HPRT-1 are suitable reference genes to evaluate effects of CM from ADSCs in C6 glioma cells. Together, these results highlight the importance of caution with gene expression data currently available in literature.

Author contributions

Conceived and designed the experiments: ICI APSB MRW. Performed the experiments: ICI, APSB, RPC. Analyzed the data: ICI, APSB, MRW. Contributed reagents/materials/analysis tools: MRW. Wrote the paper: ICI, APSB, MRW.

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CAPÍTULO II

Conditioned medium from adipose-derived stem cells (ADSCs) promotes epithelial-to-mesenchymal-like transition (EMT-like) in glioma cells in vitro

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APRESENTAÇÃO

O nosso grupo de pesquisa tem como uma das linhas de interesse, o estudo das interações entre células tumorais e MSCs. A nossa atenção se voltou para esse assunto quando começamos a avaliar a segurança da aplicação de MSCs em outros tipos de patologias e também devido a nossa outra linha de pesquisa que visa utilizar MSCs como vetores de agentes terapêuticos contra tipos tumorais. Trabalhos na literatura mostrando que MSCs fazem parte do microambiente tumoral e que as interações entre as células que compõem o estroma do tumor e células do próprio tumor poderiam levar a um fenótipo tumoral mais agressivo, nos levaram a investigar os efeitos das MSCs, atraves de seu CM na biologia dos GBMs.

Neste artigo são apresentados os resultados de nossas análises de como células de glioma C6 respondem ao tratamento com CM de ADSCs. Primeiramente, foram analisados parâmetros como viabilidade celular, ciclo celular e capacidade proliferativa. Num segundo momento, analisamos capacidade de adesão, migração e morfologia celular e nuclear das células de glioma tratadas com CM.

Antes de começarmos a desenvolver este trabalho, apenas um artigo havia analisado os efeitos do CM de células tronco no processo de EMT em GBM. Entretanto, nesse artigo foram utilizadas células tronco de cordão umbilical e foi avaliada apenas a população de células tronco do tumor. Como sabemos, células tronco de origens distintas podem agir completamente diferente umas das outras.

Desse modo, a falta de estudos nesse campo da ciência, aliada à necessidade de melhorar a compreensão a respeito dos mecanismos de invasão dos GBMs, nos levaram ao conjunto de resultados que é apresentado neste Capítulo da tese.

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Conditioned Medium from Adipose-Derived Stem Cells (ADSCs) Promotes Epithelial-to-Mesenchymal-Like Transition (EMT-Like) in Glioma Cells In vitro

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Abstract Mesenchymal stem cells (MSCs) have recently been described to home to brain tumors and to integrate into the tumor-associated stroma. Understanding the communication between cancer cells and MSCs has become fundamental to determine whether MSC-tumor interactions should be exploited as a vehicle for therapeutic agents or considered a target for intervention. Therefore, we investigated whether conditioned medium from adipose-derived stem cells (ADSCs-CM) modulate glioma tumor cells by analyzing several cell biology processes in vitro. C6 rat glioma cells were treated with ADSCs-CM, and cell proliferation, cell cycle, cell viability, cell morphology, adhesion, migration, and expression of epithelial-mesenchymal transition (EMT)-related surface markers were analyzed. ADSCs-CM did not alter cell viability, cell cycle, and growth rate of C6 glioma cells but increased their migratory capacity. Moreover, C6 cells treated with ADSC-CM showed reduced adhesion and underwent changes in cell morphology. Up-regulation of EMT-associated markers (vimentin, MMP2, and NRAS) was also observed following treatment with ADSC-CM. Our findings demonstrate that the paracrine factors released by ADSCs are able to modulate glioma cell biology. Therefore, ADSC-tumor cell interactions in a tumor microenvironment must be considered in the design of clinical application of stem cell therapy.

Keywords Adipose-derived stem cell · Conditioned medium · C6 glioma cell · Epithelial-mesenchymal transition

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Introduction

Gliomas are the most frequent primary tumors that affect the human brain. Glioblastoma (GBM) is the most common malignant form of glioma and is one of the most aggressive and lethal human cancer. The current survival rate for patients with GBM is about 1 year [1, 2], and this has not changed significantly over the last decade. GBMs are characterized by widespread invasion throughout the brain, marked heterogeneity in appearance and gene expression, and resistance to traditional and newer targeted therapeutic approaches. Despite the available treatments with surgical resection of the tumor, radiotherapy, chemotherapy, and drugs, such as temozolomide, these treatments have shown only slight effects on survival of patients and GBM remains fatal [2–4].

It is now understood that maintaining the mutual and interdependent interaction between tumor and its microenvironment is crucial to orchestrate the fate of tumor progression [5]. A tumor microenvironment is composed of a complex crosstalk of different cell types such as fibroblast, endothelial cells, mesenchymal stem cell (MSCs), and inflammatory cells as well as cytokines and growth factors secreted by these cells [5]. Inflammation is always associated with tumor development where the tissue suffers from chronic injury. Thereby, malignancy may also be considered as a nidus of chronic inflammation or "wound that never heals" [6]. Based on the property of MSCs to be recruited to injured tissues, MSCs can

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be attracted to the surrounding tumor stroma and play a role in modulating cancer progression [7].

MSCs are being broadly studied and have emerged as a great promise of the current therapeutic research. The enthusiasm about these cells is mainly due to their ability to differentiate in many cell types, capacity of self-renew, and paracrine activity that includes immunomodulatory effects [8]. Contrary to what was initially thought, the ability to differentiate is not the main feature that makes these cells attractive for therapeutic purposes. There are increasing evidences that the secretions of these cells, such as nucleotides, growth factors, cytokines, and chemokines constitute their most significant therapeutic mechanism of action [9–11]. The tumor homing ability has encouraged researchers to analyze MSCs as a tool in gene therapy, since these cells can act as gene delivery vehicles for anticancer agents [12–15]. However, evidence suggests that interactions between MSCs and cancer cells may impact upon the phenotype of the tumor cells [16–20]. Conflicting reports exist with relation to the effect of MSCs on glioma cells growth, with some studies reporting cytotoxicity against malignant glioma cells [20–23] and others suggesting a pro-tumoral effect [24–28].

Epithelial to mesenchymal transition (EMT) is a process in which cells gradually substitute their epithelial characteristics with a mesenchymal phenotype, that is associated with increased invasion and metastasis [29]. This status is characterized by increased motility, invasiveness, and elevated synthesis of extracellular matrix-degrading enzymes able to degrade the basement membrane, besides loss of cell-cell contact, reorganization of cytoskeletal proteins, and elevated resistance to apoptosis [30, 31]. Notably, the composition of the tumor microenvironment, such as growth factors and cytokines, has recently been suggested to modulate the EMT process in cancer cells [29, 32–35].

In our study, conditioned medium from adipose-derived stem cells (ADSC-CM) from rat did not change the viability, proliferation, or cell cycle distribution of glioma. However, ADSC-CM induced an epithelial-to-mesenchymal-like transition phenotype in tumor cells with increased tumor cell migration, reduced cell adhesion capacity, changed cell morphology, as well as increased expression of mesenchymal-related genes.

Thus, we propose that to ensure the safety of anticancer therapy using MSCs, the characteristics of MSCs themselves, as well as the interaction between stem cells and cancer cells, are quite important and should be investigated before using an MSC-based therapy.

Materials and Methods

Ethical Aspects

The protocols used in this study were approved by the Ethics Committee on Animal Use (CEUA) of Universidade Federal de Ciências da Saúde de Porto Alegre (UFCSPA), under the number 104/11, following the resolutions of the Conselho Nacional de Controle de Experimentação Animal (CONCEA). The NIH "Guide for the Care and Use of Laboratory Animals" (NIH publication N° 80–23, revised 1996) was followed in all experiments. The surgery of ADSC isolation was performed with all efforts to minimize the animal suffering.

Cell Culture

The C6 rat glioma cell line from ATCC was kindly provided by Dra. Ana Maria Oliveira Battastini (UFRGS, Brazil). Cells at 5–20 passages were grown in culture flasks and maintained in Dulbecco's modified Eagle's medium (DMEM) (pH 7.4) containing 1 % DMEM (Sigma, St. Louis, MO, USA), 8.4 mM HEPES, 23.8 mM $NaHCO_3$, 0.1 % amphotericin B, and penicillin (100 U/mL)/streptomycin (100 mg/mL) (Gibco BRL, Grand Island, NY, USA) and supplemented with 10 % (*v*/*v*) fetal bovine serum (FBS) (Cultilab, São Paulo, SP, Brazil). Cells were kept at 37 °C, a minimum relative humidity of 95 %, and an atmosphere of 5 % CO_2 in air.

Isolation and Culture of Adipose-Derived Stem Cells

Adipose-derived stem cells (ADSCs) were extracted from visceral adipose tissue of *Wistar* rats (6–8 weeks), as previously described [36]. Briefly, the abdominal fat tissue from rat was transferred to a petri dish, washed with phosphate-buffered saline (PBS), and dissociated mechanically with a pipette. The fat fragments were transferred to a falcon tube containing 2 mg/mL of collagenase type I, solubilized in DMEM medium without FBS, and incubated in a water bath at 37 °C for 30–45 min. After collagenase digestion, the supernatant was transferred to a new tube and centrifuged at 1000 rpm for 10 min at room temperature (RT). The supernatant was discarded, and cells were resuspended in complete medium and seeded in six-well dishes. ADSC cultures were grown in culture flasks and maintained in DMEM low glucose, containing 8.4 mM HEPES (pH 7.4), 23.8 mM $NaHCO_3$, 0.1 % amphotericin B, and penicillin (100 U/mL)/streptomycin (100 mg/mL) (Gibco BRL, Grand Island, NY, USA) and supplemented with 10 % (*v*/*v*) FBS (Cultilab, São Paulo, SP, Brazil). Cells were kept at a temperature of 37 °C and humidity of 95 %/5 % CO_2 in air.

ADSC Differentiation

Osteogenic differentiation was induced by culturing ADSCs up to 8 weeks in Differentiation SingleQuots^{TM} (Lonza, Walkersville, MD, USA) plus 5 μg/mL of ascorbic acid. In order to observe calcium deposition, cultures were washed once with PBS, fixed with 4 % paraformaldehyde in PBS for 15–30 min, and stained for 5 min at RT with alizarin red S stain

at pH 4.2. Excess of stain was removed by several washes with distilled water. To induce adipogenic differentiation, ADSCs were cultured up to 8 weeks in DMEM supplemented with 10^{-8} M dexamethasone, 2.5 mg/mL insulin, and 100 mM indomethacin. To further confirm their identity, cells were fixed with 4 % paraformaldehyde in PBS for 1 h at RT and stained with oil red O solution. Chondrogenic differentiation was performed using differentiation CDMTM basal medium and CDMTM Differentiation SingleQuots^{TM} (Lonza). Cultures were analyzed by fixing with 4 % paraformaldehyde in PBS for 20 min and staining with alcian blue (pH 2.5) (Sigma, St. Louis, MO, USA) in 3 % acetic acid for 5 min at RT. After staining, the cultures were washed with distilled water.

Immunophenotyping

The cultures of ADSCs were characterized to confirm the presence or absence of MSC surface markers using flow cytometry. Cells were trypsinized, centrifuged, and incubated for 25 min at 4 °C with phycoerythrin (PE)- or peridinin-chlorophyll protein (PerCP-Cy5.5)-conjugated antibodies with anti-rat CD45, CD29, or CD90.2 (Life Technologies, Carlsbad, CA, USA). The unconjugated antibody CD11b (Life Technologies, Carlsbad, CA, USA) was incubated with the secondary anti-mouse IgG2 FITC-conjugated antibody (Sigma-Aldrich, St. Louis, MO, USA) for an additional 25 min. Excess of antibody was removed by washing with PBS (phosphate-buffered saline). Cells were analyzed using a FACSCalibur cytometer equipped with a 488-nm argon laser (Becton Dickinson, San Diego, CA, USA) with the CellQuest software. At least 10,000 events were collected. Gating was set using unstained cells.

Conditioned Medium

For all experiments, ADSC cells between the fourth and tenth passages were seeded in T75 tissue culture flask in DMEM with 10 % (*v*/*v*) FBS low glucose, containing 8.4 mM HEPES (pH 7.4), 23.8 mM $NaHCO_3$, 0.1 % amphotericin B, and penicillin (100 U/mL)/streptomycin (100 mg/mL) (Gibco BRL, Grand Island, NY, USA) at a density of 3320 cells/cm^2. Twenty-four hours after seeding, the medium was replaced by a fresh medium and conditioned for 24 h (CM24) or 48 h (CM48). CM from flasks was harvested, filtrated on 0.22 μm filters (Millipore), and stored at −80 °C until use.

Assessment of Glioma Cell Viability

C6 cells were seeding with 3 mL of DMEM/10 % FBS without phenol red in each flask of 25 cm^2 (in triplicate) and allowed to grow until reaching a 70 % confluence. After, C6 cells were exposed to CM24 and CM48 (prepared using DMEM without phenol red to avoid color interference) during 24 and 48 h. After this time, aliquots of culture medium were taken out for viability analysis. Cell integrity was assessed by measuring the release of the cytosolic enzyme lactate dehydrogenase (LDH) into the medium. LDH activity was detected by automatic biochemical analyzer (Mindray BS-120, Shenzhen, GNG, China) with the commercial bio-kit (Bioclin, Belo Horizonte, Brazil) at 340 nm according to the manufacturer's protocol. Supernatants of cells treated for 24 and 48 h with DMEM without phenol red was used as negative control. Positive control was performed after cell lysis by addition of 1 % Triton X-100. Results were expressed as the percentage of LDH release against the positive control.

Cell Cycle Analysis

In order to evaluate a possible interference of CM in cell cycle progression, we adapted the protocol of the method described by Overton and McCoy [37]. Cells were plated in six-well plates (5×10^4 cell/well) for 1 day, and after they were treated with CM24 and CM48 for 24 and 48 h, respectively. Cells were trypsinized, centrifuged, and washed with PBS twice. Then, cells were resuspended in ice-cold ethanol 70 % (*v*/*v* in PBS) and left for 24 h at 4 °C. On the next day, fixed cells were washed with PBS and marked with a solution containing 0.3 mg/mL RNAse, 20 μg/mL propidium iodide, and 0.1 % Triton X-100 for 30 min, in the dark, at RT. Marked cells were analyzed in a FACSCalibur flow cytometer (BD Pharmingen) using the CellQuest program suite to evaluate DNA content of cells and thus cell cycle distribution of samples. Approximately 10,000 cells were analyzed in each experiment. All analyses were performed at least three times.

Proliferation Assays

The ability of CM24 and CM48 to induce cellular proliferation was evaluated by using MTT 3-(4,5 dimethylthiazol-2-thiazyl)-2,5-diphenyl-tetrazolium bromide assay (MTT, Sigma-Aldrich, St. Louis, MO, USA). Briefly, C6 cells (2×10^3 cell/well) were seeded in 96-well plates. After 24 h, cells were washed and treated with CM. After 24 or 48 h, MTT solution (5 mg/mL) was added and cells were incubated at 37 °C for 3 h. The supernatant was aspirated, and the formazan crystals were solubilized in dimethyl sulfoxide (DMSO; 100 μL). The absorbance, proportional to the number of viable cells, was measured at 570 nm by a multiwell spectrophotometer (SpectraMax Plus Microplate Spectrophotometer, Molecular Devices, US). Results were expressed as the absolute values of results from replicate wells.

To evaluate the proliferation rate in a long-term culture of C6 cells in response to treatment with CM24 and CM48, cumulative population doublings were determined in the PD assay. Cells were plated in 24-well plates at a concentration of 1.5×10^4 cells/well and exposed to CM during 24 or 48 h.

Confluent cells were passaged, and population doublings (PD) were determined according to the formula PD = [log N(t) − log N(to)]/log 2, where N(t) is the number of cells per well at time of passage, and N(to) is the number of cells seeded at the previous passage. The sum of PDs was then plotted against time of culture. Cells were followed until day 20.

Adhesion Assay

C6 cells were treated with CM24 or CM48 for 24 and 48 h, respectively. Then, cells were seeded at density of 3×10^4 cells/well in 96-well plates and incubated with CM24 and CM48 for 1 h at 37 °C with a 5 % CO_2 enriched atmosphere. The non-adherent cells were removed by washing carefully three times with PBS. Adherent cells were fixed with 4 % paraformaldehyde (PFA) for 10 min and stained for 10 min with 100 μL 0.5 % crystal violet diluted in 20 % methanol. The cells were then washed three times with Milli-Q™ water, and the stain was eluted in 100 μL 10 % acetic acid (*v/v*). Cell adhesion was analyzed by measuring optical density (OD) at 570 nm in a microplate reader [38].

Migration Assays

Scratch Wound Healing

C6 cells were plated in 24-well culture plates and grown in complete medium until reaching 80 % of confluence. Then, monolayers were scratched using a 200 μL sterile plastic pipette tip as previously described [39] and washed three times with PBS. After, cells were treated with CM24 and CM48 serum free (SF) during 24 h. Positive controls were performed with DMEM+ 10 % FBS and negative controls with DMEM SF. Scratch wound closure was monitored by phase microscopy using a ×4 objective at 0, 4, 8, 12, and 24 h.

Transwell Assay

In parallel, cell migration was also evaluated using a 24-well Transwell chamber (Greiner, Frickenhausen, Germany). For this purpose, 2.5×10^4 C6 cells were seeded in the upper chamber of a 8-μm pore size insert and allowed to migrate toward a DMEM + 10 % FBS (positive control) or DMEM without FBS (negative control) or ADSC (1.4×10^4 cells/well) present in the lower chamber. Cells were incubated for 24 and 48 h, then the non-migrating cells of the upper chamber were removed with the aid of a cotton swab and the remaining cells were fixed in methanol. Cells that migrated to the lower surface of the membrane were stained with 0.5 % crystal violet diluted in 20 % methanol and counted with a microscope (Olympus BX-50 coupled to a Moticam 2500 camera). Four visual fields were randomly chosen for each assay. The quantitation was performed by processing all obtained images using Image J software (http://imagej.nih.gov/ij/). The average number of the migrating cells in these four fields was taken as the cell migration number of the group [40].

Actin Cytoskeleton Staining

Actin cytoskeleton reorganization was assessed via filamentous actin (F-actin) staining, as reported previously [41]. Briefly, cultured C6 cells seeded on glass coverslips were treated with CM24, CM48, or TGFβ (1 μg/L) during 24 h, 48 h, and 5 days. After each treatment, cells were washed with PBS and fixed with 4 % paraformaldehyde for 24 h. Then, cells were stained with phalloidin-fluorescein isothiocyanate and the nucleus was stained with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI; 300 nM; Sigma) for 30 min. Images of the fluorescently labeled stress fibers were captured using an Olympus BX-50 with optical lens (×10/0.30 Ph1-UplanFI) coupled to a Moticam 2500 camera.

Nuclear Morphometric Analysis (NMA)

Nuclear morphometry was analyzed as described by Filippi-Chiela [42]. Briefly, cells were treated with ADSC-CM or TGFβ (24 h, 48 h, and 5 days), fixed with 4 % paraformaldehyde (*v/v* in PBS) for 30 min at room temperature, and stained with DAPI 300 nM for 30 min, followed by quantification of DAPI-stained nuclei using the Software Image Pro Plus 6.0 (Media Cybernetics, Silver Spring, MD). Images of the DAPI-labeled nuclei were captured using an Olympus BX-50 with

Table 1 Primers for EMT markers used at RT-qPCR experiments

Primer	Sense	Antisense
MMP2	5′-ACAACAGCTGTACCACCGAG-3′	5′-GGACATAGCAGTCTCTGGGC-3′
Vimentin	5′-GAGGAGATGAGGGAGTTGCG-3′	5′-GGTCAAGACGTGCCAGAGAA-3′
NRAS	5′-CACGAGCTGGCCAAGAGTTA-3′	5′-TGAGGCTTGAAAGTGGCTCG-3′
Src	5′-CTTCCTCTCACTAGGCCTGC-3′	5′-CTTCCTCTCACTAGGCCTGC-3′
TBP	5′-CGTGACGATAACCCAGAAAG-3′	5′-GGTGGAAGGCTGTTGTTC-3′

EMT epithelial-mesenchymal transition, *MMP2* matrix metalloproteinase-2, *Src* proto-oncogene tyrosine-protein kinase, *RT-qPCR* quantitative reverse transcription-polymerase chain reaction

optical lens (×10/0.30 Ph1-UplanFI) coupled to a Moticam 2500 camera. Data are presented as a plot of area versus nuclear irregularity index (NII).

RT-qPCR Analysis

Total RNA from glioma cell cultures were isolated with TRIzol LS reagent (Life Technologies, Carlsbad, CA, USA) in accordance with the manufacturer's instructions and stored at −80 °C.

The complementary DNA (cDNA) was synthesized with M-MLV reverse transcriptase enzyme (Promega, Madison, WI, USA) 3 μg total RNA in a final volume of 20 μL with a random hexamer primer in accordance with the manufacturer's instructions. cDNA was diluted to 1:10 in diethyl pyrocarbonate (DEPC) water and stored at −20 °C. Real-time PCRs were carried out in an Applied Biosystems StepOnePlus™ Real-Time PCR cycler and done in duplicate. Reaction settings were composed of an initial enzyme activation step of 20 s at 95 °C, followed by 40 cycles of 3 s at 95 °C and 30 s at 60 °C for data acquisition. Real-time PCRs were prepared in a 12.5-μL final volume composed of 6.25 μL of Fast SYBR green master mix (Applied Biosystems, Foster City, CA, USA), 0.40 μL of 10 μM primer pairs (Table 1), 3.85 μL of water, and 2 μL of diluted cDNA. After an appropriate selection using relative standard curve method, TBP was chosen as the most stable reference gene between five gene candidates and was used as a control for cDNA synthesis [43]. All results were analyzed by the $2^{-\Delta\Delta CT}$ method [44]. The data were presented as ratio of genes/TBP.

Statistics

All data were expressed as mean ± SEM, and they were analyzed using *t* test for single comparisons or ANOVA followed

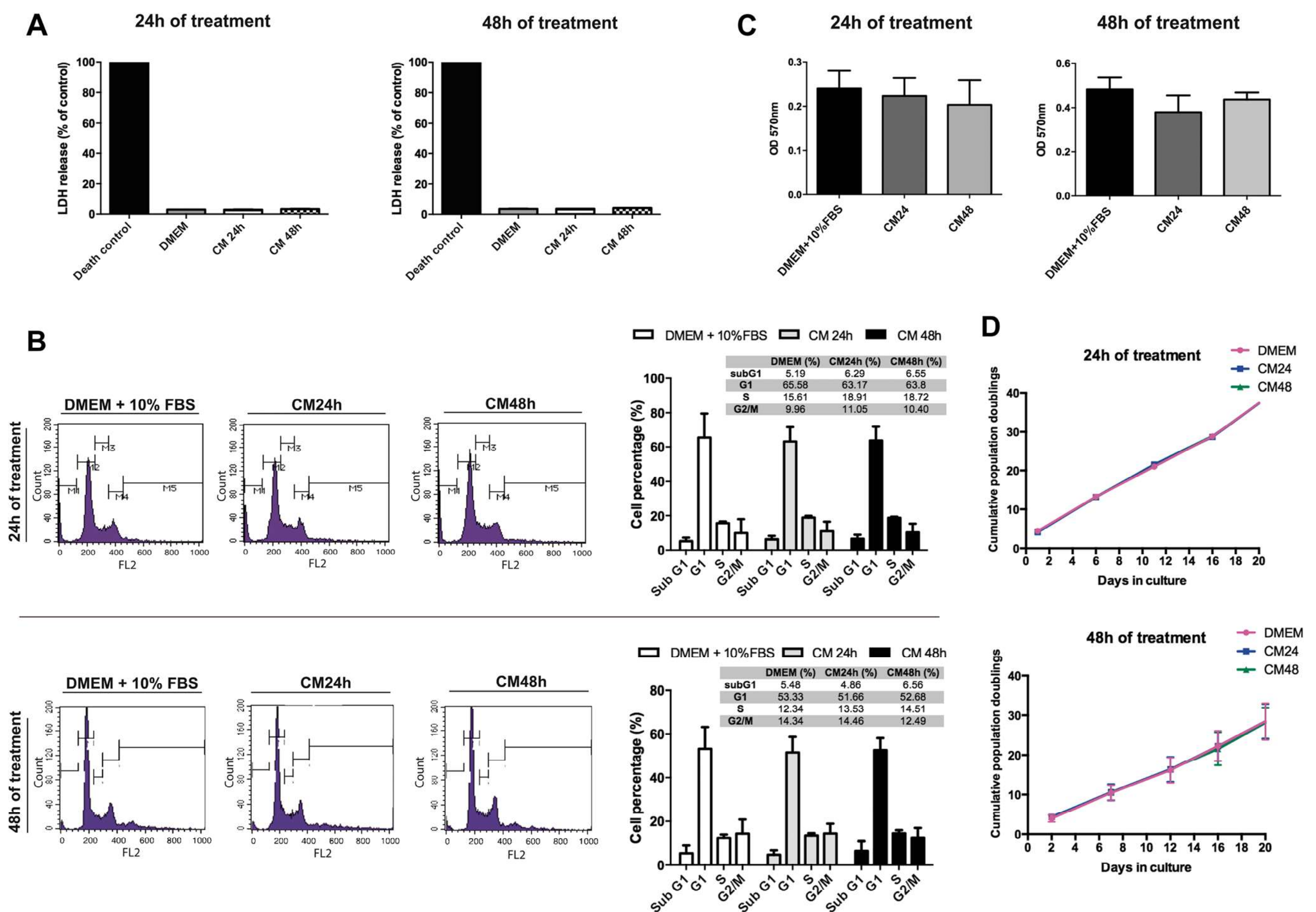
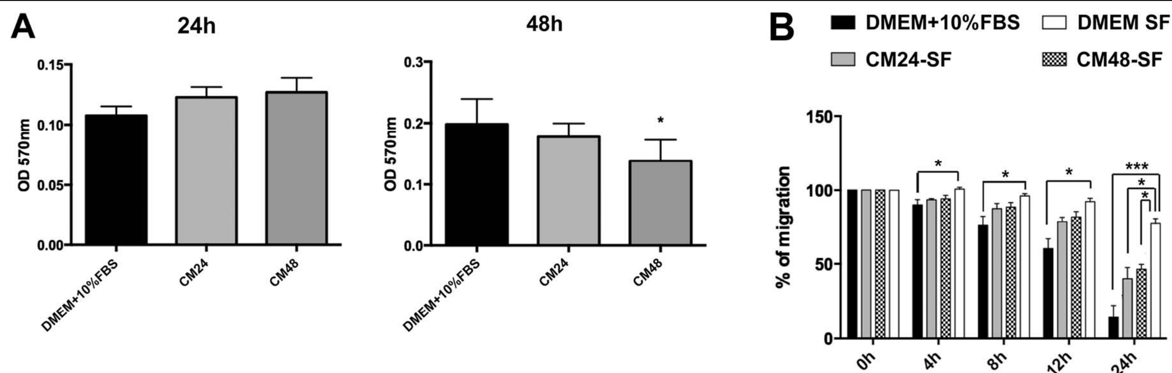
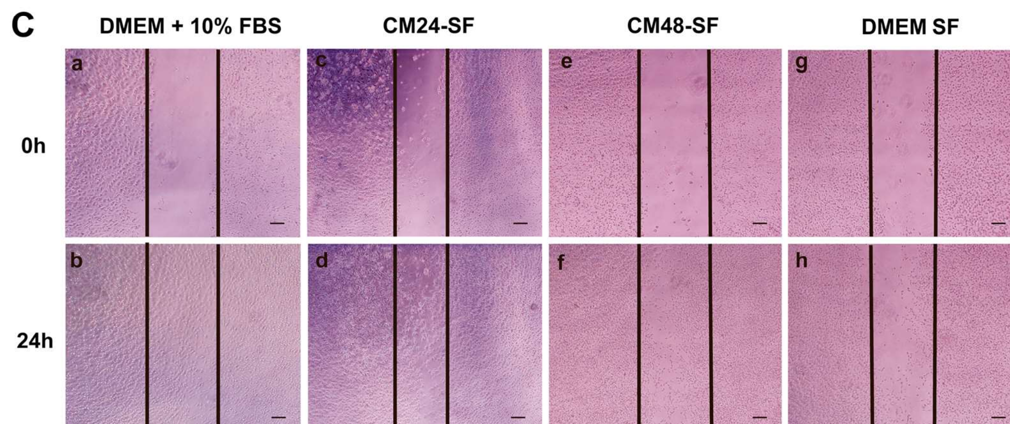


Fig. 1 Effect of ADSC-CM on LDH release, cell cycle distribution, and cell proliferation rate in C6 glioma cells. **a** The cytotoxicity of ADSC-CM was evaluated by measuring the LDH enzymatic activity on supernatant of C6 cells treated with ADSC-CM for 24 and 48 h. **b** Flow cytometry was used for determination of cell cycle distribution (sub-G1, G1/G0, S, and G2/M) in untreated and ADSC-CM-treated (24 and 48 h) C6 cells. **c** Cell cultures were exposed to ADSC-CM for 24 and 48 h, and cell proliferation was assessed by the MTT assay as described in the "Materials and Methods" section. Glioma cultures treated with 10 % FBS was taken as 100 % of cell proliferation. **d** C6 cells were cultivated in the presence of ADSC-CM for 24 and 48 h, and cumulative population doublings were plotted against time. Results are expressed in percentage and were analyzed by one-way ANOVA, followed by Tukey's multiple comparisons test. The values were considered significantly different from controls, when $P < 0.05$. All experiments were repeated at least three times


A
24h
48h
OD 570nm
DMEM+10%FBS
CM24
CM48
B
DMEM+10%FBS
DMEM SF
CM24-SF
CM48-SF
% of migration
0h
4h
8h
12h
24h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DMEM + 10% FBS
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DMEM SF
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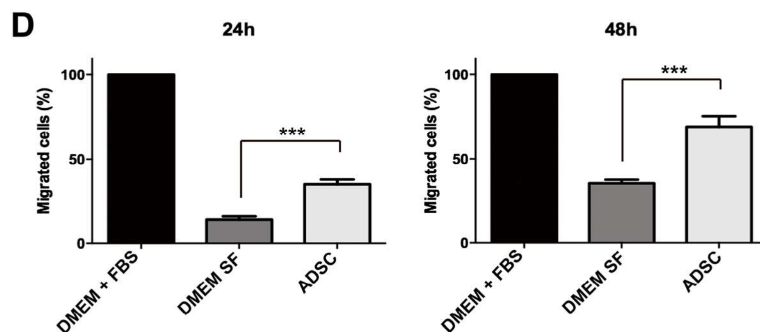
D
24h
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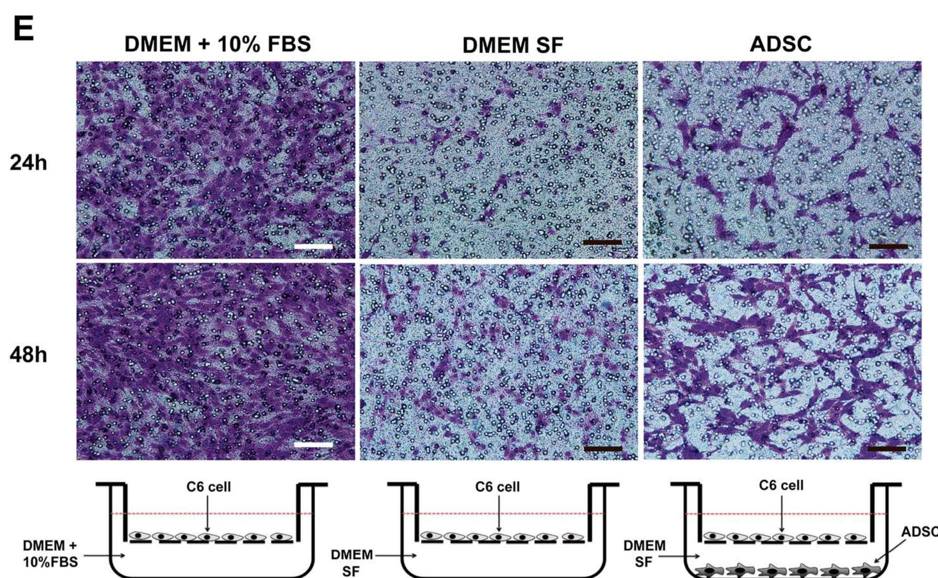
Migrated cells (%)
DMEM + FBS
DMEM SF
ADSC


E
DMEM + 10% FBS
DMEM SF
ADSC
24h
48h
C6 cell
DMEM +
10%FBS
DMEM
SF
ADSC

◀ **Fig. 2** Effect of ADSC-CM on glioma cell adhesion and migration. **a** Semi-confluent cultures of C6 cells were treated with ADSC-CM for 1 h. Then, cell adhesion was evaluated as described in the "Materials and Methods" section. **b** C6 cells wound repair after 24 h with ADSC-CM-SF. Negative controls indicates wound repair with DMEM SF. Positive control represent wound repair with 10 % FBS supplemented DMEM. The average initial wound width was measured and defined as 100 %. **c** Inverted light microscopic images of C6 cells wound repair. Negative and positive controls indicate DMEM SF and complete DMEM + 10 % FBS, respectively. Wound healing within the scrape line was recorded every day. Representative scrape lines are shown at 0 and 24 h; *dashed line* indicated the margin of the scratch. *Scale bars* 200 μm. **d** ADSC-CM affected cell migration by Transwell assay. C6 cell seeded in the upper chamber of Transwells were incubated with ADSC added to the lower chamber, and cell migration was determined during 24 and 48 h, as described in the "Materials and Methods" section. DMEM + 10 % FBS and DMEM SF were used as the positive and negative control, respectively. Cells were photographed, and average cells per field were calculated. *Columns* represent the mean percentage of migrating cells relative to the negative control. **e** Representative light microscopy image of crystal violet stained cells on the basal surface of the filter and scheme illustrating each assay. *Scale bars* 100 μm. $^{*}p<0.05$ and $^{***}p<0.001$ indicate significant differences in relation to the control group as determined by ANOVA followed by Tukey's test. All experiments were repeated at least three times

by Tukey's test for multiple comparisons of at least three independent experiments. Differences with $p<0.05$ were considered significant. Analysis of the data was performed using GraphPad Prism version 6.0 software (Graphpad, La Jolla, CA, USA).

Results

ADSCs Express Stem Cell Markers and Display Multipotent Differentiation

The ADSCs population was characterized by the CD marker profile using flow cytometry analysis. As already reported [45], ADSCs expressed CD90 (glycosylphosphatidylinositol-anchored glycoprotein) and CD29 (integrin b1 chain), whereas CD11b and CD45, hematopoietic surface markers, were negative (Supplementary Fig. 1A). In our study, ADSCs isolated from visceral adipose tissue presented the expected elongated fusiform morphology and capacity to differentiate into osteogenic, adipogenic, or chondrogenic lineages (Supplementary Fig. 1B, C and D).

ADSC-CM Neither Damages the Cell Membrane of C6 Cells Nor Induces Cell Cycle Arrest and Changes in Growth Rate of C6 Glioma Cells

First, we examined if the ADSC-CM could affect the viability of glioma C6 by treating cells with CM24 and CM48 for 24 and 48 h, respectively. At the end of incubation, LDH activity was determined in supernatants. This assay showed that ADSC-CM did not cause a significant release of LDH into culture medium of C6 cells, indicating that the factors present in CM are unable to generate loss in cell membrane integrity (Fig. 1a).

To explore the influence of ADSC-CM on cell cycle progression, the proportion of cells in the G1, S, and G2/M phases was determined in the presence or absence of CM24 and CM48 after 24 and 48 h of treatment, respectively. The flow cytometry analyses using PI for DNA labeling showed that both CM24 and CM48 were unable to change the relative content of cells in the cell cycle phases (Fig. 1b).

In order to determine the proliferation rate of C6 glioma, cell cultures were treated with CM24 and CM48 and MTT assay was performed (Fig. 1c). Exposure of glioma cells to CM for 24 and 48 h did not result in alteration of cell proliferation. After 24 or 48 h of treatment, the absorbance of cells treated with CM24 and CM48 was not statistical different from control cells.

Finally, to investigate the influence of ADSC-CM on a long-time proliferation rate, the PD was performed. After the treatment with ADSC-CM during 24 and 48 h, glioma cells were followed for 20 days. Our results showed that ADSC-CM does not affect the PD rate of C6 cells, suggesting that the factors contained in CM do not lead to a significant change in cell growth and cell senescence (Fig. 1d).

ADSC-CM-Pretreated Tumor Cells are Able to Induce EMT-Like Plasticity

ADSC-CM Decreases C6 Cell Adhesion

Epithelial-mesenchymal transition (EMT) is a recognized process often associated to development and cancer progression [46]. Enhanced invasive capacity with loss of intercellular adhesion proteins and acquisition of the more motile mesenchymal phenotype are among the characteristics of EMT [47]. Considering the importance of this process in tumorigenesis, we investigate if ADSC-CM-treated tumor cells could undergo an EMT-like process. To this end, we evaluated several parameters, starting by performing cell adhesion assay.

The results showed that ADSC-CM24 and ADSC-CM48 are unable to change glioma cell adhesion during 24 h of treatment. On the other hand, after 48 h of treatment, ADSC-CM48 promoted a decrease of 20 % in glioma cell adhesion (Fig. 2a).

ADSC-CM or ADSCs Increase Glioma C6 Cells Migration

Further, we evaluated the ability of ASCD-CM to affect tumor cell migration. First, the ability of ADSC-CM to affect glioma cell migration was tested by a scratch wound model in which C6 cells were treated with ADSC-CM-SF. Treatment of glioma C6 with CM24 or CM48 reveals a significant increase in

migration (2- and 1.7-fold, respectively) when compared with control (DMEM SF), an effect that became significant after 24 h (Fig. 2b, c).

Next, we tested in a Transwell system if the presence of ADSCs in the basal compartment could induce a stronger response than that generated by ADSC-CM. Glioma cells exhibited a 2.5-fold and 2-fold increase in cell migration when compared to negative control (DMEM SF), after 24 or 48 h of indirect co-culture of ADSCs and C6 cells, respectively (Fig. 2d, e).

Morphological Changes in C6 Glioma Cells Treated with ADSC-CM

Changes in the migratory profile of cells are tightly associated with alterations in cell spreading and cytoskeleton organization. When glioma cells were maintained in ADSC-CM, morphological changes in the majority of tumor cells could be observed. Cells treated with CM shifted from the epithelial-like cobblestone morphology to the spindle-like fibroblastoid appearance, when compared with untreated cells over the same period of time (Fig. 3).

Moreover, ADSC-CM induced characteristic reorganization of actin filaments after 24 h, 48 h, and 5 days of culture with CM, as detected by phalloidin-fluorescein labeling (Fig. 4). This result was observed mainly in CM48 treatment and on a smaller scale in CM24 treatment, but it was not observed in C6 cultured in control medium. ADSCs were observed to have F-actin filaments organized along the length of the cell as an elongated rod shape, reflecting a change in cellular polarity. Our results also showed that changes in cell morphology were accompanied by increased nuclear irregularity, mainly due to a circular to fusiform transition, suggesting that the EMT-like effect induced by CM is similar to TGFβ-induced EMT when nuclear morphology is considered, suggesting that TGFβ could be one of the factors present in CM that induces EMT-like process in C6 cells (Fig. 5).

ADSC-CM Enhance Expression of Markers Associated with EMT

Lastly, up-regulation of the EMT-associated markers in C6 cells exposed to ADSC-CM was confirmed. We analyzed

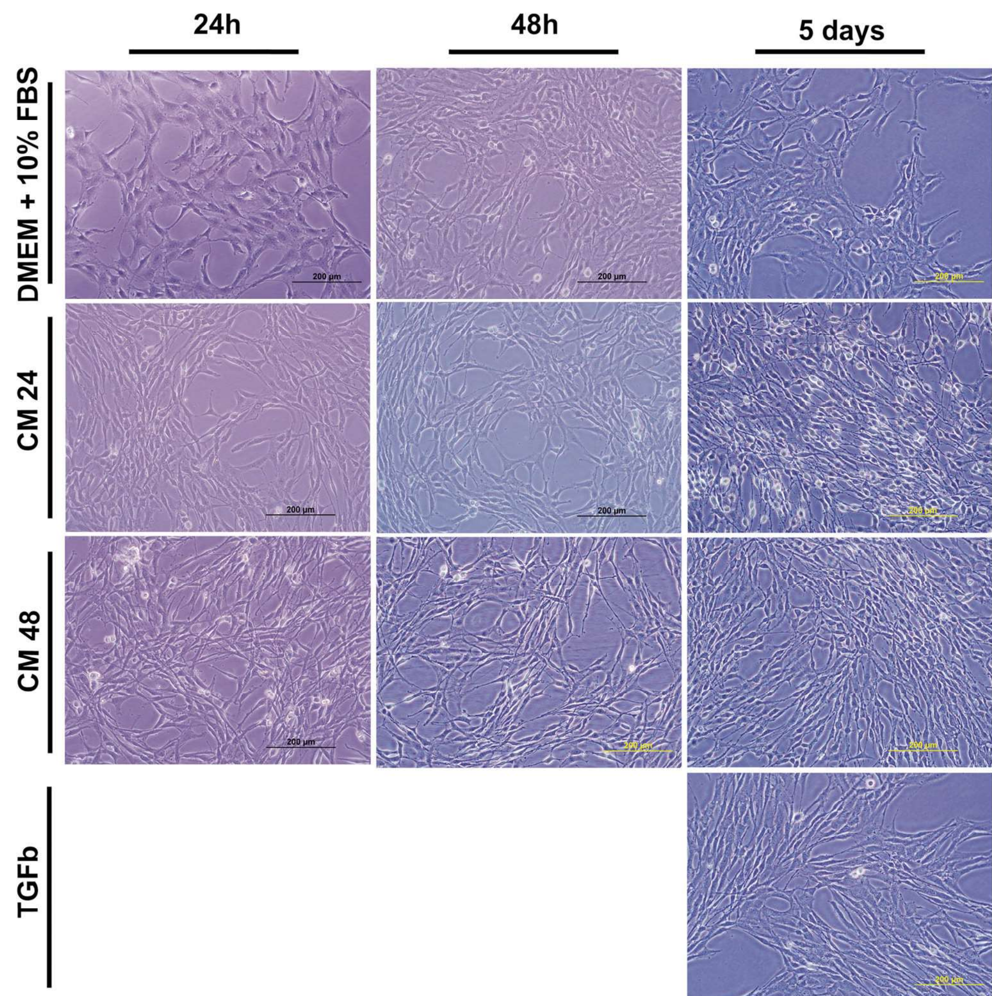
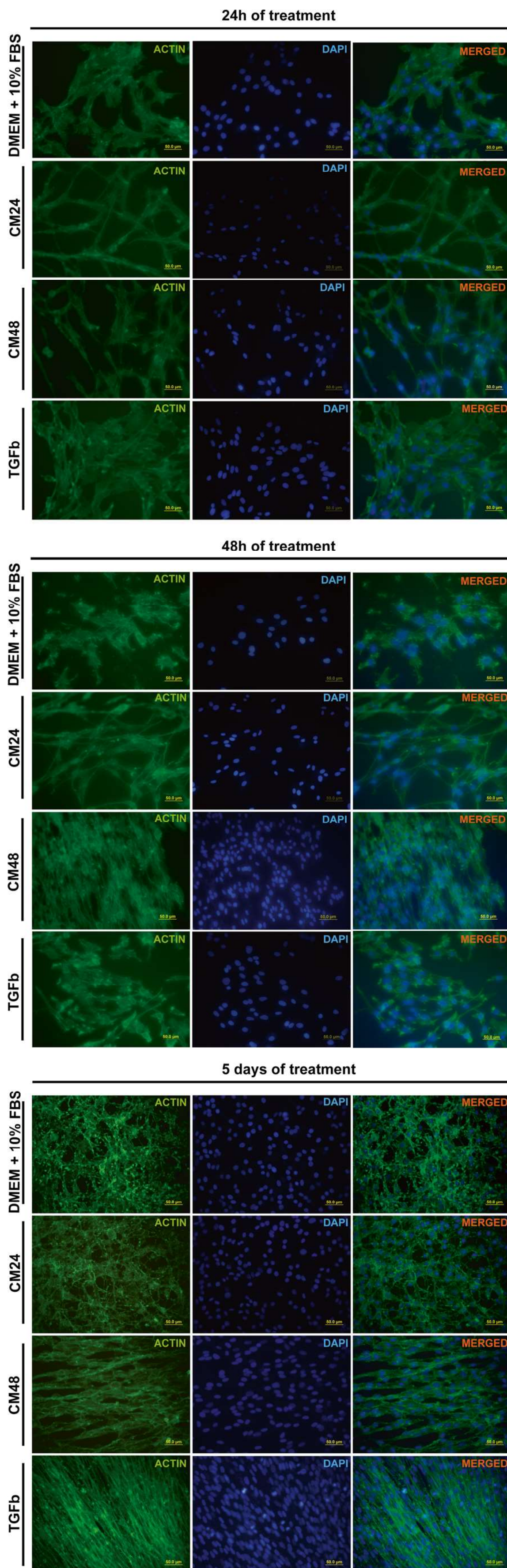


Fig. 3 Morphological appearance of C6 cells treated with ADSC-CM. Phase contrast microscopic views of C6 cells treated with ADSC-CM during 24 h, 48 h and 5 days. Positive control cells were treated for 5 days with 1 ng/mL TGF-β. *Scales bars*, 200 μm



◀ **Fig 4** F-actin fiber formation in C6 cells induced by ADSC-CM and TGF-β. Representative images of actin cytoskeleton reorganization via filamentous actin (F-actin) immunostained with DAPI and phalloidin-fluorescein. Cells were treated during 24 h, 48 h and 5 days with ADSC-CM. Negative and positive controls were performed by treatment with DMEM + 10 % FBS and 1 ng/mL TGF-β, respectively. All images presented are at ×400 magnification

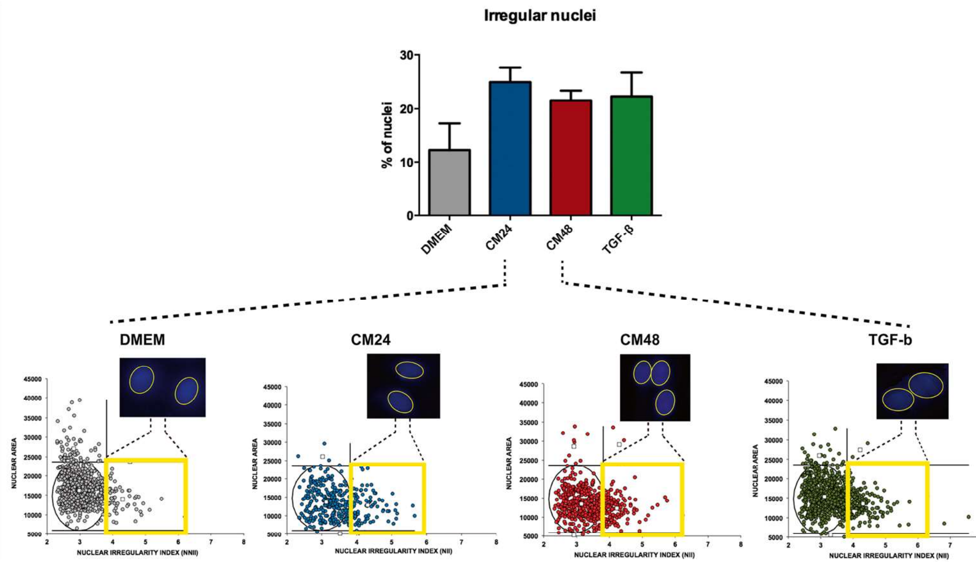
the mRNA expression of vimentin, MMP2, NRAS, and Src mRNA and found that ADSC-CM48 was able to significantly increase the expression of vimentin and NRAS after 24 h of treatment, whereas after 48 h of cell exposure to CM, the expression of MMP2, vimentin and NRAS were upregulated. The expression of Src was not altered by ADSC-CM (Fig. 6). These data further support that treatment of C6 tumor cells with ADSC-CM promotes activation of signaling pathways leading to "reprogramming" of tumor cells toward a mesenchymal phenotype.

Discussion

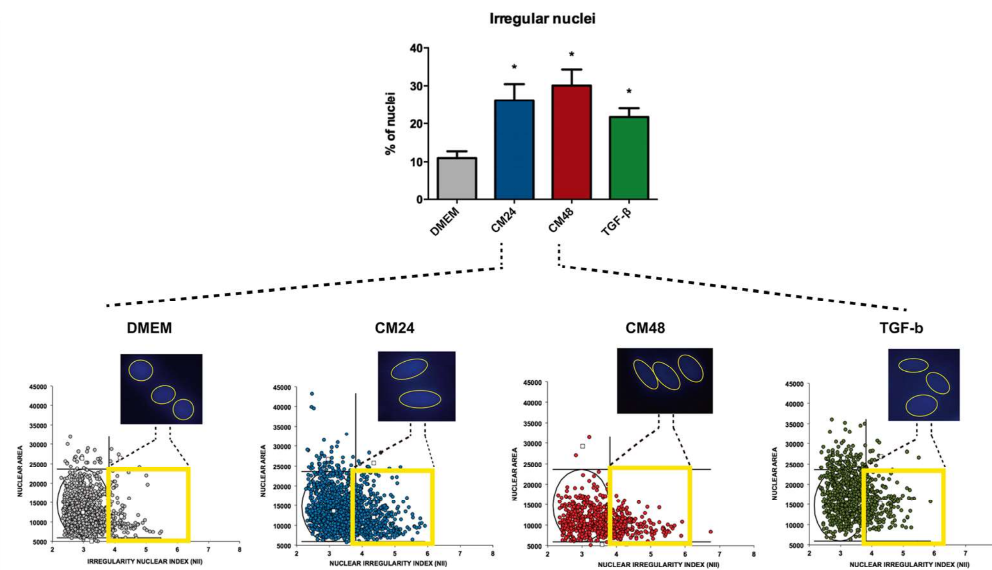
Increasing evidence has shown that MSCs play a tumor-promoting function by stimulating tumor growth, invasion, motility, and anticancer-drug resistance mainly by interacting with cancer cells and promoting EMT [19, 32, 48–51]. Since MSCs are recruited to sites of growing tumor, there is a continuous and bilateral molecular crosstalk between stromal cells and cancer cells mediated through direct cell-cell contacts or by secreted molecules [52, 53]. Although it remains unclear whether physical contact between MSCs and tumor cells is really required [54], it seems almost certain that the major mechanism of MSC participation in cancer development is related to their paracrine activity [55]. Hence, the mechanisms of the modulatory behavior of MSCs must be explored and the use of MSCs in patients with cancer should be performed with caution.

Interestingly, we observed that ADSC-CM promotes a marked increase in cancer cell migration. Recent reports have shown a significant increase in migration of breast cancer cells [48, 56], gastric cancer cells [57], hepatic cells [58], esophageal carcinoma cells [59], and colon cancer cells [60] in response to factors secreted by MSCs. Increased migration is a key event to cellular invasiveness and is involved in degradation of basement membrane and extracellular matrix as well as vessel invasion. For occurrence of this process, it is necessary a paracrine communication through extracellular signals released by stromal tumor cells, synthesis of proteins, and active intracellular signaling with cytoskeleton proteins [34]. Several genes involved in glioma invasiveness have been identified and include members of the family of metalloproteases (MMP). Expression of MMP-2 and MMP-9 correlate with invasiveness, proliferation, and prognosis in astrocytomas [61].

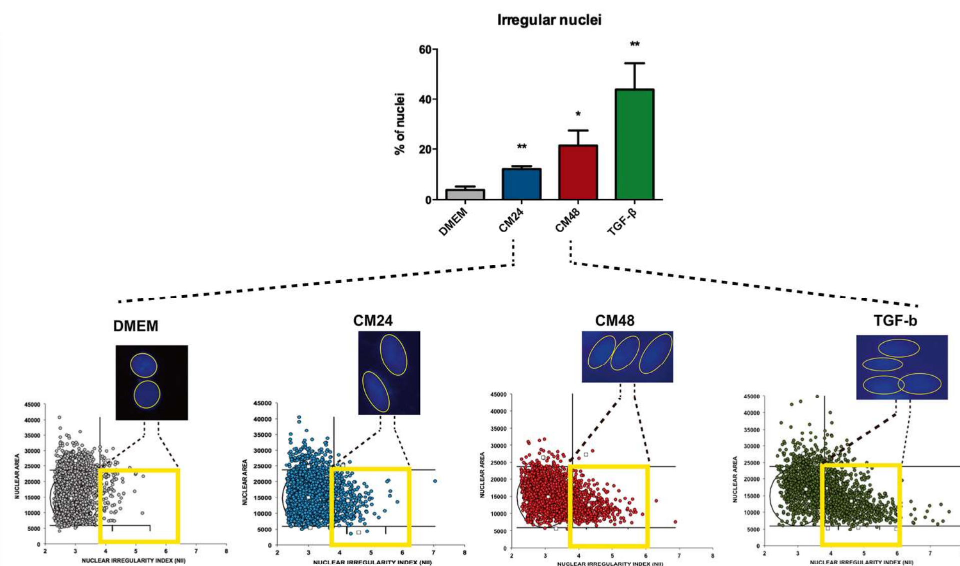
24h of treatment



48h of treatment



5 days of treatment



◀ **Fig 5** Morphologic nuclear changes in C6 cells treated with ADSC-CM. Distribution of C6 cells according to nuclear morphology. *Black circles* mark the normal nuclear morphology. Cells into the *yellow squares* represent cells with nuclear irregularity. Representative microphotographs (×40) showing the effect of CM in nuclear morphology of C6 cells. Results are expressed in percentage and were analyzed by one-way ANOVA, followed by Tukey's multiple comparisons test. The values were considered significantly different from controls, when $P < 0.05$. All experiments were repeated at least three times

Reinforcing this pro-spreading effect of MSCs in cancer cells, Karnoub et al. showed that bone marrow-derived human MSC when co-injected with breast carcinoma cells, in immunocompromised mice, are able to promote a marked enhancement in lung tumor metastasis, when compared to tumors composed only by cancer cells. Additionally, this effect on invasive potential was only observed when MSCs were injected close to the engraft tumor. These results demonstrate the importance of the tumor microenvironment and that cancer cells can respond in a transient way to signals received from their stromal microenvironment [62]. Although the metastasis of GBM already has been described in the literature [63], they remain very uncommon. The rapid relapse of tumor in GBM patients has been considered as the key factor of the lack of distant metastasis in patients with high-grade malignant gliomas [64]. However, despite metastasis outside of the CNS is not common in this type of tumor, the capacity to infiltrate and spread throughout the brain is a hallmark of GBM and is the main basis for the failure of treatment [65].

In our findings, the secretome of ADSCs was able to increase cell migration even without the physical presence of ADSCs. However, this effect was increased in the co-culture Transwell system, in which cells share the same medium, but without direct contact. This can be explained by the fact that when different cells are together in the same microenvironment, the crosstalk among them is favored. In this case, the crosstalk could be favorable to cancer progression, once cancer cells could also release factors to modulate MSCs in its favor [24].

EMT is related to a more invasive cancer cell phenotype. This process is only possible because the detachment of tumor cells from the primary tumor is facilitated through the loss of cell-cell adhesion molecules [66]. Cells of epithelial origin are in part characterized by extensive points of cell-cell contact that maintain cells as a layer, whereas mesenchymal cells present only few contact points on their surface which mediate their interaction with neighboring cells, decreasing their adherence capacity [67]. In accordance, we demonstrate that C6 cells, after treatment with ADSC-CM, have their capacity of adhesion significantly reduced. This is compatible with EMT phenotype, in which cell adhesion molecules, such as cadherin, have their expression or function changed, promoting carcinoma progression and metastasis [68].

In order to confirm whether the decreased adhesion capacity and enhanced migration ability of C6 cells after CM treatment could be associated with EMT, we evaluate other hallmark features of EMT that are loss of apico-basal polarity, reorganization of cytoskeletal architecture, and changes in cell shape [69]. Epithelial cells are highly polarized apico-basally, which is manifested through the organization of the cytoskeleton. Mesenchymal cells show front-back polarity instead of apico-basal polarity [67]. Not surprisingly, we observed a marked demodulation of actin cytoskeleton, morphology changes, and characteristic elongated shape of the nucleus in

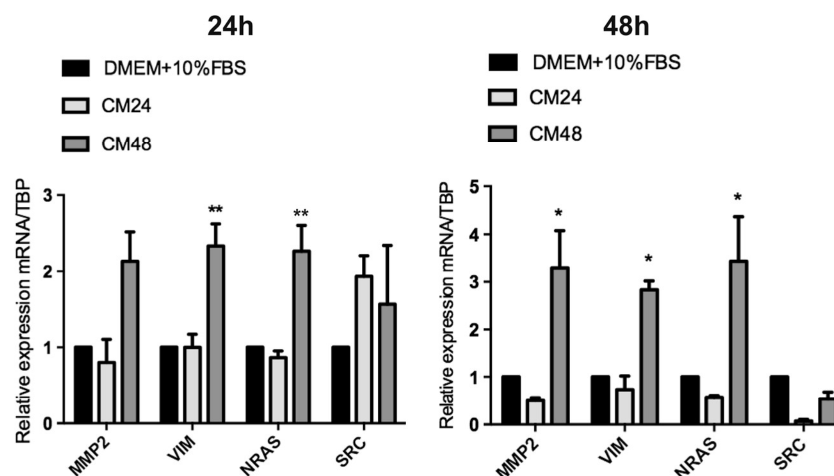


Fig 6 Expression of EMT Markers in the C6 glioma cells cultured in ADSC-CM. Quantitative RT-PCR confirmed significant increase in the mRNA expression of vimentin, MMP2, NRAS, and Src in C6 cells exposed during 24 and 48 h to ADSC-CM in comparison to C6 cells maintained under the standard culture conditions. Quantitative PCR measurements of gene expression levels are normalized against TBP levels, and expressed relatively to the control samples. The values were considered significantly different from controls, when $P < 0.05$ as determined by ANOVA followed by Tukey's test. *$p < 0.05$ and ***$p < 0.001$ indicate statistical difference from control. All experiments were repeated at least three times

CM-treated C6 cells. Therefore, we consider that the treatment with CM leads to an enhanced potential for cell motility, mainly due to cytoskeletal modulation in C6 cells.

Next, the genetic markers associated with EMT, vimentin, MMP2, NRAS, and Src were examined in C6 cancer cells following CM treatment. The results showed that with exception of the Src marker, the treatment with ADSC-CM 48 was capable to significantly increase the expression of EMT-associated genes. The matrix metalloproteinase MMP2 and the cytoskeletal protein vimentin are extensively described to be associated with increased migration after EMT [67]. Furthermore, the vimentin and MMP-2 up-regulation correlate with enhanced migration and invasion in many human tumors, including glioblastoma [70–79]. Oncogenes have also been reported to be involved with EMT [80, 81]. In malignant human melanoma, NRAS/BRAF activation drives a switch in EMT transcription factor expression, leading to changes in favor of TWIST1 and ZEB1 [82]. In human pancreatic cancer cell line, Snail, a key regulator of EMT, can be induced by TGF-β in tumor cells and this induction is highly dependent on cooperation with active RAS signals [83]. Finally, the expression of Src was not altered by ADSC-CM in our study and probably the enhanced expression of this gene is not crucial to EMT induction. Additionally, tumor cells were shown to undergo an "incomplete" EMT. In other words, cells could progress through an intermediate epithelial/mesenchymal hybrid

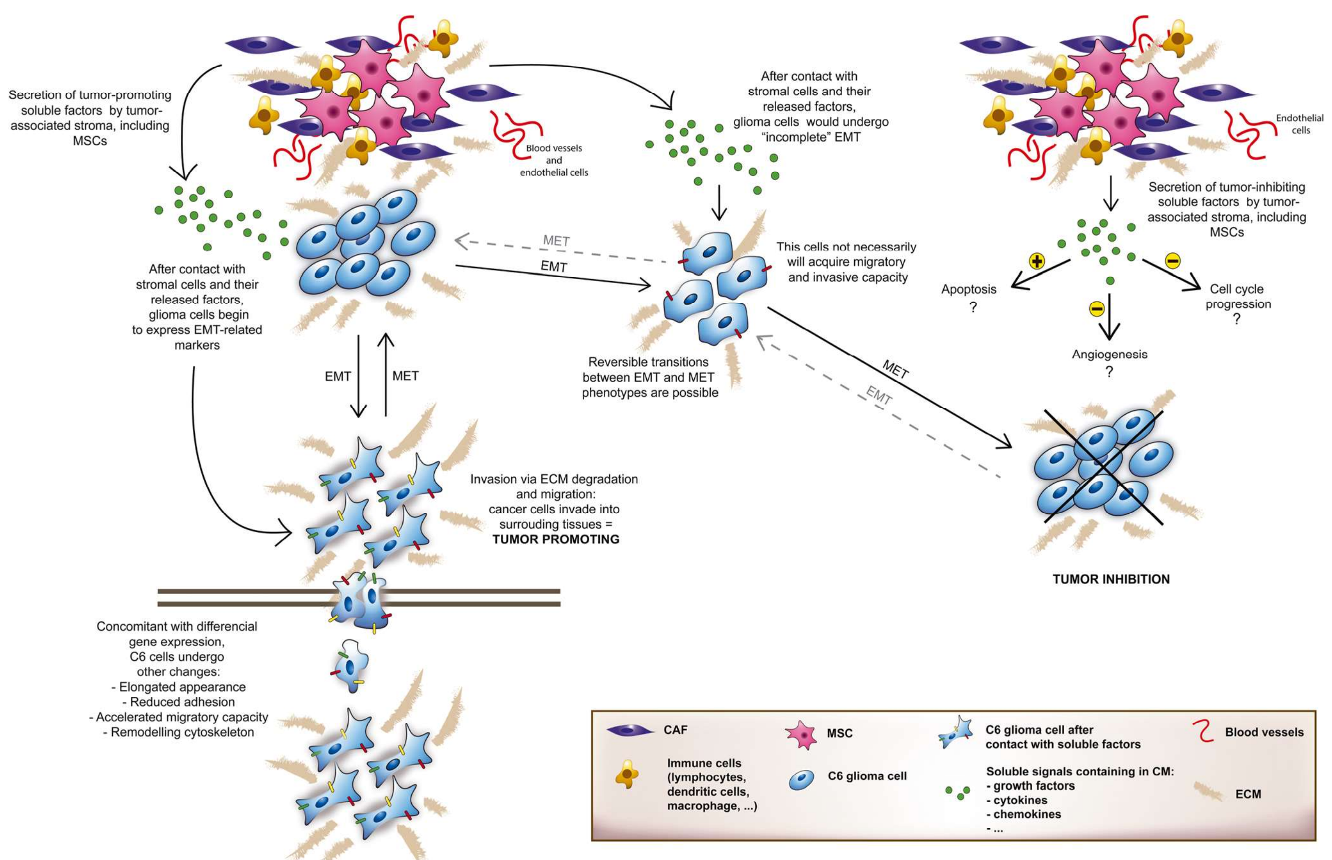


Fig 7 Schematic overview of the role of MSC-released factors in tumor progression. MSCs are known to be able to migrate and home to the tumor stroma. Within the tumor microenvironment, MSCs can deliver trophic factors, including growth factors, chemokines, and cytokines, which influence cancer cell phenotypes. MSC-derived factors can interact with receptors on C6 cell surface and activate signal transduction, resulting in loss of cell-cell contact and cobblestone appearance that lead to a more elongated cell shape, with front-back polarity. These cancer cells could show enhanced expression of EMT-related markers such as vimentin, MMP2, and NRAS. The crosstalk between MSCs and cancer cells also impact in subsequent stimulation of migratory capacity, leading to a spreading of tumor cells and cancer progression. On the other hand, factors released by MSCs can promote differences in cellular responses and trigger a partial or incomplete EMT in gliomas cells. Additionally, cancer cells can switch between epithelial or mesenchymal phenotypes several times during formation of the complex crosstalk between tumor cells and its microenvironment. In this context, one has to keep in mind that EMT is a reversible process and the initiation of signal transduction cascades that drive EMT in epithelial cancer cells manifests in different levels in distinct cell types. Furthermore, when cells acquire an intermediate state of EMT, not necessarily will these cells be compromised with cancer progression, but in opposite, these cells can stay in a senescent state and be inhibited by the surrounding stroma or return through MET to an epithelial phenotype. At the end, if the stimulus of the tumor stroma surrounding was negative, an inhibitory effect into the tumor can limit tumor progression and result in disadvantage for tumor survival, without cells initiate the EMT changes. *EMT* epithelial to mesenchymal transition, *MET* mesenchymal to epithelial transition, *CAF* cancer-associated fibroblasts, *MSC* mesenchymal stem cell, *ECM* extracellular matrix

stage and acquire a metastatic and invasive phenotype without a requirement for a complete EMT as can be seen in Fig. 7 [84, 85]. In a model of murine mammary epithelial cell line, EpH4, the activation of MEK1 signaling can induce changes associated with EMT, such as enhanced motility, invasiveness, and remodeling of the actin cytoskeleton. However, no reduction in the levels of E-cadherin was observed, nor was there any induction of mesenchymal markers, such as smooth muscle actin or vimentin (Pinkas J). The incomplete EMT could be explained by the complexity of the EMT regulation network, most often resulting in a small subset of tumor cells advancing into the EMT program. Consequently, the induction of a full EMT in cancer cells is difficult to observe [86].

It is well-established that tumor cells work to stimulate their tumor-stromal cells to create a favorable environment for their survival [87]. Then, in accordance with the hypothesis that MSCs could support tumor progression, our analysis of cell cycle and cell viability showed that ADSC-CM is unable to alter C6 viability or the progression in cell cycle, thereby allowing cell survival. The growth rate of C6 cells in our model was not changed after CM cell treatment. Until now, there is no consensus in the literature about the effect of MSCs on cancer cells proliferation, with some studies reporting inhibition [7, 28, 48], stimulation [26, 28], or no change [88]. Therefore, we consider that these conflicting findings are due differences in cell source, model of cancer tested, or species studied. There is scant information in the scientific literature regarding the association between stem cells and EMT in gliomas. One of the few works found showed that CD133+ glioma stem cell treated with human umbilical cord blood derived-MSCs presented a down regulation of Twist1 and Sox2 proteins, losing their stemness feature, by reverting EMT through induction of mesenchymal epithelial transition (MET) [89]. This study reinforces the importance to take into account the origin and type of MSC before their therapeutic application.

In summary, in the current work, we explored the interaction between ADSCs and C6 glioma cell line through ADSC-CM to understand the effects of ADSC-secretome in tumor development. This report provides evidences that molecules secreted by rat ADSCs can mediate the induction of EMT-like transformation in C6 cancer cells. Despite the recognition of the role of EMT in the cancer progression, the mechanisms explaining how stimuli-induced EMT occurs at the tumor site remains unknown. Further understanding of the complex crosstalk between MSCs and tumor cells as well as the role that paracrine communication plays in tumor progression is required to establish the effectiveness and safety of treatment strategies using cell therapy in gliomas. An overview to a better comprehension about the intricate communication between cancer cells and their microenvironment is provided in Fig. 7.

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Compliance with Ethical Standards

Competing Interests The authors declare that they have no competing interests.

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Supplementary figure

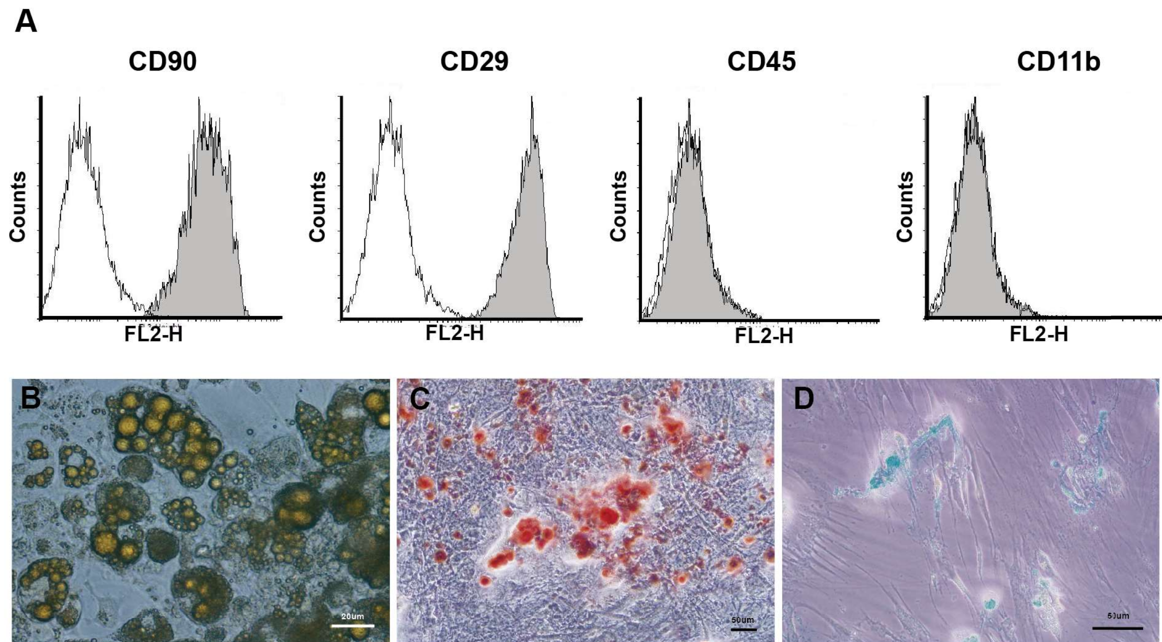

Fig. S1 ADSC characterization. The differentiation of ADSCs is shown in light microscopy. A. Immunophenotyping analysis of surface markers expression in ADCSs. Flow cytometry histograms show the expression (bold line) of selected molecules (CD90, CD29, CD11b and CD45) by ADSC populations in comparison with controls. B. ADSCs differentiated into adipocyte-like cells, which was stained by Oil Red O, the triglyceride specific dye. C. Cells differentiated into osteoblasts showing the bone matrix stained by Alizarin Red, the calcium-specific marker. D. Cells differentiated into chondrocytes showing the proteoglycan rich matrix stained by Alcian blue. B and D: Magnification, X 400. C: Magnifications, X 200.

CAPÍTULO III

Analysis of the impact of rat adipose-derived stem cells (ADSCs) and their conditioned medium (CM) on C6 glioma tumor microenvironment.

Manuscrito a ser submetido à publicação

APRESENTAÇÃO

Os resultados obtidos no Capítulo III desta tese, nos levaram a fazer alguns questionamentos:

- Será que a indução de EMT-*like* em células de GBMs submetidas ao tratamento com ADSC-CM é um processo estável e permanente?
- Qual será o efeito em termos de agressividade do pré-tratamento de células de GBM com ADSC-CM *in vivo*?
- O pré-tratamento de células de GBM com ADSC-CM poderia influenciar a expressão de marcadores de EMT *in vivo*?

Com o intuito de responder essas questões, este Capítulo da tese apresenta os resultados obtidos a partir da analise dos efeitos do CM de ADSCs, bem como, os efeitos da co-injecao de células de GBM com ADSCs em um modelo de GBM de rato.

Analysis of the impact of rat adipose-derived stem cells and their conditioned medium on C6 glioma tumor microenvironment

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Abstract

In our previous study *in vitro*, we have shown that the conditioned medium (CM) from adipose-derived stem cells (ADSCs) promotes epithelial to mesenchymal (-like) transition (EMT-like) in C6 glioma cells, suggesting that factors released by ADSCs may modulate the biology of tumor cells. However, the exact mechanism of how these interactions happen *in vivo* remains unclear. In the present study, we investigated the effects of rat ADSCs and their conditioned medium (CM) in C6 tumor growth and malignancy *in vivo*. Our histological analyses showed that the pre-treatment of C6 cells with ADSC-CM (conditioned medium from ADSCs) not alter histological characteristics, as well as, Ki67 and GFAP expression in our glioma tumor model. We further showed that neither C6 pre-treated with ADSC-CM, nor co-injection of C6 cells and ADSCs were able to induce changes on expression of epithelial to mesenchymal transition (EMT)-related genes *in vivo*. However, surprisingly, tumors originated by co-injection of C6 cells and ADSCs were larger when compared with tumors of the other experimental groups. These results can be explained by the capacity of tumor cells in modulate their microenvironment. Our data provide new insights into the way by which mesenchymal stem cells (MSCs) and tumor cells interact and highlight the importance of understanding the fate and roles of MSCs in tumor sites *in vivo*, as well as, their intricate crosstalk with cancer cells, to ensure the safe use of MSCs in cancer therapy.

Keywords adipose-derived stem cell; conditioned medium; C6 glioma cell; epithelial-mesenchymal transition

Introduction

Glioblastoma multiforme (GBM) is the most common and lethal primary brain tumor. Despite many advances, the mean survival has not significantly improved and to date, there are no efficient therapeutics available for GBM. Surgical tumor removal, radio and chemotherapy improve survival for a short period after which the tumor relapses, mainly because their highly proliferative and infiltrative nature, making the total resection practically impossible (Jones and Holland 2012, Lima, Kahn et al. 2012). Therefore, in recent years many efforts have been made to find new effective therapeutic tools to treat GBM.

Adipose-derived stem cells (ADSCs) are a promising type of multipotent adult stem cells to use for tissue and cell engineering applications. These cells are abundant and easy to obtain, what enable to utilize them for therapeutic purposes (Mitchell, McIntosh et al. 2006, Bexell, Svensson et al. 2013). In addition, ADSCs have been recognized for their ability to migrate to injury sites, including tumors. Their tropism for brain tumors can be used for delivering therapeutic molecules, such as genes, proteins, peptides, or small chemical molecules to the tumor site (Kucerova, Altanerova et al. 2007, Cavarretta, Altanerova et al. 2010, Altanerova, Cihova et al. 2012).

For a long time, it was thought that tumors were self-sufficient and independent. However, recent reports have demonstrated that tumors consist not only of the malignant cancer cells, but also of stromal cells that are mobilized from systemic circulation and integrated into the tumor to support their microenvironment. The stroma mainly consists of myofibroblasts, tumor-associated fibroblasts (TAFs), mesenchymal stem cells (MSCs), immune cells, endothelial cells, smooth muscle cells, as well as non cellular components, such as the extracellular matrix (ECM) and secreted extracellular molecules that provide support to the tumor development (Barcellos-de-Souza, Gori et al. 2013, Adjei and Blanka 2015).

MSCs, by themselves are not malignant and function to preserve normal tissue structure and function. However, through intercellular interactions or paracrine secretions by cancer cells and other stromal cells, they may regulate tumor growth by providing structural and functional support or by secreting chemokines and growth factors that affect the tumor microenvironment (Droujinine, Eckert et al. 2013, Guan and Chen 2013).

Previous studies have produced controversial results regarding the roles of MSCs in tumor stroma. Some studies have demonstrated that MSCs can inhibit tumor progression (Giuffrida, Rogers et al. 2009, Zhu, Sun et al. 2009, Chien, Hsiao et al. 2011), whereas other investigations have revealed that MSCs promote tumor progression (Karnoub, Dash et al. 2007, Beckermann, Kallifatidis et al. 2008, Sun, Roh et al. 2009, De Luca, Lamura et al. 2012). These conflicting observations may be related in part to the diversity of experimental parameters among different investigations, primary tumor origin, location, type of cells, animal model, origin of MSCs, propagation and administration of cells that can vary greatly among experiments and must be recognized (Kidd, Spaeth et al. 2008, Wong 2011).

In our previous report, we showed that conditioned medium (CM) from rat ADSC was able to induce an EMT-like process in C6 cell line. The treatment with CM was marked by an enhanced migration capacity and increased expression of vimentin, MMP-2 and NRAS. CM also promoted a reduction in adhesion and changes in cell morphology, while not interfered the proliferation and cell cycle progression (Iser, Ceschini et al. 2015).

In this study, we aimed to investigate the effect of treatment of tumor cells with ADSC conditioned medium (ADSC-CM) on the development of glioma tumor in a rat model, as well as the effect of direct co-injection of C6 cells and ADSCs. We have found that animals co-injected C6+ADSC formed significantly higher tumors compared to animals that received C6 cells pretreated with a single dose of ADSC-CM and animals that received only C6 cells. Nevertheless, we have not observed difference related with expression of Ki67 proliferation marker, GFAP or EMT-related genes.

Methods

Cell culture

The C6 rat glioma cell line from ATCC, was kindly provided by Dra. Ana Maria Oliveira Battastini (UFRGS, Brazil). Cells at 5–20 passages were grown in culture flasks and maintained in Dulbecco’s modified Eagle’s medium (DMEM) (pH 7.4) containing 1% DMEM (Sigma, St. Louis, M.O., USA), 8.4 mM HEPES, 23.8 mM $NaHCO_3$, 0.1% amphotericin B, penicillin (100U/mL)/streptomycin (100 mg/mL) (Gibco BRL, Grand Island,NY,USA) and supplemented with 10% (v/v) fetal bovine serum (FBS) (Cultilab, São Paulo, SP, Brazil). Cells were kept at 37°C, a minimum relative humidity of 95% and an

atmosphere of 5% CO_2 in air.

Isolation and culture of adipose-derived stem cells (ADSCs)

ADSCs were extracted from visceral adipose tissue of *Wistar* rats (6–8 weeks), as previously described (da Silva Meirelles, Chagastelles et al. 2006). Briefly, the abdominal fat tissue from rat was transferred to a petri dish, washed with phosphate-buffered saline (PBS) and dissociated mechanically with a pipette. The fat fragments were transferred to a falcon tube containing 2 mg/mL of collagenase Type I, solubilized in DMEM medium without FBS and incubated in a water bath at 37°C for 30-45 min. After collagenase digestion, the supernatant was transferred to a new tube and centrifuged at 1000 rpm for 10 min at room temperature (RT). The supernatant was discarded and cells were resuspended in complete medium and seeded in six-well dishes. ADSCs cultures were grown in culture flasks and maintained in DMEM low glucose, containing 8.4 mM HEPES (pH 7.4), 23.8 mM $NaHCO_3$, 0.1% amphotericin B, penicillin (100U/ml)/streptomycin (100mg/ml) (Gibco BRL, Grand Island,NY,USA) and supplemented with 10% (v/v) FBS (Cultilab, São Paulo, S.P., Brazil). Cells were kept at a temperature of 37°C, humidity of 95% / 5% CO_2 in air.

ADSC differentiation

Osteogenic differentiation was induced by culturing ADSCs up to 8 weeks in Differentiation Single Quots^{TM} (Lonza; Walkersville, MD, USA) plus 5 μg/mL of ascorbic acid. In order to observe calcium deposition, cultures were washed once with PBS, fixed with 4% paraformaldehyde in PBS for 15–30 min, and stained for 5 min at RT with Alizarin Red S stain at pH 4.2. Excess of stain was removed by several washes with distilled water. To induce adipogenic differentiation, ADSCs were cultured up to 8 weeks in DMEM supplemented with 10^{-8} M dexamethasone, 2.5 mg/mL insulin and 100 mM indomethacin. To further confirm their identity, cells were fixed with 4% paraformaldehyde in PBS for 1 hour at RT and stained with Oil Red O solution. Chondrogenic differentiation was performed using differentiation CDMTM Basal Medium and CDMTM Differentiation Single Quots^{TM} (Lonza). Cultures were analyzed by fixing with 4% paraformaldehyde in PBS for 20 min and staining with Alcian Blue (pH 2.5) (Sigma, St. Louis, M.O., USA) in 3% acetic acid, for 5 min at RT. After staining, the cultures were washed with distilled water.

Immunophenotyping

The cultures of ADSCs were characterized to confirm the presence or absence of MSC surface markers using flow cytometry. Cells were trypsinized, centrifuged and incubated for 25 minutes at 4°C with phycoerythrin (PE)- or peridinin-chlorophyll protein (PerCP-Cy5.5)-conjugated antibodies with anti-rat CD45, CD29 or CD90.2 (Life Technologies, Carlsbad, CA, USA). The unconjugated antibody CD11b (Life Technologies, Carlsbad, CA, USA) was incubated with the secondary anti-mouse IgG2 FITC-conjugated antibody (Sigma-Aldrich, St. Louis, MO, USA) for additional 25 min. Excess of antibody was removed by washing with PBS (phosphate buffered saline). Cells were analyzed using a FACS-Calibur cytometer equipped with 488 nm argon laser (Becton Dickinson, San Diego, CA, USA) with the CellQuest software. At least 10,000 events were collected. Gating was set using unstained cells.

Conditioned medium

For all experiments, ADSC cells between the 4th-10th passages were seeded in T75 tissue culture flask in DMEM with 10% (v/v) FBS low glucose, containing 8.4 mM HEPES (pH 7.4), 23.8 mM $NaHCO_3$, 0.1% amphotericin B, penicillin (100U/ml)/streptomycin (100mg/ml) (Gibco BRL, Grand Island,NY,USA) at a density of 3,320 cells/cm^2. Twenty-four hours after seeding, the medium was replaced by a fresh medium and conditioned for 48h (CM48). CM from flasks was harvested, filtrate on 0.22 μm filters (Millipore), and stored at -80ºC until use.

Glioma implantation

Rat C6 glioma cells at around 80% confluence were trypsinized (0.25% trypsin/EDTA solution), washed once in DMEM without SFB, spun down and ressuspended in the same medium. A total of 5 x 10^5 cells in a volume of 3 μl were injected using a 10 μL Hamilton microsyringe at a depth of 6.0 mm into the right striatum (coordinates with regard to bregma: 0.5 mm posterior and 3.0 mm lateral) of adult Wistar male rats (8 weeks old, 220–260 g) anesthetized by intraperitoneal (i.p.) administration of ketamine/xylazine (Takano, Lin et al.

2001). The protocols used in this study were approved by the Ethics Committee on Animal Use (CEUA) of Universidade Federal de Ciências da Saúde de Porto Alegre (UFCSPA), under the number 104/11, following the resolutions of the CONCEA (Conselho Nacional de Controle de Experimentação Animal). The NIH ‘‘Guide for the Care and Use of Laboratory Animals’’ (NIH publication Nº 80–23, revised 1996) was followed in all experiments. The surgeries were performed with all efforts to minimize the animals suffering.

Treatment of animals

The animals were divided into three groups as follows: (1) received $5x10^5$ C6 cells (control group); (2) received $5x10^5$ C6 cells treated with CM48 for 48h previously of the implant (C6+CM group) and (3) received $5x10^5$ C6 cells plus $5x10^5$ ADSCs (C6+ADSC group) (Fig. 1). At least seven rats per group were used for the immunohistochemical study and at least three rats for the RNA extraction. After 20 days of the implant of cells, the perfusion was conducted. Animals were deeply anesthetized with ketamine (90 mg/kg) and xylazine (15 mg/kg) (*i.p.*) and injected with 1mL heparin (Cristalia, Brazil). Using a peristaltic pump (50 mL/min), the animals were perfused through the left cardiac ventricle with 200 mL of saline solution followed by 200 mL of fixative solution of 4% paraformaldehyde diluted in 0.1 M phosphate buffer (PBS), pH 7.4. Brains were dissected from the skull, post-fixed in paraformaldeyde (4%) followed by 70% ethanol at room temperature until it was embedded in paraffin.

Pathological analysis and tumor volume quantification

At least three Hematoxylin and Eosin (H&E) sections (4 µm thick, paraffin embedded) from each animal were analyzed by a pathologist in blinded manner. For tumor size quantification, images were captured using an Opton camera connected to a microscope (Olympus BX 50) and the tumor area (mm^2) was determined using ImageJ software (National Institute of Health, Bethesda, MA). The total volume (mm^3) of the tumor was computed by the multiplication of the slice sections and by summing the segmented areas (Morrone, Oliveira et al. 2006).

Immunohistochemical staining

Paraffin embedded, 4 μm formalin fixed tissue sections were mounted on microscope slides and de-paraffinized for 1 h at 60°C. Endogenous peroxidase was inhibited by 5% H_2O_2 in methanol for 15 min. To block the unspecific bindings, the samples were incubated with 1% bovine serum albumin (BSA), for 30 min. Incubation with the following antibodies was performed overnight at 4°C temperature: anti-GFAP (1:50) and anti-Ki67 (1:200) followed by incubation with universal immuno-peroxidase polymer for rat tissue sections (Histofine Simple Stain Rat Max-PO - Nichirei Co., Tokyo, Japan). The immune complexes were visualized by using diaminobenzidine (EnVision kit; DAKO, Carpinteria, CA), according to the manufacturer's specifications. The Ki67 immunostained sections were then counterstained with hematoxylin.

Ki67 immunoreactivity evaluation

For this analysis, nine digitized images (x200) from tumor bulk were obtained from each animal using an Opton camera connected to a microscope (Olympus BX 50). One randomized area of interest (AOI) measuring 5,624 μm^2 was overlaid on each image. The positive and negative cells located inside this square were counted. Cells located inside this square or intersected by the upper and/or left edges of the square were counted. Cells intersected by the lower and/or right edges of the square were not counted. Data of glioma cell proliferation were presented by counting the percentage of Ki67 positive glioma cell nuclei. Sections of rat gut were used as positive controls.

GFAP immunoreactivity evaluation

The intensity of GFAP immunoreactivity was measured using semi-quantitative desitometric analysis (Xavier, Viola et al. 2005), using an Olympus BX 50 microscope coupled to an Opton camera and Image Pro Plus software (Image Pro-plus 6.1, Media Cybernetics, Silver Spring, USA). For this analysis, three digitized images (50x) from tumor bulk, tumor edge and contralateral hemisphere were obtained from each animal. The images were converted to an 8-bit gray scale (256 gray levels) and one randomized area of interest (AOIs), measuring 15,300 μm^2 was overlaid on each image and used to estimate the regional optical density (OD).

All lighting conditions and magnifications were kept constant during the process of capturing the images. Blood vessels and histological artifacts were avoided. The background correction was performed in accordance with our previously published protocol (Xavier, Viola et al. 2005).

The OD was calculated using the following formula:

OD(x,y) = -log[(INT(x,y)-BL)/(INC-BL)]

Where "OD (x,y)" is the optical density at pixel (x,y), "INT (x,y)" or intensity is the intensity at pixel (x,y), "BL" or black is the intensity generated when no light passes through the material, and "INC" is the intensity of the incidental light, which is completely white.

RT-qPCR analysis

After 20 days of the cells implant, at least three rats per group were decapited, the whole brain was removed and the tumor area was isolated. Briefly, rat glioma samples were removed and immediately preserved in RNAlater (Sigma-Aldrich Ltd.) and frozen at -80°C (for RNA extraction).

Total RNA from glioma cell cultures were isolated with Trizol LS reagent (Life Technologies, Carlsbad, CA, USA) in accordance with the manufacturer's instructions and stored at -80ºC.

The cDNA was synthesized with M-MLV Reverse Transcriptase enzyme (Promega, Madison, Wis., USA) 3 µg total RNA in a final volume of 20 µL with a random hexamer primer in accordance with the manufacturer's instructions. cDNA was diluted to 1:10 in diethyl pyrocarbonate (DEPC) water and stored at -20 ºC. Real-time PCRs were carried out in an Applied-Biosystem StepOnePlus™ Real-Time PCR cycler and done in duplicate. Reaction settings were composed of an initial enzyme activation step of 20 s at 95 °C, followed by 40 cycles of 3s at 95°C and 30s at 60°C for data acquisition. Real-time PCRs were prepared in a 12.5 µL final volume composed of 6.25 µL of Fast SYBR green master mix (Applied Biosystems, Foster City, CA, USA), 0.40 µL of 10 µM primer pairs (Table 1), 3.85 µL of water and 2µL of diluted cDNA. Normal brain was used as negative control. As previously described (Iser IC 2015), after an appropriate selection using relative standard curve method, TBP was chosen as the most stable reference gene between five genes candidates and was used as a control for cDNA synthesis (data not shown). The data were presented as ratio of genes/TBP.

Statistics

All data were expressed as mean ± SEM and they were analyzed using ANOVA followed by Tukey's test for multiple comparisons of at least three independent experiments. Differences with $p < 0.05$ were considered significant. Analyses of the data were performed using GraphPad Prism version 6.0 software (GraphPad, La Jolla, CA, USA).

Results

ADSCs express stem cell markers and display multipotent differentiation

Phenotypically, ADSCs were characterized by being negative for expression of the CD11b and CD45, hematopoietic surface markers. Flow cytometric analysis also demonstrates that cultured MSCs were positive for CD90 (glycosylphosphatidylinositol-anchored glycoprotein) and CD29 (Integrin b1 chain) (Fig 2), as expected. Such as reported previously by our group, ADSCs exhibited a spindle-shaped morphology in culture and were able to differentiate into adipocytes, chondrocytes and osteoblasts (Iser, Ceschini et al. 2015). These results were consistent with previous reports and indicated that the established cell line indeed consisted of ADSCs (Gutierrez-Fernandez, Rodriguez-Frutos et al. 2013).

Histopathological analysis

Implanted tumors have characteristics that are closer to those of human glioblastomas, with C6 cells demonstrated in most of cases to grow in the intracerebral, intraventricular and intraparenchymal spaces (data not shown).
A representative figure of H&E analysis showed that mitotic index, coagulative necrosis and vascular proliferation were very similar among the groups. In addition, pathological analysis identified pallisading cells delineating the foci of necrosis and lymphocytic infiltration, with formation of peritumoral edema and neovascularization in all groups. (Fig. 3 and Table 2).

Co-injection of C6 and ADSC enhanced tumor size in vivo

The rat glioma experimental model has been extensively used for a variety of studies, especially for investigations of glial tumor biology. If ADSCs promote or not tumor growth in rat glioma models remains unknown. In an effort to answer these question, we first treated C6 tumor cells with ADSC-CM for 48 h, followed by intracranial injection of untreated C6 tumor cells alone, hMSC-CM-treated C6 tumor cells alone or untreated tumor cells mixed with ADSC into *wistar* rats.

After 20 d of cells implantation, the hematoxylin and eosin (H&E) analysis showed that 77.8% of animals that received C6 cells presented a defined tumor mass, against 88,9% and 87.5% animals in the groups that received hMSC-CM-treated C6 tumor cells alone or untreated tumor cells mixed with ADSC, respectively. The other animals showed only cells with characteristics of a residual tumor in the site of glioma implantation. Therefore, the analysis of the tumor volume was carried out exclusively in animals presenting a tumoral mass that could be quantified. Interestingly, one animal that was co-injected with C6 cells and ADSCs was lost due to cannibalism practiced by its cage mate.

The mean tumor sizes in the group co-injected with ADSC cells was 128,2 mm^3 at 20 d after injection, in contrast to that in the control group and in the group pretreated with ADSC-CM (33.24 mm^3 and 37.54 mm^3 in size, respectively, 20 d after injection) (Fig. 4).

All animals that composed the groups to RNA analysis formed tumors 20 d after injection of cells. Interestingly, two animals of the group whose rats received C6 cells co-injected with ADSC cells developed signs indicative of systematic illness, including slaughtered, cachexia/anorexia and limited mobility. Because of this, these animals were sacrificed at the sixteenth day after the implant of cells.

Effects of ADSC-CM on tumor cell proliferation

Next, we tested whether ADSC-CM alters tumor cell volume through direct modulation of tumor cell proliferation. As expected, the mitotic index observed by H&E examination was confirmed by counting the percentage of Ki67 positive glioma cell nuclei. We found through Ki67 immunostaining that the proliferation rate of C6-derived gliomas did not statistically differ following treatment with ADSC-CM or co-injection with ADSCs (Fig. 5). These data suggest that ADSC-CM treatment or co-injection with C6 and ADSCs, per se, do not, or is not sufficient to, modify the *in vivo* proliferation program of C6 tumor cells.

Immunohistochemical analysis of GFAP activity in tumor-bearing brain

In vivo, astrogliosis involves the proliferation of astrocytes and is associated with increased expression of GFAP and a change of cell morphology into a hypertrophic state. Brain tumors also induce reactive astrogliosis in the brain, therefore, we tested whether GBM cells activate GFAP in our model (Chekhonin, Baklaushev et al. 2007, Pisati, Belicchi et al. 2007). In agreement with previous observations (Le, Besson et al. 2003, Wilhelmsson, Eliasson et al. 2003). GFAP activity was highly upregulated at the tumor edge, forming a glial scar, whereas a reduced GFAP immunoreactivity was observed in the tumor bulk. The contraletral hemisphere was used as negative control. However, in our experimental design, neither co-implant of C6 cells + ADSCs nor exposure of C6 cells to the ADSC-CM demonstrated any difference in the density of GFAP immunopositive cells around of the tumor mass when compared with C6 cells-derived tumors (Fig. 6).

Expression of EMT-related genes

EMT is a newly recognized process often seen in development and oncogenesis. Enhanced vimentin, MMP2 and NRAS expression are among the main characteristics of EMT.

We previously showed that stimulation of C6 cells with ADSC-CM leads to enhanced expression of the EMT-related genes vimentin, MMP2 and NRAS (Iser, Ceschini et al. 2015). Then, we wondered if CM-treated tumor cells or the C6+ASDCs-derived tumors cells also undergo EMT. Surprisingly, we not observed the same effect in gene expression when compared with our *in vitro* model. The expression of vimentin, MMP2 and NRAS were statistically similar in tumors derived of only C6 cells, C6+ADSC cells and C6 cells following treatment with ADSC-CM (Fig. 7). When we compared the gene expression of the all groups with normal brain (control), we observed that C6- and C6+ADSC-derived tumors presented vimentin mRNA expression approximately 20-fold and 30-fold, respectively, more than normal brain, corroborating with already presented data in the literature (Hirato, Nakazato et al. 1994). In contrast, the expression of vimentin in C6 cells pre-treated with CM is not statistically different from normal brain. The mRNA expression pattern of MMP2 *in vivo* is very similar both, in normal brain as well as in C6-derived gliomas pre-treated with

ADSC-CM or C6+ADSC- derived gliomas. In addition, the C6- derived gliomas were found to exhibit a 2-fold increase in NRAS gene expression compared with normal brain. Lastly, we observed that tumors originated from pre-treatment of C6 cells with ADSC-CM and the co-implant of C6 cells and ADSCs presented NRAS expression similar to normal brain.

Our data thus suggest that neither the physical presence nor molecules secreted by ADSCs are able to enhance the expression of EMT-related genes at RNA levels *in vivo*.

Discussion

Tumor progression occurs by a complex network in which tumor and stromal cells perform an intricate crosstalk in either direct or indirect manners. The discovery that MSCs can constitute the tumor stroma, through a strong tropism for injury sites, has attracted a great deal of interest over the past decade, to disclose the functions of MSCs in tumors microenvironment (Zhang, Daquinag et al. 2009, Barcellos-de-Souza, Gori et al. 2013, Guan and Chen 2013).

To determine the impact of MSCs on tumor development, researchers have used many tumor models. Interestingly, many studies have reported discrepant findings about the effects of MSCs on tumor growth (Pisati, Belicchi et al. 2007, Kang, Jeun et al. 2008, Yu, Jun et al. 2008, Jiao, Guan et al. 2011, Akimoto, Kimura et al. 2013, Behnan, Isakson et al. 2014). Although the specific pathways by which MSCs interact with tumor cells are unknown, many mechanisms have been reported to be responsible for these observations, such as secretion of soluble factors, vascular support, immune modulation or differentiation capacity (Cuiffo and Karnoub 2012). Currently, there are increased evidences that molecules secreted by MSCs, such as growth factors, cytokines and chemokines constitutes their most significant therapeutic mechanism of action (Caplan and Correa 2011).

Based on the property of MSCs being recruited to tumor sites, MSCs have been used as cellular delivery vehicles for antitumor agents in a broad variety of tumor types (Stoff-Khalili, Rivera et al. 2007, Xin, Kanehira et al. 2007, Kucerova, Matuskova et al. 2008, Cavarretta, Altanerova et al. 2010, Bak, Lam et al. 2011). However, it is important to determine under what conditions MSCs might enhance or inhibit tumor progression to develop safe therapeutic approaches and to understand the role of stroma in tumorigenesis.

In our previous study *in vitro*, we showed that ADSC-CM promotes EMT-like process in C6 cells, as evidencing by reduced adhesion capacity, enhanced migration ability and

increased expression of EMT-like makers (Iser, Ceschini et al. 2015) (Fig. 8). Therefore, the principal objective of this study was to explore the effective *in vivo* effect of ADSCs and ADSC-CM from rat in malignant glioma tumors.

Here we showed that neither ADSC-CM nor ADSC were able to induce increased expression of EMT-related markers *in vivo*. EMT is associated with an increased invasive potential by cancer cell, marked by expression of markers related to mesenchymal phenotype and invasion. This result draws our attention to the fact that the detection of EMT *in vivo* during disease progression is particularly difficult and remains a challenge of EMT program (Thiery and Chopin 1999, Lee, Dedhar et al. 2006). This difficulty is explained mainly because EMT is a floating process, marked by the ability of cancer cells to remain in a hybrid stage, known as "incomplete" EMT, or even return their phenotype through (mesenchymal to epithelial transition) MET (Strauss, Hamerlik et al. 2012).

Our pathological analysis demonstrated that the expression of Ki67 marker, a non-histone cell-cycle-associated antigen that is expressed during G1, S, and G2/M phases in proliferating cells, was not changed in the tumors of rats pre-treated with ADSC-CM or co-injected with C6 cells and ADSCs. This result indicates that the treatments were not able to change the C6 cell proliferation program. In contrast, recently, Zhu and collaborators (2011) reported that the pre-treatment of SGC-7901 cells, a human gastric cancer cell line, with human bone marrow MSC-conditioned medium *in vitro,* promoted tumor growth, comparable to the effect of MSC co-injection *in vivo* in immunodeficient mouse xenograft model (Zhu, Huang et al. 2011). These opposed results could be due to different cell and animal models used. Besides, it is important to consider that the immune system is very important to tumor formation and aggressiveness (Du and Barcellos-Hoff 2013). In addition, in that work, cells were injected subcutaneously in the flanks of animals, forcing them to adapt to a different microenvironment.

The process of gliosis (or "reactive astrocytosis") that is induced by brain injury involves morphological changes in astrocytic cells that can be demonstrated by immunohistochemistry for GFAP. Diffuse gliomas infiltrate into the brain tissue and stimulate their own gliosis, thus creating an altered brain microenvironment, which is more permissive to tumor growth/invasion (Chekhonin, Baklaushev et al. 2007, Fitzgerald, Palmieri et al. 2008). In our work, the formation of a glial margin at the periphery of the glioma, consisting of GFAP-positive reactive astrocytes, was shown by the immunohistochemical method. Reactive astrogliosis was observed only at the periphery of tumors, forming a gliotic margin.

Moreover, the level of GFAP expression was similar in all of our experimental groups. In accordance with histophatological results, our observations demonstrad that the treatment of C6 cells with CM and co-injection with ADSCs not alter histological characteristics of the tumors. Taken together, these results demonstrate that ADSCs and ADSC-CM treatment are not sufficient to alter the malignancy parameters in our *in vivo* GBM model. In accordance with our results, previous data of our group showed that, *in vitro,* human ADSC-CM not change steamness-related features, such as sphere forming capacity and expression of genes related to cancer stem cells (CSCs), in human GBM cell line. Besides, hADSC-CM treatment did not alter cell proliferation rate, as well as, response of GBM U87 cells to chemotherapy (Giovana R. Onzi et al. Unpublished data).

Surprisingly, although proliferation rate and astrogliosis reaction were not altered in our model, animals co-injected with C6 cells and ADSCs developed larger tumors when compared with animals that received untreated C6 tumor cells alone or CM-treated C6 tumor cells alone (Fig. 8). These results suggest that something else than the proliferative rate accounts for the difference in tumor volume. Several researchers have suggested different roles of MSCs in tumor formation, including the capacity of tumors in "educate" their stromal cells and induce malignant transformation of MSCs, both *in vitro* and *in vivo* (Rosland, Svendsen et al. 2009, Liu, Zhang et al. 2012, Pan, Fouraschen et al. 2014). Liu et al (2012) showed that besides MSCs undergoing malignant transformation after direct or indirect co-culture with C6 cells *in vitro*, when they are transplanted subcutaneously, in an *in vivo* mice xenograft model, it was observed the formation of large tumors (Liu, Zhang et al. 2012). On possible explanation for this is that MSCs became malignant cancer cells when exposed to the tumor microenvironment, suggesting that factors released from the cancer cells could have an important role in MSCs transformation. Although malignant transformation was not molecularly analyzed here, our results suggest that the increased tumor bulk is stimulated by other indirect mechanism that does not alter the proliferation program. However, the exact molecular mechanisms warrant further investigation.

When MSCs are applied in gene therapy approaches, it is desirable that MSCs are removed of the tumor environment after delivering therapeutic molecules into the tumor site, in order to avoid effects that facilitate tumor progression by intercommunication among the cells that compound the tumor stroma. The understanding of the fate and functions of MSCs in tumor sites *in vivo*, as well as, their intricate crosstalk with cancer cells, is essential to ensure the safe use of MSCs to treat different types of cancers. We sincerely believe that a

fuller understanding of the mechanisms by which MSCs interact with cancer cells will bring immense benefits to field of oncology. The wide knowledge about the role of MSCs in cancer development will allow not only the improvement of MSC-based therapies, but also, open up opportunity for developing effective therapies for preventing tumor progression.

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Figure Legends

Figure 1. Experimental design and timeline. At day 0, the animals were injected with untreated C6 cells, ADSC-CM-treated C6 cells (C6 glioma cells were previously treated with ADSC-CM for 48h) or untreated C6 cells mixed at 1:1 ratio with ADSCs into the right striatum of brain of rats by stereotaxic surgery. At day 20, the animals were perfused for histological analysis. The animals whose brains were used to molecular analysis were not perfused.

Figure 2. Immunophenotyping analysis of surface markers expression in ADCSs. Flow cytometry histograms show the expression (shaded) of selected molecules (CD90, CD29, CD11b and CD45) by ADSC populations in comparison with controls.

Figure 3. Histological analysis of implanted gliomas. The sections of implanted rat glioma were stained with hematoxylin and eosin (H&E), as described in Methods Section. Representative pictures of histological characteristics that define glioblastoma multiform, as seen in rats implanted with untreated C6 cells (a, d, g), ADSC-CM-treated C6 cells (b, e, h) and untreated C6 cells mixed with ADSCs (c,f,i). Pseudopalisading necrosis, microvascular proliferation (arrows) and mitosis (arrows) were observed. Scale bars = 10 μm (a, b, c); 50 μm (i); 100 μm (d, e, f, g, h).

Figure 4. Tumor size of implanted gliomas. Animals were treated, as described in "Methods" Section. The animals were killed 20 days after implantation of cells and glioma sections were dissected and analyzed for tumor size. (a) Photographs of rat brain slices of implanted gliomas of untreated C6 cells, ADSC-CM-treated C6 cells and untreated C6 cells mixed with ADSCs. The gliomas are marked with a circle. (b) Tumor size quantification of implanted gliomas. The values were represented as means ± S.E.M of at least seven animals per group. Data were analyzed by ANOVA followed by post-hoc comparisons (Tukey's test). *$p<0.05$ and **$p<0.01$

Figure 5. Immunohistochemical staining of Ki67 in implanted gliomas. (a) Representative pictures of immunohistochemical analysis in rats implanted with untreated C6 cells, ADSC-CM-treated C6 cells and untreated C6 cells mixed with ADSCs. (b) Percentages of positive

(Ki67+) and negative (Ki67-) cells to brown nuclear expression of Ki67. The results are presented as mean values ± S.E.M., as determined by one-way ANOVA followed by post-hoc comparisons (Tukey). Scale bars = 20 μM.

Figure 6. Immunohistochemical staining of GFAP in implanted gliomas. Reactive astrocytosis was assessed by immunostaining for GFAP positive cells. Representative pictures of immunohistochemical analysis of tumor bulk and edge of rats implanted with untreated C6 cells (a,d), ADSC-CM-treated C6 cells (b,e) and untreated C6 cells mixed with ADSCs (c,f). (g) Percentages of positive GFAP immunostaining cells. BT = tumor bulk; TE = tumor edge; CH = contralateral hemisphere. The results are presented as mean values ± S.E.M., as determined by one-way ANOVA followed by post-hoc comparisons (Tukey`s test). Scale bars = 100 μM.

Figure 7. Expression of EMT markers in implanted gliomas. Quantitative RT-PCR of (a) vimentin, (b) MMP2 and (c) NRAS genes in tumors of rats implanted with untreated C6 cells, ADSC-CM-treated C6 cells and untreated C6 cells mixed with ADSCs. Quantitative PCR measurements of gene expression levels are normalized against TBP levels, and expressed relatively to the control samples (normal brain). The values were considered significantly different from controls, when $P<0.05$ as determined by ANOVA followed by Tukey's test. $^*p<0.05$ indicate statistical difference from control. All experiments were repeated at least three times.

Figure 8. Schematic overview of how ADSCs and their CM affect glioma cells *in vivo*. The co-injection of ADSCs and glioma cells (A) into the brain of rats originates bigger tumors (1) when compared with animals that received glioma cells previously treated with conditioned medium from ADSCs (B) or only glioma cells (C) (2 and 3). These findings raising the possibility that C6 cells are able to induce malignant transformation in ADSCs *in vivo*.

Table 1. Primers for EMT markers used at RT-qPCR experiments

Primer	Sense	Antisense
MMP2	5'-ACAACAGCTGTACCACCGAG-3'	5'-GGACATAGCAGTCTCTGGGC-3'
Vimentin	5'-GAGGAGATGAGGGAGTTGCG-3'	5'-GGTCAAGACGTGCCAGAGAA-3'
NRAS	5'-CACGAGCTGGCCAAGAGTTA-3'	5'-TGAGGCTTGAAAGTGGCTCG-3'
TBP	5'-CGTGACGATAACCCAGAAAG-3'	5'-GGTGGAAGGCTGTTGTTC-3'

EMT: epithelial-mesenchymal transition; MMP2: matrix metalloproteinase-2; RT-qPCR: quantitative reverse transcription-polymerase chain reaction.

Table 2. Histological characteristics of implanted gliomas.

	C6 cell group (n=9)	C6+ADSC-CM group 2 (n=9)	C6+ADSC (n=8)
Coagulative necrosis	7/9 (77.8%)	7/10 (70%)	7/8 (87.5%)
Intratumoral hemorrhage	1/9 (11.1%)	0/10 (0%)	1/8 (12.5%)
Lymphocytic infiltration	8/9 (88.9%)	9/10 (90%)	7/8 (87.5%)
Peritumoral edema	8/9 (88.9%)	9/10 (90%)	7/8 (87.5%)
Peripheric pseudopalisading	4/9 (44.5%)	5/10 (50%)	7/8 (87.5%)
Mitotic index: mitosis/HPF	2.65±0.99	0.75±0.16	4.28±0.95

The histological variables (coagulative necrosis, intratumoral hemorrhage, lymphocytic infiltration, peritumoral edema and peripheric pseudopalisading) were regarded as present or absent. Mitosis was counted in ten high power fields (HPF) of the periphery of the tumor, and the average of this counting was used as mitotic index (means ± S.E.M).

Figure 1

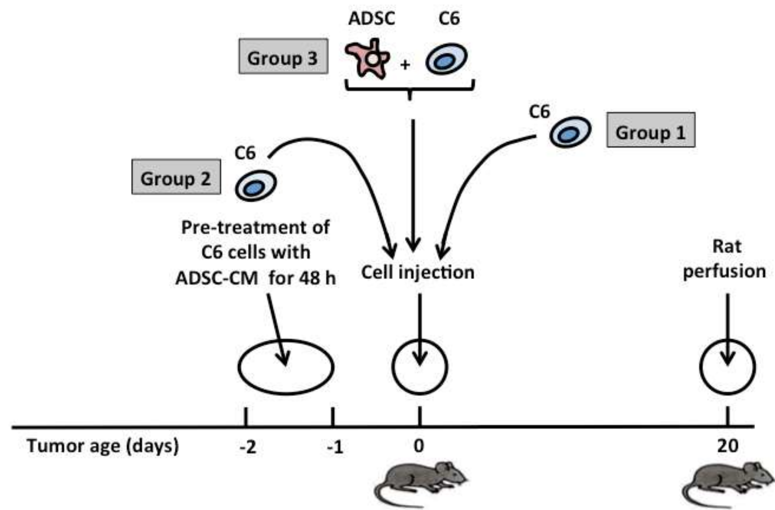


Figure 2

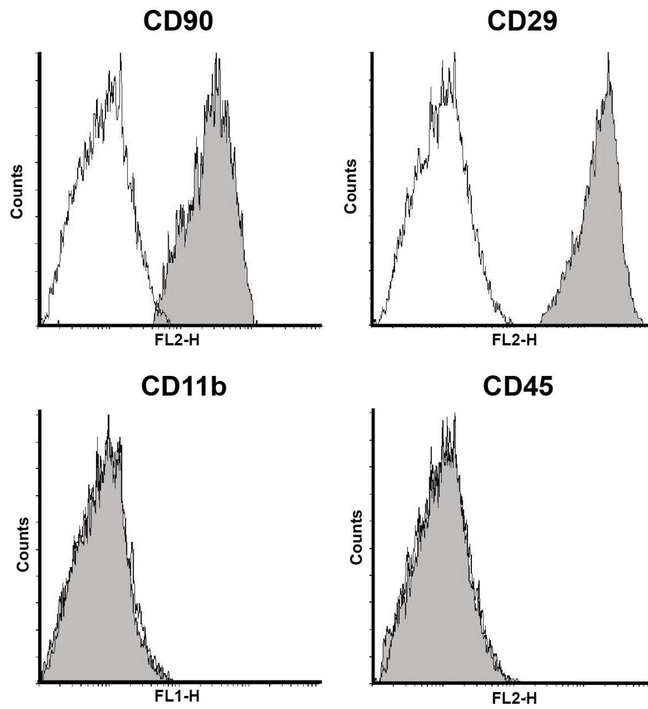


Figure 3

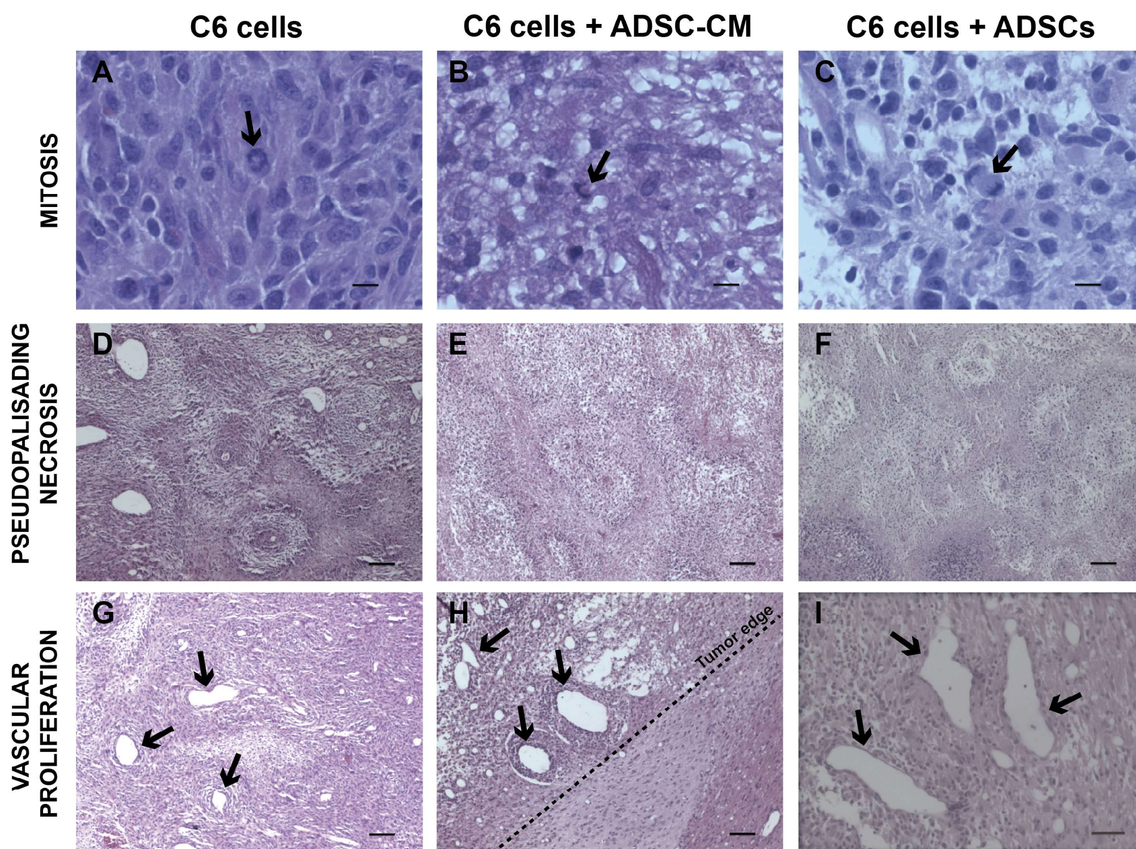


Figure 4

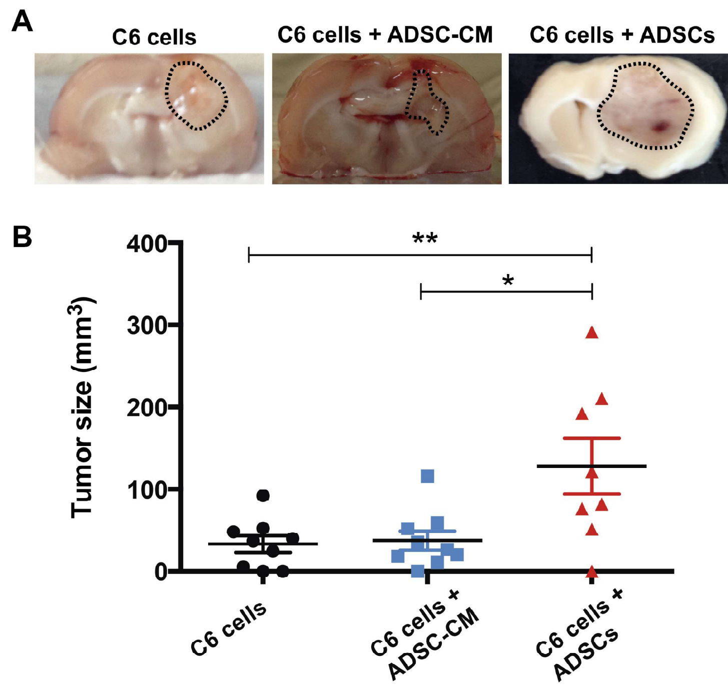


Figure 5

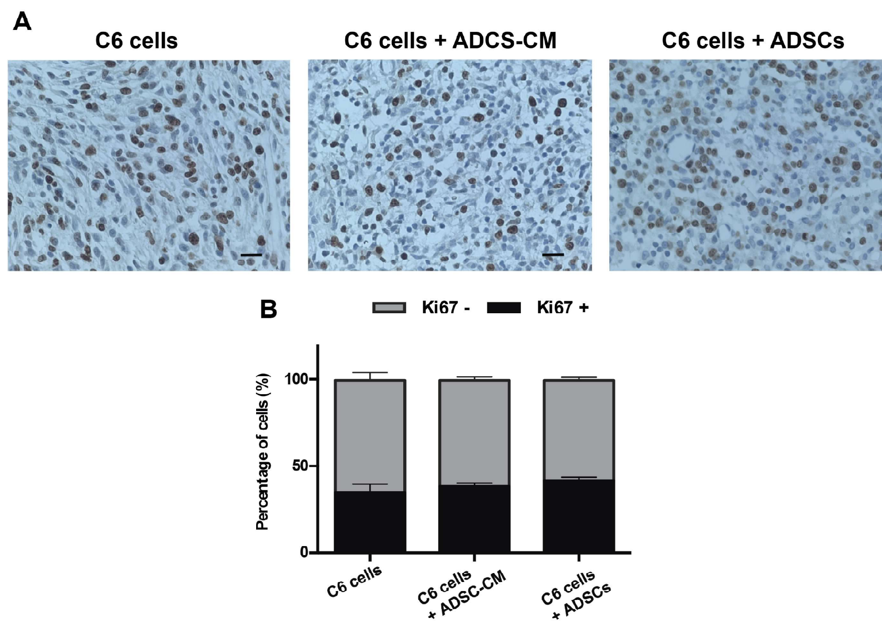


Figure 6

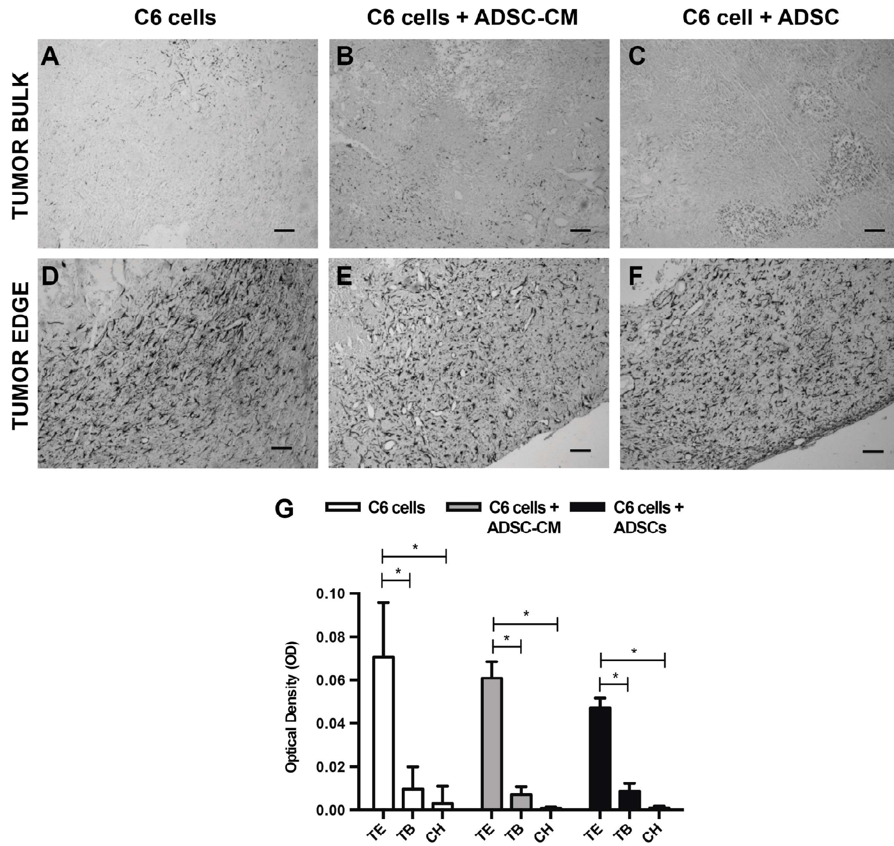


Figure 7

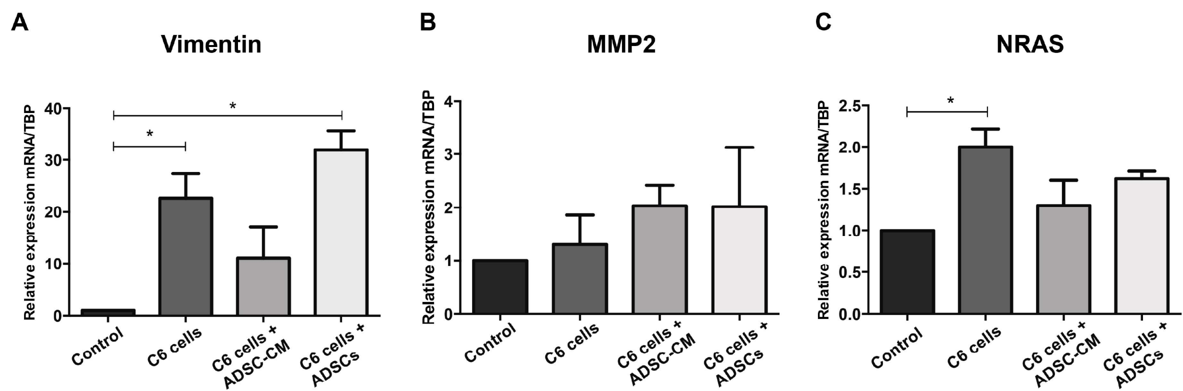
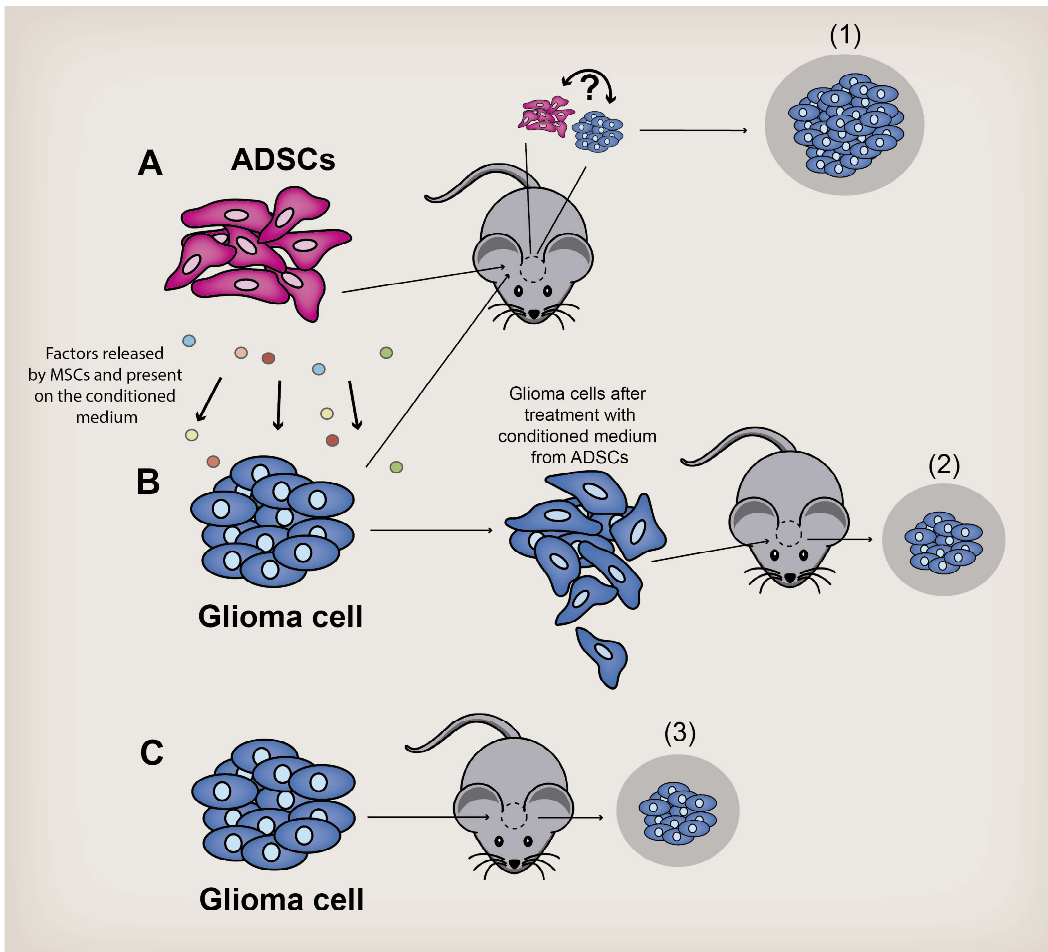


Figure 8



CAPÍTULO IV

The epithelial to mesenchymal transition-like in glioblastoma: An updated systematic review and in silico investigation

Artigo de Revisão publicado no periódico ***Medicinal Research Reviews***

APRESENTAÇÃO

Neste Capítulo será apresentado um artigo de revisão sistemática no qual são descritas as principais vias de sinalização, moléculas e efetores envolvidos no processo de EMT em GBM.

Diante dos resultados obtidos nos Capítulos II e III desta Tese, decidimos fazer uma busca na literatura para comparar os nossos resultados com resultados encontrados por outros pesquisadores em diferentes desenhos experimentais, bem como obter um *overview* do conhecimento atualmente disponível sobre o processo de EMT em GBM. O objetivo foi fazer uma descrição dos mecanismos moleculares que regem a EMT em GBMs, focando nos principais fatores de transcrição, vias de sinalização e moléculas descritas como reguladoras de EMT. O artigo também aborda a importância das células tumorais circulantes, das células tronco tumorais (CSCs) e o envolvimento das MSCs nesse processo. Também é descrito o papel dos miRNAs como moléculas responsáveis pela regulação e fino ajuste da indução ou inibição de EMT em GBM. Os mecanismos pelos quais microambientes de hipóxia induzem EMT também são abordados nessa revisão, bem como o papel da EMT na indução de resistência à terapia. Além disso, o artigo apresenta um panorama da principais drogas, compostos e moléculas capazes de inibir EMT em células de GBM, provendo, assim, um *overview* dos principais agentes terapêuticos inibidores de EMT em GBM existentes na literatura.

Por fim, realizamos análises de bioinformática com o intuito de responder importantes questões ainda não bem compreendidas sobre o processo de EMT em gliomas.

The Epithelial-to-Mesenchymal Transition-Like Process in Glioblastoma: An Updated Systematic Review and In Silico Investigation

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Abstract: Glioblastoma multiforme (GBM) is the most aggressive form of brain cancer due to its highly invasive nature that impedes the surgical removal of all tumor cells, making relapse inevitable. However, the mechanisms used by glioma cells to invade the surrounding tissue are still unclear. In this context, epithelial-to-mesenchymal transition (EMT) has emerged as a key regulator of this invasive state and although the real relevance of this program in malignant glioma is still controversial, it has been strongly associated with GBM malignancy. EMT is a very complex process regulated by several families of transcriptional factors through many signaling pathways that form a network that allows cancer cells to acquire invasive properties and penetrate the neighboring stroma, resulting in the formation of an advantageous microenvironment for cancer progression and metastasis. In this systematic review, we focus on the molecular mechanisms of EMT including EMT-factors, drug resistance, miRNA, and new therapeutic strategies. In addition, we address controversial questions about mesenchymal shift in GBMs with a bioinformatics analysis to show that in terms of epithelial and mesenchymal phenotype, the majority of GBMs samples analyzed have a profile more mesenchymal than epithelial. If induced, this phenotype can be shifted toward an even more mesenchymal phenotype in an EMT-like process in glioma cells. A better understanding of the molecular regulation of the EMT during tumor spreading will help to provide potential therapeutic interventions to target this program when treating GBM. © 2016 Wiley Periodicals, Inc. Med. Res. Rev., 00, No. 0, 1–43, 2016

Key words: glioblastoma multiforme; epithelial-to-mesenchymal transition; EMT-like; transcriptome analysis

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1. INTRODUCTION

Malignant gliomas are the most common type of primary brain tumors and glioblastoma multiforme (GBM) is the most aggressive (WHO grade IV) and common of the human gliomas.[1,2] Despite extensive investigations, 88% of all GBM patients die within 1 year after diagnosis and the 5-year survival rate is less than 5%. One of the reasons for this high mortality is the resistance of GBM to therapeutic intervention (surgical resection, followed by radiation therapy and chemotherapy), mainly due to the singular nature of these tumors, marked by diffuse growth, intratumoral genetic heterogeneity, and microvascular proliferation.[2,3] Thereby, GBM remains one of the most challenging malignancies worldwide and many attempts at novel therapeutic approaches have had little impact on survival.[2,4]

The invasive characteristic of GBMs is, at least in part, due to their high migratory potential to invade the surrounding tissue. Epithelial-to-mesenchymal transition (EMT) has been pointed as one of the mechanisms that confer to GBM cells this invasive property.

EMT is a biological process in which a polarized sheet of epithelial cells undergoes multiple biochemical changes that culminates in a mesenchymal phenotype, characterized by cells with weak cell adhesions and enhanced migratory capacity. Besides, these cells display altered morphology and resistance to chemotherapy and anoikis.[5,6] EMT is classified into three different subtypes based on the biological context in which they occur. EMT type 1 is associated with implantation, embryo formation, and organ development. The second type of EMT is associated with wound healing, tissue regeneration, and organ fibrosis. Finally, EMT type 3 occurs in neoplastic cells and is associated with cancer progression.[5]

In the late 1960s, the first formal description about EMT was published by Hay et al. They showed the importance of this event during normal embryogenesis, with the migration of epithelial-derived cells from the surface to the interior of the embryo, to form the mesoderm during gastrulation.[7,8] In the 80s, Greenburg and Hay described epithelial-to-mesenchymal changes in cell phenotype of adult and embryonic epithelia.[9] Only in the 1990s, accumulating evidence indicated that a similar process observed during embryogenesis was also associated with cancer progression.[10] The recognition of mesenchymal shift in GBM was more recent, but was crucial to the understanding that the mechanisms that promote EMT in cancer also may be of great relevance in GBM progression.[11,12]

The signals that trigger EMT in cancer cells include different members of the transforming growth factor (TGF) superfamily, epidermal growth factor (EGF), fibroblast growth factor (FGF), hepatocyte growth factor (HGF), and hypoxia-inducible factor (HIF), among others.[13] Furthermore, multiple signaling pathways are involved in EMT process, such as mitogen-activated protein kinase (MAPK), phosphoinositide 3-kinase (PI3K), Akt, SMADs, Wnt/β-catenin, and Ras.[14] Receptor-mediated signaling in response to specific ligands triggers the activation of multiple intracellular effector molecules and transcriptional regulators, such as Snail (Snail1 or SNAI1), Slug (Snail2 or SNAI2), zinc-finger E-box-binding homeobox (ZEB)1/2, and Twist1/2, which in turn, regulate changes in gene-expression patterns that orchestrate EMT. These changes are marked by increased expression of genes normally expressed in mesenchymal cells, such as N-cadherin (CDH2), vimentin, and fibronectin, associated with downregulation of the epithelial cell surface markers and cytoskeleton components such as E-cadherin (CDH1), claudins, occludins, and cytokeratins. Furthermore, other genes involved in motility, proliferation, differentiation, and survival also change their expression during EMT. At the end, cells undergoing EMT lose their apical–basolateral polarization and acquire a fibroblast-like morphology, which, in the case of cancer cells, increases their capacity to migrate, spread, and disseminate to the surrounding tumor tissue or distant sites.[6,10,13,15]

It is well established that the capacity of cancer cells to survive and invade depends, at least partially, on the acquisition of mesenchymal characteristics. However, taking into account that

secondary tumors or metastasis histopathologically resemble primary tumors, from which they are derived, it seems evident that the capacity of tumor cells to form metastasis depends on their ability to revert back to an epithelial phenotype, which is known as mesenchymal-to-epithelial transition (MET) and is crucial to the conclusion of metastasis.[10,15]

Although EMT suggests trans-differentiation, "EMT-like" processes can vary from an intermediate phenotype to a phenotype that is less epithelial and more mesenchymal. Then, cancer cells do not need to undergo a full conversion to a mesenchymal phenotype to reach the morphologic and phenotypic changes necessary for metastasis. Notwithstanding, shifts between epithelial and mesenchymal phenotypes can occur many times along the process of cancer progression, suggesting that EMT is not an "all or nothing" event.[16–19] Given these observations, EMT that was initially described as "epithelial-to-mesenchymal transformation" has been defined by "epithelial-to-mesenchymal transition," reflecting its transient nature.[20]

In this systematic review, we provide an overview from the literature and transcriptomic databases to better understand the recent findings that reinforce the importance of considering the epithelial and mesenchymal characteristics and their dynamic transitions in glioma biology. We also summarize how the current knowledge available in the scientific literature might be useful to design novel therapeutic strategies, by modulating these characteristics to reduce tumor growth and recurrence.

This study describes a "literature overview" about EMT in glioblastoma (Supporting Information Fig. 1) as well as an investigation based on transcriptomic databases, whose details are provided in the Supporting Information. The main findings of the works selected are provided in Tables I–VIII.

2. THE EMT-LIKE PROCESS IN NORMAL GLIAL CELLS

Epithelial cells form close contacts with each other and an apicobasal organization through adherens junctions, desmosomes, and tight junctions (TJs) at lateral surfaces. In addition, the epithelial cell layer keeps overall communication through gap junctional complexes, remaining separated from adjacent tissues by a basal membrane.[21,22] In contrast, brain tissue does not have this morphological organization and brain tumors, as well as several other types of tumors, form a very disorganized and aberrant mass of cells, which contributes to further accentuate the differences when compared with normal epithelial cells.

Astrocytes are polarized cells that regulate the metabolic state of the brain. These cells express multiple cell adhesion molecules (CAMs) and ECM receptors to interact with a variety of surrounding molecules and cells, with which they communicate through GAP junctions. This interconnected position of astrocytes makes them perfectly positioned to respond to multiple signals in their microenvironment. For example, astrocytes respond to all forms of central nervous system (CNS) insults through a process known as reactive astrogliosis, which has become a pathological hallmark of CNS structural lesions.[23,24] Astrogliosis changes the molecular expression and morphology of astrocytes, causing scar formation. This fills the space that results from neuronal loss, influencing the wound healing and neuronal regeneration processes following brain injury.[25]

The process of astrogliosis shares some similarities with EMT. For example, TGFβ1 has been shown to be increased in reactive astrocytes after CNS wounds.[25] Besides, this factor may attract fibroblasts into the lesion site, regulate the deposition of ECM proteins and synthesis of matrix metalloproteinases (MMPs), and control angiogenesis in the scar.[26] In addition, many genes and molecules implicated in EMT are upregulated during glial scar formation, such as CD44, matricellular proteins, IL-1, IL-6, IL-8, FGF, IGF, EGF, vimentin, nestin, and MMP9,

Table I. EMT Transcription Factors

Reference	Cell type	*In vivo* or *in vitro*	EMT-related molecules/ genes	Importance in GBM
Han et al.[55]	U87 and GBM05 cell lines	*In vitro*	Snail1	Promotes cell proliferation, migration, and invasion
Myung et al.[56]	KNS42, U87 and U373 cell lines	*In vitro*	Snail1	Promotes cell proliferation, migration, and invasion; enhanced EMT markers expression
Yang et al.[57]	Human tissue samples of GBM and U87, U251, U343, and T98 cell lines	*In vitro* and *in vivo*	Slug/Snail2	*In vitro*: promotes cell proliferation, migration, and invasion; *in vivo*: angiogenesis and tumor growth
Siebzehnrubl et al.[61]	Human tissue samples and hGMB L0, L1, and L2 cell lines	*In vitro* and *in vivo*	ZEB1 and E-N-cadherin	Promotes invasion and chemoresistance
Qi et al.[62]	Human GBM samples and cell lines U87 and U251	*In vitro*	ZEB2	Overexpressed in human GBM samples; promotes cell proliferation, migration, invasion and inhibits cell apoptosis in glioma cells
Mikheeva et al.[65]	Human tissue samples; human primary GBM cancer-initiated cells (GBM4, GBM6); and T98G, SNB19, SF767, and U87 cell lines	*In vitro* and *in vivo*	Twist1 and E-N-cadherin	Promotes EMT markers expression and invasion
Camand et al.[91]	Human GBM and U138 and U373 cell lines	*In vitro*	N-cadherin	Induces reduced invasive cell properties

EMT, epithelial-to-mesenchymal transition; GBM, glioblastoma.

besides numerous signaling pathways (Stat3, NF-κB, MAPK, PI3k/Akt).[25,27] Most of these factors can contribute to the formation of glial scar and facilitate astrocyte migration.[25,28–31]

In face of these results and keeping in mind that EMT occurs during wound healing in some epithelial tissues, it would be possible to suspect that a process similar to EMT could also be involved in glial scar formation; however, the literature is not very informative regarding the

Table II. Master Signaling Pathways

Reference	Cell type	*In vivo* or *in vitro*	EMT-related molecules/ genes	Importance in GBM	Agents	Mechanism of action	Effect and efficacy
Lv et al.[72]	U87 cell line	*In vitro*	SDF1/CXCR4	Promotes EMT markers expression, proliferation, and migration	–	–	–
Zhu et al.[73]	U87 cell line	*In vitro*	CXCR4	Promotes invasion and adhesion; stimulates expression of EMT markers	–	–	–
Liao et al.[74]	U251 cell line	*In vitro*	SDF1/CXCR4, Survivin	Cell cycle progression; induces expression of EMT markers	–	–	–
Kahlert et al.[77]	Primary tumor-derived cells; NCH421k, NCH644, HSR-GBM1; U87 cell lines; and GBM human tissue samples	*In vitro*	WNT/beta-catenin	Promotes cell migration and invasion; induces EMT markers expression	–	–	–

Continued

Table II. Continued

Reference	Cell type	*In vivo* or *in vitro*	EMT-related molecules/ genes	Importance in GBM	Agents	Mechanism of action	Effect and efficacy
Jin et al.[78]	U87 cell line	*In vitro* and *in vivo*	Frizzled 4	Promotes expression of EMT markers; induces formation of neurosphere and invasiveness	–	–	–
Lu et al.[80]	Murine VEGFKO cell line and primary human GBM cells (GBM43, SF7996, and SF8161)	*In vitro* and *in vivo*	HGF/cMET	Enhances EMT markers expression; promotes cell invasion	–	–	–
Chi et al.[81]	–	*In vivo* (case report)	HGF/cMET	–	Crizotinib	Targets cMET receptor	Improvement of prognosis of patient with GBM
Talasila et al.[82]	Human GBM and U87 cell line	*In vitro* and *in vivo*	EGFR	Enhances EMT markers expression	–	–	–

EMT, epithelial-to-mesenchymal transition; GBM, glioblastoma; SDF1, stromal-derived factor 1α; HGF, hepatocyte growth factor; EGFR, epidermal growth factor receptor.

Table III. Other molecules involved in EMT in GBM

Reference	Cell type	*In vivo* or *in vitro*	EMT-related molecules/ genes	Importance in GBM
Tao et al.[95]	Human tissue samples of GBM and U87 and U251 cell lines	*In vitro*	TPM3	Promotes cell migration and invasion; induces expression of EMT markers
Heldring et al.[96]	U87 cell line	*In vitro* and *in vivo*	NCoR	Inhibits invasion, growth capacity, and EMT markers expression in nonadherent tumor cells
Guo et al.[98]	U87 cell line	*In vitro*	ID1	Promotes cell proliferation, migration, and inhibit adhesion; induces EMT markers expression
Perez-Pinera et al.[99]	U373 cell line	*In vitro*	Pleiotrophin (PTN)	Promotes cell adhesion, cytosqueleton reorganization, and EMT
Mikheev et al.[100]	Human GBM samples	*In vitro*	Periostin	Induces cell invasion, migration, and adhesion; correlated with tumor grade and recurrence
Lee et al.[106]	Human GBM samples and human glioma cell lines U118, U87, U343, U251	*In vitro* and *in vivo*	KITENIN	Overexpressed in human GBM samples; promotes invasion and migration; associated with decreased survival; induces expression of EMT markers
Lin et al.[109]	Human GBM samples and U87 cell line	*In vitro* and *in vivo*	H3R	Upregulated in GBM and glioma cell lines; promotes proliferation, invasiveness, and the expression of EMT markers

TPM3, tropomyosin 3; ID1, DNA binding 1 protein; PTN, pleiotrophin; H3R, histamine receptor 3; NCoR, nuclear receptor corepressor; EMT, epithelial-to-mesenchymal transition; GBM, glioblastoma.

Table IV. Circulating tumor cells, cancer stem cells markers, and stem cells in EMT

Reference	Cell type	*In vivo* or *in vitro*	EMT-related molecules/ genes	Importance in GBM
Sullivan et al.[113]	Human GBM cells	*In vitro*	Several EMT-related genes	Circulating GBM cells have mesenchymal phenotype, are invasive, and may invade outside the brain
Zarkoob et al.[127]	GBM human tissue samples	*In vitro*	CD133	Correlation with mesenchymal subtype of GBM
Cheng et al.[128]	Human tissue samples	*In vitro*	CD44	Related with EMT gene signature
Kim et al.[129]	GBM human tissue samples	*In vitro*	CD44	Related with tumor invasiveness and poor prognosis
Nevo et al.[130]	0923 and 1228A1 human glioma stem cell lines	*In vitro*	OLIG2	Induces EMT markers expression
Velpula et al.[64]	Human tissue samples and U87MG, U251 cell lines, and xenografts (4910 and 5310)	*In vitro* and *in vivo*	Sox2 and Twist1	Inhibited by human umbilical cord blood derived-MSC; promotes EMT markers expression, migration, and invasion;
Iser et al.[131]	C6 cell line	*In vitro*	Vimentin, MMP2 and NRAS	ADSC-CM promotes EMT-like process in GBM cell line

EMT, epithelial-to-mesenchymal transition; GBM, glioblastoma; OLIG2, oligodendrocyte transcription factor.

connection of these two processes in adult normal astrocytes, neither only the EMT process in astrocytes.

3. THE EPITHELIAL AND MESENCHYMAL CHARACTERISTICS OF CELL OF ORIGIN OF GLIOMAS AND THEIR DYNAMICS DURING GLIOMAGENESIS

The investigations of EMT in GBM are relatively recent and only a limited amount of information is available about the differences and peculiarities between the classical EMT that occurs in epithelial tumors and the glial–mesenchymal changes that occur in high-grade gliomas. Candidate cells for originating gliomas are many, and the forerunners are the stem cells that naturally occur in the adult brain, neural stem cells (NSCs), and oligodendrocyte precursor cells (OPCs),[32,33] although more differentiated cells are not completely excluded.[34] Glial cells

Table V. miRNA and EMT

Reference	Cell type	*In vivo* or *in vitro*	EMT-related molecules/ genes	Importance in GBM
Serna et al.[142]	Human GBM samples	*In vitro*	miR-200c	Downregulated in tumors with high level of EGFR
Yan et al.[149]	Human GBM samples and cell lines U87MG and U251	*In vitro*	miR-10a	Overexpressed in GBM; promotes cell migration and invasion
Rathod et al.[150]	Human GBM samples and glioma stem cell lines HNGC-2 and NSG-K16	*In vitro* and *in vivo*	miR-34a	Downregulated in GBM; induces decreased proliferative and migratory potential; induces cell cycle arrest and apoptosis
Liao et al.[151]	U87, U251, U87AR and U251AR cell lines	*In vitro* and *in vivo*	miR-203	Inhibits slug; reverses EMT; and sensitizes cells to chemotherapy
Wang et al.[144] and He et al.[146]	Human GBM samples and cell lines	*In vitro* and *in vivo*	miR-181	Downregulated in GBM; inhibits EMT-markers, cell invasion and proliferation
Zhao et al.[145]	Human GBM samples and U87, U251, andA172 cell lines	*In vitro*	miR-154	Downregulated in GBM; inhibits EMT-markers, cell invasion and migration
Pang et al.[143]	Human GBM samples and U87MG andA172 cell lines	*In vitro*	miR-590-3p	Downregulated in GBM; inhibits EMT-markers, cell invasion and migration
Cai et al.[148]	Human GBM samples and U87 and U251 cell lines	*In vitro*	miR-124	Downregulated in GBM; inhibits proliferation, invasion, and migration
Li et al.[147]	A172 and U87 cell lines	*In vitro*	miR-663	Downregulated in GBM; inhibits EMT-markers, cell invasion and migration

EMT, epithelial-to-mesenchymal transition; GBM, glioblastoma; micro-RNA, miR; EGFR, epidermal growth factor receptor.

Table VI. Hypoxia and EMT

Reference	Cell type	*In vivo* or *in vitro*	EMT-related molecules/genes	Importance in GBM
Joseph et al.[160]	U87, U251, and SNB75 cell lines	*In vitro*	HIF-1α and ZEB1	Mediate increased invasion and EMT markers expression during hypoxia
Brown et al.[161]	Human tissue samples of GBM	*In vitro*	HIF-1 alpha, c-Met, CD133 e CD44	Hypoxia induces EMT/steamness characteristics
Xu et al.[162]	Human GBM samples, U87 and U251 human cell lines, and C6 rat cell line	*In vitro*	HIF1α and HIF2α	Gene markers of hypoxia and EMT were upregulated in bevacizumab treated tumors; hypoxia promotes cell proliferation, expression of EMT genes, and cell migration

EMT, epithelial-to-mesenchymal transition; GBM, glioblastoma; HIF-1, hypoxia-inducible factor.

are developmentally derived from the neuroepithelial lineage, thus presenting a behavior that differs from classical epithelial cells and therefore gliomas do not undergo type 3 EMT during tumorigenesis. Nonetheless, gliomas adopt a phenotype that could be considered mesenchymal under several aspects and therefore the term "glial-to-mesenchymal transition (GMT)" or EMT-like process has been proposed.[35]

In order to position the glial normal cells and gliomas in an epithelial/mesenchymal transcriptional scale, we developed a mesenchymal and epithelial metasignature based on TGFβ-induced EMT in lung adenocarcinoma cells and immortalized bronchial epithelial cells (Supporting Information Fig. 2A–C). As expected, normal epithelial cells and lung adenocarcinoma cells moved to a more mesenchymal phenotype if treated with TGFβ (Fig. 1, left). The majority of cell types from mouse normal brain (available in: http://web.stanford.edu/group/barres_lab/brain_rnaseq.html)[36] are highly mesenchymal, except OPCs, which are somewhat more epithelial, but still considerably below normal and tumoral epithelial cells (Fig. 1, middle). In this scale, most gliomas are positioned in the range of epithelial cells treated with TGFβ, supporting a more mesenchymal phenotype with only a few cases of tumors with a more epithelial phenotype (Fig. 1, right). The most commonly used glioma cell lines also present a predominant mesenchymal signature, reflecting, to some extent, the epithelial and mesenchymal translational signatures found in GBM tumors (Supporting Information Fig. 3).

Taken together, the data above highlight that glial cells are a very particular type of cells with very specialized functions that in many aspects are broadly different from epithelial cells, despite their neuroectodermal developmental origin. Thus, cells of origin do not undergo a significant modification toward a more mesenchymal phenotype during gliomagenesis either maintaining their mesenchymal-like features or even gaining some epithelial characteristics.

Table VII. EMT in therapy resistance

Reference	Cell type	*In vivo* or *in vitro*	EMT-related molecules/ genes	Importance in GBM
Chockalingam et al.[165]	U87 cell line	*In vitro*	MCSF (CSF-1)	Induces EMT markers expression and drug resistance
Yan et al.[166]	Human GMB cell lines SWOZ1, SWOZ2 and BCNU resistant-SWOZ2	*In vitro*	E-cadherin, vimentin, and MMP-9	BCNU-resistant cells undergo EMT; induces enhanced migratory and metastatic potential
Piao et al.[163]	U87- bevacizumab resistant	*In vitro* and *in vivo*	Several EMT-related genes	Enhanced EMT markers expression and genes associated with inflammation; enhanced cell migration and invasion
Meng et al.[167]	Human tissue samples	*In vitro*	Several EMT-related genes	Enhanced EMT markers expression in radioresistant phenotypes
Kubelt et al.[168]	Human GBM samples and T98G cell line	*In vitro*	Several EMT-related genes	Temozolamide induces expression of EMT markers in GBM cell line
Mahabir et al.[35]	Human tissue samples and T98G and KMG4 cell lines	*In vitro* and *in vivo*	Snail1	Enhanced by irradiation; promotes migration, invasion and EMT markers expression

EMT, epithelial-to-mesenchymal transition; GBM, glioblastoma; MCSF, macrophage colony stimulating factor; BCNU, carmustine; MMP-9, matrix metallopeptidase 9.

4. THE PLASTICITY OF EPITHELIAL AND MESENCHYMAL PHENOTYPES OF GLIOMAS

Gliomas, as any other type of cancer, present high plasticity, which includes the alteration of their epithelial and mesenchymal phenotypes, that is, EMT-like and MET-like processes. Tumor cells from the invasive front, for example, in general execute a program of invasion marked by detachment of tumor mass, adhesion to the extracellular matrix (ECM) via specific receptors, degradation of ECM components, and at the end, spreading in a nondelineated form and invasion into the brain tissue. This is not a metastatic process, but may result in a widespread dissemination.[37] In addition, during tumor spreading, both epithelial cancer cells and glioma cells are capable of crossing barriers. While epithelial tumor cells detach and spread through basement membrane (BM), that is a special type of ECM composed mainly by laminin, proteoglycans, and collagen IV, glioma cells invade the ECM, which is a complex mixture of glycosaminoglycans, laminin, fibronectin, tenascin, nidogen, fibrillar collagens, and elastin surrounding tumor cells.[38,39]

Table VIII. Targeting EMT pathway in GBM

Reference	Cell type	*In vivo* or *in vitro*	EMT-related molecules/ genes	Importance in GBM	Agents	Mechanism of action	Effect and efficacy
Shi et al.[169]	U87 and LN229 glioblastoma cells line	*In vitro* and *in vivo*	miR-21	Overexpressed in GBM	AC1MMYR2	Blocked dicer enzyme and upregulate PTEN, PDCD4, and RECK	Induces E-cadherin expression and inhibits mesenchymal markers; suppresses proliferation, survival, and invasion
Ren et al.[170]	U87 cell line	*In vitro* and *in vivo*	miR-21/CDK5	–	AC1MMYR2	Targeting CDK5/RAP1, CDK5 activator P39 AND pFAK	Treatment of AC1MMYR2 combined with taxol suppresses tumor migration and invasion ability; inhibits EMT markers expression
Zhang et al.[171]	U87 and U251 cell lines	*In vitro*	–	–	FTY720	Targeting PI3K/AKT/ mTOR/p70S6K signaling pathway	Inhibits cell migration, invasion and EMT markers expression
Lin et al.[172]	GBM8401 cell line	*In vitro*	–	–	Osthole	Suppresses PI3K/ Akt pathway	Inhibits IGF-1-induced cell migration and EMT

Continued

Table VIII. Continued

Reference	Cell type	*In vivo* or *in vitro*	EMT-related molecules/ genes	Importance in GBM	Agents	Mechanism of action	Effect and efficacy
Guo et al.[173]	U87 cell line and human GBM samples	*In vitro*	–	–	Oleanolic acid	Suppresses MAPK/ERK pathway	Inhibits tumor migration and invasion
Fu [174]	Human GBM samples	*In vitro*	–	–	NPV-LDE-225 (Erismod-egib)	Suppresses SHH signaling pathway	Inhibits EMT, cell motility, invasion, and migration of GBM cells; induces apoptosis
Joo et al.[175]	U87MG cell line	–	–	–	Honokiol	Regulates adhesion by inhibiting VCAM-1 and inhibit EMT by downregulation of Snail, β-catenin, and N-cadherin and upregulation of E-cadherin	Inhibits adhesion to BMECs cells and invasion
Tian et al.[176]	Human U251 and U87 cell lines	*In vitro* and *in vivo*	ZEB1 and Vimentin	–	125I seed irradiation	Activation of ROS-mediated signaling pathway	Inhibits cell migration and invasion; inhibit EMT markers expression
Song et al.[178]	U87MG cell line	*In vitro* and *in vivo*	–	–	Sj170 peptide	Increased Snail expression	Induces tumor growth *in vivo*, cell proliferation and migration *in vitro*; induces EMT markers expression

EMT, epithelial-to-mesenchymal transition; GBM, glioblastoma; PTEN, phosphatase and tensin homolog; PDCD4, programmed cell death protein 4; RECK, reversion-inducing-cysteine-rich protein; CDK5, cell division protein kinase 5; MAPK, mitogen-activated protein kinases; SHH, sonic hedgehog.

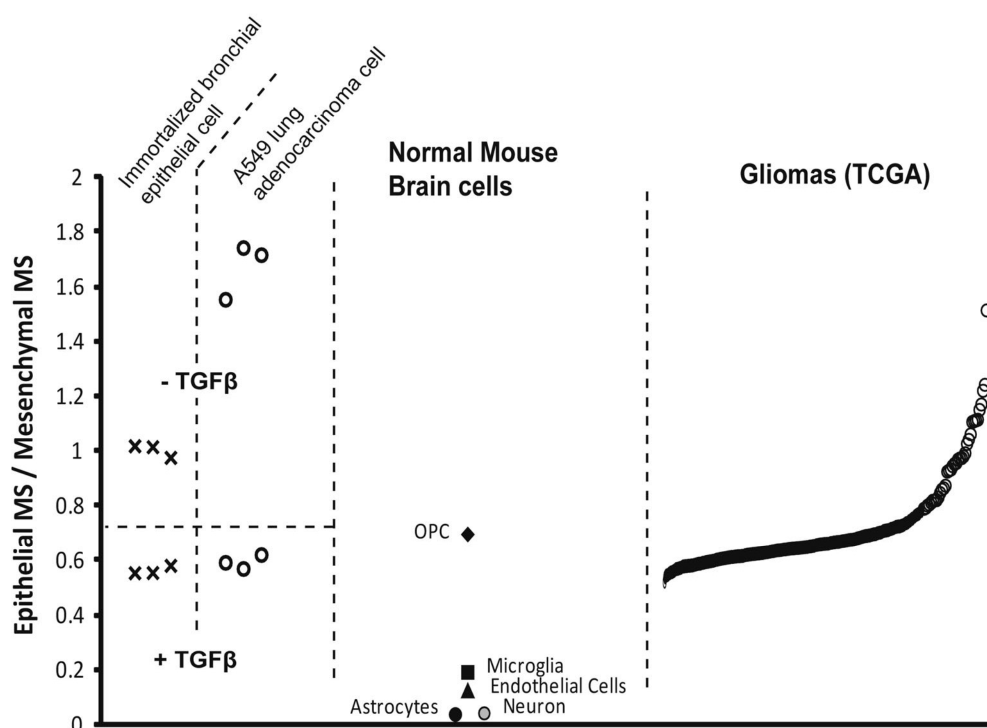

Figure 1. Epithelial and mesenchymal transcriptomic characteristics of the glioblastomas in comparison with glial cells and classical epithelial tumors. Ratio of epithelial to mesenchymal metasignature (MS) of normal and tumoral epithelial cells untreated and treated with TGFβ (left), normal mouse brain cells (middle) and gliomas from the TCGA (right).

The broad expression of CAMs is a known characteristic of epithelial cells[37,40] and, interestingly, malignant astrocytomas express different isoforms of neural CAM (NCAM), which is correlated with reduced migration and invasion of glioma cells *in vitro* as well as *in vivo*[37] However, despite this evidence, the functional roles of cell–cell adhesion molecules in glioma biology are complex and their ability to induce or inhibit tumor invasion is dependent of many factors, such as posttranslational modifications, interactions with specific ligands, and microenvironment stimuli.[41]

Claudins are essential proteins in the formation of TJs in epithelial and endothelial cells. In a similar way of epithelial cells, the expression of claudin-1 and claudin-5 has been reported in human GBM samples and their decreased expression is correlated with higher tumor grade.[42–44] These observations are important, because in cancer cells, during EMT, some transcription factors, such as Snail, are able to repress claudin expression, which helps drive Snail1-induced EMT.[45]

Furthermore, it has been demonstrated that glioma cells possess functional gap junctions that can induce proliferation, uncontrolled migration, and invasion.[46] In addition, this gap junctional link favors the communication among glioma cells, normal astrocytes, and hMSCs.[47,48]

The findings presented above give us an idea that epithelial and mesenchymal characteristics are very plastic in GBM cells and these tumors may transit in a broad phenotype spectrum during tumorigenesis and/or tumor progression.

5. MOLECULAR MECHANISMS OF EMT-LIKE PROCESS IN GBM

Collaboration between different transcription factors is a hallmark of EMT induction. These factors, which are represented mainly by ZEB1, ZEB2, Twist, Snail1 and Snail2/Slug, regulate the expression of a variety of genes, repressing the epithelial and promoting the mesenchymal phenotype.[10] These various signal networks are discussed in more detail below and in Figure 2.

A. EMT Transcription Factors

1. Snail1 (or SNAI1)/Slug (or Snail2/SNAI2)

Snails are well-known transcriptional repressors closely involved in EMT by inhibiting epithelial-related genes upon binding to E-box DNA sequences through their carboxy-terminal zinc-finger domains. Because of their central importance in promoting EMT, Snails expression is controlled by multiple signaling pathways, such as Wnt, TGFβ, HIF-1α, and PI3K/Akt.[45,49]

In addition to transcriptional regulation, Snails are tightly regulated by posttranslational modifications that control their nuclear localization or degradation. This control is mediated by glycogen synthase kinase-3β (GSK3β) that promotes Snail phosphorylation, directing Snail to ubiquitination and proteolytic destruction, thus preventing its nuclear accumulation and inhibiting its ability to activate EMT. Mutation in GSK3β phosphorylation sites or activation of specific signaling pathways, such as TGFβ/SMAD, PI3K/Akt, MAPK, and Wnt, prevent Snail phosphorylation and degradation, then increasing the half-life of the Snail protein, ensuring that it stays constitutively nuclear, thus facilitating EMT.[50–54]

In human GBM cell lines, the silencing of Snail1 (*SNAI1*) by siRNA reduced the proliferation, invasion, and migration of GBM cells *in vitro* in part through decreased expression of vimentin and increased expression of E-cadherin.[55,56] Slug (Snail2/*SNAI2*) is also involved in malignancy processes, since this gene is overexpressed in primary specimens of human GBM, and its expression correlates with invasive phenotype and tumor grade in patients with GBM.[57] In an *in vivo* model, in which U251 GBM cell line overexpressing Slug was injected into the flanks of nude mice, it was observed that tumors with a higher expression of Slug showed a significantly increased growth rate and vascular proliferation, when compared with controls, supporting the notion that a Slug/Snail2-induced mesenchymal phenotypes leads to a more aggressive tumor phenotype.[57]

2. ZEB1/ZEB2

ZEB1 and ZEB2 are transcription factors containing two zinc-finger domains that are highly conserved. They bind to E-boxes in regulatory regions, contributing to repression of epithelial genes as well as activation of mesenchymal genes, such as collagens, smooth muscle actin, and vimentin.[58] ZEB expression is induced in response to TGFβ and Wnt proteins, and growth factors that activate RAS-MAPK signaling. Furthermore, ZEB proteins have emerged in oncology as factors with special roles in carcinogenesis, tumor invasiveness, and drug resistance.[10,59,60] The transcription factor ZEB1 has important function in GBM progression, acting as a protumoral factor and is inversely correlated with survival in GBM patients.[61] When ZEB1 knockdown GBM cell lines were inoculated in brain of mice, the tumors formed are less invasive when compared with cells expressing ZEB1. In this same work, ZEB1-underexpressing cells showed increased sensibility to temozolomide (TMZ), the standard drug use to treat GBM.[61]

ZEB2 is also related with EMT in GBM. The ZEB2 expression analysis in human GBM samples showed that high levels of this factor are correlated with fast tumor progression in GBM patients. In *in vitro* analysis, ZEB2 downregulation using siRNA enhanced E-cadherin expression, whereas it inhibited β-catenin, vimentin, N-cadherin and Snail, and well-known

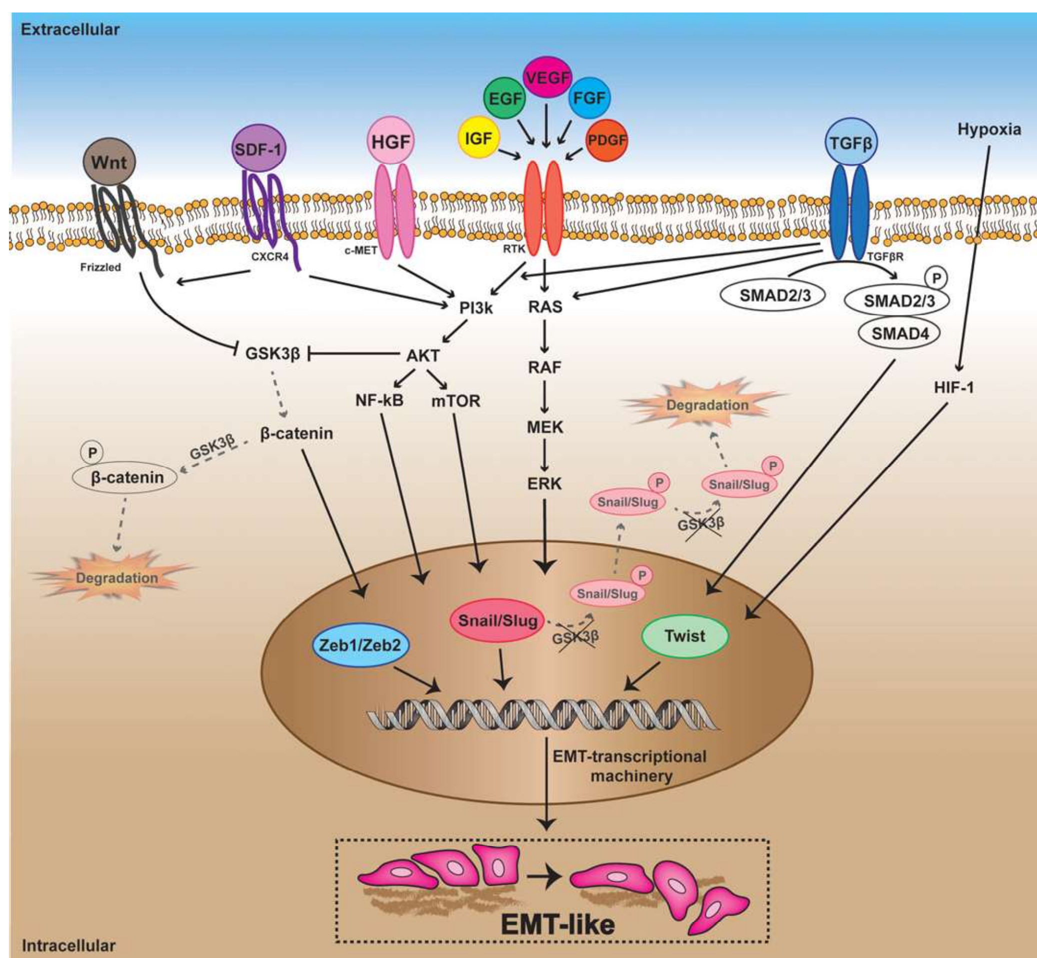

Figure 2. Summary of signaling pathways involved with EMT in GBM. Several growth factors can induce EMT through receptor tyrosine kinases (RTKs), including EGF, FGF, HGF, IGF, PDGF, and VEGF. The pathway RAS-RAF-MEK-ERK (also known as MAPK) is the major signaling cascade that can be activated by RTKs in response to growth factors. Once activated, this pathway induces EMT by increasing the expression of EMT transcription factors and regulates cell motility and invasion. In normal conditions, β-catenin present into the cytoplasmic compartment is readily phosphorylated (P) by glycogen synthase kinase 3 beta (GSK3β), resulting in its degradation by the ubiquitin-proteasome pathway. However, in many types of cancer, GSK3β is mutated or inhibited by signaling pathways such as PI3K/AKT and Wnt. Thus, β-catenin accumulates and is translocated to the nucleus, where it forms a complex with lymphoid enhancer-binding factor 1 and T-cell factor to regulate transcription factors, promoting EMT. Snail activities, localization, and stability are also regulated by posttranslational modifications. GSK3β phosphorylates (P) Snail1, which is exported from the nucleus and again phosphorylated after their subsequent degradation. Besides inhibiting GSK3β, the PI3K/Akt pathway (which can be activated by RTK receptors, such as CXCR4 or c-MET) can also activate mechanistic target of rapamycin (mTOR) or NF$\kappa\beta$, thus leading to transition from epithelial to mesenchymal phenotype. TGFβ induces EMT through SMAD-mediated and non-SMAD signaling. In SMAD-mediated signaling TGFβ signals through a complex of type I and type II receptors (TβRI and TβRII) to activate SMAD2 and SMAD3, which then combine with SMAD4. The trimeric SMAD complex translocates into the nucleus and cooperates with transcription factors, increasing their activity as repressors or activators of EMT-related genes, as well as activating their expression. TGFβ can also contribute to EMT to induce non-SMAD signaling pathways through activation of PI3k/Akt and MAPK signaling pathways. Finally, the rich hypoxia tumor microenvironment can also promote EMT in glioma cells through induction of hypoxia-inducing factor (HIF-1), which activates the expression of transcription factors Twist and Zeb. Broadly, all these signaling pathways act in cooperation with the main EMT transcription factors and at the end, induce the necessary cellular changes to allowing cancer cells undergo EMT or EMT-like process.

EMT markers and lead to a suppression of cell proliferation, migration, and invasion, as well as induced cell death in glioma cell lines U87 and U251.[62]

3. Twist

Essential transcription factors in EMT include Twist1 and Twist2, the basic helix-loop-helix transcription factors, whose domain mediates their binding to target genes in DNA, promoting or repressing transcription of several genes and leading to induction of mesenchymal phenotype in epithelial cells.[63] Underexpression of Twist1 inhibits EMT by reducing the expression of N-cadherin, vimentin, β-catenin, and Sox2 (an NSC marker). Similarly, knockdown of Sox2 decreased Twist1 expression, suggesting that both Twist1 and Sox2 are necessary to maintain the stemness in $CD133^+$ glioma stem cells (GSCs), obtained from U251 and U87 cell lines, or xenografts 4910 and 5310 cells (see below the "Cancer stem cells markers" section where the link between cancer stem cells and EMT in malignant gliomas is described) and induce EMT.[64]

The overexpression of Twist1 promoted invasion of SNB19 and T98G GBM cell lines in SCID-NOD mice brain. Besides, these cells showed enhanced migratory capacity and reorganization of actin cytoskeleton, consistent with process of glioma invasion. Moreover, upregulation of Twist1 is consistent with enhanced expression of EMT-related genes, including FN1 (fibronectin 1), POSTN (perostin), MMP2, Slug, and HGF.[65]

B. Master Signaling Pathways Driving EMT in GBM

1. TGFβ

TGFβ signaling pathway is well known for its growth inhibitory effect in normal cells and early carcinomas, being considered a tumor suppressor factor. The tumor suppressive role or cytostatic effect of TGFβ has been well described in multiple target tissues. However, different types of human tumors are resistant to the TGFβ cytostatic effects, due to genetic and epigenetic modifications in components of TGFβ pathway, or to the activation of pro-oncogenes.[66]

Two signaling pathways have been identified as mediators of TGFβ effects. The first involves Smad proteins whereas the second is Smad-independent.[66] The Smad-dependent signaling occurs when TGFβ binds on type I (TβRI or TGFR1) and type II (TβRII or TGFR2) receptors to activate Smad2 and Smad3, which then combine with Smad4 within the cytoplasm. The trimeric Smad complex translocates into the nucleus where it acts as a DNA site-specific transcriptional regulator cooperating in the repression (i.e., E-cadherin, occludin) or activation (i.e., Snail, Zeb, Twist, fibronectin, and vimentin) of target genes. Interestingly, the signaling triggered by TGFβ not only activates the expression of transcription factors essential for EMT, but also cooperates with these transcription factors to increase their transcriptional activities. On the other hand, the Smad-independent signaling pathway induced by TGFβ leads to activation of PI3K-Akt-mTOR1 or MAPK signaling.[49,66,67]

Intriguingly, during tumor progression, cancer cells selectively evade the antiproliferative signaling of TGFβ, but at the same time, preserve a functional TGFβ receptor and Smad system, resulting in decreased adhesion capacity, increased migration ability, invasion, and dissemination. However, the exact mechanism that allows cancer cells to avoid the cytostatic signaling of TGFβ is still unknown.[68] Recently, Bruna et al. showed that TGFβ/Smad signaling promotes proliferation via induction of PDGF-B (platelet-derived growth factor subunit B) expression in some glioma cells with an unmethylated PDGF-B gene. Besides, in other types of glioma cells that have the PDGF-B gene promoter methylated, the expression of p-Smad2 is lower. In addition, the tumors formed are less aggressive and proliferative when compared to gliomas lacking the methylated gene. In face of these results, we can deduce that the status of methylation of the PDGF-B gene appears to be critical for the pro-oncogenic role of TGFβ.

This may be one explanation as to why the anti-proliferative response of TGFβ signaling is lost during tumor progression.[68]

In GBM, the cooperation of Smads with co-activators, such as FoxO (Forkhead transcription factors), can be responsible by the cytostatic effect of TGFβ signaling. The cooperation of Smads and FoxO generates a *p21Cip1*-activation dependent of TGFβ. *p21Cip1* is a cyclin-dependent kinase inhibitor; consequently, this network mediates cell cycle arrest at G1. However, the complex FoxO/Smad can be inhibited by FoxG1, a transcription factor with repressor activities. PI3K growth-promoting pathway acts combined with FoxG1 and both mediate the capacity of GBM cells to block tumor suppressor effects of TGFβ.[69] In this cancer type, TGFβ can also be a potent inductor of EMT. The exposure of U87 and U251 GBM cells to this factor induces a mesenchymal transdifferentiation via Smad2 signaling. After treatment with TGFβ, GBM cells underwent morphological changes, followed by enhanced expression of mesenchymal markers, migration, and invasion *in vitro* and *in vivo*. Interestingly, the ZEB1 expression was enhanced in a TGFβ-dependent manner, in contrast with other transcription factors, such as Snail1, Slug/Snail2, and Twist, whose expression was not changed.[70]

2. SDF1/CXCR4

The cytokine CXCL12 (also known as stromal-derived factor 1α; SDF1-α) and its receptor CXCR4 are overexpressed in many cancer types and this abnormal expression is strongly associated with enhanced proliferation, migration, and invasion. Besides, SDF-1/CXCR4 signaling promotes EMT by activating the MEK/ERK, PI3K/Akt, or Wnt/β-catenin pathway.[71]

Lv et al. showed that the treatment of U87 GBM cell line with stromal cell-derived factor (SDF-1) upregulates vimentin, Snail, and N-cadherin and downregulates E-cadherin. In contrast, the repression of CXCR4 by siRNA reduced the expression of these genes related withmesenchymal phenotype. The knockdown of CXCR4 was also able to decrease U87 cell migration and proliferation induced by SDF-1. These results were further substantiated by activation of pathways that play important roles in glioma progression, such as PI3K/Akt and ERK.[72] In a very similar study, Zhu et al. also showed the suppression of EMT in CXCR4 knockdown U87 cells. In this study, besides the EMT-markers analyzed by Lv et al., it was shown that β-catenin, TGF-β1, p-Smad2, and p-Akt and Twist were also downregulated by repression of CXCR4 gene.[73] In U251 GBM cell line, the action of SDF-1/CXCR4 via PI3K/Akt and ERK pathways, besides stimulating EMT, leads to enhanced survivin expression (one of inhibitors of apoptosis proteins) and cell cycle progression.[74]

*3. Wnt/**β**-Catenin Pathway*

The canonical Wnt signaling pathway is mediated by β-catenin. β-Catenin has a dual role in EMT, operating as a component of adherens junctions, linking E-cadherin to the cytoskeleton and also working as a driver of target gene transcription when translocated to the nucleus. In the absence of Wnt, GSK-3β phosphorylates β-catenin and targets it for ubiquitination and degradation, thus maintaining cytoplasmic β-catenin at a low level. The binding of Wnt ligands to Frizzled (FZD) receptors results in the inhibition of GSK-3β, therefore preventing β-catenin phosphorylation and leading to accumulation of its cytoplasmic form that can then translocate to the nucleus and regulate target transcription genes, simultaneously decreasing cadherin-mediated adhesion.[6,49,75] The canonical Wnt/β-catenin pathway was already associated with glioma progression and reduction on overall patient survival, raising the interest about its possible involvement with EMT.[76]

The evaluation of 30 specimens from patients with the aggressive mesenchymal subgroup of GBM (classified by Verhaak et al.—more details in section 7) showed significantly higher canonical Wnt/β-catenin pathway activation (by analysis of nuclear β-catenin distribution) in

invasive front of the tumor. Besides, the activation of Wnt/β-catenin pathway in GBM cell lines increased the expression of EMT-related transcription factors ZEB1, Twist, Snail, and Slug, resulting in significant increased migration/invasion in these cells.[77]

Expression of FZD4 receptor was found enhanced in an established, highly invasive glioma cell line (U87R4). These invasive cells showed mesenchymal-like morphology (via Snail and vimentin induction), glioma stem cell-like features, resistance to apoptosis (evidenced by decreased expression of caspase-3), and resistance to anticancer drug that induce cell death, when compared with a less aggressive cell line (U87L4).[78]

4. Tyrosine Kinase Receptors

The HGF receptor cMET, a tyrosine kinase receptor that acts as a proto-oncogene, activates multiple cellular pathways and promote proliferation, migration, invasion, survival, and poor prognosis in several tumors, including GBM.[79] Lu et al. found that in a model of orthotopically implanted GBM cell line, the animals that received cells in which VEGF expression was silenced developed tumors that were non-angiogenic, but extremely diffuse and invasive, forming clusters along the normal blood vessels into the brain parenchyma. These invading clusters showed strongly positive stain to p-cMET (active receptor), indicating that the loss of VEGF induced cMET activation in invasive tumor cells. As expected, VEGF expression can block HGF/cMET signaling. In addition, the inhibition of VEGF expression and cMET activation in GBM cells induces upregulation of EMT markers Snail and N-cadherin, while it suppresses E-cadherin.[80] These results could explain a case report of a patient with a cMET amplified GBM. This patient was treated with the standard drug TMZ, combined with cediranib, a VEGF receptor tyrosine kinase inhibitor. Interestingly, after five cycles of treatment, the lesion increased and the disease progressed. However, when he was treated with crizotinib, an inhibitor of cMET, a substantial reduction of tumor size was observed; however, these results were not permanent and after five cycles, the disease progressed.[81] These results reinforce that personalized therapy to GBM could be more successful to inhibit tumor progression. So, it seems that cMET could be a good target for new therapies against some subset of GBMs with cMET amplification.

In addition to cMET receptor, other tyrosine kinase receptors might be important for invasion in GBM. For instance, the EGF receptor (EGFR), implicated in cell invasion, proliferation, and angiogenesis, can promote invasive tumor growth by a non-angiogenic mechanism. GBM cells exhibit infiltrative and diffuse growth; however, the cancer invasion not necessarily is accompanied by enhanced angiogenesis. It was shown recently that the activation (pEGFR) of wt-EGFR promoted invasion and infiltration, independent of angiogenesis, both in animal model and human samples of GBMs, whereas loss of its activity resulted in prolonged survival of animals, enhanced epithelial-like phenotype and downregulation of vimentin, snail and twist, evidencing the induction of an MET-like process. Moreover, as high-grade tumor cells exhibit high plasticity, when challenged, EGFR knockdown tumor cells, acquired the ability to induce an angiogenic growth program and escape the invasion block mechanisms.[82]

C. E-to-N-Cadherin Switch

E-cadherin, a type I classical cadherin, is the major cadherin expressed in epithelial tissues and a key component in the formation of cell–cell adherens-type junctions in these tissues, but its expression appears to be limited in both GBM and normal brain samples, including neuronal cells, astrocytes, and oligodendrocytes.[83–87] In most of epithelial carcinomas, EMT is executed by an E-to-N-cadherin switch, marked by the loss of E-cadherin and increased N-cadherin expression. This "cadherin switch" has been accepted as the most widely used marker for EMT in carcinomas.[10]

E-cadherin has been found in a rare subset of GBMs with epithelial or pseudoepithelial differentiation and has been correlated with poorer prognosis compared to patients who do not express E-cadherin. In addition, E-cadherin expression promoted invasiveness when tumor cells were implanted orthotopically in mouse brain. Interestingly, N-cadherin was broadly and stably expresses in GBM samples and cell lines, including GBMs with epithelial or pseudoepithelial differentiation,[83] suggesting that enhanced expression of N-cadherin seems not to be associated with GBM invasiveness, in contrast with what occurs in epithelial tumors.[61,83]

These data provide additional support to the idea that E-cadherin is not limited to a tumor suppressor role in tumor development and either this E-to-N-cadherin switch is not essential for EMT in gliomas or gliomas do not undergo a classical EMT. In addition, the literature findings indicate that during EMT or EMT-like process, epithelial- and mesenchymal-related genes are not simply turned on or off, but probably they are modulated in a less organized fashion, creating different phenotypes that regulate tumor biology. Then, probably, epithelial-like or mesenchymal-like characteristics in GBM involve the regulation of several genes and proteins, not just cadherins.

Despite the role of cadherins in GBM invasiveness to be an intriguing topic, few studies have focused on this issue.[84,85,88] Interestingly, it seems that this is a controversial issue also in tumors of epithelial origin. Previous studies in ovarian carcinoma (typically epithelial tumors) have shown that the expression of E-cadherin in these tumors is responsible for inducing tumor cell growth, invasiveness, and metastases via MEK/ERK pathway.[89,90] Taken together, these results have uncovered the alternative roles for E-cadherin in tumor progression, and could explain the absence of epithelial-mesenchymal cadherin switch in GBM.

Regarding N-cadherin, the hypothesis is that the role of N-cadherin in GBM is not associated with its increased expression, but with its differential distribution in cell membrane, which can alter the tumor cell capacity of adhesion and motility.[61] It has been observed that not only the switch between E- and N-cadherin did not happen in astrocytomas, but also N-cadherin is downregulated in human GBM samples when compared with normal brain and in U138 and U373 GBM cell lines when compared with normal astrocytes. At the end, the decreased N-cadherin expression in glioma cells contributes to a faster and less directed migration when compared with normal astrocytes. In contrast, overexpression of N-cadherin is associated with retrieval of cell polarity and inhibition of migration.[91] Mikheeva et al. supported these results showing that not only GBM cell lines overexpressing Twist1 do not undergo the canonical E-to-N-cadherin switch, but also this "switch" is not required to promote an invasive mesenchymal cellular phenotype in GBM cells.[65] Asano et al. showed the effects of N-cadherin expression on invasion and metastasis *in vitro* and *in vivo*, by overexpressing N-cadherin in the rat C6 glioma cell line, which normally has low levels of N-cadherin. They found that upregulation of N-cadherin resulted in a decreased invasive capacity *in vivo* and *in vitro*.[84] In another study, Shinoura et al. did not find correlation between invasiveness and expression of N-cadherin, in highly invasive GBM tumors, compared with minimally invasive tumors, showing that the levels of N-cadherin expression do not restrict the invasion of GBM.[88]

Siebzehnrubl et al. showed that there are differences in the distributions of N-cadherin on cell membrane, but not in its level of expression in ZEB1 knockdown cells. This differential localization would be responsible by alterations in tumor cell adhesion and invasion capacity. In their work, they showed that ZEB1 knockdown cells presented concentration of N-cadherin to the juxtaposed membranes between adjacent cells, contrasting to the control cells that expressed uniformly N-cadherin on the cell membrane. This re-distribution of N-cadherin could be responsible for greater cell motility of controls.[61] This hypothesis is also supported by Perego et al., who presented data showing that the degree of organization and stability of N-cadherin may be more important for the invasive properties of gliomas than differences in its expression levels.[92]

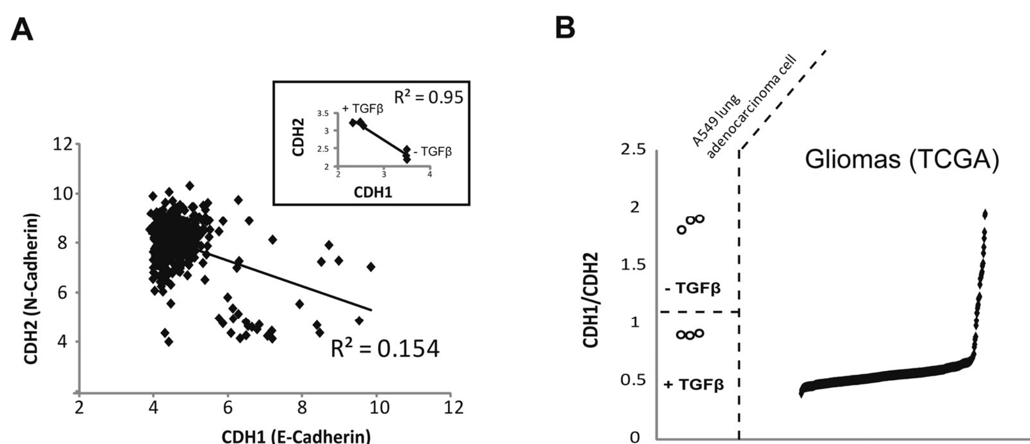

Figure 3. E- and N-cadherin expression in GBMs. (A) Correlation of the expression of CDH1 and CDH2 mRNA in glioblastomas from the TCGA and from lung cancer cells untreated or treated with TGFβ (insert) and (B) ratio of CDH1 and CDH2 expression of the same samples.

As a result of these complex context-specific interactions, the expressions and functions of E- and N-cadherin have been differentially reported for GBM. In face of this, we decided to investigate in transcriptomic databases the existing relation between E-cadherin and N-cadherin in human GBM samples from TCGA, in comparison with an epithelial cancer. We analyzed the relation between E- and N-cadherin in human GBM in relation to human A549 lung adenocarcinoma cell line treated or not with 5 ng/mL of TGFβ for 72 hr as a model of EMT.[93,94] A representative scatter plot (Fig. 3A) illustrates graphically that there is a strong mutually exclusive expression of E- and N-cadherin in lung cancer cells in which EMT was induced by TGFβ (insert), as expected for an epithelial derived-tumor. On the other hand, the scatter plot of GBM samples shows a very weak or nonassociation ($R^2 = 0.15$) between E- and N-cadherin, supporting the lack of a transcriptionally concerted switch between these two genes. The Figure 3B shows the distribution of the E/N cadherin ratio in GBMs, comparing with the ratio in lung cancer treated or not with TGFβ. The majority of GBM samples have a ratio that is even lower that the TGFβ treated lung cancer cells, although in some samples this value reached the level observed in the untreated lung cancer cells.

D. Other Molecules Involved in EMT in GBM

1. Tropomyosin 3 (TPM3)

TPM3, an actin-binding protein, has been reported to act as an oncogene in GBM. Using GBM cell lines, it was showed that the downregulation of TPM3 using siRNA led to a reduction of cell invasion and migration and this effect was associated with decreased expression of MMP-9 and Snail. Additionally, after evaluating data of CGGA (Chinese Glioma Genome Atlas), the same group revealed that patients presenting high levels of TPM3 had a significantly higher mortality, when compared with patients with lower levels. Interestingly, the TPM3 expression is also positively correlated with MMP family genes and EMT-like factors, such as Snail, Slug, Twist1, and Zeb2.[95]

2. NCoR

NCoR is a known transcriptional repressor involved in maintaining the undifferentiated state of NSCs. More recently, it has emerged as an EMT regulator. The knockdown of NCoR in U87

cells promoted an increase in the expression of MMP-7, MMP-12, vimentin, and Twist being compatible with an EMT-like invasive phenotype. In parallel, the downregulation of NCoR modulated autophagy, by enhancing the expression of autophagic genes in GBM cell lines.[96]

3. DNA Binding 1 Protein (ID1)

DNA binding 1 protein (ID1) is overexpressed in many types of cancer and is involved with proliferation, invasiveness, metastasis, and neo-angiogenesis. However, its main role in cancer progression is its capacity to inhibit cell differentiation, maintaining cells in an immature/stem cell-like state and inducing oncogenic transformation.[97] In U87 GBM cell line, ID1 knockdown dramatically reduced cell invasion and proliferation that was accompanied by morphological changes, and robust reduction in expression levels of E-cadherin, β-catenin, c-Myc, and cyclin D1 as well as enhanced capacity of cell adhesion.[98]

4. Pleiotrophin (PTN)

There are many factors well known to stimulate the EMT in cancer cells, such as TGFβ and HGF. However, more recently, new factors that may play the same role have been emerged. For example, the cytokine PTN, when binds to (RPTP)β/ζ receptor (a tyrosine phosphatase receptor) stimulates the degradation and reorganization of cytoskeletal proteins, loss of cell–cell adhesion, and induction of a morphology related to EMT in U373 cells.[99] The suggested mechanism is that the ligation of PTN to (RPTP)β/ζ receptor causes an inhibition of its endogenous protein tyrosine phosphatase activity, allowing the phosphorylation of several proteins by tyrosine kinases that in turn bind at the same sites that would be dephosphorylated by (RPTP)β/ζ when activated.[99]

5. Periostin

Periostin is a secreted matricellular protein important for EMT that correlates with tumor grade and recurrence, and inversely correlates with survival, in human GBM samples. *In vitro* analysis showed that periostin binds to integrins activating the FAK-mediated signaling pathways and leading to increased invasion and adhesion. Periostin knockdown markedly impaired survival of GBM stem-like cells *in vivo*. Besides, TCGA analysis showed that the expression of periostin was increased in mesenchymal and classical GBM subtypes when compared with proneural and neural GBMs.[100]

6. Kitenin

KAI1/CD82, a metastatic suppressor gene, is a member of the transmembrane 4 superfamily (tetraspanin) and is downregulated during the malignant progression of several tumors.[101–103] It was shown that an alternatively spliced variant of KAI1 mRNA (that lacks exon 7 at the COOH-terminal region) loses its activity as metastatic suppressor, when compared with the wild-type KAI1. Then, suggesting that the COOH-terminal region of KAI1 is important for the effects of KAI1 on cell motility and growth.[104]

In 2004, Lee et al. identified a member of tetraspanin proteins, named as "KAI1 COOH-terminal interacting tetraspanin," KITENIN. This protein member when interacts, specifically, with the COOH-terminal region of KAI1 promotes invasion and metastasis.[105] Accordingly, analysis of samples from human GBM tissues and GBM cell lines revealed that KITENIN expression was higher in tumor samples when compared with controls, which translated in poor prognosis. In orthotopic intracranial mouse tumor model, the median survival was significantly shorter in animals transplanted with cells overexpressing KITENIN. In addition, in GBM cells, the expression of KITENIN is strongly associated with expression of N-cadherin, ZEB1, ZEB2,

Snail, and Slug, well-known EMT markers. Finally, the expression of CD133 is also positively associated with KITENIN expression, reinforcing a link between EMT and glioma stemness.[106]

7. Histamine Receptor 3 (H3R)

Histamine exerts many different biological effects in human cells, modulating cell proliferation, embryonic development, and tumor growth. It acts through the activation of specific histamine receptors (H1, H2, H3, and H4) that differ in their tissue expression patterns and functions.[107]

Although many studies regarding the modulatory roles of histamine in tumor progression have been reported, the effect of histamine in development of some types of tumors remains controversial.[108] Interestingly, in samples from high-grade human GBM was detected a higher expression of H3R when compared with low-grade GBM, pointing to a role of histamine receptors in GBM progression. In U87 cells the blockade of H3R by siRNA or the antagonist CPX, decreased the proliferation, migration, and invasion capacity of glioma cells *in vitro*. Similar results were found in an *in vivo* model, in which rats that received U87MG cells transfected with H3R siRNAs presented smaller tumors when compared with controls. Besides, after downregulation of H3R the EMT was inhibited and the expression of Snail, Slug, Twist, vimentin, and N-cadherin was negatively regulated. Not surprisingly, the signaling pathways PI3K/Akt and MEK/ERK play a role in the H3R-mediated EMT progression, leading to a tumor invasion.[109]

E. Circulating Tumor Cells (CTCs)

According to Paget (1989), who first suggested the "seed and soil" theory, the sites where metastases develop do not occur randomly, but the tumor cells (*the seed*) have special affinity for certain organ sites (*the soil*) with a receptive microenvironment. Thus, tumor metastasis is dependent on local properties that are inherent to the environment where the CTCs anchor.[110] Dissemination of cancer cells happens mainly through the bloodstream or lymphatic system. Once cancer cells reach the circulation, the mechanism of EMT will drive epithelial cells to avoid anoikis (anchorage independent cell death). Then, the reverse process of EMT, MET, will govern growth of metastasis in the target tissue.[111,112] Although GBM cells rarely metastasize, circulating brain tumor cells can be detectable within the bloodstream (Fig. 4). In one study that evaluated blood samples of patients with GBM, in 39% of them, CTCs were identified in the blood. Interestingly, CTCs of all patients analyzed showed robust expression of genes related with the aggressive mesenchymal GBM subtype (described by Verhaak et al.), such as *SERPINE1, TGFB1, TGFBR2*, and *VIM*. Furthermore, one case of patient with lymph node and pulmonary GBM metastatic lesions was reported. The blood analysis of this patient revealed a higher number of mesenchymal CTCs subtype, when compared with other subtypes. Similar result was found in metastatic lesions, where mesenchymal phenotype was the GBM cells subtype predominantly observed.[113]

F. Cancer Stem Cells Markers

More than a decade ago, the existence of a small population with both stem cell-like properties and tumor-initiating ability was for the first time identified in acute myeloid leukemia[114] and, subsequently, in several solid tumors.[115–120] These populations with stem cell-like properties were named "cancer stem cells (CSCs)," indicating that only a subset of cancer cells within a tumor possess the ability of self-renewal and multipotency, thus also termed "tumor initiating cells."[121,122]

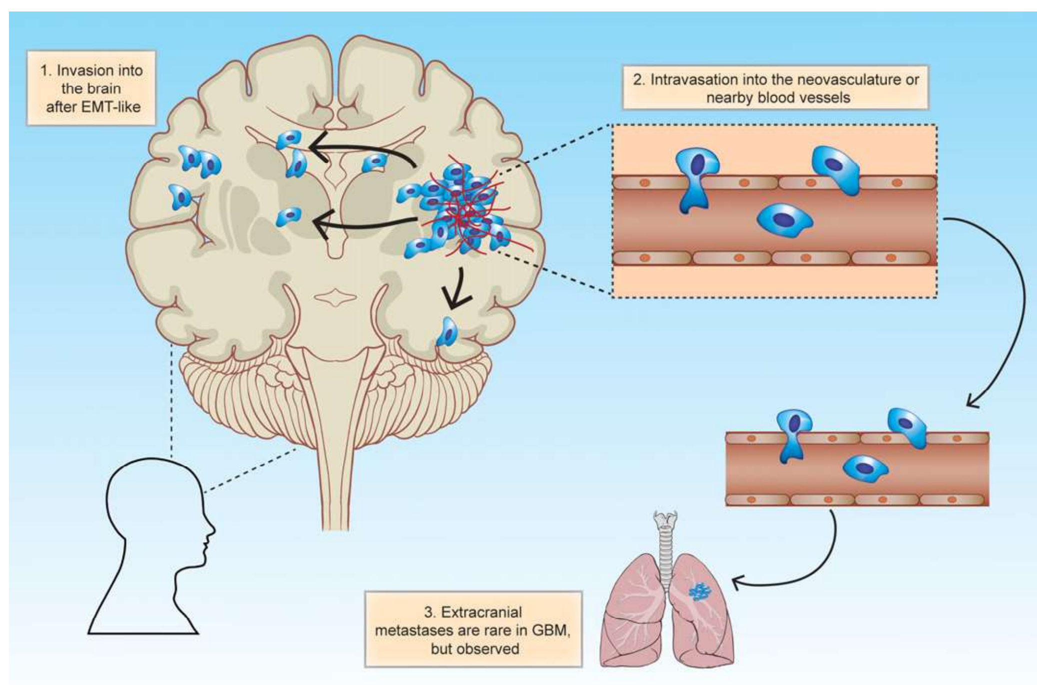

Figure 4. Epithelial and mesenchymal states and their transitions (EMT and MET) affect several aspects of GBM tumors. A more mesenchymal state favors tumor invasions (box 1), tumor intravasion into blood vessels (box 2) whereas a more epithelial phenotype is required for the rare cases in which glioblastoma multiforme (GBM) metastasize (box 3). During malignant transformation, GBM tumor cells undergo an uncontrolled process of proliferation that can culminate in loss of adhesion to their neighboring cells and invasion to the surrounding tissue. GBMs are tumors locally invasive and infiltrative. In spite of this, eventually, tumor cells acquire capacity to spread through the blood circulation, in a process called of intravasation. The events of local invasion and intravasation, as well as in other tumors, are marked by decreased expression of epithelial marker proteins and increased expression of mesenchymal marker proteins, culminating in EMT or EMT-like process. During the course of circulating tumor cells (CTCs) within the bloodstream, some cells may eventually extravasate from the circulation into the surrounding tissue. The process between extravasation and invasion to the stroma of the host organ is marked by a transition of tumor cells from a mesenchymal toward an epithelial phenotype, known as mesenchymal-to-epithelial transition (MET). Metastasis formation is one option that CTCs can follow, but as in GBMs this usually does not occur and probably some of these cells remain quiescent or dormant.

These CSCs are considered the cellular "drivers" of the tumorigenic process in many tumor types. The current knowledge suggests that brain tumor stem cells (BTSCs) are responsible for the highly invasive and resistant potential of many human brain tumors.[123] EMT appears to be involved in the process that leads to the acquisition of stemness by tumor cells. Through this process, cells acquire an invasive phenotype that may contribute to tumor recurrence and metastasis.[124] Therefore, CSCs might promote tumor progression and invasion of tumors by undergoing EMT.[10,125]

In 2010, using data obtained from The Cancer Genome Atlas (TCGA), Verhaak et al. classified GBMs into four genetic subtypes mesenchymal, classical, neural, and pro-neural. They present distinct features, each of them being characterized by the differences in particular genes.[126] It is important to keep in mind that the mesenchymal classification of Verhaak is different from our mesenchymal metasignature that was developed through the comparison between TGFβ-treated and nontreated lung cancer cells. Ever since, many efforts have been made to better describe the particularities of each type of GBM. Zarkoob et al., analyzing an

EMT gene signature, originated from a study using mammary cancer cells induced to undergo an EMT, by expressing Gsc, Snail, Twist, TGF-β1 and knocking down E-cadherin, showed that the mesenchymal subtype of GBM presented the closest correlation with both EMT and CD133 gene signatures. The surface protein CD133 is a marker of stem and tumor-initiating cells in GBM. Its expression together with EMT genes could be associated with invasiveness and poor prognosis of this subpopulation.[127] In other study, it was shown that CD44, a stem marker that is predominantly expressed in mesenchymal subtype of GBM, is strongly related with the EMT signature.[128] In addition, the analysis of data of TCGA indicated that the expression of EMT gene signature and CD44 is directly associated with tumor invasiveness and poor prognosis in patients with GBM after therapy.[128,129]

Nevo et al. showed that invading glioma cells present low expression of EMT markers, whereas OLIG2 (glioma stem cell marker) expression is increased, suggesting that once tumor cells have invaded, its mesenchymal features are attenuated. Interestingly, although invading cells express higher levels of OLIG2 when compared with EMT markers, the downregulation of OLIG2 in GSC line led to a reduced expression of EMT-factors Twist and Slug.[130] In accordance with these results, recently it was shown that knockdown of ZEB1 in GBM cells inhibits the stem cell regulators SOX2, CD133, and OLIG2,[61] reinforcing the close relation between EMT and stemness. These findings support the notion that as soon as tumor cells move away from the tumor area to infiltrate brain parenchyma, tumor stem cells features are regulated in synchrony with epithelial/mesenchymal features and this co-regulation can be important for tumor invasion

G. The Role of Stem Cells in EMT

It is known that stem cells release several cytokines and chemokines capable to modulate cancer progression. Stem cells can apparently regulate EMT in cancer cells as evidenced by the modulation of Twist1, a key EMT-effector, by co-culture of GSCs, a CD133$^+$ enriched population of glioma cell lines with human umbilical cord blood stem cells (hUCBSC). The treatment of GSCs with hUCBSC reduced the invasive potential of GSCs *in vitro* and *in vivo* and decreased N-cadherin, β-catenin, vimentin, Sox2 and Twist1 expression, but increased the E-cadherin levels. In other words, the hUCBSC treatment was able to promote a more epithelial phenotype in the GSCs.[64]

We showed, on the other hand, that conditioned medium (CM) from rat adipose-derived stem cell (ADSC) was able to induce an EMT-like process in C6 glioma cell line, which included an enhanced migration capacity and increased expression of vimentin, MMP-2, and NRAS. CM also promoted a reduction in adhesion and changes in cell morphology that resemble the changes observed with TGFβ.[131]

H. miRNA and EMT

MicroRNAs (miRNA) are noncoding single strands of 18–25 nucleotides that regulate gene expression by targeting mRNAs to degradation and/or repression of translation, leading to partial or full silencing of the protein-coding gene.[132] miRNAs play a key role in diverse biological processes, including development, cell proliferation, differentiation, and apoptosis. This wide range of action is mainly due to the fact that 20∼30% of all human mRNAs are controlled by miRNAs.[133]

In recent years, many studies have reported that miRNAs play a critical role in cancer as well as in EMT. Different miRNA signatures can help to detect several cancers subtypes and can be useful as a prognostic predictor.[134] These miRNAs dynamically impact the regulation of the epithelial–mesenchymal state. In addition, by targeting the cancer-related pathways, such as

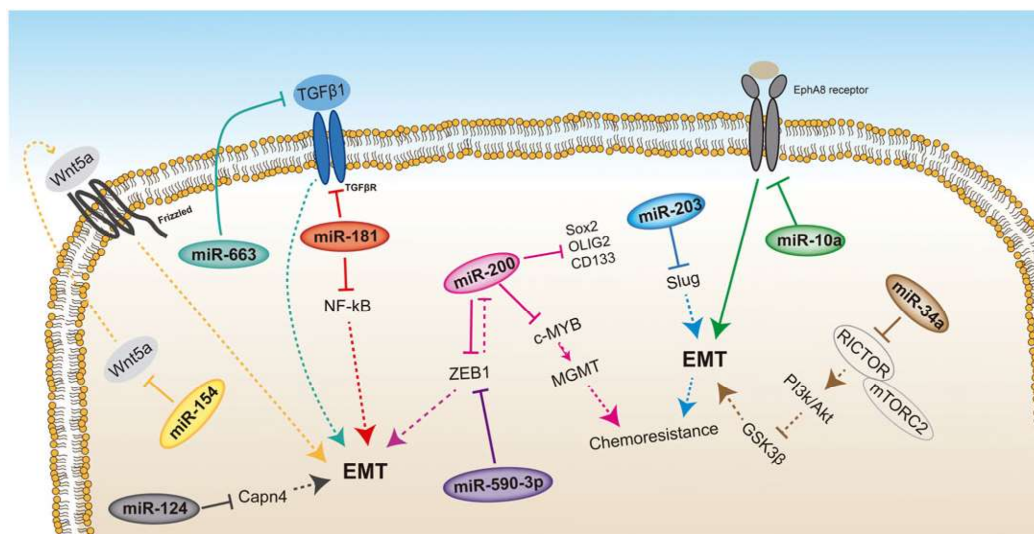

Figure 5. miRNAs regulate GBM invasion and progression through EMT. miRNAs can repress EMT by targeting ZEB (miR-590-3p, miR200), Slug (miR-203), Wnt5a (miR-154), TGFβ (miR-663), TGFβ receptor and NF-kB (miR-181), Rictor (miR34a), and Capn4 (miR-124), whereas miR10a can induce EMT by repressing the expression of the tyrosine kinase receptor EphA8, respectively. EMT-inducing genes can also regulate the expression of miRNAs, such as miR-200, in a double-negative feedback mechanism. This miRNA has impact on chemoresistance and stemness. EMT, epithelial–mesenchymal transition; TGFβR, transforming growth factor receptor; MGMT, *O*-6-methylguanine-DNA methyltransferase; RICTOR, rapamycin-insensitive companion of mammalian target of rapamycin; mTOR, mammalian target of rapamycin; Capn4, calpain small subunit 1.

proteins involved in invasion, migration, proliferation, DNA damage response and steamness, miRNAs can govern the epithelial/mesenchymal plasticity and, thus, promote or inhibit the spreading ability of cancer cells.[10, 135–137]

Many studies already reported the importance of miRNA to regulate mechanisms involved in glioma tumorigenesis, as recently reviewed by Moller,[138] in our search, we observed a large number of papers linking miRNA, GBM, and EMT, which are summarized in Figure 5. The miR-200 family is composed of five miRNA sequences and regulates negative tumor development and EMT, mainly by targeting ZEBs.[139–141] The analysis of an expression profile from whole-genome miRNA of human GBM samples showed that the loss of miR-200c expression is related with EGF receptor (EGFR) amplification and ZEB1 overexpression, suggesting the involvement of ZEB1 in tumor aggressiveness.[142]

Another miRNA that targets ZEB genes is miR-590-3p, whose expression levels are downregulated in both GBM tissue samples and GBM cell lines. In contrast, when overexpressed, miR-590-3p inhibited the motility, invasion, and expression of epithelial genes in glioma cell lines through targeting ZEB1 and ZEB2 genes.[143]

miR-181 family and miR-154 also act as GBM suppressors and their expression is associated with poor prognosis in GBM patients. In addition, the expression of these miRNAs is related with reduced migration and invasion of GBM cell lines, as well as induction of MET by stimulation of epithelial genes and repression of mesenchymal genes. *In vivo*, GBM cells stably expressing miR-181b formed smaller and less-invasive tumors when compared with controls. The repression of EMT by miR-181 family and miR-154 is explained based on the inhibitory effect of these miRNAs on TGFβ and NF-κB, and Wnt pathways, respectively.[144–146]

TGFβ pathway is also targeted by miR-663 that normally is downregulated in human GBM tissues and cell lines, when compared to normal cells. It was observed that the overexpression

of miR-663 in A172 and U87 tumors cells leads to reduction of TGFβ1 levels, consequently inhibiting mesenchymal characteristics by decreasing migratory, invasive, and proliferative ability of this cells.[147]

miR-124 has also been found to be downregulated in human GBM tissues, and its expression is inversely correlated with EMT. The overexpression of miR-124 in U87 and U251 cells leads to inhibition of calpain small subunit 1 (Capn4), a protein that has been correlated with the invasion of several types of tumors, besides decreased expression of phospho-FAK, MMP2, vimentin, and N-cadherin, as well as impaired cell migration and invasion this cencer cells.[148]

The microRNA-10a (miR-10a) also regulates EMT. It promotes cell migration and invasion of GBM cell lines by negatively regulating the expression of Eph tyrosine kinase receptor A8 (EphA8). miR-10a overexpression or EphA8 inhibition induces EMT, as evidenced by changes in cell morphology. Besides, the expression of E-cadherin at protein level was upregulated, whereas vimentin and ICAM-1 were downregulated in U251 and U87 cells, when miR-10a was blocked.[149]

miRNA-34a was able to reduce proliferation and induce cell cycle arrest and apoptosis in HNGC-2 and NSG-K16 cell lines. They belong to the mesenchymal subtype of GBM, presenting positivity for expression of mesenchymal markers Twist1, ZEB2, and COL5A1, and absence of expression of proneural related genes. HNGC-2 and NSG-K16 cells overexpressing miRNA-34a formed significantly reduced tumors, when compared to control cells in an *in vivo* xenograft model. The authors showed that Rictor, a component of the mTORC2 complex, is a target for miRNA-34a. The inhibition of Rictor by miRNA-34a blocks the Akt phosphorylation/activation and inhibits the nuclear activation of β-catenin; at the end, inhibiting Wnt signaling pathway and consequently proliferation, invasion, and tumorigenicity of GBM cells.[150]

miRNA-203 is another miRNA with important role in carcinogenesis. In GBM, miRNA-203 targets Slug and inhibits EMT, decreasing the resistance to chemotherapy. miR-203 was significantly downregulated in the imatinib-resistant GBM cells. As expected, these resistant cells exhibit mesenchymal characteristics, such as reduced E-cadherin expression, upregulation of the mesenchymal genes ZEB1 and vimentin, enhanced invasiveness, and increased cell viability. miR-203 was also found downregulated in samples from patients diagnosed with GBM grade III/IV, when compared with grade I/II samples. Also, there is an inverse correlation between miR-203 expression level and Slug mRNA level in human GBM specimens.[151]

I. Hypoxia and EMT

Hypoxia is a common event in solid tumors, including GBM. It is a condition in which the growth rate of cancer cells outstrips its blood supply, generating cells deprived of oxygen, due to a limited blood flow.[152] This exposure to low levels of oxygen can produces necrotic areas, one of the hallmarks of GBM. The necrotic areas are typically surrounded by "pseudopalisading" cells, marked to be severely hypoxic cells overexpressing HIF-1.[153] These typical areas of hypoxia in GBMs have been associated with poor prognosis and have a negative impact on survival of patients.[154,155] Investigations indicate that tumor cells are able to adapt to hypoxia condition, giving rise to resistant cells. This condition allows survival and consequently results in a more malignant phenotype, probably through an EMT-like process. This could explain why the beneficial effect of anti-angiogenic therapy is transient, with initial responses being frequently followed by intense progression of the disease.[75,156–159]

Hypoxia induced a mesenchymal shift in human GBM cell lines, recognized by morphological changes, enhanced invasive/migratory capacity and overexpression of mesenchymal markers (fibronectin and COL5A1). ZEB1 seems to be responsible for mediating the

hypoxia-induced mesenchymal shift, since the ZEB1-knockdown cells, when exposed to hypoxia, did not shift the phenotype and lost the invasive potential.[160] Morphoproteomics analysis of GBM human samples revealed an association among HIF-1α signaling, cMet, CD133 and CD44. This association supports the idea that the hypoxia state can lead to a more mesenchymal/stemness potential in GBM cells.[161] This is also supported by the increase in the mesenchymal markers MMP2, Zeb1, Zeb2, Snail, Slug, and Twist in tumor samples of patients treated with the anti-VEGF antibody bevacizumab. These samples also exhibited a marked increase in cellularity, cell proliferation, and spindle-shaped mesenchymal morphology. The data suggest that the treatment with anti-angiogenic therapy produces a more hypoxic environment that through inhibitor of HIF-1/2α activates an EMT-like process in GBM.[162–164]

J. EMT in Therapy Resistance

Despite many efforts to find different molecularly targeted agents against many tumor types, in combination or not, to traditional anticancer therapy, no therapy at the moment has proven to be fully effective. The different therapeutic modalities for glioma treatment, including radiotherapy and chemotherapy, with alkylating drugs such as TMZ, which are the gold standard treatment regimes for GBM, may lead concomitantly to severe tumor progression, by promoting EMT-like process. Therefore, the acquisition of EMT-like program by treated cancer cells may provide an explanation for the still high recurrence rate of GBM tumors.[21,75]

For instance, ZEB1 was able to induce TMZ resistance in human GBM cell lines through the induction of c-MYB, miR-200, and MGMT (O(6)-methylguanine-DNA-methyltransferase). MGMT, an important DNA repair protein and the main enzyme involved in chemoresistance, is positively regulated by c-MYB, a proto-oncogene that binds to the MGMT promoter in GBM cells. c-MYB, in turn, is inhibited by miR-200, that acts in a negative feedback loop with ZEB1 (Fig. 5). In this context, mice engrafted with ZEB1 knockdown GBM cells showed an increased survival rate, after TMZ treatment, when compared with control animals. This is explained because decreased expression of ZEB1 allowed the inhibition of c-MYB (and therefore MGMT) by miR-200.[61]

Another link between chemoresistance and EMT was provided by experiments using the human U87 GBM cell line. Macrophage colony stimulating factor (MCSF), a pro-tumoral cytokine, associated with cancer progression, is also associated with drug resistance. U87 cells overexpressing MCSF were shown to be more resistant to treatment with 5-fluorouracil (5-FU) than control U87 cells. Simultaneously, these cells acquired a mesenchymal phenotype, with enhanced expression of N-cadherin, vimentin, Notch-1, and morphology changes, consistent with EMT induction.[165] In addition to U87, other human glioma cell lines, resistant to BCNU (carmustine) showed alterations related with EMT, such as fibroblast-morphology, enhanced migratory and invasive capacity, and decreased E-cadherin expression, with concomitant enhanced expression of vimentin and MMP-9.[166]

It is known that CSC is considered to be more resistant to toxic injuries and chemoradiation therapy than differentiated cells.[75] In accordance, intrinsic BCNU-resistant glioma cells present abundant presence of "stem-cell-like" cancer cells. They intrinsically formed neurospheres *in vitro* and showed stable growth rates, characteristic of cancer stem-like cells.[166] Piao et al. also showed that GSC line and U87 cell line with acquired resistance to bevacizumab (anti-VEGF therapy) presented higher expression of mesenchymal gene signatures and were more invasive, when compared with nonresistant cells.[163]

Meng et al. assessed human patients with GBM, from GEO (Gene expression omnibus) and TCGA datasets and observed that radioresistance is associated with EMT gene signature. They crossed 31 signature genes related to radiosensitivity in radiotherapy-treated patients and

observed that radiosensitive patients tended to have improved overall survival, when compared with radioresistant patients. Moreover, several mesenchymal gene sets were strongly associated with the radioresistant profile, supporting the idea that EMT plays an important role in resistance to radiation.[167]

In clinically recurrent human GBM, markers related with EMT, including vimentin, fibronectin, CD44, collagen, and MMPs, were increased.[35] This finding reinforces the theory that EMT-like process can be induced by irradiation. In accordance, GBM cell lines, after irradiation, presented an increased expression of Snail. Snail also promotes migration and invasion via MMP-2 activity in postirradiated GBM cell lines. These processes were possibly triggered through the activation of ERK1/2 and inactivation of GSK3β, in a TGFβ-dependent manner.[35] Kubelt et al. in a similar work using T89G glioma cells after TMZ treatment showed that TMZ was able to induce expression of Twist1, DSP, vimentin, Snail1, β-catenin, TGFβ R1, and L1CAM, all markers involved in EMT process.[168]

Taken together, these results indicate that the choice of treatment for patients should take into account the particular set of cells that comprise the tumor, once these different subgroups of cells express several combinations of mesenchymal and/or epithelial markers in an intricate pattern. However, in general researchers should invest in therapies that would block the mesenchymal shift during treatment.

K. Targeting EMT Pathway in GBM

Shi et al., after screening a set of small molecules, based on the 3D structure of the Dicer binding site on pre-miR-21, showed that the chemical compound AC1MMYR2 is a potent inhibitor of miR-21. This was used to suppress proliferation, migration, and invasion and promote apoptosis in human U87 GBM cell line. The expression of the epithelial marker E-cadherin was increased, whereas the expression of the mesenchymal markers N-cadherin, b-catenin, ZEB1, ZEB2, and MMP9 was reduced after AC1MMYR2 treatment. Similar effects were observed in a U87 glioma intracranial model in which was observed a significant difference in tumor volume of AC1MMYR2-treated group, in comparison with the controls. The expression of proliferation and mesenchymal markers was also decreased in AC1MMYR2-treated group relative to the control group.[169]

The same research group reported, 2 years later, that AC1MMYR2 also might be used as an adjuvant treatment with taxol in metastatic cancers. AC1MMYR2 reverted the EMT in U87 cells, caused by treatment with high dose of taxol, mainly by attenuating CDK5/P-FAK^{Ser732} activity. Moreover, EMT-related markers β-catenin and vimentin were dramatically decreased in a therapy combining the AC1MMYR2 with taxol, when compared with taxol only. Most importantly, a significant reduction of lung metastasis was observed in orthopic breast cancer transplantation mice model treated with the same combination.[170]

Recently, FTY720, a synthetic compound produced by modification of a metabolite from the fungus *Isaria sinclairii* was shown to reduce proliferation, migration, and invasion of U251 and U87 glioma cell lines, through the PI3K/Akt/mTOR/p70S6K signaling pathway. Besides, FTY720 significantly reduced mesenchymal phenotype, by decreasing the expression of MMP-2, MMP-9, vimentin, and N-cadherin.[171]

Another compound that inhibits EMT via downregulation of PI3K/Akt is osthole, a coumarin derivative, isolated from the fruit of *Cnidium monnieri*. Osthole is able to block the IGF-1-induced fibroblast-like morphology, cell migration, upregulation of N-cadherin, vimentin, Snail, and Twist and suppressed expression of E-Cadherin, ZO-1, β-catenin, MMP-2 and MMP-9 in a dose- and time-dependent manner in GBM8401 cells.[172]

EMT is also target of oleanolic acid, a compound described to inhibit migration and invasion of GBM human samples and cell lines. Oleanolic acid decreased the expression of

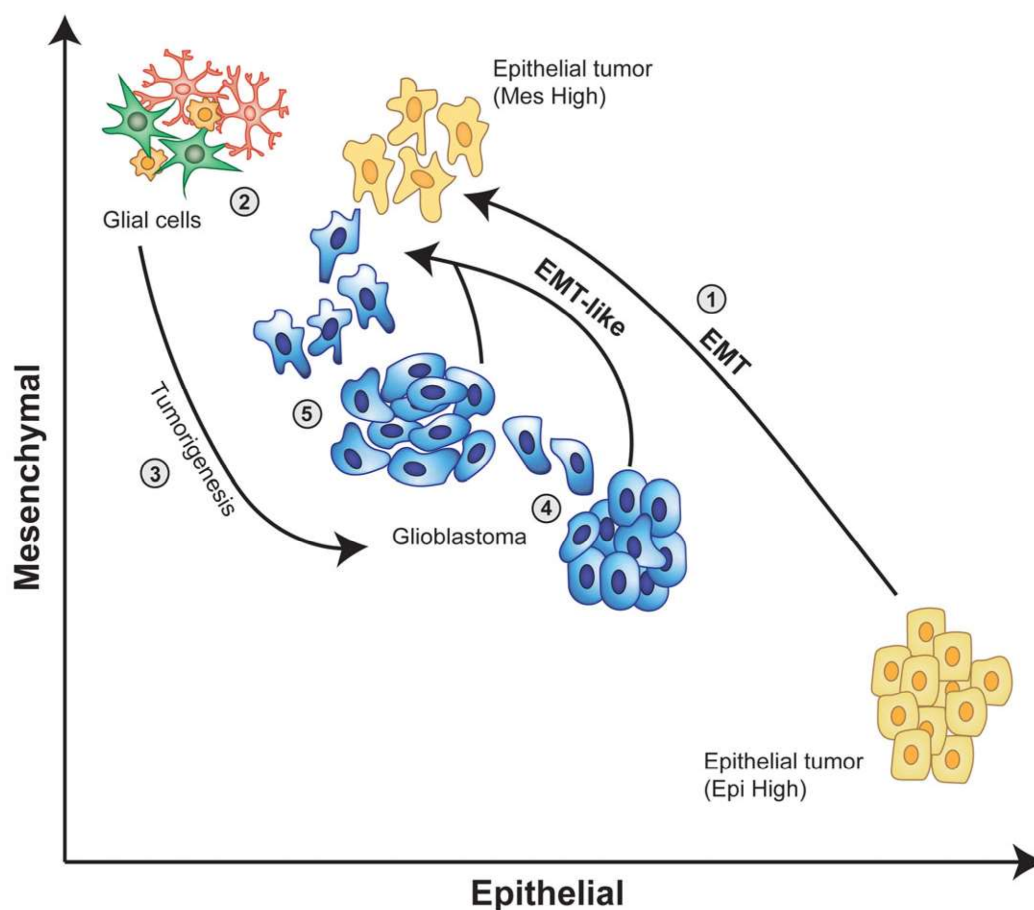

Figure 6. Position of the glioblastomas in an epithelial–mesenchymal spectrum in comparison with glial cells and classical epithelial tumors. The epithelial tumors after stimuli can undergo EMT with a clear migration from the epithelial to mesenchymal phenotype (1). Normal glial cells are positioned toward a high mesenchymal and low epithelial phenotype (2) and during tumorigenesis move to a slightly less mesenchymal phenotype (3). The GBM cells are distributed in an intermediate spectrum dislocated to a more mesenchymal phenotype (4) and can move to a more mesenchymal phenotype in an EMT-like process induced by the microenvironment (5).

EMT-related markers N-cadherin, vimentin, and twist1, while enhanced the expression of E-cadherin. These effects were related to the inhibition of MAPK/ERK pathway.[173]

Fu et al. studied another molecule termed NPV-LDE-225 that is a potent and specific Hedgehog pathway inhibitor, through binding and antagonizing Smoothened. This compound, used in topical creams for the treatment of basal cell carcinoma, suppressed epithelial-mesenchymal transition in GSCs by upregulating E-cadherin and inhibiting N-cadherin, Snail, Slug, and Zeb1, through modulation of the miR-200 family. NPV-LDE-225, in addition, inhibited motility, invasion, and expression of Snail, Slug, Zeb1, N-cadherin, and genes related with pluripotency in brain GBM cells. One mechanism proposed in this work was that NVP-LDE-225 inhibits EMT, by upregulating the miR-200 family. The downregulation of miR-200a/b/c prevented the inhibitory effects of NVP-LDE-225 on cell migration and invasion.[174]

Among the compounds that have emerged with antitumor activity is a bioactive constituent of the bark of *Magnolia officinalis,* known as Honokiol. In an *in vitro* study, this compound inhibited the adhesion of U87 cells to brain microvascular endothelial cells (BMECs), by

inhibiting VCAM-1. Indeed, honokiol inhibited the invasion of U87 through BMECs, by reducing membrane permeability and EMT processes. In the end, reduction of the mesenchymal markers Snail, β-catenin, and N-cadherin and increase in the levels of the epithelial marker E-cadherin were observed.[175]

Although radioative[125] I seed has been used in cancer treatment, as GBM, its effect in EMT process still had not been analyzed. Very recently, Tian et al. showed that ^{125}I seed inhibits EMT and cell growth in GBM cell lines, more efficiently than X-ray. Colony-formation ability, viability, and cell proliferation were significantly reduced by ^{125}I seed irradiation, whereas apoptotic cell death and cell cycle arrest were markedly induced. Moreover, ^{125}I seeds inhibited invasion and migration, as well as downregulated ZEB1 and vimentin. Interestingly, inhibition of GBM cell growth and EMT were induced by ^{125}I seeds with the involvement of a ROS-mediated signaling pathway. These effects were confirmed *in vivo* experiments, once ^{125}I seeds efficiently inhibited xenograft tumor growth and EMT.[176]

Although, scorpion venom has been recognized as a potent approach against tumor progression,[177] surprisingly, in GBM, Sj7170, a recombinant peptide isolated from the venom of the scorpion, *Scorpiops jendeki,* promoted cell proliferation, cellular migration and invasion of glioma U87, and also enhanced tumor growth in an U87 xenografted tumor *in vivo*. Sj7170 also induced EMT as evidenced by change in cell morphology and cytoskeleton of U87 cells and enhanced expression of Snail.[178] This venom seems to be an exception among peptides isolated from venom of different species of scorpions. Thus, although animal venoms are composed of a variety of active principles, which may inhibit cancer progression, this specific peptide is not a good candidate for use in cancer therapy for GBM.

6. CONCLUSION

In this review we have discussed the complex molecular signaling pathways involved in controlling the epithelial and mesenchymal phenotypes during GBM formation, progression, and response to therapy. These phenotypes are controlled on multiple levels, including transcriptional repression, posttranslational modifications, and cell signaling. The crosstalk of different pathways is responsible for an intricate network, which can induce cells to acquire a mesenchymal phenotype. Understanding the interplay of these molecular routes will allow the development of novel therapies to potentially prevent or reverse EMT prior to tumor advancing.

The studies described in this review aimed provide a picture of the current state of the art of the EMT-like regulatory mechanisms in GBM. Although the attention to EMT-related molecules and their function has increased over the last years, the potential roles of them in inducing malignancy, invasion, and metastasis are virtually unexplored.

Different to what occurs to epithelial tumors, whose mechanisms of invasion and metastasis have been better explored, the process of EMT in GBM has only recently been accessed and little knowledge is available in current literature. Moreover, as glioma is not a tumor of epithelial origin, there is no consensus about the EMT biomarkers that are common between gliomas and epithelial tumors. While E-to-N-cadherin switch is a well-established event in carcinomas during EMT, in GBM the mesenchymal molecular and cellular changes occur in an independent way of a "cadherin switch." This can be explained mainly because repressors of E-cadherin are able to regulate also other invasion and migratory-associated genes. Interestingly, the expression of these other molecules appears to be sufficient to induce at least a partial EMT in GBMs.

Although the available literature supports that glial tumors can transit between more epithelial and more mesenchymal phenotypes, we realized that there are some questions not yet clearly defined, such as the following: In terms of epithelial/mesenchymal gene expression, are

GBMs similar to tumors of epithelial origin? In our in silico analysis, we revealed that GBM are in an intermediate state between epithelial and mesenchymal phenotype, with some samples looking more like epithelial tumors, but the vast majority of GBMs more inclined towards the mesenchymal features (Fig. 6). These peculiarities associated with the fact that metastasis, which is one of the most important outcomes of EMT in epithelial tumors, is not a common event during GBM progression, make the gliomas a peculiar type of cancer.

We believe that the heterogeneity of glioma genotypes, phenotypes, and neighboring tissue architecture allows that a miscellany of tumor invasion mechanisms could be used by tumor cells at different stages of tumor progression, which would explain why gliomas do not follow the rules of a typical EMT. In addition, the differences in them from the epithelial tumors do not appear to be limiting to GBM undergoing EMT, since it is very probable that they can surpass to a more mesenchymal state during EMT-like process.

It is evident that the scientific community is joining efforts to target EMT in several types of cancer. This concern is mainly due to the evident importance of this process in cancer progression. However, clearly, more studies are necessary to enhance the understanding of cancer biology and answer many questions about the role of EMT in cancer development.

In this light, we consider it of great importance to make a profound analysis to understand and determine exactly, which are the key genes related with EMT in GBM, and what in the future will allow us to produce more refined researches in order to target specific molecules and pathways to effectively inhibit glioma dissemination.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web site:

Supplementary Figure 1. (**A**) Papers identified in PubMed/Medline database regarding the EMT in glioblastoma (terms used in the search: "Epithelial Mesenchymal Transition" or "Epithelial-to-mesenchymal Transition" AND "Glioblastoma"). (**B**) Methodological flow chart of the search strategy in PubMed and Scopus databases.

Supplementary Figure 2. Volcano plot representing the differentially expressed genes in GSE17708 and GSE40374. The genes with fold-change greater than 1.5 (**A**) or 5 (**B**) and adjusted p-values>0.05 were highlighted in red. Correlation analysis between the epithelial/mesenchymal ratio of the meta-signatures GSE17708 e GSE40374 in normalized RNA-seq data of glioblastoma patients (**C**).We also applied the GSE17708 MS to different glioma cell lines obtained from GSE4536. We observed that both, GBM samples and glioma cell lines follow the same tendency of a more mesenchymal profile (Supplementary Figure 3).

Supplementary Figure 3. Position of glioma cell lines with published transcriptome in the epithelial/mesenchymal metasignature graph (right) (For comparison, data from TGFβ-induced EMT in lung cancer and epithelial cell as well as gliomas from TCGA were copied from Figure 1) (left).

Supporting Information S1. Answers to reviewers.

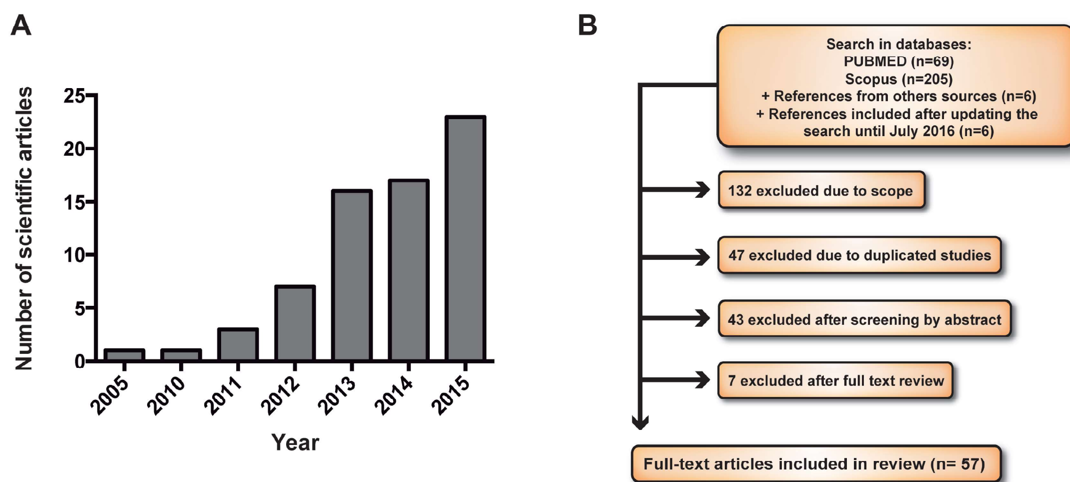
Suplemmentary material

Methods and Results

Literature search strategy

This study describes a "literature overview" about EMT in glioblastoma. In Supplementary Fig. 1A it is possible to observe the increased interest in the recent years of the scientific community on the role of EMT-related processes in GBM development. Despite the relevance of this biological process in GBM progression and invasion, the wide and updated compilation of new findings about this topic is not provided in the literature.

This literature search was conducted in the PubMed and Scopus databases, combining the medical subject heading (MeSH) terms: "epithelial mesenchymal transition" OR "epitelial to mesenchymal transition" AND "glioblastoma". All studies until May 2015 were included (a new round of searching was conducted in July 2016 to update the review). No language restriction was applied. By this search strategy, 274 papers were identified. Six additional citations obtained by manual search were also included. After reviewing their abstracts, 53 articles were reviewed in full-text form. After analysis of these studies, 7 were excluded for be not eligible. Six additional citations were included after the second round of searching conducted in July 2016. At the end, 57 papers that examined EMT in glioblastoma were selected (Supplementary Figure 1B and Tables 1-8).



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Metasignatures construction and validation

In order to select genes differentially expressed during EMT, we created two distinct meta-signatures from data obtained from the Gene Expression Omnibus (GEO). Both databases used cell treated with TGFβ to induce EMT in normal epithelial cells or in lung adenocarcinoma cells.

In the first transcriptome microarray dataset (GSE17708 accessed on 06.01.2016), A549 lung adenocarcinoma cells treated with TGFβ for 72h (GSM442049, GSM442050 and GSM442051) where used as the mesenchymal phenotype and the untreated cells (GSM442026, GSM442027 and GSM442028) as the epithelial phenotype. These two groups were compared using the GEO2R software to produce a fold-change value for each gene and a p-value adjusted by the Benjamini and Hochberg method. To select the most differentially expressed genes between the groups, we used the genes with fold-change greater than 1.5 and adjusted p-values smaller than 0.05 (-log (p value) > 1.3) (Supplementary Figure 2a). Thereby, we created a meta-signature for each condition (epithelial and mesenchymal), using the average expression of 25 genes expressed in the TGFβ-treated condition (mesenchymal genes) and the average expression of 14 genes expressed in the untreated condition (epithelial genes) (Supplementary Figure 2a). As expected, average expression of the epithelial genes (Epithelial MS 17708) was high in untreated and low in treated cells and the average expression of the mesenchymal genes (Mesenchymal MS 17708) was high in treated and low in untreated cells (Supplementary Figure 2a, insert).

As a second strategy, we used transcriptome microarray data from immortalized bronchial epithelial cells (GSE40374 accessed on 01.06.2016). Data from untreated cells (GSM992456, GSM992457 and GSM992458) and cells treated for 6 days with TGFβ (GSM992459, GSM99260 and GSM992461), were analyzed as described above, except that the only genes with a fold-change greater than 5 and adjusted p-values smaller than 0.05 (-log (p value) > 1.3) were used (Supplementary Figure 2b). Thereby, we created two meta-signature based on the average expression of 49 mesenchymal genes and 20 epithelial genes (Supplementary Figure 2b). Again, the expression of the Epithelial MS 40374 the mesenchymal meta-signature Mesenchymal MS 40374 behaved as expected (Supplementary Figure 2b, insert).

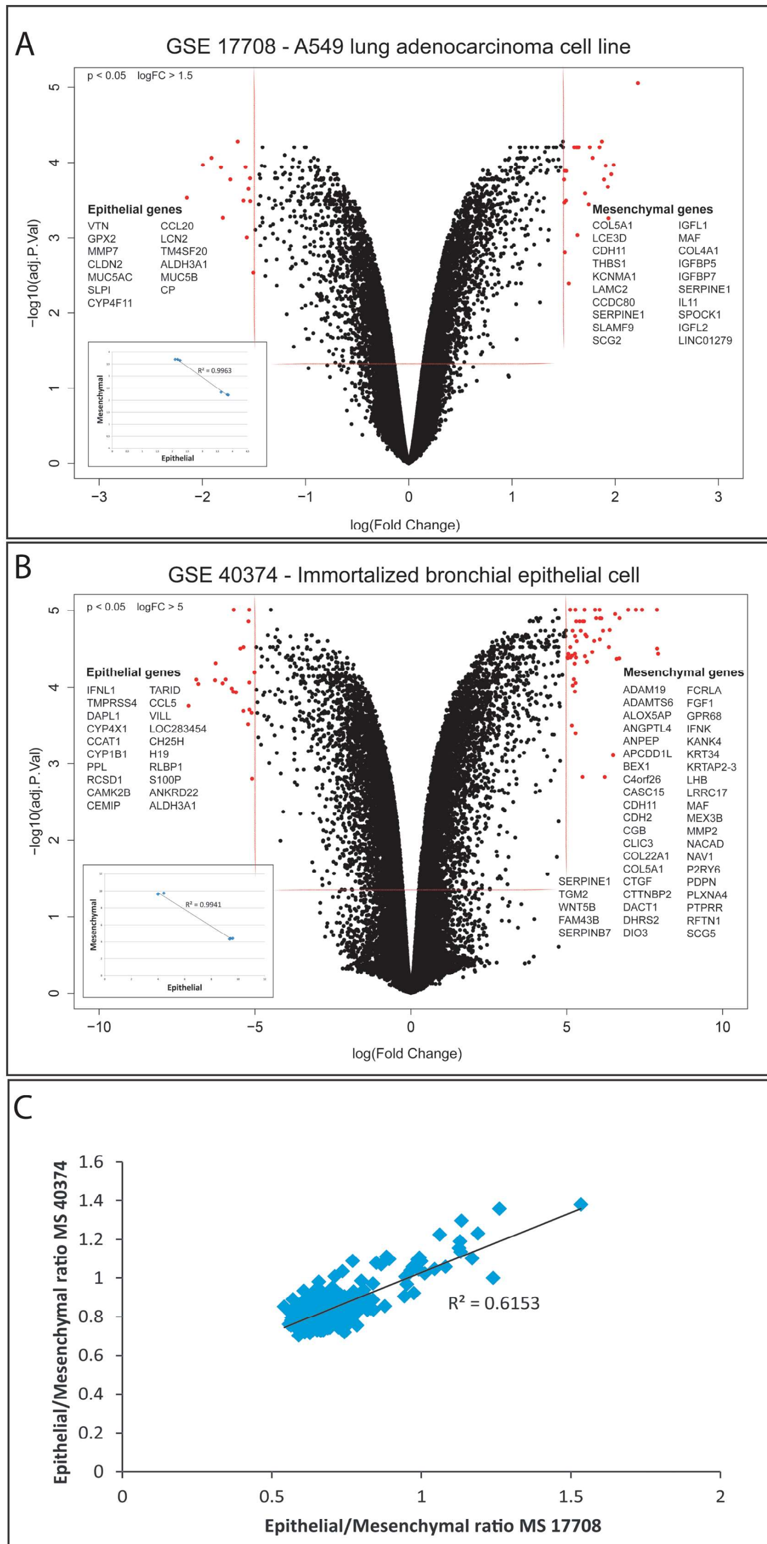
In order to compare the meta-signatures generated in the different datasets, we performed a correlation analysis between the epithelial/mesenchymal ratio of the meta-signatures GSE17708 and GSE 40374 using normalized RNA-seq data from glioblastoma patients based upon data generated by the TCGA Research Network: http://cancergenome.nih.gov/ downloaded on 09.06.2015. The two metasignatures generated from cancer and normal cells treated with TGFβ generated a good correlation in this complex dataset (Supplementary Figure 2C).

E-/N-Cadherin relation in glioblastoma patients RNA-Seq data

We evaluated the relation between the E-/N-cadherin ratio in RNA-seq data of glioblastoma patients and the relationship between these ratio in the samples from A549 lung adenocarcinoma cell line (GSE17708) untreated and treated with TGFβ as well as glioblastoma samples from TCGA (Fig. 3).

Use of the meta-signature in GBM and comparison with glial cells

We applied the epithelial and mesenchymal meta-signatures generated in the GSE17708 in samples from glioblastoma patients from the TCGA Research Network. We also used normalized RNA -Seq data of normal glial cells samples of mice, obtained from GSE52564 accessed on 06.07.2016. In order to compare the results obtained in different transcriptome datasets, we used the epithelial/mesenchymal ratio of the different samples in all datasets and the distribution of the ratio is shown in Fig 1.



Supplementary Figure 2. Volcano plot representing the differentially expressed genes in GSE17708 and GSE40374. The genes with fold-change greater than 1.5 (**A**) or 5 (**B**) and adjusted p-values>0.05 were highlighted in red. Correlation analysis between the

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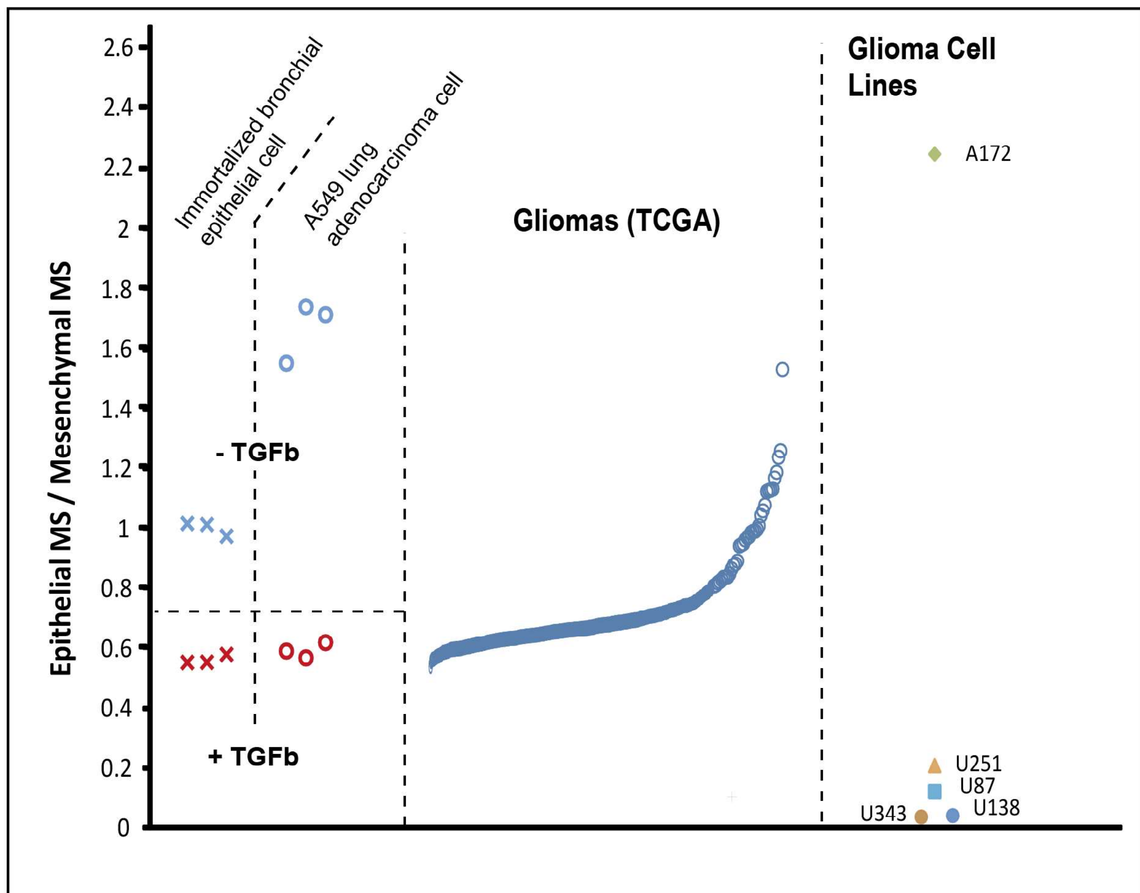
We also applied the GSE17708 MS to different glioma cell lines obtained from GSE4536. We observed that both, GBM samples and glioma cell lines follow the same tendency of a more mesenchymal profile (Supplementary Figure 3).



Supplementary Figure 3. Position of glioma cell lines with published transcriptome in the epithelial/mesenchymal metasignature graph (right) (For comparison, data from TGFβ-induced EMT in lung cancer and epithelial cell as well as gliomas from TCGA were copied from Figure 1) (left).

DISCUSSÃO

GBM é o tumor mais maligno do SNC, apresentando alto grau de agressividade e altas taxas de remissão, o que fazem com que a sobrevida dos pacientes acometidos por esse tumor seja de aproximadamente um ano (Lima, Kahn et al. 2012). Chama a atenção o fato de que apesar da intensa pesquisa que vem sendo feita nessa área da oncologia, nenhum tratamento disponível até o momento foi capaz de aumentar significativamente a sobrevida dos pacientes.

Diante dos escassos avanços que têm sido alcaçados nos últimos anos a fim de aumentar a sobrevida de pacientes com GBM, muitos pesquisadores têm direcidonado esforços, através das mais diversas abordagens, a fim de compreender melhor os mecanismos que levam esse tipo de tumor a tornar-se tão devastador. Como células tronco têm sido constante foco de estudo em modelos de terapia gênica e celular, inclusive para o tratamento de gliomas, o nosso principal objetivo na presente Tese, foi avaliar os efeitos do CM de ADSCs em células de glioma C6.

No capítulo I desta Tese, foram avaliados genes candidatos para serem usados como genes de referência em células de glioma C6 tratadas com ADSC-CM. Essa análise permitiu que nossos posteriores experimentos, através do método de RT-qPCR fossem realizados sem o risco de uma interpretação equivocada, dos efeitos biológicos estudados em nosso modelo. Reações de RT-qPCR precisam ser normalizadas, principalmente porque existem variações entre subpopulações de células de diferentes linhagens e em diferentes condições experimentais. Essas diferenças celulares podem alterar os padrões de expressão de mRNA nas células. Além disso, muitos tratamentos aos quais as células são expostas, podem alterar o perfil de expressão de mRNA. Esses vieses precisam ser levados em consideração quando realizadas análises quantitativas de expressão gênica (Bustin, Benes et al. 2005). *Então, porque não escolher qualquer gene constitutivo para ser utilizado como normalizador?* A resposta a essa pergunta, é porque um bom gene normalizador precisa sofrer a menor variação possível dentro do desenho experimental testado e sua expressão precisa ser mantida estável entre as amostras. *Mas a expressão de genes constitutivos não é estável?* Genes constitutivos não apresentam obrigatoriamente uma expressão estável. Desde meados dos anos 70 e 80 já havia sido mostrado que genes constitutivos muito utilizados em análises moleculares, não eram estáveis. Por exemplo, foi mostrado que o gene HPRT possui expressão diferencial em diferentes regiões do SNC, além disso, β-actina possui expressão alterada em diferentes amostras de pacientes com leucemia e, por fim, foi visto que diferentes quantidades de mRNA

geraram perfis de expressão do gene GAPDH muito similares em diferentes tecidos de rato (Huggett, Dheda et al. 2005).

Ao fazermos uma revisão na literatura, percebemos que os trabalhos publicados utilizando técnicas de RT-qPCR em amostras de glioma C6, não mencionavam qualquer tipo de processo de validação de genes normalizadores. Vale ressaltar que esses trabalhos utilizaram diferentes tipos celulares, além de diferentes condições experimentais e tratamentos. Em 2009, a fim de atingir um consenso em relação a melhor forma de conduzir análises de experimentos de PCR quantitativo, foi publicado um guia (MIQE - Minimum Information for Publication of Quantitative Real-Time PCR Experiments) que reporta os principais parâmetros que devem ser observados antes de realizar o experimento. Esses parâmetros incluem integridade do RNA, volume de reação, concentração de cDNA/RNA e curvas de calibração (Bustin, Benes et al. 2009). Esse guia pode vir a auxiliar muitos pesquisadores a alcançarem resultados mais reprodutíveis.

Em nossas análises, verificamos que o gene constitutivo mais estável para ser utilizado isoladamente em nosso modelo experimental é o *TATA box binding protein* (TBP). Entretanto, quando utilizados em combinação, TBP e *hypoxanthine-guanine phosphoribosyltransferase I* (HPRT) se mostraram os mais estáveis.

A partir desses resultados, nossos experimentos de RT-qPCR conduzidos no Capítulo II desta Tese, foram realizados utilizando o gene normalizador TBP. Para os experimentos do Capítulo III, realizamos uma nova validação (uma vez que, apesar de termos utilizado células C6, nossas amostras foram oriundas de tumores extraídos do cérebro de animais *in vivo*) para garantir a confiabilidade de nossos dados. Nessa segunda análise, identificamos novamente o gene TBP como sendo o mais estável (dados não mostrados).

EMT é um mecanismo chave nos processos de invasão tumoral e está fortemente associado com alto grau de agressividade e metástases (Thiery 2002). Recentemente, esse mecanismo tem sido associado também com mau prognostico e invasão de células de GBM (revisado no Capítulo IV).

O processo de EMT está fortemente sujeito à modulação pelo microambiente tumoral. Acredita-se que o esse microambiente, juntamente com os tipos celulares que o compõem, como MSCs, são os grandes propulsores da progressão maligna, principalmente por darem suporte físico e conter fatores que estimulam a propagação das células do tumor (Hanahan and Weinberg 2011).

No capítulo II, mostramos que o CM de ADSCs de rato induz um processo semelhante

a EMT em células de glioma C6.

Alguns artigos têm mostrado que MSCs são capazes de induzir EMT em variados tipos de células tumorais. Bhattacharya e colaboradores (2012) mostraram que o co-cultivo direto de hMSCs e células de linhagem de carcinoma hepático induziu EMT nas células tumorais pelo aumento da expressão de Snail, Slug e vimentina, além de alterar as morfologia celular (Bhattacharya, Mi et al. 2012). Outro estudo, utilizando células de câncer pancreático, mostrou que quando BM-MSCs são co-cultivadas com células tumorais, elas são capazes de se diferenciar em miofribroblastos (fibroblastos ativados). Além disso, induzem fenótipo relacionado com CSCs e aumento da expressão de genes relacionados com EMT nas células do tumor. *In vivo*, as MSCs diferenciadas promovem crescimento do tumor e resistência à terapia (Kabashima-Niibe, Higuchi et al. 2013). Outros trabalhos já mostraram também que o tratamento de células de tumor de mama com CM de MSCs promove EMT, que foi evidenciado pelo aumento da expressão de genes relacionados com o fenótipo mesenquimal, como N-caderina e vimentina nas células tumorais (Klopp, Lacerda et al. 2010, Martin, Dwyer et al. 2010).

Nossos resultados mostraram que o tratamento de células C6 durante 48h com CM de ADSC promoveu uma redução na capacidade celular de adesão, o que nos levou a especular que essas células poderiam estar entrando em um processo permissível à invasão. Uma das características da progressão tumoral é a redução da capacidade de adesão célula-célula e célula-ECM. A perda da expressão de E-caderina, uma das mais importantes moléculas de adesão, é uma das marcas da EMT. N-caderina, ao contrário de E-caderina, está relacionada com aumento da motilidade e invasão das células tumorais. Essa proteína encontra-se aumentada na maioria dos tumores que passam por EMT (Cavallaro and Christofori 2001). Esse "switch" de expressão entre E- e N-caderina não foi confirmado ocorrer em GBM, entretanto, sabe-se que células de glioma passam pelo mesmo processo de perda de adesão, entretanto, através de mecanismos ainda desconhecidos (Mahabir, Tanino et al. 2014).

Quando analisamos o perfil migratório das células de glioma, vimos que o co-cultivo indireto com ADSCs promove o aumento da migração das células tumorais. Esse aumento de motilidade foi acompanhado por alterações morfológicas e nucleares, compatíveis com o fenótipo de EMT. Essas alterações são clássicas modificações pelas quais células em EMT passam (Lee, Dedhar et al. 2006). Associado a isso, observamos aumento na expressão de vimentina, MMP2 e NRAS.

Proteínas RAS são oncogenes capazes de regular proliferação, sobrevivência e diferenciação celular. Existem três proteínas RAS altamente homologas (NRAS, HRAS e KRAS) que interagem com os mesmo efetores, uma vez que seus domínios de ligação a efetores são idênticos. Mutações que resultam em ativação de RAS estão associadas com aproximadamente 30% de todos os tumores humanos malignos (Parikh, Subrahmanyam et al. 2007). Essas proteínas estão fortemente associadas com EMT (Lamouille, Xu et al. 2014, Lindsey and Langhans 2014). RAS induz EMT por aumentar a expressão dos clássicos fatores de transcrição de EMT, regulando, assim, invasão e metástase (Lamouille, Xu et al. 2014)(Figura 8).

MMPs também são fortemente associadas com EMT (Thiery 2002, Lee, Dedhar et al. 2006). Essas proteínas podem degradar quase todos componentes da ECM e possuem um crucial papel em invasão de GBM. Níveis elevados de MMP2 e MMP9 são aumentados em gliomas, quando comparados com cérebro normal. MMP2 não somente degrada ECM, mas também regula processos celulares, como proliferação, adesão e migração, facilitando, assim, EMT (Chintala, Tonn et al. 1999).

Vimentina é uma das mais clássicas moléculas relacionadas com EMT. Ela é uma proteína estrutural do citoesqueleto e constitui os filamentos intermediários das células mesenquimais. Sua expressão é regulada principalmente pela via Wnt/β-catenina e está relacionada com migração e invasão em EMT (Kokkinos, Wafai et al. 2007).

De acordo com nossos resultados, embora a expressão do oncogene Src também esteja relacionada com EMT, seus níveis não foram alterados em nosso modelo. Alguns pesquisadores acreditam que invasão e metástase é um processo que resulta de uma incompleta EMT. As diferentes repostas aos estímulos que induzem EMT resultam em um amplo *spectrum* de fenótipos, que vão desde o puramente epitelial até o puramente mesenquimal. Acredita-se que algumas células só são capazes de metastizar porque adquirem um fenótipo parcial de EMT (Christiansen and Rajasekaran 2006).

Ainda nesse Capítulo, observamos que o CM de ADSCs de rato não altera a viabilidade celular, taxa de proliferação e ciclo celular de células de glioma C6 *in vitro*. Xu e colaboradores (2012) já haviam demonstrado resultados semelhantes utilizando células de linhagem de câncer de mama humano (MCF7). Nesses estudos, foi mostrado que o co-cultivo indireto, através de uso de *transwell*, de células tumorais e hADSCs não alterou a taxa de proliferação e o ciclo celular das células MCF7, mas, em contrapartida, induziu EMT (Xu, Wang et al. 2012). Durante o processo de tumorigênese, a ativação de oncogenes e vias de

sinalização promovem a proliferação das células tumorais, entretanto, quando o tumor já está estabelecido e começa o processo de invasão e metástase, a hiperproliferação é desestimulada, permitindo assim, que as células tumorais migrem. Ou seja, o aumento da proliferação celular é requerido somente nos estágios iniciais da tumorigênese e pode sofrer redução durante a invasão e EMT (Evdokimova, Tognon et al. 2009). Isso poderia explicar os nossos resultados relacionados à proliferação.

Além disso, vale ressaltar que diferentemente do que alguns autores haviam mostrado (Dasari, Velpula et al. 2010, Ho, Toh et al. 2013), em nosso modelo ADSC-CM não alterou a viabilidade das células tumorais.

Juntos, esses resultados nos fizeram propor um modelo no qual moléculas secretadas por ADSCs e presentes em seu CM, são capazes de ativar vias de sinalização relacionadas com EMT em GBM e, desse modo, induzir um processo de EMT-*like* em células de glioma (Figura 7). Entretanto, como essas alterações *in vitro* iriam se refletir num modelo *in vivo* é a próxima questão a ser respondida.

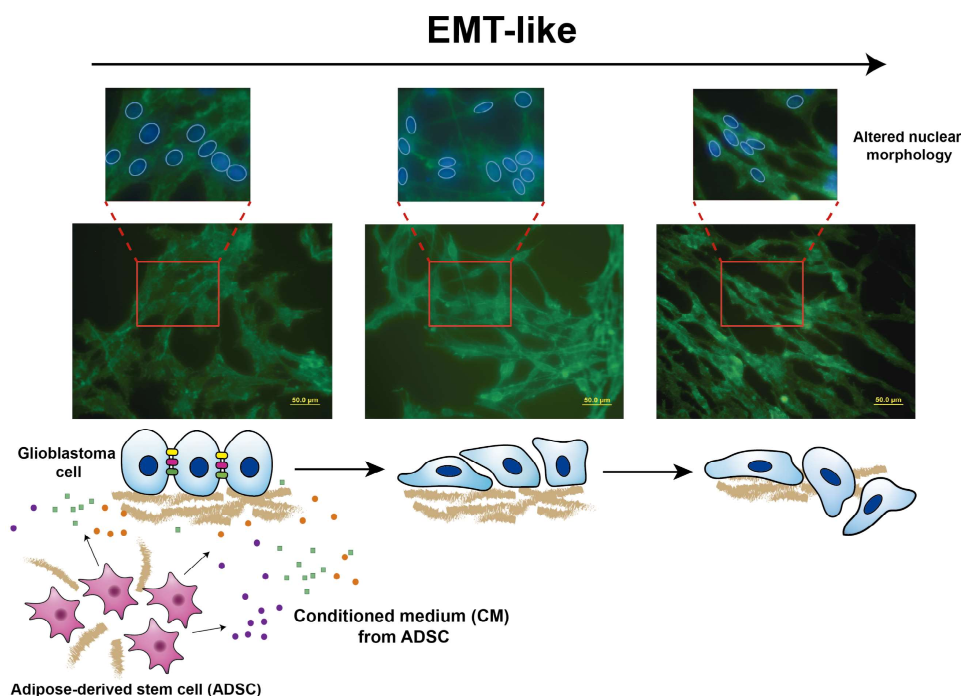


Figura 7. Esquema resumindo os resultados encontrados no capítulo III desta tese.

No Capítulo III desta tese, nosso principal objetivo foi investigar se os nossos resultados obtidos *in vitro* no capítulo II iriam se repetir num modelo de implante de glioma

em ratos.

Muitos pesquisadores já tentaram resolver a intrigante questão que envolve a comunicação celular entre MSCs e células tumorais. Ao contrário do que foi mostrado por Zhu e colaboradores (2011), no nosso modelo, observamos que o CM de ADSCs, bem como as próprias ADSCs, não foram capazes de potencializar a capacidade proliferativa das células tumorais, como evidenciado pela expressão de Ki67, que não foi alterada nos grupos de animais que receberam somente células C6 em comparação com os animais que receberam C6+ADSCs ou C6 pré-tratada com ADSC-CM. Além disso, diferentemente do que observamos *in vitro*, o CM de ADSCs também não foi capaz de aumentar a expressão de vimentina, MPP2 e NRAS. Curiosamente, o co-implante de C6 e ADSCs também não provocou alteração na expressão desses marcadores.

Zhu mostrou em um modelo de camundongo, que o tratamento de células de carcinoma gástrico com uma única dose de CM de hMSC gerou tumores de tamanho aumentado e mais vascularizados, semelhantes aos obtidos a partir da co-injeção de MSCs e células tumorais. Além disso, *in vitro*, o tratamento com CM induziu EMT nas células de tumor (Zhu, Huang et al. 2011). Kabashima niibe (2013) e Laurenzana (2015) também mostraram evidências de que MSCs possam induzir EMT *in vivo* (Kabashima-Niibe, Higuchi et al. 2013, Laurenzana, Biagioni et al. 2015).

Entretanto, embora já se tenha observado EMT *in vivo* (Santisteban, Reiman et al. 2009, Jahn, Law et al. 2012), esse continua sendo um desafio para os pesquisadores, principalmente devido à transitoriedade do processo de EMT. Isso faz com que frequentemente as células tumorais permaneçam em um estado híbrido, ou incompleto de EMT, tornando muito difícil detectar esse processo *in vivo* (Christiansen and Rajasekaran 2006). Karnoub, por exemplo, mostrou que embora células de carcinoma de mama ao receberem estímulos pró-EMT, passando a expressar altos níveis de marcadores mesenquimais e baixos níveis de E-caderina, elas permanecem em um estado parcial de EMT, detectado principalmente pela retenção de sua morfologia epitelial (Karnoub, Dash et al. 2007). Além disso, outro trabalho mostrou que a indução de EMT em células de carcinoma de mama promove aumento da expressão de Snail, mas não induz aumento da migração das células tumorais (Lundgren, Nordenskjold et al. 2009). Esses dados sugerem que o estado parcial de EMT pode ser mais comum do que se imagina.

Em nosso trabalho, observamos que a co-injeção de ADSCs e células C6 induziu a formação de tumores maiores quando comparados com os grupos que receberam células C6

não tratadas ou tratadas com ADSC-CM. Surpreendentemente, esse efeito não foi resultado do aumento da proliferação de células C6, como comprovado por avaliação da expressão do marcador de proliferação Ki67. Diante disso, acreditamos que outro mecanismo possa ser o responsável pelo aumento do volume tumoral observado em nossos resultados. Uma hipótese seria a possibilidade de transformação maligna das ADSCs.

Atualmente é consenso que células tumorais são capazes de "educar" as demais células presentes no microambiente do tumor.Essas células "educadas" podem adquirir funções pro-tumorigênicas (Quail and Joyce 2013). Por exemplo, Rubio e colaboradores (2008) mostraram que MSCs podem ser espontaneamente transformadas por células tumorais (através de MET), gerando células pobremente diferenciadas e com características mesenquimais fortemente reprimidas (Rubio, Garcia et al. 2008). Isso além de evidenciar que MSCs podem se transformar em células malignas, também poderia explicar porque nem sempre a indução de EMT em células tumorais *in vitro*, será observada *in vivo*. Outro exemplo de que células tumorais podem influenciar as células tronco, foi mostrado por Karnoub e col. (2007), no qual perceberam que quando MSCs e células de carcinoma de mama são injetadas na mesma região subcutânea de camundongos, as células tronco são capazes de induzir potencial metastático nas células tumorais. Entretanto, quando as células são injetadas contralateralmente, o mesmo efeito não é observado, indicando que MSCs podem aumentar o potencial metastático das células tumorais apenas quando fisicamente próximas (Karnoub, Dash et al. 2007).

Em resumo, diante dos resultados apresentados neste tarabalho, podemos propor um modelo de interação entre ADSCs e células de glioma de rato:

In vitro as ADSCs podem liberar fatores que permanecem presentes em seu CM e que, quando em contato com as células tumorais, induzem uma mudança fenotípica, semelhante a EMT. Durante esse processo, ocorre o aumento da expressão do oncogene NRAS, que por sua vez, é capaz de interagir com fatores de transcrição relacionados à EMT e induzir a expressão de genes como MMP2 e vimentina. O aumento da expressão de MMP2 pode levar à degradação de proteínas da matriz extracelular, facilitando assim, a invasão, que também é estimulada pelo aumento da expressão de vimentina. Alem disso, as células tumorais adquirem uma morfologia fibroblastóide, que é acompanhada por alteração na morfologia nuclear. *In vivo*, esse processo aparece nao ocorrer. Muito provavelmente porque em nosso modelo, após o tratamento com o CM, as células tumorais permanecem crescendo durante 20 dias no cérebro dos animais, desse modo, podemos supor que seja necessário um estímulo

constante para que a EMT seja induzida. Além disso, a presença do microambiente tumoral, com as demais células que o compõem, pode influenciar nesse processo. Apesar do CM de ADSCs nao influenciar o desenvolvimento de gliomas *in vivo*, a co-injeção de ADSCs com células de glioma C6, induz aumento tumoral, que não é provocado por aumento na taxa de proliferação celular. Isso nos faz acreditar que, supostamente, as células de glioma poderiam estar modulando a biologia das ADSCs e talvez até mesmo induzindo-as a adquirir um fenótipo maligno, o que auxiliaria a sustentar o desenvolvimento tumoral (Figura 8).

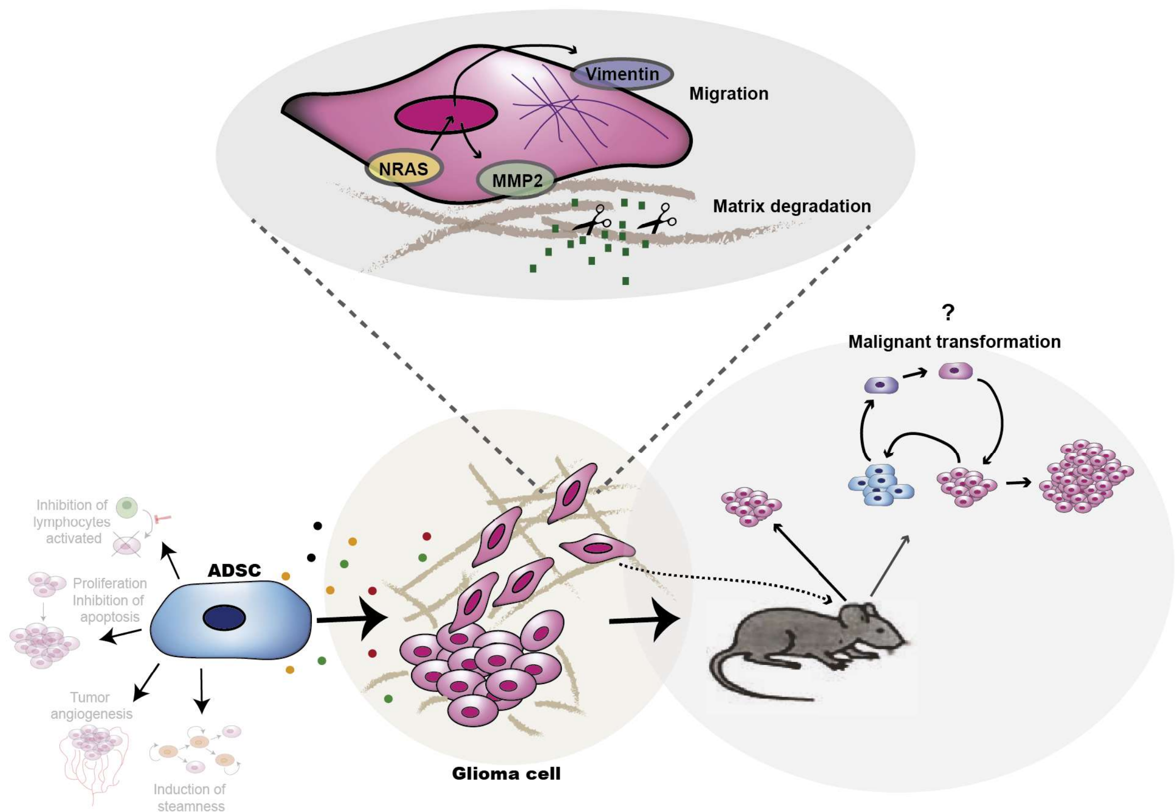


Figura 8. Modelo de interação entre ADSCs e células de glioma C6, proposto a partir dos resultados observados neste trabalho.

Desde que começamos a estudar EMT em nosso laboratório, percebemos que haviam poucos estudos analisando esse processo em GBM. Diante dessa realidade, decidimos fazer uma busca e reunir os trabalhos disponíveis na literatura em um artigo de revisão, a fim de melhor compreendermos a EMT em gliomas. Fomos especialmente motivados a desenvolver este trabalho pelo fato de que EMT foi primeiramente descrito em tumores de origem epitelial e, sendo os gliomas de origem neuroepitelial, nos pareceu necessário discutir as

peculiaridades desse processo em GBM. Surpreendentemente, nenhum outro autor havia antes analisado as diferenças entre a tradicional EMT, que ocorre em tumores de origem epitelial e a EMT que ocorre em tumores de origem não-epitelial.

No trabalho apresentando no Capítulo IV, foi feita uma revisão sistemática da literatura a fim de descrever os principais mecanismos que induzem ou inibem EMT em GBM e de maneira inédita, discutir as singularidades da EMT nesse tipo tumoral.

Primeiramente foram abordados os mais clássicos fatores de transcrição relacionados com EMT em tumores de origem epitelial, como Snail/Slug, Twist e ZEB1/ZEB2, mostrando que eles também possuem grande importância na progressão de GBM. Em outros tipos tumorais, como carcinoma de próstata (Smith and Odero-Marah 2012), ovário (Haslehurst, Koti et al. 2012), mama (Smith, Burton et al. 2014), colorretal (Fan, Samuel et al. 2012), entre outros, esses fatores também são apontados como reguladores de EMT. Muitas vias de sinalização são acionadas durante EMT, entre elas, PI3K/Akt, TGFβ/Smad, Ras/MEK, Wnt/β-catenina. Essas múltiplas vias cooperam para ativar a expressão dos fatores de transcrição relacionados acima que, por sua vez, irão inibir a expressão de marcadores epiteliais, como citoqueratinas e E-caderina e aumentar a expressão de vimentina e fibronectina, promovendo motilidade celular e invasão.

Outra questão que tem recebido muita atenção dos pesquisadores nos últimos tempos e que foi levantada neste trabalho, foi o *crosstalk* entre EMT e CSCs. Os trabalhos revisados mostram que EMT parece induzir um fenótipo mais indiferenciado em algumas células tumorais, estimulando a população "tronco" contida no tumor. Como CSCs têm sido relacionadas com recorrência e resistência à terapia em GBM (Lima, Kahn et al. 2012), novos agentes terapêuticos poderiam ser estudados a fim de atingir a população de CSCs ou bloquear o estímulo gerado pela EMT.

Também apresentamos as principais moléculas terapêuticas estudadas com capacidade de afetar a sinalização envolvida em EMT e, desse modo, bloquear, pelo menos em parte, a progressão tumoral. Algumas dessas abordagens parecem promissoras, entretanto, mais estudos serão necessários para que se possa vencer alguns desafios, como por exemplo, a dificuldade que se tem de acessar EMT *in vivo*, uma vez que nem sempre a indução controlada de EMT que observamos *in vitro* no laboratório ocorrerá de maneira idêntica no organismo vivo. Isso ocorre porque em geral, o que se observa *in vivo* é um processo parcial de EMT, com algumas vias de sinalização sendo inibidas e outras mantendo-se ativadas, dificultando assim, a caracterização desse processo nos diferentes tipos tumorais e nos

diferentes estadiamentos da doença. Outro desafio é encontrar a molécula ou via de sinalização decisiva para a ocorrência de EMT nos tumores. Como a EMT envolve múltiplos fatores, os pesquisados ainda precisam descobrir qual(ais) a(s) molécula(s) chave(s) para bloquear esse processo.

Entretanto, o que mais nos chamou atenção durante o desenvolvimento desse trabalho, foi a inconsistência de resultados relacionados à expressão de E- e N-caderina em linhagens de GBM, bem como em amostras de tumor humano, principalmente após a indução de EMT. Levando em conta que a perda da expressão de E-caderina, com o consequente aumento da expressão de N-caderina, é a principal e mais clássica característica de EMT durante a progressão tumoral, nos perguntamos por que esse *switch* entre E- e N-caderina não ocorre de forma unânime e consistente nos GBMs.

Uma das explicações que nos surgiu foi que como gliomas são, possivelmente, originados de células da glia, ou seja, células neuroepiteliais, muito provavelmente, o processo de EMT ao qual os gliomas estão sujeitos deva diferir da EMT clássica a qual estão sujeitos os tumores de origem tipicamente epitelial.

Como não encontramos dados suficientes na literatura, que pudessem nos responder essa questão, decidimos fazer uma análise *in silico*, na qual comparamos amostras de células tumorais e células sadias retiradas de bancos de dados.

Quando comparamos amostras de GBM humano com uma metassinatura de carcinoma de pulmão humano (um tumor de origem tipicamente epitelial) induzido a EMT através do tratamento com TGFβ, percebemos que enquanto a diferença de expressão de E- e N-caderina é clara nos tumores de pulmão antes e depois da indução de EMT, essa relação é praticamente inexistente nos GBMs. Além disso, diferentemente do que se poderia imaginar, a maioria dos GBMs, ao contrário dos carcinomas de pulmão, apresentam naturalmente um perfil mais mesenquimal do que epitelial. Apesar disso, acreditamos que seja possível que os gliomas consigam, através de um processo de EMT-*like,* migrar para um estado mais mesenquimal.

Esses resultados nos chamam atenção pois, de forma inédita, conseguimos mostrar que diferentes tipos tumorais podem passar por diferentes tipos ou graus de transição mesenquimal e que essas diferenças podem ser cruciais para a progressão tumoral e futuro desenvolvimento de moléculas terapêuticas.

Apresentamos aqui, pela primeira vez de forma experimental, que possivelmente GBMs não passam por um processo clássico de EMT e que, portanto, em gliomas a denominação mais correta seria EMT-*like* ao invés de EMT. Além disso, nos parece que

talvez as alterações fenotípicas, mesmo que parciais, caracterizadas por um processo de “ida e volta”, possam ser mais necessárias para o processo de malignidade e agressividade relacionada com EMT em GBMs do que a completa alteração fenotípica caracterizada pela clássica EMT.

CONCLUSÕES

Geral

Neste trabalho foi possível concluir que EMT é um processo importante para invasão e migração de GBMs. Além disso, fatores presentes no MC de ADSCs podem induzir um processo de EMT-*like* em células de glioma *in vitro.* Entretanto, esse efeito não foi observado *in vivo.* Apesar disso, a presença física de ADSCs no ambiente tumoral gerou tumores maiores *in vivo*.

Específicas

Capítulo I

- A validação de genes normalizadores é um passo crucial para a realização de experimentos de RT-qPCR;
- O gene normalizador mais estável para ser usado em amostras de células de glioma C6 tratadas com CM de ADSCs é o TBP ou TBP em associação com HPRT.

Capítulo II

- ADSC-CM não altera viabilidade celular, proliferação e ciclo celular de células C6;
- ADSC-CM aumenta a migração e reduz capacidade de adesão de células C6;
- ADSC-CM altera a morfologia celular e nuclear de células C6, induzindo um fenótipo fibroblastóide;
- ADSC-CM aumenta a expressão dos genes vimentina, NRAS e MMP2 em células C6;

Capítulo III

- O pré-tratamento de células C6 com ADSC-CM ou a co-injeção de células C6 com ADSCs não altera a expressão do marcador de proliferação Ki67, nem do marcador relacionado com gliose reativa, GFAP;

- O pré-tratamento de células C6 com ADSC-CM não altera o volume do tumor formado em cérebro de ratos;
- A co-injeção de células C6 com ADSCs promove um aumento do volume tumoral *in vivo.*
-

Capítulo IV

- GBMs parecem não passar por um processo clássico de EMT, diferindo dos tumores de origem epitelial;
- A EMT em GBMs não é necessariamente carcaterizada pela perda da expressão de E-caderina e aumento da expressão de N-caderina.
- EMT-*like* pode ser induzida em GBM através da ação de vários fatores e inúmeras vias de sinalização intracelular;
- EMT-*like* pode favorecer a sobrevivência celular, migração, invasão e contribuir para a agressividade de GBM;
- A modulação de EMT-*like* em GBM parece ser uma boa estratégia terapêutica.

PERSPECTIVAS

- Analisar quais fatores dentro do MC de ADSCs poderiam causar os efeitos observados no Capítulo III;
- Investigar os efeitos do MC de ADSCs em outros mecanismos celulares importantes como, autofagia, senescência,resistência à terapia e dano ao DNA;
- Investigar os efeitos, in vitro, do co-cultivo direto de C6 e ADSCs na biologia das células tumorais e mesenquimais;

  No nosso modelo in vivo:
- Avaliar as curvas de sobrevida dos animais tratados ou nao com MC e ADSCs;
- Avaliar se outras vias de sinalização podem ser afetadas com os tratamentos de MC e ADSC;
- Co-cultivar diretamente células C6 e ADSCs antes do implante em ratos e observar parâmetros de malignidade.

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ANEXOS

Anexo I – Carta de aprovação da Comissão de Ética no uso de Animais (CEUA)**.**

REPÚBLICA FEDERATIVA DO BRASIL
MINISTÉRIO DA EDUCAÇÃO

UFCSPA
UNIVERSIDADE FEDERAL DE CIÊNCIAS DA SAÚDE DE PORTO ALEGRE

CEUA –COMISSÃO DE ÉTICA NO USO DE ANIMAIS

PARECER CONSUBSTANCIADO DE PROJETO DE PESQUISA E ENSINO

1) PROTOCOLO Nº: 056/11 **Parecer 104/11**

2) DATA DO PARECER: 14/12/2011

3) TÍTULO DO PROJETO:

Caracterização do Sistema Purinérgico em células-tronco e sua aplicação terapêutica in vivo

4) PESQUISADOR RESPONSÁVEL:

Márcia Wink

5) RESUMO DO PROJETO:

O projeto visa investigar e comparar a expressão das enzimas ectonucleasese seus receptores em CTMs indiferenciadas e diferenciadas em osteócitos e adipócitos, buscando uma maior compreensão dos aspectos básicos das células. Após feita esta expressão os pesquisadores propõem avaliar o potencial das CTMs como ferramenta terapêutica.

6) OBJETIVOS DO PROJETO:

O projeto esta dividido em 2 fases bem distintas e por conseqüência os objetivos também.
Fase 1: In vitro
Avaliar a expressãos odos receptores purinérgicos em CTMs diferenciadas e não diferenciadas, bem como investigar a atividades de degradação de nucleotídios extracelulares na superfície da CTMs.
Fase 2: In vivo
Utilizar as CTMs como transportadoras da enzima NTPDase 1 solúvel até. O sítio do tumor a fim de bloquear a retroalimentação positiva purinérgica através da degradação do ATP extracelular.

7) FINALIDADE DO PROJETO: [ ] Ensino [x] Pesquisa

8) ITENS METODOLÓGICOS E ÉTICOS DO PROJETO:

Título [x] Adequado [ ] Comentários

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Introdução	[x] Adequada	[ ] Comentários
Objetivos	[x] Adequados	[ ] Comentários
Relevância e Justificativa	[x] Adequados	[ ] Comentários
Materiais e Métodos	[x] Adequados	[ ] Comentários
Cronograma para execução da pesquisa	[x] Adequado	[ ] Comentários
Orçamento e fonte financiadora	[x] Adequados	[ ] Comentários
Referências Bibliográficas	[x] Adequadas	[ ] Comentários

9) O PROJETO ESTÁ ADEQUADO À LEGISLAÇÃO VIGENTE: [x] Sim [ ] Não

10) INFORMAÇÕES RELATIVAS AOS ANIMAIS:

Grau de dor/estresse: **B** [ ] **C** [ ] **D** [x] **E** [ ]

Justifique:

Investigara eficácia da terapia gênica em células tumorais. Para esse fim, além dos objetivos in vitro do estudo se faz necessários experimentos in vivo para que os resultados possam ser extrapolados para organismos vivos e se a proposta tumoral possui aplicabilidade.

Espécie: Ratus novergicus **Número Amostral:** 99

Redução Amostral: [ ] Sim [x] Não

Justifique:

Substituição de Metodologia: [ ] Sim [x] Não

Se achar necessário, justifique e sugira uma nova metodologia:

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Aprimoramento da Metodologia: [ ] Sim [ ] Não
Se achar necessário, justifique e sugira aprimoramentos da metodologia:

Acomodação e manutenção dos animais: [x] Adequada [ ] Inadequada
Se achar inadequada cite abaixo as melhorias necessárias:

Manipulação dos animais: [x] Adequada [ ] Inadequada
Se achar inadequada cite abaixo as melhorias necessárias:

Não falam sobre o treinamento dos pesquisadores, principalmente da aula.

Analgesia dos animais (se aplicável): [x] Adequada [ ] Inadequada
Se achar inadequada cite abaixo as melhorias necessárias com analgésico substituto:

Anestesia dos animais (se aplicável): [x] Adequada [ ] Inadequada
Se achar inadequada cite abaixo as melhorias necessárias com anestésico substituto:

Eutanásia dos animais (se aplicável): [x] Adequada [ ] Inadequada
Se achar inadequada cite abaixo as melhorias necessárias com metodologia substituta:

Local de Realização (Biotério/Labotarório):UFCSPA

Outra instituição. Qual?

11) CRONOGRAMA DE UTILIZAÇÃO DE ANIMAIS

Data: 2014	**Espécie** Ratus novergicus	**Sexo M e F**	**Quantidade 99**

12) RECOMENDAÇÃO:

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[x] Aprovado

[ ] Com Pendência

[ ] Não aprovado

Comentários gerais sobre o projeto: