UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

Redução da proliferação celular e aumento da expressão de marcadores neurais de células-tronco de glioblastoma humano expostas a um inibidor de histona deacetilase.

Felipe de Almeida Sassi

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Prof. Dr. Rafael Roesler

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“Qualquer pessoa que tenha experiência com o trabalho científico sabe que aqueles que se recusam a ir além dos fatos raramente chegam aos fatos em si.”

Th. H. Huxley
(naturalista inglês, 1825-1895)

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LISTA DE ABREVIAÇÕES E SIGLAS

SNC – Sistema Nervoso Central *(Central Nervous System)*

GBM – Glioblastoma Multiforme

OPCs – Precursores de Oligodendrócitos *(Oligodendrocytes Precursor Cells)*

NSCs – Células-tronco neurais *(Neural Stem Cells)*

SVZ – Zona Subventricular *(Subventricular Zone)*

OMS – Organização Mundial da Saúde

TMZ – Temozolamida *(Temozolamide)*

VEGF – Fator de Crescimento Vascular *(Vascular endothelial growth factor)*

FDA – *(Food and Drug Administration)*

CSC – Células-tronco tumorais *(Cancer Stem Cells)*

ECM – Matriz Extracelular *(Extracellular Matrix)*

GFP – Proteína Fluorescente Verde *(Green Fluorescent Protein)*

DNA – Ácido Desoxirribonucleico *(Deoxyribonucleic Acid)*

RNA – Ácido Ribonucleico *(Ribonucleic Acid)*

HATs – Histonas Acetil-Transferases

HDACs – Histonas Deacetilases

HDACis – Inibidores de HDACs *(Histone Deacetylase Inhibitors)*

TSA – Tricostatina A *(Trichostatin A)*

SAHA – *(Suberoylanilide Hydroxamic Acid)*

NaBu – Butirato de Sódio *(Sodium Butyrate)*

LIF – Fator Inibidor da Migração dos Leucócitos *(Leukemia inhibitory factor)*

RESUMO

Os glioblastomas multiforme (GBM), são tumores cerebrais, que por sua malignidade, aliada ao seu rápido crescimento e frequente recorrência, exigem uma maior investigação da comunidade científica. Novas terapias devem afetar as células-tronco tumorais (CSC), as quais são responsáveis pela resistência e progressão tumoral. Neste trabalho fizemos o uso da Tricostatina A (TSA), um inibidor de histonas deacetilase, para se obter a modulação epigenética, e portanto, manipulação da expressão gênica, da linhagem celular U87-MG de GBM, utilizada aqui como um modelo para a pesquisa com CSC. Observamos a redução da proliferação e sobrevivência das tumoresferas de U87, as quais são formadas por CSC, seguida de alterações morfológicas nas células tratadas. A diferenciação das U87 foi confirmada pelo aumento dos níveis de marcadores neuronais e gliais, tais como NeuN e GFAP. Além disso, mostramos evidências de senescência celular após o tratamento com TSA. Nenhum efeito sobre a migração celular foi encontrado após a modulação epigenética. Portanto, os nossos resultados mostram a influência da TSA na diferenciação, proliferação e sobrevivência de CSC de glioma e também na indução da senescência celular, demonstrando o potencial da TSA na terapia dos tumores cerebrais

ABSTRACT

Glioblastoma multiforme (GBM), because of its fast growth and recurrence, require further investigation by the scientific community in order to find promising new therapies for these tumors, specially affecting their Cancer stem cells (CSC), which drive many tumorigenic processes. In this work we have made use of the HDAC inhibitor Trichostatin A to achieve the epigenetic modulation of the U87-MG GBM cell line, as a model for CSC research. We have observed reduction of the U87 tumorspheres, which are enriched for CSC, proliferation and survival followed by morphological changes both in the treated tumorspheres and in single cells. Enhanced on the U87 differentiation was confirmed by increased levels of neuronal and glial markers such as NeuN and GFAP. Furthermore we showed evidences of cellular senescence after the TSA treatment. No effect on cell migration was found after TSA treatment. Therefore, these results demonstrate a plethora effects on differentiation, proliferation, survival of glioma cells and induction of cellular senescence by TSA, making TSA a promising agent for glioma therapy.

CAPÍTULO I

1. TUMORES DO SISTEMA NERVOSO CENTRAL

Entre os diversos tipos de cânceres que acometem os humanos, aqueles de origem e localização no sistema nervoso central (SNC) representam apenas 2% do total (Buckner *et al*., 2007). Apesar de representarem uma pequena parcela, tais tumores destacam-se pelas suas altas taxas de morbidade e mortalidade, como resultado da fragilidade e limitação espacial do SNC. Ainda assim, a incidência desses tumores é de 20,5 por 100 mil habitantes norte-americanos ao ano (Dolecek *et al*., 2012). Também em 2007 foram estimados 20.500 novos casos de tumores cerebrais benignos e malignos no Estados Unidos, dos quais aproximadamente 12.740 poderiam resultar em óbito (Jemal *et al*., 2007). Já em 2011, foram estimados 22.340 novos casos e 13.110 possíveis óbitos (Siegel *et al*., 2011).

No Brasil, os óbitos por câncer cerebral corresponderam a 4,4% em 1998 do total de mortes por câncer (Monteiro *et al*., 2003), além disso a taxa bruta de mortalidade por tumores do SNC apresentou um aumento de 59,0% ao longo do período de 1980-1998.

Dissecando esses dados em tipos histológicos o tipo mais frequente é o meningioma não-maligno seguido do **glioblastoma multiforme (GBM),** o tipo de glioma mais agressivo e também o mais comum (Dolecek *et al*., 2012). Sabe-se também que o grupo glioma, como um todo, representa 30% de todos os tumores do SNC e 81% dos tumores malignos em adultos jovens (**Figura 1**). A importância do tipo histológico se reflete, ainda, nos dados epidemiológicos, como pode ser observado na taxa de sobrevivência: o tipo benigno de glioma astrocitoma pilocítico mantém uma taxa de 5 anos de sobrevivência de 94%, enquanto para os GBM essa taxa cai para menos de 5%.

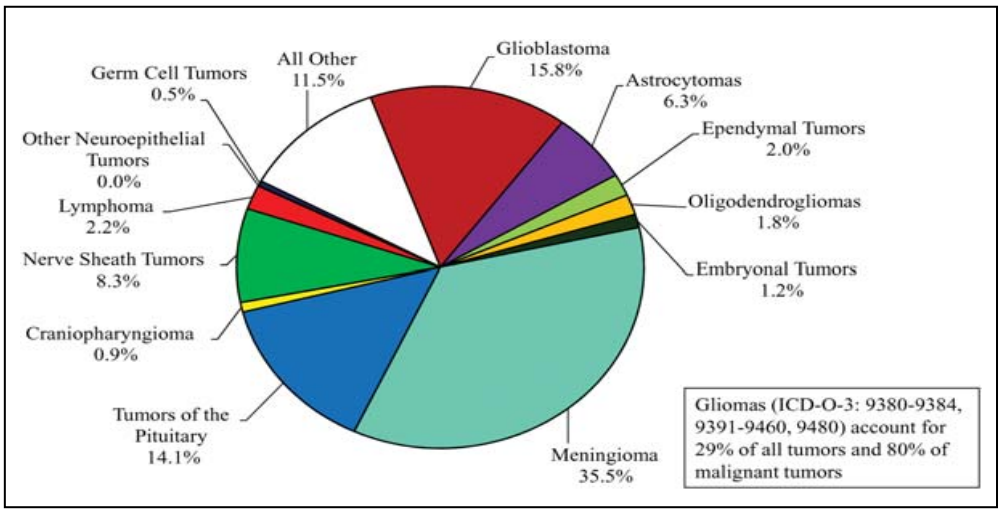


Figura 1: Distribuição dos tumors cerebrais por histologia. Fonte: *Neuro-Oncology* (Dolecek *et al*., 2012).

A **Tabela 1** resume de forma simplificada as principais características epidemiológicas dos GBM. A análise desses dados torna evidente a importância do estudo de novas abordagens terapêuticas para esse tipo de glioma, o qual por consequência da sua malignidade, mantém altos os índices de fatalidades na população (Dolecek *et al*., 2012).

Tabela 1 - Características Epidemiológicas do Glioblastoma

Glioblastoma Multiforme:
⇒ **Representam 80% dos tumores cerebrais malignos**
⇒ **Mortalidade de 4,28 por 100 mil ao ano (2005-2009, EUA)**
⇒ **Taxa de sobrevivência de 5 anos menor que 5%**
⇒ **24,560 novos casos nos Estados Unidos em 2013**

2. GLIOMAS

Os gliomas são tumores derivados da transformação maligna de precursores neurogliais, tais como precursores de oligodendrócitos (OPCs), ou células-tronco neurais (NSCs) residentes majoritariamente na Zona Subventricular (SVZ) do

encéfalo, na qual ocorre neurogênese pós-natal (Liu *et al.;* Siebzehnrubl *et al.*, 2011).

Resumidamente, as células gliais participam de funções vitais do SNC: além da sua função básica de suporte mecânico elas ainda mantêm um papel na função sináptica, formação da mielina, reparo pós-lesão e no desenvolvimento do SNC (Brodal, 2010). Além disso, a glia possui a capacidade mitótica, ausente nos neurônios, sendo este um dos motivos para estar suscetível a transformação neoplástica. Os estudos na área ainda reduzidos no que diz respeito a causas da transformação, já que os fatores ambientais conhecidos são poucos, assim como alterações hereditárias relacionadas ao surgimento de gliomas. Os mecanismos e características celulares e moleculares da tumorigênese dos gliomas serão abordados no **Capítulo II**, com mais detalhes.

2.1. Classificação dos gliomas

O gliomas podem receber diversas classificações dependendo do critério escolhido (**Figura 2**): de acordo com o tipo celular com o qual mais se assemelha fenotipicamente (astrócitos, oligodendrócitos, células ependimais ou misto), seguindo a classificação da Organização Mundial da Saúde (OMS) em baixo (tipos I e II) ou alto (III, IV) grau, de acordo com a sua malignidade e prognóstico e também pela sua localização (Louis *et al*., 2007).

Mais recentemente, estudos moleculares de alta tecnologia vêm sendo empregados para a classificação de centenas de amostras de gliomas de acordo com as suas semelhanças genéticas e moleculares. Os tipos são classificados basicamente por diferenças nas expressão gênica. Dois estudos foram importantes para a classificação em subgrupos moleculares: Verkaak *et al*., 2010 e Yan *et al*., 2012. O primeiro classifica em 4 tipos principais sendo o pró-neural de melhor prognóstico e o mesenquimal com prognóstico pobre, incluindo GBM. O segundo estudo classifica os gliomas em 3 grupos sendo o G1 similar ao pró-neural e o G3 similar ao mesenquimal (**Figura 2**).

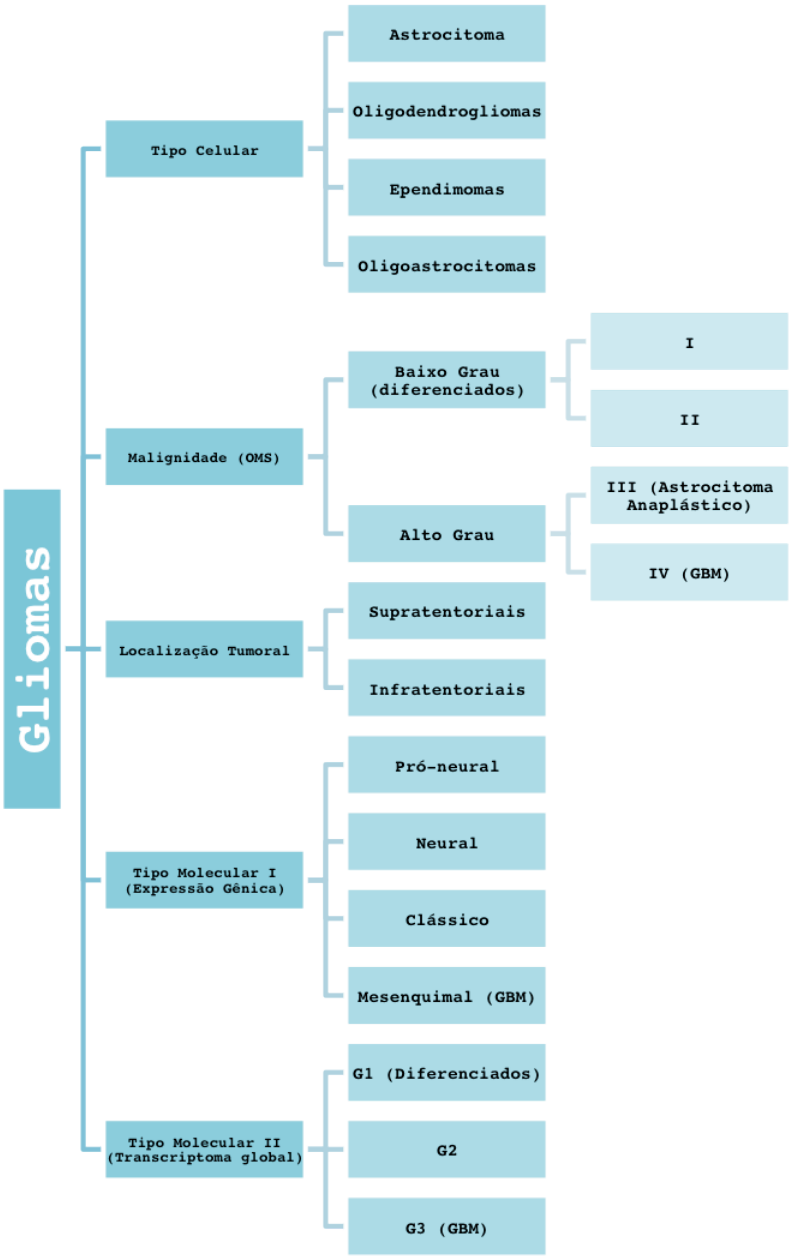


Figura 2: Diagrama hierárquico representando as diferentes classificações dos gliomas

2.2. Tratamento de glioblastomas

Na maior parte dos casos, quando viável, primeiramente é feita a ressecção cirúrgica da massa tumoral. Seguido dela, é feito o tratamento com radioterapia em conjunto com o quimioterápico temozolamida (TMZ), o qual mostrou aumento

na taxa de sobrevivência de 16% com pouca toxicidade no grupo tratado com as duas terapias no estudo de Stupp *et al.,* 2005. Ainda assim, após o tratamento é comum a recidiva do câncer e a TMZ mostrou melhora na sobrevida de 12 para 14 meses apenas.

Fazendo uso da alta vascularização dos GBM e da alta expressão do Fator de Crescimento Vascular (VEGF), anticorpos monoclonais anti-receptor de VEGF (Bevacizumab, Avastin®) pareciam uma escolha adequada para o tratamento. Com esse racional o Bevacizumab foi utilizado em dois ensaios clínicos prospectivos e mostrou melhora em pacientes com recidiva, sendo aprovado pela *Food and Drug Administration* EUA (FDA) como um agente único no GBM recorrente (revisto por Chamberlain, 2011), todavia, o tratamento de GBM *in vivo* com um anticorpo contra VEGFR-2 inibiu a angiogênese mas também aumentou a invasividade tumoral ao longo da microvasculatura (Kunkel *et al*., 2001). Esses dados deixam evidentes as possíveis desvantagens do uso do Bevacizumab e a necessidade do uso de um agente antiinvasivo em conjunto com a terapia (Nakabayashi *et al.*, 2010).

Atualmente os efeitos do Bevacizumab ainda estão sendo melhor avaliados, já que em outros tipos de tumor essa terapia não apresentou efeitos benéficos. Portanto, a malignidade dos GBM, que combinada ao rápido crescimento e efeitos colaterais severos no SNC, além da constante recidiva subsequente às atuais terapias disponíveis, exige um maior esforço da comunidade científica para a busca de novas terapias mais promissoras para os gliomas malignos.

3. CÉLULAS-TRONCO TUMORAIS

Os tumores podem seguir dois modelos de crescimento tumoral: o primeiro é o modelo de crescimento estocástico (revisto por Clevers, 2011), no qual todas as células da massa tumoral têm igual probabilidade de evoluir geneticamente e adquirir as características descritas como "marcos tumorigênicos", as quais são necessárias para evolução do tumor (Hanahan e Weinberg, 2011). Esse modelo também é chamado de evolução clonal. O segundo é denominado de "modelo hierárquico" e é baseado na capacidade de apenas alguns subgrupos celulares

proliferarem indefinidamente, gerando subsequentes populações celulares mais diferenciadas e menos proliferativas. É notável que esse conceito também é designado às populações de células-tronco normais dos tecidos sadios, com a diferença que as células-tronco tumorais (CSC) possuem as diversas capacidades de uma célula maligna e ainda com o diferencial de poderem iniciar novas metástases ou novos tumores quando isoladas e injetadas em modelos animais de transplante de xenoenxerto (Clevers, 2011 e O'Brien *et al*., 2010).

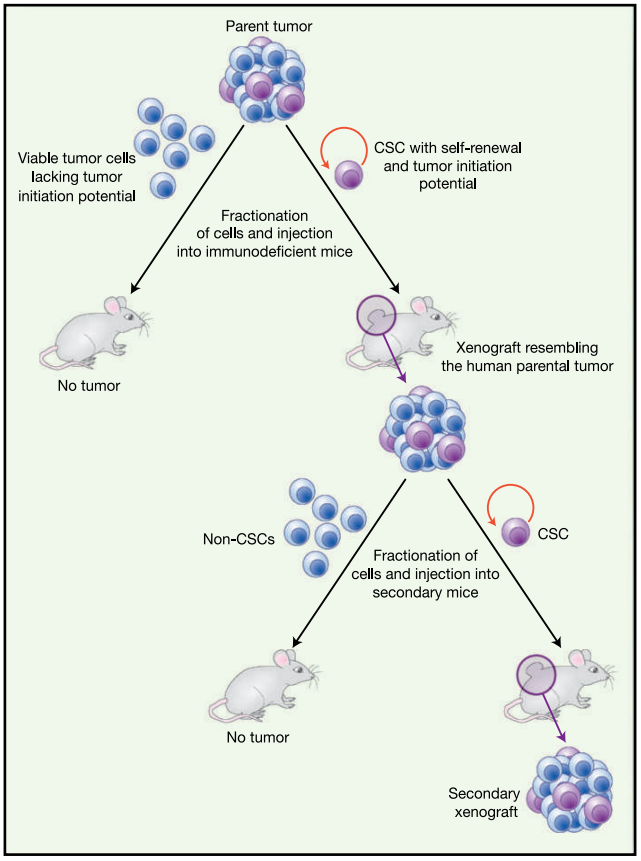


Figura 3: Ensaio de xenoenxerto utilizado para avaliação da capacidade tumorigênica em tumores hierárquicos. Fonte: *Clinical Cancer* Research (O'Brien *et al.*, 2010).

3.1. Células-tronco de gliomas

Em 2003, uma população de CSCs foi detectada em culturas de tumores cerebrais, incluindo gliomas (Singh *et al.,* 2003 e 2004). Através do ensaio de xenoenxerto foram identificadas células capazes de iniciar tumores *in vivo*.

Inicialmente, apenas a fração celular carregando o antígeno característico de células-tronco CD133 foi capaz de dar origem a novos tumores. Mais ainda, essa subpopulação apresentou uma capacidade acentuada para a proliferação, auto-renovação e diferenciação.

Nos GBM o isolamento dessas células mostrou-se possível não só pelo isolamento por citometria de fluxo ou, *Fluorescence-activated cell sorting* (FACS), mas também pela capacidade dessas células de proliferarem em suspensão, independentes de adesão ao substrato. Quando elas são cultivadas em meio apropriado, ocorre a formação de estruturas denominadas de tumoresferas (similares às neuroesferas geradas pelo cultivo *in vitro* de NSCs), as quais são enriquecidas para este tipo celular (Yuan *et al.,* 2004). Posteriormente, em outro estudo (Liu *et al.,* 2006), as células $CD133^+$ isoladas por FACS e capazes de gerar tumoresferas demonstraram maior expressão gênica de marcadores associados a precursores neurais (como, por exemplo, Nestina e MELK), resistência à apoptose (e também à anoikis). Neste mesmo estudos células $CD133^+$ foram associadas a tumores recorrentes e mostraram-se mais resistentes a quimioterápicos usuais em contrapartida com as $CD133^-$.

Mais recentemente, Lathia *et al.,* 2011, comprovaram pela primeira vez a funcionalidade das CSCs dentro dos GBM: após injetarem um pequeno número de CSCs derivadas de GBM primários em um animal imunodeficiente formaram-se tumores exibindo uma hierarquia celular. Tendo em vista que essas células eram transformadas para expressar proteína verde fluorescente - *green fluorescent protein* (GFP) - foi possível rastrear as células injetadas e as suas proles dentro do tumor ao longo do tempo. Os autores concluíram que as CSCs, ao contrário das células $CD133^-$, são responsáveis por propagar tumores heterogêneos *in vivo*, mesmo em pequenas quantidades. Esse estudo frisa o papel dessas células na recidiva tumoral após diversas terapias, já que apenas um pequeno número dessas células é capaz de gerar um tumor complexo, caso elas sobrevivam. A **Figura 4** resume as principais características e funções das células-tronco tumorais (também abordadas no **Capítulo II**), demonstrando a sua importância como alvo terapêutico.

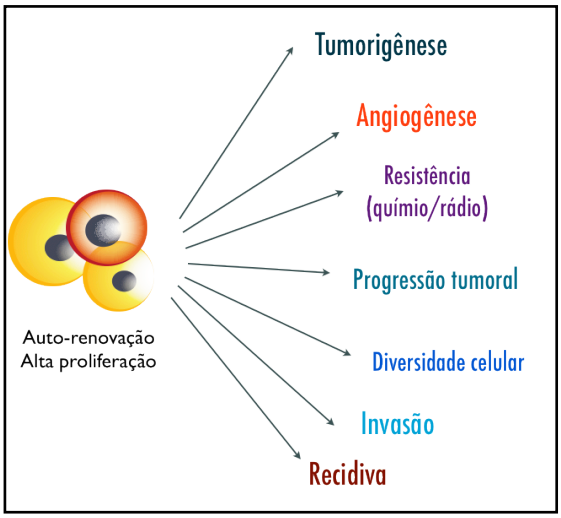


Figura 4: As principais características e funções das células-tronco tumorais.

4. MODULAÇÃO EPIGENÉTICA

O estudo da epigenética compreende os mecanismos pelos quais a expressão gênica é regulada sem a alteração da sequência genômica. Tais mecanismos são determinantes para o destino das células, já que permitem a adaptação às variações das condições ambientais e podem ser herdados (Cavalli, 2006).

A cromatina contém diversas proteínas que são necessárias para a sua montagem e empacotamento em eucromatina ou heterocromatina, bem como para a replicação e transcrição do DNA e modificações pós-transcricionais das histonas (Li, 2002). Entre os principais tipos de alterações epigenéticas encontram-se: a metilação do DNA, as modificações de histonas e os RNAs reguladores não-codificantes (Hsieh e Gage, 2005). Essas modificações, por conseguinte, ditam o quanto reguladores transcricionais podem ter acesso ao DNA, regulando programas específicos da transcrição gênica.

A unidade fundamental da cromatina, o nucleossomo, consiste em 147 pares de bases de DNA genômico enrolado em torno de um octâmero de quatro histonas centrais (H2A, H2B, H3 e H4), enquanto a histona H1 é a histona de ligação entre dois nucleossomos. As caudas N-terminais das histonas

nucleossomais estão sujeitas a várias modificações, incluindo acetilação, metilação, fosforilação e ubiquitinação. Essas modificações de histonas podem, a princípio, ser mantidas através da divisão celular (tanto mitose quanto meiose) e são, assim, consideradas como mecanismos de epigenética hereditária (Carafa *et al.,* 2013).

4.1. Desregulação epigenética no câncer

A complexidade dos fatores envolvidos na tumorigênese vai muito além das alterações genéticas, como já se é sabido. Mudanças no microambiente e, como consequência, na regulação epigenética das células pré-malignas parecem estar associados com diversos aspectos da manutenção tumoral.

Mudanças no padrão de metilação do DNA representam uma das mais estudadas alterações epigenéticas no câncer (Ducasse e Brown, 2006): hipometilação nas sequências repetidas dos telômeros já foram associadas a instabilidade gênomica, a qual é um fator associado à malignidade tumoral. Além disso, hipometilação em genes específicos está associada a invasão e metástase tumoral, bem como em promotores de genes supressores tumorais, causando o silenciamento desses genes.

O nível de acetilação das histonas em regiões promotoras também foi demonstrado contribuir com a desregulação da expressão gênica, estando associada com a carcinogênese e progressão tumoral (Ducasse e Brown, 2006).

4.2. Acetilação de histonas

A acetilação é uma modificação covalente pós-transcricional que ocorre com maior frequência nas lisinas das proteínas, neste caso, nas histonas. Mais especificamente elas são encontradas nas caudas amino-terminais dessas proteínas por adição do grupo acetila pelas histonas acetil-transferases (HATs). As formas mais comuns de acetilação ocorrem nas lisinas 9 ou 14 das histonas 3 (H3K9ac, H3K14ac) promovendo o relaxamento do nucleossomo por diminuir a

interação de carga positiva das caudas das histonas com o esqueleto de fosfato do DNA, o qual é carregado negativamente.

As enzimas histonas desacetilases (HDACs) têm atividade inversa: por diminuição da acetilação das caudas das histona, o DNA é empacotado em cromatina condensada, o que acaba por reprimir a transcrição gênica (**Figura 5**; Hsieh e Gage, 2005).

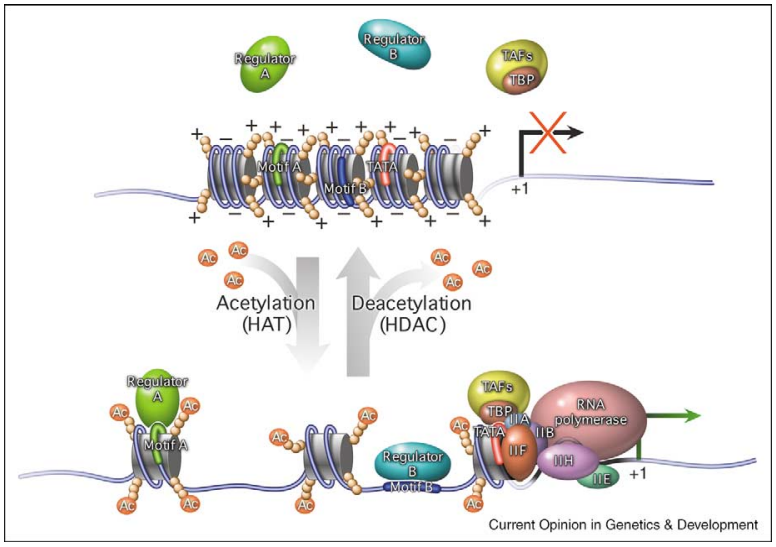


Figura 5: Regulação da expressão gênica através da acetilação das histonas. Fonte: *Current Opinion in Genetics & Development (*Hsieh e Gage, 2005).

A acetilação das histonas regula diversos aspectos da transcrição gênica associados ao desenvolvimento e câncer. No que diz respeito a diferenciação neuronal, estudos já demonstraram que o recrutamento de HDACs para os promotores de genes que regulam a neurogênese e diferenciação neural é essencial para a repressão dos mesmos genes em células não-neurais, demonstrando que a manutenção da acetilação é fundamental para a diferenciação celular (Li, 2002).

4.3. Histona desacetilases (HDACs)

Em humanos já foram descritos 18 HDACs diferentes, os diversos tipos dessa enzima existem para uma regulação mais fina da expressão gênica. Dessa forma diferentes grupos de enzima agem em grupos de genes específicos. Os tipos humanos de HDACs podem ser classificados em cinco classes principais

com base na similaridade na sequência de HDACs de levedura e na sua função (Xu *et al.,* 2007). A **Tabela 2** foi adaptada de Dokmanovic *et al.,* 2007, e resume a classificação atual das HDACs. A Classe I é a principal, baseada na distribuição ubíqua dos seus representantes e no seu principal papel de reguladora da acetilação global. Classe III é a única classe que não utiliza zinco no sítio catalítico e seus representantes são denominadas Sirtuínas.

Tabela 2: Classificação das HDACs

Classe	HDACs	Localização
I	1, 2, 3, 8	Núcleo
IIA	4, 5, 7, 9	Núcleo/Citoplasma
IIB	6, 10	Citoplasma
III	SIRT1-7	----------
IV	11	Núcleo/Citoplasma

Apesar de as mutações de genes que codificam as HDACs raramente serem encontrados em tumores (Lafon-Hughes *et al.,* 2008), a expressão alterada e o recrutamento aberrante de HDACs já foram relatados: a super-expressão de HDAC1, HDAC2, HDAC3, HDAC6 e SIRT7 foi identificada em câncer de mama, cólon, próstata, tireóide, colo do útero e gástrico.

A Classe I de HDAC se destaca como um alvo terapêutico promissor, já que seus representantes parecem regular diversos aspectos da malignidade tumoral. A enzima HDAC1 foi identificada primeiramente em ratos tendo a sua expressão regulada por fatores de crescimento e sendo altamente expressa em células proliferativas e transformadas (Bartl *et al.,* 1997). De acordo com Jurkin *et al.,* 2011, o *knockdown* de HDAC1, mas não de HDAC2, afetou o crescimento de células de osteossarcoma humano, câncer de mama e HeLaS3. Especificamente HDAC2 parece ter função anti-apoptótica, já que o *knockdown* de HDAC2 em células cancerígenas resultou em um fenótipo mais diferenciado e causou aumento de apoptose causada por níveis aumentados de p21. Além disso, o *knockdown* de HDAC2 em células de câncer de mama induziu a atividade de

ligação de p53 levando, então, à inibição da proliferação e senescência celular (Harms e Chen, 2007).

4.3.1. Inibidores de HDAC

Ainda não está claro se a desregulação epigenética é uma causa ou consequência da câncer. Apesar disso, a constatação dessa anormalidade surge como uma nova oportunidade terapêutica através da reversão das aberrações epigenéticas.

Os inibidores de HDACs (HDACis) incluem grupos de moléculas capazes de inibir a atividade de HDACs clássicas (I e II), por se ligarem aos sítios catalíticos contendo zinco dessas enzimas induzindo diversos efeitos nas células. Devido à sua capacidade de reverter aberrações epigenéticas, há um crescente interesse no desenvolvimento dessas moléculas, já que elas apresentam um enorme potencial para a regulação gênica, podendo, assim, ser utilizadas para potencializar os efeitos de outros medicamentos ou apenas como agentes únicos.

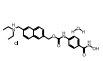
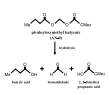
Em células de câncer essas drogas são eficazes na indução de parada do ciclo celular, apoptose, diferenciação celular e autofagia (Carafa *et al.,* 2013). Além disso, a inibição da atividade de HDAC também parece ser seletiva, já que ela levou à diminuição da proliferação e aumento de apoptose em células transformadas, mas não em células normais (Warrener *et al.,* 2003). Warrener e colaboradores também observaram que o uso de HDACis incluindo a Tricostatina A (TSA), SAHA e butirato de sódio (NaBu) leva à saída prematura da mitose, causando defeitos no *checkpoint* do fuso mitótico levando à apoptose por parada prolongada em G1 dependente ou não de p53.

Além de modular o ciclo celular, o HDACi ácido valpróico foi capaz de diferenciar progenitores multipotentes em neurônios e suprimir a diferenciação glial. O tratamento de OPCs com TSA também inibiu a diferenciação oligodendrocítica incluindo traços morfológicos e marcadores específicos (Hsieh e Gage, 2005).

Devido a esses efeitos diversos, HDACis disponíveis atualmente obtiveram resultados promissores em estudos clínicos de Fase I, II e III em mais de um tipo de câncer (de Almeida *et al.,* 2011).

A tabela a seguir foi extraída de Dokmanovic *et al.,* 2007, e classifica os principais HDACis de acordo com a sua estrutura e potência, mostrando algumas informações relevantes sobre esses compostos. Basicamente os HDACis podem ser classificados em hidroxamatos (TSA), peptídeos cíclicos, ácidos alifáticos (NaBu) e benzamidas (os mais específicos).

Tabela 3: HDACis (fonte: Dokmanovic, 2007**)**

Class	Compound	Structure	HDAC Target (Potency)	Effects on Transformed Cells	Stage of Development (Reference)
Hydroxamates	TSA		Class I and II (nmol/L)	TD; GA; A; AI; AE	N/C
	SAHA, Zolinza, vorinostat		Class I and II (μmol/L)	TD; GA; AI; AE; MF; AU; S; PP; ROS-CD	Merck Food and Drug Administration approved for CTCL (4)
	CBHA		N/A (μmol/L)	GA; A; AI; AE	Merck (4)
	LAQ-824		Class I and II (nmol/L)	GA; A; AI	Novartis phase I (discontinued)
	PDX-101		Class I and II (μmol/L)	GA; A	TopoTarget phase II (57)
	LBH-589		Class I and II (nmol/L)	GA; A; ROS-CD	Novartis phase I (51)
	ITF2357		Class I and II (nmol/L)	GA; A; AI	Italfarmaco phase I (56)
Cyclic peptide	PCI-24781 Depsipeptide (FK-228)	NA 	Class I and II (NA) Class I (nmol/L)	N/A TD; GA; A; AI; AE; MF; ROS-CD	Pharmacyclics phase I Gloucester Pharmaceuticals phase IIb for CTCL and PTCL (63) phases I and II
Aliphatic Acids	Valproic Acid		Class I and IIa (mmol/L)	TD; GA; A; S	Abbot phase II
	Phenyl butyrate		Class I and IIa (mmol/L)	TD; GA; A; AI; AE	Phase II
	Butyrate		Class I and IIa (mmol/L)	TD; GA; A; AI; AE	Phase II
	AN-9		N/A (μmol/L)	TD; GA; A	Titan Pharmaceuticals phase II
Benzamides	MS-275		HDAC1, HDAC2, HDAC3 (μmol/L)	TD; GA; A; AI; AE; ROS-CD	Schering AG phase II (51)
	MGCD0103		Class I (μmol/L)	TD; GA; A	Methylgene phase II (60)

Abbreviations: GA, growth arrest; TD, terminal differentiation; A, apoptosis; AI, cell death by activating intrinsic apoptotic pathway; AE, cell death by activating extrinsic apoptotic pathway; MF, mitotic failure; AU, autophagic cell death; S, senescence; PP, polyploidy; ROS-CD, reactive oxygen species–facilitated cell death; N/A, not available; CBHA, M-carboxycinnamic acid bishydroxamate; CTCL, cutaneous T-cell lymphoma; CTCL, peripheral T-cell lymphoma.

Como pode ser observado em suas estruturas TSA, SAHA, e LAQ824, tem três componentes básicos (**Tabela 3**): (1) um radical de ácido hidroxâmico que quela o zinco ativo e por ligações de hidrogênio desloca a molécula de água nucleofílica presente no sítio ativo, (2) um espaçador hidrofóbico com dimensões capazes e (3) um tampão hidrofóbico que bloqueia a entrada do sítio ativo (Drummond, 2004). A TSA é geralmente considerada um inibidor de HDAC não específico, uma vez que tem um Ki semelhante para todas as isoformas examinadas e por isso pode ser denominada como um pan-inibidor.

Um estudo em câncer de pulmão, câncer de mama e células de melanoma, o qual comparou a atividade de TSA com o HDACi depsipeptídeo (Chang et al., 2012), mostrou que TSA apresenta uma maior especificidade para células de câncer em relação a células normais quando comparadas ao inibidor seletivo de Classe I depsipeptídeo, podendo essa ser um melhor agente anticâncer quando comparada a outros HDACis. Curiosamente os diferentes HDACis mostraram sensibilidades divergentes para cada linhagem celular estudada, causando também diferentes fenótipos, o que destaca a capacidade dessas moléculas de afetarem também outros aspectos da regulação gênica, que não a acetilação das histonas, causando os demais efeitos nas células como a citotoxicidade.

Apesar da grande eficácia que os HDACis vem mostrando em ensaios clínicos e pré-clínicos, poucos, como o SAHA, já foram aprovados para o uso clínico. Ainda que não haja nenhum ensaio clínico utilizando a TSA para terapia antitumoral, ela parece oferecer uma ótima combinação de eficácia e especificidade para diversos tumores (Chang *et al*., 2012). Atualmente são raros os trabalhos científicos que estudam a TSA em GBM e nenhum estudo na literatura mostra os efeitos dessa molécula em CSC, incluindo as células-tronco de glioma. Por essas razões é necessária uma investigação direcionada para os efeitos do HDACi TSA em células de GBM humano e, visando a sua eficácia terapêutica, é preciso também averiguar se esse inibidor é capaz de afetar as células-tronco tumorais, as quais são reconhecidas por dirigirem a progressão e resistência desses tumores.

OBJETIVOS

Tendo em vista a busca de uma nova terapia mais promissora para os gliomas malignos, os quais contribuem para os altos os índices de fatalidades na população, este trabalho visa avaliar o tratamento de células de glioblastoma humano com o inibidor de histonas desacetilases (HDACi) Triscostatina A (TSA) e mais especificamente:

1. Avaliar os efeitos na viabilidade, proliferação e sobrevivência celular do tratamento com TSA na linhagem celular de glioblastoma humano (GBM) U87-MG.
2. Avaliar se o tratamento com TSA afeta a viabilidade, proliferação e sobrevivência das células-tronco tumorais na linhagem U87-MG através da análise de tumoresferas enriquecidas para essas células, as quais foram demonstradas participarem da progressão e resistência tumoral.
3. Avaliar os mecanismos moleculares pelos quais a TSA atua nessas células, levando em consideração a gama de efeitos já descritos para outros HDACis em outras linhagens celulares de tumores.

CAPÍTULO II

ARTIGO DE REVISÃO

O artigo ***" Glioma Revisited: From Neurogenesis and Cancer Stem Cells to the Epigenetic Regulation of the Niche "*** foi submetido e aceito pela revista *Journal of Oncology* e será apresentado a seguir em sua forma original.

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Review Article

Glioma Revisited: From Neurogenesis and Cancer Stem Cells to the Epigenetic Regulation of the Niche

Felipe de Almeida Sassi,[1,2] Algemir Lunardi Brunetto,[1,3,4] Gilberto Schwartsmann,[1,4,5] Rafael Roesler,[1,2,4] and Ana Lucia Abujamra[1,3,4,6]

[1] *Cancer Research Laboratory, University Hospital Research Center (CPE-HCPA), Federal University of Rio Grande do Sul, 90035-903 Porto Alegre, RS, Brazil*
[2] *Laboratory of Neuropharmacology and Neural Tumor Biology, Department of Pharmacology, Institute for Basic Health Sciences, Federal University of Rio Grande do Sul, 90035-903 Porto Alegre, RS, Brazil*
[3] *Children's Cancer Institute and Pediatric Oncology Unit, Federal University Hospital (HCPA), 90035-903 Porto Alegre, RS, Brazil*
[4] *National Institute for Translational Medicine (INCT-TM), 90035-903 Porto Alegre, RS, Brazil*
[5] *Department of Internal Medicine, School of Medicine, Federal University of Rio Grande do Sul, 90035-903 Porto Alegre, RS, Brazil*
[6] *Medical Sciences Program, School of Medicine, Federal University of Rio Grande do Sul, 90035-903 Porto Alegre, RS, Brazil*

Correspondence should be addressed to Ana Lucia Abujamra, aabujamra@hcpa.ufrgs.br

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Gliomas are the most incident brain tumor in adults. This malignancy has very low survival rates, even when combining radio- and chemotherapy. Among the gliomas, glioblastoma multiforme (GBM) is the most common and aggressive type, and patients frequently relapse or become refractory to conventional therapies. The fact that such an aggressive tumor can arise in such a carefully orchestrated organ, where cellular proliferation is barely needed to maintain its function, is a question that has intrigued scientists until very recently, when the discovery of the existence of proliferative cells in the brain overcame such challenges. Even so, the precise origin of gliomas still remains elusive. Thanks to new advents in molecular biology, researchers have been able to depict the first steps of glioma formation and to accumulate knowledge about how neural stem cells and its progenitors become gliomas. Indeed, GBM are composed of a very heterogeneous population of cells, which exhibit a plethora of tumorigenic properties, supporting the presence of cancer stem cells (CSCs) in these tumors. This paper provides a comprehensive analysis of how gliomas initiate and progress, taking into account the role of epigenetic modulation in the crosstalk of cancer cells with their environment.

1. Introduction

Gliomas are the most common brain tumor in adults, with very low survival rates, even when combining radio- and chemotherapy. Among the gliomas, glioblastoma multiforme (GBMs) is the most common and aggressive type, and patients frequently relapse or become refractory to conventional therapies. GBMs are usually detected upon the incidence of neurological symptoms, rendering it a disease that is diagnosed already at an advanced stage. Other glioma types include astrocytomas, oligodendrogliomas, and mixed oligoastrocytomas, which are characterized according to their histological features.

How is it that such a malignancy arises in this carefully orchestrated organ, where cellular proliferation is barely needed to maintain its function? This question has intrigued scientists until very recently, when the discovery of the existence of proliferative cells in the brain overcame such doubts. Even so, the precise origin of gliomas still remains elusive. Fortunately, with new advances in molecular biology, researchers have been able to depict the first steps of glioma formation and to accumulate knowledge about how neural stem cells and its progenitors become gliomas. Indeed, GBMs are composed of a very heterogeneous population of cells, which exhibit a plethora of tumorigenic properties, supporting the presence of cancer stem cells (CSCs) in these tumors.

In this paper a comprehensive analysis of how gliomas initiate and progress will be depicted. Several reports have already described each aspect of glioma formation separately. Here, however, we promote an overall landscape of this process, considering how the tumor environment, including its epigenetic mechanisms, may contribute to this disease, providing new insights for better therapeutic approaches.

It is important to understand normal physiological conditions before understanding the pathology itself. Therefore, this paper describes both physiological and pathological conditions together, to better relate the tumor microenvironment studies. There are five parts to this paper, ranging from the historical perspectives of the most relevant works in the field to a discussion of the role of epigenetic modulation in the crosstalk of cancer cells with their environment.

2. Part I: Physiological Neurogenic Niches

Before understanding the dynamics of tumor environment and its relationship with cancer cells, it is necessary to depict the physiological conditions in the brain which permit cellular proliferation and stemness, which are necessary for malignant transformation. Many normal stem cell niches from different tissues provide bright information about tumor stem cell behavior, in part because very often tumor stem cells are derived from stem cells of the same tissue of origin and because they may require the same signals to maintain themselves and proliferate in their microenvironment.

Although adult neurogenesis has been extensively discussed over the last century, it was only in 1998 that researchers in the field found *in vivo* evidence for human neurogenesis by screening postmortem brain tissues with the mitotic label bromodeoxyuridine (BrdU) [1], reviewed in [2]. Those findings have pushed brain tumor research to a new level, since it was clear that the brain indeed possessed a source of stem cells, corroborating the thoughts that tumors are most likely originated from cells capable of proliferation (the other possible way being through dedifferentiation, in other words, when a more differentiated cell acquires the phenotype of a stem cell).

Adult neurogenesis is a complex process comprising the activation of a pool of stem cells, the proliferation of precursors, and the differentiation and functional maturity of the newborn cells. Postnatal neuronal production seems to be important not only in pathological conditions, such as epilepsy, ischemia, schizophrenia, and tumorigenesis, but also in normal functions such as learning, memory, and migration (reviewed in [2]). In the adult mammalian brain, neurogenesis is restricted to two areas. The most examined and largest niche is the subventricular zone (SVZ) of the forebrain, followed by the subgranular zone (SGZ) of the hippocampus.

2.1. Components of Neurogenic Niches. The SVZ is located in the lateral walls of the lateral ventricles and can be divided into four distinct layers based on its histological structure. The third layer is the most relevant one, since it consists of three distinct astrocyte cell types which participate in neurogenesis: stem cell astrocytes (type B cells, expressing glial fibrillary acidic protein; GFAP+) are more likely to be quiescent; however, they can be stimulated to generate neuroblasts (type A cells, GFAP−/Dlx2+/doublecortin+) through the rapidly dividing transit-amplifying cells (type C cells, GFAP−/Dlx2+) [3]. Neuroblasts originated in the SVZ migrate long distances along the rostral migratory stream (RMS) to the olfactory bulb (OB), where the majority differentiate into granule cells and a small population become periglomerular cells [3, 4]. Beside neurons and astrocytes, oligodendrocytes can also be generated in the adult SVZ [5] (reviewed in [6]), and the role of oligodendrocytes precursors in gliomagenesis will be further discussed in the paper. Type B astrocytes have a characteristic apical process contacting the ventricle and a basal process extending to the underlying blood vessels [7]. In addition, they express neural stem cell markers (such as CD133 and nestin) and are labeled with proliferative markers such as Ki67 and phosphohistone H3 [8]. This subpopulation of slowly dividing neural stem cells (NSCs) can proliferate *in vivo*; moreover they can form neurospheres with multipotential and self-renewal abilities *in vitro* [7, 9], reviewed in [2, 8].

The neurosphere assay is the current gold standard for determining the presence of NSCs [10]. By culturing the cells in serum-free, growth factor-supplemented media in low adherent conditions, stem cells divide continually, forming undifferentiated and multipotent spheres denominated neurospheres, which can be dissociated and replated to expand the culture and select the cells with self-renewal capacity. Neurospheres have been isolated from both the SVZ and SGZ, and they were capable of generating cells with neuronal, oligodendrocyte, and glial markers [11]. The neurosphere assay, which will be discussed in this paper, is also important for evaluating the stemness of brain tumor stem cells (tumorsphere assay) as well.

As thoroughly discussed by Quiñones-Hinojosa and collaborators [2, 12], the SVZ is a complex microenvironment composed by different cell types interacting among themselves and with various extracellular molecules that promote neurogenesis. Beside astrocytes, microglia, and oligodendrocytes, endothelial cells also participate in the niche and directly interact with NSCs to enhance neurogenesis *in vitro*. The extracellular matrix (ECM) components, such as tenascin-C, basal lamina components, and chondroitin sulfate proteoglycans [13], also contribute to the neurogenic environment by binding, presenting, and sensitizing various growth and signaling factors to neural precursors (Figure 1(a)).

Furthermore, the cell surface carbohydrate Lewis X (LeX)/CD15 is an epitope that is expressed in all spheres-forming cells from the SVZ and which is shed into their environment, being shown to play an important role in the neurogenic niche modulation by capturing factors from the blood vessels [14].

The hippocampus is another area of the mammalian brain that continues to produce neurons postnatally. Also using BrdU labeling, Kuhn and colleagues [15] confirmed that, in the adult rat brain, neuronal progenitor cells divide at the border between the hilus and the granule cell layer.

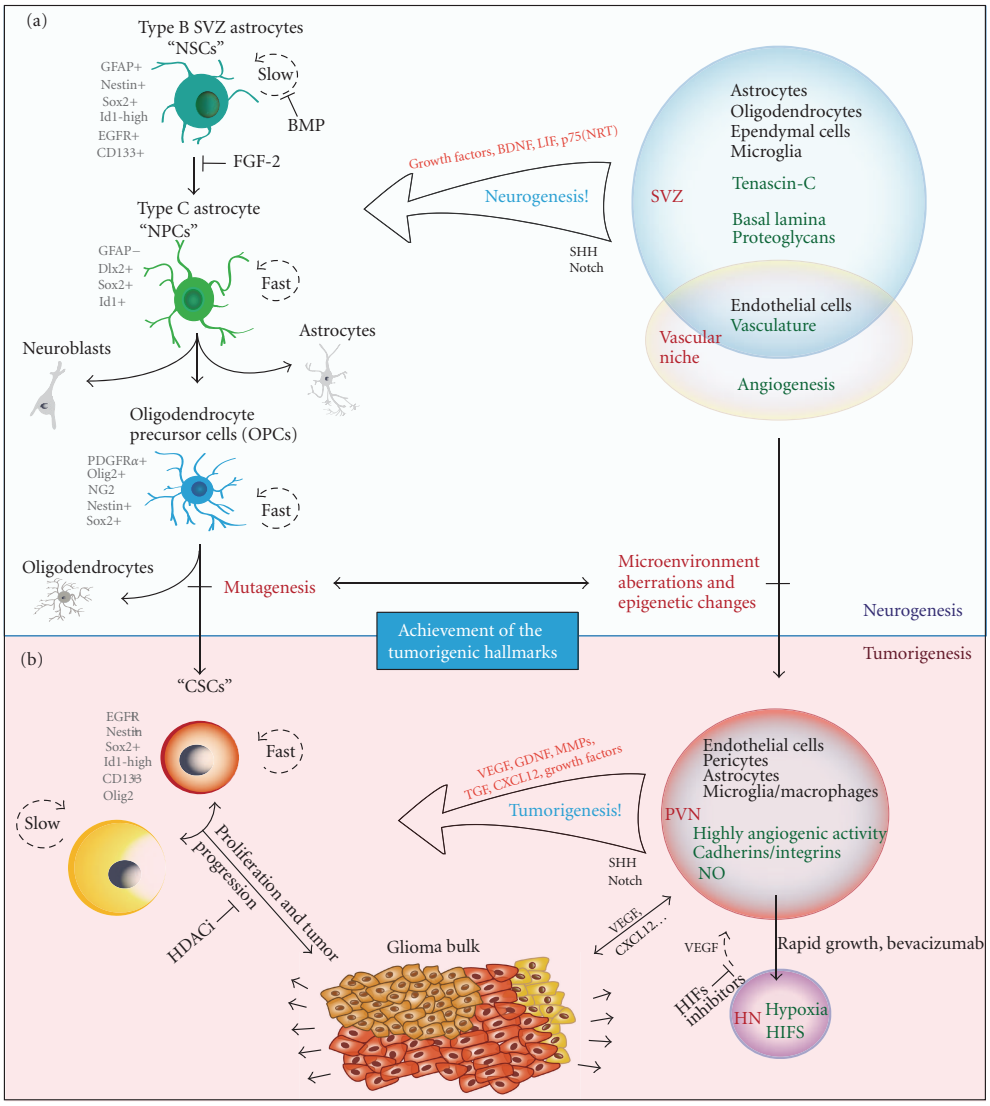


Figure 1: A summary of gliomagenesis. (a) The interactions between the neurogenic niche (subventricular zone, SVZ) and neural stem cells (NSCs) highlighting the most relevant cell types and the secreted factors that affect the neural proliferation. Oligodendrocyte progenitors are more likely to undergo malignant transformation. (b) The role of cancer stem cells (CSCs) in tumor progression. There are different subpopulations of CSCs, which may contribute to tumor heterogeneity. Histone deacetylase inhibitors (HDACis) may be effective against CSCs by promoting their differentiation. The perivascular niches (PVNs) provide growth factors that enhance CSC proliferation and self-renewal. Because of the rapid tumor growth, hypoxic niches (HNs) are formed and, through the action of HIFs, secrete VEGF, which in turn may lead to new vascular niches.

The newborn granule cells are capable of extending axonal projections along the fiber tract to their natural target area, the hippocampal CA3 region. Two populations of astrocytes have been defined in the SGZ: the radial astrocytes, which extend processes into the granule cell layer, are nestin+ and are able to divide, and the horizontal astrocytes, which extend basal processes under the granule cell layer and are nestin− and S100+ (reviewed in [6]).

In 1998, Eriksson and colleagues [1] finally detected BrdU-labeled cells in the adult human hippocampus, which were quantified in the granule cell layer and the subgranular zone of the dentate gyrus and in the hilus (CA4 region). BrdU-labeled cells also coexpress neuronal markers, indicating the presence of proliferating neural progenitor cells. The newly generated cells were able to survive and differentiate into cells with neuronal morphological and

phenotypic characteristics. Cells are generated daily in the young adult rodent dentate gyrus with a fraction integrating into the neuronal circuitry [16]. Current evidence suggests, however, that the proliferating cells of the hippocampus are multipotent progenitor cells instead of NSCs [17, 18].

2.2. Neurogenic-Associated Signaling. Most knowledge about the neurogenic niche in the hippocampus came from aging, learning, and memory studies regarding neurogenesis in animal models [14, 15, 18]. Data from numerous studies suggests that precursor activation and neurogenesis are intimately linked to activity levels at the synapse, such as that in the case of voluntary exercise and other novel external sensory experiences [19]. Moreover, long-term potentiation (LTP) has been shown to increase the proliferation of neural precursors in the dentate gyrus [20, 21]. Activation could be through the release of growth factors within the neurogenic niche. Brain-derived neurotrophic factor (BDNF) release is known to be enhanced with electrical activity; therefore it could mediate the synaptic stimulation.

Neurotrophic factors are known to regulate many aspects of the neural cell cycle. BDNF administered to rat SVZ-derived neuroblasts *in vitro* promoted the long-term survival of these cells. Furthermore, following intraventricular infusion of BDNF, other groups also observed increases in the number of newly formed neurons in adjacent structures (reviewed in [22]). In BDNF-null mice, defects in SVZ neurogenesis are not detectable until at least 2 weeks after birth [23]. This indicates that SVZ-derived stem cells destined for the OB may not depend upon BDNF signaling during embryonic and early postnatal development, becoming sensitive to extrinsic factors, such as BDNF, only after birth. Consequently, BDNF is a relevant factor for the survival of adult stem cells and its progeny.

Another neurotrophic receptor participating in adult neurogenesis is the orphan receptor p75(NTR), a member of the tumor necrosis receptor superfamily. p75(NTR) exerts its potent effects on nervous system development through a variety of mechanisms (reviewed in [22]). A high degree of colocalization was found between p75(NTR) and nestin, a marker that labels proliferating cells within the SVZ and RMS. *In vitro* assays show that this population of cells is responsible for the production of all neurospheres and that p75(NTR)-positive cells alone are neurogenic. Beside that, p75(NTR)-null mice show a 70% reduction in their neurogenic potential *in vitro* [22, 24].

It is not a surprise that growth factors play an important role in neuronal proliferation and survival. The fibroblast growth factor (FGF)-2, epidermal growth factor (EGF), transforming growth factor (TGF), ciliary neurotrophic factor (CNTF), and the vascular endothelial growth factor (VEGF) all are able to augment neural proliferation and interfere with neurogenesis (reviewed in [22]). When these growth factors are administrated intraventricularly, they are capable of increasing cellular proliferation, and when their receptors are blocked or knocked down, neurogenesis is significantly affected [25–31].

Platelet-derived growth factor (PDGF) is another key factor, which is known to be a regulator of oligodendrocyte production. PDGFRα+ astrocytes are present in the human SVZ [32], and almost 80% of SVZ astrocytes express PDGFRα [33]. Studies on the effects of PDGF signaling on neural progenitor cell differentiation demonstrate a proliferating effect on these cells and an inhibition of differentiation [34]. Furthermore, endogenously produced PDGF ligand was detected in cultures, suggesting that this pathway is regulating the proliferation of neural progenitor cells [31]. The vascular-derived molecules also show to locally regulate the adult NSC niche. Some of these molecules include leukemia inhibitory factor (LIF), BDNF, VEGF, and PDGF (reviewed in [35]).

Beside all of these promitotic regulators, studies in rats have elucidated the NSC quiescent mechanism. Researchers have found that quiescent NSCs are induced by autocrine production of bone morphogenetic proteins (BMPs), which induce terminal astrocyte differentiation without EGF and FGF2. Accordingly, the BMP antagonist, noggin, can replace conditioned medium to sustain continuous self-renewal. Noggin can also induce dormant cells to reenter the cell cycle, upon which they reacquire neurogenic potential. The crosstalk between FGF-2 and BMP, which is required to suppress terminal astrocytic differentiation and maintain stem cell potency during dormancy, is crucial to regulate NSCs propagation, dormancy, and differentiation [36] (Figure 1(a)).

Another marker has recently been shown to regulate NSC proliferation. High expression of Id1, a dominant-negative helix-loop-helix transcriptional regulator, identifies a rare population of GFAP+ astrocytes with stem cell attributes among the SVZ. The rare, long-lived, and relatively quiescent $Id1^{high}$ astrocytes self-renew and generate migratory neuroblasts that differentiate into OB interneurons. Cultured $Id1^{high}$ neural stem cells can self-renew asymmetrically and generate a stem and a differentiated cell expressing progressively lower levels of Id1. Id1+ cells, which were also GFAP+ and nestin+, were also evident in the subgranular layer [37].

Hence, adult neurogenic niches directly rule neuronal production and stem cell maintenance. In contrast, an inhibitory environment that is refractory to neurogenesis is present throughout most of the brain, since primary cells from neurogenic areas transplanted into nonneurogenic regions exhibit very limited neurogenesis (reviewed in [6]).

3. Part II: Gliomagenesis

Malignant gliomas, as with any other tumor type, may originate from a complex sequence of events that are necessary to allow the development of these aberrant organs within a normal tissue environment. Multistep tumor formation comprises a cascade that starts with a series of mutations in a pool of susceptible cells and ends with a whole tumor microenvironment that was built during this process and which allows cancer survival and progression. In this context, it is important to comprehend not only the features that a cell acquires to become a cancer cell, but also the role of the microenvironment components, such as different cell types and extrinsic molecules, in the tumor formation process.

3.1. First Steps towards Tumorigenesis. Any tumor must achieve basic requirements to successfully develop and grow. Hanahan and Weinberg [38] have logically classified the requirements for tumorigenesis into six basic hallmarks, some of them very remarkable in GBM. First, through the production and release of growth-promoting signals, such as those required for neurogenesis, glioma cells manage to power their own cell cycle and to modulate their own environment in a self-sufficient fashion. Evading growth suppressors comes as an important hallmark as well, since this ability complements the first one: cancer cells must deactivate programs that inhibit cellular proliferation, such as programs that depend on the action of tumor suppressor genes like those which encode for the RB (retinoblastoma-associated) and TP53 proteins. Both proteins act by regulating cellular programs such as proliferation, senescence, and apoptosis. TP53 is also a key regulator of another relevant hallmark, the capacity of resisting cell death signals. In addition, it is widely known that several GBM cell lines present mutant TP53, with variable levels of the protein [39].

In consequence of their rapid growth, tumors demand a larger amount of nutrients and oxygen when compared to normal tissues. These needs are illustrated by the tumor-associated neovasculature, generated by the process of angiogenesis. GBMs are highly angiogenic, and their neoformed vessels are thought to arise from the sprouting of preexisting brain capillaries. Nonetheless, recent findings [40] demonstrate that a population of glioblastoma stem-like cells (GSCs) may originate lineages other than neural lineages. Like normal neural stem cells, which are able to differentiate into functional endothelial cells *in vitro* and *in vivo* [41], *in vitro* cultures of GSCs in endothelial conditions generated progeny with phenotypic and functional features of endothelial cells. The authors have also demonstrated that a significant number of endothelial cells in glioblastoma present the same genomic alteration as tumor cells, indicating that a significant portion of the vascular endothelium has a neoplastic origin. In addition, GSCs closely interact with the vascular niche and promote angiogenesis through the release of VEGF and the chemokine stromal-derived factor 1 (CXCL12) [40]. Therefore, the constituents of signaling cascades and their crosstalks with the tumor microenvironment are crucial for cancer initiation and progression.

Although the necessary components for malignant transformation have been elucidated, the search for the originating cell that leads to glioma formation is still a work in progress. In the past years gliomas were thought to be originated from a transformation of the cell type that is predominant in each tumor, but that remained a speculation. Recently, as adult neurogenesis has been more carefully examined, it was shown that there are still mitotic niches in the postnatal brain. Researchers then focused their efforts on depicting the role of NSCs and neural precursors in glioma formation.

However, studies regarding the first steps towards glioma formation were hampered by the fact that cancer is a dynamic and progressive disease. Consequently, established tumors are just the endpoint of a complex cascade and provide no consistent clues of how they behaved before fully developed. In a very clarifying review [42], Clevers has depicted the role of cancer stem cells (CSCs) in tumor initiation and progression. It was proposed that, as these cells acquire oncogenic mutations, they hierarchically generate subpopulations of cells that have growth advantages among the others, enhancing tumor heterogeneity and dynamics and eventually extinguishing prior subpopulations of cells. Even if the subpopulation derived from the cell of origin persists along the tumor lifespan, its molecular blueprints will be significantly modified as a result of the high genetic instability of cancer cells, hindering its identification. Fortunately, new insights regarding gliomagenesis are emerging, making use of the knowledge acquired from the brain neurogenic niches and the appearance of genetically modified animal models to circumvent the difficulties of working with established tumors. Researchers could, then, for the first time, assess the formation regarding gliomas from the beginning.

3.2. The Subventricular Zone as a Tumorigenic Niche. Since the microenvironment in most areas of the brain is repressive to neurogenesis (reviewed in [43]), the neurogenic niches are probably the most vulnerable sites for the growth of transformed cells, since they are abundant in growth factors and thus permissive to proliferation. In addition, they harbor the brain cells with the most proliferative potential, cells that have a higher chance of becoming cancer cells than others [44]. Siebzehnrubl and colleagues [44] propose that the cell of origin of most gliomas may come from the SVZ since this is the largest neurogenic niche, containing the most proliferative cells in the adult brain. Regarding tumor localization, there is evidence that the majority of malignant astrocytic tumors contact the lateral ventricles [45]. The localization of tumors, most of which are benign, away from the lateral ventricles could be explained in part by the existence of progenitor cells away from the niche [46], in contrast with results from another research group which show that more than half of the GBMs studied were radiographically distinct from the ventricles, probably arising from the subcortical white matter and expanding towards the SVZ [47].

Alcantara Llaguno and collaborators [48] used a tamoxifen-inducible nestin−creERT2 transgene to deliver floxed tumor suppressors to the SVZ stem/precursor cells expressing nestin. The results showed that all adult mice subjected to SVZ targeting developed astrocytomas, thus establishing that mutation of these astrocytoma-relevant tumor suppressors in the neurogenic compartment *in vivo* is sufficient to induce tumor formation. They showed that, in contrast to normal adult neural stem cells that are strictly confined to the SVZ or SGZ, tumors arising from these cells or their progeny are not restricted to these niches and indeed migrate away from their normal locations, thus accounting for the presence of tumors elsewhere in the forebrain.

3.3. The Search for the Primordial Cell. It is known that stem cells are usually quiescent [49] and proliferate only when demanded. This confers a protective mechanism against transformation, since the more frequently a cell divides, the bigger the chance for it to accumulate mutations and

become a cancer cell. On the other hand, progenitors derived from NSCs are a pool of proliferating cells required for neurogenesis. These cells still have the potential to generate different lineages, such as oligodendrocytes and astrocytes, and because they are both multipotent and fast proliferating, they have the highest probability for transformation into a highly malignant tumor [44]. Indeed, type B cells in the SVZ are mainly quiescent and have been found resilient to transformation by c-myc, illustrating that quiescence confers a mechanism of protection [50]. A critical step in neurogenesis which enhances the odds for transformation is the transition of stem cells to transitory amplifying progenitors, a stage involving chromatin rearrangement and a switch from a cell that rarely proliferates to a cell that rapidly proliferates. If genetic lesions are not repaired and persist within these cells, they become incorporated into the dividing population, increasing the chance of further lesions. Whenever a glial progenitor cell reaches the tumorigenic hallmarks, it can result in the dedifferentiation to a more multipotent lineage, such as initiating cancer stem cells, leading to a high-grade glioma. Heterogeneous tumors may also arise from different cell types, because transforming events can affect more than one cell at once. The microenvironment may also increase the probability of transformation in adjacent cells by the release of growth factors [44].

In the same way, gliomas with differing genetic signatures may originate from different cell subtypes [51, 52]. A variety of mutations have been described in human astrocytomas: some of them disrupt cell cycle and apoptosis regulation (INK4A, CDK4, RB, TP53) while others participate in growth factor receptor signaling (EGF, PDGF, PTEN) [52]. More specific genetic models with expression targeted to individual cell types in the SVZ are leading to new insights in brain tumor formation. Such studies exploit particular genetic lesions in the mouse to generate animal models that mimic human malignancy, allowing the investigation of tumor development. Through cre/lox technology, mouse strains with germline or somatic heterozygous mutations at the TP53, NF1, and PTEN tumor suppressor sites developed high-grade astrocytomas with 100% penetrance [53]. TP53, NF1, and PTEN mutations are among the most frequent mutations reported for astrocytomas [50].

In a study by Lee and colleagues [53], human fetal NSCs underwent tumorigenic transformation through the introduction of genes such as v-myc and H-Ras, which resulted in heterogeneous glial tumors with some characteristics of cancer stem cells (small numbers of nestin+ neural stem-like cells). Considering the crucial functions of p53 in protecting cells against oncogenic transformation in a variety of cellular systems, the lower p53 transcriptional activity observed in v-myc-expressing cells may be responsible for the oncogenic transformation induced by the combination of both v-myc and H-Ras genes. Furthermore, this process did not occur when the cells lost neural stemness because of differentiation, indicating that the expression of factors responsible for H-Ras-induced oncogenic transformation may vary according to neural stemness characteristics. This may account for the differing susceptibility to oncogenic transformation between differentiated glial cells and NSCs.

3.4. Glial Progenitors as a Plausible Cell of Origin. Although many researchers have successfully focused their studies towards depicting the role of NSCs in gliomagenesis, a remarkable effort has been made along the same lines as those proposed by Siebzehnrubl and colleagues, highlighting the glial progenitor population as being much more susceptible to neoplastic transformation. Some relevant results were pointed by Canoll and Goldman in their review [46], such as the *in vivo* evidence that adult glial progenitors have the proliferative and self-renewing capacity needed to form malignant tumors. These results were obtained by studies that made use of infecting progenitors in the adult white matter with retroviruses that express PDGF, generating tumors that closely resembled human glioblastoma and that were composed of cells bearing the immunophenotype of oligodendrocyte progenitors (olig2+/NG2+/PDGFRα+). They also emphasized that glial precursors can be found throughout the brain and can behave in a malignant manner when overstimulated with high levels of growth factors such as PDGF and EGF. Such findings also point out the possibility that cancer stem cells can arise from glial progenitors beside the NSCs with SVZ origins.

Perhaps the most elucidating study regarding the cellular origins of gliomas emerged in 2011 by Liu and colleagues [54] (commented in [55]). Through mosaic analysis with double markers (MADM), they generated a mouse genetic mosaic system to analyze aberrations in individual cell lineages before the final transformation, allowing for the screening of the cell of origin. When mutations are introduced in stem/progenitor cells, it is extremely difficult to distinguish whether initial mutant cells directly transform or whether they simply pass on mutations to more restricted progeny that can undergo further malignant transformation and dedifferentiation into a cancer stem cell. After initiating p53/NF1 mutations sporadically in NSCs, they analyzed mutant NSCs and all of their progeny at pre-malignant stages. The MADM technique allowed Liu et al. to discriminate between cells and its progeny with oncogenic mutations by utilizing a GFP tracer from normal counterparts utilizing a RFP tracer over time. Only mutant NSCs generated neoplastic oligodendrocyte precursor cells (OPCs) which were PDGFRα+. All other NSCs-derived cell types, including NSCs themselves, remained mostly unaffected by the disruption of the two tumor suppressive pathways. When p53/Nf1 inactivation is targeted specifically to OPCs, tumors form as NSCs-derived gliomas. Interestingly, these tumors acquired the expression of NSCs genes, which could be misleading during analysis in further stages of the tumor development. The findings demonstrate that, in p53/Nf1 mutation-driven gliomas, mutations may initially occur in either NSCs or OPCs, but only OPCs provide the suitable cellular context needed for transformation.

Their studies emphasize the importance of the intersection between genetic mutations and the signaling context within the cell of origin. Furthermore, they showed that OPCs are particularly sensitive to p53/NF1 mutations, whereas NSCs and other brain cell types are much less responsive, opposing the results obtained by the genetically modified animal model in Lee's studies [53]. Liu's findings

have also pointed that nestin−driven mutagenesis results in OPC transformation away from the SVZ, where NSCs reside, with lesions starting at the gray matter and later migrating to the SVZ and white matter, where the tumor fully developed, perhaps with the benefit of the neurogenic niche. It is interesting to see malignant gliomas arising from the gray matter and moving further out, since Canoll's groups have previously shown that the transformation of glial progenitors by PDGF can also result in malignant gliomas in the white matter [56].

4. Part III: The Brain Tumor Microenvironment

Cells are continually receiving information from their microenvironment concerning how they should behave, and in the same way cancer cells cannot survive alienated from the surrounding tissues [38]. Once cancer cells start to propagate in their cradle and are established as developed tumors, they manage to construct a complex network in their own microenvironment. In the same way stromal cells from normal tissue restrict the tumor's malignant behavior, cancer cells collaborate for its survival. These feedbacks from both parts are determining for tumor progression or regression. Therefore, there is a constant communication and an intimate relationship between the tumor niche and its surroundings. This network consists of different cellular types beside the cancer cells themselves, being of note the extracellular matrix proteins and soluble signaling factors and cytokines.

Among the various cells present in the tumor bulk, the majority of nontransformed cells in gliomas are tumor-associated macrophages (TAMs, from nonneuronal tissues) and microglia. Macrophages are the predominant inflammatory cells infiltrating gliomas and most of the time microglia stand in the tumor bulk periphery [57]. Evidence suggests that the immune function of microglia might be suppressed when these cells are located inside the tumor, as a result of inflammatory cytokine production, such as interleukin-10 (IL-10), IL-4, IL-6, TGF-β, and prostaglandin E2 by cancer cells (reviewed in [58]). TGF-β in particular suppresses the activation and proliferation of microglia. Beside that, there is also an impact of microglia on glioma migration which might be related to the production of membrane type 1 metalloproteinase. Microglia in the glioma microenvironment are also a primary source of interleukin 1b (IL-1b), which can enhance gene expression of TGF-β [59]. Increased transcription of TGF-β can, thus, lead to suppression of antiglioma responses by inhibiting the immune response and blocking antitumor activity [60]. TGF-β can also lead to angiogenesis (by enhancing VEGF expression), proliferation (by enhancing EGFR expression), and invasion (by stimulating MMP-9 production) [61]. Therefore, these reports show that when microglia are in a glioma context, they acquire a phenotype that can support tumor development and progression.

The vast metabolic and nutritional needs of gliomas are supplied by constant angiogenic activity, which makes these tumors highly vascularized. The formation of new vessels is a result of the secretion of VEGF by the tumor cells directly and by fibroblasts and inflammatory cells in the stroma. Macrophages also release a number of factors that influence endothelial cell behavior, including VEGF, hepatocyte growth factor (HGF), MMP-2, and cytokines and interleukins [62]. The rapid neovascularization, typical of cancers, often leads to the production of abnormal and non-uniform vessels which eventually produce hypoxic niches that stimulate additional VEGF production [63]. Endothelial cells thus have a relevant role in the progression of brain tumors. Beside promotion and regulation of angiogenesis, they also release factors that maintain the stemness of CSCs, a topic that will be better examined further on in this paper.

Next to the vascular endothelium, there are nontransformed astrocytes, which exert a trophic role in the tumor microenvironment. They secrete a number of neurotrophic factors, including transforming growth factor (TGF-α), CXCL12, and glioma-derived growth factor (GDNF). These neurotrophic factors have been described as capable of driving the invasive properties of GBM cells and other aspects of tumor progression, such as angiogenesis, metastasis, and survival of other cancer types [64–66]. Astrocytes are widely recognized components of the blood-brain barrier (BBB), conferring barrier tight junctions with brain endothelial cells. Immunostaining experiments of the astrocyte-endothelial interface of the BBB suggest that tumors induce specific changes in endothelial cells. Abnormal astrocyte-endothelial interactions lead to the remodeling of the ECM, which can thus facilitate tumor invasion [67].

Another endogenous nontransformed cell type that interacts with gliomas is the neural precursor cell (NPC). These cells were demonstrated to migrate towards primary brain tumors over large distances, even when there are only few cancer cells (reviewed in [58]). Large numbers of NPCs were derived from the SVZ and home into pathologic brain tissue and possibly to tumors as well because of CXCR4 expression (the receptor for CXCL12) [68]. Several labeling techniques were used to track endogenous NPCs and identify their presence near gliomas. The genetically labeled cells were accumulated in many cellular layers around gliomas, and further experiments indicated that the precursors were exerting antitumorigenic actions, diminishing glioma proliferation, and leading to glioma cell apoptosis [58, 68, 69]. Chirasani et al. identified the bone morphogenetic protein-7 (BMP7) as an NPC-derived paracrine tumor suppressor that induces the differentiation of human GSCs [70]. Regarding these properties of NPCs, researchers began to explore manipulated NPCs to delivery cytokines, enzymes, and viral particles specifically to cancer cells [71].

Finally, the neural ECM consists of a unique microenvironment within the CNS, with specific molecules and structure. As it is known, the first difference is the absence of fibroblasts and collagen nearly throughout the brain. In turn, the brain ECM is composed mainly of hyaluronan, proteoglycans, tenascin-C, and thrombospondin, which confer a high state of hydration and loose connections (reviewed in [72]). The composition of the ECM in brain tumors is significantly altered. Within primary brain tumors, components such as vitronectin, osteopontin, tenascin-C, SPARC and BEHAB can be found, and some of them are

upregulated and modulate brain tumor growth, proliferation and invasion (reviewed in [72]).

5. Part IV: Glioma Stem Cells and Their Niches

5.1. Glioma Stem Cells Properties and Signaling. Behind tumor initiation, establishment, and dynamic evolution, there is a group of cells that plays a central role in all of these stages: glioma stem cells (GSCs). These cells have been isolated and characterized as a heterogeneous cell population that have unique features, making them a relevant key in tumor survival. They also show marked capacity for proliferation, self-renewal, and differentiation [73]. Characteristic GSCs can be defined according to their ability to efficiently reconstitute the original tumor when transplanted into immunocompromised mice (xenograft assay) [42]. Furthermore, they should express markers that are also expressed by the normal stem cells in the tissue of origin.

CD133 (prominin-1) is a transmembrane glycoprotein that is normally expressed in hematopoietic stem cells, endothelial precursor cells, and NSCs [74–76]. The CD133+ subpopulation of GSCs was demonstrated to present a more malignant behavior: the frequency of CD133+ cells was shown to increase with tumor grade, and its frequency is related to tumor recurrence [77]. Moreover, radioresistant tumors displayed higher percentage of CD133+ cells than the parent cell population, since GSCs could repair the damages more rapidly and efficiently than matched nonstem cells. Therefore, these data demonstrate that CD133+ cells may play an important role in GSC resistance to chemo- and radiotherapy [78]. CD133 is also informative for GSC division mode: in the research conducted by Lathia et al. [79], CD133 was the only marker among others (such as Bmi-1, nestin, CD15, Sox2, and Olig2) that could be asymmetrically segregated, as a result of localized CD133 expression and its positioning against the mitotic axis. The symmetric expansion mode will increase the GSC pool in the tumor, whereas asymmetric cell division will increase cellular heterogeneity of the tumor while maintaining the GSC pool. Other stem cell markers were not cosegregated with CD133. Their study also demonstrated that, in CD133− cells, CD15 could serve as a GSC marker, since this population survive better and proliferate faster as compared to their negative counterparts, complementing some part of CD133 function.

Intrinsic regulation of GSCs occurs through key proliferative and survival pathways including c-Myc, Oct4 (POU5F1), Olig2, and Bmi1, which are known to regulate embryonic stem cell proliferation as well [80]. In the same way notch, sonic hedgehog (SHH), and Wnt are important for the proliferation and stemness of NSCs, as well as for other cancer cells (Figure 1(b)). In Kondo's review [81], three pathways were depicted: Notch receptors are involved in a number of biological functions, including cell proliferation, differentiation, survival, and tumorigenesis [82]. There is also accumulating evidence that Notch activation not only maintains the multipotentiality of NSCs, but also promotes their differentiation into astrocytes. Regarding tumors, the depletion of Notch1 by RNAi blocks glioma proliferation *in vivo* and *in vitro* [83], suggesting that Notch signaling is involved in gliomagenesis, as well as in normal brain development.

SHH signaling is also involved in proliferation, development, and tumorigenesis [84]. Proteins that participate in the SHH pathway, such as Gli, Ptc1, and Smo, are all expressed in the SVZ, suggesting that SHH signaling may be essential for the maintenance of NSCs. Ectopic activation of Hedgehog in the central nervous system is likely to lead to brain tumor formation, and Gli1 is highly activated in many brain cancers [84, 85] (reviewed in [81]). Mutations in the SHH pathway are associated with medulloblastomas, which are primary brain tumors common in children. Hedgehog signaling is active in gliomas and contributes to GSCs function (reviewed in [80]), and its ligands are required for GSCs self-renewal as well as tumorigenesis. Treatment of GSCs with the Hedgehog inhibitor cyclopamine inhibits proliferation and self-renewal while increasing apoptosis [86]. Furthermore, CD133+ glioma cells overexpress genes involved in Notch and SHH pathways. These pathways contribute to the chemoresistant phenotype of CD133+ glioma cells, as their antagonism leads to an additive effect when used in combination with temozolomide (TMZ), which is an oral alkylating antineoplastic agent used for the treatment of GBM [87]. The authors showed that the therapeutic effect of TMZ was enhanced by inhibiting the Notch and SHH pathways with the antagonists GSI-1 and cyclopamine. More importantly, simultaneous treatment involving TMZ with both of these compounds led to a significant increase in CD133+ glioma cytotoxicity when compared to treatment with any of these agents alone.

The Wnt family coordinates several developmental processes, including cell proliferation and cell fate, via secreted proteins [88] (reviewed in [81]). Wnt1 and 3a, for example, are expressed in the ventricular and SVZ in the developing brain. Furthermore, the Wnt-β-catenin pathway is also involved in NSCs proliferation [89], and its disregulation has been implicated in many medulloblastomas [90] (reviewed in [2]). These findings suggest that hyperactivation of Wnt signaling may promote brain tumourigenesis.

Extrinsically, GSCs are regulated by growth factors as well as cell-cell and cell-extracellular matrix (ECM) interactions. GSC behavior is constantly affected by external signals from the niche, including neighboring stromal, immune, and nonstem tumor cells. Such signals will trigger the intrinsic pathways above described and will thus regulate CSCs function and properties. Some of these extrinsic pathways are well described: the signal transducer and activator of transcription 3 (STAT3), a member of the STAT family of transcription factors, is important in GBM, tumorigenesis, central nervous system development, and embryonic stem cell (ESC) biology. STAT3 is activated by a wide variety of cytokines and growth factors. STAT3 target genes regulate many cellular processes, including proliferation and apoptosis, and constitutive activation of STAT3 has been observed in many human cancers [91, 92]. Sherry and collaborators [93] have found that treatment of GSCs with two small molecules which prevent DNA binding of STAT3 inhibits, cell proliferation and the formation of new tumorspheres

from single cells. Genetic knockdown of STAT3 using a short hairpin RNA also inhibits GSCs proliferation and tumorsphere formation. Markers of neural stem cell, such as Olig2 and nestin, also decrease upon STAT3 inhibition, suggesting that STAT3 is required for maintenance of the stem-like characteristics of these cells.

The RTK (receptor tyrosine kinase) family of receptors mediates the effects of multiple oncogenic growth factor pathways, among which the EGFR is one of the best characterized in gliomas. The signal initiated by RTKs activates the Akt pathway, which promotes survival, proliferation, invasion, and secretion of proangiogenic factors. Pharmacologic inhibitors of Akt attenuate GSC tumorsphere formation, induce apoptosis, and substantially delay intracranial tumor formation [80]. Eyler and collaborators [94] have demonstrated that GSCs are more dependent on Akt signals than matched nonstem glioma cells. Treatment with an Akt inhibitor more potently reduced the numbers of viable brain cancer stem cells relative to matched nonstem cancer cells associated with a preferential induction of apoptosis and a suppression of neurosphere formation. Akt inhibition also reduced the motility and invasiveness of all tumor cells, but had a greater impact on cancer stem cell behavior.

Bone morphogenetic proteins (BMPs) is another family of growth factors that are crucial to regulate differentiation, proliferation, and apoptosis of NSCs [95]. Findings by Sun et al. highlight an extrinsic regulatory network, comprising BMPs, BMP antagonists, and FGF-2 signals, which govern proliferation, dormancy, and differentiation of rat NSCs and which can be manipulated to enable long-term clonogenic self-renewal. BMP induces NSC growth arrest through the canonical effectors Smads, but, in the presence of FGF-2, terminal differentiation is blocked and stem cell potency preserved. These findings indicate that NSC propagation, dormancy, and differentiation are regulated by counterbalancing BMP and FGF signals [36]. The same regulatory network should also be important for GSCs. Indeed, treating GSCs with BMPs *in vivo* markedly delays tumor growth and reduces tumor invasion. These data suggest that selective activation of BMP pathways may reduce the tumorigenic capacity of GSCs [96].

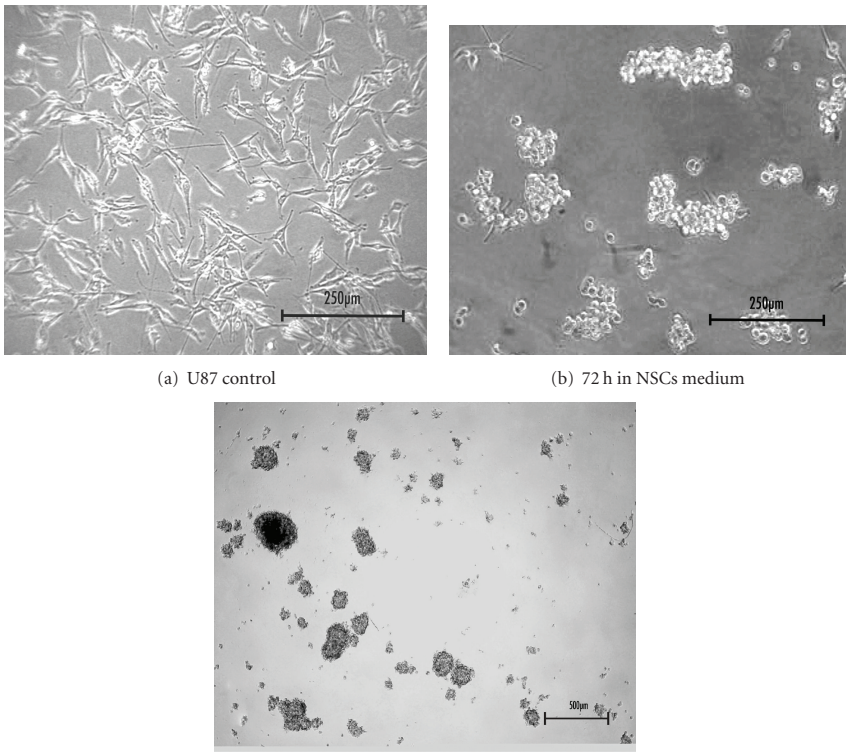
5.2. CSCs Contribute to Glioma Cellular Heterogeneity. Remarkably, GBM consists of morphologically diverse cells expressing a wide variety of differentiated and undifferentiated markers [42, 52]. Models that explain the origin of tumor heterogeneity and their capacity to undergo fast malignant progression can be adapted to GBM: the first one consists of a stochastic model in which all tumor cells have a random probability of developing mutations to permit tumor maintenance, and the second is based on a hierarchical model in which sustained tumor growth is restricted to selected subpopulations, such as CSCs [97]. Studies on acute myeloid leukemia have brought useful knowledge concerning the CSC model that could be applied to other tumor types: it is suggested that the tumor is originated from leukemic stem cells that, regarding their self-renewal capacity, are superior in a hierarchical manner to its subsequent progenitors, which are locally restricted to the stem cell niche [42, 98]. However, these models are not mutually exclusive: a single tumor may contain multiple CSC clones that are genetically distinct as a result of the stochastic model, but these cells will always have a common ancestor, the cell that sustained the first oncogenic mutation.

As Clevers has pointed out [42], in order for a certain cancer type to fit into the CSC model, it has to be demonstrated that the primary tumor has different capacities for tumor initiation among the tumor cell subsets, therefore illustrating the presence of CSCs. Singh and collaborators have reported that in human brain tumors there is a cluster of CD133+ cells that could initiate new brain tumors in immunodeficient mice, while CD133− cells could not [73]. Like normal NSCs, GSCs can form spheres when cultured in serum-free medium supplemented with EGF and FGF and could be induced to differentiate into all neuronal lineages expressing mature neuron markers, astrocytes, and oligodendrocytes (reviewed in [99]). Figure 2 illustrates the resulting tumorspheres, obtained in our laboratory by switching the usual medium to the NSC medium which allows enrichment of stem cell and pluripotency markers through cell growth in suspension [100–102], without the use of cell sorting, which only selects specific GSC subpopulations.

In spite of the recent controversy concerning the use of self-renewal as a tumorigenic marker [103] and the difficulties that are intrinsic to this methodology (such as culture artifacts) [98], the use of the tumorsphere assay to select for GSCs is still widely accepted. However, in order to obtain a more reliable understanding of GSCs behavior, *in vivo* studies concerning the GSC tumor niche should be considered. With that in mind, GSC niches are going to be depicted next.

5.3. The Perivascular Niche (PVN). Since most CSCs usually inhabit a microenvironment very similar to the ones of normal stem cells, we are encouraged to explore both niches to develop new approaches to cancer treatments which specifically target CSCs and their communication with the microenvironment. In GBM GSCs have been localized in two distinct niches, which are going to be discussed in this part of the paper.

The study of neurogenic niches in mammalians has led to the first thoughts regarding the existence of a particular niche in brain tumors in which CSCs could reside. These studies provided solid reports about the importance of the vasculature for neurogenesis: the vascular compartment within the neural stem cell niche was shown to have the unique capacity to regulate neural stem and progenitor cells through direct contact and paracrine signaling by endothelial and mural cells, also integrating systemic signals into the local microenvironment via distribution of soluble factors in the circulation to regulate stem cell niche behavior (reviewed in [35]). These thoughts, together with the fact that the most aggressive brain tumors present an overwhelming angiogenic activity (endothelial hyperplasia and microvascular proliferation) [104], have led scientists to investigate in more detail the location of GSCs within the tumor, making use of NSC markers such as those discussed above.



(a) U87 control

(b) 72 h in NSCs medium

(c) Tumorspheres after two passages

Figure 2: Glioma tumorspheres. (a) U87 glioma cell line in DMEM + 10% of FBS. (b) Tumorspheres of U87 cells after 3 days in NSCs medium. (c) Tumorspheres after two dissociations.

In 2007, Calabrese and colleagues [105] published an elegant report elucidating the role of the vasculature in brain tumor stem cells (BTSCs). Their data support the hypothesis that vascular niches in brain tumors are abnormal and contribute directly to the generation of GSCs and tumor growth (Figure 1(b)). They have found that many of the vessel-associated nestin+ tumor cells are proliferating and interacting with endothelial cells and that endothelial cells maintain self-renewal of BTSCs in culture and promote the initiation and growth of orthotopic brain tumor xenografts (with GFP-labeled CD133+ cells). Endothelial cocultures also demonstrated that endothelial cells maintain self-renewing and undifferentiated BTSCs. In addition, several molecular signaling events from endothelial cells and other stromal cells within the perivascular microenvironment appear to regulate the stem cell-like properties of resident BTSCs, in a very similar way as that seen in the NSCs niches (reviewed in [58]).

Very recently, human glioma tissue samples were analyzed by immunohistochemistry assays by He and collaborators [106]. They noted that CD133+ and nestin+ niches are localized perivascularly in all glioma tissues and that blood vessels were also nestin− and CD133+. Both CD133+ blood vessels and nestin+ blood vessels have an important role in maintaining glioma stem cell niche structure. Moreover, the abundance of CD133+ niches and nestin+ niches increases significantly as tumor grade increases.

It is important to point out that the relationship between GSCs and their microenvironment is reciprocal: GSCs are able to modulate the same microenvironment that produces the signals that regulate themselves. For example, GSCs secrete VEGF, which stimulates endothelial cell growth to support a local vascular environment. In turn, endothelial cells express Notch ligands which stimulate Notch receptors, which are essential for GSCs maintenance (reviewed in [107]). GSCs have a stronger capacity for promoting angiogenesis, partially through amplified secretion of VEGF, compared to noncancer stem cells [108]. Treating GSCs with the VEGF-neutralizing antibody bevacizumab attenuates their ability to promote angiogenesis both *in vitro* and *in vivo*, which in turn markedly inhibits the GSC tumorigenesis (reviewed in [83]).

The perivascular niche has also been shown to regulate GSC phenotype through regulation of the Notch pathway in these cells (reviewed in [76]). Blockade of this pathway has been demonstrated to deplete GSC population through reduced proliferation and increased apoptosis, as well as through increase in the sensitivity of GSCs to radiation-induced cell death, underscoring the importance of Notch in the regulation of GSCs. Nitric oxide (NO) is another factor

in the PVN with the capacity to enhance the self-renewal characteristics of BTSCs. NO also activates Notch signaling in the BTSCs to enhance their self-renewal characteristics *in vitro* and their tumorigenic capacities *in vivo*. Further, eNOS, an enzyme that synthesizes NO from the vascular endothelium, is elevated in the platelet-derived growth factor (PDGF)+ subset of gliomas, and suppression of eNOS activity, which corresponded to a decrease in Notch signaling in these tumors, prolonged survival of tumor-bearing mice [109] (reviewed in [58]).

Surrounding the vasculature are large-body, GFAP-expressing astrocytes and smooth muscle actin-expressing fibroblastic pericytes that intimately associate with tumor endothelia. Macrophages are also located in this region and are recognized to play a significant role in tumor progression of many tumor types [58]. In the normal neurogenic microenvironments, rates of cell proliferation are quite low. On the other hand, researchers found that many of BTSCs in niches were proliferating, differentiating, and benefiting from the protection of their niche through the adherence of stem cells to the niche by cadherin- and integrin-mediated cell adhesion, molecules which are enriched in GSCs [82, 105, 106].

It is thus clear that the tumor microvasculature generates specific niche microenvironments that promote the establishment and maintenance of BTSCs. As well as regulating stem cell proliferation and cell-fate decisions, niches also play a protective role, shielding stem cells from environmental insults, like chemo- and radiotherapy [81, 105]. The dynamics of the PVN structure have only recently been elucidated however. Beside the usual evidences of angiogenesis and vasculogenesis (tumor vasculature arising from sprouting and proliferation of endothelial cells from local vessels and colonization of circulating endothelial or other cells primarily from the bone marrow, resp.) [110], the reports from Ricci-Vitiani et al. and Wang et al. [40, 111] show that beside the already known features of GSCs, they are also capable of transdifferentiating into endothelial cells. Wang's group has demonstrated that a subpopulation of endothelial cells within glioblastomas harbor the same somatic mutations identified within tumor cells, such as amplification of EGFR and chromosome 7. The stem-cell-like CD133+ fraction includes a subset of vascular endothelial-cadherin (CD144+) cells that show characteristics of endothelial progenitors capable of maturing into endothelial cells [111].

The Ricci-Vitiani group has also demonstrated that a variable number (range 20–90%) of endothelial cells in glioblastoma carry the same genomic alterations as tumor cells, indicating that a significant portion of the vascular endothelium has a neoplastic origin. The vascular endothelium contained a subset of tumorigenic cells that produced highly vascularized anaplastic tumors [40].

In 2011, Lathia and collaborators [112], in a very elegant study, have provided the first direct evidence for tumor propagation by a solid GSC tumor subpopulation *in vivo*. Making use of live imaging, they showed that a small fraction of tumor cells that resided perivascularly initiated a heterogeneous tumor. Through xenotransplantation models, they were able to evaluate the GSC behavior in a niche context, avoiding culture artifacts and considering the niche interactions with components such as the vasculature and stroma. They investigated the behavior of GSCs and nonstem tumor cells in an identical microenvironment, transplanting differentially labeled human GSCs and nonstem tumor cells derived from the same parental tumor into the same recipient mouse and monitored their *in vivo* behavior over time using intravital microscopy. The results showed that GSCs (10% of the total number of transplanted cells) outgrew the nonstem cells population. Intriguingly, the resulting tumors had an overwhelming majority of cells that were derived from GSCs. Furthermore, GSCs and their descendants (YFP-labeled cells) were in proximity to the vasculature. Analysis of Sox2 expression, a GSC marker, showed that 25.9% of transplanted GSCs and their descendants were Sox2+ as compared to 0.1% of nonstem tumor cells and their descendants. Hence it was determined that the transplanted tumor cells contained stem-like cells with capacity to self-renew. Their results also suggest that the *in vivo* environment provides instructive cues to recreate an equilibrium of differentiation status and thus cellular heterogeneity.

5.4. The Hypoxic Niche (HN). Hypoxic niches spontaneously arise in malignant tumors as a result of the fast tumor growth that exceeds its neovascularization [113]. Furthermore, with increasing tumor size, tumor perfusion declines because of the severe morphological and functional alterations of the tumor microcirculation [114] (reviewed in [115]). Whenever the vasculature inefficiently irrigates a tissue, the resultant reduction in tissue oxygen tension often leads to neovascularization to satisfy the tissue's needs [116]. VEGF mRNA levels are increased after exposing different cell cultures to hypoxia, but return to background levels when the normal oxygen supply is resumed. VEGF was then identified as the main factor that mediates this feedback response, functioning as a hypoxia-inducible angiogenic factor [117].

In 1993 researchers were unraveling the cellular response to hypoxia in cancer cells [118]. They found that transcription of the human erythropoietin (EPO) gene is activated in Hep3B cells exposed to hypoxia and that the hypoxia-inducible factor 1 (HIF-1) was the nuclear factor whose DNA binding activity was induced in such conditions (hypoxia prevents proteasomal degradation of cytosolic HIFs). Therefore, they were the molecular mediators of hypoxia. About ten years later, by the time that scientists found CSCs in brain tumors, there was a solid concern about how oxygen levels influence tumor behavior. What they did not know was that the recently discovered subpopulation with stem cell characteristics within the tumor would be ruling this behavior. What they did not know was that the recently discovered subpopulation with stem cell characteristics within the tumor would be ruling this behavior; at the time, it was observed that hypoxia was associated with tumor aggression [119]. Some of the mechanisms they thought to be underlying the relation between hypoxia and tumor aggression were the hypoxic regulation of cytokine and growth factor release, such as VEGF, the regulation of tumor suppressors and oncogenes, and the modulation of invasion-associated cytokines, such as MMP [118].

Rankin and Giaccia [120] have recently reviewed the role of hypoxia in tumorigenesis, given that the expression of both HIF-1α and HIF-2α are commonly increased in a variety of human tumors. Their study pointed out that HIFs can promote tumorigenesis by the regulation of several hallmarks, such as angiogenesis, metabolism, proliferation, metastasis, and differentiation. The last one is relevant, since HIF indirectly regulates proliferation and differentiation through interactions with other signaling proteins such as c-Myc and Notch, both important for the CSC maintenance. In addition, it is known that normal stem cells reside in regions of low oxygen pressure, such as the hypoxic niche (HN) in the bone marrow, where hematopoietic stem cells proliferate [121].

A very intriguing research by Heddleston and collaborators [122] shows that hypoxia induces the expression of key stem cell genes, specifically Nanog, Oct4, and c-Myc, in nonstem cancer cells (the same genes Yamanaka used to reprogram fibroblasts to induce pluripotent stem cells [123]). Furthermore, they showed that inducing HIF-2α expression alone can reprogram differentiated, nonstem cancer cells towards an undifferentiated state, similar to neurospheres, since HIF-2α may directly regulate core stem cell pathways that are essential in CSC maintenance.

Another clarifying work by Seidel and colleagues [124] specifically explored the relationship between GSCs and hypoxia. In this research, the authors have isolated and characterized GSCs using a side population assay, defining a differential signature that made it possible to track cells through immunohistochemistry. Signature gene expressions, such as CD133, were located in perinecrotic (hypoxic) areas and in perivascular niches as well. HIF-2α overexpression, instead of HIF-1α, resulted in a significative increase in the levels of all side population markers tested, as well as of the established HIF-2α target, Oct4. HIF-2α knockdown in a primary GBM cell line completely blocked the upregulation of the side population signature genes following hypoxia, demonstrating how hypoxia controls the expression of several genes that regulate stem cells.

Regarding the actual therapeutics concerning both niches, the highlights are laid on the humanized monoclonal antibody against vascular endothelial growth factor (VEGF)-A, bevacizumab, which was the first antiangiogenic agent to be approved for cancer therapy in patients with metastatic colorectal cancer, nonsquamous non-small-cell lung cancer, and metastatic breast cancer (reviewed in [125]).

Since glioblastoma are highly vascularized cancers and have high expression of VEGF, bevacizumab seemed a proper choice for treatment. It was shown to improve patient outcomes in combination with chemotherapy in recurrent GBM in two distinct prospective phase 2 studies, granting approval by the US Food and Drug Administration (FDA) as a single agent in recurrent GBM (reviewed in [126]). However, bevacizumab used for metastatic breast cancer has not been shown to provide a benefit for delay in tumor growth and in improving overall survival, forcing the FDA to revoke the agency's accelerated bevacizumab approval for HER2-negative breast cancer. Bevacizumab, however, remained on the market, since it has been approved for the treatment of other cancer types [127].

It is important to point out that the tumor response against an antiangiogenic agent may differ between tumor types and subtypes, and, as a result, the complex mechanisms involved in antiangiogenic therapy are still being uncovered. Treatment of glioblastoma *in vivo* with an antibody against VEGFR-2 has inhibited angiogenesis but has also increased tumor invasiveness along host microvasculature [128]. Since high-grade gliomas often show a remarkable brain invasion capacity, this finding has emphasized the need of a combination of different treatment regimens against glioblastoma. To illustrate, two studies have successfully combined antiinvasive and antiangiogenic therapy against high-grade gliomas. Nakabayashi and colleagues made use of the MMP inhibitor MMI-166 which significantly inhibited the invasive and angiogenic activities of glioma cells *in vitro* and *in vivo*, leading to tumor growth inhibition *in vivo* [129]. Another group tested the effects of sunitinib on orthotopic models of GBM *in vitro* and *in vivo*. Sunitinib is an oral multitargeted tyrosine kinase inhibitor with both antiangiogenic and antitumor activities due to selective inhibition of various receptor tyrosine kinases. The drug exhibited potent antiangiogenic activity; however, the antiinvasive activity of sunitinib was observed only *in vitro*, since it was not effective in overcoming the invasion increase caused by its antiangiogenic activity [130].

Recently, Conley and colleagues [131] have found, through the generation of intratumoral hypoxia in human breast cancer xenografts, that the antiangiogenic agents sunitinib and bevacizumab increase the cancer cell population. Furthermore, *in vitro* studies revealed that stem or progenitor cell enrichment is primarily mediated by hypoxia, specifically by HIF1α. These are very interesting results, since they demonstrated that antiangiogenic agents are able to disrupt tumor vasculature, and therefore the PVN, but meanwhile, they create neohypoxic niches, which in turn can generate new GSCs [122] and reestablish the proliferative niche, pointing out the dynamics of the PVN and HN crosstalk, and even more so because they are able to interconvert (Figure 1(b)). These findings show the importance of employing converging therapeutical strategies into both niches by, for example, aiming at both VEGF and HIFs together.

Indeed, Rapisarda et al. [125] tested the hypothesis that HIF-1α inhibition in a hypoxic-stressed tumor microenvironment generated by the administration of antiangiogenic agents may result in a more pronounced therapeutic effect. The activity of bevacizumab, either alone or in combination with the HIF-1α inhibitor topotecan, was evaluated in the glioblastoma cell line U251-HRE (containing a hypoxic responsive element) xenografts. The luciferase expression in U251-HRE xenografts is dependent on the presence of a functional HRE sequence. The authors then designed the experiments to test whether topotecan inhibited HIF-1-dependent luciferase expression and tumor growth in U251-HRE xenografts. The combination of a low dose of topotecan with bevacizumab synergically inhibited tumor growth. The addition of topotecan to bevacizumab was also

associated with significant inhibition of proliferation and with induction of apoptosis (not seen with bevacizumab alone). Importantly, they showed that the increased cytotoxic activity by bevacizumab did not account for the increased antitumor effects observed. The effects of the combination of the two drugs are explained by the inhibition of the hypoxic responses usually triggered by bevacizumab. Interestingly, there was also a reduction in angiogenesis relative to either agent alone, possibly as a result of these two agents inhibiting converging angiogenic pathways controlled by HIF-1 transcriptional activity, such as the VEGF pathway.

On the other hand, it is intriguing that, although HIF-1α inhibition alone does not significantly affect GSC maintenance [122, 124], it still can directly modulate the GSC niche, indirectly affecting the GSC population. Therefore it would be of great value to evaluate effects of both VEGF and HIF-1α inhibition on GSC population. Furthermore, the effects of VEGF and HIF-2α inhibition on tumor growth and aggressiveness remain to be explored, since until now HIF-2α has been considered the main regulator of GSC in the HN [124].

Overall, there has been a significant progress in studies regarding GSC niche. The hypoxic environment was shown to regulate many aspects of GSC signaling, but little is known about they behave *in vivo* in such niches. The complex mechanisms involved in hypoxic responses and in antiangiogenic therapy, and its consequence specially in GSC maintenance must be further examined to better explore antiglioma therapy.

6. Part V: Epigenetic Control at the Niche

Epigenetics are referred to as the mechanisms by which gene expression is regulated without altering the genomic sequence. Epigenetic regulation can thus shape cell fate allowing adjustment to varying environmental conditions (reviewed in [131]). These molecular signals act on chromatin of not only one cell, but in the whole microenvironment [132], promoting cell-type-specific changes through the acquisition of distinct programs for gene expression. This process renders this mechanism of great importance to the developing tissue stability and homeostasis, which are accomplished by the maintenance of cellular memory (the heritable patterns of gene expression), through genomic imprinting.

Chromatin contains several proteins that are required for its assembly and packaging into euchromatin or heterochromatin, as well as for DNA replication and transcription, DNA and histone modification, and DNA repair or recombination (reviewed in [133]). The main epigenetic mechanisms include DNA methylation, histone modifications (acetylation and methylation), and regulatory noncoding RNAs (reviewed in [134]).

6.1. Epigenetic Mechanisms. Recent studies have highlighted the active role of histone modifications in gene expression regulation (reviewed in [135]). The covalent posttranscriptional changes at their amino-terminal tails by acetylation, phosphorylation, methylation, and ubiquitylation dictate how much access transcriptional regulators have to the DNA (reviewed in [133]). Lysine acetylation promotes nucleosome relaxation by decreasing the interaction of positively charged histone tails with the negatively charged DNA phosphate backbone. Histone deacetylases (HDACs) have an opposite activity: by deacetylating histone tails, the DNA is packed into condensed chromatin (nucleosomes) which, as a result, represses gene transcription (reviewed in [134]).

Many epigenetic studies focused on embryonic stem cell (ESC) maintenance and differentiation, relating it to embryonic development. Specific epigenetic marking by histone modifications is already known to occur in multipotent stem cells because of the binding of transcription factors involved in lineage choice (reviewed in [136]). Transcription factors that are expressed in ESCs (including Oct-4, Nanog, and Sox-2) would have a similar role in establishing epigenetic marks.

Concerning neuronal differentiation, Li and colleagues [134] have summarizedthe epigenetic influence on neuron-specific gene expression. They highlight that the recruitment of HDACs to neuronal gene promoters is essential for the repression of the same genes in nonneuronal cells and that the maintenance of histone acetylation is important for neuronal differentiation. Epigenetic mechanisms control lineage-specific gene expression for the generation of different neural cell types. Mechanisms such as DNA methylation keep GFAP repressed in neurons, but this can also be reverted in response to microenvironment changes. Furthermore, multipotent neural progenitor cells differentiate predominantly into neurons in the presence of the HDAC inhibitor (HDACi) valproic acid (VPA), and the silencing of some neuronal-specific genes can be reverted by treatment of the HDACi trichostatin A (TSA) [134].

Results from our laboratory show that this action may be effective against GSC propagation. Treatment for 72 hours with TSA was sufficient to decrease tumorsphere formation after medium shift to NSC medium in the human glioma cell line U87-MG, as measured by the tumorsphere formation assay (Figure 3). This result shows that acetylation may be essential for GSC stemness and maintenance.

6.2. Epigenetics in Tumors. Since chromatin structure responds to environmental cues and it is tightly regulated in several ways at the molecular level, tumors clearly originate from not only genetic alterations, but also from epigenetic aberrations in its microenvironment. Indeed epigenetics regulate many aspects of tumor behavior, including initiation, proliferation, and metastasis of the primary tumor [137].

As fully reviewed by Dey [138], cancer cells present aberrations in their DNA methylation pattern. Hypomethylation at centromeric repeat sequences has been linked to genomic instability. Furthermore, hypomethylation has also been associated with the activation of genes required for invasion and metastasis. On the other hand, local hypermethylation of individual genes has been associated with aberrant gene silencing, such as the repression of tumor suppressor genes. Beside that, evidences show aberrant loss or gain of histone methyltransferase (HMTase) activity in tumorigenesis and proliferation of cancer cells [138]. Moreover, histone acetylation/deacetylation in promoter regions contributes to

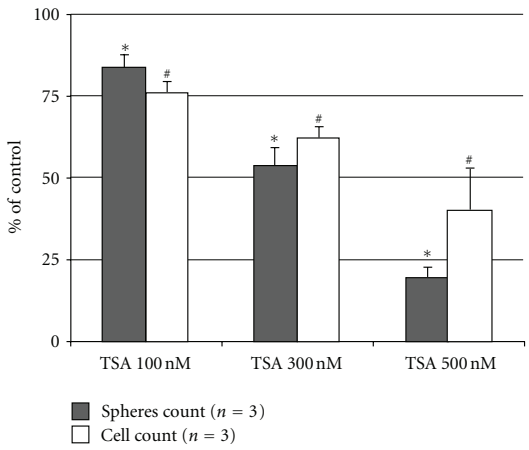


Figure 3: Tumorsphere formation assay upon treatment of U87-MG cells with Trichostatin A (TSA). A seventy-two-hour treatment with the histone deacetylase inhibitor TSA affects tumorsphere formation and cellular proliferation after medium shift to NSC medium. Bar represents control percentage. *represents $P < 0.02$ for 100 nM and $P < 0.001$ for 300 and 500 nM for spheres count, and #represents $P < 0.046$ for 100 nM $P < 0.011$ for 300 nM and $P < 0.001$ for 500 nM for cell count as measured by trypan blue. One-way ANOVA, followed by Turkey's post hoc test were used for statistical analysis, where P-values <0.05 were considered significant.

the disregulation of gene expression and has also been associated with carcinogenesis and cancer progression [138].

In addition, DNA methylation patterns are useful as biomarkers for glioblastoma. The most relevant mark is the methylation status of the MGMT (O6-methylguanine–DNA methyltransferase, a DNA repair protein) gene promoter. When the MGMT promoter region is epigenetically silenced, it is associated with a favorable outcome after temozolomide chemotherapy in patients with newly diagnosed glioblastoma, suggesting that it could be further studied as a biomarker with prognostic value [139].

Hegi and colleagues [140] have also associated epigenetic marks to glioma cells: in their work, the authors analyzed the methylation status of both DNA cytosine methyltransferases (DNMTs) and specific tumor suppressor genes promoters and compared them with normal brain samples in order to confirm that tumor suppressor genes are hypermethylated and silenced in gliomas [141]. With their results, the authors propose that overexpression of DNMT1 and DNMT3B in gliomas is a result of a significant hypomethylation occurring in the euchromatin region of its gene promoters. The increase of DNMT activity, in turn, causes hypermethylation of various tumor suppressor gene promoters, leading to the epigenetic inactivation of those genes, enhancing the proliferative capacity of glioma cells and harboring a poor prognosis in gliomas. The authors propose that overexpression of DNMTs may serve as a marker for cancer cells and as a potential target for future cancer therapy [140]. This work is an example of how epigenetic aberrations cause genomic instability which contribute to the achievements of the tumorigenic hallmarks in gliomas, illustrated in Figure 1.

6.3. Epigenetic Plasticity. Epigenetic plasticity is often illustrated by the normal stem cell lineage commitment. Likewise, differentiated cells are also able to be epigenetically reprogrammed into a stem-like chromatin state, as seen in iPSCs (induced pluripotent stem cells) reprogramming, and to transdifferentiate into a disparate lineage (such as when glioma cells generate endothelial cells). The dedifferentiation of cancer cells into CSCs has also been described as epigenetic plasticity. Furthermore, the HDACi VPA has been shown to facilitate the induction of pluripotency by chromatin remodeling [142] (reviewed in [143]). VPA was also shown to be involved in neuronal differentiation of NSC, regulating neurogenesis [144].

Therefore, the ability of cells to alter their state by modulating gene expression has also been observed in differentiation-altering, microenvironment-associated plasticity [143]. This means that gene expression of cancer cells can be altered, as well as its phenotype, by alternating its microenvironment. To illustrate, transition from 2D to 3D culture reduced epigenetic plasticity in platinum-resistant CP70 ovarian cancer cells [143]. Furthermore, the influence of the tumor microenvironment components over the maintenance of the cancer cells is reinforced when tumor cells are placed in a nonmalignant environment. Melanoma cells, when plated on top of ESC-derived extracellular matrices, remarkably differentiate into sphere-forming melanocytes, and the opposite (ESC plated on top of melanoma-derived extracellular matrices) is also true [145, 146]. Human ESCs show the ability to suppress the tumorigenic phenotype by the secretion of Lefty (which is exclusively expressed in ESCs), which neutralizes the expression of Notch in aggressive tumor cells.

It still remains unclear whether abnormal epigenetic regulation is a cause or consequence of cancer. Evidences demonstrate that the environment itself can modulate epigenetic plasticity, so abnormal signals from the microenvironment could predict and sensitize a potential cell for oncogenic transformation. Other results show that cancer cells or CSCs maintain the epigenetic signature of normal stem cells, which could favor malignant transformation. On the other hand, epigenetic disregulation is often a consequence of chromatin regulatory protein abnormalities, such as histones and HDACs, which are encoded by the very same DNA sequences that they regulate. Therefore, these alterations could arise as result of the genetic instability related to cancer.

Either way, epigenetic regulation of cancer cell gene expression offers us the opportunity to modulate these responses, since these are very dynamic changes, as opposed to the permanent genetic mutations, which therefore require complex therapeutic approaches, such as gene therapy and enzymatic reposition. GBM, specially, shows remarkable plasticity, and may be susceptible to epigenetic modulators such as HDACi, which are able to diminish the tumorigenic potential of cancer cells [147–149], all the while offering

new insights into how glioma cells respond to treatment. In addition, epigenetics can modulate the PVN and the HN. Hypoxic microenvironments may influence local epigenetic alterations, leading to inappropriate silencing and reawakening of genes involved in cancer, the main mechanism being loss of global methylation [150]. Potential cellular factors that link HDACis to the repression of HIF function have been proposed: type I/II HDAC inhibitors repress HIF function by either reducing functional HIF-1α levels or repressing HIF-α transactivation [149]. TSA, for example, is among several HDACi reported to repress angiogenesis *in vitro* and *in vivo* [151, 152]. VEGF is also epigenetically regulated [153], and together with the inhibition of HIF response, scientists can aim for the modulation of the GSC microenvironment to develop new therapeutic strategies.

7. Final Remarks

The knowledge about how neurogenesis functions in physiological conditions and maintains neuronal plasticity (which allows for physiological adaptations) lies on understanding the peculiarities of the mitotic niches that allow for stem and progenitor cells to proliferate and generate new cells. Depicting the function of normal stem cells and their relationship with their surroundings (a crucial crosstalk for tissue homeostasis) facilitates the understanding of cancer stem cell functions. Hence, it can awake new insights into cancer therapy, because accumulating evidences point out to CSCs as the main culprit. It is clear that both physiological and pathological stem cell niches share similar features, such as hypoxic and angiogenic signaling, as well as several other pathways which enable cancer cells to proliferate and self-renew with no limitations.

Through the study of neurogenesis, researchers could also shed light into the origins of glioblastoma. Such incurable malignancies are very heterogeneous and dynamic, hampering the complete elucidation of tumor biology during the first stages of their inception. The characterization of neural progenitors in specific brain niches lead to studies which focused on specific cell types. Through the advent of modern techniques, it was also possible to trace markers and cells along a certain period. As mentioned above, the cell of origin for GSCs is still under debate, but it is now becoming clear that they may arise from OPCs and NSCs from the neurogenic niches. Likewise, they may arise from mature cells that acquired the ability to self-renew as a result of oncogenic mutations; it is important to point out that this still remains an open question.

The way by which the microenvironment affects its cells and vice versa is still being uncovered, but the deeper the scientists unravel the idiosyncrasies of epigenetic regulation, the more is understood about how a cell responds to each context. This notion is already raising new promising pharmacological approaches for cancer therapy, since reverting epigenetic aberrations possibly inhibit the cancer-prone state (Figure 1(b)). Modulators such as histone deacetylases inhibitors, which are already being employed in clinical trials for several malignancies, are capable of differentiating CSCs, diminishing their malignant potential. Furthermore, new discoveries regarding the inhibition of angiogenic factors, such as VEGF, and the blockade of signals which arise from the hypoxic niche are also promising for targeting CSC niches. Even though much work still needs to be accomplished in order for researchers to uncover the dynamics of tumor microenvironments with its cells, this area has provided important information regarding tumor behavior, and new therapeutic approaches can now focus not only on the tumor itself, but also on its surrounding tissue.

Conflicts of Interest

The authors hereby report that there are no conflicts of interest that may have influenced the discussion presented herein.

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CAPÍTULO III

ARTIGO DE DADOS

O artigo ***"THE HISTONE DEACETYLASE INHIBITOR TRICHOSTATIN A REDUCES PROLIFERATION AND INDUCES NEURONAL DIFFERENTIATION OF U87-DERIVED HUMAN GLIOBLASTOMA STEM CELLS"*** será submetido à revista *Neuroscience* e será apresentado na forma de manuscrito, conforme solicitado pela revista.

Neuroscience

Research Paper

THE HISTONE DEACETYLASE INHIBITOR TRICHOSTATIN A REDUCES PROLIFERATION AND INDUCES NEURONAL DIFFERENTIATION OF U87-DERIVED HUMAN GLIOBLASTOMA STEM CELLS

FELIPE DE ALMEIDA SASSI a, b, c, LÍLIAN CAESAR a, d, MARIANE JAEGER a, b, c, PATRÍCIA LUCIANA DA COSTA LOPEZ a, b, c, CAROLINE BRUNETTO DE FARIAS a, c, d, CAROLINA NÖR a, b, c, ANA LUCIA ABUJAMRA a, c, d, GILBERTO SCHWARTSMANN a, c, e, ALGEMIR LUNARDI BRUNETTO a, c, e AND RAFAEL ROESLER a, b, c

[a] *Cancer Research Laboratory, University Hospital Research Center (CPE-HCPA), Federal University of Rio Grande do Sul, Porto Alegre, RS, Brazil*

[b] *Laboratory of Neuropharmacology and Neural Tumor Biology, Department of Pharmacology, Institute for Basic Health Sciences, Federal University of Rio Grande do Sul, Porto Alegre, RS, Brazil*

[c] *National Institute for Translational Medicine, Porto Alegre, RS, Brazil*

[d] *Children's Cancer Institute (ICI-RS), Porto Alegre, RS, Brazil*

[e] *School of Medicine, Federal University of Rio Grande do Sul, Porto Alegre, RS, Brazil*

Correspondence to: R. Roesler, Department of Pharmacology, Institute for Basic Health Sciences, Federal University of Rio Grande do Sul, Rua Sarmento Leite, 500 (ICBS, Campus Centro/UFRGS), 90050-170 Porto Alegre, RS, Brazil. Tel: +55 51 3308 3183; fax: +55 51 3308 3121.

E-mail address: rafael.roesler@pq.cnpq.br (R. Roesler).

Abbreviations: ATCC, American Type Culture Collection; BSA, bovine serum albumin; DAPI, 4′, 6-diamino-2-phenylindole; DNER, Delta/Notch-like epidermal growth factor-related receptor; EGF, epidermal growth factor; FGF, fibroblast growth factor; GBM, glioblastoma multiforme; GSC, glioblastoma stem cell; HDAC, histone deacetylase; HDACi, histone deacetylase inhibitor; NMA, Nuclear Morphometric Analysis; NSC, neural stem cell; NaB, sodium butyrate; OPC, oligodendrocyte precursor; RT-PCR, reverse transcriptase polymerase chain reaction; SAHA, suberoylanilide hydroxamic acid; TSA, trichostatin A.

Abstract – Glioblastomas (GBMs) are proposed to contain a subpopulation of glioblastoma stem cells (GSCs) that sustain tumor progression and therapeutic resistance. Epigenetic alterations have been increasingly implicated in GBM pathogenesis, and epigenetic modulators including histone deacetylase inhibitors (HDACis) have been investigated as potential therapies. However, the effects of HDACis on GSCs remain poorly understood. Here we show that the HDACi trichostatin A (TSA) reduces proliferation and colony sizes, induces alterations in nuclear morphology consistent with cell senescence, and increases the protein content of differentiation markers, but does not affect cell migration, in cultured human U87 GBM cells. In U87-derived GSCs expanded in a tumorsphere assay, TSA reduced sphere formation, induced neuron-like morphology changes, increased mRNA levels of neuronal differentiation, and reduced mRNA content of stemness and pluripotency markers. These findings indicate that HDACis inhibit proliferation and survival and induce differentiation of both non-stem GBM cells and GSCs, and provide evidence for the development of HDACis as anti-GSC therapeutics.

Key Words: histone deacetylase, trichostatin A, epigenetics, brain tumor stem cell, glioblastoma, brain cancer

INTRODUCTION

Glioblastoma multiforme (GBM, or grade IV glioma) is the most common and aggressive type of brain tumor. The prognosis for GBM remains poor despite optimal clinical treatment, with a median overall survival of 12-15 months (Schwartzbaum et al., 2006; Wen and Kesari, 2008). The cell of origin for GBM remains a matter of debate, with evidence pointing to neural stem cells (NSCs), oligodendrocyte precursors (OPCs), or dedifferentiated neurons and astrocytes (Wang et al., 2009; Alderton, 2011; Friedmann-Morvinski et al., 2012; Lee et al., 2012). GBM displays cellular heterogeneity, and are proposed to contain a subpopulation of glioblastoma stem cells (GSCs), which share several characteristics of normal NSCs. Thus, these cells presenting stem-cell like properties, also called tumor-initiating cells, are basically defined by their self-renewal properties and capability of recapitulating the whole tumor cell population (Singh et al, 2004; Vescovi et al., 2006; Sutter et al., 2007; Flores et al., 2009; Hadjipanayis and Van Meir, 2009).

Both genetic and epigenetic alterations in NSCs and their progenitors may give rise to GBM. Epigenetic alterations likely to play a role in GBM pathogenesis include changes in mechanisms related to histone modifications (Nagarajan and Costello, 2009). Histone acetylation is a type of posttranslational alteration importantly involved in regulating gene expression through chromatin remodeling (Kouzarides, 2007). One study found that, in GBM samples, histone H3 acetylation was increased in comparison to non-neoplasic brain tissue. In addition, the levels of expression of genes encoding different types of histone deacetylase (HDAC),

which increase histone acetylation, was shown to be negatively correlated with glioma grade (Lucio-Eterovic et al., 2008). Histone acetylation can be pharmacologically enhanced by HDAC inhibitors (HDACis) such as trichostatin A (TSA), suberoylanilide hydroxamic acid (SAHA), sodium butyrate (NaB), valproic acid, 4-phenylbutyrate, and MS275. These agents can display anticancer activities via multiple consequences of increased histone acetylation, including the promotion of cell differentiation and increased expression of tumor suppressor genes (Bolden et al., 2006; Xu et al., 2007). A number of studies have indicated that HDACis inhibit glioma cell proliferation by inducing cell cycle arrest and apoptosis *in vitro*, reduce tumor growth *in vivo* in experimental models of GBM, and might potentiate the effects of radiotherapy and cytotoxic agents (Eyüpoglu et al., 2005; Komata et al., 2005; Wetzel et al., 2005; Yin et al., 2007; Bangert et al., 2012). Based on this evidence, the effects of the HDACis valproic acid and vorinostat in adults or children with GBM have been examined in clinical studies (Masoudi et al., 2008; Galanis et al., 2009; Berendsen et al., 2012; Tsai et al., 2012).

Given that cancer stem cells are proposed to be crucial for maintaining tumor growth, increasing effort has focused on investigating the effects of experimental therapies in this specific subset of cells. However, the effects of HDACi in GSCs remain poorly characterized. Sphere-formation assays have been used to obtain cultures enriched in cancer stem cells (Pastrana et al., 2011). GSCs give rise to tumorspheres when GBM cell lines are cultured in the presence of growth factors under conditions appropriate for stem cell propagation (Yu et al., 2008; Qiang et al., 2009; Hong et al., 2012). Here we show the effects of an

HDACi, TSA, on cell proliferation, tumorsphere formation, differentiation, and senescence, in cultured human GBM cells of the U87 cell line.

EXPERIMENTAL PROCEDURES

Cell culture

The human GBM cell line U87-MG (U87) was obtained from ATCC - American Type Culture Collection (Rockville, USA). Cells were maintained in tissue culture flasks at 37 °C with humidified atmosphere and 5 % CO_2. The culture medium, which was changed every 2/3 days, was prepared with DMEM Low Glucose (Gibco BRL, Grand Island, USA), 1 % penicillin and streptomycin (Gibco BRL), 0.1 % fungizone (Gibco BRL) supplemented with 5 % fetal bovine serum (FBS, Gibco BRL), with pH adjusted to 7.4 (Flores et al., 2008). Culture medium appropriate for stem cell propagation contained DMEM F12 (Gibco BRL), 1 % penicillin and streptomycin, 0.1 % fungizone supplemented with 20 ng/ml fibroblast growth factor (FGF, Sigma-Aldrich, St. Louis, USA), 20 ng/ml epidermal growth factor (EGF, Sigma-Aldrich), 10 ng/ml LIF (Sigma-Aldrich), N2 0.5X and 50 µg/mL bovine serum albumin, BSA; (Sigma-Aldrich), B27 (Gibco, BRL) 0.1 x with pH adjusted to 7.4. GBM tumorspheres were obtained by the substitution of the usual medium to the stem cell medium as described by Yu et al. (2008).

TSA treatment and cell proliferation and viability assays

TSA was purchased from Sigma-Aldrich. The concentrations of TSA were 100, 300, 500, 800, or 1,000 nM, depending on each specific experiment. Concentration ranges were based on previous studies (Egler et al. 2008; Bajbouj

et al., 2012; Wang et al, 2012). TSA was initially dissolved in 100% ethanol and then further diluted in water to the same concentration of ethanol of the largest dose of TSA for each experiment. Control cells were exposed to ethanol at the maximal concentration used in TSA-treated cells for each particular experiment. U87 cells were plated at a low-density 24 h before the experiments and then were cultured for 48 h to 72 h while exposed to different TSA concentrations. After the treatment cells were trypsinized and counted with trypan blue in a Neubauer chamber for viability and proliferation measurement. Cell viability was calculated as the number of viable cells divided by the total number of cells within the grids on a hemacytometer. Cells were considered non-viable if they stained with trypan blue.

Tumorsphere formation assay

For tumorsphere formation, 96-well plates and DMEM F12 with growth factors plus different concentrations of TSA were used. U87 cells were trypsinized and counted in Neubauer chamber. Two thousand cells were plated containing the treatment or control in 6 wells each in a 96-well plate. Control cells were exposed to the culture medium plus ethanol at the appropriate concentration to match the group treated with the highest dose of TSA. After 72 h, the plate was analyzed using an inverted optical microscope, in which the number of spheres was counted and photographs were taken for cell morphology analyses. Spheres were considered cells that formed a group, which were not adhered to and had at least

20 cells. All neurorspheres were then trypsinized and counted as described above for the proliferation and viability measurements.

Analysis of tumorsphere number and sizes

Tumorspheres were plated in sextuplicates in 96-well plates and grown for 48 h, then counted with an inverted microscope and treated with 300, 500, 800 or 1000 nM TSA or ethanol as a control. After 48 h of treatment, wells were photographed and the pictures were analyzed with the ImageJ software, making use of the spheres' contrast with the background. The tool enabling analyzes of particles was used in order to measure final sphere number, area covered by spheres, sphere size, and proportion of larger spheres among the total number (larger spheres were considered dark color condensed spheres, with an area larger than 900 pixels or ~300uM).

Clonogenic assay

Two hundred and fifty U87 cells were plated in 6-well plates and treated for 48 h with 100, 300, or 500 nM of TSA. To examine the colony-forming capability of cells from tumorspheres, mature spheres (at least two passages) were dissociated, stem cell medium was replaced by DMEM 10% BFS (to promote adhesion), and 750 cells were plated in each well. Twenty four hours later, cells were treated with TSA at 300 or 500 nM of TSA for 48 h. At least 2 wells were counted for each dose. After treatment, cells were maintained for 10 days, with the medium being changed every 2 days. The cells were then fixed with 70% ethanol

and counterstained with 0.5% crystal violet for 5 minutes. Photographs of the plates were taken and the sphere number and size were analyzed using the ImageJ software as previously described by Cai et al. (2011).

Reverse transcriptase polymerase chain reaction (RT-PCR)

U87 tumorspheres were cultured in the presence of 1000 nM TSA for 48 h. Total RNA extraction was performed using Trizol reagent (Invitrogen, São Paulo, Brazil), in accordance with the manufacturer's instructions, followed by DNAseI treatment (Invitrogen, Carlsbad, USA) and reverse transcribed with Superscript First-Strand (Invitrogen). The human Gria2, GLAST, Oct4 and β-actin primers used for RT-PCR amplification were designed according to the corresponding Gene Bank sequence (**Table 1)**.

Semiquantitative RT-PCR conditions were optimized to determine the number of cycles that would allow product detection within the linear phase of mRNA transcript amplification. The expression of β-actin was measured as an internal control. All assays were carried out in a total volume of 10 µl using 40 cycles for amplification that consisted of 10 min at 94 °C, denaturation at 95 °C for 60 s, annealing at ~60 °C for 40 s, and extension of primers at 72 °C for 40s, followed by a final extension at 72 °C for 5min using GoTaq® Green Master Mix (Promega, Fitchburg, USA). The products were electrophoresed through 1.0% agarose gels containing 1% ethidium bromide (Biotium, Hayward, USA) and visualized with ultraviolet light. Fragments' lengths were confirmed using a 50-bp DNA ladder (Invitrogen) and the relative expression of the genes was determined

by densitometry using freeware ImageJ for Mac. Each experiment was performed in replicate using RNA isolated from independent cell cultures, and representative findings are shown. For each set of PCR reactions, a negative control was included. Semiquantitative data are shown as percent changes relative to β-actin (the lowest value among replicates in the control group was taken as 100%).

Western blot

Cultured U87 cells were lysed and prepared for western blotting as previously described (Zamin et al., 2009). After 20 µg of protein was separated by SDS-PAGE and electroblotted, the PVDF membranes were incubated overnight with primary antibodies against NeuN (1:500; Millipore, Billerica, USA) and GFAP (1:500; Dako Cytomation, Fort Collins, USA). Incubation of all of the primary antibodies was followed by incubation with the appropriate horseradish peroxidase-conjugated secondary antibody (1:2000; Cell Signaling, Beverly, USA) for 2 h at 4°C. Chemoluminescence was detected by X-ray films (Kodak X-Omat, Rochester, USA). Band density was analyzed using ImageJ.

Cell senescence associated to SA-β-galactosidase

U87 cells were plated at low confluence in sextuplicates of 96-plate wells and treated with TSA at 100 or 500 nM for 48 h. For the SA-*β*-galactosidase staining, we used the Senescence Cells Histochemical Staining Kit (Sigma-Aldrich), following the manufacturer's instructions. Cells were exposed to the

solution containing the β-galactosidade substrate, X-Gal, for 6 h. Cells were then washed with PBS and 50 µl of a solution of marking core (containing Triton X-100, PBS and 4′, 6-diamino-2-phenylindole, DAPI) was added for 15 min. Photographs were obtained using inverted fluorescence microscope Carl Zeiss and the images were analyzed using the ImageJ 1.42q.

Nuclear morphometric analysis (NMA)

The DAPI staining used for quantifying the cell senescence was also used to quantify the images using the Software Image Pro Plus 6.0 (IPP6 - Media Cybernetics, Silver Spring, USA). The parameters examined were roundness, aspect, radius ratio and area box, which were quantified and grouped in an index, named Nuclear Irregularity Index (NII), which is composed by the sum of aspect, radius ratio and roundness, subtracted by the value of area box. Data are presented as a plot of Area versus NII in which normal nuclei were considered the nuclei inside 2 standard deviations (SD) of the mean of a population of nuclei obtained from untreated cells. Nuclei were considered large and regular if above 2 SD of size and below 3SD of NII and irregular when above 3SD of the large population or above 5 SD of the normal sized population (Filippi-Chiela et al., 2012).

Cell migration assay

About 120.000 U87 cells were plated in each well of a 6-well plate. Twenty-four h later, 2 wells were used for each TSA dose (control, TSA 100 nM or 500 nM). Cells were treated for 48 h and then 3 scratches were made in each well with a 100-uL tip. A mark was drawn with a pen to make sure that all the pictures would be taken in the same spot. Pictures were taken in an inverted microscope at the time of the scratches and 10 h later. Ten measurements of each picture regarding the distance between both borders of the scratch, before and after 10 h, were made with ImageJ 1.43u. The average differences between t=0 h and t=10 h were shown as percent of control for estimated cell migration (Liang et al., 2007).

Statistics

All experiments included in the analyses were repeated at least 3 times. All data are expressed as mean ± standard error of mean (S.E.M.) and were analyzed using one way analysis of variance (ANOVA) followed by Tukey' s post-hoc tests for multiple comparisons when appropriate. Statistical analyses were performed using GraphPad INSTAT software, (GraphPad Software, San Diego, USA).

Table 1. Forward and reverse primers used for RT-PCR amplification.

Gene	Primer sequences	Amplicon size (bp)
Gria2	*Forward:* 5' CACTTCGGAGTTCAGACTG 3' *Reverse:* 5' GCCTCTGTCACTGTCATAG 3'	316

GLAST	*Forward:* 5’ TCTTCTCCATGTGCTTCGG 3’ *Reverse:* 5’ CTTGCAGCAACCCTCCAAT 3’	321
Sox2	*Forward:* 5’ ACACCAATCCCATCCACACT 3’ *Reverse:* 5’ GCAAACTTCCTGCAAAGCTC 3’	224
Prominin-1	*Forward:* 5’ ACCAGGTAAGAACCCGGATCAA 3’ *Reverse:* 5’ CAAGAATTCCGCCTCCTAGCACT 3’	100
Notch1	*Forward:* 5’ CCGCCTTTGTGCTTCTGTT 3’ *Reverse:* 5’ TCCTCCTCTTCCTCGCTGTT 3’	490
c-Myc	*Forward:* 5’ TTCGGGTAGTGGAAAACCAG 3’ *Reverse:* 5’ CAGCAGCTCGAATTTCTTCC 3’	203
Oct4	*Forward:* 5’ AACATGTGTAAGCTGCGGC 3’ *Reverse:* 5’ TTGAATGCATGGGAGAGCC 3’	496
Musashi	*Forward:* 5’ ACAGCCCAAGATGGTGACTC 3’ *Reverse:* 5’ CCACGATGTCCTCACTCTCA 3’	191
β-actin	*Forward:* 5’ GAGACCTTCAACACCCCAG 3’ *Reverse:* 5’ GCTACAGCTTCACCAGCAG 3’	190

RESULTS

TSA inhibits proliferation and reduces colony sizes in U87 human GBM cell cultures

We first examined the effect of HDAC inhibition by TSA on the proliferation of human U87 GBM cells using the trypan blue cell counting assay. Treatment with TSA at 100, 300, or 500 nM, but not at 30 nM, for 72 h significantly reduced mean cell number by 31, 54 and 58% respectively (all $P < 0.001$ compared to control cells), whereas cell viability was not affected (**Fig. 1A**).

A colony-forming assay was performed to examine cell survival in U87 cells exposed to TSA at 100, 300, or 500 nM. Although the TSA-induced reduction in the number of colonies formed 10 days after treatment did not reach statistical significance, TSA produced a mean decrease of 30, 73, and 53% respectively, in colony sizes (all $P < 0.001$ compared to controls) (**Fig. 1B, 1C**). The results suggest that TSA inhibits the proliferation and survival of U87 GBM cells.

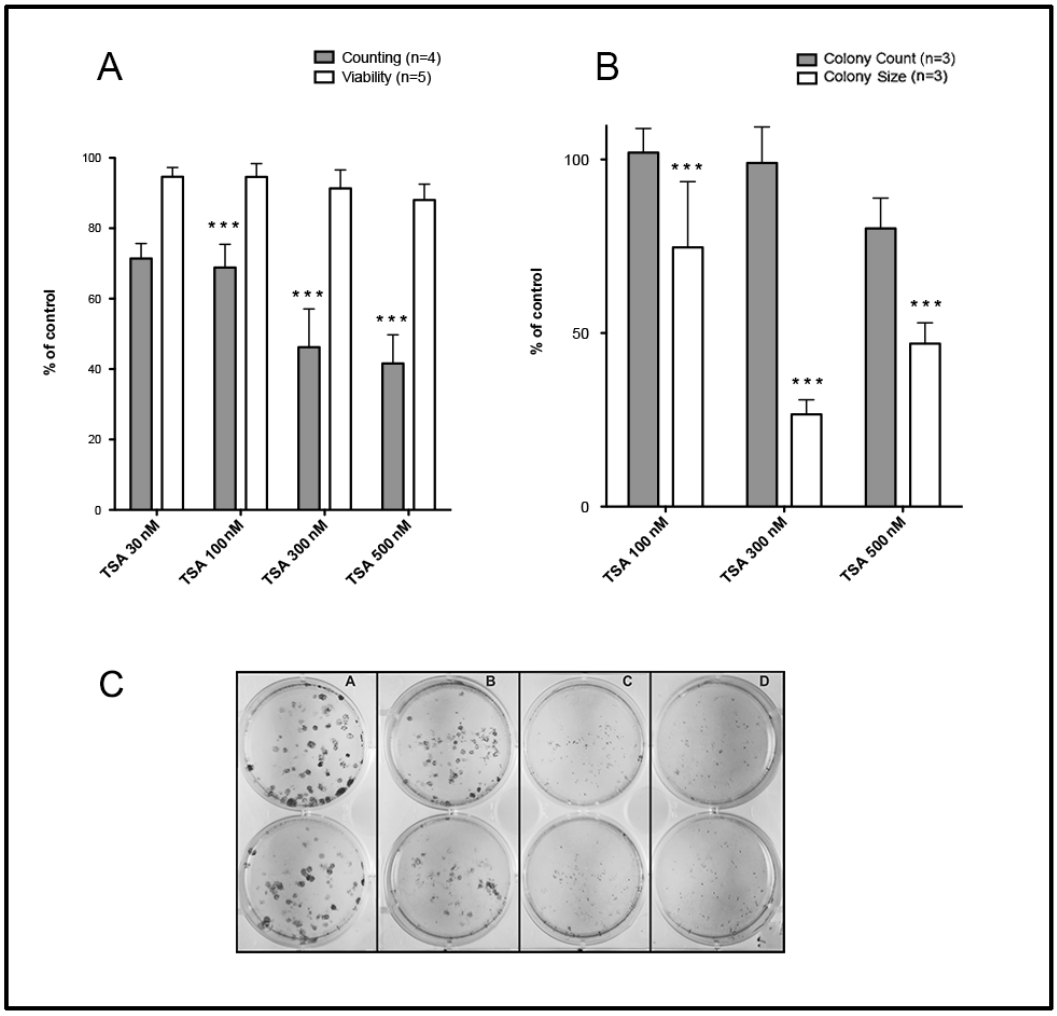

Fig. 1. The HDAC inhibitor TSA reduces proliferation and survival of human U87 GBM cells. (**A**) Cells were culture and treated with TSA at 30, 100, 300, or 500 nM for 72 h. Cell number and viability were measured by a trypan blue cell counting assay. Data are percent mean ± S.E.M. of total number of cells or number of viable cells relative to controls; the mean value among replicates in control cells was taken as 100%; n = 5 independent experiments; *** $P < 0.001$ compared to control cells. (**B**) Cells were treated with TSA at 100, 300, or 500 nM for 48 h, and colonies were allowed to form for 10 days. Data are percent mean ± S.E.M. of the number of colonies or colony size relative to controls; the mean value among replicates in control cells was taken as 100%; n = 3 independent experiments; *** $P < 0.001$ compared to control cells. (**C**) Representative image of colony formation in the different experimental conditions (*A*, control; *B*, TSA 100 nM; *C*, TSA 300 nM; *D*, TSA 500 nM).

Increased protein content of differentiation markers in U87 cells treated with TSA

We next examined the possible effects of TSA on cell differentiation analyzing the protein levels of the glial marker GFAP and the neuronal marker NeuN in U87 cells by Western blot. There was an increase in GFAP in cells treated with TSA at 100 or 500 nM for 48 h, and NeuN was detected only in cells treated with 500 nM TSA (**Fig.2**). The results suggest that TSA promotes the differentiation of U87 cells.

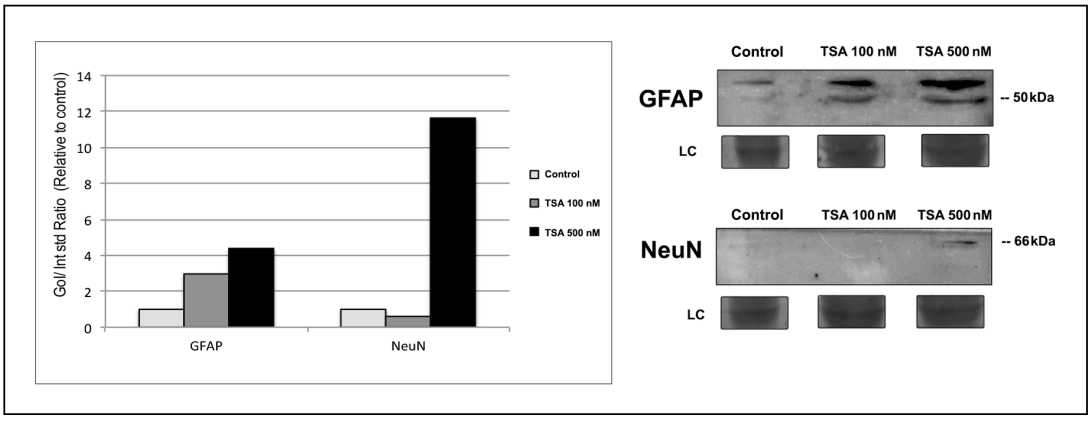

Fig. 2. TSA induces alterations in the content of glial and neuronal markers in human U87 GBM cells. A representative Western blot analysis showing increased levels of the glial marker GFAP in cells treated with 100 or 500 nM TSA for 48 h, and detection of the neuronal marker NeuN only in cells treated with 500 nM TSA. Data in the graph are shown as protein levels of the Gene of Interest (GoI) divided by the loading control (LC).

TSA induces alterations in nuclear morphology in U87 cells

A nuclear morphometric analysis (NMA, as recently described by Filippi-Chiela et al*.,* 2012) was used to assess possible TSA-induced alterations in nuclear morphology associated with cell death or senescence. The analysis of DAPI-stained nuclei revealed an increased number of cells with large and regular nuclei in the cells exposed to 500 nM TSA for 48 h ($P < 0.05$ compared to controls), suggesting the induction of senescence (**Fig. 3A-3C**). No other alterations were found. The presence of senescent cells was confirmed by a SA-β-galactosidase activity assay (**Fig. 3D**), in which senescent cells displayed morphological alterations (e.g., multiple nuclei and flat cytoplasm) similar to those observed in the NMA analysis.

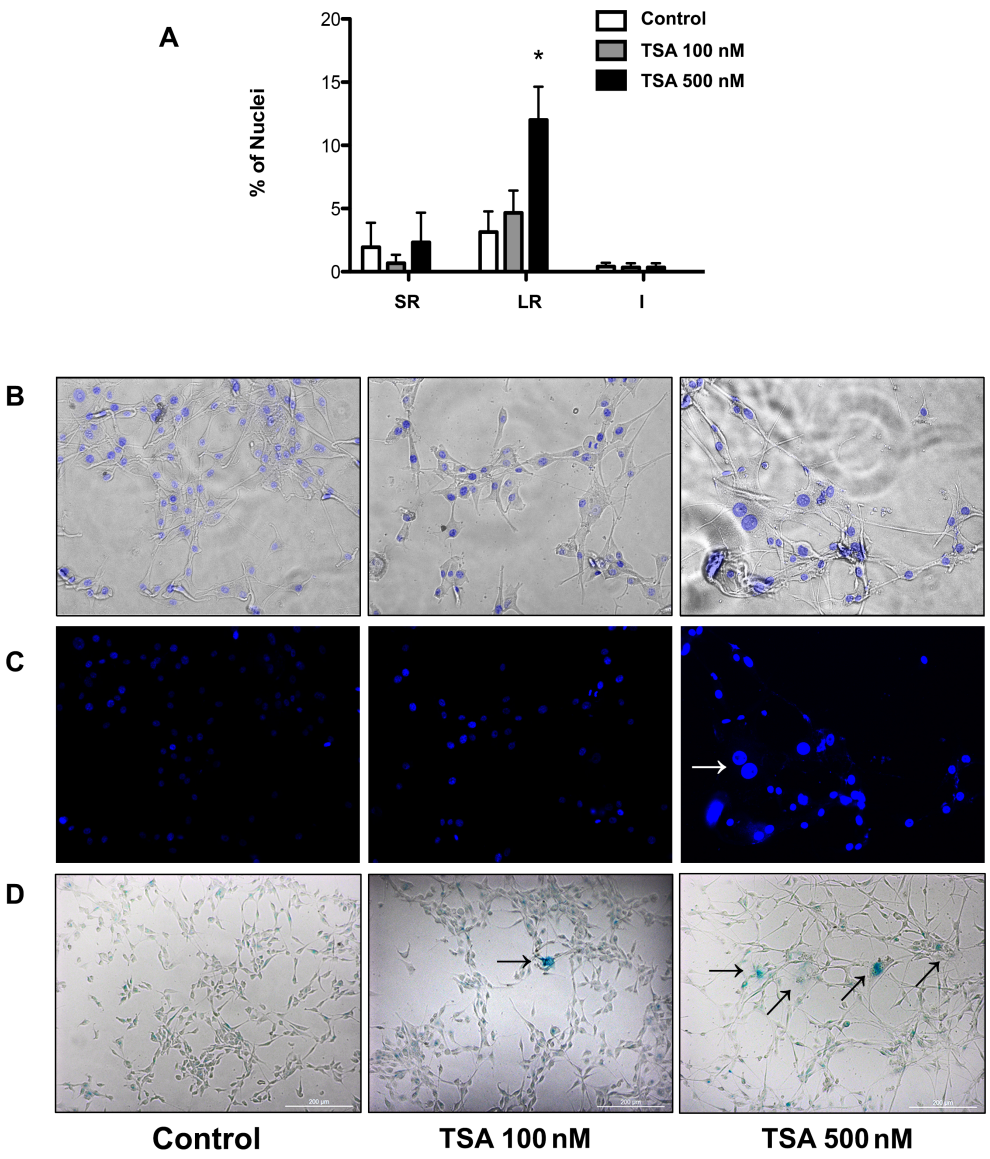

Fig. 3. TSA alters the nuclear morphology of human U87 GBM cells. Cells were treated with TSA (100 or 500 nM) for 48 h for NMA analysis. The area (in pixels) and Nuclear Irregularity Index (NII) of each nucleus were measured using DAPI images. (**A**) Percent mean ± S.E.M. of apoptotic cells showing small and regular nuclei (SR), senescent cells showing large and regular nuclei (LR), and cells with irregular nuclei (I); *n* = 3 independent experiments; * *P* < 0.05 compared to control cells. (**B**, **C**) Representative images used for NMA showing (**B**) DAPI-stained nuclei merged with bright field and (**C**) DAPI alone (x 20). (**D**) Representative image from SA-β-galactosidase activity assay showing the senescent morphology in treated cells. Arrows indicate senescent cells (x 10).

TSA does not affect U87 cell migration

To further examine the effects of TSA on GBM cell function, a scratch wound healing assay was used to allow the observation of cell migration *in vitro*. TSA failed to significantly alter the number of cells migrating towards the wound center (data not shown) or the migration index (**Fig. 4**), defined as the difference between wound sizes at t = 0 and t = 10 h, which represents the overall migration of cells.

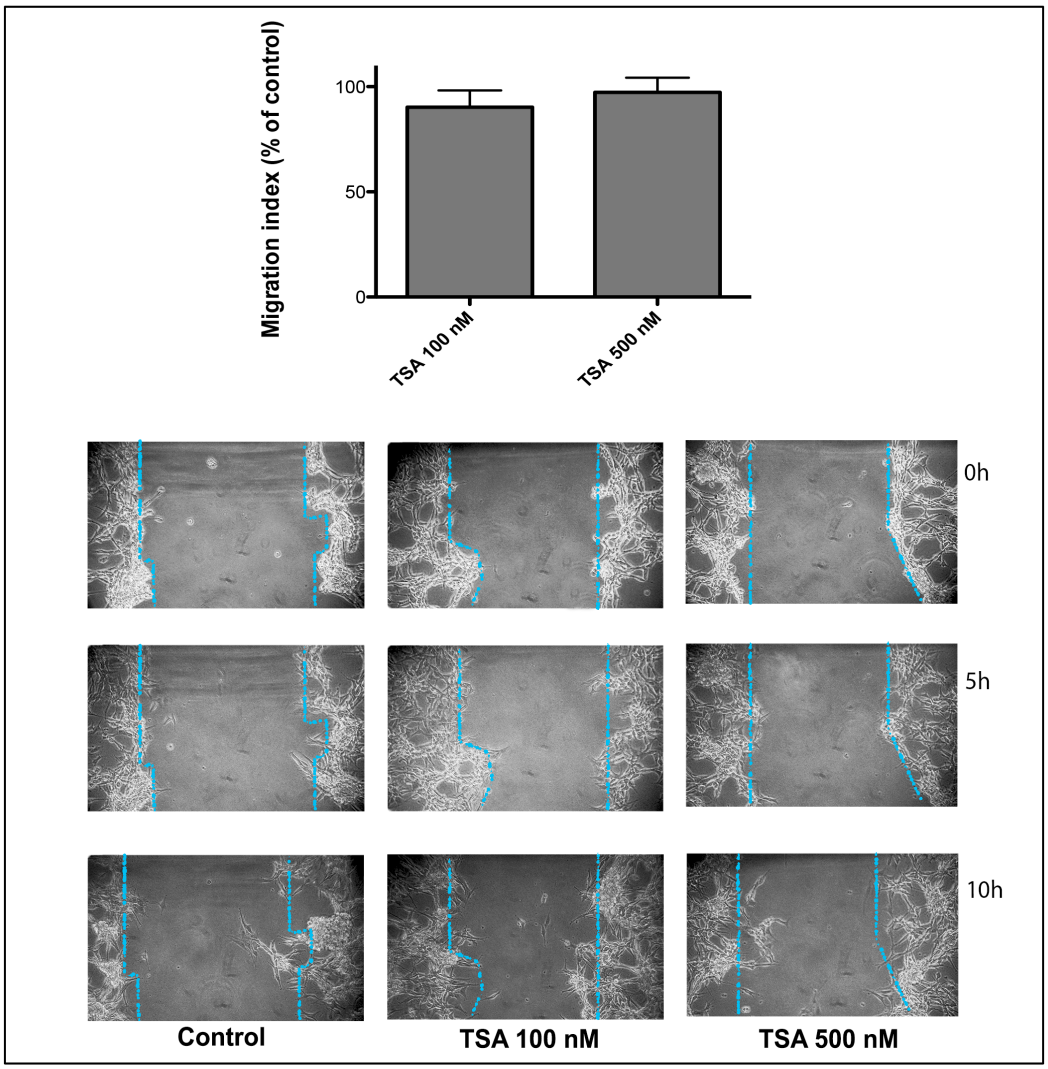

Fig. 4. TSA does not affect the migration of human U87 GBM cells. Cells were cultured and treated with TSA (100 or 500 nM) for 48 h for migration measurement by a scratch wound assay. Photographs were taken at t = 0 h and t = 10 h after scratching. The distances between wound borders were measured using ImageJ. Top, data in the graph are shown as percent mean ± S.E.M. migration index, defined as the difference of wound size between t = 0 and t = 10 h; *n* = 3 independent experiments. Bottom, representative images of photographs taken at t = 0 h, t = 5 h, and t = 10 h illustrating cell migration in control and TSA-treated cells (x 10).

TSA reduces tumorsphere formation and sizes in U87 cell cultures

We then went on to investigate the consequences of TSA treatment on stem-like GBM cells obtained by inducing tumorsphere formation in U87 cell cultures. Cultivating U87 cells with appropriate growth factors for stem cell expansion led to consistent tumorsphere formation within 72 h of treatment. TSA (100, 300, or 500 nM) induced a pronounced reduction in the number of tumorspheres counted at 72 h (mean reduction to 83.9, 54.5, and 19.9%, respectively; TSA 100 nM, $P < 0.02$; TSA 300 or 500 nM, $P < 0.001$ compared to control cells). Cells in tumorspheres were then dissociated for trypan blue cell counting analysis. TSA induced a significant decrease in the number of tumorsphere-derived cells (TSA 100 nM, $P < 0.05$; TSA 300 nM, $P < 0.02$; and TSA 500 nM, $P < 0.001$ compared to controls) (**Fig. 5A**).

Mature tumorspheres were obtained after two passages during tumorsphere expansion (**Fig. 5C**). These spheres were dissociated and then re-cultured at low confluence in regular U87 medium supplemented with serum to provide cell adhesion. After adhesion cells were treated for 48 h with TSA at 300 or 500 nM. The treatment significantly decreased cell survival expressed by mean colony size (mean reduction was 72 % in cells treated with TSA 300 nM and 61% in cells treated with TSA 500 nM ($P < 0.0001$, compared to control) (**Fig. 5B**, **5D**).

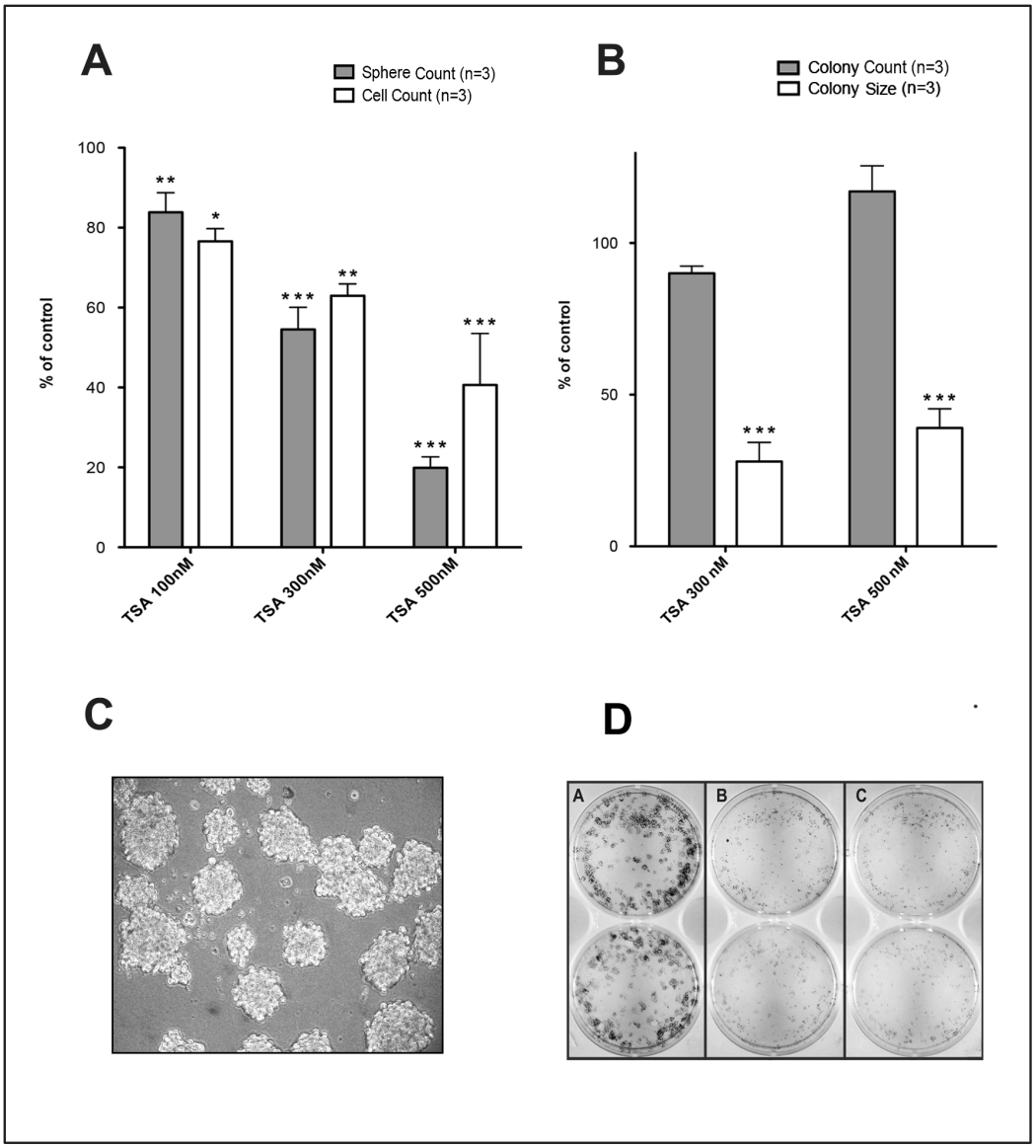

Fig. 5. TSA reduces the proliferation of putative GSCs assessed by the number and sizes of tumorspheres derived from human U87 GBM cells. (**A**) Cells were grown in the presence of growth factors under conditions appropriate for stem cell expansion and treated with TSA (100, 300, or 500 nM) for 72 h. Data are percent mean ± S.E.M. number of spheres and tumorsphere-derived dissociated cells; n = 3 independent experiments; * $P < 0.05$ ** $P < 0.02$, and *** $P < 0.001$ compared to control cells. (**B**) Tumorspheres were dissociated, re-cultured, and treated with TSA (300 or 500 nM) for the assessment of colony formation. Data are percent mean ± S.E.M. number of colonies and colony size; n = 3 independent experiments; *** $P < 0.0001$ compared to control cells. (**C**) Representative photomicrograph of untreated tumorspheres used for the colony-forming assay prior to dissociation (x 10). (**D**) Representative images of colonies from (*A*) control cells and cells exposed to TSA at (*B*) 300 or (*C*) 500 nM.

We further explored the effects of TSA on GBM tumorspheres by analyzing tumorsphere sizes and area covered by tumorspheres in culture plates. Mature tumorspheres were treated for 48 h with TSA at 300, 500, 800, or 1,000 nM. Higher doses were included because pilot experiments indicated that mature tumorspheres showed resistance to TSA (data not shown). TSA significantly reduced the number of spheres classified as “large” (i.e., 300 uM or more), with the highest dose leading to a mean 51% reduction ($P < 0.001$) (**Fig. 6A**). In addition, TSA at the higher doses used produced significant decreases in mean tumorsphere size (TSA 800 nM, $P < 0.01$; TSA 1,000 nM, $P < 0.001$) (**Fig. 6B**), number of tumorspheres (TSA 1,000 nM, $P < 0.01$) (**Fig. 6C**), and total area occupied by tumorspheres (TSA 800 nM, $P < 0.01$; TSA 1,000 nM, $P < 0.001$) (**Fig. 6D**). Representative photographs of control cells and a culture treated with 1,000 nM TSA are shown in **Fig. 6E** and **Fig. 6F**, respectively. Together, the results suggest that TSA inhibits the proliferation, survival, or permanence in a ‘stem-like’ state, of putative GSCs, in U87 cultures.

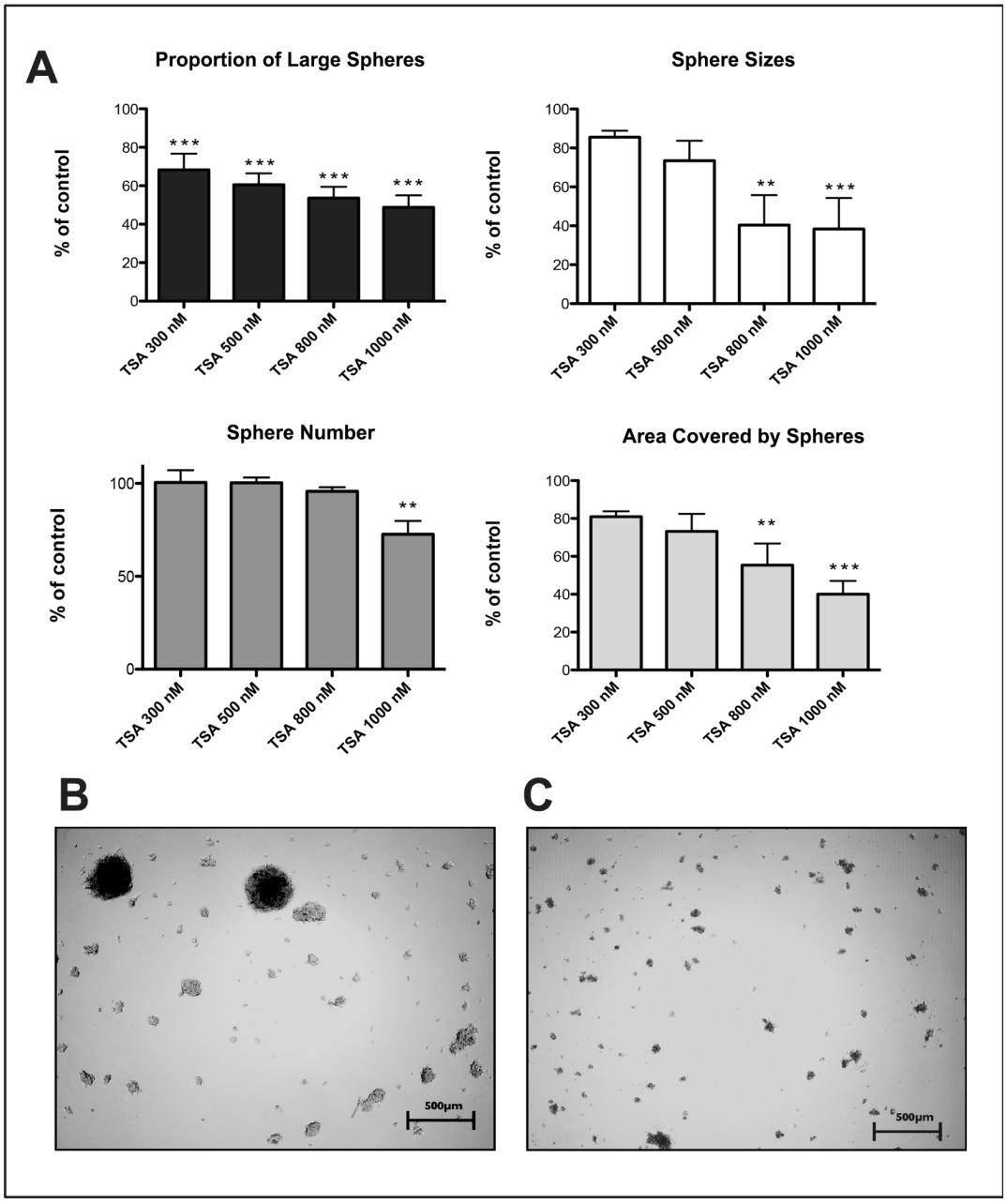

Fig. 6. TSA reduces the size of tumorspheres derived from human U87 GBM cells containing putative GSCs. Tumorspheres were treated with TSA (300, 500, 800, or 1,000 nM) for 48 h. The (**A**) proportion of large spheres, (**B**) sphere sizes, (**C**) sphere number, and (**D**) area covered by spheres were analyzed using ImageJ. Data are percent mean $\pm$ S.E.M.; the mean value among replicates in control cells was taken as 100%; $n = 3$ independent experiments; ** $P < 0.01$ and *** $P < 0.001$ compared to control cells. Representative images of cultures (**E**) in the untreated condition and (**F**) exposed to 1,000 nM TSA are shown (x 4).

Neuron-like morphology, increased mRNA levels of differentiation markers, and reduced mRNA levels of stemness in tumorsphere-derived GSCs treated with TSA

To evaluate whether TSA promoted the differentiation or loss of stemness of U87 cells constituting tumorspheres, a morphological analysis followed by measurement of mRNA levels for differentiation and stemness markers was performed. During tumorsphere formation, we observed an increase in the number of adherent single cells and a reduction in the number of floating spheres, an alteration that was more pronounced as the concentration of TSA increased (**Fig. 7A-7D**). Adherent cells showed a distinct neuron-like morphology with long and bipolar cell extensions and thinner cytoplasm (**Fig. 7E-7G**). Obvious morphological alterations were seen also in mature tumorspheres treated with TSA for 48 h compared to control spheres (**Fig. 7H, 7I**).

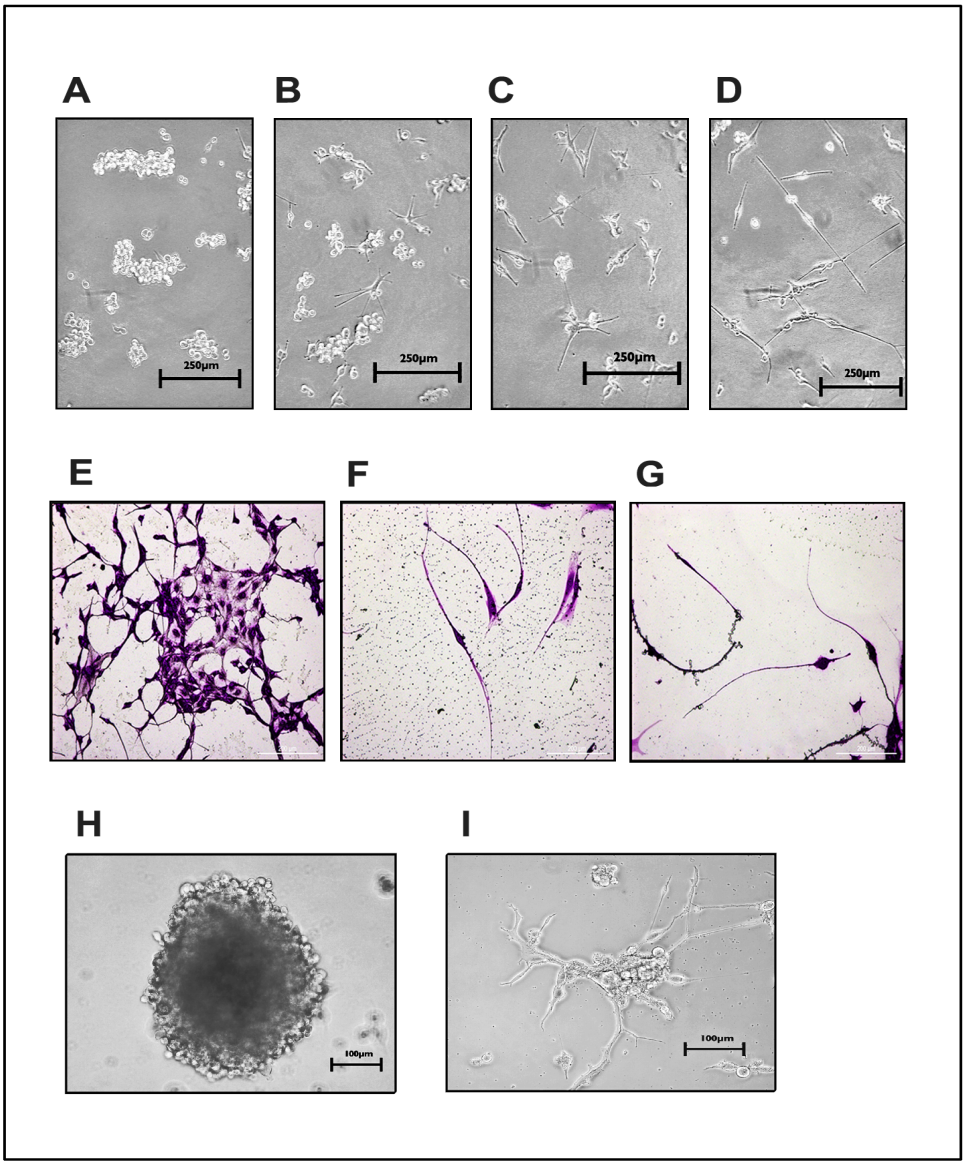

Fig. 7. TSA induces neuron-like morphological alterations in putative GSCs derived from GBM tumorspheres. U87 cells were grown under conditions appropriate for stem cell expansion and treated with TSA (100, 300, or 500 nM) for 72 h. An increase in the number of adherent single cells and a reduction in the number of floating spheres was observed with increasing concentrations of TSA (**A**, control; **B**, TSA 100 nM; **C**, TSA 300 nM; **D**, TSA 500 nM; (x 10). (**E-G**) Representative microphotographs of adherent tumorsphere-derived GSC showing long and bipolar extensions and thinner cytoplasm (x 20). Representative microphotographs of (**H**) a control mature tumorsphere obtained after two passages during tumorsphere expansion and (**I**) a tumorsphere treated with 1,000 nM TSA for 48 h illustrate TSA-induced morphological changes in mature tumorspheres (x 40).

RT-PCR analysis indicated that TSA (1000 nM for 48 h) led to an increase in the mRNA levels of neuronal marker Gria2 in mature tumorspheres. On the other hand, mRNA levels for the glial marker GLAST were reduced after exposure to TSA. The pluripotency and stemness markers Sox2, c-Myc and Oct4 decreased, as well as the GSC markers Prominin-1 (CD133) and Notch1. However, mRNA for another stem cell marker, Musashi, was increased by TSA treatment (**Fig. 8**). The results suggest that TSA might promote the neuronal differentiation of U87 cells forming tumorspheres. Most importantly, GBM cells from tumorspheres show stem cell-like characteristics that are altered, with the mRNA content of most stemness markers being reduced, by TSA.

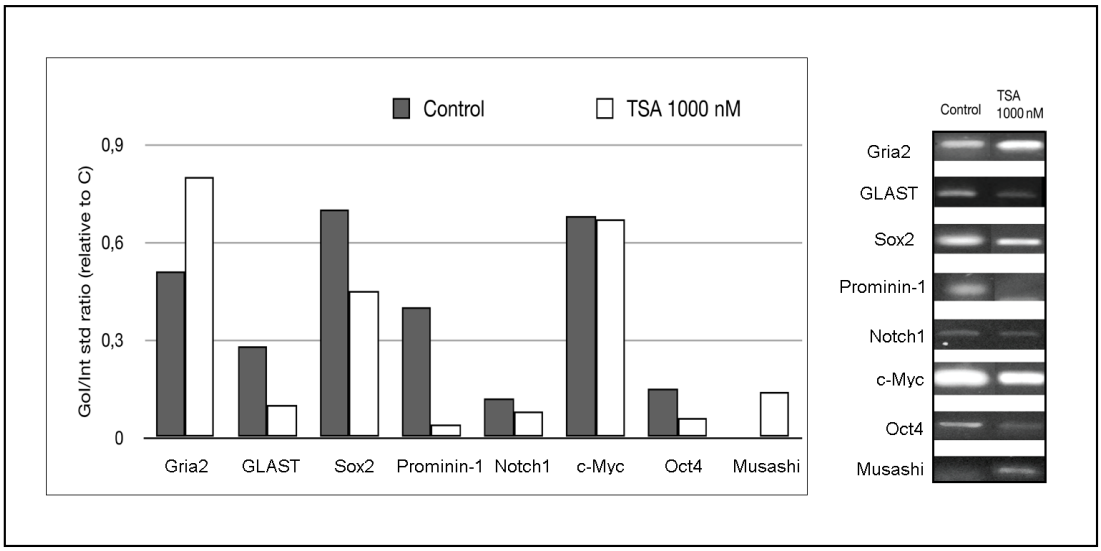

Fig. 8. TSA induces neuronal differentiation and loss of stemness in putative GSCs derived from mature U87 GBM tumorspheres. A representative RT-PCR analysis of mRNA for Gria2, GLAST, Sox2, Prominin-1, Notch1, c-Myc, Oct4, and Musashi is shown. Cells from mature tumorspheres were treated with TSA at 1,000 nM for 48 h. Data in the graph are shown as mRNA levels of the Gene of Interest (GoI) divided by the internal standard control (β-actin) relative to control cells.

DISCUSSION

In recent years, increasing attention has been focused on epigenetic alterations in cancer and the antitumor effects of agents modulating epigenetic phenomena, particularly HDACis. In GBM, epigenetic inactivation of a wide variety of genes associated with tumor suppression, cell cycle regulation, invasion, and apoptosis has been described (reviewed by Martinez, 2012). Moreover, other mechanisms of interplaying genetic and epigenetic alterations in carcinogenesis have been increasingly revealed. For instance, inactivating mutations can target genes that control the epigenome, leading to alterations in DNA methylation patterns (You and Jones, 2012). This highlights the potential of candidate therapeutics aimed at epigenetic-mediated regulation of gene expression, of which HDACis are currently the best studied (Dawson and Kouzarides, 2012).

Previous studies in experimental glioma have found that HDACis can induce apoptosis and cell cycle arrest and reduce tumor growth (Eyüpoglu et al., 2005; Komata et al., 2005; Wetzel et al., 2005; Yin et al., 2007; Bangert et al., 2012). We found that the HDACi TSA reduced proliferation without significantly affecting the viability of U87 GBM cells, suggesting dissociation between effects on proliferation and viability. This is somewhat in contrast to the recent results reported by Bajbouj et al. (2012), in which TSA produced a small inhibition of both proliferation and viability in U87 cells. In addition to inhibiting proliferation, TSA in our experiments produced a distinct effect on colony formation, reducing the sizes but not the number of colonies in U87 cell cultures. In addition, we found increased content of markers of both glial and neuronal differentiation in TSA-

treated cells, which is consistent with previous evidence of differentiation promotion by HDACis in glioma cells (Benitez et al., 2008; Svechnikova et al., 2008; Boulay et al., 2009). Furthermore, we used the recently described NMA analysis (Filippi-Chiela et al*.,* 2012) as well as the SA-β-galactosidase assay to provide the first evidence for senescence induced by an HDACi in GBM cells. Finally, we found no alterations in GBM cell migration exposed to TSA, although recent evidence from experiments using prostate cancer cells has supported the possibility that HDACis alter cell migration (Kong et al., 2012).

The molecular consequences of HDAC inhibition in GBM cells playing a role in its growth inhibitory effects remain to be further elucidated. The cyclin-dependent kinase inhibitor p21 (WAF1) was recently identified as the major player in cell cycle arrest induced by TSA in U87 cells. In addition, TSA exposure resulted in an up-regulation of p53 and down-regulation of cell cycle regulators including cdk4, cdk6, and cyclin D1. The effects of TSA in U87 cells might be triggered by increased acetylation of H3 and H4 histones and increased binding of acetylated H4 binding (Bajbouj et al., 2012).

The little improvement in chemotherapeutic success in the treatment of GBM might have to do with the presence of GBM stem-like cells (GSCs) capable of sustaining tumor growth, relapse, and therapeutic resistance. Thus, the development of effective therapies may involve focusing on the investigation of how the GSC subpopulation responds to candidate agents (Vescovi et al., 2006; Hadjipanayis et al., 2009; Roesler et al., 2010). However, few previous studies have examined the effects of HDACis on GSCs. HDACis combined with the proteasome inhibitor bortezomib have been recently shown to kill GSCs (Asklund

et al., 2012). Moreover, one study (Sun et al., 2009) showed that HDACis inhibited growth and induced differentiation and apoptosis in GBM-derived tumorspheres. The effects seemed to be mediated by HDACi-induced expression of the Delta/Notch-like epidermal growth factor-related receptor (DNER). We used tumorsphere formation as a standard *in vitro* assay to investigate the proliferation of GSCs (Singh et al., 2004; Li et al., 2009; Guryanova et al., 2011). The likelihood of the stem cell-like phenotype of our cultured cells was indicated both by tumorsphere formation and the detection of stem cells markers (Oct4, Prominin-1, Sox2, c-Myc, Notch1). Consistent with a preliminary observation (Sassi et al., 2012), TSA reduced the formation of tumorspheres in U87 cell cultures. Our analyses included the use of a novel measurement method to show that TSA reduced tumorsphere sizes and the area covered by spheres in the cultures. Moreover, the possibility that TSA induced neuronal differentiation was supported by neuron-like morphological alterations, increased mRNA expression of the neuronal marker Gria2, and decreased mRNA levels of most pluripotency and stemness markers evaluated. Finally, the possible involvement of GSCs in drug resistance is supported by our finding that affecting mature tumorspheres required the use of higher doses of TSA compared to the ones able to affect non-GSC U87 cells.

Together, these findings strongly support the possibility that TSA inhibited the proliferation and induced neuronal differentiation of GSCs. It should be noted, however, that some caveats limit this interpretation. First, although we confirmed the stem cell phenotype of our putative GSCs by tumorsphere formation, marker expression, and differentiation induction (Guryanova et al., 2011), we did not carry

out an *in vivo* tumor propagation experiment, which is taken as the gold standard for the functional definition of GSCs. Second, although the tumorsphere formation assay is accepted as a standard and useful experimental approach for the propagation of GSCs, recent evidence has raised the possibility that glioma cells lacking sphere-forming ability *in vitro* can display a high tumorigenic potential (Barrett et al., 2012; Read and Wechsler-Reya, 2012). Even taking these considerations into account, we provide consistent *in vitro* evidence for an anti-proliferative and pro-differentiation activity of TSA in GSCs.

Our study provides a rationale for the evaluation of the effects of TSA and other HDACis on GSC-driven GBM initiation and progression in *in vivo* models. Also, since no epigenetic therapy is likely to be effective in isolation, another aim of future studies should be to characterize the possible synergistic effects of HDACis combined with cytotoxic chemotherapeutics or different classes of molecularly targeted therapies. In this regard, some studies using medulloblastoma and leukemia cells suggest that combinations of an HDACi with the topoisomerase II inhibitor etoposide might be particularly effective (Tsai et al., 2000; Sonnemann et al., 2006; dos Santos et al., 2009; Nör et al., submitted manuscript). In GBM cells, valproic acid was shown to synergistically enhance etoposide-induced cytotoxicity (Das et al., 2007). Reductions in proliferation of brain tumor cells have also been reported with the use of HDACis combined with agents that act on tyrosine kinase receptors (Marino et al, 2011).

CONCLUSION

In summary, our results support and extend previous findings indicating that HDACis hinder the proliferation and survival of GBM cells, and indicate that TSA can decrease the expansion and induce neuronal differentiation of GSCs. These results provide a foundation for further investigation and development of HDACis as anti-GSC therapeutic agents.

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CAPÍTULO IV

DISCUSSÃO

1. *RATIONALE*

Tendo em vista os dados epidemiológicos relacionados a malignidade dos GBM (**Tabela 1**), a qual é refletida nos altos índices de fatalidades na população, tem-se como urgente a necessidade de se estudar novas abordagens terapêuticas, com a finalidade de se encontrar terapias mais promissoras para esse câncer.

O tratamento dos GBM manteve-se praticamente inalterado até aproximadamente duas décadas atrás, quando ocorreu a implementação do uso do quimioterápico TMZ para os tumores gliais. Apesar da aceitação, o impacto para os pacientes não foi como o esperado, em relação à taxa de sobrevivência.

Surgiu, recentemente, uma nova opção no tratamento desses tumores: a descoberta e o estudo de um grupo de células que poderia atuar como alvo terapêutico no tratamento antitumoral. Esse grupo, denominado células-tronco tumorais, possui características únicas, relacionadas não só às altas taxas de proliferação desses gliomas, mas também à resistência quimio e radioterápica, à infiltração e recidiva desses tumores.

Também, nos últimos anos, entrou em evidência a modulação epigenética como parte da terapia de diversos cânceres. Mudando o padrão de metilação ou acetilação, por exemplo, das caldas das histonas, pode-se alterar diversos aspectos da expressão gênica, e portanto, reverter aberrações epigenéticas, mudando o fenótipo celular.

Este trabalho foi realizado levando em consideração este *rationale*, conduzido para estudar o efeito da Tricostatina A (TSA), um HDACi, o qual aumenta a acetilação global das células, causando diversos efeitos antitumorais, principalmente em células malignas. Mais especificamente, os experimentos foram conduzidos para verificar se a TSA era eficaz afetando CSC de glioblastomas e quais efeitos esta droga estaria ocasionando.

2. ANÁLISE DOS RESULTADOS (CAPÍTULO III)

No presente trabalho, avaliaram-se os efeitos da TSA na linhagem celular de glioblastoma humano U87-MG (U87), utilizada como um modelo para o estudo das CSC. Primeiramente foi avaliado o tratamento de TSA na monocamada de U87 com doses que variavam de 30-500 nM. A contagem de células foi utilizada para avaliar a proliferação e viabilidade celular, após o tratamento. A TSA mostrou afetar essas células com doses iguais ou superiores a 100 nM, causando redução significativa na proliferação celular acompanhada de alterações fenotípicas (Fig. 1, Fig. 4). Os resultados mostram que a TSA é capaz de afetar o crescimento celular, mas não necessariamente afeta a viabilidade celular. Isso significa que a TSA poderia estar atuando via mecanismos de parada do ciclo celular e senescência, ou ainda induzindo um aumento da diferenciação celular (ou de células diferenciadas), caracterizando um efeito citostático e não citotóxico. Além disso, a TSA reduziu eficazmente a sobrevivência de células após 10 dias depois de 48 horas de tratamento, o que sugere que as células podem manter os seus efeitos induzidos pela TSA durante um longo período após à exposição ao HDACi (Fig. 1B).

Esses resultados foram também observados quando os mesmos experimentos foram repetidos com as tumoresferas de U87 (Fig. 2A-B). Neste caso, através do ensaio de formação de esferas e do ensaio clonogênico com células derivadas de tumoresferas, foi possível observar que os efeitos de TSA se estendem também à população de CSC, como pode ser visto também no tratamento de esferas maduras, as quais são mais resistentes (Fig.3 e Fig. 4), e pela redução dos níveis de mRNA para marcadores de células-tronco, incluindo o marcador de CSC CD133 (Prominin-1) (Fig. 5A). Min *et al*., 2012, mostraram recentemente que a TSA pode afetar nas linhagens celulares de câncer de ovário, a população de CSC (expressando CD133), mas esse feito varia para cada linhagem, como resultado da sua interferência, também nos padrões de metilação das células. O nosso estudo mostrou, no entanto, que, para a linhagem U87 de GBM, a TSA reduziu os níveis do mRNA de CD133 em conjunto com os marcadores de pluripotência (como Sox2 e Oct4) das tumoresferas tratadas. Além disso, Asklund

et al., 2012, já demonstraram que as combinações do inibidor de proteossoma Bortezomibe com HDACis podem afetar culturas de CSC de GBM (TB101 e R11) e as suas capacidades de formar colônias. Aqui mostramos pela primeira vez que a TSA, por si só, é capaz de reduzir a proliferação de células de glioma afetando, também, a população CSC.

A fim de melhor compreender os efeitos fenotípicos observados na morfologia das células de U87 tratadas (Fig. 4), foi investigada a expressão de marcadores de diferenciação neural. Como a expressão do marcador neuronal AMPA2 (subunidade do receptor GluR2) já foi descrita como aumentada em U87 após o tratamento com TSA (Ekici *et al*., 2012), fez-se o uso do mesmo marcador neste estudo (Fig. 5A). Aqui verificou-se que a TSA na concentração de 1000 nM foi eficaz para aumentar os níveis de mRNA do AMPA2 em tumoresferas de glioma. Este resultado foi suportado pelo surgimento da expressão da proteína de NeuN , uma proteína nuclear de neurônio, após 48h de tratamento com TSA 500nM (Fig. 5B).

A hipótese de diferenciação neuronal também foi apoiada pelas alterações marcantes nas esferas maduras, as quais tornaram-se aderentes (mesmo na ausência de soro) após o tratamento com TSA por 48h (Fig. 4I). Além disso, encontramos aumento dose-dependente nos níveis de GFAP, um marcador para a diferenciação de astrócitos (Fig. 5B), o qual foi controverso com a diminuição dos níveis de mRNA do marcador glial GLAST (Fig. 5). Em seu estudo, Svechnikova *et al*., 2008, também constatou que as linhagens de GBM diferenciaram em resposta à TSA. Nas células U-343 MGa, 100 nM de TSA durante quatro semanas, resultou no aumento da diferenciação astrocítica, com uma elevada expressão de GFAP e morfologia característica. De acordo com esses resultados, eles também observaram que o tratamento com 100 nM de TSA durante 5 dias reduziu a expressão Nestina consideravelmente e ainda mais a expressão de Vimentina. Para um efeito mais rápido, os mesmos autores utilizaram TSA 500 e 1000 nM por 48h em células U-343 MG. Neste caso eles encontraram uma redução de 60 e 80%, respectivamente, na expressão de Vimentina, a qual está inversamente correlacionada com a diferenciação celular.

Existem na literatura diversos trabalhos relativos aos efeitos de TSA (e outros

HDACis) na indução de apoptose por meio da ativação de caspase 3 e de p53 em linhagens celulares de GBM (Svechnikova *et al*, 2008; Hsu *et al*, 2011), câncer de pulmão (Zhang *et al*., 2009) entre outros. Além disso, a parada do ciclo celular foi também descrita como um efeito comum após o tratamento com TSA. Por exemplo, um estudo mostrou que a presença de TSA em células U87 mostrou além da redução de proliferação celular, parada no ciclo celular com acúmulo em G1/S. Este efeito foi acompanhado por um significativo aumento da expressão da proteína p53. Neste estudo, entretanto a TSA não induziu apoptose em U87 (de forma similara aos nossos resultados: Fig.1 e Fig. 6), mas sim a translocação nuclear de *p21(WAF1)*, provocando a parada do ciclo (Bajbouj *et al*, 2012.).

A parada do ciclo celular, no entanto, não é sinônimo de senescência (Blagosklonny, 2011). Em culturas de células tumorais, a parada pode levar a senescência, por conflitar com o resultado da estimulação oncogênica. Isso pode levar a um estado de hipertrofia celular (células maiores), que por sua vez leva à ativação compensatória dos lisossomos, autofagia e positividade para beta-Gal (Blagosklonny, 2011). Os nossos resultados confirmam que a TSA pode induzir a senescência celular, tal como foi observado com a análise morfométrica nuclear, a presença de células com núcleos grandes, citoplasma plano, e positividade para beta-Gal (Fig.6) com 500 nM de TSA por 48h. Esses efeitos são prováveis resultados da parada no ciclo celular.

Por último, analisamos também os efeitos da TSA sobre a migração celular. Em discordância com o trabalho de Kong *et a*l. (2012) com células de tumor de próstata, não encontramos nenhuma diferença na migração das células com ambas as concentrações de TSA analisada: 100 e 500 nM, durante 48 horas. Isto pode indicar, como resultado preliminar, que TSA não leva ao aumento da agressividade do tumor, tal como foi sugerido pelos autores.

3. CONCLUSÃO

Os nossos resultados mostram um grande número de efeitos de TSA sobre a diferenciação, proliferação, sobrevivência de células de glioma e também na indução de senescência celular, fazendo com que a TSA possa servir como um agente promissor para a terapia dos GBM. O mais importante é que a atividade do HDACi afetou também as células-tronco tumorais da linhagem celular estudada, o que suporta o uso de TSA por si só, ou em combinação com outras drogas e terapias, como visto em muitos estudos na literatura. Como ensaios clínicos ainda não foram realizados com TSA, mais estudos são necessários para avaliar a sua utilização clínica.

4. PERSPECTIVA

Tem-se como perspectivas imediatas deste trabalho a combinação do uso de TSA com outras drogas utilizadas no tratamento de GBM, tais como a Temozolamida, a Vincristina e também drogas que fazem dano ao DNA como a Tetraciclina e drogas que afetas as topoisomerases, como o Etoposídeo. A ação dessas drogas pode ser sinergicamente melhorada pelos efeitos da TSA na acetilação e na indução de apoptose. Além disso, como perspectivas futuras, efetuaremos testes *in vivo* em modelos animais, para uma melhor avaliação clínica da droga.

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ANEXO

Curriculum Vitae

Felipe de Almeida Sassi

LOCAL DE NASCIMENTO: Porto Alegre, RS, Brasil

DATA DE NASCIMENTO: 02 de Outubro de 1988

ENDEREÇO: Av. Caí 303/113 Porto Alegre, RS, Brasil

TELEONE: +555193319577

Endereço para acessar este CV (lattes): http://lattes.cnpq.br/0370418209211299

E-mail: fesassi@gmail.com

Formação

- **UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL, UFRGS, 2011-2013**

  Mestrado em Biologia Celular e Molecular - "Redução da proliferação e sobrevivência celular por diferenciação neuronal de células-tronco de glioblastoma humano expostas a um inibidor de histona deacetilase." (*Orientador*: Prof. Dr. Rafael Roesler)
- **UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL, UFRGS, 2007-2010**

  Bacharelado em Biomedicina - "Aplicações de células-tronco mesenquimais no tratamento da hipóxia- isquemia cerebral neonatal em modelo animal" (*Advisor*: Prof. Dr. Patricia Helena Lucas Pranke)

Prêmios e Distinções

- **APRESENTAÇÃO ORAL NO 10º CONGRESSO INTERNACIONAL EM BIOLOGIA CELULAR. RIO DE JANEIRO, BRASIL, 2012.**
- **BOLSA DE MESTRADO PELO CONSELHO NACIONAL DE DESENVOLVIMENTO CIENTÍFICO E TECNOLÓGICO.**
- **FUNDO DE INCENTIVO À PESQUISA E EVENTOS (FIPE) DO HOSPITAL DE CLÍNICAS DE PORTO ALEGRE, 2011-2013**
- **MELHOR TRABALHO DE MESTRADO NO SIMPÓSIO NOVAS FRONTEIRAS DA BIOLOGIA CELULAR, SOCIEDADE BRASILEIRA DE BIOLOGIA CELULAR, 2011**
- **AUXÍLIO FINANCEIRO PARA MISSÃO CIENTÍFICA INTERNACIONAL DE CURTA DURAÇÃO, PRÓ- REITORIA DE PÓS-GRADUAÇÃO UFRGS.**

Publicações

- **SASSI, F. A.; BRUNETTO, A. L.; SCHWARTSMANN, G.; ROESLER, R.; ABUJAMRA, A.** "GLIOMA REVISITED: FROM NEUROGENESIS AND CANCER STEM CELLS TO THE EPIGENETIC REGULATION OF THE NICHE." **JOURNAL OF ONCOLOGY, VOLUME 2012, ARTICLE ID 537861, 20 PAGES DOI:10.1155/2012/537861**
- **SASSI, F. A. ; CAESAR, L.; JAEGER, M.; LOPEZ, P. L. C.; DE FARIAS, C. B.; NÖR, C.; SCHWARTSMANN, G. ; BRUNETTO, A. L. ; ABUJAMRA, A.; ROESLER, R.** "THE HISTONE DEACETYLASE INHIBITOR TRICHOSTATIN A REDUCES PROLIFERATION AND INDUCES NEURONAL DIFFERENTIATION OF U87- DERIVED HUMAN GLIOBLASTOMA STEM CELLS" **NEUROSCIENCE, SUBMETIDO.**

Experiência Científica

- ***PESQUISA DE MESTRADO:*** **LABORATÓRIO DE PESQUISA EM CÂNCER, CPE-HCPA, UFRGS, BRASIL, 2011-2013**

  Projeto: "Sinalização celular por neuropeptídeos e neurotrofinas em células-tronco de tumores cerebrais" (*Orientador*: Prof. Dr. Rafael Roesler; Bolsa CNPq)

  Descrição: obtenção de células-tronco tumorais de linhagens de gliomas, caracterização molecular de tumor-esferas de gliomas (RT-PCR, qPCR), desenvolvimento de uma metodologia para a medição do tamanho de tumor-esferas com o *software* ImageJ; manipulação epigenética das células tumorais; avaliação da viabilidade e sobrevivência celular / apoptose / senescência / diferenciação neural

- ***ESTÁGIO CURRICULAR:*** **LABORATÓRIO DE SINALIZAÇÃO E PLASTICIDADE CELULAR, DEPARTAMENTO DE BIOFÍSICA, UFGRS, BRASIL , 2011**

  Projeto: "Células-tronco tumorais: indicadores de malignidade?" - Ênfase Molecular (*Orientador*: Prof. Dr. Guido Lenz)

  Descrição: cultura de células; RT-PCR; *Western Blot*; citometria de fluxo; silenciamento por *short-hairpin RNA*.

- ***TRABALHO DE CONCLUSÃO DE CURSO*****: LABORATÓRIO DE ISQUEMIA CEREBRAL, DEPARTAMENTO DE BIOQUÍMICA, UFGRS, BRASIL, 2009-2010**

  Projeto: "Engenharia de Tecidos: o uso de células-tronco e da nanotecnologia para a regeneração de tecidos em modelos animais de lesões neurológicas" *(Orientador:* Prof Dr. Carlos Alexandro Netto*)*

  Descrição: manipulação de ratos; cirurgia em modelo animal de isquemia cerebral; injeções de células, testes de comportamentais; manipulação do tecido cerebral; preparação de lâminas histológicas; imunofluorescência; microscopia confocal

- ***PESQUISA DE INICIAÇÃO CIENTÍFICA:*** **LABORATÓRIO DE PESQUISA BÁSICA, INSTITUTO DE PESQUISA COM CÉLULAS-TRONCO (IPCT), BRASIL, 2008-2009**

  Projeto: "Uso de nanotecnologia na bioengenharia para produzir pele, através do cultivo de células-tronco em matrizes produzidas por *electrospinning*" (*Orientador:* Prof Dr. Patrícia Helena Lucas Pranke*)*

  Descrição: Produção de matrizes de PLGA [poli (ácido láctico-co-ácido glicólico)] por electrospinning, obtenção e estabelecimento de linhagens primárias de células-tronco mesenquimais; microscopia eletrônica

- ***PESQUISA DE INICIAÇÃO CIENTÍFICA:*** **DEPARTAMENTO DE GENÉTICA, INSTITUTO DE BIOCIÊNCIAS, UFRGS, BRASIL, 2007-2008**

Projeto: "Estudo de associação entre polimorfismos e genes candidatos no transtorno de déficit de atenção e hiperatividade (TDAH) em adultos" (*Orientador:* Prof Dr. Claiton Henrique Dotto Bau)

Descrição: PCR, genotipagem, extração de DNA, meta-análise

Formação Complementar

- **MICROSCOPIA AVANÇADA, 10º CONGRESSO INTERNACIONAL EM BIOLOGIA CELULAR. RIO DE JANEIRO, BRASIL, 2012.**
- **XVII ALEXANDER HOLLAENDER COURSE, URUGUAY, 2012**

  Environmental genetics, epigenetics and genomic instability: Capacity building on new analytical tools
- **SINALIZAÇÃO CELULAR NO CÂNCER, UFRGS, BRAZIL, 2011**
- **CUIDADOS NO MANEJO DE ANIMAIS DE LABORATÓRIO, FFFCMPA, BRASIL, 2007**
- **PROFICIÊNCIA NA LÍNGUA INGLESA (TOEFL, SCORE 101-IBT),**

Apresentação de trabalhos

- **SASSI, F. A. ; LAUREN VALENTIM ; PATRICIA HELENA LUCAS PRANKE ; CARLOS ALEXANDRE NETTO .** DENTAL PULP STEM CELL-TREATMENT ASSOCIATED WITH RECOVERY OF COGNITIVE OUTCOME IN A NEONATAL HYPOXIA-ISCHEMIA ANIMAL MODEL. **2011. (APRESENTAÇÃO DE TRABALHO/CONGRESSO).**
- **SASSI, F. A. ; NOR C. ; ABUJAMRA, A. ; ROESLER, R. .** BRAIN CANCER STEM CELLS ARE AFFECTED BY AN HISTONE DEACETYLASE INHIBITOR. **2011. (APRESENTAÇÃO DE TRABALHO/CONGRESSO).**
- **SASSI, F. A. ; PATRICIA HELENA LUCAS PRANKE ; CARLOS ALEXANDRE NETTO ; LAUREN VALENTIM ; LISIANE BERNARDI ; NICE ARTENI .** DENTAL PULP STEM CELL TREATMENT ASSOCIATED WITH RECOVERY OF COGNITIVE OUTCOME IN A NEONATAL HYPOXIA-ISCHEMIA ANIMAL MODEL. **2010. (APRESENTAÇÃO DE TRABALHO/CONGRESSO).**
- **SASSI, F. A. ; LAUREN VALENTIM ; PATRICIA HELENA LUCAS PRANKE .** APLICAÇÃO DE CÉLULAS-TRONCO MESENQUIMAIS NO TRATAMENTO DA HIPÓXIA-ISQUEMIA CEREBRAL NEONATAL EM MODELO ANIMAL. **2010. (APRESENTAÇÃO DE TRABALHO/OUTRA).**
- **SASSI, F. A. ; CARLOS ALEXANDRE NETTO ; DAIKELLY IGLESIAS ; DANIELA STEFFENS ; ZANATTA, G.GEANCARLO ZANATTA ; MARIA DA GLÓRIA TRAMUNT ; PATRICIA HELENA LUCAS PRANKE .** ISOLAMENTO E CARACTERIZAÇÃO DE CÉLULAS-TRONCO MESENQUIMAIS E SUA ASSOCIAÇÃO COM NANOTECNOLOGIA PARA USO NA ENGENHARIA DE TECIDOS. **2009. (APRESENTAÇÃO DE TRABALHO/OUTRA).**
- **DAIKELLY IGLESIAS ; DANIELA STEFFENS ; SASSI, F. A. ; ZANATTA, G.GEANCARLO ZANATTA ; MARIA DA GLÓRIA TRAMUNT ; PATRICIA HELENA LUCAS PRANKE .** USO DE CÉLULAS-TRONCO MESENQUIMAIS HUMANAS NA ENGENHARIA DE TECIDOS VISANDO O TRATAMENTO DA LESÃO DE MEDULA ESPINHAL. **2009. (APRESENTAÇÃO DE TRABALHO/OUTRA).**
- **MARIA DA GLÓRIA TRAMUNT ; SASSI, F. A. ; DANIELA STEFFENS ; DAIKELLY IGLESIAS ; ZANATTA, G.GEANCARLO ZANATTA ; PATRICIA HELENA LUCAS PRANKE ; CARLOS ALEXANDRE NETTO .** OBTENÇÃO, CULTIVO E CARACTERIZAÇÃO DE CÉLULAS-TRONCO MESENQUIMAIS HUMANAS DE CORDÃO UMBILICAL PARA O TRATAMENTO DA HIPÓXIA-ISQUEMIA CEREBRAL NEONATAL EM RATOS. **2009. (APRESENTAÇÃO DE TRABALHO/OUTRA).**
- **DANIELA STEFFENS ; CARLOS ALEXANDRE NETTO ; DAIKELLY IGLESIAS ; SASSI, F. A. ; ZANATTA, G.GEANCARLO ZANATTA ; MARIA DA GLÓRIA TRAMUNT ; PATRICIA HELENA LUCAS PRANKE .** AVALIAÇÃO DA VIABILIDADE CELULAR DURANTE O PROCESSO DE ELECTROSPINNING COM O OBJETIVO DE USAR A NANOTECNOLOGIA PARA A ENGENHARIA DE TECIDOS. **2009. (APRESENTAÇÃO DE TRABALHO/OUTRA).**