UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

CENTRO DE BIOTECNOLOGIA

PROGRAMA DE PÓS-GRADUAÇÃO EM BIOLOGIA CELULAR E MOLECULAR

Modulação da sobrevivência e proliferação de células de câncer: mecanismos relacionados ao estado da cromatina e ao nicho tumoral

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Orientador: Rafael Roesler

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Muita gente pequena,

em muitos lugares pequenos,

fazendo coisas pequenas,

mudarão a face da terra.

Provérbio africano

ÍNDICE GERAL

Lista de Abreviaturas

ALDH (*aldehyde dehydrogenase*)

Akt (*Protein kinase B*)

AMPA (*α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid*)

APC (*adenomatous polyposis coli*)

BDNF (*brain-derived neurotrophic factor*, fator neurotrófico derivado de cérebro)

BTSC (*brain tumor stem cells*)

CDK (*cyclin-dependent kinases*)

CSC (*cancer stem cells*)

CTT (células-tronco tumorais)

CXCL1 (oncogene relacionado ao crescimento)

CXCL8 (*interleukin-8*)

EGF (*epidermal growth factor*)

FDA (*Food and Drug Administration*)

GLAST (*glutamate aspartate transporter*)

GNP (*granule neuron precursors*)

GP130 (glicoproteina 130)

HAT (*histone acetyl transferases*, histona acetiltransferases)

HDAC (*histone deacetylases*, desacetilases de histonas)

HDACi (*histone deacetylases inhibitor*, inibidor de desacetilases de histonas)

HNSCC (*head and neck squamous cell carcinoma*, carcinoma de células escamosas de cabeça e pescoço)

HPV (*human papillomavirus*)

IL-6 (*interleukin*-6, interleucina 6)

JAK (*janus kinase*)

MB (*medulloblastoma*)

MTT [*3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide*)]

NaB (*sodium butyrate*, butirato sódico)

NCI (*National Cancer Institute*)

NFP (*neurofilament protein*)

NSC (*neural stem cells*)

Oct4 (*octamer-binding transcription factor 4*)

PNET (*primitive neuroectodermal tumors*)

rh-IL-6 (*recombinant human interleukin-6*)

SAHA (*suberoylanilide hydroxamic acid*)

SCID (*severe combined immunodeficient mouse*)

SFRP (*secreted frizzled-related proteins*)

SHH (*sonic hedgehog pathway*)

SNC (sistema nervoso central)

STAT3 (*signal transducers and activators of transcription-3*)

SUFU (*suppressor of fused analogue*)

TSA (*trichostatin A*)

VEGF (*vascular endothelial growth fator*)

Wnt (*wingless pathway*)

Lista de Figuras

Introdução

Capítulo I

Capítulo II

Capítulo III

Lista de tabelas

Capítulo I

Resumo

Câncer é a principal causa de morte nos países desenvolvidos e a segunda causa de morte nos países em desenvolvimento. O trabalho de diversos grupos de pesquisa tem sugerido que os tumores estão organizados em uma hierarquia de células, na qual uma pequena fração apresenta propriedades de células-tronco. Essas células tem se mostrado resistentes à quimioterapia convencional, dependentes da sinalização do microambiente tumoral e responsivas à terapia diferenciativa. Aqui nós mostramos que butirato sódico (NaB), um inibidor de desacetilase de histonas, diminui a proliferação celular e a formação de colônias em linhagens celulares de meduoblastoma humano. Estes efeitos foram acompanhados de um aumento da expressão de RNAm Gria2, um marcador de diferenciação neuronal, em duas das três linhagens celulares testadas. A formação de neuroesferas também foi impedida com a exposição de uma linhagem crescida em meio de cultura apropriado para células-tronco e NaB. NaB se mostrou capaz de potencializar os efeitos do quimioterápico etoposídeo e do fator neurotrófico derivado de cérebro (BDNF) na inibição da viabilidade celular de meduloblastoma. Além disso, nós observamos que o tratamento com cisplatina aumenta a proporção de células-tronco tumorais (CTT), identificadas por ALDH+CD44+, em células de carcinoma de cabeça e pescoço, quando tratadas também com interleucina 6 (IL-6), uma citocina liberada pelo nicho perivascular. O mesmo tratamento promoveu a proliferação, sobrevivência e auto-renovação de CTTs *in vitro* ao aumentar o número de esferas em placas de baixa aderência e a expressão de Bmi-1 em western blots. A fosforilação do transdutor de sinal e ativador de transcrição 3 (STAT3), um indicativo de propriedades de CTTs, induzida por IL-6 não foi afetada pelo tratamento com cisplatina em células de HNSCC, enquanto que a indução de fosforilação de quinases reguladas por sinais extracelulares (ERK), indicativo de processo de diferenciação, por IL-6 foi

parcialmente inibido por cisplatina. Células resistentes a baixas doses de cisplatina também expressaram mais Bmi-1 do que células nunca expostas ao quimioterápico. Experimentos *in vivo* corroboram os achados *in vitro*, uma vez que tumores humanos induzidos em camundongos imunocomprometidos apresentaram aumentada proporção de células ALDH+CD44+ após tratamento dos animais com cisplatina. O anticorpo contra o receptor de IL-6 foi capaz de reverter a indução de expressão de Bmi-1 por cisplatina e IL-6. Em conjunto, esses resultados sugerem que a modulação de mecanismos epigenéticos das células tumorais e de sinais provenientes do nicho tumoral são novos alvos promissores para o desenvolvimento de terapias adjuvantes contra o câncer.

Palavras-chave: células-tronco, HDAC, interleucina 6, resistência à quimioterapia, meduloblastoma, carcinoma de células escamosas de cabeça e pescoço.

Abstract

Cancer is the leading cause of death in economically developed countries and the second cause of death in developing countries. Work from a number of laboratories strongly suggests that tumors are organized as a hierarchy based on a subset of cancer cells that have stem-cell properties. These cells have been shown to be resistant to conventional therapy, dependent on contextual signals within the tumor microenvironment and, to be responsive to differentiation therapy. Here we show that sodium butyrate (NaB), a histone deacetylase inhibitor, decreases cell proliferation and colony formation in human medulloblastoma cell lines. These effects were accompanied by an increased mRNA expression of Gria2, a neuronal differentiation marker, in two out of three cell lines tested. In addition, neurosphere formation was impaired by NaB exposure in a cell line submitted to stem cells proper media. NaB also may potentiate the effect of etoposide chemotherapy and BDNF (Brain-derived neurotrophic factor) on the inhibition of medulloblastoma cells viability. Moreover, we observed that cisplatin treatment increased the proportion of cancer stem cells (CSC), identified by $ALDH^{high}CD44^{high}$ *cells, in head and neck squamous cell carcinoma (HNSCC), when treated together with recombinant human IL-6 (rhIL-6). The same regimen promoted proliferation, self-renewal and survival of CSC in vitro as seen by the increase in orosphere number formed in ultra-low attachment plates, and Bmi-1 expression induction in western blots. IL-6–induced signal transducer and activator of transcription 3 (STAT3) phosphorylation (indicative of stemness) was unaffected by treatment with cisplatin in HNSCC cells, whereas IL-6–induced extracellular signal-transducer kinases (ERK) phosphorylation (indicative of differentiation processes) was partially inhibited by cisplatin. Cells resistant to lower doses of cisplatin also expressed more Bmi-1. In vivo experiments corroborated in vitro findings by showing increased proportion of*

ALDH^{high}CD44^{high} cells in xenograft tumors of mice treated with cisplatin. An antibody against the receptor of IL-6 was able to revert the induction of Bmi-1 expression seen in cells treated with cisplatin plus IL-6. Taken together, these results suggest that the modulation of the epigenetic states of the cancer cell and modulation of signals provided by the niche are promising new molecular targets for the development of adjuvant therapy for cancer.

***Keywords**: stem cells, HDAC, IL-6, resistance to therapy, medulloblastoma, head and neck squamous cell carcinoma*

INTRODUÇÃO

O câncer é a doença que mais mata nos países economicamente desenvolvidos e a segunda causa de morte em países em desenvolvimento. Baseado na estimativa da GLOBOCAN, apenas em 2008, foram diagnosticados 12,7 millhões de novos casos e cerca de 7,6 milhões de pessoas morreram de câncer no mundo (Global Health Observatory – GHO 2008, fonte: Organização Mundial da Saúde). Este cenário é o resultado do aumento da expectativa de vida e de hábitos que predispõe ao câncer como fumo, falta de atividade física, consumo de alimentos processados, e consume de álcool em demasia (Jemal *et al*., 2011).

De maneira a definir melhor o câncer, Hanahan & Weinberg em 2000 elaboraram uma publicação de referência que reune as principais características dos tumores. A tumorigênese seria um conjunto de alterações genéticas que levaria a uma progressiva transformação de células normais em células altamente malignas com características de auto-suficiência de sinais reguladores de proliferação, insensibilidade a sinais inibitórios de proliferação, evasão da apoptose, potencial replicativo ilimitado, angiogênese própria, invasão e metástases (Hanahan & Weinberg 2000). Entretanto, os tumores não parecem ser apenas um conjunto de células tumorais altamente proliferativas, e sim, assemelham-se a um órgão formado por diferentes tipos celulares e devidamente organizado capaz de interagir com o microambiente e superar as defesas do hospedeiro para garantir sua progressão e disseminação. As células que formam a população heterogênea do tumor diferem significativamente entre si quanto a habilidade de proliferar e formar novos tumores. Em muitos tumores as células-tronco tumorais constituem uma minoria de células com capacidade de auto-renovação ilimitada. Estas células são responsáveis por dar origem às células maduras e amplificadoras que constituem a massa tumoral. Células-tronco do tumor dependem do

seu microambiente imediato formado por estroma, células inflamatórias e vasculatura tumoral a fim de garantir sua sobrevivência e disseminação através de metástases (**Figura 1**; Al-Hajj & Clarke, 2004; Hanahan & Weinberg 2011; Krishnamurthy & Nör 2012).

Apesar da extensa pesquisa já realizada acerca da biologia tumoral, muito ainda resta a ser descoberto para que no futuro alcancemos uma realidade de cura do câncer.

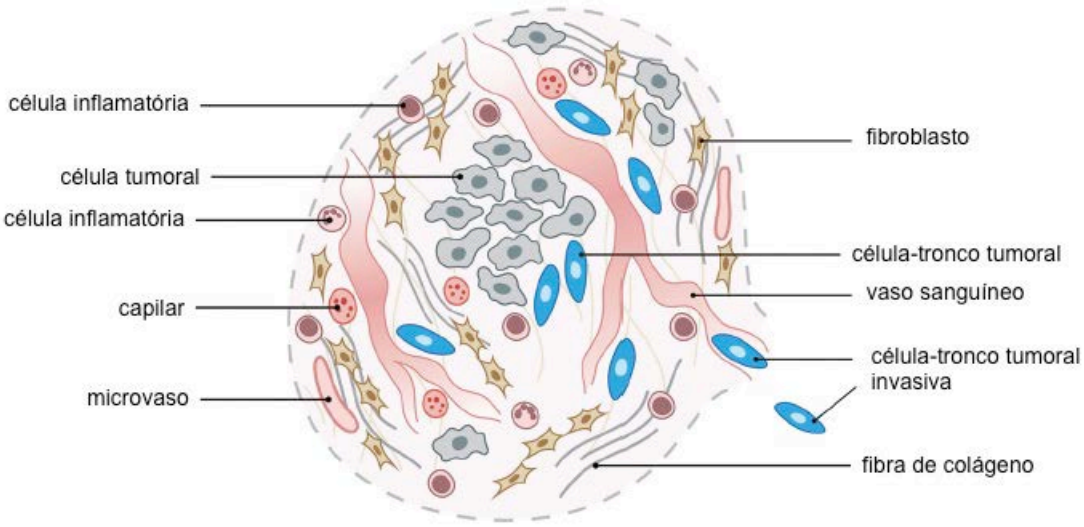

Figura 1: O tecido tumoral é organizado como um `órgão`, no qual o microambiente do tumor é formado por uma variedade de tipos celulares, além das células tumorais, as celulas inflamatórias, fibroblastos e vasos sanguíneos. As células-tronco tumorais são raras e se localizam principalmente nas bordas invasivas da massa tumoral, junto a vasos sanguíneos. Adapatado de Krishnamurthy & Nör, 2012.

Epigenética

A observação de mutações ou deleções de genes e frequentes anormalidades cromossômicas em tumores, fez com que o câncer fosse considerado uma doença genética. De fato, a perda de função de genes supressores tumorais e ou a

hiperativação de oncogenes tem sido identificada em amostras de câncer humano, bem como em modelos animais (Bolden *et al*., 2006; Hanahan & Weinberg 2000). Entretanto, a genética por si só não é capaz de explicar toda a diversidade de fenótipos de uma população, nem mesmo a suscetibilidade a doenças e o desenvolvimento de um processo complexo como o câncer (Esteller, 2008). A epigenética é definida como sendo as alterações herdáveis de expressão gênica que não são acompanhadas por mudanças na sequência de DNA. O controle da expressão gênica por mecanismos epigenéticos demonstrou-se crucial no surgimento e progressão do câncer (Bolden *et al*., 2006). A expressão aumentada ou o silenciamento de genes pode impulsionar os primeiros eventos de expansão aberrante de células aumentando o risco de subsequentes alterações genéticas e epigenéticas que promovam a formação do tumor (Lund & Lohuizen 2004; Jones & Baylin 2007). Por exemplo, o silenciamento do gene suppressor tumoral p16ink4A é um dos eventos epigenéticos comuns em estágios iniciais de diversos tumores como mama, colorretal e pulmão (Belinsky *et al*., 1998; Jones & Baylin 2007). Além disso, a via de sinalização Wnt (do inglês, *wingless pathway*) encontra-se ativada na tumorigênese de câncer colorretal em função da hipermetilação de genes da família SFRP (do inglês, *secreted frizzled-related proteins*) que codificam proteínas inibidoras desta via. A superativação de Wnt pode induzir as células a desenvolverem novas mutações que ativem outras proteínas envolvidas desta via como APC (do inglês, *adenomatous polyposis coli*) ou β–catenina e assim levar a progressão do câncer colorretal (Baylin & Ohm 2006; Jones & Baylin 2007).

A modulação epigenética ocorre a nível de nucleossomo. Os nucleossomos são formados por cerca de duas voltas de DNA ao redor de um octâmero de histonas composto por 2 subunidades de cada histona, H2A, H2B, H3 e H4. As caudas N-terminais das histonas se projetam para fora do nucleossomo e servem como substrato para a modulação epigenética (Lund & van Lohuizen, 2004).

A marca epigenética mais conhecida é a metilação do DNA. A metilação do DNA se dá pela adição de um grupo metil ao DNA no carbono 5 do anel citosina-pirimidina que precede uma guanina. Esta reação é catalisada por enzimas metiltransferases que transferem grupamentos metil a partir de S-adenosilmetionina. De maneira geral, o DNA dos tumores humanos é hipometilado em regiões pobres em genes o que confere instabilidade cromossômica e pode favorecer a ocorrência de recombinações mitóticas levando a deleções e translocações. Por outro lado, o DNA tumoral é hipermetilado nas regiões promotoras de genes supressores tumorais, inibindo a transcrição desses genes (Esteller, 2008; Liao *et al*., 2014).

A modulação da cromatina através da modificação da estrutura das histonas exerce um importante papel na regulação epigenética. Entre as principais modificações das caudas amino-terninais de histonas estão a acetilação, metilação, fosforilação, ubiquitinação, entre outras (**Figura 2**) (Monneret 2005; Bolden *et al*., 2006). A regulação da acetilação de histonas se dá pelo equílibrio dinâmico entre a atividade de dois grupos de enzimas de funções opostas. As acetiltransferases (do inglês, *histone acetyl transferases* - HAT*)* que transferem grupamentos acetil a resíduos de lisina das porções amino-terninais de histonas, relaxando a cromatina e facilitando a ligação de fatores de transcrição e assim favorecendo a transcrição gênica; e as histonas desacetilases (do inglês, *histone deacetylases* - HDAC) que removem os grupamentos acetil levando a condensação da cromatina e a repressão da transcrição (Monneret, 2005).

As enzimas HDACs estão superexpressas em diversos tipos tumorais e a hipoacetilação global de H4 é uma característica comum em tumores humanos (Johnstone, 2002; Bolden *et al*., 2006).

a

Cromatina condensada: repressão transcricional

b

Cromatina relaxada: ativação transcricional

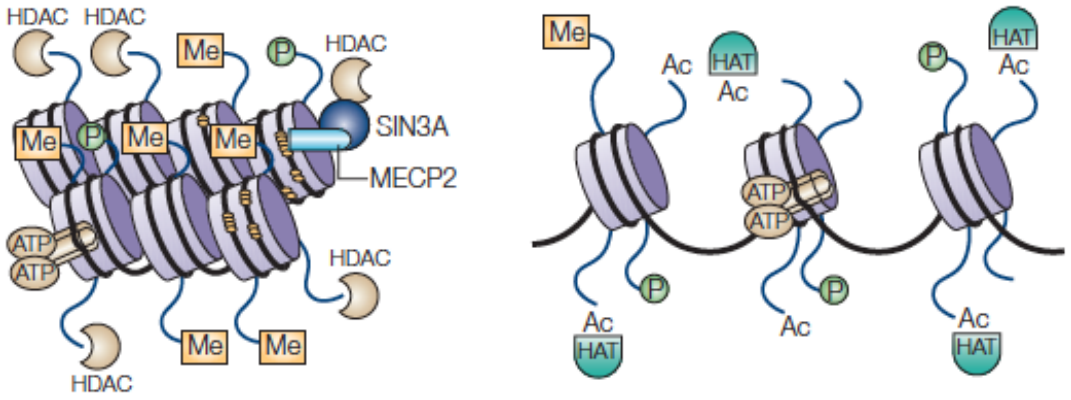

Figura 2: A regulação da transcrição através da modulação da cromatina. Nucleossomos consistem do DNA (linha preta) e das histonas (lilás), estrutura que pode ser alterada por metilação (Me), fosforilação (P), ou acetilação (Ac). (**a**) A metilação do DNA e a desacetilação de histonas condensam a cromatina e inibem a transcrição. (**b**) A acetilação de histonas e a demetilação do DNA relaxam a cromatina e ativam a transcrição. Adaptado de Johnstone, 2002.

Ao contrário de mutações em genes, a regulação epigenética do câncer pode ser revertida com fármacos moduladores da cromatina a fim de restaurar a função normal dos genes.

As desacetilases de histonas tem se mostrado um alvo promissor de intervenção farmacológica no câncer. Butirato sódico (NaB), um inibidor de desacetilase de histonas (HDACi) mostrou atividade de inibição da proliferação, estimulação da diferenciação e indução de apoptose em células tumorais (Ruefli *et al*., 2001; Munster *et al*., 2001). HDACis agem inibindo as enzimas histona-desacetilases resultando em um aumento de histonas acetiladas e assim, promovendo a reativação da expressão de genes envolvidos na diferenciação celular, genes supressores tumorais como CDKN1A,

das enzimas apoptóticas caspase 3 e 9 e de outros genes pró-apoptóticos como Bax, Puma e Noxa (Johnstone, 2002; Xu *et al*., 2003; Bolden *et al*., 2006).

A atividade antitumoral de HDACis foi observada em células de diferentes tipos de câncer humano como neuroblastoma, mama, próstata, e câncer renal e em modelos animais de neoplasias humanas como câncer colorretal, pulmão, pâncreas, ovário, assim como próstata, revelando o potencial clínico desses agentes (Saito *et al*., 1999; Sonnemann *et al*., 2006). Alguns desses compostos já encontram-se em fase de estudo clínico e um fármaco desse grupo, o vorinostat (ou do inglês, *suberoylanilide hydroxamic acid*, SAHA), está aprovado pelo FDA (*Food and Drug Administration*) para o tratamento de linfoma de células T cutâneo (Lane & Chabner, 2009; Slingerland *et al*., 2014). Além deste, a romidepsina, um pró-fármaco que age pela interação do grupamento tiol com o átomo de zinco no sítio de ligação dependente de zinco da HDAC, bloqueando sua atividade, foi aprovada pelo FDA em 2009 para uso em linfoma de células T cutâneas. Em 2011, a mesma foi aprovada para tratamento de linfoma de células T periféricas (Slingerland *et al*., 2014). Em contrapartida, os resultados dos estudos clínicos desses fármacos em tumores sólidos não foram tão positivos.

Tem sido demonstrado que HDACis são capazes de aumentar a eficácia de outros regimes terapêuticos como radiação ionizante (Camphausen *et al*., 2005) ou quimioterapia (Kim *et al*., 2003), e teriam efeitos mínimos no tecido normal, uma vez que as células transformadas seriam mais sensíveis ao efeito indutor de apoptose de HDACIs, comparado com as células normais (Lane & Chabner, 2009).

Um estudo prévio do nosso grupo de pesquisa demonstrou que NaB aumenta o efeito citotóxico de citarabina e etoposídeo em células T linfoblásticas (dos Santos *et al*., 2009).

Células tronco tumorais

É sabido que muitos dos tecidos normais adultos são mantidos por células-tronco que promovem a expansão e diferenciação nos tipos celulares particulares de cada órgão. As células-tronco são definidas pela extensa capacidade de auto-renovação que permite a manutenção do *pool* de células indiferenciadas, e pela habilidade de sofrerem diferenciação a fim de gerar os elementos funcionais do tecido (**Figura 3**). O processo de auto-renovação é extremamente regulado para manter o número de células-tronco estável (Al-Hajj *et al*., 2004; Blanpain *et al*., 2004).

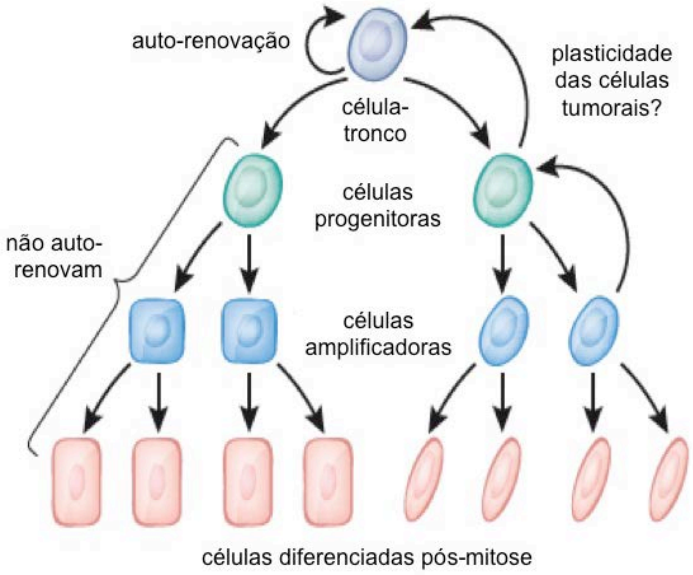

Figura 3: As células tronco tem duas funções principais. Primeiro, elas tem a função de se diferenciarem nas células maduras do órgão. Segundo, as células tronco durante a replicação podem gerar novas células iguais a elas num processo chamado de auto-renovacao. Auto-renovacao é um processo de divisão celular em que pelo menos uma das células filhas tem igual capacidade de diferenciar e de replicar da mesma forma que a célula-tronco original. As células progenitoras não possuem a capacidade de auto-renovação e a cada divisão celular se tornam mais diferenciadas e com menor capacidade de replicação. Adaptado de Gupta *et al*., 2009.

Acredita-se que a população de células do câncer também contenha uma pequena fração de células multipotentes e auto-replicativas, as células-tronco tumorais (CTT) (Al-Hajj *et al*., 2003; Singh *et al*., 2004; Veeragavu *et al*., 2008).

Embora exista uma hipótese estocástica para a tumorigenese, na qual todas as células tumorais tem igual probabilidade de iniciar e propagar um tumor, há um crescente número de evidências que leva à aceitação da hipótese da existência de células-tronco tumorais. De acordo com esta hipótese, existe uma hierarquia entre as células malignas e apenas as células tronco tumorais são capazes de iniciar um novo tumor (Reya *et al*., 2001).

As demais células que formam a massa tumoral são as células altamente proliferativas, as células amplificadoras, e as células diferenciadas pós-mitóticas, todas derivadas das células-tronco, mas que não detém o potencial tumorigênico (Gupta *et al*., 2009; Valent *et al*., 2012).

As células progenitoras, embora tenham uma extensa capacidade de proliferação, estão eventualmente destinadas a se diferenciar e parar o ciclo celular. A cada divisão celular, as células geradas a partir das células progenitoras seriam progressivamente mais diferenciadas e a sua capacidades de proliferação diminuiria (Al-Hajj & Clarke, 2004).

A hipótese da célula-tronco tumoral parte dos conceitos da embriogênese e das células-tronco normais para explicar o processo de carcinogênese. As principais características da hipótese da célula tronco tumoral são: (1) apenas uma pequena fração das células tumorais tem potencial tumorigênico quando transplantadas em animais imunocomprometidos; (2) a sub-população de células tronco tumorais pode ser

separada das demais células pela expressão distinta de marcadores celulares; (3) os tumores gerados pelas células tronco tumorais contém toda a população de células, tumorigênicas e não-tumorigênicas, do tumor original; e (4) as células tronco tumorais podem ser transplantadas por múltiplas gerações mostrando a capacidade de auto-renovação desta população (Clarke & Fuller, 2006; Prince & Ailles, 2008).

Os primeiros estudos a demonstrarem que sub-populações enriquecidas de células tronco tumorais podem ser obtidas através do perfil de marcadores de superfície celular e que estas células uma vez transplantadas em animais imunocomprometidos, geram tumores semelhantes ao original, podendo ser transplantadas em série, foram os estudos em leucemia (Lapidot *et al*., 1994) e em tumores sólidos de mama (Al-Hajj *et al*., 2003) e sistema nervoso central (SNC – Singh *et al*., 2003). O conceito da célula tronco tumoral tem sido amplamente aplicado a diversos outros tipos de câncer.

A origem das células-tronco tumorais ainda não é consenso, se de células-tronco normais ou de células mais diferenciadas que se desdiferenciam. Entretanto, as CTTs se assemelham às células-tronco normais ou células progenitoras que formam o tecido de origem correspondente (Al-Hajj *et al*., 2004; Lobo *et al*., 2007).

A regulação epigenética também está envolvida na iniciação tumoral envolvendo as CTTs. Genes como p16, SFRP e APC supressor tumoral, que normalmente controlam a auto-renovação de células-tronco adultas, estão silenciados em células tronco ou precursoras tumorais mantendo essas células em estado tronco-indiferenciado e promovendo a expansão clonal anormal (Kinzler & Vogelstein 1996; Valenta *et al*., 2006; Reynolds *et al*., 2006). Estas células, ao expressarem baixos níveis de genes controladores de auto-renovação estariam comprometidas com as vias de sobrevivência e facilitariam a ocorrência de mutações que favorecem a progressão

tumoral. A massa tumoral resultante é composta de uma subpopulação de CTT e uma progenie neoplásica (Jones & Baylin 2007).

Mais recentemente, tem se demonstrado que as células-tronco e não tronco de um tumor apresentam perfis epigenéticos distintos (Vlerken *et al*., 2012; Tsai & Baylin, 2011). Em câncer de mama e fígado, as CTT apresentam níveis baixos de metilação no DNA e de marcas em histonas como H3K27me3, se comparadas com as células mais diferenciadas desses tumores. Em câncer de cabeça e pescoço, as CTT identificadas como CD44+ possuem um padrão epigenético único de metilação em 22 genes comparadas com as células não tronco CD44- (Vincent & Seuningen 2012). Em pacientes com câncer de ovário, a hipometilação de ATG4A e HIST1H2BN em células tronco ou iniciadoras de tumor predizem um pior prognóstico (Liao *et al*., 2014)

As células-tronco tumorais exibem propriedades de auto-renovação, proliferação ilimitada, alta taxa de adesão, migração e metástases, além de serem capazes de regenerar toda a população de células do tumor (Al-Hajj *et al*., 2003; Clarke *et al*., 2006; Gupta et al., 2009; Singh *et al*., 2004; Tu *et al*., 2009).

Recentemente, tem se sugerido que as células-tronco tumorais também estejam envolvidas com resistência à quimioterapia convencional. Como as CTTs são células de proliferação lenta, as terapias atuais que agem em células altamente proliferativas e assim diminuem o volume tumoral, podem não afetar as CTTs (Korkaya *et al*., 2012).

Já foi demonstrado que as CTTs são capazes de sobreviver após o tratamento antitumoral em câncer de pulmão (Bertolini *et al*., 2009; Liu *et al.*, 2012), glioblastoma (Chen *et al*., 2012) e câncer de mama (Korkaya *et al*., 2012).

As CTTs possuem alta capacidade de efluxo de fármacos através dos transportadores ABC ou, *ATP-binding cassette transporters* (Zhou *et al*., 2001; Schatton *et al*., 2008), propriedade a qual é comumente utilizada para identificar CTTs *in vitro* (Goodell *et al*., 1997; Scharenberg *et al*., 2012).

Considerando o papel crucial das CTTs na progressão tumoral, se torna evidente a necessidade de novas terapias que tenham como alvo as CTT a fim de melhorar o desfecho de pacientes com câncer. No entanto, como as CTT compartilham de algumas similaridades com as células-tronco normais, o desafio do tratamento é a especificidade às CTT. Ou seja, se faz necessária a identificação de fatores que são importantes para a manutenção das CTTs e não para as células-tronco normais (Yilmaz *et al*., 2006).

Terapias-alvo direcionadas às enzimas moduladoras da cromatina podem significar uma importante nova alternativa terapêutica. Por exemplo, Bmi-1, oncogene da família *polycomb ring finger* tem sido considerado um promissor novo alvo terapêutico. O silenciamento desse gene, por siRNA ou por fármacos moduladores de epigenética como os inibidores de HDAC butirato sódico e ácido valpróico, aumenta a sensibilidade das células tumorais para a quimio e radioterapia inibindo o crescimento do tumor (Vincent & Seuningen 2012).

Microambiente tumoral

Uma importante diferença entre as CTTs e as células-tronco normais é o nível de dependência e regulação do seu microambiente imediato. As células-tronco de vários tecidos se localizam em microambientes ou nichos formados por diversas células diferenciadas. Estas células promovem um ambiente protetor a partir da liberação de

fatores que controlam a manutenção, auto-renovação e diferenciação das células-tronco (Blanpain *et al*., 2004; Fuchs *et al*., 2004; Moore & Lemischka 2006). Shen e colaboradores (2004) mostraram que as células-tronco neurais estão presentes em nichos vasculares e que as células endoteliais secretam fatores que mantém a capacidade de auto-renovação e o potencial neurogênico dessas células.

Células-tronco tumorais também foram localizadas em nichos vasculares (Calabrese *et al*., 2007). Num estudo com tumores cerebrais, incluindo meduloblastoma e glioblastoma, os autores identificaram CTTs Nestina+/CD133+ localizadas predominantemente em áreas de aumentada densidade vascular, denominada de CTT-associada a vasos, enquanto que células CD133- encontravam-se distribuídas aleatoriamente no tumor (**Figura 4**).

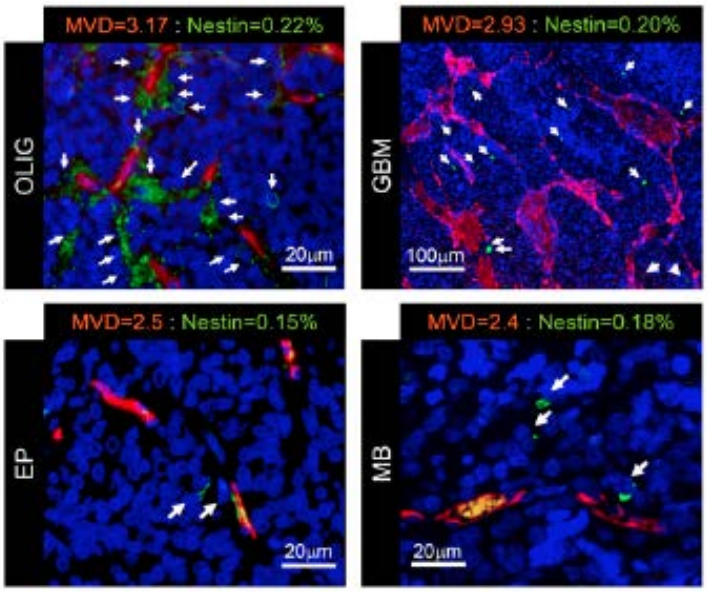

Figura 4: **As CSC estão localizadas próximo a capilares sanguíneos em gliomas**. As CTT, identificadas pelo marcador Nestin+, encontram-se localizadas na proximidade dos capilares do tumor, identificados pelas células CD34+ (MVD, densidade de microvasos; Adaptado de Calabrese *et al*., 2007).

Enquanto as células-tronco normais possuem um rígido controle dos mecanismos celulares de proliferação, as CTT adquiriram a capacidade de superar este controle. A desregulação de fatores provenientes do nicho pode levar ao descontrole da proliferação de CTT e tumorigênese (Clarke & Fuller, 2006). A regulação do microambiente tumoral parece envolver um diálogo entre as CTT e as células vasculares endoteliais com o objetivo de induzir angiogênese. As células tumorais liberam uma variedade de quimiocinas e fatores de crescimento que induzem alterações no estroma local que juntos provocam o recrutamento e proliferação de células da medula óssea para o desenvolvimento de novos vasos sanguíneos (Kaplan *et al*., 2006). Foi verificado em carcinoma de células escamosas que, numa interação contato-dependente, as células tumorais expressam a proteína *Jagged1* que leva a ativação da sinalização de *Notch* nas células endoteliais adjacentes levando ao aumento no crescimento do tumor (Zeng *et al*., 2005). E de um modo independente de contato, a liberação do fator de crescimento vascular endotelial (do inglês, *Vascular Endothelial Growth Fator* - VEGF) pelas células endoteliais associadas ao tumor induz a expressão de Bcl-2, oncogene relacionado ao crescimento (CXCL1) e interleucina 8 (CXCL8) nas células tumorais o que também induz o aumento do tumor (Kaneko *et al*., 2007). Por sua vez, Calabrese e colaboradores (2007) verificaram em tumores do SNC que o aumento da quantidade de células endoteliais e vasos sanguíneos que suprem o tumor leva ao aumento do número de CTT auto-replicativas e da malignidade do tumor.

As CTT interagem e também são reguladas por componentes do nicho. Essas interações envolvem citocinas comuns a processos inflamatórios, como IL-1, IL-6 e IL-8. As vias de sinalização ativadas por citocinas, como STAT3 e NF-κ B auxiliam na auto-renovação de CTTs (Zou, 2005).

A partir das evidências de que as CTT residem em nichos perivasculares e que são fortemente afetadas pela sinalização proveniente do microambiente, intervenções terapêuticas direcionadas à ruptura da comunicação entre as CTT e as células adjacentes pode levar a uma maior sensibilização das CTT às terapias existentes, bem como também, parece ser um caminho promissor na busca de novos fármacos para o câncer.

Carcinoma de células escamosas de cabeça e pescoço (HNSCC)

O Carcinoma de células escamosas de cabeça e pescoço (do inglês, *head and neck squamous cell carcinoma* – HNSCC) compreende as lesões malignas de origem epitelial da cavidade oral, laringe e faringe.

Estima-se que HNSCC seja o oitavo tipo mais comum de câncer, com mais de 250 mil novos casos por ano e responsável por quase 130 mil mortes em 2008 (Jemal *et al*., 2011).

Os principais fatores de risco associado ao desenvolvimento de HNSCC são consumo de tabaco e álcool, além de falta de higiene oral e histórico familiar. É estimado que o consumo de tabaco e álcool seja responsável por 72% dos casos de HNSCC, sendo 4% por álcool somente, 33% por tabaco somente e 35% por tabaco e álcool combinados (Hashibe *et al*., 2009). E mais recentemente, a infecção pelo papilomavirus humano (do inglês, *human papillomavirus* – HPV) tem se mostrado um importante agente causador de HNSCC, principalmente em homens com menos de 50 anos (Jégu *et al*., 2013).

O tratamento padrão consiste de ressecção cirúrgica e radioterapia. A adição de quimioterapia à base de platinas ao esquema terapêutico contribuiu para a remissão inicial do tumor e para um melhor controle local da doença. Entretanto, a falta de um tratamento mais alvo-específico, permite altas taxas de recorrência e metástases, fazendo com que a melhora na sobrevida dos pacientes não seja significativa (Forastiere, 2008; Sano & Myers, 2007).

Sabe-se que a toxicidade de cisplatina se dá pela formação de ligações cruzadas entre porções de DNA, entre DNA e proteínas, e entre fitas de DNA, levando à ativação de diversas vias de transdução de sinal como reconhecimento e reparo ao dano ao DNA, repressão do ciclo celular e morte celular programada, apoptose (Siddik *et al*., 2003; Kelland, 2007).

O câncer de cabeça e pescoço é um problema de saúde pública, sendo que apenas nos Estados Unidos foram gastos cerca de 3,6 bilhões de dólares em 2010 (*National Cancer Institute* - NCI – 2009/2010). Portanto, o diagnóstico precoce, o melhor entendimento da biologia desses tumores e a busca de alvos terapêuticos mais eficazes se tornam prioridades.

Já se sabe que mutações no gene p53 estão presentes em metade dos pacientes e que o receptor de EGF (do inglês, *epidermal growth fator receptor* – EGFR) está superexpresso em quase a totalidade desses tumores, sendo um fator de baixo prognóstico (Poeta *et al*., 2007; Kalyanakrishna & Grandis, 2006). A expressão de VEGF pelas células tumorais em HNSCC leva a um aumento da proliferação e migração de células endoteliais, e está relacionada a sobrevivência dos pacientes (Kaneko *et al*., 2007).

HNSCC é um tumor altamente vascularizado, mas que responde apenas modestamente a terapias antiangiogênicas sugerindo a existência de interações potentes entre as células endoteliais e células tumorais que mantenham o sistema vascular do tumor e assim, contribuam para a resistência à terapia (Kaneko *et al*., 2007). Isto possivelmente se deve a fatores liberados pelas células endoteliais que ativam as vias de STAT, ERK e Akt em HNSCC. Estas vias estão envolvidas no controle do ciclo celular, sobrevivência e migração das células tumorais (Neiva *et al*., 2009) e são consideradas determinantes para a regulação da proliferação celular e agressividade do tumor (Fenic *et al*., 2007; Mishima *et al*., 2002).

A interleucina 6 (IL-6) é uma citocina que afeta uma variedade de funções biológicas, como resposta imune, inflamação, hematopoese e oncogênese, a partir da regulação de crescimento celular, sobrevivência e diferenciação (Van Snick, 1990). A IL-6 é uma das principais ativadoras da via STAT3 (signal transducer and activator of transcription 3) (**Figura 5**) (Hirano *et al*., 2000).

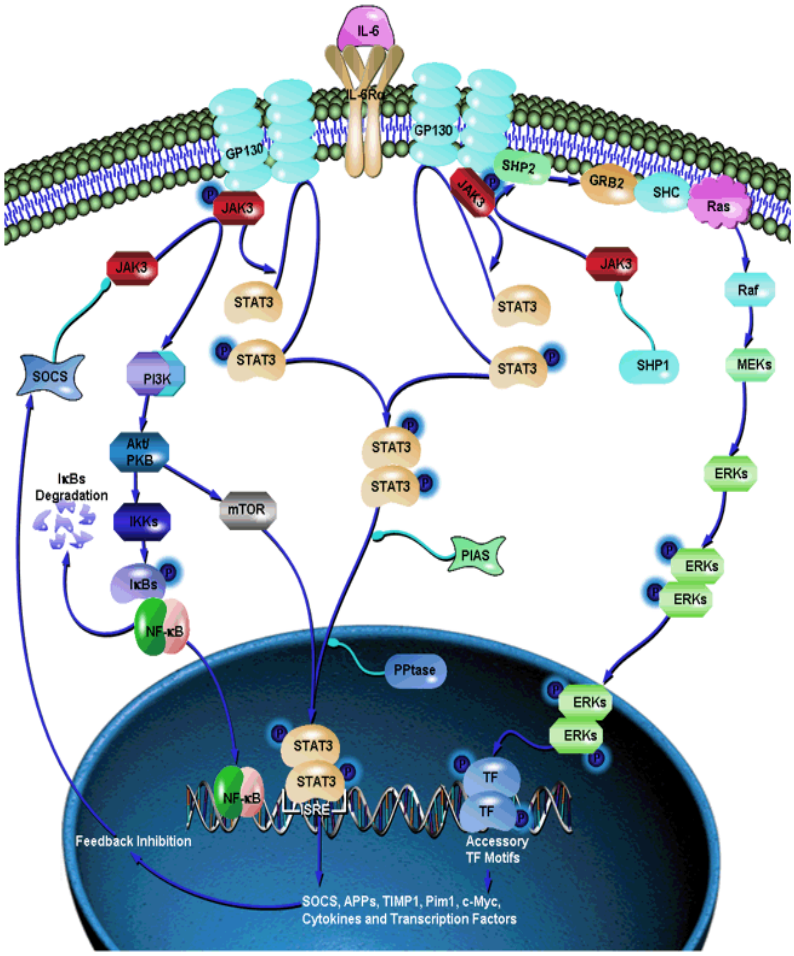

Figura 5: Via de sinalização da IL-6. A IL-6 se liga a um receptor composto de 2 subunidades, uma ligante-específico e o GP130 (Glicoproteina 130), que é compartilhado com outras citocinas da família de IL-6. A ligação de IL-6 ao receptor resulta na ativação das JAK (*janus kinase*) cinases e da sinalização mediada por Ras. A via das JAK leva a ativação de, principalmente, STAT3 (*Signal Transducers and Activators of Transcription-3*), que translocada ao núcleo ativa genes de sobrevivência e de ciclo celular. A via da PI3K/Akt também pode ser ativada por IL-6. Adaptado de Sabiosciences IL-6 pathway (www.sabiosciences.com/pathway.php?sn=IL-6_Pathway)

Níveis séricos aumentados de IL-6 foram detectados em pacientes com mau prognóstico e com recorrências de tumores de HNSCC (Duffy *et al*., 2008).

Foi demonstrado que IL-6 está super-expressa em células endoteliais, que super-expressam a proteína Bcl2, e que o meio de cultura proveniente delas induz o aumento da fosforilação de STAT3 e ERK nas células tumorais, protegendo as mesmas de *anoikis*, a morte celular devido à perda de adesão ou retirada do nicho (Neiva *et al*., 2009). Além disso, as células endoteliais foram capazes de provocar um gradiente quimiotático que induz a motilidade das células tumorais na direção dos vasos sanguíneos (Warner *et al*., 2008).

Trabalhos recentes demonstram que HNSCC também segue a teoria da célula tronco tumoral, em que uma sub-população de células é altamente tumorigênica. Estas células seriam as responsáveis também por recorrências e metástases.

As células-tronco tumorais de HNSCC foram identificadas pela primeira vez através da expressão de CD44, um marcador de células-tronco em tumores epiteliais, por Prince e colaboradores em 2007. Neste trabalho, 20 de 31 implantes de células CD44+ formaram tumores em camundongos imunocomprometidos, enquanto que apenas um dentre 40 implantes de células CD44- formou tumor. Além disso, as células CD44+ apresentaram um perfil mais primitivo, ao expressarem o marcador citoqueratina 5/14, e as células CD44- se assemelharam mais a células diferenciadas do epitélio escamoso. Os tumores oriundos das células CD44+ apresentaram a mesma heterogeneidade de células do tumor original e puderam ser transplantados em série, demonstrando as principais características de células tronco (Prince *et al*., 2007).

A expressão da enzima em células-tronco de várias origens, aldeído-desidrogenase (ALDH) (Ginestier *et al*., 2007), caracteriza células tumorigênicas em HNSCC (Chen *et al*., 2009).

As células ALDH1+/CD44+ fazem parte de uma sub-população de células que exibem as propriedades de CTT, como auto-renovação e capacidade de regenerar o tumor e encontram-se localizadas em proximidade com vasos sanguíneos. Fatores secretados por células-endoteliais promovem a proliferação e auto-renovação de CTT com aumento da expressão de Bmi-1. Além disso, foi verificado que a eliminação seletiva das células endoteliais resulta num decréscimo significativo da quantidade de CTT *in vivo* (Krishnamurthy *et al*., 2010).

Meduloblastoma

O tumor de sistema nervoso central (SNC) é o segundo câncer mais prevalente em crianças, depois da leucemia, mas permanece sendo a principal causa de morte relacionada ao câncer em crianças (Siegel *et al*., 2012; Northcott *et al*., 2012). Meduloblastoma é o tipo mais comum de tumor cerebral na infância (Gajjar *et al*., 2012). Meduloblastoma é um tumor embrionário que se desenvole na camada externa do cerebelo a partir de células granulosas precursoras de neurônios e faz parte do grupo dos tumores primitivos neuroectodérmicos do SNC (do inglês, *primitive neuroectodermal tumors* – PNET) (Marino, 2005; Bartlett *et al*., 2013).

O meduloblastoma tem uma inerente capacidade de disseminar-se por metástases. Um terço dos pacientes tem evidências de disseminação no SNC ao diagnóstico. Além disso, um pequeno mas considerável número de pacientes desenvolve metástases extraneurais nos ossos, medula óssea, linfonodos, fígado ou pulmão (Gilbertson, 2004).

O índice de cura dos pacientes com meduloblastoma pode chegar a 80% com o tratamento que consiste de radioterapia crânio-espinal (crianças com mais de 3 anos),

cirurgia e esquemas combinados de quimioterápicos convencionais (Bartlett *et al*., 2013). Entretanto, a maioria dos sobreviventes sofre com os efeitos colaterais causados pelo tratamento, como danos ao desenvolvimento cerebral, déficit neurólogico, neuroendócrino e psico-social (Northcott *et al*., 2012).

Recentemente, estudos genômicos permitiram a classificação de meduloblastomas em 4 sub-grupos molecularmente distintos, o Wnt, sonic hedgehog (SHH), grupo 3 e grupo 4. Estes grupos podem ser discriminados também pelos padrões histológicos, pelas diferenças clínicas e pelos índices de cura dos pacientes (Gajjar *et al*., 2012; Northcott *et al*., 2012).

O sub-grupo Wnt compreende 10% dos diagnósticos. É o de melhor prognóstico, com índice de cura superior a 95% e raramente produz metástases. Este sub-grupo se caracteriza por mutacões nos genes CTNNB1 e APC que promovem a estabilização e a localização nuclear de β-catenina, também frequentemente apresenta mutacões no gene TP53 (Lindsey *et al*., 2011). A via de sinalizacão de Wnt compreende um conjunto altamente conservado de aproximadamente 20 proteínas secretadas que atuam no desenvolvimento embrionário, na diferenciação celular e na regulação de células-tronco. O principal evento decorrente da ativação de Wnt é o acúmulo intracelular de β-catenina. Uma vez no núcleo, esta proteína ativa a expressão de genes como c-Myc, cyclin D, MMP-7 e CD44 (Eberhart *et al*., 2000; Carlotti *et al*., 2008). As mutações presentes no sub-grupo Wnt geram uma forma da proteína que está predominantemente no núcleo. E o seu acúmulo é usado como biomarcador de meduloblastoma na clínica (Northcott *et al*., 2012).

O sub-grupo SHH é caracterizado por apresentar um prognóstico intermediário com taxas de sobrevivência de 60 a 80%, e é gerado por mutações nos genes dos reguladores negativos da via de Sonic Hedghog (SHH), patched 1 (PTCH1) e SUFU (do inglês, *suppressor of fused analogue*). A sinalização por SHH está envolvida na

formação do SNC, especialmente no cerebelo, onde controla a proliferação das células precursoras granulares, e neste sub-tipo de meduloblastoma encontra-se constantemente ativada. Antagonistas da sinalização de SHH estão sendo testados em estudos clínicos para meduloblastomas, entretanto, apesar de mostrarem um efeito inicial pronunciado, logo os tumores adquirem resistência ao tratamento (Carlotti *et al*., 2008; Northcott *et al*., 2012).

O grupo 3 é caracterizado pela amplificação da expressão dos proto-oncogenes da família MYC sendo que os pacientes deste grupo tem o pior prognóstico dos 4 grupos. O grupo 4 é o sub-grupo mais comum, de prognóstico intermediário. A ocorrência de amplificação dos proto-oncogenes MYCN e CDK6 (do inglês, *cyclin-dependent kinase 6*) ocorre frequentemente neste grupo (Northcoth *et al*., 2012).

Embora os meduloblastomas tenham sido classificados de acordo com suas características moleculares, o desenvolvimento de terapias-alvo ainda está apenas começando. E, em função da já antecipada ocorrência de resistência, outros alvos moleculares, além das vias de sinalização alteradas em cada sub-grupo, precisam ser investigados.

A partir das crescentes descobertas a cerca das bases moleculares e etiologia dos meduloblastomas, tem se tornado evidente que o desenvolvimento normal do cérebro e a tumorigênese compartilham dos mesmos eventos. Genes que estão envolvidos no processo de desenvolvimento normal do cérebro são os mesmos que estão aberrantemente expressos durante a tumorigênese. Ambos processos são decorrentes de alterações na proliferação e morte celular, diferenciação, motilidade e angiogênese (Marino, 2005; Manoranjan *et al*., 2012).

O ponto de partida de ambos processos parece estar centrado nas propriedades de auto-renovação e diferenciação das células-tronco. As células progenitoras da camada granular externa do cerebelo são precursoras neuronais que retém a

capacidade de expandirem-se significativamente durante o desenvolvimento pós-natal antes de sofrerem diferenciação terminal. A transição entre o estado mitótico ativo indiferenciado e o estado pós-mitótico diferenciado dessas celulas é fortemente regulado (Reya *et al*., 2001; Marino, 2005). De acordo com a hipótese da célula-tronco tumoral, a fração de células do tumor com propriedades tronco tem potencial ilimitado de auto-renovação, enquanto as demais células neoplásicas tem potencial proliferativo limitado, embora rápido ou estão completamente diferenciadas e já nao detém a capacidade proliferativa (Singh *et al*., 2004; Fan & Eberhart, 2008). Evidências clínicas indicam que meduloblastomas seguem esse mesmo padrão. Meduloblastomas nodulares/desmoplásicos contém dois compartimentos distintos, um contendo as células altamente proliferativas e indiferenciadas e, o outro contendo as células não-proliferativas que diferenciaram em neurônios e que formam os nódulos. Os tumores extensamente nodulares, com menor compartimento de tronco-progenitoras são menos agressivos (Fan & Eberhart, 2008).

Terapias que promovam a regulação da proliferação de CTT ou que promovam a diferenciação neuronal em meduloblastomas são, portanto, potenciais recursos para tratamentos mais efetivos.

As CTT em meduloblastoma tem sido identificadas pela presença de proteínas de membrana como CD133 e CD15 bem como pela capacidade de formar esferas não aderentes em culturas livres de soro fetal bovino (Read *et al*., 2009; Singh *et al*., 2003). Trabalhos do grupo de Singh e colaboradores demonstraram que as células CD133+ isoladas de tumores primários foram capazes de proliferar e de se auto-renovar na forma de esferas em cultura, além de se diferenciar gerando os tipos celulares presentes no tumor original (Singh *et al*., 2003). Além disso, quando implantadas em camundongos imunocomprometidos, as células CD133+ foram capazes de induzir a formação de tumores, ao contrário das células CD133- que falharam na geração de

tumores, mesmo quando números superiores de células foram injetadas (Singh *et al*., 2004).

Mecanismos epigenéticos tem importante papel nos eventos transcricionais que ocorrem durante a diferenciação de células tronco normais a células mais especializadas. No desenvolvimento do câncer e de acordo com a teoria da CTT, modificações epigenéticas aberrantes podem ser utilizadas pelas células tumorais para gerar a população heterogênea de células do tumor. Alguns inibidores de HDAC e DNMT poderiam interferir neste processo, levando a diferenciação das CTT (Tsai & Baylin, 2011; Vlerken *et al*., 2012; Vincent & Seuningen, 2012).

Justificativa

O conceito de CTT, em que as células dos tumores estão organizadas numa hierarquia baseada no grau de diferenciação e capacidade de proliferação, em que as CTT constituem um subgrupo unico capaz de perpetuar a população de células malignas indefinidamente, tem uma considerável importância clínica. Este modelo também oferece uma explicação plausível para o fato de que muitos tratamentos são efetivos inicialmente na redução da massa tumoral mas que não impedem a recorrência dos tumores.

As CTTs são em geral menos sensíveis ao tratamento que as células da massa tumoral geradas por elas. Assim, o tumor que parece ter sido eliminado em um primeiro momento, reaparece mais tarde, como resultado das CTT que sobreviveram. Além da inerente resistência das CTT às terapias anticâncer tradicionais, acredita-se que alterações genéticas e epigenéticas ocorram continuamente nas CTT e conferem novas propriedades de resistência que são transmitidas as demais células da progenie. As

interações entre as CTT com seu microambiente, pode também conferir uma proteção ao tratamento convencional (Valent *et al*., 2012).

Portanto, a busca por terapias inovadoras deveria levar em consideração este cenário. Por isso, a proposta deste trabalho é investigar tanto a sobrevivência das CTT pós quimioterapia, como avaliar a modulação epigenética e do nicho vascular como possíveis novas estratégias terapêuticas para o câncer.

Sendo assim, este trabalho está dividido em duas etapas, pois contou com dois modelos diferentes de estudo, sendo realizado em dois laboratórios independentes.

A primeira etapa consistiu da modulação epigenética de células tumorais e CTTs utilizando o modelo de meduloblastoma, sendo realizado no Laboratório de Pesquisas em Câncer do Hospital de Clínicas de Porto Alegre (HCPA) e supervisionado pelo professor Dr. Rafael Roesler (orientador). Este trabalho serviu de base para dois artigos cientifícos, que constam nos Capítulos I e II desta tese.

A segunda etapa consistiu da avaliação da resistência das CTTs pós quimioterapia e da influência da citocina IL-6 liberada pelo nicho na proteção das CTTs. Neste trabalho, o modelo usado foi o de HNSCC, sendo realizado no *Angiogenesis Research Laboratory* do *Department of Cariology, Restorative Sciences and Endodontics* da *University of Michigan School of Dentistry*, sob orientação do prof. Jacques Eduardo Nör, PhD. Os dados gerados neste trabalho, foram organizados em um manuscrito que será submetido para publicação, e encontra-se no Capitulo III desta tese.

Hipótese Geral

A hipótese geral dessa tese é de que a modulação epigenética das células tumorais e a intervenção no controle do nicho tumoral, tendo como foco principal as CTT, represente um racional de sucesso na busca por novos fármacos antineoplásicos

e mecanismos de superação de resistência a quimioterapia tradicional.

Objetivos

Avaliar a atividade antitumoral *in vitro* de um inibidor de desacetilase de histona, butirato sódico, em meduloblastoma focando em seu efeito sobre a proliferação de células-tronco, na forma de neuroesferas e, o efeito de um quimioterápico convencional, cisplatina, sobre a população de células-tronco de carcinoma de células escamosas de cabeça e pescoço *in vitro* e *in vivo*, bem como a influência de IL-6, uma citocina proveniente do nicho vascular, neste efeito.

Capítulo I

Dada a necessidade de desenvolvimento de novos tratamentos em meduloblastoma e o crescente potencial terapêutico demonstrado por HDACIs em câncer, os objetivos deste Capítulo são:

1. Avaliar o efeito da exposição ao NaB na viabilidade celular das linhagens de meduloblastoma DAOY, ONS76 e D283;
2. Avaliar o efeito de NaB sobre a sobrevivência celular (formação de colônias) das linhagens de meduloblastoma DAOY, ONS76 e D283;
3. Avaliar o efeito de NaB na diferenciação das células das linhagens de meduloblastoma DAOY, ONS76 e D283;
4. Padronizar o ensaio de formação de neuroesferas a partir das linhagens de meduloblastoma;
5. Avaliar o efeito de NaB sobre a formação de neuroesferas.

6. Avaliar o efeito da combinação de NaB com o quimioterápico etoposídeo nas linhagens de meduloblastoma DAOY, ONS76 e D283.

Capítulo II

1. Avaliar o efeito da combinação de NaB com o fator neurotrófico derivado de cérebro (*Brain Derived Neurotrophic Factor*, BDNF), cuja atividade citotóxica foi previamente demonstrada por nosso grupo (Schmidt *et al*., 2009), nas linhagens de meduloblastoma DAOY e ONS76.

Capítulo III

Tendo em vista o impacto clínico negativo da ocorrência de resistência à quimioterapia de uso corrente, cabe a investigação do envolvimento das CTT com esse processo e de potenciais mecanismos passíveis de intervenção farmacológica que estejam envolvidos. Sendo assim, os objetivos desse capítulo do trabalho são:

1. Avaliar o efeito do quimioterápico cisplatina sobre a proporção de CTT em linhagens celulares de HNSCC;
2. Avaliar o efeito de cisplatina sobre a proliferação de CTT *in vitro*, através da formação de oroesferas a partir de células de HNSCC;
3. Avaliar o efeito da cisplatina na sobrevivência de CTT através da expressão do marcador Bmi-1;
4. Gerar células resistentes a cisplatina a partir de células de HNSCC e avaliar a expressão de marcadores de CTT e a tumorigenicidade dessas células *in vivo*;
5. Avaliar o efeito de cisplatina nas vias de sinalização de sobrevivência celular, Akt e ERK em células de HNSCC e nas células resistentes;
6. Avaliar o efeito de cisplatina na proporção de CTT *in vivo*;

7. Avaliar a influência de IL-6, e de sua via de sinalização mediada por STAT3, na proteção das CTT de HNSCC, após tratamento com cisplatina, em todos os parâmetros acima;
8. Avaliar a inibição do receptor de IL-6 como possível intervenção farmacológica em HNSCC.

CAPÍTULO I

The histone deacetylase inhibitor sodium butyrate promotes cell death and differentiation and reduces neurosphere formation in human medulloblastoma cells

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The Histone Deacetylase Inhibitor Sodium Butyrate Promotes Cell Death and Differentiation and Reduces Neurosphere Formation in Human Medulloblastoma Cells

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Abstract Increasing evidence suggests that alterations in epigenetic mechanisms regulating chromatin state play a role in the pathogenesis of medulloblastoma (MB), the most common malignant brain tumor of childhood. Histone deacetylase (HDAC) inhibitors, which increase chromatin relaxation, have been shown to display anticancer activities. Here we show that the HDAC inhibitor sodium butyrate (NaB) markedly increases cell death and reduces colony formation in human MB cell lines. In addition, NaB increased the mRNA expression of Gria2, a neuronal differentiation marker, in D283 and DAOY cells and reduced the number of neurospheres in D283 cell cultures. Finally, NaB reduced the viability of D283 cells when combined with etoposide. These data show that NaB displays pronounced inhibitory effects on the survival of human MB cells and suggest that NaB might potentiate the effects of etoposide. In addition, our study suggests that HDAC inhibition might promote the neuronal differentiation of MB cells and provides the first evidence that an HDAC inhibitor might suppress the expansion or survival of MB cancer stem cells.

Keywords Histone deacetylase · Epigenetics · Chromatin · Brain tumor stem cell · Medulloblastoma · Brain cancer

Introduction

Brain tumors represent the leading cause of cancer-related mortality in children. Medulloblastoma (MB) is the most common malignant childhood brain tumor. Although multimodal therapy has improved cure rates, about 30 % of patients still have a low chance of being cured and survivors often

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experience long-term neurocognitive and neuroendocrine dysfunctions [1–3]. MB shows predominantly neuronal differentiation, and recent advances in the understanding of its cellular origin have indicated that different MB subtypes may arise from neural stem cells (NSCs) or cerebellar granule neuron precursors (GNPs) that undergo genetic alterations [4–6]. Also, brain tumor stem cells (BTSCs), a subset of cancer cells capable of self-renewing and maintaining tumor growth, might originate from NSCs or GNPs and play a crucial role in MB initiation and development [7–9].

Accumulating evidence suggests that alterations in epigenetic mechanisms regulating chromatin state are involved in the pathogenesis of MB. Among other posttranslational modifications, histone acetylation and methylation play a key role in controlling chromatin remodeling, thus altering gene expression [10]. Recent genomic analyses have shown that alterations in genes involved in chromatin modification are found across different molecularly defined subtypes of MB. These alterations include truncating mutations in the KDM6A gene encoding a histone 3 lysine 27 demethylase [11], homozygous deletions of several genes with established roles in histone lysine methylation, and amplification of the histone acetyltransferase gene *MYST3* [12].

An increasingly used approach for targeting epigenetic alterations and chromatin modification mechanisms in cancer is to test histone deacetylase (HDAC) inhibitors as antitumor agents. The interplay between HDACs and acetyltransferases (HATs) controls histone acetylation: HATs transfer acetyl groups to amino-terminal lysine residues in histones, resulting in local chromatin relaxation and increased access to genomic DNA, thus favoring gene expression, whereas HDACs remove acetyl groups, leading to chromatin condensation and transcription repression [10, 13]. Early studies in the 1970s reported that sodium butyrate (NaB) could inhibit cancer cell growth by increasing histone acetylation [14, 15]. NaB is a potent HDAC inhibitor that causes hyperacetylation of histones H3 and H4 in a range of mammalian cells [16]. Many HDAC inhibitors have now been developed and extensively investigated as candidate anticancer agents in experimental models, several have entered clinical trials, and one (suberoylanilide hydroxamic acid, SAHA) has been approved by the FDA for the treatment of cutaneous T cell lymphoma [13, 17, 18]. The anticancer mechanisms of HDAC inhibitors likely include promoting cell differentiation and increasing the expression of tumor suppressor genes [13]. In MB cells, HDAC inhibitors including SAHA, valproic acid, MS-275, and trichostatin A (TSA) have been shown to reduce growth, promote cell death, and increase the sensitivity to the effects of ionizing radiation, etoposide, and the kinase inhibitor sorafenib in vitro [19–24]. In addition, valproic acid inhibited the in vivo growth of DAOY and D283 human MB cells xenografted into nude mice, and its antitumor effect correlated with H3 and H4 hyperacetylation [25]. However, the effects of NaB in MB cells remain relatively poorly understood. One study indicated that NaB could increase cell death, alter the cell cycle, and stimulate caspase-3 and caspase-9 activities in DAOY and UW228-2 cells, as well as induce sensitization to ionizing radiation and TRAIL-induced apoptosis in DAOY cells [19]. However, we previously found that NaB was able to reduce the viability of DAOY cells only when combined with the neurotrophin brain-derived neurotrophic factor [26]. Moreover, although there is some recent evidence that HDAC inhibitors can inhibit the growth of BTSCs in other types of brain cancer, namely, gliomas and ependymomas [27–29], previous studies have not characterized the possible effects of HDAC inhibition on the proliferation of MB stem cells. Here we show the effects of NaB on cell death, colony-forming capability, and differentiation of three human MB cell lines (DAOY, D283, and ONS76). In addition, we report findings indicating that NaB can reduce the number of MB stem cells and sensitize MB cells to etoposide.

Materials and Methods

Cell Culture and Treatments

DAOY and D283 human MB cells were originally obtained from the American Type Culture Collection (Rockville, MD, USA), and ONS76 was originally obtained from the National Institute of Biomedical Innovation—Japanese Collection of Research Bioresources (Osaka, Japan). All three cell lines were kindly donated by Dr. Michael D. Taylor (The Hospital for Sick Children, Toronto, Canada). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Carlsbad, USA) containing 2 % (*w*/*v*) H-glutamine and 10 % (*v*/*v*) fetal bovine serum (Sorali, Campo Grande, Brazil), gentamicin (4 mg/ml; Nova Pharma, Jardim Anápolis, Brazil), ampicillin (50 mg/ml; Nova Pharma, Jardim Anápolis, Brazil), and fungizone (250 mg/kg; GIBCO, Grand Island, USA). Cells were kept at a temperature of 37 °C, a minimum relative humidity of 95 %, and an atmosphere of 5 % CO_2 in air.

Cells were treated immediately after seeding with NaB (1.0, 2.0, 5.0, 7.5, or 10 mM; Sigma-Aldrich, St. Louis, USA) for 72 h. Etoposide (1.0 μM) was added 24 h after NaB and maintained for 48 h. The doses of NaB were chosen on the basis of previous in vitro studies using cultured medulloblastoma cell lines [19, 26].

Cell Death

Cell death was examined with Trypan blue cell counting [21, 23]. Cells were seeded at a density of 12×10^4 cells/well

in DMEM 10 % FBS into six-well polystyrene tissue culture-treated flat-bottom microtiter plates (TPP, Switzerland) in triplicates. After treatment, the medium was removed, cells were washed with Hanks' Balanced Salt Solution (HBSS, Invitrogen), and 500 μl of 0.25 % trypsin/EDTA solution was added to detach the cells. Cell suspension was homogenized with 0.4 % Trypan blue 1:1 and counted immediately in a hemocytometer.

Cell Viability

Cell viability was measured with the 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Sigma-Aldrich) assay [26]. Cells were seeded at a density of 5×10^3 cells/well into 96-well polystyrene tissue culture-treated plates in quadruplicates. After treatment, the medium was removed and replaced with 1× MTT 5 mg/ml solution diluted in cell medium. The plates were further incubated at 37 °C under 5 % CO_2 in air for 4 h as previously described [30]. The plate was left at room temperature until completely dry. Dimethyl sulfoxide was added and the absorbance was read at 492 nm in a multiplate reader. The viability was determined as the percentage absorbance of treated cultures compared with those of untreated control cultures.

Colony Formation

For evaluation of colony-forming capability, the colony formation assay was performed [22, 31]. First, cells were cultured in six-well plates for 72 h with NaB and then reseeded at a density of 500 cells/well as duplicates in a six-well plate without treatment. Cells were maintained for 7 to 10 days until colonies were formed. Media was changed every 2 days. Cells were then fixed with 70 % ethanol and counterstained with 0.1 % crystal violet. Colonies of at least 50 cells were counted using freeware ImageJ 1.37 for Windows. The effect on treated colonies of three independent experiments was shown as mean percent of control.

Neurosphere Formation

Neurosphere formation was used as an established experimental assay to evaluate cancer stem cell proliferation [32], using the general methods described in previous studies [33–36]. The D283 line was chosen based on pilot experiments indicating that it allowed more consistent and reproducible sphere formation after 4 days, compared to the other cell lines (data not shown). D283 cells growing as monolayer were detached with 0.25 % trypsin/EDTA solution, washed with HBSS for serum removal, and resuspended in DMEM/F12 media without serum supplemented with 20 ng/ml epidermal growth factor (Sigma-Aldrich), 20 ng/ml basic fibroblast growth factor (Sigma-Aldrich), B-27 supplement 1X (Gibco, Life Technologies), N-2 supplement 0.5X (Gibco, Life Technologies), 50 μg/ml bovine serum albumin (Sigma Aldrich), and antibiotics, as previously described. Cells were monitored daily until sphere formation. Treatment with NaB was added straight after plating 3×10^3 cells in 96-well plates with the stem cells media. Spheres were counted 5 days after treatment under an inverted microscope at× 4 magnification. A sphere was counted if it had at least 15 cells. On the 8th day, spheres were trypsin-dissociated and replated in 10 % FBS DMEM.

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

The total RNA from the three cell lines was extracted using Trizol reagent (Invitrogen), in accordance with the manufacturer's instructions, quantified in NanoDrop (Thermo Scientific) and reverse-transcribed with superscript® III First-Strand Synthesis supermix (Invitrogen). The α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor subunit Gria2 and the glutamate aspartate transporter (GLAST) were used as differentiation markers for neurons and astrocytes, respectively. Octamer-binding transcription factor 4 (Oct4) was used as a stem cell marker. The expression of β-actin was measured as an internal control. The human Gria2, GLAST, Oct4, and β-actin primers used for RT-PCR amplification were designed according to the corresponding GenBank sequence and are shown in Table 1.

The PCR conditions for Gria2, GLAST, Oct4, and β-actin experiments were 2.5 mm $MgCl_2$, 0.1 μM for each primer, 0.2 mM DNTPs, 1 U Taq Platinum (Invitrogen), and 1 μl cDNA template. All assays were carried out in a total volume of 15 μl using 35 cycles for amplification that consisted of 1 min at 95 °C, denaturation at 94 °C for 30 s, annealing at 58–60 °C, accordingly to the specific primer, for 30 s, and extension of primers at 72 °C for 45 s, followed by a final extension at 72 °C for 10 min. The products of Gria2 (316 bp), GLAST (321 bp), Oct4 (496 pb), and β-actin (190 bp) were electrophoresed through 1.0 % agarose gels containing ethidium bromide (Biotium, Hayward, USA) and visualized with ultraviolet light. The fragments' length was confirmed using a Low DNA Mass Ladder (Invitrogen) and the expression of Oct4 relative to β-actin was determined by densitometry using freeware ImageJ 1.37 for Windows. For each set of PCR reactions, a negative control was included. Semiquantitative data are shown as relative numbers in relation to β-actin.

Statistics

Data are shown as the mean ± standard error of mean (SEM) of three to four independent experiments. Data were analyzed by one-way analysis of variance, followed when appropriate by

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

Gene	Primer sequences	PCR product size (bp)
Gria2	Forward: 5′ CACTTCGGAGTTCAGACTG 3′ Reverse: 5′ GCCTCTGTCACTGTCATAG 3′	316
GLAST	Forward: 5′ TCTTCTCCATGTGCTTCGG 3′ Reverse: 5′ CTTGCAGCAACCCTCCAAT 3′	321
Oct4	Forward: 5′ AACATGTGTAAGCTGCGGC 3′ Reverse: 5′ TTGAATGCATGGGAGAGCC 3′	496
β-Actin	Forward: 5′ GAGACCTTCAACACCCCAG 3′ Reverse: 5′ GCTACAGCTTCACCAGCAG 3′	190

the Tukey post hoc test, using SPSS version 16.0. Values of $P<0.05$ were considered significantly different.

Results

NaB Promotes Cell Death in Human MB Cell Lines

We first examined the effect of NaB on the death of human MB cells using the Trypan blue cell counting assay. Treatment for 72 h with NaB at 1.0 and 2.0 in DAOY and ONS76 cells, and at 5.0, 7.5, and 10.0 mM in all three cell lines, significantly reduced cell number (Fig. 1). The maximal mean decrease, produced by NaB at 10.0 mM, was 87.7 % in D283 cells, 93.4 % in DAOY cells, and 91.7 % in ONS76 cells (all Ps<0.001 for comparisons between cells treated with 10.0 mM NaB and controls).

NaB Reduces the Colony-Forming Capability of Human MB Cell Lines

We next verified whether NaB could influence the colony-forming capability of MB cells. The cells were exposed to NaB for 72 h and then replated without treatment. Colonies were formed after 7 to 9 days, depending on the cell line. Colony formation was significantly reduced by NaB at 2.0, 5.0, 7.5, and 10.0 mM in ONS76 cells; by the doses of 5.0, 7.5, and 10 mM in D283 cells; and only at 10.0 in DAOY cells. The lowest dose (1.0 mM) had no effect in any cell line (Fig. 2). Again the maximal effect was obtained with 10.0 mM NaB (mean reduction compared to controls for D283, 91.4 %, $P<0.001$; DAOY, 74.5 %, $P<0.05$; and ONS76, 57.1 %, $P<0.05$).

mRNA Levels of Differentiation Markers in Human MB Cells Treated with NaB

RT-PCR analysis indicated that NaB at 10 mM for 72 h led to an increase in the mRNA levels of the AMPA receptor subunit Gria2, a marker of neuronal differentiation, in D283 and DAOY cells ($P<0.05$ compared to control cells). Expression of the glial marker GLAST was detected in all three cell lines, regardless of whether they were exposed to NaB (Fig. 3).

NaB Reduces the Number of Neurospheres in D283 MB Cell Cultures

Neurosphere assays are now widely used to investigate the proliferation of NSCs and glioma and MB BTSCs [9, 32–34, 37]. However, the present study is the first to characterize neurosphere formation in D283 cells. Culturing D283 cells in appropriate culture medium for expansion of stem cells consistently promoted neurosphere formation (Fig. 4a–e). Dissociating and replating the spheres in 10 % FBS DMEM resulted in the culture reversing to a monolayer pattern (Fig. 4f). A representative RT-PCR analysis showing expression of the stem cell marker Oct4 in neurospheres is shown in Fig. 4g. Cells in spheres had a 23.5 % higher content of mRNA for the stem cell marker Oct4 relative to non-spheres ($P<0.05$ compared to controls).

NaB at all doses tested (1.0, 2.0, 5.0, 7.5, and 10 mM) significantly reduced the number of spheres counted 5 days after treatment compared to controls (all $Ps<0.001$), with the dose of 10 mM producing the maximal mean reduction of 98.3 % (Fig. 5a). In addition, a reduction in neurosphere volume was evident in cultures treated with NaB (Fig. 5b). These results suggest that MB stem cells can be enriched in D283 cell cultures, and NaB might reduce the presence of BTSCs in D283 cell cultures.

NaB Combined with Etoposide Reduces the Viability D283 MB Cells

The final experiment was carried out to verify whether NaB could potentiate the effects of etoposide on MB cells. Neither NaB (5.0, 7.5, or 10.0 mM) nor etoposide (1.0 μM) given alone significantly affected the viability of D283 cells as measured by the MTT assay. However, the combination of NaB at 10 mM and etoposide resulted in a mean reduction of 41.7 % in viability ($P<0.05$ compared to controls) (Fig. 6).

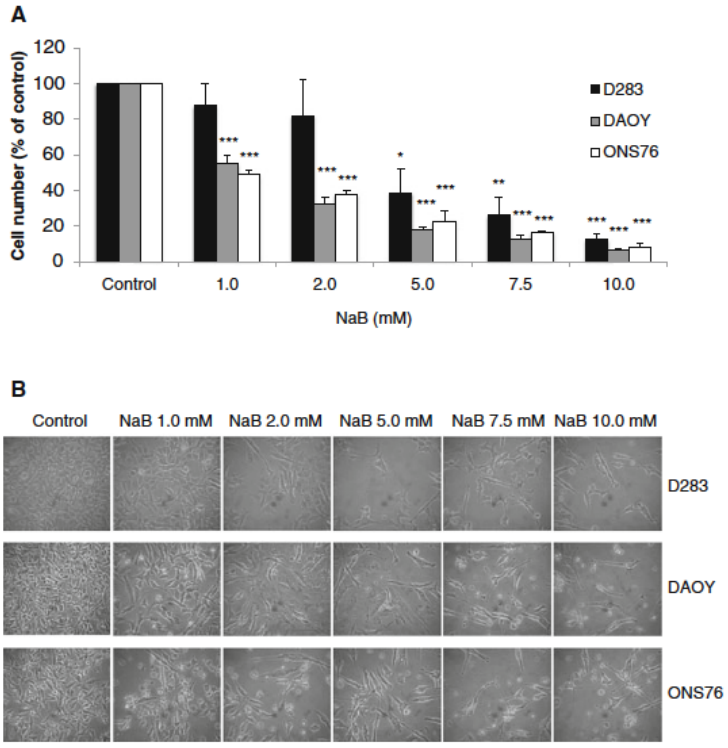

Fig. 1 The HDAC inhibitor NaB induces cell death in human MB cells. D283, DAOY, and ONS76 cells were cultured and treated with NaB (1.0, 2.0, 5.0, 7.5, or 10 mM) for 72 h, as described in "Materials and Methods." Cell death was measured by Trypan blue cell counting. **a** Data are mean ± SEM percentage of control (the average value among replicates in control cells was taken as 100 %); **b** representative photomicrographs of control cells and cells treated with different doses of NaB; $n=4$ independent experiments performed in quadruplicates; $^{*}P<0.05$; $^{**}P<0.01$; and $^{***}P<0.001$ compared to control cells

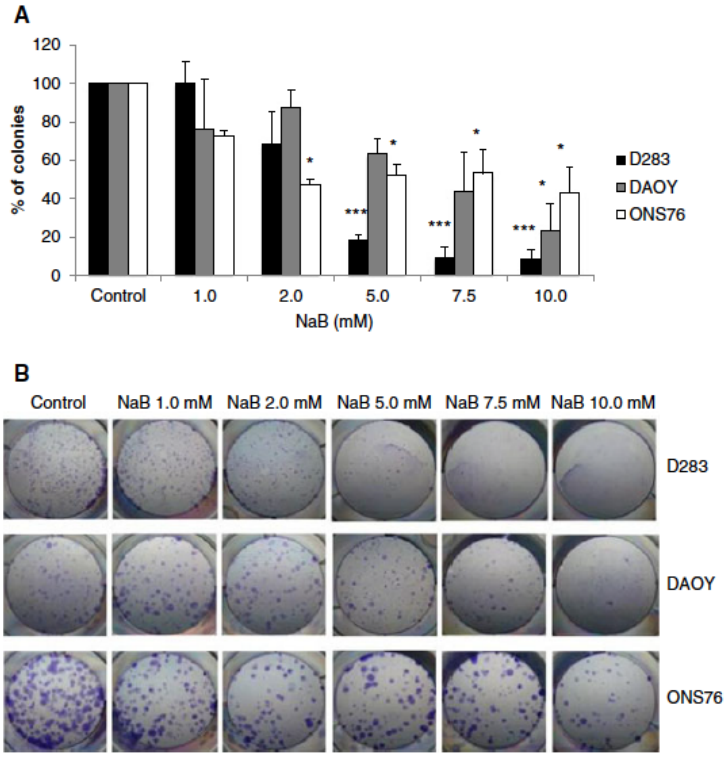

Fig. 2 The HDAC inhibitor NaB reduces the colony-forming capability of human MB cells. D283, DAOY, and ONS76 MB cell lines were cultured and treated with NaB (1.0, 2.0, 5.0, 7.5, or 10.0 mM) for 72 h, and then remained in culture in the absence of NaB for additional 7 (D283) or 9 days (DAOY and ONS76). **a** Data are mean ± SEM % surviving fraction (the average value of number of colonies in the control group was taken as 100 %); $n=3$ independent experiments performed in duplicates; $^{*}P<0.05$; $^{***}P<0.001$ compared to control cells. **b** Representative images of colonies formed from D283, DAOY, and ONS76 cell under the different treatment conditions

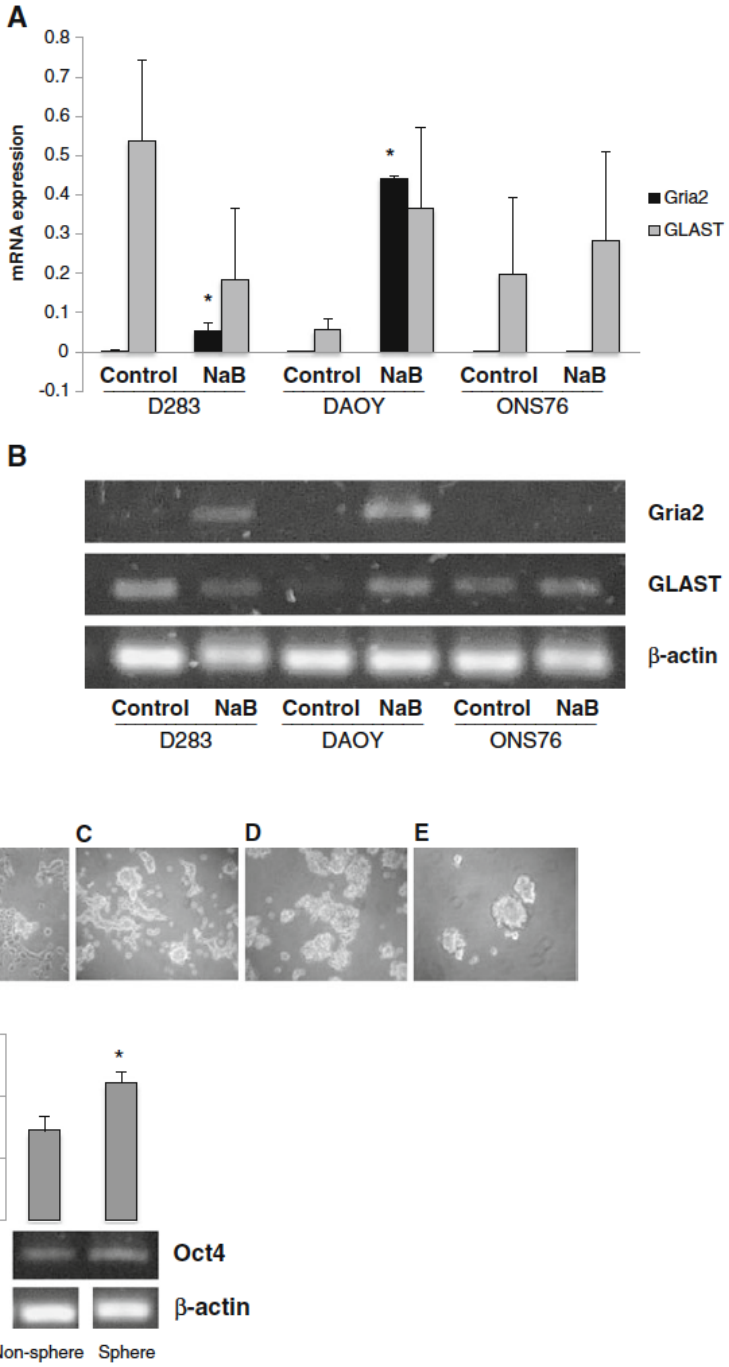

Fig. 3 The HDAC inhibitor NaB increases the mRNA levels of Gria2 in D283 and DAOY MB cells. D283, DAOY, and ONS76 MB cells were treated with NaB (10 mM) for 72 h. **a** Data are mean ± SEM % mRNA expression relative to β-actin (taken as =1.0); $n=3$ independent experiments; $^{*}P<0.05$ compared to control cells. **b** Representative RT-PCR analysis of Gria2 and GLAST

Fig. 4 Neurosphere formation in D283 cell cultures. Cells were cultured in appropriate medium for stem cell generation during 7 days as described in "Materials and Methods." Representative photomicrographs depicting neurosphere formation in **a** 0, **b** 2, **c** 4, **d** 5, and **e** 7 days. **f** The cells expanded again as a monolayer when the medium was replaced by 10 % FBS on day 8. **g** Representative RT-PCR showing mRNA expression of Oct4 in neurospheres and non-spheres in D283 cell cultures at day 5; a 23.5 % higher level of Oct4 expression is observed in neurospheres. Data in the graph are shown mean ± SEM expression of Oct4 relative to β-actin (taken as =1.0); $n=3$ independent experiments; $^{*}P<0.05$ compared to control cells

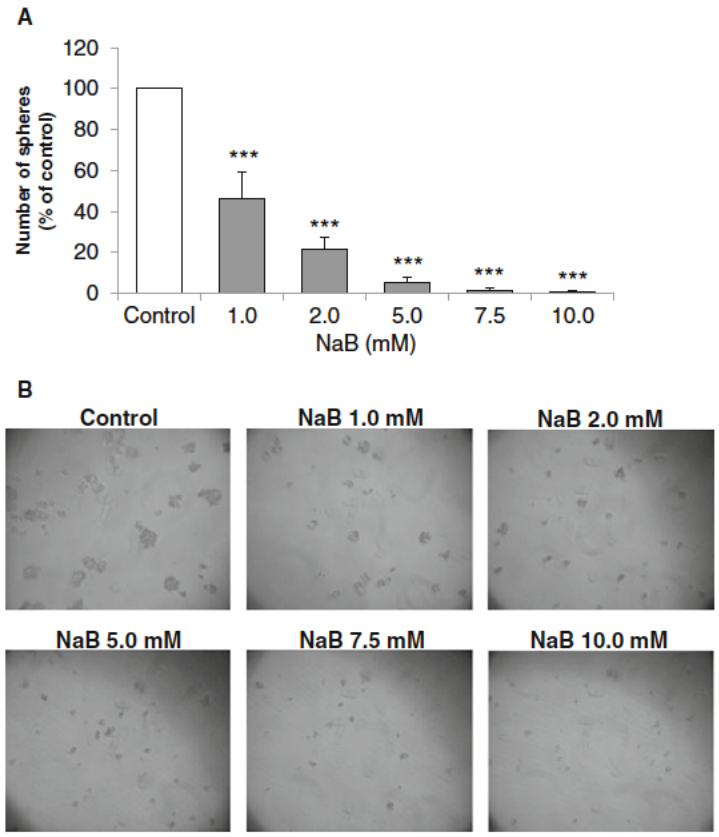

Fig. 5 The HDAC inhibitor NaB reduces the number of neurospheres in human D283 MB cell cultures. Cells were cultured in appropriate medium for stem cell generation during 7 days and treated with NaB (1.0, 2.0, 5.0, 7.5, or 10.0 mM) as described in "Materials and Methods." **a** Number of neurospheres after 5 days of treatment with NaB. Data are mean ± SEM of three different experiments performed in decaplicates. The mean value for control cells was taken as 100 %. ***$P<0.001$ compared to control cells. **b** Representative photomicrographs of neurospheres at 5 days under the different treatment conditions. Note the reduced neurosphere sizes in NaB-treated cultures

Discussion

We have found that NaB at a range of concentrations induces a pronounced decrease in cell number, indicating a promotion of cell death, in cultures of three different human MB cell lines. In addition, NaB significantly reduced the cells' colony-forming capability. These findings support and extend previous studies showing that HDAC inhibitors kill MB cells and sensitize them to apoptosis [19–24]. The mechanisms underlying the anticancer effects of HDAC inhibitors are multiple and include alterations in the expression of genes regulating several aspects of cell cycle, apoptosis, and angiogenesis. These mechanisms also likely include an increased expression of genes associated with differentiation [13]. In MB, transcriptional repression of genes promoting neuronal differentiation, which can result in maintaining the cells in a more "stem-like" state, might play a crucial role in tumorigenesis [38, 39]. Recent evidence indicates that the WNT/β-catenin pathway, which is aberrantly activated in 25 % of MBs, might generate experimental MBs primarily by inhibiting neuronal differentiation in cerebellar progenitor cells [39]. The antitumor effects of phenylbutyrate, which induces hyperacetylation of H3 in D283 and DAOY cells, were associated with an increased expression of markers of neuronal and glial differentiation [40], suggesting that HDAC inhibitors can inhibit the growth of MBs by promoting differentiation. Consistent with this evidence, we found that NaB induced an increase in the expression of Gria2 mRNA in

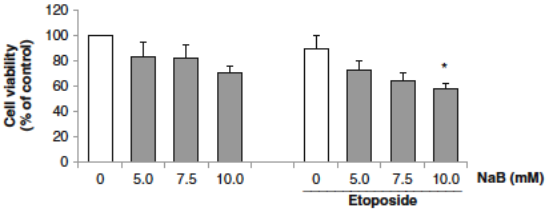

Fig. 6 The HDAC inhibitor combined with etoposide reduces the viability of D283 human MB cells. Cells were cultured and treated with NaB (5.0, 7.5, or 10 mM) for 72 h, and etoposide (1.0 μM) was added 24 h after NaB and maintained for 48 h, as described in "Materials and Methods." Cell viability was measured by the MTT assay. Data represent the mean ± SEM of five different experiments performed in quintuple wells each. The mean value for control cells was taken as 100 %. *$P<0.05$ compared to control cells

D283 and DAOY cells, which may be due to selective elimination of undifferentiated cells or active differentiation of MB cells with stem cell features. Expression of Gria2 (also known as GluR2) is found in differentiated granule cells, but not stem cells, in the hippocampal dentate gyrus, supporting its use as a marker of neuronal differentiation and maturation [41]. Further studies should evaluate the functional consequences of Gria2 expression in MB cells.

An additional mechanism has been recently proposed for the effects of HDAC inhibitors in MB, in which HDAC directly binds to the oncogenic transcription factor Gli1 and promotes its deacetylation [42, 43]. This oncogene is a crucial effector of the Hedgehog pathway, a signaling system strongly implicated in the pathogenesis of MB [44, 45]. Activation of the Hedgehog pathway leads to increased HDAC1 expression, which in turn promotes direct Gli1 deacetylation and activation, and possibly results in other consequences of increased HDAC activity in MB [42, 43]. Endogenous inhibition of HDAC1 is provided by the tumor suppressor REN^{KCTD11}, which increases HDAC ubiquitination and proteasomal degradation through a mechanism dependent on the SCF-like E3 ubiquitin ligase complex [43, 46]. Two novel genes, KCASH2 and KCASH3, have been recently identified and characterized as REN^{KCTD11} homologues that are deleted in human MB and act by suppressing both HDAC and Hedgehog activity, resulting in growth inhibition in experimental MB [47]. This evidence illustrates how the role of HDAC-related mechanisms may be more complex and crucial to the genesis and progression of MB than previously thought and highlight the need to understand how HDAC inhibitors interact with endogenous mechanisms that modulate HDAC activity and Hedgehog-mediated signaling.

The different sensitivities of the different MB cell lines to NaB observed in our experiments might be related to phenotypic differences between cell lines. For example, DAOY cells express epidermal growth factor receptors (EGFR) and a number of antigens associated with glioma, but not neurofilament protein (NFP), whereas D283 cells show more neuroblastic features and expression of NFP but not EGFR or most glioma-associated antigens tested, indicating that these cell lines might represent biologically distinct subgroups of MB [48].

Our results using the neurosphere formation assay provide preliminary evidence suggesting that HDAC inhibitors might suppress the formation or survival of MB stem cells. In addition, we describe for the first time the generation of neurospheres in D283 cell cultures. However, several caveats limit the interpretation of this set of findings. First, although neurosphere formation (which is a currently established assay for cancer stem cell propagation) [32] and marker expression were used to examine the stem cell phenotype, we did not carry out an in vivo tumor propagation experiment, which is taken as the gold standard for the functional definition of BTSCs. Recent studies in glioma have indicated that cells lacking sphere-forming ability in vitro can still display tumorigenic potential; thus, sphere formation would not be an ideal parameter defining stem cells [49, 50]. Second, the experiment was limited to a single cell line, namely D283, in which we were able to induce sphere formation. Finally, the stem cell marker we used, Oct4, was also detected, although at a lower level, in non-spheres. It is noteworthy that a recent study found that Oct4 was detectable in 48 % of analyzed MB samples from patients and indicated that Oct4 expression status might be used as a predictor of poor clinical outcome in MB patients [51]. Because of these limitations, our findings using MB neurosphere formation serve as preliminary evidence that should be followed by further studies aimed at characterizing the effects of HDACs in MB stem cells, including the use of in vivo models. Also, future experiments should examine whether these basic findings can be reproduced with the use of other HDACs, including those that are more likely to show clinical usefulness in MB.

NaB reduced MB cell viability, evaluated by the MTT assay, only when combined with etoposide. Although NaB alone at 10 mM was capable of producing pronounced inhibitory effects in the experiments using cell counting and clonogenic assays, it did not affect viability when measured by the MTT assay. This discrepancy may be related to differential sensitivities between the MTT assay and other assays to detect the effects of NaB on MB cells, since factors related to metabolic activity, cell volume, and the presence of colored compounds can interfere with the outcome of the MTT assay and limit its sensitivity to detect loss of cell viability or proliferation [52, 53]. Despite this caveat, our result is consistent with one previous study showing that another HDAC inhibitor, SAHA, induced sensitization of DAOY cells to etoposide but not vincristine [19] and supports the view that HDAC inhibitors can interact with etoposide in inhibiting MB growth. We previously reported that NaB selectively potentiated the cytotoxic effect of etoposide (but not that of bleomycin, doxorubicin, vincristine, or methotrexate) in human lymphoblastic leukemia cells [54]. Moreover, the concentration of etoposide necessary to inhibit the growth of drug-resistant cell lines was consistently reduced by combining etoposide with TSA, which by itself did not affect cell viability [55]. Etoposide is a topoisomerase II (topo II) inhibitor currently used in the treatment of several cancer types [56]. Many clinical trials have investigated etoposide as a chemotherapeutic agent in the treatment of MB in children [57, 58]. One possibility to explain the preferential interaction of HDAC inhibitors with etoposide in inhibiting cancer cell proliferation is a direct association between HDAC and topo II. HDAC1 and HDAC2 have been shown to interact directly with topo II to form functionally coupled complexes, and HDAC and topo II modify each other's activity in vitro and

in vivo. It is possible that HDAC targets topo II to chromatin regions undergoing histone acetylation, facilitates chromosome condensation by topo II, and is directed by topo II to specific chromosomal sites [55]. Together, this evidence supports the combination of HDAC inhibitors and etoposide as a promising anticancer strategy that should be further investigated in experimental models and clinical studies.

Conclusion

In summary, the present study shows pronounced inhibitory effects of the HDAC inhibitor NaB on the survival of human MB cell lines and provides further evidence that HDAC inhibition reduces MB growth. Our findings suggest that NaB might contribute to the neuronal differentiation of MB cells and indicate preliminary support for the possibility that an HDAC inhibitor might hinder the proliferation or induce death of MB stem cells. Moreover, the results also support the combination of HDAC inhibitors with etoposide as a potential anticancer strategy in MB.

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Capítulo II

The histone deacetylase inhibitor sodium butyrate in combination with brain-derived neurotrophic factor reduces the viability of DAOY human medulloblastoma cells

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BRIEF COMMUNICATION

The histone deacetylase inhibitor sodium butyrate in combination with brain-derived neurotrophic factor reduces the viability of DAOY human medulloblastoma cells

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Abstract
Purpose Histone deacetylase inhibitors (HDACis) are a promising class of anticancer agents for the treatment of brain tumors. HDACis can increase the expression of brain-derived neurotrophic factor (BDNF) in brain cells. We have previously shown that BDNF reduces the viability of medulloblastoma cells. The aim of the present study was to examine the effect of the HDACi sodium butyrate (NaB) combined with human recombinant BDNF (hrBDNF), on the viability of human medulloblastoma cell lines.
Methods DAOY and ONS76 medulloblastoma cells were treated with NaB, hrBDNF, or NaB combined with hrBDNF. Cell viability was measured with the MTT assay.
Results NaB combined with hrBDNF significantly reduced the viability of DAOY medulloblastoma cells. In ONS76 cells, NaB alone reduced viability, but the effect was not potentiated by hrBDNF.
Conclusion These findings provide early evidence for a rationale supporting further evaluation of HDACis and BDNF as a new combinatorial approach to inhibit the growth of medulloblastoma.

Keywords Histone deacetylase inhibitors · Sodium butyrate · Epigenetics · Brain-derived neurotrophic factor · Medulloblastoma · Brain tumors

Introduction

Alterations in gene expression produced by epigenetic changes have been increasingly implicated in cancer initiation and progression. Remodeling of chromatin is a key epigenetic mechanism regulating gene expression. Histone acetyltransferases (HATs) and histone deacetylases

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(HDACs) play opposing roles in modifying chromatin. HATs increase the access to genomic DNA by relaxing chromatin structure, whereas the activity of HDACs leads to chromatin condensation and transcriptional repression. HDACs are promising molecular targets for anticancer therapies aimed at reversing epigenetic modifications in tumors, and HDAC inhibitors (HDACis) have been developed as potential anticancer agents [1].

Epigenetic changes, including inactivation of tumor suppressor genes, have been described in medulloblastoma [2]. Medulloblastoma, the most common brain tumor of childhood, is a highly malignant neoplasia, for which more effective therapies are urgently needed. HDACis have been evaluated as potential anticancer agents in both in vitro and in vivo experimental models of medulloblastoma [3–7]. For instance, the HDACi sodium butyrate (NaB) enhanced the susceptibility of human medulloblastoma cell lines to ionizing radiation and apoptosis induced by tumor necrosis factor-related apoptosis-induced ligand [4].

In cultured neurons and glial cells, NaB and other HDACis upregulate gene transcription for the neurotrophin brain-derived neurotrophic factor (BDNF) [8, 9]. In addition, treatment with NaB increases brain protein levels of BDNF in rats [10]. BDNF signaling through its receptor, TrkB, is a novel target in cancer therapy [11]. We have previously shown that treatment with human recombinant BDNF (hrBDNF) reduced the viability of human medulloblastoma cells in vitro [12]. This evidence suggests that HDACi and BDNF might interact to produce antitumor effects in medulloblastoma. Previous studies have not examined this possibility.

In order to carry out an early screening of potential additive effects of an HDACi and BDNF in medulloblastoma, in the present study we examined cell viability using the 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay in DAOY and ONS76 human medulloblastoma cells treated with NaB, hrBDNF, or NaB combined with hrBDNF.

Materials and methods

Cell culture and treatments

The human medulloblastoma cell lines were kindly donated by Dr. Michael D. Taylor (The Hospital for Sick Children, Toronto, Canada). DAOY cells were originally obtained from the American Type Culture Collection (Rockville, MD, USA), and ONS76 was originally obtained from the Institute for Fermentation (Osaka, Japan). The cells were plated into 96 multiwell plates (TPP) at a density of 5×10^3 cells per well in quadruple and grown and maintained in Dulbecco's modified Eagle's medium (Gibco BRL, Carlsbad, USA) containing 2% (*w*/*v*) H-glutamine and 10% (*v*/*v*) fetal bovine serum (Sorali, Campo Grande, Brazil). Cells were treated 24 h after plating with hrBDNF (0.074, 0.74, or 7.4 nM; Sigma-Aldrich, St. Louis, USA), or immediately after plating with NaB (5, 7.5, or 10 mM; Sigma-Aldrich) combined 24 h later with hrBDNF (0.74 nM). Drug doses were chosen on the basis of previous studies [4, 12]. Cells were kept at a temperature of 37°C, a minimum relative humidity of 95%, and an atmosphere of 5% CO_2 in air.

MTT assay

Cell viability was measured by MTT (Sigma-Aldrich) 48 h after treatment with BDNF. The medium was removed and replaced with 1×MTT 5 mg/mL solution diluted in cell medium. The plates were further incubated at 37°C under 5% CO_2 in air for 4 h as previously described [12]. The plate was left at room temperature until completely dry. Dimethyl sulfoxide was added, and the absorbance was read at 492 nm in a multiplate reader. The viability was determined as the percentage absorbance of treated cultures compared with those of untreated control cultures.

Statistics

Data are shown as mean±standard error of the mean (SEM) number of cells. The mean values for control cells were taken as 100%. Data were analyzed by one-way analysis of variance, followed by the Tukey post hoc test, using the SPSS program, version 16.0. Values of $p<0.05$ were considered to indicate significant differences.

Results

We first performed a dose–response curve of hrBDNF alone in order to compare the results with previous experiments [12] in which a different culture protocol was used (see "Discussion" below). Treatment with hrBDNF did not affect the viability of DAOY cells (Fig. 1a). When NaB was combined with hrBDNF, neither NaB nor hrBDNF affected viability when used alone. However, cell viability was significantly reduced (by 41%, $p<0.001$) when cells were treated with 10 mM NaB combined with hrBDNF at 0.74 nM (Fig. 1b). The results indicate that NaB in combination with hrBDNF can reduce the viability of DAOY medulloblastoma cells.

In the ONS76 cell line, viability was not significantly affected by hrBDNF (Fig. 2a). NaB at either 7.5 and 10 mM produced a decrease of around 30% in viability (both *p*s <0.05 compared to control cells) when given alone. When combined with hrBDNF at 0.74 nM, NaB at 7.5 and 10 mM reduced viability by about 34.1 and 32.8%,

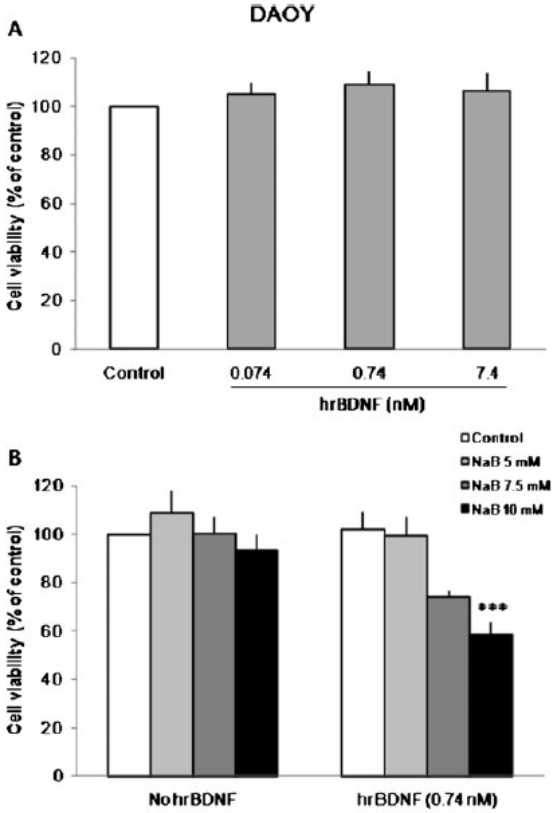

Fig. 1 The HDACi sodium butyrate (*NaB*) in combination with human recombinant brain-derived neurotrophic factor (*hrBDNF*) reduces the viability of DAOY human medulloblastoma cells. hrBDNF alone did not significantly affect viability (**a**). Cell viability was significantly reduced by NaB (10 mM) combined with hrBDNF (0.74 nM) (**b**). Data represent the mean±SEM of four to five different experiments performed in quadruple wells each. The mean value for control cells was taken as 100%; ***$p<0.001$ compared to control cells

respectively (both *ps* <0.01). There were no significant differences between groups treated with NaB alone and cells treated with NaB plus hrBDNF (Fig. 2b). The results indicate that NaB induced a dose–dependent decrease in the viability of ONS76 cells, and this effect was not modified by co-treatment with hrBDNF.

Discussion

HDACis have been shown to induce cell death, reduce viability, and increase sensitivity to radiation and other drugs in medulloblastoma cells [3–7, 13–15]. Because HDACis increase accessibility to chromatin, they might be particularly useful in combination therapies with agents that target DNA or enzymes associated with the DNA [1, 4, 16]. Our results indicate that the HDACi NaB can reduce the viability of ONS76, but not DAOY, cells in vitro when used alone.

Signaling triggered by BDNF has recently been put forward as a target in cancer. BDNF might contribute to increase cancer cell survival, proliferation, and resistance to chemotherapy [11]. Unexpectedly, in a previous study, we found that, in human medulloblastoma cell lines, BDNF inhibits, rather than increases, cell viability in DAOY and D283 cells, under a culture condition in which, different from the present study, cell quiescence was induced by serum deprivation [12]. Further research is required to examine the mechanisms underlying the inhibitory effect of BDNF on viability of medulloblastoma cells. Nonetheless, enhancing BDNF signaling should be further investigated

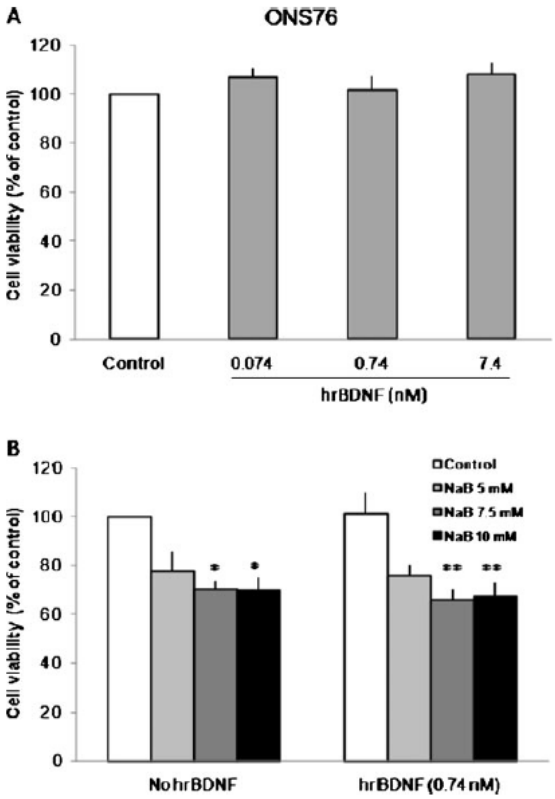

Fig. 2 The HDACi sodium butyrate (*NaB*) alone or combined with human recombinant brain-derived neurotrophic factor (*hrBDNF*) reduces the viability of DAOY human medulloblastoma cells. hrBDNF alone did not significantly affect viability (**a**). Cell viability was significantly reduced by NaB (7.5 or 10 mM) alone or combined with hrBDNF (0.74 nM) (**b**). Data represent the mean±SEM of four to five different experiments performed in quadruple wells each. The mean value for control cells was taken as 100%; *$p<0.05$, **$p<0.01$ compared to control cells

in preclinical models as a possible therapeutic approach in medulloblastoma [12, 15]. Because HDACis increase BDNF gene expression and protein levels in brain cells [8–10], we aimed at examining the effects of NaB combined with hrBDNF on the viability of medulloblastoma cells. We found that, in the DAOY cell line, the combination of NaB and hrBDNF produced a significant reduction in cell viability, while either agent alone had no effect. This finding suggests that NaB and hrBDNF might share a common pathway in inhibiting the viability of DAOY cells. In ONS76 cells, hrBDNF neither affected viability when used alone nor modified the inhibitory effect of NaB. The differential effects of NaB and hrBDNF between DAOY and ONS76 cells, observed in the present study as well as in a previous report [12], might be related to phenotypic differences between these cell lines. ONS76 cells show features of neuronal differentiation, whereas DAOY cells express glioma-associated antigens, but not the neuronal marker neurofilament protein [17, 18], and these two cell lines have been shown to display opposite responses to Rac-1 inhibition [19].

Conclusions

The main conclusions of the present report can be summarized as follows: (1) the inhibitory effect of NaB on viability of ONS76 cells is consistent with the view that HDACis can produce antitumor effects in medulloblastoma; (2) the finding that hrBDNF combined with NaB reduced the viability of DAOY cells is consistent with our previous finding [12] suggesting that, in medulloblastoma cells, BDNF might serve as an inhibitory rather than a pro-survival signal; and (3) the results suggest for the first time that, at least under some conditions, HDACis in combination with BDNF might reduce the growth of experimental medulloblastoma. This study provides early evidence for a rationale supporting further evaluation of HDACis and BDNF as a new combinatorial approach in medulloblastoma.

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Ethical standards All experimental procedures were approved by the institutional research ethics committee (GPPG-HCPA). The present study does not involve the use of experimental animals or materials obtained from patients.

Conflict of interest The authors declare that they have no conflict of interest

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Capítulo III

Cisplatin induces Bmi-1 and enhances the stem cell fraction in head and neck cancer

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Cisplatin induces Bmi-1 and enhances the stem cell fraction in head and neck cancer

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Abstract

Increasing evidence suggests that cancer stem cells (CSCs) are the drivers of tumor initiation and progression, as well as of recurrence and distant metastasis. Increasing evidence indicates that head and neck squamous cell carcinoma (HNSCC) contain a subpopulation of highly tumorigenic, multi-potent and self-renewing cells. The slow proliferative CSC could likely scape from conventional chemotherapy and explain the poor prognosis of HNSCC patients. Factors secreted by endothelial cells have been proposed to protect CSC. Here, we hypothesized that cisplatin treatment spare CSCs and that IL-6, a chemokine secreted by the vascular niche, contributes to cisplatin resistance in HNSCC. We observed an increase in the proportion of putative CSCs identified by ALDH+CD44+ in cells treated with cisplatin and IL-6. Cisplatin combined with IL-6 also promoted the proliferation, self-renewal and survival of CSCs *in vitro* as indicated by an increase in orosphere number formed in ultra-low attachment plates and Bmi-1 expression induction in western blots. Cells resistant to lower doses of cisplatin also expressed more Bmi-1. In addition, STAT3 phosphorylation was enhanced in cells treated with IL-6, and was not affected by treatment with cisplatin. In contrast, ERK phosphorylation was induced by IL-6 alone, but was inhibited when cells were exposed to cisplatin. *In vivo* experiments corroborated the *in vitro* findings by showing an increased proportion of ALDH+CD44+ cells in xenografted tumors treated with cisplatin. Taken together with observation that IL-6R antibody can revert the induction of Bmi-1 expression by cisplatin and IL-6, these results suggest that IL-6 secreted by endothelial cells plays a role in the development of cisplatin resistance involving stem cells in HNSCC. The IL-6 receptor should be further evaluated as a possible new molecular target for the development of adjuvant therapies.

Introduction

Drug resistance is a critical problem in cancer treatment. Head and neck squamous cell carcinoma (HNSCC), one of the most common type of cancer that afflicts over half a million people around the world each year, has a poor patient survival due to recurrences from primary tumors (Chen 2009; Jemal *et al*., 2011). The mainstay of treatment for head and neck tumors in the past several decades has been surgery and radiation. The addition of platinum based drugs to therapy meant an important improvement in local disease control and organ preservation, however, the overall survival rate has not been changed due to chemoresistance and incidence of distant metastasis (Genden *et al*., 2003; Forastiere, 2008; Sano *et al*., 2007).

The clinical scenario indicates that some of the head and neck tumor cells are unaffected by treatment and have the capability to migrate and initiate a new tumor. It has been known that the tumor bulk, as well as normal tissues, comprises a heterogeneous cell types collection based on a differentiation pattern. Recently, resistance to therapy has been attributed to the existence of a small subpopulation of less-differentiated, multi-potent and self-renewing cells, the cancer stem cells (CSCs) (Clarke *et al*., 2006; Gupta *et al*., 2009; Tu *et al*., 2009). CSCs are proposed to be capable to initiate and maintain tumor growth and also disseminate from the primary tumor to promote metastasis (Reya *et al*., 2001; Al-Hajj *et al*., 2003; Korkaya *et al*., 2012). Because CSC are slow proliferative cells, current therapies that shrink tumor by affecting tumor bulk may frequently spare these cells.

The existence of a small cell population with stemness and self-renewal properties in HNSCC, described before (Prince *et al*., 2007; Krishnamurthy and Nör, 2012), motivated our present study of drug resistance involving the survival of stem cells after treatment in experimental HNSCC. Therapies that act more specifically against CSCs, when associated to the current therapeutic scheme may result in more efficient

tumor eradication of HNSCC by avoiding metastatic spread.

Evidence that CSCs can survive after anticancer treatment has been recently reported in cancer types including lung cancer (Bertolini *et al*., 2009; Liu *et al*., 2012), glioblastoma (Chen *et al*., 2012) and breast cancer (Korkaya *et al*., 2012). The emerging findings of increased CSC compartment after chemotherapy are in agreement with the concept that CSCs are key regulators of tumorigenesis and recurrence from treatment failure (Visvader & Lindeman, 2008). Therefore, taking into account the proposed crucial role of CSCs for cancer progression, it becomes evident that the specific targeting of these cells is urgently needed to promote better outcomes for cancer patients.

Considerable research efforts have been directed towards CSCs over the past years, however their efficient elimination remains a challenge because CSC share many characteristics with normal stem cells which are responsible for tissue maintenance. Indeed, CSCs have a marked capacity of drug efflux via the ATP-binding cassette (ABC) transporters (Zhou *et al*., 2001; Schatton *et al*., 2008), which is commonly used to identify stem cells *in vitro* (Goodell *et al*., 1997; Scharenberg *et al*., 2012). Since the direct targeting of CSCs seems to be difficult, other approaches should be considered.

It has been found that CSCs are dependent on the tumor microenvironment for their growth and survival (Lobo *et al*., 2007, Polyak *et al*., 2009). HNSCC CSCs reside in perivascular niches and maintain a molecular dependence upon cells from the blood vessels (Calabrese *et al*., 2007; Krishnamurthy *et al*., 2010). Thus, the blockade of the crosstalk between endothelial cells and CSCs means an interestingly possibility for targeted therapy. In a previous work, we demonstrated that interleukin-6 (IL-6), interleukin-8 (CXCL8), and epidermal growth factor (EGF) expression are upregulated in endothelial cells co-cultured with HNSCC cells. Indeed, blockade of endothelial cell-derived IL-6, CXCL8, or EGF inhibited key pathways of tumorigenesis like STAT3, Akt, and ERK in tumor cells, respectively. Consequently, survival and migration of cancer

cells were impaired (Neiva *et al*., 2009). Among these signaling molecules, IL-6 and also its receptor, IL-6R, and the JAK/STAT3 pathway are implicated in the malignancy of cancer stem cells, which is well described for breast cancer (Sansone *et al.,* 2007; Liu *et al.,* 2011; Marotta *et al.,* 2011; Korkaya *et al*., 2012). In HNSCC, there is a strong correlation between serum Interleukin-6 (IL-6) levels and a poor patient survival (Riedel *et al*., 2005; Duffy *et al*., 2008).

In this study, we examined the effect of cisplatin, the main chemotherapeutic drug used in HNSCC, on CSCs and the contribution of IL-6 in the process of acquired resistance. CSCs were identified as CD44+ cells, which were shown to have increased tumorigenicity and self-renewal properties in HNSCC by Prince and colleagues in a landmark publication (2007); and cells with high aldehyde dehydrogenase 1 activity (ALDH+ cells). ALDH activity appears to enrich for CSC more efficiently than the expression of surface antigens in cell sorting. The majority of the ALDH high cells are also CD44 high and it seems that ALDH activity defines a subset of HNSCC CD44 high cells with increased tumorigenicity (Clay *et al*., 2010). Also, ALDH activity may be related to resistance of progenitor cells towards chemotherapy because of its capacity of breaking down cytotoxic drugs (Ginestier *et al*., 2007). CSC self-renewal was evaluated by Bmi-1 expression. Here we resort to complementary approaches that included analyses and sorting of HNSCC cells and established cisplatin resistant cells, generation of xenograft tumors with human tumor cells and vascularized with human endothelial cells stably transduced with shRNA-IL-6 (or empty vector controls), and treatment of mice bearing tumors with cisplatin and posterior analysis of the ALDH+CD44+ cells proportion. Together, these data suggest that cisplatin treatment increases the CSC population in HNSCC, and IL-6 might play a role in development of resistance. Further research is needed on the possible therapeutic inhibition of the IL-6 pathway for adjuvant therapy in HNSCC patients.

Methods

Cell culture

HNSCC cell lines (UM-SCC-1, UM-SCC-22A, UM-SCC-22B; gift from Dr. Carey, University of Michigan) were cultured in Dulbecco`s Modified Eagle Medium (DMEM), 10% fetal bovine serum (Gibco Invitrogen), 100 U/ml penicillin-streptomycin (Invitrogen; Grand Island, NY, USA). Before using, the cell lines were evaluated for Mycoplasma contamination through a Mycoplasma Detection Kit (PlasmoTest®, InvivoGen, San Diego, CA) and all cell lines were Mycoplasma free. Cisplatin resistant sub lines were generated from UM-SCC-22B cells accordingly to the methods described previously (Hong *et al*., 1988; Kelland *et al*., 1992; Negoro *et al*., 2007). Briefly, the parent cell line was submitted to repeated subcultures in the presence of increasing concentrations of cisplatin from 0.5 μM to 12 μM during a 3-month period. Each concentration was applied twice and exposure was continuous over 3 days, drug was then removed, and cells were exposed again when they had regained their normal growth properties. Four sub lines were collected over the process, UM-SCC-22B resistant to 1 μM cisplatin (UM-SCC-22BCis1), UM-SCC-22B resistant to 4 μM cisplatin (UM-SCC-22BCis4), UM-SCC-22B resistant to 6 μM cisplatin (UM-SCC-22BCis6) and UM-SCC-22B resistant to 12 μM cisplatin (UM-SCC-22BCis12), and maintained with 0.5 μM, 1 μM, 2 μM and 4 μM cisplatin, respectively. Maintenance treatment was removed at least two passages before using the cells in experiments. Human dermal microvascular endothelial cells (HDMEC; Cambrex, Walkersville, MD) were cultured in endothelial cell growth medium-2 (EGM2-MV; Lonza, Walkersville, MD, USA). The identity of all tumor cell lines was confirmed by genotyping at the University of Michigan DNA sequencing core facility. Cells derived from tumors, i.e. primary sample or xenographts, were obtained

immediately, within 12 hours after surgery. Tumors were cut into small pieces and minced with a sterile scalpel until being able to pass a 25 ml pipette tip, then suspended in a 9:1 DMEM-F12 (Hyclone, Waltham, MA) and collagenase/hyaluronidase (Stem Cell Technologies; Vancouver, BC, Canada) solution and incubated for one hour at 37°C. The tumor pieces were mechanically dissociated by passing them through a 10 ml pipette every 15 minutes for mechanical dissociation. The mixture was filtered through a 40 µm nylon mesh (BD Falcon; Franklin Lakes, NJ, USA) and suspended in low glucose DMEM containing 20% FBS to stop collagenase/hyaluronidase action, as previously described by Krishnamurthy *et al*., 2010. The single cell suspensions obtained were then submitted to flow cytometry analysis or sorting. The primary tumor was obtained from patient properly consented undergoing surgery for removal of HNSCC.

Proliferation assay (sulforhodamine B reaction)

Sulfohrodamine B (SRB) cytotoxicity assay was done as described (Zeitlin *et al*., 2006). Briefly, cells were seeded at 2^x10^3 cells per well in 96-well plates, allowed to attach overnight, and treated with cisplatin for 48 to 96 hours. Human recombinant IL-6, when used, was pre-treated for one hour. Cells were fixed with 10% trichloroacetic acid, stained with 0.4% SRB (Sigma-Aldrich) in 1% acetic acid, and plates were read in a microplate reader at 560 nm (GENios; Tecan). Data were obtained from triplicate wells per condition and are representative of 3 independent experiments.

Flow cytometry

Single cell suspensions obtained either from trypsinization of cells lines or digestion of tumor specimens from patients/xenographts was re-suspended at 1^x10^6 cells/ml PBS. The Aldefluor kit (Stem Cell Technologies) was used to identify cells with high ALDH activity. As described before (Krishnamurthy *et al*., 2010), cells were

suspended with activated Aldefluor substrate (BAA) or the negative control (DEAB, a specific ALDH inhibitor) for 45 minutes at 37°C. Then, cells were exposed to anti-CD44 antibody (clone G44-26BD; BD Pharmingen; Franklin Lakes, NJ, USA) and lineage markers (*i.e.* anti-CD2, CD3, CD10, CD16, CD18; BD Pharmingen) pre-conjugated with PE-cy5 to detect cells of the hematopoietic origin and eliminate them. Mouse cells were identified using anti-H2K antibody (BD Biosciences; Franklin Lakes, NJ, USA) and eliminated. The 7-Aminoactinomycin (7-AAD, BD Pharmingen) was used to select live cells.

Orosphere assay

Orospheres (i.e. non-adherent spheroids of HNSCC-derived cells) were generated from 1 cell per well in 96 wells ultra-low attachment plates (Corning) or 1,500 cells in 6 wells ultra-low attachment plates (Corning), as indicated. Cells were plated right after sorting and maintained in low glucose DMEM, 10% fetal bovine serum (Invitrogen) and 100 U/ml Penicillin-streptomycin (Invitrogen) or 100 U/ml Antibiotic Antimycotic Solution (AAA) for one overnight before treatment. Cells were treated with cisplatin 0.02, 0.2 and 2 µM with or without rhIL-6 for 12 days when the spheres were counted under microscope. New treatment was added every 4 days.

Western blot

UM-SCC-1 ($300x10^3$ cells) and UM-SCC-22A, UM-SCC-22B ($700x10^3$ cells) cell lines were plated in 6 cm dishes, serum starved overnight, and treated with cisplatin in the indicated concentrations with or without 20 ng/ml rhIL-6 or 10 µg/ml anti-IL-6R (Chugai Pharmaceuticals, Tokyo) and western blots were performed. For the cell signaling western blots cells were treated for 30 min and for Bmi-1 western blots treatment was for 24h. Primary antibodies were as follows: mouse anti-human phospho-

STAT3, rabbit anti-human STAT3, rabbit anti-human phospho-ERK1/2, mouse anti-human ERK1/2, rabbit anti-human phospho-Akt, rabbit anti-human Akt, rabbit anti-human Bmi-1 (Cell Signaling Technology, Danvers, MA); and mouse anti-glyceraldehyde-3-phosphate dehydrogenase (Chemicon, Millipore, Billerca, MA). Immunoreactive proteins were visualized by SuperSignal West Pico chemiluminescent substrate (Thermo Scientific, Rockford, IL).

Stable short hairpin RNA transduction

Lentivirus expressing a short hairpin RNA (shRNA) construct for silencing IL-6 (Vector Core, University of Michigan) was generated in human 293T cells (human embryonic kidney cells) transfected using the Calcium Phosphate method, as described (Kaneko *et al*., 2006). Scrambled oligonucleotide sequence was used as control (shRNA-C). Supernatants were collected 48 hours after transfection and HDMEC cells were infected with it in a 1:1 dilution medium containing 4 μg/ml polybrene (Sigma-Aldrich, St. Louis, MO). Cells were selected for 2 weeks and maintained in EGM2-MV supplemented with 1 μg/ml puromycin (InvivoGen, San Diego, CA). Down-regulation of IL-6 was confirmed by ELISA (Quantikine; R&D Systems).

SCID mouse model of human tumor angiogenesis

Xenographt tumors vascularized with functional human microvessels were generated in severe combined immunodeficient (SCID) mice (CB 17 SCID, Harlan Charles River Taconic; Germantown, NY, USA) as described (Nör *et al*., 2001). Briefly, 1^x10^5 tumor cells were seeded with 9^x10^5 HDMEC in poly-(L-lactic) acid (PLLA; Medisorb, Nicosia, Cyprus) biodegradable scaffolds. Mice were anesthetized with ketamine and xylazine and bilateral scaffolds were implanted subcutaneously in the dorsum of each mouse. Mice were monitored daily for tumor growth and treatment was

started when the average volume of the tumors reached 200 mm^3. Mice implanted with UM-SCC-22B cells and HDMEC shRNA-C or shRNA-IL6 received 3 intraperitoneal injections of 5 mg/kg cisplatin or vehicle seven days apart.

At termination of the experiment, mice were euthanized and tumors were retrieved, measured, weighted and processed for histology and flow cytometry. The care and treatment of experimental animals was in accordance with University of Michigan institutional guidelines. Tumor size was calculated using the formula: volume (mm3) = $LxW^2/2$ (L, length; W, width).

Histology

Tumors were retrieved and fixed overnight in 10% buffered formalin at 4°C, and processed for histology. The pathology of tumors was evaluated by a trained pathologist blinded to the treatment conditions. The mitotic index was determined by counting mitotic figures in 5 high-power fields per tumor section (Balch *et al*., 2009).

Statistical Analysis

One-way ANOVA was performed using the SigmaStat 16.0 software (SPSS, Chicago, IL). Statistical significance was determined at $*P<0.05$ (unless otherwise specified). Statistics for the *in vivo* studies was performed by a Biostatistician with appropriate software using generalized estimating equation (GEE).

Results

Cytotoxicity of cisplatin on head and neck carcinoma cells is not affected by IL-6

The improved control of local disease with platinum-based drugs has been accompanied by an increase in the incidence of distant metastasis of head and neck cancers. This suggests that some of the HNSCC cells survive after treatment and are

capable to re-populate the tumor in a new location. The overall hypothesis underlying this study was that the tumor cells react to an unfavorable microenvironment generated by chemotherapy by acquiring a more invasive and aggressive phenotype through the survival of the CSCs. In a previous study of our group, we described the existence of CSCs in vascular niches (Krishnamurthy *et al*., 2010), and the endothelial cell-derived IL-6 signaling, which promotes tumor initiation and tumor growth in HNSCC (Krishnamurthy, unpublished data).

In this study, we first looked at the overall survival of cell lines treated with cisplatin and rhIL-6 together. To test this, we performed SRB assay using two HNSCC cell lines, UM-SCC-1 and UM-SCC-22B, pre-treated with rhIL-6 for one hour, and then exposed to cisplatin and rhIL-6 for 72 additional hours. The pre-treatment with the rhIL-6 scheme was chosen in order to mimic the physiological condition, in which IL-6 is already present in the tumor microenvironment at the moment of cisplatin administration. The IC50 for cisplatin was around 2 μM for UM-SCC-1 and UM-SCC-22B as seen in Supplemental Figure 1A-C, confirming our previous observations of cisplatin effects in HNSCC cell lines (Naoki *et al*., 2009). We had expected that the addition of rhIL-6 could revert part of the cytotoxic effect of cisplatin. However, when we co-incubated cells with rhIL-6 the proliferation of both cells did not change (Figure 1A and B). This observation could be confirmed with western blots showing the expression of three signaling molecules, i.e., STAT3, Akt, and ERK, which play critical roles in the control of cell cycle, survival, proliferation, and migration of tumor cells (Song and Grandis, 2000; Zeng *et al*., 2002; Chang *et al*., 2003). STAT3 signaling, together with Janus kinases, is the main downstream target of IL-6, and besides of inflammatory responses during biological processes, also plays an important role in oncogenesis by interfering in cell proliferation as well as differentiation, angiogenesis and apoptosis. STAT3 signaling is often up-regulated during carcinogenesis, especially during the interaction of tumor cells and

immune cells within the tumor microenvironment (Hodge *et al*., 2005; Leemann-Neil *et al*., 2011). The Akt pathway has also been implicated in tumorigenesis and the ERK pathway has been described as an important regulator of tumor growth and a key target for cancer therapy (Vivanco and Sawyers 2002; Roberts and Der, 2007). Western blot for phosphorylated and total STAT3, ERK and Akt was performed in UM-SCC-1 and UM-SCC-22B cells serum-starved overnight and exposed to cisplatin (0-5 μM) and rhIL-6 (20 ng/ml) for 30 min. In a previous study, we have observed that the induction of phosphorylation of STAT3, ERK and Akt in HNSCC cell lines occurs primarily between 15 and 60 minutes and the signal peak is around 30 minutes (Neiva *et al*., 2009). STAT3 phosphorylation was induced with rhIL-6 treatment in the two cell lines, but decreases when cisplatin was added in increasing concentrations (1, 2.5 or 5 μM) in UM-SCC-1, however remains unaffected in UM-SCC-22B (Figure 1C). This means that in UM-SCC-22B IL-6-STAT3 pathway still works in the presence of cisplatin. For this reason, UM-SCC-22B cell line was chosen for further experiments. Recombinant human IL-6 treatment also induces phosphorylation of Akt and ERK in both cell lines, signal which was decreased by the addition of increasing concentrations of cisplatin (Figure 1C). It is known so far that the cytotoxicity of cisplatin is derived from the formation of DNA–protein and DNA–DNA interstrand, and primarily intrastrand crosslinks, therefore leading to the activation of different signal-transduction pathways, such as DNA-damage recognition and repair, cell-cycle arrest, and programmed cell death/apoptosis (Siddik, 2003; Kelland, 2007). Since the inhibitory effect of DNA adducts of cisplatin on the G1-phase cyclin-dependent kinases (CDKs) is a later event in the sequence of checkpoint activation (He *et al*., 2011), and is likely facilitated by the Cdk4 inhibitor p16INK4A (Shapiro *et al*., 1998), significant accumulation of cells in the G1 phase is seen infrequently, largely because cells remain trapped in G2/M. CDK4 expression also decreased in both cell lines (Figure 1C). In a second experimental design, we

established the cisplatin dose in 2 μM and submitted UM-SCC-22B cells to a higher concentration of rhIL-6 (50 ng/mL) for 30 minutes. Human recombinant IL-6 in a higher amount was capable to revert cisplatin ERK phosphorylation. Interestingly, we observed Akt phosphorylation with cisplatin alone (Figure 1D). Considering that STAT3 and ERK are involved in cell survival, these results suggest endothelial cell-secreted IL-6 plays a role in the development of Cisplatin resistance in HNSCC and IL-6 receptor can be an interesting new molecular target for the development of adjuvant therapy.

Cisplatin and IL-6 promote self-renewal of head and neck cancer stem-like cells *in vitro*

The CSC hypothesis provides a coherent rational for the establishment of recurrences in head and neck cancers (Mimeault *et al*., 2010). In a previous study, we demonstrated that endothelial cell-derived IL-6 promotes survival of head and neck cancer stem cells *in vivo* and self-renewal and invasion of HNCSC *in vitro* (Krishnamurthy *et al*., unpublished data). As we could not find a protective activity of IL-6 over the total population treated with cisplatin, as shown by the SRB data and, by the western blots, in which probably, not all, but some of the cells have ERK phosphorylation with rhIL-6 treatment or Akt induction by cisplatin as shown in Figure 1D. Now we looked at the effect of Cisplatin and IL-6 on the stem-cells proportion of UM-SCC-22B cells. FACS analysis revealed a 2 fold increase in the proportion of ALDH+CD44+ cells after treatment with Cisplatin (2 μM) alone, whereas rhIL-6 (20 ng/mL) induced a 2.5 fold increase in the stem cell proportion, comparing to untreated cells (Figure 2 A and B). Cells were treated for 24 hours before analysis and all the death cells were gated out using the 7AAD marker, as previously described (Krishnamurthy and Nor, 2012). Combining cisplatin and IL-6 treatment we found a synergistic effect inducing a 5.5 fold increase in the stem cell fraction (Figure 2B). Cell numbers for the cisplatin dose-

response curve in 3 time points and treatment conditions right before flow analysis are shown in Supplemental Figure 2A and B.

To address the propagation of HNSCC that retain stemness and self-renewal *in vitro*, we sorted ALDH+CD44+/ALDH-CD44- cells and performed the orospheres assay, first described by Krishnamurthy and Nör (2012). Further, we submitted cells sorted from a primary head and neck tumor sample to the orosphere method. The tumor was digested and submitted to FACS the day after surgery and single ALDH+CD44+ cells were plated in each well of low attachment plates and treated either with cisplatin or IL-6 and in combination for 12 days until spheres were formed. In this experiment we observed a higher number of wells containing spheres in the cisplatin plus IL-6 condition, when compared to the cisplatin treatment only (Figure 2C). Representative spheres found in this experiment are shown in Figure 2D.

Head and neck carcinoma cells overexpressed stem cells markers after Cisplatin and rhIL-6 treatment.

To address the role of cisplatin and IL-6 on the self-renewal of stem cells in HNSCC, we analysed the expression of Bmi-1 by western blot. Bmi-1, a member of the polycomb group family of transcriptional regulators, is strongly linked to carcinogenesis and its high expression in epithelial cancers is associated with poor patient outcome. Bmi-1 was first identified in hematopoietic stem cells for its essential role in stem cell fate decisions and now is related to the self-renewal capacity of several types of normal and cancer stem cells (Park *et al*., 2003; Wu *et al*., 2011).

We had previously demonstrated (Krishnamurthy *et al*., 2010) that Bmi-1 is a good marker to identify HNSCC stem cells *in vitro*, as ALDH+CD44+ cells strongly express the self-renewal marker Bmi-1, when compared to the ALDH-CD44- cells. Moreover, conditioned medium from endothelial cells was able to enhance Bmi-1

expression over time, indicating that factors secreted by the niche induce self-renewal in CSCs. Besides of that, Bmi-1 might be related to resistance to cisplatin as it has been demonstrated that Bmi-1 knockdown sensitized cells to cisplatin induced apoptosis through inhibition of PI3K/AKT pathway or through induction of caspase activity in other types of tumors (Wu *et al*., 2011, Wang *et al*., 2011). However, there are no reports in the literature referring to the expression of Bmi-1 after cisplatin chemotherapy. Here, we analyzed the protein lysates from UM-SCC-22A cells, a cell line obtained from a hypopharynx primary tumor and, UM-SCC-22B, the neck metastasis from the same patient. Western blot analysis showed an increase in Bmi-1 expression in both cell lines treated with IL-6 (20 ng/mL) for 24 hours when compared to the untreated cells (Figure 3.A). In addition, cells treated with cisplatin alone expressed a higher amount of the stem cells survival marker in two different doses, 0.2 μM and 2 μM, the last one which is responsible for 50% cell death (IC50 ~ 2 μM). Lysate was collected immediately after 24 hours treatment only from the attached cells, not including dead floating cells. By treating cells with rhIL-6 and cisplatin simultaneously we could observe a higher Bmi-1 expression in comparison with each drug alone or with control cells. The synergistic effect was seen in the lower doses of cisplatin, 0.02 μM for UM-SCC-22B and 0.02 μM and 0.2 μM for UM-SCC-22A.

Cancer cells that are exposed to IL-6 have shown enhanced capacity to invade the extracellular matrix and increased drug resistance. These malignant features are linked to the capacity of IL-6 triggering a physiological mechanism of repair in the cancer tissue by stimulating proliferation and self-renewal of CSCs (Sansone *et al*., 2007)**.** To address whether the effect of cisplatin on the CSC compartment is maintained after treatment, we generated cisplatin resistant cell lines. The UM-SCC-22B cell line was cultured in increasing concentrations of cisplatin, 0.5, 1, 3, 4, 6, 8 and 12 μM. The sub-lines resistant to 1 μM (UM-SCC-22BCis1), 4 μM (UM-SCC-22BCis4), 6 μM (UM-SCC-

22BCis6) and 12 μM (UM-SCC-22BCis12) of cisplatin were collected during the process (Figure 3B) and tested for stemness markers. Results verified at the protein level shows an increased expression of Bmi-1 in the cisplatin resistant cell UM-SCC-22BCis1 if compared to the parental UM-SCC-22B (Figure 3C). In the presence of 2 μM cisplatin, UM-SCC-22B presents higher expression of the stem cell survival marker, confirming previous data, however the other resistant cell lines do not show differences in comparison to untreated cells (Figure 3 C). In the presence of IL-6, UM-SCC-22BCis1 cells seem to express more Bmi-1 than the parental cell UM-SCC-22B. When Cisplatin is combined to IL-6, UM-SCC-22B cells showed an increase in Bmi-1 expression in comparison with IL-6 alone (Figure 3C).

To confirm our data, we chose another stem cells marker, Oct4, a transcription factor involved in the maintenance of pluripotency in embryonic stem cells (Nichols *et al*., 1998; Niwa *et al*., 2000, Yamanaka, 2006). From the western blots showed in Figure 3D, we suggest that the expression of Oct4 follows the same pattern as the ones observed for Bmi-1. UM-SCC-22BCis1 cells expressed more Oct4 than the parental cell line, in untreated cells. Oct4 expression in the cisplatin resistant cell lines seems not to be affected by cisplatin treatment. Also, UM-SCC-22B cells expressed more Oct4 if submitted to IL-6 and Cisplatin together, than to IL-6 alone (Figure 3D).

Head and neck cisplatin resistant cells take longer to generate tumors

To specifically compare the tumor initiation capability of the cisplatin resistant cell, UM-SCC-22BCis12, with the parental line, UM-SCC-22B, *in vivo* we implanted in immune-deficient mice either 100,000 cells of UM-SCC-22B or 100,000 cells of UM-SCC-22BCis12 with 900,000 endothelial cells, HDMEC (n=11 in each group), as previously described (Nör *et al*., 2001) (Figure 4A). The tumor initiation graph demonstrated a significantly early initiation of tumors in the UM-SCC-22B + HDMEC

group than the UM-SCC-22BCis12 + HDMEC group (Supplementary figure 3). Tumor initiation was evaluated by both tumor palpability and the time of the scaffold to reach 200 mm^3. The average of the tumor volumes demonstrates the earlier initiation and greater tumor volume in the UM-SCC-22B + HDMEC group compared to the xenografts generated from the UM-SCC-22BCis12 + HDMEC group in the 18^{th} and the 20^{th} days after implantation (Figure 4B). Moreover, tumors in the UM-SCC-22BCis12 + HDMEC group took 21 days longer to reach 200 mm^3 than tumors in the UM-SCC-22B + HDMEC group (Figure 4B). These data suggest that cells previously submitted to chemotherapy have initially their tumorigenicity compromised by treatment, but after time, can regain tumor initiation capability and generate tumors with the same volume of tumors generated by tumors from untreated cells.

Trying to understand the differences in the tumorigenicity pattern of the cisplatin resistant cell and the parental line, we evaluated STAT3 and ERK phosphorylation by Western Blot. The activation of STAT3, the canonical transduction pathway of IL-6, has been related to carcinogenesis due to its association with cancer survival, invasion and metastasis (Burke *et al*., 2001, Aziz *et al*., 2010). It has been frequently found that STAT3 is constitutively activated in different human cancers (Burke, *et al*., 2001; Kusaba, *et al*., 2006). High STAT3 phosphorylation was correlated with shorter survival compared with low STAT3 phosphorylation in esophageal cancer (Wang *et al*., 2012), suggesting its importance in tumor progression. We found that rhIL-6 (20 ng/mL for 30 min) induced STAT3 phosphorylation in the Cisplatin resistant cells, but in a less degree than in the parental line (Figure 4C). Interestingly, ERK phosphorylation was increased in Cisplatin resistant cells compared to parental line, in untreated cells (Figure 4C). Exposure of the different cells to rhIL-6 increased ERK phosphorylation in UM-SCC-22B cells, but decreased gradually in UM-SCC-22BCis1, UM-SCC-22BCis4, UM-SCC-22BCis6 and UM-SCC-22BCis12, in comparison to untreated cells (Figure 4C).

Phosphorylation events of the mitogen-activated protein kinase (MAPK) pathway, which comprises the key signaling components RAS-RAF-MEK-ERK-MAPK, play important role in tumorigenesis. Alteration of the MAPK pathway has been reported as the most frequent pathway dysregulated in human cancer. This pathway regulates multiple critical cellular functions including proliferation, survival, differentiation, motility, and angiogenesis. Also, marked activation of the MAPK pathway can also suppress cellular growth in a wide variety of normal and cancer cells by inducing cellular senescence (Michaloglou *et al*., 2005; Campisi 2005). This mechanism of senescence is usually modulated by cyclin dependent kinase inhibitors (e.g. p27Kip1), and it is adopted by normal cells to overcome oncogene activation (Campisi, 2005, Ben-Porath and Weinberg, 2005). Moderate levels of MAPK pathway activation could induce abnormal cellular functions leading to transformation and immortalization of mouse melanocytes, increased *in vitro* colony formation, and elevated Erk1/2 activities (Inamdar *et al*., 2010). We have tested here the early effects of cisplatin and IL-6 exposure on the cells previously treated with Cisplatin, once Western blot was performed after 30 minutes treatment. ERK phosphorylation was decreased in UM-SCC-22BCis12 cells, what may explain why these cells took longer time to generate tumors *in vivo*. Thus, there may are later events that transform cells to re-enter the cell cycle.

Moreover, we generated orospheres from UM-SCC-22BCis12 sorted cells. The ALDH+CD44+ and ALDH-CD44- cells were sorted and plated in 24 wells low attachment plates at a density of 1,500 cells per well. Cells were treated and maintained in culture for 4 days until spheres were formed. In the ALDH+CD44+ cells derived spheres we observed an increase in sphere number in the cisplatin 2 μM plus IL-6 20 ng/mL condition (Figure 4D). Whereas, in the ALDH-CD44- cells derived spheres we also found an increase in number of spheres, but when IL-6 20 ng/mL was combined to cisplatin 0.2 μM (Figure 4E).

Cisplatin treatment increases stem cell population in head and neck xenografted tumors

In order to evaluate the effect of cisplatin treatment on the stem cells compartment *in vivo*, we generated xenografts of tumors again in immunodeficient mice and used the same criteria of tumors having a mass of 200 mm^3 (Figure 5A). We therefore implanted tumor cells, UM-SCC-22B cells, together with endothelial cells, either silenced for IL-6 (HDMEC-shRNA-IL-6) or control (HDMEC-shRNA-C). The effectiveness of silenced IL-6 expression was confirmed by ELISA (Figure 5B). The tumor volume over time demonstrates that UM-SCC-22B + HDMEC-shRNA-C group initiated tumors earlier and were greater in volume than the UM-SCC-22B + HDMEC-shRNA-IL-6 group (Figure 5C).

Once the average tumor volumes reached 200 mm^3, we divided each group into treated and control mice. The treated mice received cisplatin 5 mg/kg intraperitonealy once a week and the control mice received PBS in the same volume as the drug. Mice were sacrificed the day after the third injection (Figure 5A). Here, we used cisplatin at maximum tolerated dose (MTD) for the mice, as shown by a decrease in weight of treated mice in both groups (Supplementary figure 4).

Tumor volume was statistically lower in UM-SCC-22B + HDMEC-shRNA-IL-6 Cisplatin treated group than in UM-SCC-22B + HDMEC-shRNA-C control group and in day 8 (P=0.040), day 10 (P=0.002), day 12 (P=0.001) and day 14 (P=0.034) (Figure 5D). Interestingly, between untreated mice, tumor volume was significantly lower in UM-SCC-22B + HDMEC-shRNA-IL-6 group in the last day (day 14, P=0.017) (Figure 5D). The analysis of the cancer stem cell fraction revealed a significant increase in the proportion of ALDH+CD44+ cells in the tumors arised from the mice treated with Cisplatin in both groups (Figure 5E-H). In general, tumor xenografts were characterized by large islands

of tumor cells surrounded by connective tissue. Tumor cells presented basophile cytoplasm, increased nuclear-to-cytoplasm diameter ratio, as well as marked cellular pleomorphism (Figure 5I). We observed a significant overall difference in mitotic index (Figure 5J, $P < 0.0002$). Pairwise analysis revealed that the mitotic index of shC tumors treated with cisplatin was higher than the mitotic index of each one of the other experimental conditions.

IL-6 receptor blockade reduces the survival and stemness of head and neck carcinoma cells

To investigate the possible use of IL-6 inhibition as an adjuvant therapy to decrease tumor stem cells population and therefore avoid recurrences in head and neck cancer, we looked at the protein expression of the stem cell marker, Bmi-1, and the survival markers STAT3, Akt and ERK in UM-SCC-22B cells. First, we evaluated the effect of the neutralizing antibody to IL-6 and the antibody against IL-6 receptor (IL-6R Ab, 5 and 10 ng/mL) on the main signaling pathway of IL-6, STAT3. Indeed, the induction of phosphorylation of STAT3 by IL-6 was decreased in the presence of the neutralizing antibody to IL-6 and, as well as the antibody to IL-6R, the last one seems to have a dose-dependent effect (Figure 6A). Akt and ERK phosphorylation were also decreased in cells co-incubated with rhIL-6 and the antibodies against IL-6 and IL-6R (Figure 6A). From these results, we suggest IL-6 inhibition, either through the blockade of the ligand IL-6 or its receptor, may interfere in cell proliferation and survival of head and neck cells *in vitro*.

We next assessed the effect of IL-6 and IL-6R antibodies on the expression of Bmi-1 in UM-SCC-22A and UM-SCC-22B cells, as well as the previously Cisplatin treated cells, UM-SCC-22BCis1 and UM-SCC-22BCis12 cells. We observed a decrease in Bmi-1 expression in both UM-SCC-22A and UM-SCC-22B cells only with the antibody

against IL-6R. A slightly decrease in Bmi-1 was seen in UM-SCC-22BCis1 cells, but UM-SCC-22BCi12 cells Bmi-1 expression seems to be unaffected by IL-6R blockade (Figure 6B).

Discussion

The outline of this study was centered on two main questions. First, does the CSCs compartment increase in the tumor cell population after treatment with chemotherapy, specifically cisplatin? Second, is IL-6, a cytokine secreted by the tumor vasculature, involved in the protection of CSCs during cisplatin treatment?

In fact, there are some reports that tumor stem cells proportion can be enriched with effective anti-cancer drugs. In lung cancer, adherent A549 cells showed an 8-fold enrichment of cancer-initiating cell fraction, identified as CD133+ cells, as a result of a cytotoxic concentration exposure of cisplatin (Bertolini *et al*., 2009). Moreover, Liu and colleagues (2012) showed that CD133+ cells were significantly increased in the relapsed tumors of lung cancer patients who have received cisplatin treatment. In glioblastoma, a subpopulation of endogenous glioma cells with CSC properties and relatively quiescent is responsible for re-growth a tumor after treatment with temozolomide (Chen *et al*., 2012). Indeed, long term trastuzumab treatment generates highly enriched CSCs in breast cancer cells resistance-induced by PTEN knock down. Interestingly, the expansion of the CSC population seems to be mediated through an inflammatory response of IL-6 (Korkaya *et al*., 2012).

It is well established that IL-6, through the activation of signal transducer and activator of transcription 3, act by blocking apoptosis in cells during inflammatory processes, permitting them to keep alive in unfavorable environments. The same pathway is employed by the tumor cells to maintain proliferation and neoplastic growth,

by avoiding apoptotic deletion (Hodge *et al*., 2005). Thus, it has been postulated that IL-6 participate in resistance to chemotherapeutic drugs (Conze *et al*., 2001; Ara *et al*., 2009). More recently, paracrine IL-6 signaling derived from the tumor surrounding vasculature in response to therapy has been related to the survival of tumor cells in lymphomas and lung carcinomas (Gilbert and Hemann, 2010; Franses *et al*., 2011).

We have previously demonstrated that ALDH+ cells are localized in close proximity to blood vessels and that the endothelial cells initiate signaling events that enhance the survival and self-renewal of stem cells in head and neck tumors. Thus, the selective ablation of the tumor associated blood vessels was sufficient to decrease the proportion of the head and neck CSCs *in vivo* (Krishnamurthy *et al*., 2010). Moreover, the secretion of IL-6 by the endothelial cells activated the IL-6R/STAT3 pathway in the head and neck CSCs identified by the double positive cells ALDH+CD44+, resulting in increased cell survival *in vitro* and *in vivo* (Krishnamurthy *et al*., unpublished data). These earlier studies were focused on the influence of the endothelial-secreted factors specifically on the head and neck CSCs. In the present study, we focused on the influence of IL-6 in the whole population of tumor cells. Here we show for the first time that IL-6 promoted expression of the stem cell self-renewal marker Bmi-1 in head and neck cells. Increased Bmi-1 expression was confirmed in another cell line, UM-SCC-1, after 24h rhIL-6 treatment, as shown in Supplemental Figure 5A. The CD44 stem cell marker was also increased in the presence of IL-6 (Supplemental Figure 5A). CD44+ cells possess not only a marked capacity of proliferation, tumorsphere formation, migration and invasion, but also overexpression of genes involved in drug resistance (Okamoto *et al*., 2009).

Together, these data support the possibility that the effect of IL-6 is directed towards the stem cell compartment, and we demonstrate that IL-6R co-localizes with ALDH1 in primary samples. Indeed, IL-6 did not increase cell proliferation in the entire

cell population, as shown here in Figure 1A and B. Interestingly, Neiva and collaborators (2009) did not observe a significant impact on HNSCC growth when IL-6 was blocked, suggesting that the effect of IL-6 is restricted to a particular group of cells, less proliferative, and not the whole tumor cell population.

The effect of cisplatin on the expression of Bmi-1 was evaluated and we observed an increase in two cell lines, also seen in UM-SCC-1 cells, confirmed by the expression of CD44 (Supplemental Figure 5B). Even cells pre-treated with cisplatin in low concentrations for a long period still demonstrated higher expression of Bmi-1, suggesting that the effect of the drug persists over time and, is not a transitory response to the drug.

The additive effect of IL-6 on the survival of stem cells treated with cisplatin was seen as a trend in flow cytometry analysis, in which the proportion of ALDH+CD44+ cells is higher when cisplatin was treated together with IL-6 then alone. The same was seen in the orosphere formation assay. The number of orospheres formed from single cells of a primary tumor was significantly increased in the presence of cisplatin and IL-6, compared to cisplatin alone. The Bmi-1 expression also show a synergistically effect of cisplatin and IL-6 exposed together in both cell lines tested. Indeed, orospheres from UM-SCC-22BCis12 also formed in a higher number when treated with combined regimen, either in ALDH+CD44+ cells and ALDH-CD44- cells. Interestingly, we found that UM-SCC-22BCis12 cells take longer time to generate tumors compared to the parental UM-SCC-22B cells, this observation is in contrast with a recent publication that describes a tamoxifen-resistant cell line with a higher tumorigenicity *in vivo* compared to the parental line in breast cancer (Dubrovska *et al*., 2012).

We also demonstrated that silencing the IL-6 gene in the endothelial cells alone resulted in a delay in tumor initiation and growth of tumor xenografts generated from UM-SCC-22B cells. Moreover, the analysis of the cancer stem cells in the xenografts

showed a significant increase in the cisplatin treatment group indicating that cisplatin increases the survival of the head and neck cancer stem cells *in vivo*. However, no difference was observed in the stem cell proportion between the shRNA-IL-6 and shRNA-C groups. This fact suggests the possibility of other endothelial cell or tumor cell secreted factors that might compensate the IL-6 knockdown and contribute to the self-renewal and survival of cancer stem cells in response to cisplatin treatment. Indeed, the number of mitotic cells was higher in tumors generated by UM-SCC-22B cells co-implanted with HDMEC-shRNA-C in cisplatin treated mice compared to tumors arose from mice that received vehicle and, both treated and control mice of the HDMEC-shRNA-IL-6 group. The mitotic index has been considered as a potent indicator of cancer patient survival (Bogunovic *et al*., 2009; Baak *et al*., 2009). Interestingly, cisplatin treatment was able to induce mitosis in tumor cells, which effect was revert by IL-6 knockdown in the cells of the tumor vasculature. Although the mechanism of chemotherapy resistance needs to be further investigated, taken together, our data suggest that cisplatin possibly induce CSC self-renewal in HNSCC, and IL-6 might be involved in this process.

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Legends for figures

Figure 1: **Analysis of proliferation and survival pathways in HNSCC cell lines.** Survival test performed in 3 time points (24h, 48h and 72h) by SRB assay in UM-SCC-1 (**A**) and UM-SCC-22B (**B**) cells. IL-6 was pre-treated for 1h before co-incubation with Cisplatin. IC50 of the cells are around 2µM. Experiments were done in quadruplicate wells per condition. Each graph is representative of 3 independent experiments. (**C**) Western blot for phosphorylated and total STAT3, ERK, Akt and CDK4 in UM-SCC-1 and UM-SCC-22B cells serum-starved overnight and exposed to cisplatin and 20 ng/ml rhIL-6 for 30 min. (**D**) Western blot for phosphorylated and total STAT3, ERK, and Akt in UMSCC-22B serum-starved overnight and exposed to cisplatin and 20 ng/ml rhIL-6 for 30 min.

Figure 2: **Analysis of head and neck cancer stem-like cells proportion and survival *in vitro*.** Flow cytometry analysis of UM-SCC-22B cells treated with Cisplatin and rhIL-6 alone or together for 24 hours. Representatives analysis is depicted in (**A**) and the quantification of triplicates of treatment is depicted in (**B**). Orospheres arised from a primary sample, in (**C**) ALDH+CD44+ single cells cultured in low attachment plates for 12 days with treatment. (**D**) Photomicrographs of representative orospheres from ALDH+CD44+ cells from primary sample. Statistical analysis was one-way ANOVA followed by Tukey pos-hoc test and the differences between groups shown were $P < 0.05$.

Figure 3: Stem cell markers expression in Head and neck carcinoma after Cisplatin and IL-6 treatment. (**A**) Western blot depicting the expression of Bmi-1 in UM-SCC-22A and UM-SCC-22B cells treated with Cisplatin and rhIL-6 for 24h. (**B**)

Representative photomicrographs of the cell lines UM-SCC-22A and UM-SCC-22B, and the generated Cisplatin resistant cells UM-SCC-22BCis1, UM-SCC-22BCis4, UM-SCC-22BCis6 and UM-SCC-22BCis12. Western blot depicting the expression of Bmi-1 (**C**) and Oct4 (**D**) in the cisplatin resistant cells treated with cisplatin and rhIL-6 for 24 h.

Figure 4: Head and neck Cisplatin resistant cells take longer time to generate tumors. (**A**) Schematic representation of the approach used for testing the tumorigenicity UM-SCC-22B and UM-SCC-22BCis12 *in vivo*. (**B**) Graph showing the tumor volume average obtained by the implantation of UM-SCC-22B and UM-SCC-22BCis12 cells (n=11 in each group) during days. (**C**) Western blot for phosphorylated and total STAT3 and ERK in UMSCC-22BCis resistant cells serum-starved overnight and exposed to 20 ng/ml of rhIL-6 for 30 min. Orospheres arising from UM-SCC-22BCis12 ALDH+CD44+ (**D**) and ALDH-CD44- (**E**) cells (1,500 cells per well were plated in 24 wells ultra low attachment plates and cultured for 4 days with or without treatment). Statistical analysis was one-way ANOVA followed by Tukey pos-hoc test and the differences between groups shown were $P < 0.05$.

Figure 5: **Cisplatin treatment increases stem cell population in head and neck xenograph tumors.** (**A**) Schematic representation of the approach used for testing the effect of Cisplatin treatment on tumors obtained by the implantation of UM-SCC-22B cell with primary endothelial cells (HDMEC) HDMEC-shRNA-C or HDMEC-shRNA-IL-6. (**B**) Graph showing Interleukin-6 levels in HDMEC-shRNA-C and HDMEC-shRNA-IL-6 obtained by ELISA. Asterisk depicts $P < 0.0001$. (**C**) Graph shows the tumor volume average obtained by the implantation of UM-SCC-22B with HDMEC-shRNA-C and UM-SCC-22B with HDMEC-shRNA-IL-6 (n=12 in each group). (**D**) Graph shows the tumor

volume average after treatment of the UM-SCC-22B+HDMEC-shRNA-C and UM-SCC-22B+HDMEC-shRNA-IL-6 tumors. Cancer stem fraction (ALDH+CD44+Lin-cells) in the UM-SCC-22B+HDMEC-shRNA-C (**E** and **F**) and UM-SCC-22B+HDMEC-shRNA-IL-6 tumors (**G** and **H**). (**I**) Representative images of histologic sections stained with hematoxilin and eosin (top row, 100; botton row, 400). (**J**) Graph depicting the average of mitotic cells per mm2 (mitotic index) determined by a trained pathologist blinded for experimental conditions. The average of mitosis per mm^2 was analyzed by one-way ANOVA followed by Tukey post-hoc test. *P* value indicates a significant difference across the 4 groups ($p < 0.0002$).

Figure 6: **Blockade of IL-6 receptor reduce survival and stemness of head and neck carcinoma cells.** (**A**) Western blot for phosphorylated and total STAT3, Akt and ERK in UM-SCC-22B treated for 30 minutes. (**B**) Western blot depicting the expression of Bmi-1 in UM-SCC-22A, UM-SCC-22B, UM-SCC-22BCis1 and UM-SCC-22BCis12 cells treated for 24hours. Treatment for both experiments was rhIL-6 and antibody against IL-6 or IL-6 receptor, IgG was used as a control.

Supplementary figures

Supplementary Figure 1: **Cytotoxic profile of cisplatin in head and neck tumor cell lines.** The cytotoxicity was determined by the SRB assay on UM-SCC-1 (**A**) and UM-SCC-22B (**B**) cells after 48, 72 and 96 hours of treatment. (**C**) IC50 values obtained with 72h of Cisplatin exposure. Experiments were done in quadruplicate wells per condition. Each graph is representative of 3 independent experiments.

Supplementary Figure 2: **Effect of cisplatin and rhIL-6 on cell number of UM-SCC-22B cell line.** (**A**) UM-SCC-22B cells counted by Trypan blue exclusion method after 24, 48 and 72 hours of cisplatin treatment (0-5µM). Only the alive cells were counted. Experiments were performed in triplicate wells per condition, and graph is representative of three independent experiments. (**B**) UM-SCC-22B cells counted by the trypan blue exclusion method right before flow cytometry analysis. Cells were treated either with single drug alone or both drugs simultaneously for 24 hours.

Supplementary Figure 3: Relative occurrence of time to palpability of tumors (i.e., the time at which tumors are initiated) in the UM-SCC-22B and UM-SCC-22BCis12 with HDMEC tumors.

Supplementary Figure 4: Graph shows the mouse weight during treatment.

Supplementary Figure 5: Western blot analysis of Bmi-1 and CD44 in UM-SCC-1 cells after treatment with IL-6 (**A**) and cisplatin (**B**) for 24 hours.

Figure 1

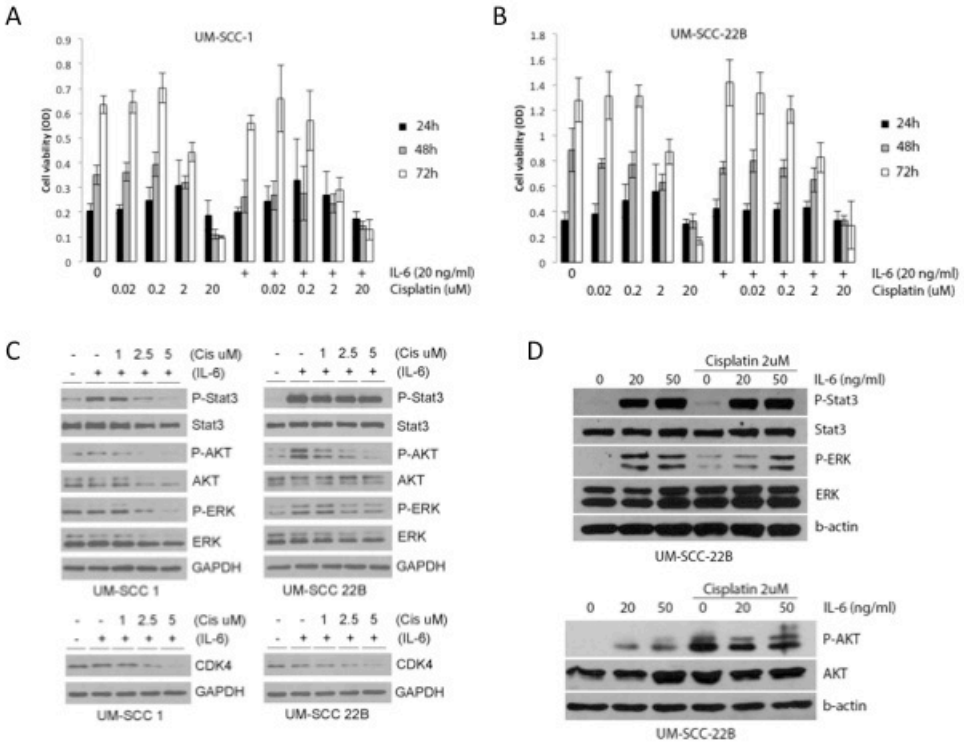

A
UM-SCC-1
Cell viability (OD)
24h
48h
72h
IL-6 (20 ng/ml)
Cisplatin (uM)
B
UM-SCC-22B
C
(Cis uM)
(IL-6)
P-Stat3
Stat3
P-AKT
AKT
P-ERK
ERK
GAPDH
UM-SCC 1
UM-SCC 22B
CDK4
D
Cisplatin 2uM
IL-6 (ng/ml)
b-actin

Figure 2

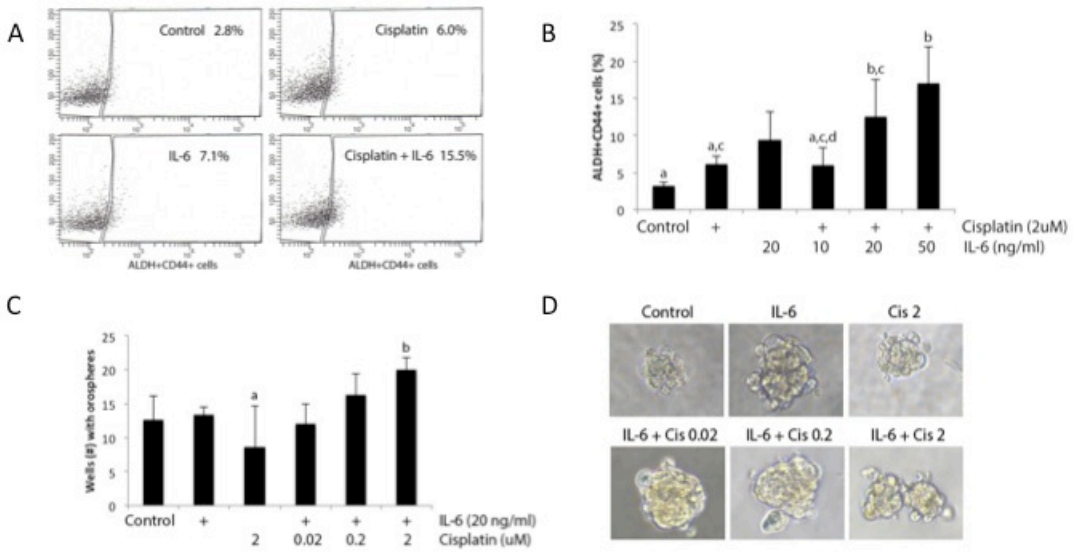

A
Control 2.8%
Cisplatin 6.0%
IL-6 7.1%
Cisplatin + IL-6 15.5%
ALDH+CD44+ cells
ALDH+CD44+ cells
B
ALDH+CD44+ cells (%)
a
a,c
a,c,d
b,c
b
Control
Cisplatin (2uM)
IL-6 (ng/ml)
C
Wells (#) with orospheres
Control
IL-6 (20 ng/ml)
Cisplatin (uM)
D
Control
IL-6
Cis 2
IL-6 + Cis 0.02
IL-6 + Cis 0.2
IL-6 + Cis 2

Figure 3

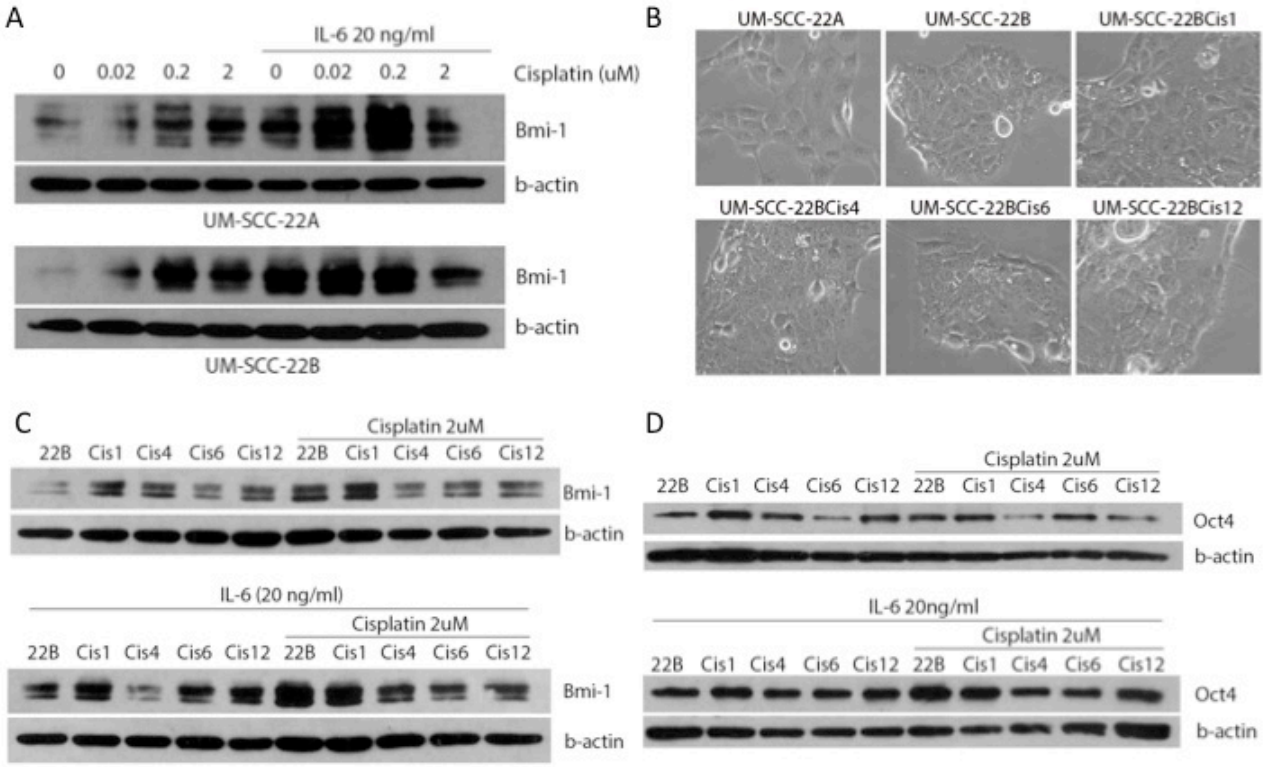

A
IL-6 20 ng/ml
0 0.02 0.2 2 0 0.02 0.2 2
Cisplatin (uM)
Bmi-1
b-actin
UM-SCC-22A
Bmi-1
b-actin
UM-SCC-22B
B
UM-SCC-22A
UM-SCC-22B
UM-SCC-22BCis1
UM-SCC-22BCis4
UM-SCC-22BCis6
UM-SCC-22BCis12
C
Cisplatin 2uM
22B Cis1 Cis4 Cis6 Cis12 22B Cis1 Cis4 Cis6 Cis12
Bmi-1
b-actin
IL-6 (20 ng/ml)
Cisplatin 2uM
22B Cis1 Cis4 Cis6 Cis12 22B Cis1 Cis4 Cis6 Cis12
Bmi-1
b-actin
D
Cisplatin 2uM
22B Cis1 Cis4 Cis6 Cis12 22B Cis1 Cis4 Cis6 Cis12
Oct4
b-actin
IL-6 20ng/ml
Cisplatin 2uM
22B Cis1 Cis4 Cis6 Cis12 22B Cis1 Cis4 Cis6 Cis12
Oct4
b-actin

Figure 4

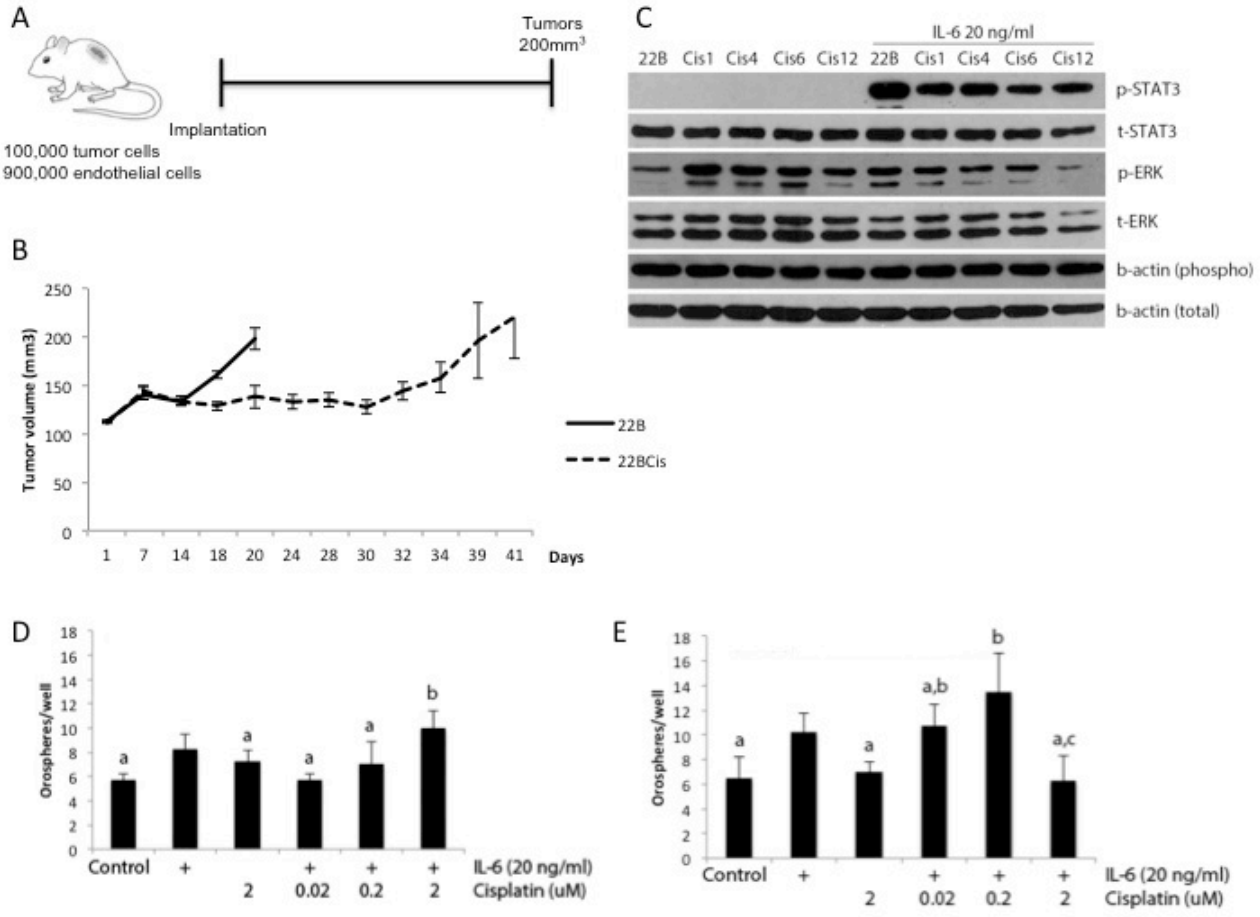
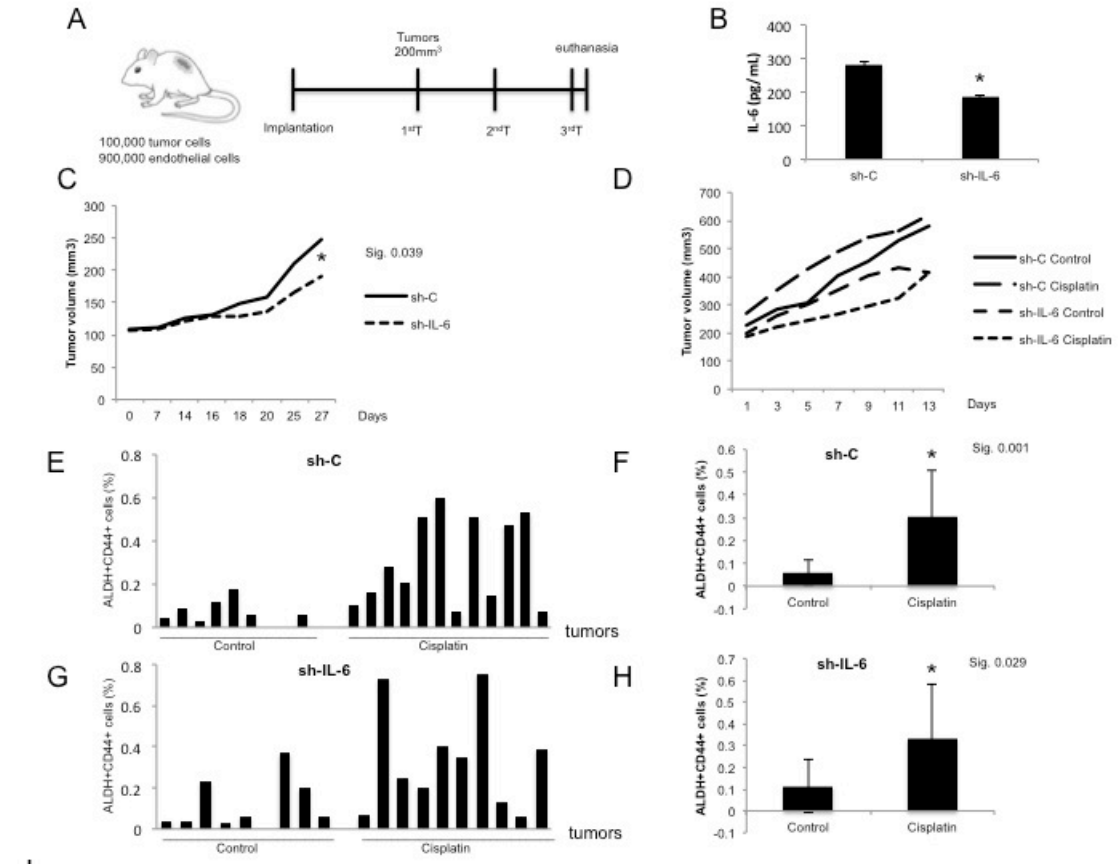
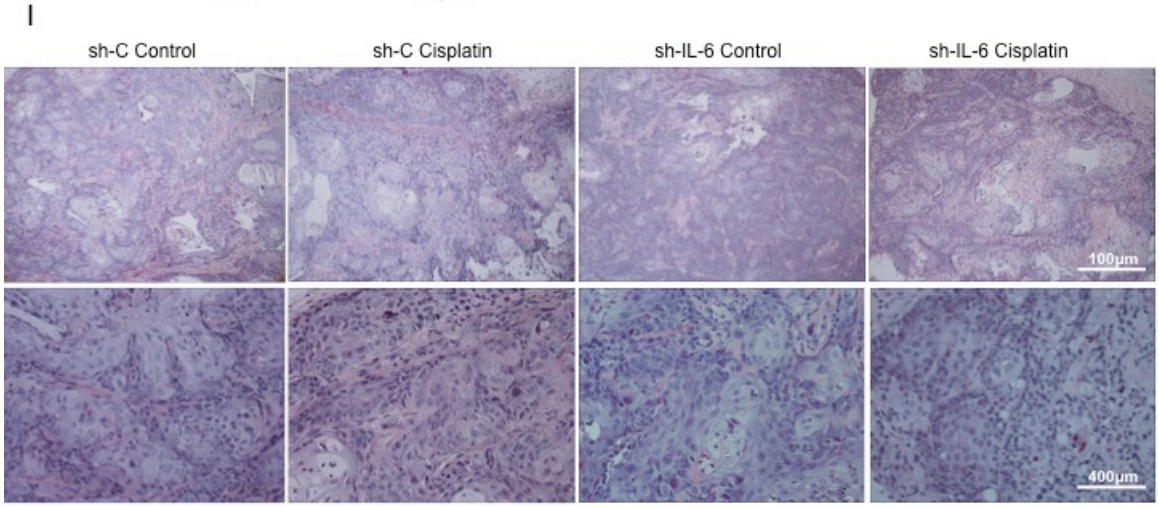

A
Tumors
200mm3
Implantation
100,000 tumor cells
900,000 endothelial cells
B
Tumor volume (mm3)
250
200
150
100
50
0
1 7 14 18 20 24 28 30 32 34 39 41
Days
22B
22BCis
C
IL-6 20 ng/ml
22B Cis1 Cis4 Cis6 Cis12 22B Cis1 Cis4 Cis6 Cis12
p-STAT3
t-STAT3
p-ERK
t-ERK
b-actin (phospho)
b-actin (total)
D
Orospheres/well
18
16
14
12
10
8
6
4
2
0
a a a a b
Control + 2 + 0.02 + 0.2 + 2
IL-6 (20 ng/ml)
Cisplatin (uM)
E
Orospheres/well
18
16
14
12
10
8
6
4
2
0
a a a,b b a,c
Control + 2 + 0.02 + 0.2 + 2
IL-6 (20 ng/ml)
Cisplatin (uM)

Figure 5


A
Tumors
200mm3
euthanasia
Implantation
1stT
2ndT
3rdT
100,000 tumor cells
900,000 endothelial cells
B
IL-6 (pg/mL)
sh-C
sh-IL-6
C
Tumor volume (mm3)
Sig. 0.039
sh-C
sh-IL-6
Days
D
Tumor volume (mm3)
sh-C Control
sh-C Cisplatin
sh-IL-6 Control
sh-IL-6 Cisplatin
Days
E
sh-C
ALDH+CD44+ cells (%)
Control
Cisplatin
tumors
F
sh-C
Sig. 0.001
ALDH+CD44+ cells (%)
Control
Cisplatin
G
sh-IL-6
ALDH+CD44+ cells (%)
Control
Cisplatin
tumors
H
sh-IL-6
Sig. 0.029
ALDH+CD44+ cells (%)
Control
Cisplatin


I
sh-C Control
sh-C Cisplatin
sh-IL-6 Control
sh-IL-6 Cisplatin
100µm
400µm

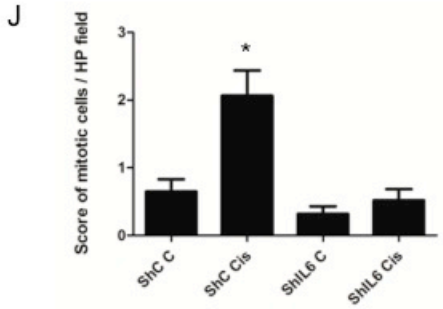
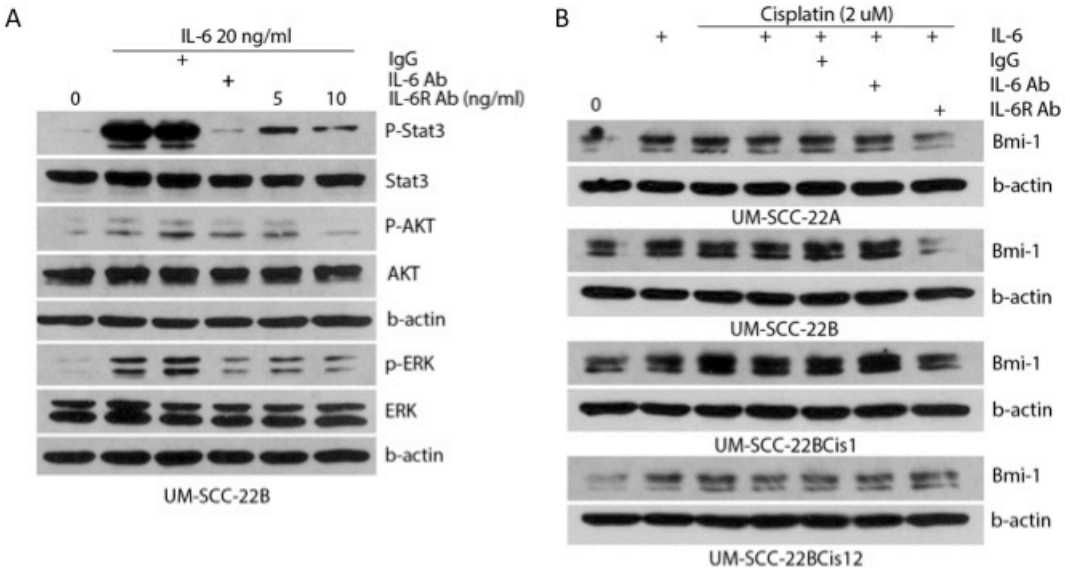
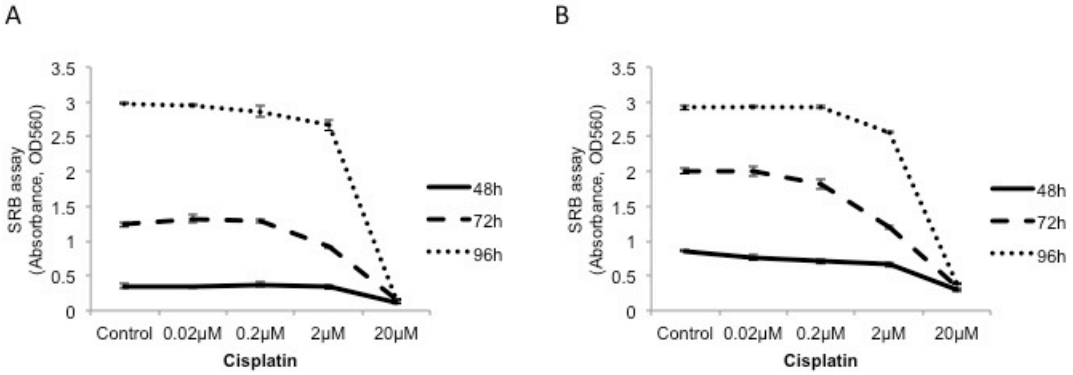

J
Score of mitotic cells / HP field
ShC C
ShC Cis
ShIL6 C
ShIL6 Cis

Figure 6


A
IL-6 20 ng/ml
+
+
IgG
IL-6 Ab
0
5
10
IL-6R Ab (ng/ml)
P-Stat3
Stat3
P-AKT
AKT
b-actin
p-ERK
ERK
b-actin
UM-SCC-22B
B
Cisplatin (2 uM)
+
+
+
+
+
IL-6
+
IgG
+
IL-6 Ab
0
+
IL-6R Ab
Bmi-1
b-actin
UM-SCC-22A
Bmi-1
b-actin
UM-SCC-22B
Bmi-1
b-actin
UM-SCC-22BCis1
Bmi-1
b-actin
UM-SCC-22BCis12

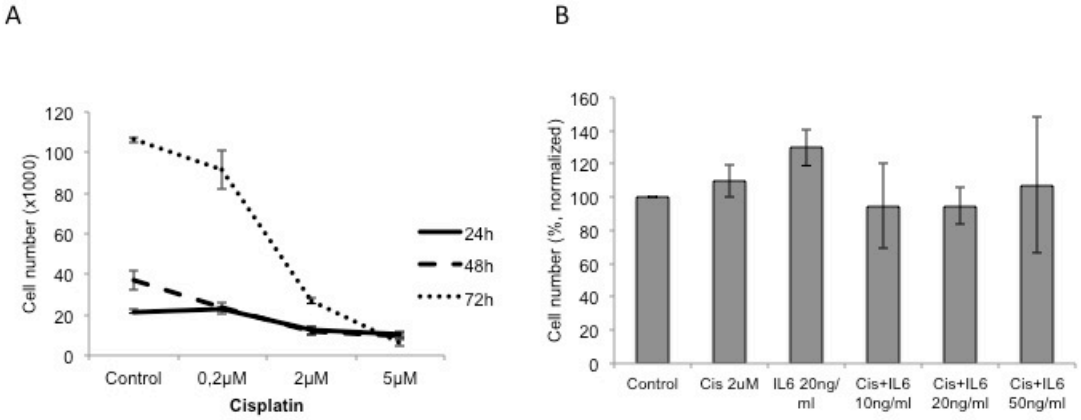
Supplementary figure 1



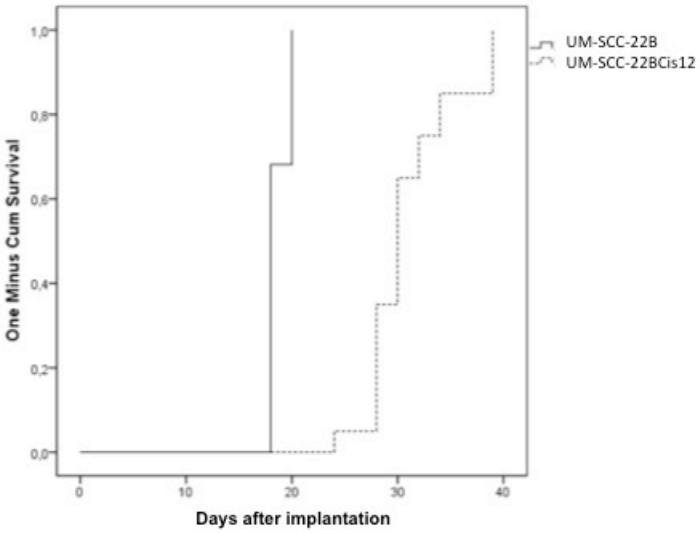
C

Cell line	IC50 (μM)
UM-SCC-1	2.305
UM-SCC-22B	2.400

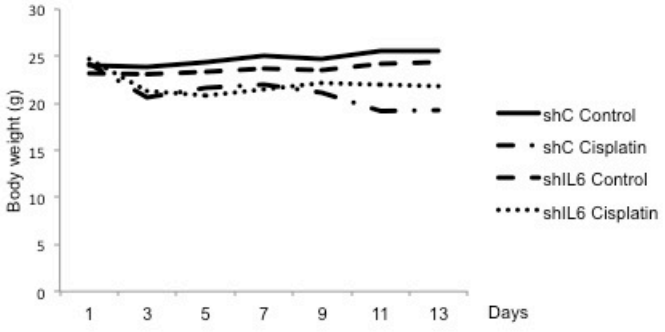
Supplementary figure 2



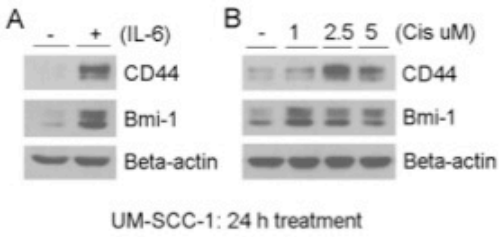
Supplementary figure 3



Supplementary figure 4



Supplementary figure 5



DISCUSSÃO GERAL

Atualmente, o câncer tem sido cada vez mais visto como um órgão aberrante (Al-Hajj & Clarke, 2004). Os tumores contêm uma população de células com propriedades de células-tronco que dirigem o seu crescimento. Estas células, as células-tronco tumorais, possuem capacidade de divisão assimétrica, gerando a progenie de células diferenciadas com as mais variadas carasterísticas morfológicas, antígenos célula-específicos, e propriedades funcionais características do tumor (Bjerkvig *et al*., 2005). Além disso, as CTTs também estariam envolvidas na recorrência dos tumores (Bao *et al*., 2006). As CTTs apresentam analogias funcionais com as células-tronco normais. Ambas as células se caracterizam pela capacidade de auto-renovação, proliferação e diferenciação, sendo esses eventos fortemente regulados nas células normais, e aberrantes nas CTT (Varghese *et al*., 2008).

Num trabalho de comparação entre o comportamento de células-tronco neurais humanas e células-tronco de gliomas, foi demonstrado que ambas respondem a sinais de diferenciação apresentando drástica diminuição da proliferação. As células-tronco tumorais se diferenciam mais rapidamente que as células-tronco neurais normais. Em se tratando de diferenciação, as células-tronco normais, deram origem a neurônios e células da glia normais, enquanto que as células-tronco tumorais produziram células morfologicamente aberrantes expressando marcadores gliais e neuronais na mesma célula (Varghese *et al*., 2008). De acordo com este trabalho, as células-tronco normais e tumorais diferiram em suas capacidades de proliferação e no padrão e na velocidade de diferenciação.

No **Capítulo I** desta tese, nós demonstramos que o inibidor de desacetilase de histonas, butirato sódico foi capaz de aumentar os níveis de RNAm da subunidade Gria2 do receptor AMPA em duas linhagens celulares de meduloblastoma, D283 e

DAOY. Este marcador está normalmente expresso em neurônios funcionais podendo ser usado como marcador de diferenciação neuronal. Este indicativo de diferenciação das células expostas a um modulador de cromatina foi acompanhado da redução do número de células viáveis, verificado por método de contagem por *Trypan blue*, e da redução da capacidade de proliferação das células após o tratamento, verificada pela formação de novas colônias, após exposição do composto por 72h.

A diferenciação verificada no trabalho de Varghese e colaboradores (2008) também foi acompanhada de decrécimo do índice proliferativo de células de glioma, verificado pelo corante Ki-67. Assim, a terapia-alvo direcionada a diferenciação das células tumorais levaria ao resultado almejado combinando redução do volume tumoral e diminuição da tumorigenicidade das células tumorais remanescentes, podendo assim evitar as recorrências do tumor primário e metástases.

Entretanto, se faz necessário salientar que o mecanismo de diferenciação celular não é o único pelo qual butirato sódico parece atuar frente às células tumorais em meduloblastoma. Sonnemann e colaboradores (2006) demonstraram que butirato sódico, dentre outros inibidores de desacetilases de histonas, induz morte celular por apoptose através da ativação das enzimas caspase-3 e 9 em duas linhagens celulares, DAOY e UW228-2.

Além disso, butirato sódico agiu de modo sinérgico com etoposídeo, um quimioterápico inibidor da topoisomerase II utilizado para o tratamento de diversos tumores, na inibição da proliferação de D283. Doses inefetivas tanto do inibidor de HDAC, quanto do inibidor de topoisomerase II, quando combinadas no ensaio de MTT reduziram significativamente a viabilidade das células em cultura. Estudos clínicos já investigaram etoposídeo como agente terapêutico em meduloblastomas (Geyer *et al*., 2005; Ruggiero *et al*., 2010). A possível explicação para o efeito encontrado está na associação direta entre HDAC e a topoisomerase II. HDACs tem demonstrado interagir

diretamente com a topoisomerase II formando complexos funcionais que tem como alvo regiões específicas da cromatina, onde as histonas estejam sofrendo acetilação e assim, facilitando a condensação cromossomal pela topoisomerase II (Tsai *et al*., 2000).

No **Capítulo II**, foi demonstrado também o efeito sinérgico de butirato sódico e BDNF na diminuição da viabilidade de DAOY, medido pelo método de MTT. Alguns estudos prévios ja demonstraram a expressão de BDNF e seu receptor TrkB em amostras de tumores de meduloblastoma (Tajima *et al*., 1998) e linhagens celulares (Schmidt *et al*., 2010), entretanto o seu efeito no crescimento desses tumores nunca havia sido avaliado.

A expressão de BDNF está relacionada com a sobrevivência, regeneração e plasticidade neuronal das células normais do cérebro. Em células C6 de glioma de rato, butirato sódico promoveu diferenciação das células gliais através do aumento da expressão de BDNF induzida por serotonina nessas células. A diferenciação das células da glia se mostrou importante na preservação da integridade das redes neurais, assim como melhorou a funcionalidade das células neuronais. Este dado sugere que existe uma associação entre a diferenciação das células da glia e o aumento da produção de BDNF no cérebro (Morita *et al*., 2009).

O efeito benéfico de butirato sódico na proliferação, migração e diferenciação neuronal verificado após injúria isquêmica depende da sinalização de BDNF-TrkB. BDNF aumenta a sobrevivência e diferenciação das células progenitoras da zona subventricular *in vitro* e aumenta o número de novas células *in vivo*. BDNF exerce um papel fundamental na neurogênese. A administração de BDNF no ventrículo lateral de ratos adultos leva à formação de novos neurônios em diversas porções do cérebro (Kim *et al*., 2009).

No **Capítulo I**, nós demonstramos que butirato sódico diminui a formação de neuroesferas *in vitro*. Ensaios de formação de esferas tem sido utilizados para estudar

CTT *in vitro*. As condições de cultivo de CTT cerebrais foram adaptadas de condições originalmente definidas para as células-tronco neurais normais e, normalmente, partem de meios de cultura livres de soro contendo fator de crescimento epidermal (do inglês, *epidermal growth factor* – EGF) e fator de crescimento de fibroblasto (do inglês, *fibroblast growth factor* – FGF) (Pastrana *et al*., 2011). Células cultivadas neste meio formam colônias de células, as neuroesferas, que predominantemente expressam marcadores de precursors neurais como nestina e CD133. No entanto, as esferas são heterogêneas e além das CTTs, também contêm células progenitoras e um pequeno número de células diferenciadas. Assim, as esferas são mais corretamente definidas como culturas de células precursoras, na qual co-existem as CTTs e as células progenitoras. As células progenitoras se distinguem das CTTs por possuírem limitada capacidade de auto-renovação e aumentada capacidade de proliferação (Dirks, 2008).

No modelo de neuroesferas, a presença de CTTs é apenas inferida retrospectivamente, quando as esferas são replaqueadas múltiplas vezes e originam células mais diferenciadas. No **Capítulo I**, nós demonstramos que as esferas obtidas a partir do cultivo da linhagem de meduloblastoma D283 em meio de cultura apropriado expressaram uma maior quantidade do RNAm de Oct4, um marcador de células-tronco embrionárias, comparado às células da monocamada. Após dissociação das esferas e recolocação das células em meio de cultura com soro, as mesmas restabeleceram o crescimento em monocamada. No entanto, as esferas não puderam ser re-plaqueadas mais que 2 vezes. De fato, a habilidade das CTTs neurais de formar esferas é variável. As células de glioblastoma, em geral, são facilmente cultivadas na condição de neuroesferas, podendo ser mantidas por longos períodos nesta condição. No entanto, as neuroesferas obtidas a partir de meduloblastomas são difíceis de serem estabelecidas e só permanecem como neuroesferas em cultura por períodos curtos (Dirks, 2008).

Embora, as populações de células ricas em CTTs possam ser inferidas pela capacidade de formação de esferas *in vitro*, o padrão-ouro na identificação experimental de CTT se dá através da capacidade dessas células de induzir a formação de tumores em animais imunocomprometidos, e de expressar marcadores de superfície celular específicos (Dirks, 2008).

As CTTs tem sido definidas com base em sua capacidade de formar tumores em animais hospedeiros, de se auto-renovar e de gerar células diferenciadas (não tronco) (Gupta *et al*., 2009). Sendo que a representatividade das CTTs numa população de células tumorais poderia ser determinada pelo número necessário de células para formar um novo tumor (Al-Hajj *et al*., 2003; Singh *et al*., 2004).

Inicialmente, as CTTs foram descritas como sendo uma pequena sub-população de células, ou um pequeno reservatório de células auto-sustentáveis e que mantém o tumor. Entretanto, os primeiros experimentos que mostraram a existência de CTT em tumores, não excluíram a possibilidade de que a proporção de CTT pudesse variar drasticamente de acordo com o tipo de tumor e condições em que elas se encontrassem. Sendo que mais recentemente, tem se sugerido que a proporção de CTT esteja intimamente ligada ao tipo celular de origem, ao microambiente tumoral, ao acúmulo de mutações e ao estágio de progressão maligna em que se encontra o tumor. A malignidade do tumor é, em grande parte, determinada pelo grau de diferenciação das células do tumor, podendo se sugerir que reguladores de diferenciação sejam determinantes na biologia das CTTs (Gupta *et al*., 2009).

Entretanto, embora as CTTs se diferenciem em células não tronco, o processo contrário, no qual as células não-tronco seriam reprogramadas a CTT, não pode ser descartado. A alta plasticidade fenotípica que existe nas populações de células tumorais, se comparado com os tecidos normais, e a sinalização proveniente do

microambiente tumoral permite, em alguns casos, a interconversão de células não-tronco em células-tronco no tumor (Gupta *et al*., 2009).

Diversos trabalhos tem demonstrado que as CTTs se apresentam mais resistentes às terapias convencionais do que as células não tronco do tumor. Estes achados sugerem que as estratégias terapêuticas atuais estejam voltadas apenas para as células não tronco e que as CTTs sobreviveriam após o tratamento levando ao desenvolvimento de novos tumores (Bertolini *et al*., 2009; Liu *et al*., 2012; Korkaya *et al*., 2012).

Em HNSCC, um dos primeiros estudos a mostrar uma hierarquização das células tumorais foi apresentado por Pierce e colaboradores em 1971. Neste trabalho, as células de carcinoma de células escamosas indiferenciadas foram marcadas com timidina tritiada e acompanhadas quanto a habilidade de diferenciação. Estas células, altamente malignas, puderam ser transplantadas em ratos e foram capazes de gerar células diferenciadas e aparentemente benignas.

Estudos subsequentes demonstraram que as linhagens celulares derivadas de tumores primários de HNSCC, contém uma subpopulação de células com propriedades clonogênicas que é mantida ao longo das passagens, podendo ser utilizadas para estudos de CTTs *in vitro* (Mackenzie, 2004; Harper *et al*., 2007).

No **Capítulo III**, nós demonstramos o aumento da proporção de CTTs em células de HNSCC após o tratamento com cisplatina e IL-6 em análise por citometria de fluxo, bem como o aumento da formação de orosferas *in vitro* de uma amostra de tumor primário, quando submetida ao tratamento combinado. Esses dados sugerem que a quimioterapia padrão não elimina as CTTs e que fatores provenientes do nicho, como a citocina IL-6 atuem na proteção das CTTs.

A expressão do marcador de sobrevivência de CTTs, Bmi-1, está aumentada em células tratadas com cisplatina e a expressão aumenta ainda mais com o co-tratamento

de IL-6. Bmi-1 foi originalmente identificado como um colaborador do oncogene Myc em leucemia de camundongos exercendo um efeito oncogênico através da regulação negativa de inibidores de CDK, $p16_{ink4a}$ e $p19_{Arf}$ interferindo principalmente na via de p53. No âmbito das células-tronco, foi demonstrado que Bmi-1 é indispensável para a manutenção tanto de células-tronco hematopoiéticas quanto de CTTs de leucemia (Park *et al*., 2003) bem como de populações tronco-neurais (Lund & Lohuizen, 2004). Bmi-1, é um modulador epigenético da transcrição gênica que regula a auto-renovação celular através da inibição da expressão de diversos genes. Múltiplos processos estão envolvidos no evento de auto-renovação, como sobrevivência, replicação e diferenciação. Assim, Bmi-1 se torna um alvo importante no estudo de CTTs *in vitro* (Clarke, 2005). Facchino e colaboradores (2010), demonstraram que Bmi-1 pode conferir resistência à radioterapia em CTTs neurais ao promover o reparo ao dano de DNA, sugerindo um papel da regulação epigenética não apenas na tumorigenicidade das CTTs, como também na ineficácia da terapia.

Células resistentes a baixas doses de cisplatina apresentaram aumento da expressão de Bmi-1 e do marcador de célula-tronco embrionária, Oct4, por si só mesmo sem a presença do quimioterápico.

Acredita-se que as células tumorais interajam com o seu microambiente imediato a fim de criarem um nicho protetor que permita um estado quiescente das células e, assim, sejam capazes de evadir o efeito da terapia. Nesta condição, as células acumulariam alterações genéticas que confeririam um fenótipo de resistência permanente (Zahreddinean & Borden 2013). Nós observamos no experimento de avaliação de tumorigenicidade *in vivo* que as células resistentes a cisplatina levaram um tempo maior que as células parentais para a formação de tumores em camundongos imunocomprometidos xenotransplantados.

No experimento *in vivo*, a partir da indução de tumores humanos em animais imunocomprometidos, nós demonstramos que o silenciamento de IL-6 nas células endoteliais co-implantadas com as células tumorais fez reduzir o volume tumoral. Após o tratamento dos animais com cisplatina, a análise dos tumores por citometria de fluxo revelou um aumento na proporção de CTTs na população total de células viáveis.

Duffy e colaboradores, propuseram que IL-6 poderia ser utilizado como biomarcador de sobrevivência e recorrência em pacientes com tumores de cabeça e pescoço, uma vez que níveis séricos desta citocina estão diretamente relacionados a um mau prognóstico. Além disso, o principal alvo intracelular de IL-6, a via de STAT3, encontra-se constitutivamente ativada em HNSCC (Seethala *et al*., 2008).

Demonstramos também que o uso de anticorpo contra o receptor de IL-6 foi capaz de diminuir a expressão de proteínas envolvidas na proliferação e sobrevivência celular Akt, ERK e STAT3, induzida por IL-6. E que o mesmo anticorpo reduz a expressão de Bmi-1 induzida por cisplatina e IL-6.

Sendo assim, se mostra crucial a busca de terapias que tenham como alvo específico as CTT. Entretanto, como existe a possibilidade de células não-tronco do tumor se reprogramarem em CTT a partir de estímulos do microambiente, o regime terapêutico ótimo necessitaria a incorporação de agentes tanto contra as CTTs, quanto às células não-tronco do tumor. Terapias que interfiram nos mecanismos de proteção do nicho tumoral podem aumentar a eficácia dos tratamentos antitumorais. Mesmo os efeitos dos agentes indutores de diferenciação poderiam ser comprometidos pela sinalização protetora de CTTs proveniente das células do microambiente. Portanto, o direcionamento da terapia aos mecanismos intrínsecos e extrínsecos de manutenção das CTTs, a partir da combinação dos efeitos de diferenciação e controle dos sinais do nicho, levaria a uma depleção mais aficaz do pool de CTTs.

CONCLUSÕES

Butirato sódico diminui a proliferação e sobrevivência celular de linhagens de meduloblastoma, DAOY, ONS76 e D283, com indicativo de diferenciação neuronal em DAOY e D283.

A linhagem celular de meduloblastoma D283 é capaz de formar neuroesferas com maior expressão de Oct4, podendo ser utilizada como modelo de estudo *in vitro* de células-tronco tumorais de meduloblastoma.

Butirato sódico diminui a formação de neuroesferas de D283.

Butirato sódico atua de modo sinérgico com etoposide na redução da viabilidade celular de D283.

Butirato sódico atua de modo sinérgico com BDNF na redução da viabilidade celular de DAOY.

O tratamento de cisplatina combinado a hrIL-6 aumenta a sub-população de células-tronco, ALDH+CD44+, em células de HNSCC *in vitro*.

Cisplatina combinada a hrIL-6 aumenta o número de esferas formadas a partir de células ALDH+CD44+ de uma amostra de tumor primário.

O tratamento com cisplatina aumenta a expressão de Bmi-1, um marcador de auto-renovação de células-tronco tumorais em linhagens de HNSCC. Este efeito é potencializado por tratamento concomitante com hrIL-6. O anticorpo contra o receptor de IL-6 reverte o aumento da expressão de Bmi-1 causada por exposição das células a cisplatina ou IL-6.

Células resistentes a baixas doses de cisplatina tem expressão aumentada de Bmi-1.

Tumores humanos gerados em animais imunocomprometidos a partir de células resistentes a cisplatina, levam mais tempo para se formarem, se comparado com a célula parental.

Tumores humanos gerados em animais imunocomprometidos a partir de células tumorais co-implantadas com células endoteliais normais ou silenciadas para IL-6 apresentam uma maior proporção de células ALDH+CD44+ após tratamento dos animais com cisplatina.

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Curriculum Vitae

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Última atualização do currículo em 11/04/2013

Resumo

Graduada em Farmácia pela Universidade Federal do Rio Grande do Sul (2004) com habilitação em farmácia industrial (2006) e mestre em Ciências Farmacêuticas pela Universidade Federal do Rio Grande do Sul (2006). Atuou na indústria farmacêutica como pesquisadora de desenvolvimento de novos produtos na empresa Cristália Produtos Químicos Farmacêticos LTDA (2006 - 2008). Tem experiência na implementação e condução de estudos de estabilidade de medicamentos. Doutorado em andamento no Programa de Pós-Graduação em Biologia Celular e Molecular, com estágio sanduíche na Universidade de Michigan. Tem experiência na busca de moleculas com atividade antitumoral.

Dados pessoais

Nome	Carolina Nör
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Formação acadêmica/titulação

2009	Doutorado em Biologia Celular e Molecular. Universidade Federal do Rio Grande do Sul, UFRGS, Porto Alegre, Brasil com **período sanduíche** em University of Michigan (Orientador : Jacques Eduardo Nör) Título: Modulação da sobrevivência e proliferação de células de câncer: mecanismos relacionados ao estado da cromatina e ao nicho tumoral Orientador: Rafael Roesler Co-orientador: Jacques Eduardo Nör Bolsista do(a): Conselho Nacional de Desenvolvimento Científico e Tecnológico
2004 - 2006	Mestrado em Ciências Farmacêuticas. Universidade Federal do Rio Grande do Sul, UFRGS, Porto Alegre, Brasil

Título: Análise química e taxonômica de espécies de *Hypericum* e avaliação da atividade antiangiogênica, Ano de obtenção: 2006
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2004 - 2006 Graduação em Habilitação em Farmácia Industrial.
Universidade Federal do Rio Grande do Sul, UFRGS, Porto Alegre, Brasil

1999 - 2004 Graduação em Farmácia.
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Formação complementar

2010 - 2010 Curso de curta duração em Biologia Molecular na Prática Médica.
Sociedade Brasileira de Oncologia Pediátrica, SOBOPE, São Paulo, Brasil

2009 - 2009 Curso de curta duração em Simpósio de Terapias Inovadoras.
Hospital de Clínicas de Porto Alegre, HCPA, Porto Alegre, Brasil

2009 - 2009 Extensão universitária em Células-Tronco Mesenquimais e Bioengenharia.
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SINDUSFARMA, SINDUSFARMA, Brasil

2008 - 2008 Curso de curta duração em Estudos de Estabilidade de Fármacos e Medicamentos.
HSV Consultores Associados, HSV, Brasil

2007 - 2007 Curso de curta duração em Quimiometria e Estatística básica.
blue square - Business Solutions, BLUE SQUARE, Brasil

2007 - 2007 Curso de curta duração em Estabilidade Farmacêutica.
Universidade de São Paulo, USP, Sao Paulo, Brasil

2007 - 2007 Curso de curta duração em Estudo de Estabilidade de Produtos Farmacêuticos.
Expolabor, EXPOLABOR, Brasil

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Federação Brasileira de Indústria Farmacêutica, FEBRAFARMA, Brasil

2006 - 2006 Curso de curta duração em Curso Básico em Pesquisa Clínica.
Pontifícia Universidade Católica do Rio Grande do Sul, PUCRS, Porto Alegre, Brasil

2005 - 2005	Curso de curta duração em Curso de Manipulação Genética. Hospital de Clínicas de Porto Alegre, HCPA, Porto Alegre, Brasil
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2003 - 2003	Curso de curta duração em Avaliação Farmacológica e Toxicológica de Plantas. Universidade Federal do Rio Grande do Sul, UFRGS, Porto Alegre, Brasil
2001 - 2001	Curso de curta duração em Tecnologia de Produção Aplicadas à Fitoterápicos. Sindicato dos Farmacêuticos do Estado de Santa Catarina, SINDFAR-SC, Brasil
2000 - 2000	Curso de curta duração em Farmácia Hospitalar. Universidade Federal do Rio Grande do Sul, UFRGS, Porto Alegre, Brasil

Atuação profissional

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Vínculo institucional

2009 - Atual	Vínculo: Bolsista , Enquadramento funcional: Aluno de doutorado, Regime: Dedicação exclusiva
2003 - 2004	Vínculo: Bolsista , Enquadramento funcional: Bolsista Iniciação Científica , Carga horária: 20, Regime: Parcial
2001 - 2002	Vínculo: Bolsista , Enquadramento funcional: Bolsista Iniciação Científica , Carga horária: 20, Regime: Parcial

2. Cristália Produtos Químicos Farmacêticos LTDA - CRISTALIA

Vínculo institucional

2007 - 2008	Vínculo: Funcionário , Enquadramento funcional: Pesquisador, Carga horária: 42, Regime: Dedicação exclusiva
2006 - 2007	Vínculo: Estágio Curricular , Enquadramento funcional: Estagiário , Carga horária: 42, Regime: Dedicação exclusiva

3. University of Michigan - UMICH

Vínculo institucional

2005 - 2005	Vínculo: Visitor , Enquadramento funcional: Visiting Graduate Researcher , Carga horária: 40, Regime: Dedicação exclusiva

4. Hospital Nossa Senhora da Conceição - GHC

Vínculo institucional

2003 - 2003	Vínculo: Estágio Curricular , Enquadramento funcional: Estagiário , Carga horária: 20, Regime: Parcial

Projetos

2011 - 2012	Investigação do papel da citocina derivada de células endoteliais IL-6 na aquisição de resistência a cisplatina em carcinoma de células escamosas de cabeça e pescoço Descrição: Avaliação da influência de IL-6 na aquisição de propriedades de célula-tronco em células tratadas com quimioterapia cisplatina. Experimentos in vitro e in vivo. Situação: Concluído Natureza: Projetos de pesquisa Integrantes: Carolina Nör; Jacques Eduardo Nör (Responsável)
2009 - Atual	Papel de Inibidores de Desacetilase de Histona e Associações com Outros Fármacos na Proliferação de Linhagens de Meduloblastoma Descrição: Avaliação do efeito de Butirato Sódico, um inibidor de desacetilase de histona na proliferação das linhagens de meduloblastoma DAOY, ONS76 e D283-MED, bem como sua combinação com quimioterápicos de uso corrente no tratamento de meduloblastoma. Situação: Em andamento Natureza: Projetos de pesquisa Integrantes: Carolina Nör Caroline Brunetto de Farias; Ana Lucia Abujamra; Gilberto Schwartsmann; Algemir Lunardi Brunetto; Rafael Roesler (Responsável) Financiador(es): Conselho Nacional de Desenvolvimento Científico e Tecnológico-CNPq, Fundação Central Sul Americana para o Desenvol. de Drogas Anti-Câncer-SOAD, Hospital de Clínicas de Porto Alegre-HCPA
2009 - Atual	Papel de Receptores de Neuropeptídeos e Vias de Sinalização Envolvidas no Crescimento de Meduloblastomas Descrição: Caracterização da expressão de NMBR e VPAC1, seus papéis na proliferação celular e suas interações com as vias de sinalização da MAPK e PI3K em linhagens celulares de meduloblastomas. Situação: Em andamento Natureza: Projetos de pesquisa Integrantes: Carolina Nör Caroline Brunetto de Farias; Ana Lucia Abujamra; Gilberto Schwartsmann; Algemir Lunardi Brunetto; Rafael Roesler (Responsável); Mariane da Cunha Jaeger

Financiador(es): Conselho Nacional de Desenvolvimento Científico e Tecnológico-CNPq, Fundação Central Sul Americana para o Desenvol. de Drogas Anti-Câncer-SOAD, Hospital de Clínicas de Porto Alegre-HCPA

Revisor de periódico

1. Journal of Dental Research

Produção bibliográfica

Artigos completos publicados em periódicos

1. **Nör C**, Sassi FA, Farias CB, Schwartsmann G, Abujamra AL, Lenz G, Brunetto AL, Roesler R. (2013). The Histone Deacetylase Inhibitor Sodium Butyrate Promotes Cell Death and Differentiation and Reduces Neurosphere Formation in Human Medulloblastoma Cells. Molecular Neurobiology. , v.x, p.xxx - xxx, 2013.
2. **Nör C**, Farias, Farias CB, Abujamra AL, Schwartsmann G, Brunetto AL, Roesler R. (2011). The histone deacetylase inhibitor sodium butyrate in combination with brain-derived neurotrophic factor reduces the viability of DAOY human medulloblastoma cells. Child's Nervous System v.27, p.897 - 901, 2011.
3. **Nör C**, Bernardi, APM, Haas JS, Schripsema J, Rech S, von Poser GL. (2008). Phenolic Constituents in Hypericum Flowers. Natural Product Communications v.3, p.237 - 240, 2008.
4. Fenner R, Sortino M, Kuze Rates SM, Dall'Agnol R, Ferraz A, Bernardi AP, Albring D, **Nör C**, von Poser GL, Schapoval EE, Zacchino S. (2005). Antifungal activity of some Brazilian species. Phytomedicine (Stuttgart) v.12, p.236 - 240.
5. Dall'Agnol R, Ferraz A, Bernardi, APM, Albring D, **Nör C**, Schapoval EE, von Poser GL. (2005). Bioassay guided isolation of antimicrobial benzopyrans and phloroglucinol derivatives from Hypericum species. Phytotherapy Research v.19, p.291 - 293.
6. **Nör C**, Albring D, Ferraz A , Schripsema J, Pires V, Sonnet P, Guillaume D, von Poser GL. (2004). Phloroglucinol derivatives from four Hypericum species belonging to the Trigynobrathys section. Biochemical Systematics and Ecology v.32, p.517 - 519.
7. Dall'Agnol R, Ferraz A, Bernardi, APM, Albring D, **Nör C**, Sarmento L, Lamb L, Haas M, von Poser GL, SCHAPOVAL, Schapoval EE, (2003). Antimicrobial activity of some *Hypericum* species. Phytomedicine (Stuttgart) v.10, p.511 - 516.

Trabalhos publicados em anais de eventos (resumo)

1. JAEGER, M. C., **NÖR, C.**, FARIAS, C. B., ABUJAMRA, A. L., SCHWARTSMANN, G., BRUNETTO, A. L., ROESLER, R. Expressão de Neuromedina B e seu Receptor em Células de Meduloblastoma Humano In: XII Congresso Brasileiro de Oncologia Pediátrica, 2010, Curitiba. **Resumos dos Trabalhos**. , 2010.
2. ALMEIDA, V. R., ZANIRATTI, G. G., PRUSH, D. S., **NÖR, C.**, FARIAS, C. B., ABUJAMRA, A. L., SCHWARTSMANN, G., BRUNETTO, A. L., Zuardi, A. W., HALLAK, J. E. C., CRIPPA, J. A. S., ROESLER, R.

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